HORIBA
Scientific

DAS-6

Operation Manual
Part number J81119 rev. A

DAS6

Fluorescence decay analysis software

User guide

HORIBA Scientific

USA: HORIBA Horiba Scientific Inc., 3880 Park Avenue, Edison, NJ 08820-3012, Toll-Free: **+1-866-Horiba Scientific**
Tel: +1-732-494-8660, Fax: +1-732-549-5125, E-mail: info@Horiba Scientific.com, www.Horiba Scientific.com
France: HORIBA Horiba Scientific S.A.S., 16-18, rue du Canal, 91165 Longjumeau Cedex,
Tel: +33 (0) 1 64 54 13 00, Fax: +33 (0) 1 69 09 93 19, www.Horiba Scientific.fr
Japan: HORIBA Ltd., JY Optical Sales Dept, Higashi-Kanda, Daiji Building, 1-7-8 Higashi-Kanda
Chiyoda-ku, Tokyo 101-0031, Tel: +81 (0) 3 3861 8231, www.jyhoriba.jp
Germany: +49 (0) 89 4623 17-0 **Italy:** +39 0 2 57603050 **UK:** +44 (0) 20 8204 8142

Table of contents

Copyright

Version

This version of the manual is intended for use with versions of DAS beginning 6.6

Technical support

All requests for technical support **must** be accompanied by the log file relating to the session during which the problem occurred. Please refer to Appendix D for more information.

1. An introduction to DAS6

Thank you for choosing a HORIBA Scientific product.

DAS6 is HORIBA's software for analysis of time domain fluorescence lifetimes. Data files measured using "DataStation" on HORIBA Scientific instruments can be opened directly and facilities are provided to import data from other instruments and software packages.

DAS6 provides multi-exponential fitting plus a number of modules for analysis of more specialised fluorescence decay processes. There is an optional module allowing for a Non Extensive Decay Distribution fit.

The key features of DAS6 are:

- Modular analysis format
- Foundation analysis for up to 5 exponentials
- Wide range of advanced modules (see below)
- Comprehensive statistical information
- Importing of other data formats
- Exporting of data and fits

Decay modules include:

- Exciplex kinetics
- Distribution
- Exponential series
- Förster energy transfer
- Yokota-Tanimoto energy transfer
- Micellar quenching
- Anisotropy analysis

- Batch exponential analysis
- Global exponential analysis

Optional decay modules include:

- Non Extensive Distribution

For full specifications of the DAS Software, please refer to Appendix C.

1.1 Upgrading to DAS6 from earlier versions

If you are upgrading from an earlier version of DAS6, you first need to un-install your current version. This can be done by running the setup program (see section 2). If an earlier version is present, you will be given the choice to “Repair” or “Remove” the software. In this case select “Remove”. After removing the previous version, install DAS6 by following the instructions in section 2.

WARNING

Once files have been opened using the latest version of DAS6, they cannot be used with earlier versions of the program. If you are running DAS6 on multiple PCs or sharing files with other laboratories you should ensure that the same version of DAS6 is being used on all computers.

1.2 Getting Started

This manual is intended for both beginners and advanced users as a guide to getting the best from the DAS6 decay analysis modules. It is not intended to be a textbook on either fluorescence spectroscopy in general or the analysis of fluorescence lifetimes as a scientific discipline.

First install DAS6 by following the instructions in section 2.

Users already familiar with the measurement and analysis of time-domain fluorescence lifetime data should then proceed to section 3. It is recommended that beginners read the appendix before proceeding.

The following references provide useful information on fluorescence spectroscopy in general:

"*Photophysics of Aromatic Molecules*", J B Birks, Wiley-Interscience, (1970).

"*Principles of Fluorescence Spectroscopy - 2nd Edition*", J R Lakowicz , Plenum, (1999).

The following references provide useful information on the measurement of fluorescence lifetimes:

"*Time-Domain Fluorescence Spectroscopy Using Time-Correlated Single-Photon Counting*", D J S Birch and R E. Imhof, in "*Topics in Fluorescence Spectroscopy – Vol. 1*", Ed. J R Lakowicz, Plenum, (1991).

"*The Measurement of Lifetimes in Atoms, Molecules and Ions*", R E Imhof and F H Read, Rep. Prog. Phys., 40, 1-104, (1977).

2. Installing DAS6

The distribution CD-ROM is affixed to the following page of this user guide.[1]

The distribution CD may contain a number of folders with various software products for your fluorescence lifetime instrument, as illustrated below. To install DAS6, please run the “SETUP.EXE” program from the DAS6 folder.

Always check for a “README.TXT” file. If present, this will contain important information which may have been added after production of this manual.

The Microsoft Windows Installer is the default installation tool for this software. The Installer is compatible with Windows XP (32 bit)/ Vista (32 bit)/ Windows 7 (32 / 64 bit).

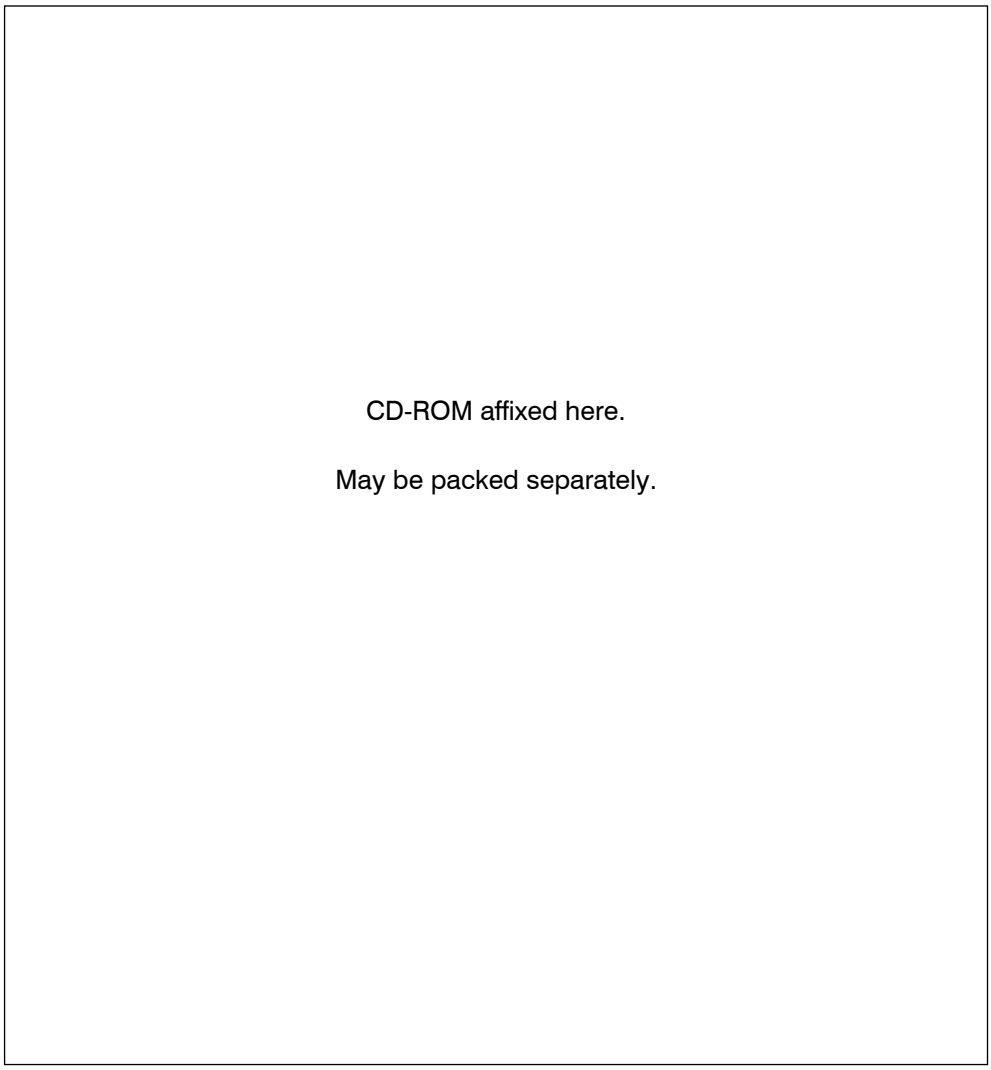
If the Microsoft .NET framework is not already present on the target PC then it will be installed first from the CD, before the application files.

When the DAS6 application files have been installed, a new file type will have been set up on the target PC along with a “.DAS” document association. The default menu items for the new software can be found on the Windows Start menu under

`Programs → HORIBA Scientific → DAS6`

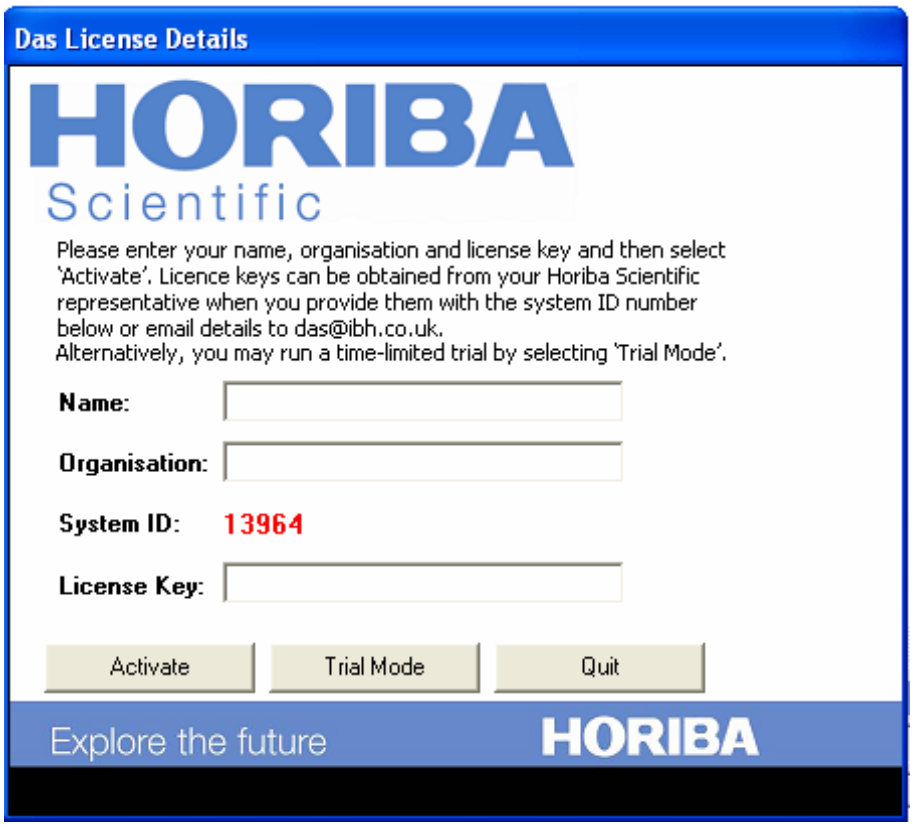
The DAS6 software (once installed) requires to be registered. Please see the next section for more details.

[1] Unless supplied with instrumentation, in which case see the CD-ROM supplied with the “FluoroHub User Guide” or FluorEssence .



2.1 Registering your software

The first time **DAS6** is started it will display a registration/demo screen similar to the one below:



The software requires a password. This password is based on your unique 5 digit **System ID Code (SIC),** shown in the registration window. To obtain your password please email the System ID Code to your local Horiba Scientific sales office or email das@ibh.co.uk. Your password will be sent by return email (usually within three working days). Please include your full contact information and details of how and when you obtained the software. In the meantime you may continue to use the software in Trial Mode for up to thirty days – see section 2.2.

IMPORTANT

This software will run only on the registered PC.

Please make sure that the installation computer is the computer that the software will actually be used on as only one full unlock password will be issued per license purchased and this password will not unlock the software on any other PC. The System Identification Code is unique to the PC that the software is running on. Please keep your unlock password in a safe place for use if the software needs to be reinstalled or updated.

Once you have received the unlock password it should be entered into the License Key box on the registration screen along with your Name and Organisation.

The "Activate" button should be clicked to activate the software. If the password is correct, the software will start. On subsequent executions a splash screen will be displayed and the registration window is not shown again. Please contact your local HORIBA Scientific sales office if you need more information.

2.2. Trial Mode

The trial version will run with all features enabled for 30 days. On subsequent executions the registration screen will show, but as long as there is time left in the evaluation period the "Trial Mode" button may be clicked.

After 30 days, the software will cease to work.

3. The DAS6 Interface

DAS6 software can be started in two ways:

- from the Windows Start menu
  `Start → Programs → HORIBA Scientific → DAS6 Analysis`
- double-clicking any ".DAS" file in a Windows Explorer window

New DAS files can be created by using our DataStation acquisition software (supplied with instruments from HORIBA Scientific), by importing data files from older v4.2 IBH files or other formats, or by using the DAS File Creation Utility (DFCU). The creation of DAS files is described in more detail in section 10. For use with this manual, a number of example .DAS files have been included in the examples folder.

While reading this section, it is recommended that you open file "Exp1.das" by double-clicking its icon. You will be presented with a display similar to that shown below, which has the main workspace areas indicated and which will be described below.

3.1 Workspace Areas

- The **Information Area** contains various details about the data in the currently loaded file; the look of this area and the information shown changes depending on the type of file loaded (i.e. lifetime, anisotropy, global or batch). Examples of information displayed are; peak counts and peak channels, counts within cursor ranges, time calibration and background values.

  The “comments” tab allows display of optional information which might have been saved by DataStation as part of the measurement.

  The drop-down list, at the bottom of the information area, allows selection of the fit module. The list shows only modules appropriate to the files type loaded.

- The **Data Tree** shows a graphical representation of the loaded data file.

- The **Data and Fit Graph** shows:
  - the instrumental response (referred to a “prompt”) – blue dots
  - the fluorescence response (referred to as “decay”) - red dots
  - the fitted function – green line

- The **Residuals Display** shows the deviation, in standard deviations, between the decay and the fitted function. This section of the screen can also show the autocorrelation function for a fit (see the description of the autocorrelation tool in section 3.3 below).

- The **Fit Results** Display shows the results for any stored fit selected from the Data Tree. The data shown here includes information about the data, selected fit algorithm, input parameters to the fit and the calculated results of the fit. This data can be opened in a resizable window by clicking on the "Open in Window" text at the top-right of the Fit Results Display.

- The **Range Panel** allows the cursor values (in channels) to be adjusted, the cursors can also be dragged using the mouse. Generally each measured or intermediate data item in the tree has a low-high cursor pair associated with it that allow ranges to be defined. Saved fits do not have cursors. At most two pairs of cursors are visible at any one time -- usually a prompt pair and a decay pair. If two pairs are visible they can be "tied" so that their values are equal - in this case only the decay pair can be moved and the prompt pair will follow automatically. Cursor ranges should always be set before performing a fit calculation.

3.2 Menus and toolbars

The standard toolbar at the top of the application window is shown here. There are minor changes when "Batch" and "Global" modules are used and these are described in the corresponding module description sections.

Most of these toolbar buttons are also contained within the menus and a description of the functionality of each button is described within the menu description below.

There are only four menus: "File", "Edit" "Tools" and "View" and these closely adhere to standard windows operation. Note that keyboard shortcuts are available for many menu items and these are indicated at the right of the menus items.

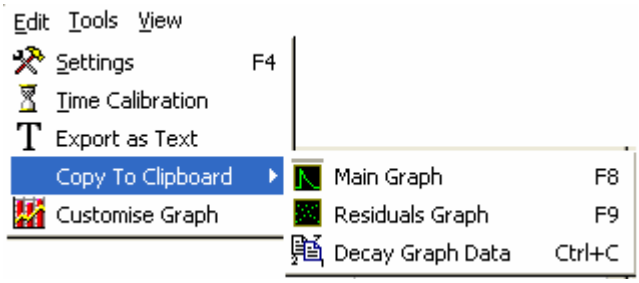
The **"File"** menu is shown below.

- "New" Creates a new DAS file – this is dealt with in more detail in section 9.
- "Open Open an existing DAS file.
- "Save As" allows you to save a copy of the file.
- "Print Setup" opens the standard windows component for printer selection and set-up. Note that the printer selected becomes the system's default printer.

- "Print" will show the Print Setup dialog box to allow selection of printing preferences before printing begins. The toolbar button will print to the default printer without showing the Print Setup dialog box.

- "Exit" closes DAS6.

The **"Edit"** menu is shown below.



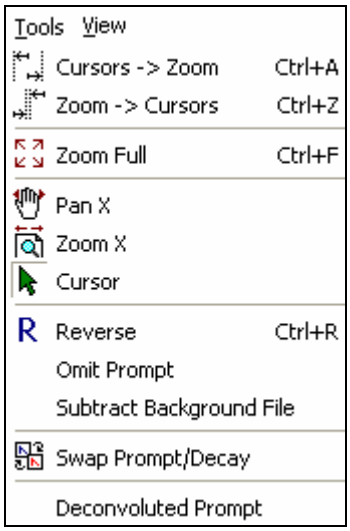
- "Settings" Set the general defaults for folders and files.

- "Time Calibration" allows a time calibration value (i.e. nanoseconds/channel) to be entered or edited. DAS files created by DataStation will most likely already contain a time calibration value. However, files created using the "New" function might not have a value set.

- "Copy to Clipboard" copies information to the clipboard so that it can be pasted into other applications such as word processors and spreadsheets. It is possible to copy the main decay graph and the residuals graph or copy the data for the decay graph .

- "Export as Text" allows information to be output as a text file. The information written to the file depends on what is being viewed in the main "Data and Fit Graph" window. If a fit is being viewed, the fit results and data are written.

The **"Tools"** menu is shown below.



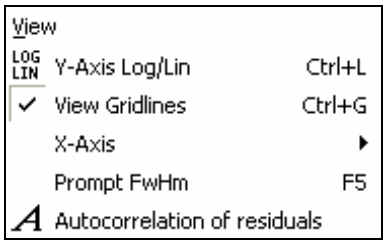
- "Cursors -> Zoom" set the cursors to match the current zoom range.
- "Zoom -> Cursors" set the zoom range to match the current cursors.
- "Zoom Full" Zoom the main graph to its full extent (depends on the data range of the loaded file).
- "Pan X" Pan on the X axis of the main graph (click, hold and drag the mouse to pan).
- "Zoom X" Zoom on the X axis of the main graph (click, hold and drag the mouse to select a range).
- "Cursor" Switch to cursor mode (you can also double-click anywhere on the graph when in zoom or pan mode to switch to cursor mode).
- R "Reverse" Reverse the measured data. This is useful when the data have been measured in "reverse mode" and the time axis therefore needs to be reversed before the data can be analysed. This function can only be used if there are no stored results (i.e. no fits have yet been performed).
- "Omit Prompt" is used when a tail fit is required in the exponential fits instead of a reconvolution fit. Normally, if the DAS file contains a prompt and a decay, the exponential fit module would expect to perform a reconvolution fit. This selection forces the module to ignore the prompt and perform a tail fit.
- "Subtract Background File" allows data in a second DAS file to be subtracted from the current data and the result to be saved as a new DAS file. This is useful when a "blank" is run for comparison against a sample measurement. However, this function should be used with caution as it does not take into consideration the data weights so the integrity of the statistical information in any

subsequent analysis is lost. The DAS files should have the same structure (i.e. same number of data points in decays and prompts, if present).

- "Swap Prompt/Decay" allows reversal of the prompt and decay data. Sometimes, in error, the prompt and decay are measured in the wrong order. This selection allows the error to be corrected. It can only be used if there are no stored results (i.e. no fits have yet been performed or all stored fits have been deleted).

- "Deconvolute Prompt" allows a second exponential decay to be used as a prompt. The function enables a working prompt for decay analysis to be calculated from the exponential decay measurement using fast Fourier transforms. This function is described in more detail in the chapter "When a Prompt Cannot be Measured"

The "**View**" menu is shown below.



- LOG LIN "Y-Axis Log/Lin" Toggle the main graph Y-axis between logarithmic and linear scales.

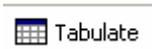
- "View Gridlines" is a checkbox which enables/disables the display of gridlines in the "Data and Fit Graph" area.

- "X-Axis" activates a pull-down menu which controls the information displayed on the X-Axis. The default is to display "channel number", but the axis can also be displayed in time units.

- "Prompt FwHm" activates a pop-up box which displays the width (FWHM) of the prompt.

- *A* "Autocorrelation of residuals" Toggle the display in the residuals box between residual and autocorrelation information. The default is to display residuals (in green) and autocorrelation (in blue). The display reverts to residuals each time a new "Stored Result" is selected.

Other toolbar buttons



Tabulate fitted parameters or lifetimes

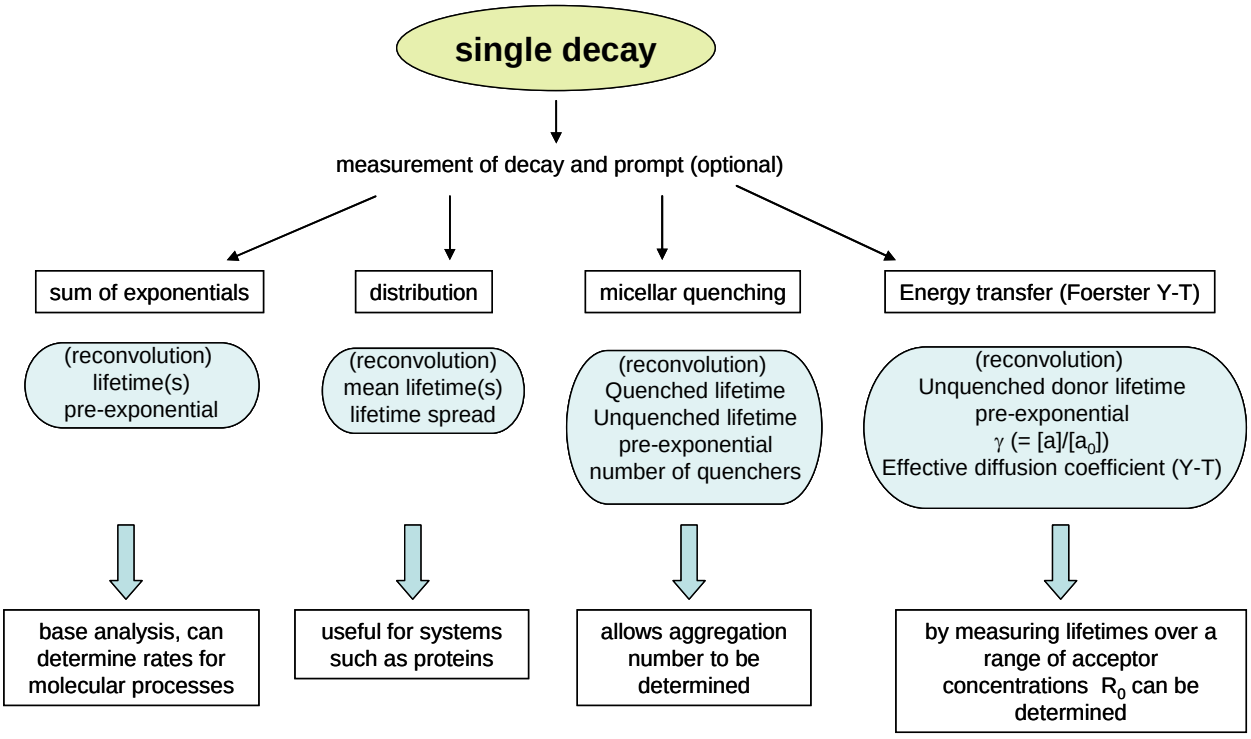
Application information - show the application "about" box, which contains product version, copyright information and allows license to be updated.

This is not a tool but a display area. It shows the size of the time window being viewed.

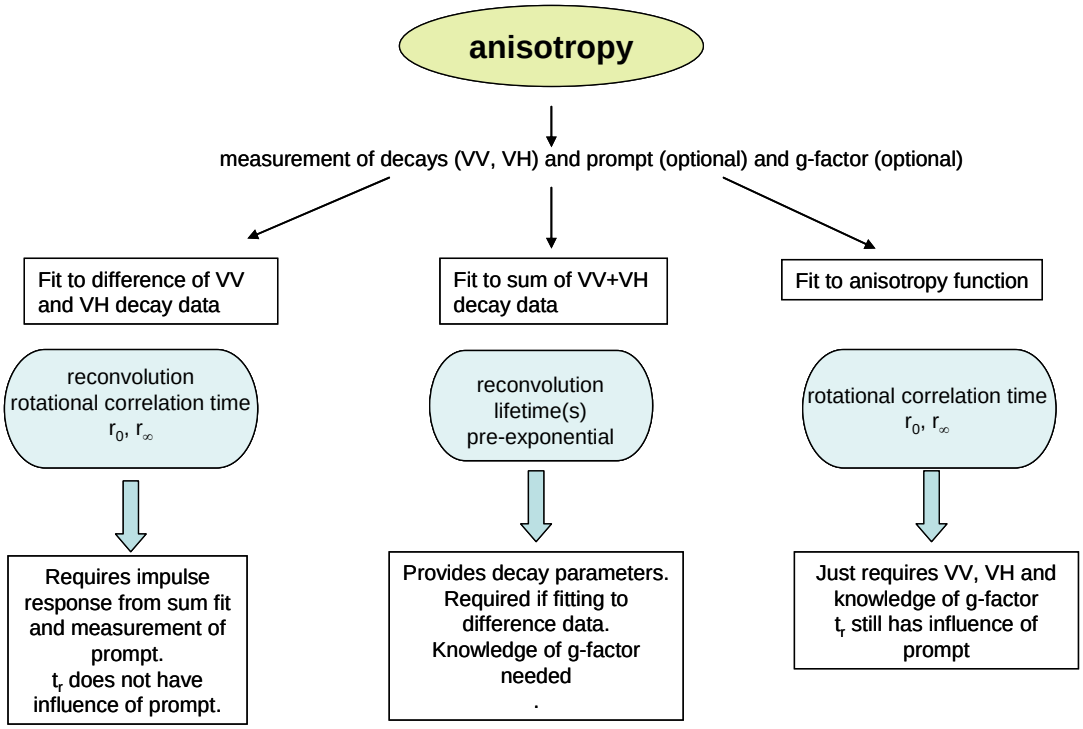
4. Measurement types and performing a Fit

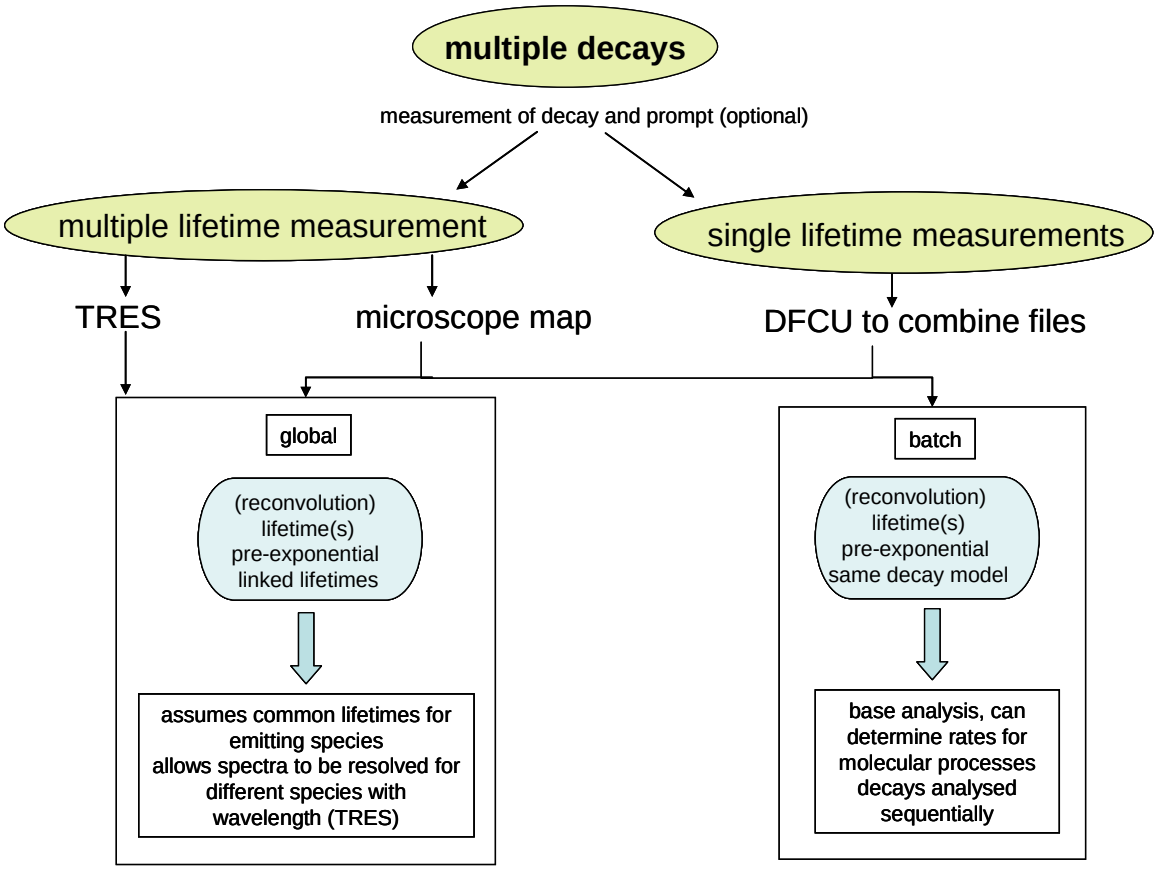
4.1 Measurement Types

The data analysis method is largely determined by the design of the experiment and influenced by the molecular processes occurring in the sample. The following three diagrams below give a rough guide to the form of analysis open for different measurement techniques, as well as a brief description of the data input required for the fit procedures.



V – vertical polariser
H –horizontal polariser




multiple decays
measurement of decay and prompt (optional)
multiple lifetime measurement
single lifetime measurements
TRES
microscope map
DFCU to combine files
global
(reconvolution)
lifetime(s)
pre-exponential
linked lifetimes
assumes common lifetimes for emitting species
allows spectra to be resolved for different species with wavelength (TRES)
batch
(reconvolution)
lifetime(s)
pre-exponential
same decay model
base analysis, can determine rates for molecular processes
decays analysed sequentially

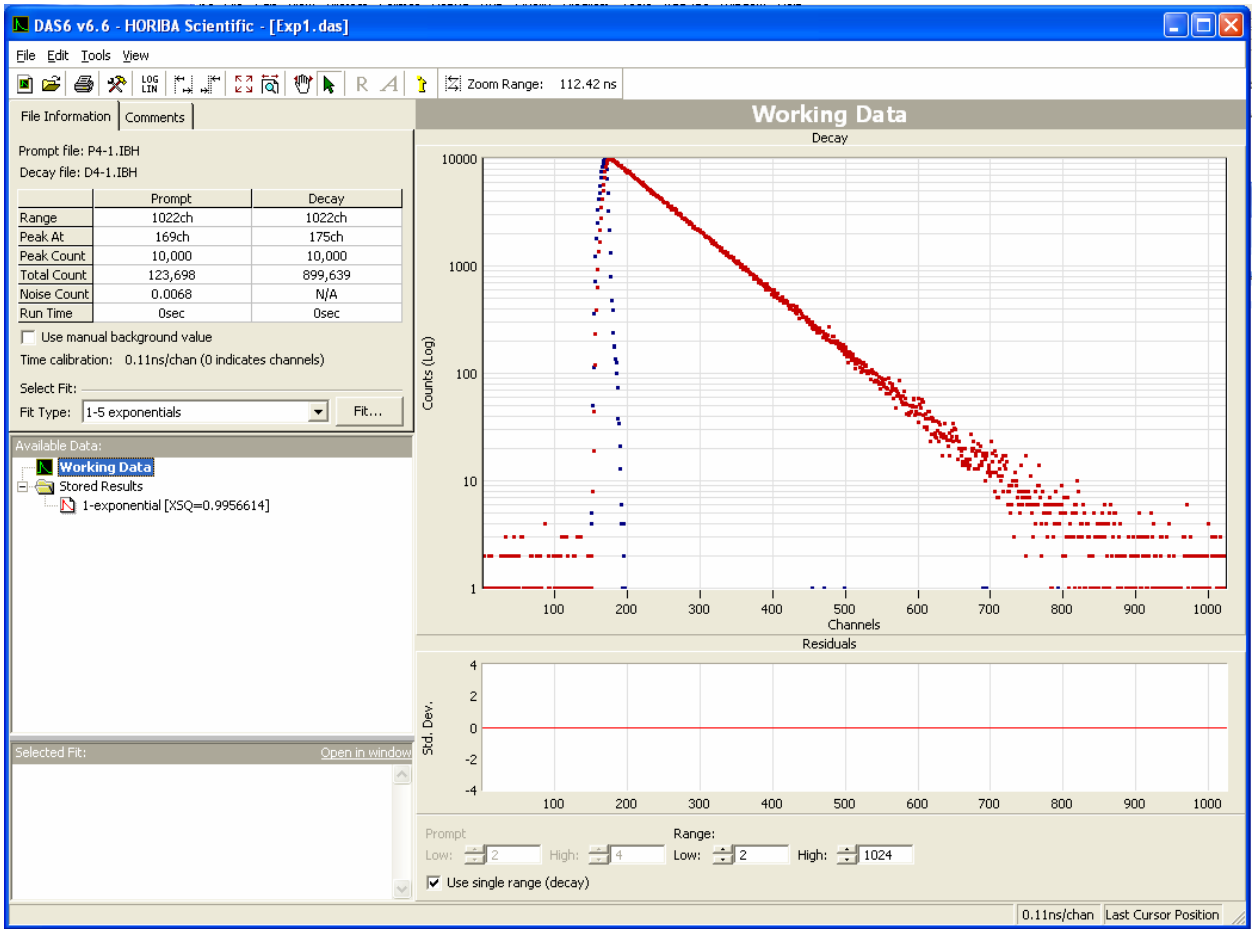
4.2 Performing a Basic Fit

This section is intended as a quick start and covers the most commonly used analysis: exponential decays with and without the presence of scattered light.

4.2.1 Example with a single exponential decay component

This example will use the “Exp1” DAS file, which can be found in the examples folder. The example demonstrates the analysis of a single exponential lifetime.

1. Click the "Open DAS File" tool and select the “Exp1” DAS file. The prompt and decay files will be loaded along with an example fit. The display should be similar to the screenshot below.

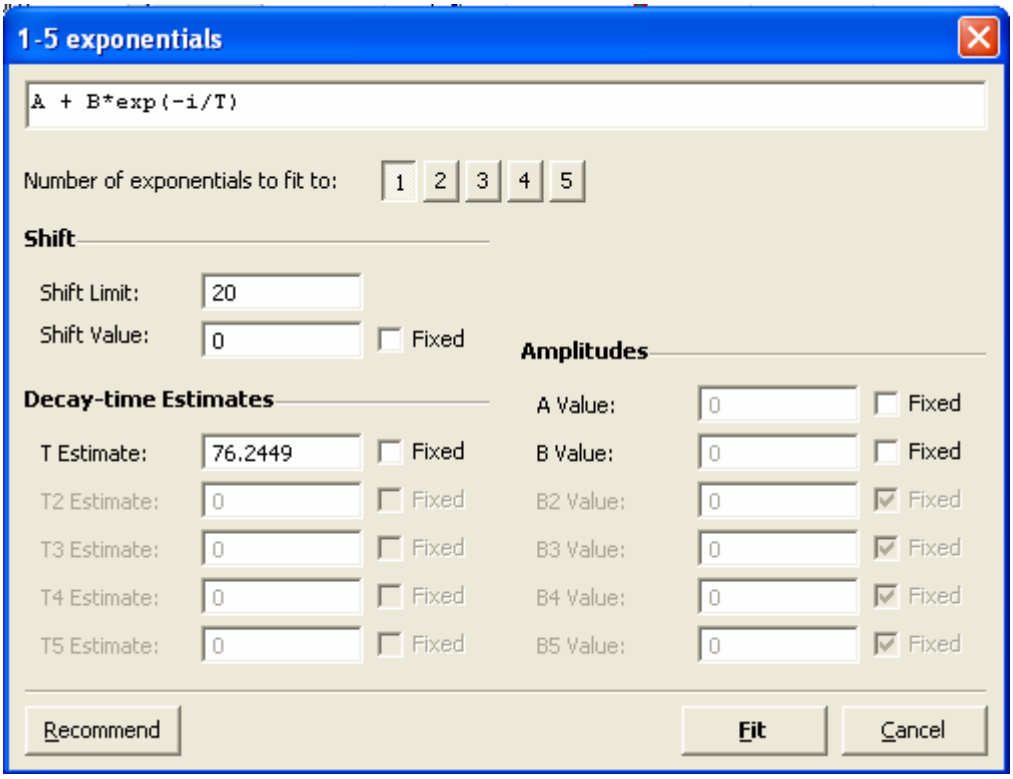


2. Using the zoom tool select the area of interest (i.e. from just before the rising edge to where the decay merges with the background level). Make sure that the range of the cursors includes a sensible amount of data to analyse. You must now set the cursors to the zoomed range by

using the “cursor to zoom” tool . You can see the channel information for the zoomed range in the “Range Panel” below the graphs and also in the “Information Area” to the left of the main graph. If you make a mistake, zoom out using the tool and start again.

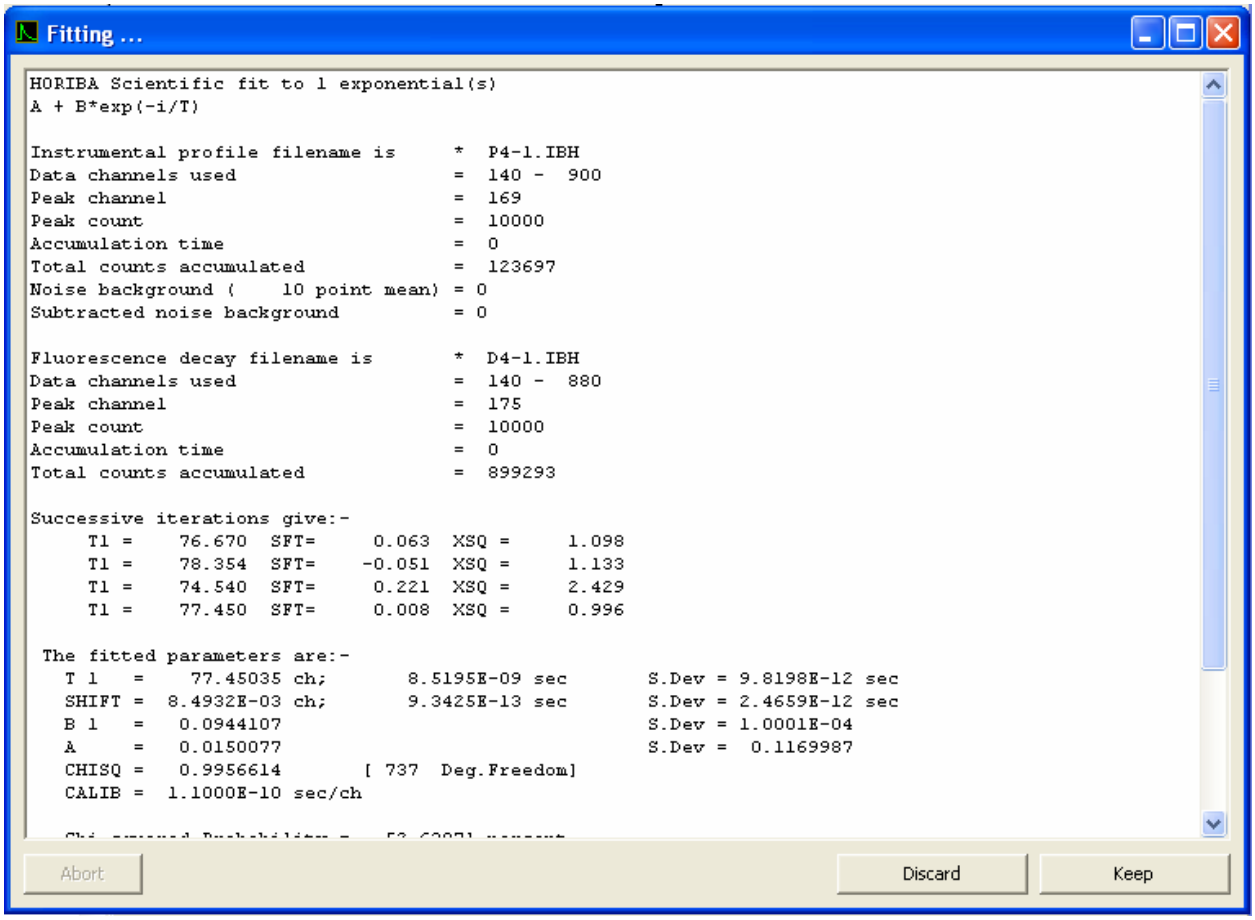
The example fit uses “low” and “high” values of 140 and 900 respectively. These values can be typed directly into the “Range Panel”, if preferred.

3. Select the fit type “1-5 exponentials” from the drop-down list at the bottom of the “Information Area” and then click the “Fit” button to the right of the list. A form for entering the fit parameters will appear similar to the screenshot below.



4. At the top of the screen select 1 exponential to fit. A starting guess for the lifetime is automatically recommended, however your own guess may be entered. Note that times are in channels. (Time calibration of given at bottom right of main form) If you make changes you can revert back to default values, by selecting the Recommend button.

5. Click on the "Fit" button to start the fit. A new window will appear that shows the progress of the fit calculation and the intermediate results. A screenshot is shown below. To view more information, the window can be resized or the scroll bars can be used.



6. When the fit has completed, the final results are shown and the graphs updated to show the fitted function curve and the residuals. At this point you can choose to discard the results or to keep them. If you keep the results you will be asked for a name for them and they will then be stored in the “Data Tree” as “Stored Results” and can be recalled at any time.

This is a very simple example and you should experiment as much as possible to get comfortable with using your new analysis software. The correct lifetime value is 8.5 nanoseconds.

The DAS file contains all the information for prompt, decay and fits and is automatically saved each time a new stored fit is added, when a different file is opened and before the application exits. There is no need to explicitly save the file.

Before continuing, select your fit (by clicking on it in the “Stored Fits” tree) and then click the “Open In Window” tag at the top right of the “Fit Results Display”. A new window containing the fit results will open. Close the window after viewing.

This would be a good time to experiment with the “Print”, “Export as Text” and “Copy to Clipboard” functions.

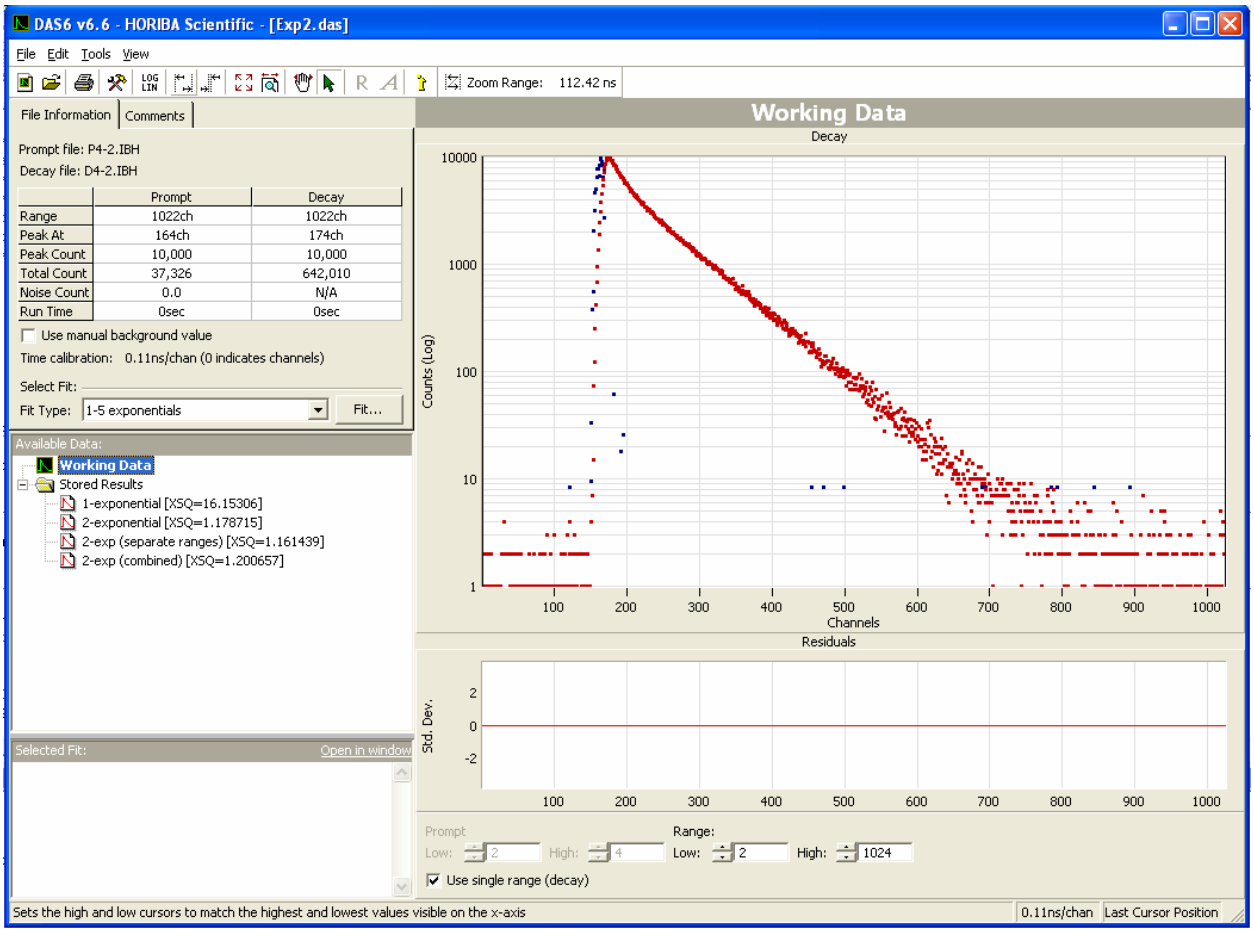
4.2.2 Example with 2 exponential decay components

This example will use the “Exp2” DAS file, which can be found in the examples folder. The data consists of two exponential decays each starting with the same intensity (i.e. B value). The decay as a function of time is given by:

$$F(t) = A + B_1 \exp\left(-t/T_1\right) + B_2 \exp\left(-t/T_2\right)$$

In this example, the lifetime values T_1 and T_2 are 2.0 ns and 8.5 ns.

When the DAS file is opened, you should see a screen similar to the one shown below. It contains the data and five fit examples. The fit examples will be discussed below.



1-exponential

We could run a single exponential fit in the same manner as described in section 4.2. However, for experience we will use a different method for this example. Start by entering values of 140 and 800 in the

"Range Low" and "Range High" boxes then click on the main window. You will see that the high and low cursors have moved to the specified positions. Next, click "Fit" to get the parameters window and run the fit by clicking "Recommend" then "Fit". You have just reproduced the first fit in the "Stored Results" tree and can either "Keep" or "Discard" it.

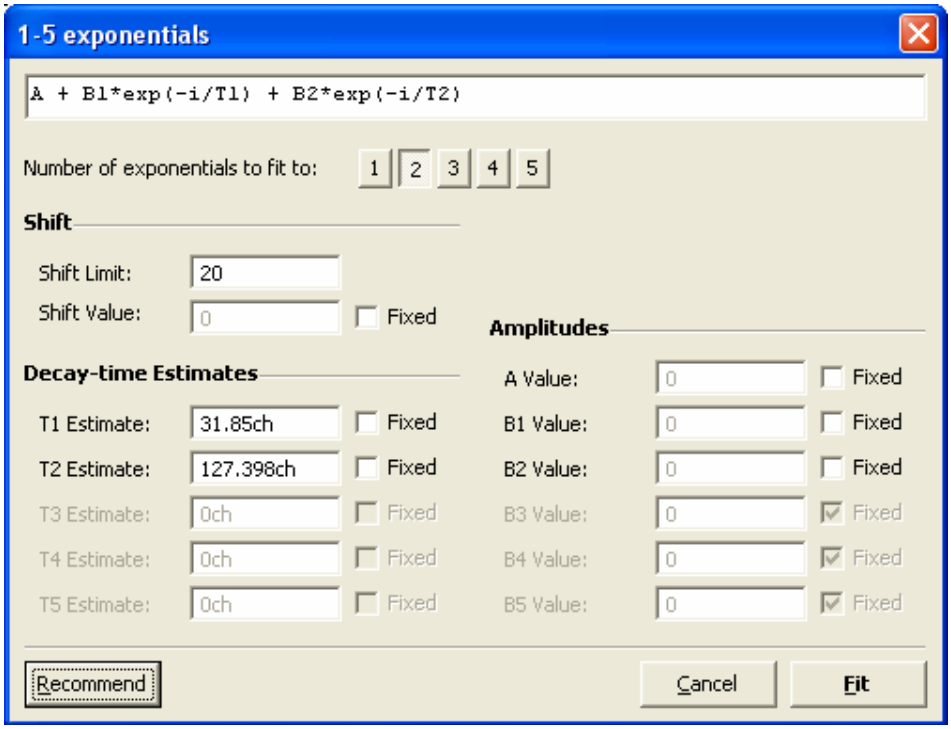
Now highlight the "1-exponential" fit in the "Stored Results" tree. The main plot, residuals plot and χ^2 (XSQ) value all indicate a poor fit:

- Main plot – fitted function clearly differs in places from measured data.
- Residuals plot – for a good fit, these should be randomly distributed around zero and within the range of a few standard deviations.
- χ^2 value – for a good fit this should be near to 1.0 (<1.2 is usually ok provided the residuals are random).

Click "Open in Window" to view the results - The lifetime value of 7.2 ns has little credibility in this case.

2-exponential

We will now repeat the process and fit to a two exponential decay model. Click on "Working Data" and then check that the range is still set at channels 140 to 800. Then click on "Fit", select "2" as the number of exponentials and click "Recommend" to get starting guesses. The parameters window should look similar to the one below.



Click “Fit” to complete the process. You have just reproduced the second fit and can either “Keep” or “Discard”.

Now highlight the “2-exponential” fit” in the “Stored Results” tree. The main plot, residuals plot and χ^2 (XSQ) value all indicate a good fit.

- Main plot – fitted function closely follows the measured data.
- Residuals plot – the residuals appear to be randomly distributed around zero and all are within the range of a few standard deviations.
- χ^2 value – the value is near to 1.0 (i.e. <1.2).

Click “Open In Window” to view the results - The lifetime values of 1.92 ns and 8.46.ns are in good agreement with those expected. Also, we were expecting equal B values and you will see that these are in reasonable agreement.

This is a good point at which to explain the [Rel.Ampl.] (relative amplitude) figures displayed alongside the B1 and B2 values. If we have a 1 ns and a 2 ns lifetime starting at the same intensity, we will see more photons from the 2 ns decay because it emits for longer. The actual number of photons is proportional to “B” multiplied by “T”. The relative amplitudes are therefore the percentage of photons coming from the different decays, $B_iT_i / \sum B_iT_i$

It will be evident that, if the decays are from different chromophores, this is the ratio of the photons coming from each.

In the present example we expect to see 19% of photons from the 2 ns decay and 81% from the 8.5 ns decay, which is in close agreement with the fit.

2-exp (separate ranges)

Although it is very quick and easy to perform the above fits, they assume that there is meaningful data throughout the same ranges of the prompt and decay. However, in this example, the “Prompt” has nothing but noise in channels above 200 and these are best discarded.

Click on “Working Data” then un-check the “Use single range” box in the “Range Panel”. You can now enter separate range values for prompt and decay. Enter values of 150 to 200 for the prompt, and 140 to 800 for the decay.

If you now run a two exponential fit, you should obtain the results for the fourth example.

Again, the values are all good.

2-exp (combined)

In this example, separate ranges are used for the prompt and decay. Also, the A value is fixed. Again, the values are all good.

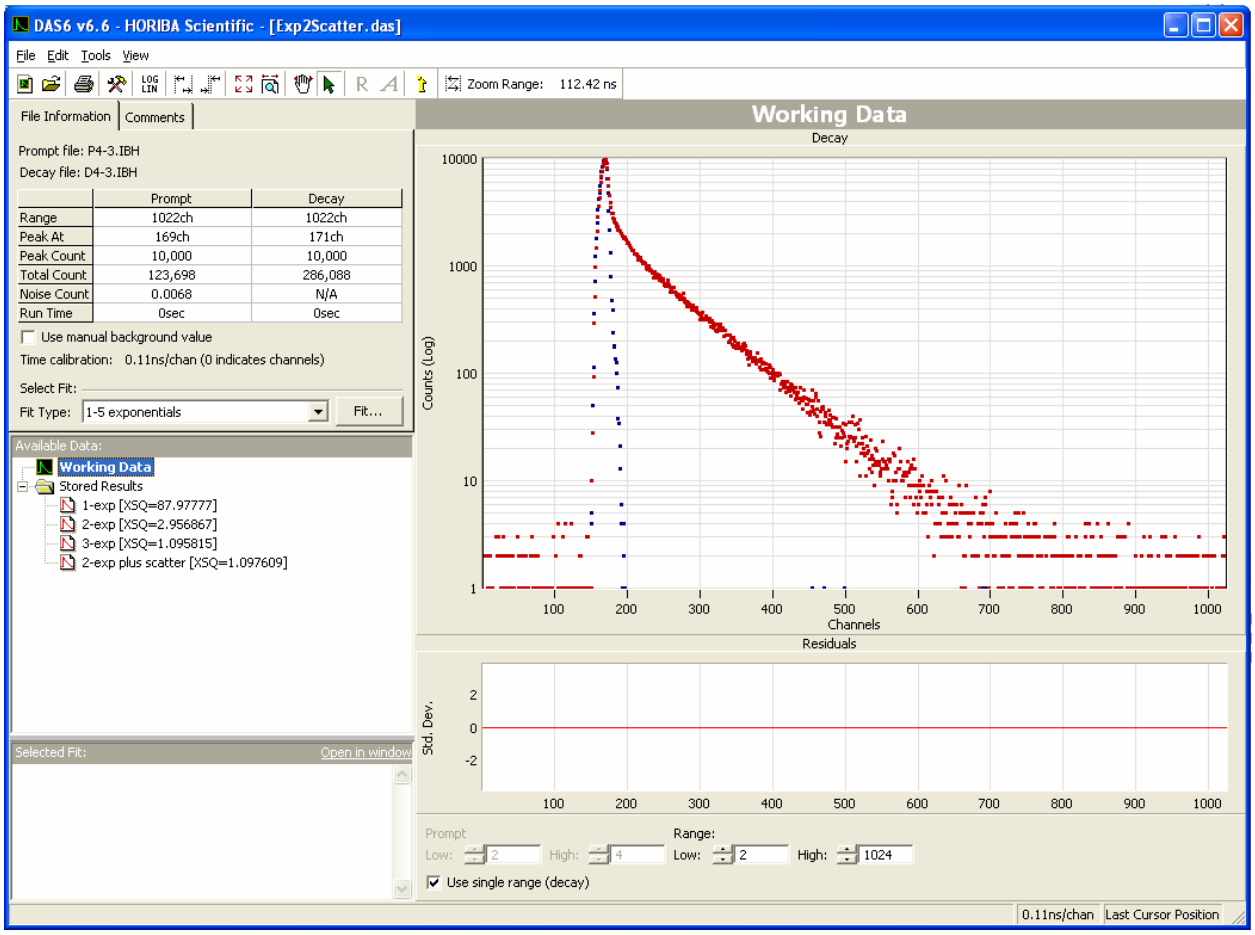
You will see from the five fits in this section that there are some subtle effects associated with fixing vales and selecting data ranges. This is something that you should experiment with. Scientifically, it is always best to separate the ranges and to fix whatever values can sensibly be fixed. However, a great deal of valid, preliminary investigation can be performed quickly and reliably by letting the default settings do the work and hence avoiding the extra effort involved.

4.2.3 Example with scattered light

This example will use the “Exp2Scatter” DAS file, which can be found in the examples folder. The data consists of two exponential decays each starting with the same intensity (i.e. B value) but in this example there is an additional, significant scattered light component. As in section 4.2, the lifetime values are 2.0 ns and 8.5 ns.

After working through the examples in sections 4.1 and 4.2, you should now be familiar with running simple exponential fits. This section therefore simply describes the fits included in the file. You can repeat the analyses for yourself using the starting conditions that you will find in the in the “Stored Results”.

When the DAS file is opened, you should see a screen similar to the one shown below. It contains the data and four fit examples. The examples will be discussed below.



1-exp

This is simply the fit to a single exponential decay model. Looking at the residuals, this is clearly not the correct decay model. This fit has been included for comparison with the ones below it.

2-exp

Again, this is clearly not the correct decay model. However, looking at the residuals, it is a much closer fit than the single exponential decay model. In fact, the residuals are a very good guide as to whether there is more information available or not.

Looking at the residuals, you can see that there is more information present because they are not randomly distributed around zero.

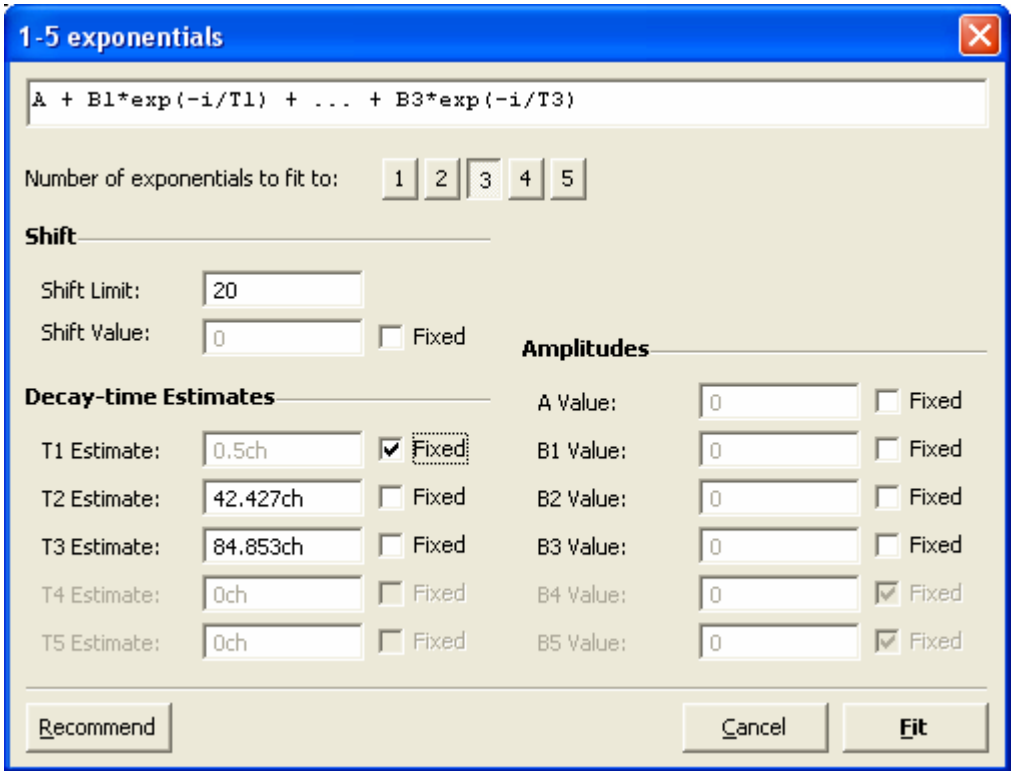
3-exp

Here is where the situation gets interesting. You can think of scattered light as a delta function or as a very fast decay component. If you examine the “Stored Results” for the “3-exp” fit, you will see that this decay model has found lifetimes of 1.9 ns, 8.4 ns and 13.1 ps. The last value is, of course, due to scattered light.

Although the fit to a three exponential model was successful in this case, it is not always successful and sometimes the fit fails as the lifetime value gets shorter and shorter. A safer way to deal with scattered light is to deliberately fix one of the lifetime components at a fast value. It is usually recommended to fix T_1 at a value of 0.5 channels.

2-exp plus scatter

The final fit in the set shows the situation when T_1 is deliberately fixed at 0.5 channels. In this case the parameter input screen is similar to the one shown below.



Examining the lifetime values and B values found by the fit, you will see that it has found reasonable values for both lifetimes and intensities.

4.3 Interpreting the results of the analysis

The meaning of the parameters recovered from the DAS6 analysis will depend on the sample application. Generally speaking DAS6 will return the following parameters (although it is strongly advised to see the specific section related to the required decay model for other recovered parameters)

A value = background offset

B value = pre-exponential function which relates to how much of an emitting species there is.

T value = lifetime

The magnitude of the **B values** returned in the fit are dependent on whether or not reconvolution is used in fitting the data. Without reconvolution the values are in counts and can easily be linked to the peak number of counts (ie for a single exponential its magnitude should be ~ the count value in the start channel). The value after reconvolution is not in counts, but still reflects the amount of a particular emitting species.

There are two main ways in which the B values are represented

i. As a relative amplitude (Rel. Ampl.), in which the pre-exponential is weighted by the lifetime, this is useful when comparing with steady state spectra

ii. By normalising (B) values, this is useful when performing in depth analysis on multi-component systems.

If we consider the decay to be represented as a sum of exponential components

$$I(t) = \sum_{i}^{n} B_i \exp(-t/T_i)$$

Then the relative amplitude (fractional) is

$$f = \frac{B_i T_i}{\sum_i B_i T}$$

while the normalised pre-exponential value is

$$B_i = \frac{B_i}{\sum_{i=1}^{n} B_i}$$

The former weights the “amount” of an emitting species by the lifetime and allows a more direct comparison with steady state data, while the latter provides information concerning the relative concentrations of emitting species. DAS6 gives both of these values, as well as the “raw” B value.

It should be noted that the T values (lifetimes) can also be interpreted as rates (k =1/T). Sometimes when comparing results it is helpful to make use of an average lifetime. This can be calculated in two main ways, depending on the application of the result. These can be found in the paper by A. Sillen and Y. Engelborghs. Photochem. *67*, 475-486 (1998). To summarise, for most applications the average lifetime can be calculated as the simple sum of normalised pre-exponential multiplied by the lifetime.

$$\tau_{ave} = \sum_{i=1}^{n} B_i T_i$$

This is the output of the average lifetime given by DAS6. Alternatively (for example for use in Stern-Volmer analysis) it can be obtained from

$$\tau_{ave} = \frac{\sum_{i=1}^{n} B_i T_i^2}{\sum_{i=1}^{n} B_i T_i}$$

DAS6 provides **errors** for these recovered values, as indicated by the standard deviation. In practice it is normal to use three standard deviations for the error on a given value.

In order to tell if the fitting model gives a good fit to the measured data the reduced chi-squared (sometimes written as $\chi 2$ or XSQ) is used. This is probably best employed in conjunction with the weighted residuals to evaluate the goodness of fit.

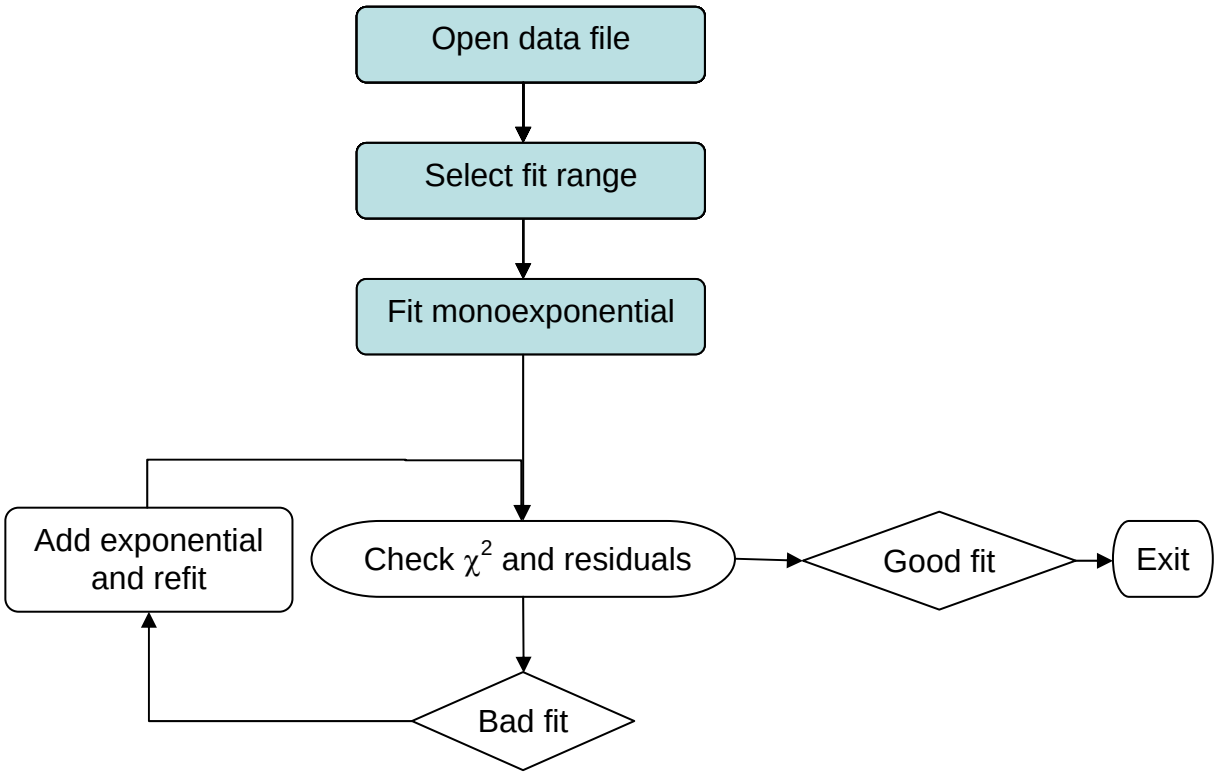
4.3.1 How do I know if I have a good data fit?

The usual criteria to assess whether a fit is satisfactory is to use the reduced chi-squared value and to see if there are any trends in the weighed residuals. Also the fitting of an additional exponential decay component should not produce any significant improvement. The returned values should also be sensible. Generally, when using reconvolution, the shortest measurable lifetime that can be returned is approximately 10 times less than the instrumental full width at half maximum. The following should be considered in assessing the fit data, along with any prior knowledge of the system studied.

- Chi-squared value below 1.2, not meaningfully improved by adding an extra decay component.
- Randomly distributed weighted residuals are expected for a good fit.
- Do the lifetime(s) correspond to previous reports (measurements or literature)?
- Lifetime values of 10^{-11} seconds (0.01 ns) or less than one data channel should be treated with caution and may relate to scattered excitation light reaching the detector.
- Large (e.g. more than ~3 channels) or negative shifts may indicate a problem depending on the detector and wavelengths used.
- Excessive errors (s. dev) on the data.

- Negative pre-exponential components (B values) are not necessarily a sign of a bad fit, they can indicate the presence of an excited state process and are referred to as “rise times”.

General process for lifetime analysis:



A good fit is determined by examining the χ^2 and residuals and using any prior knowledge of the system. Check the size of the residuals and whether they are random, check if adding another exponential reduces the value of χ^2.

5. Decay Analysis Modules

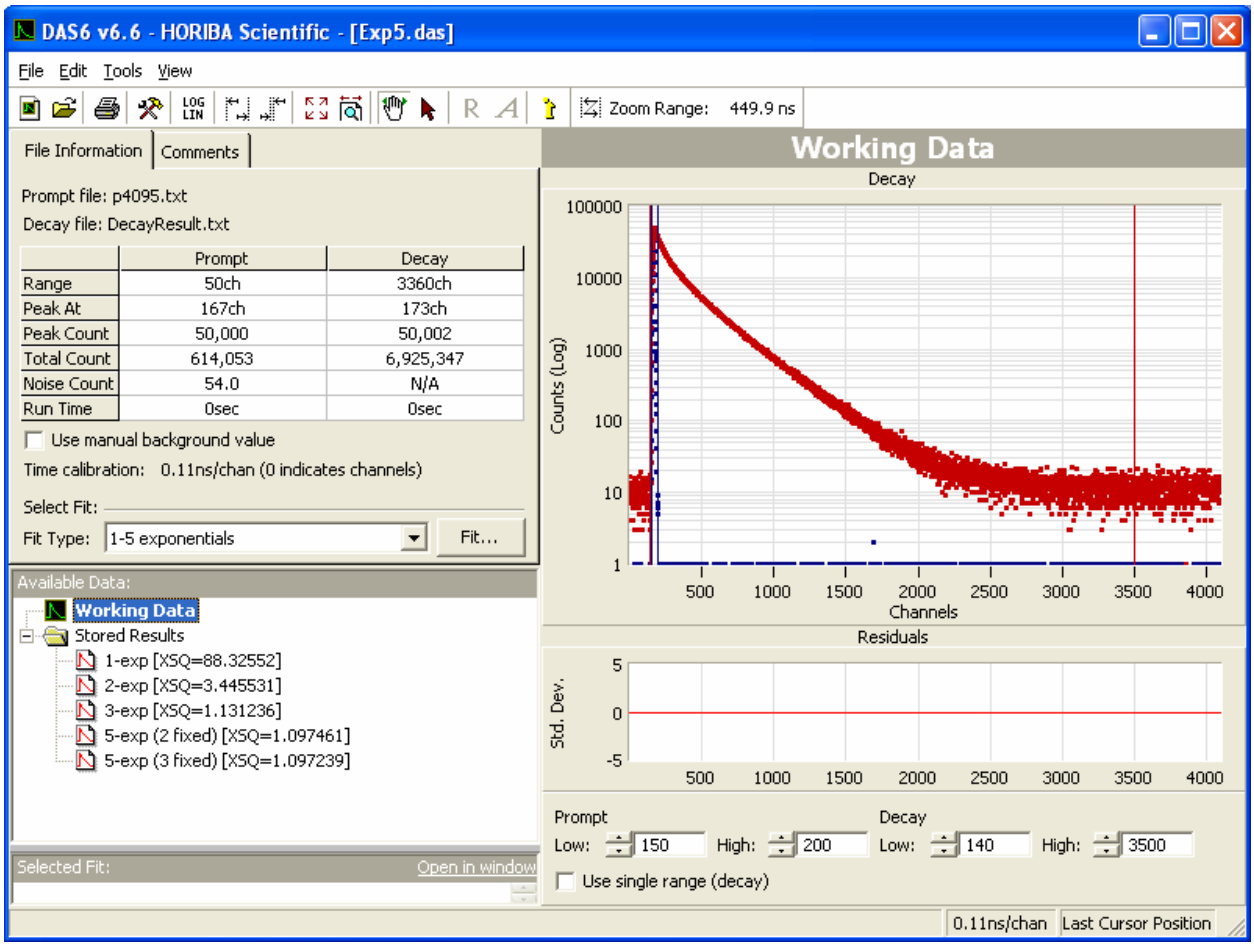
Each of the DAS6 modules provides functionality for a different fitting function (or family of functions).

5.1 Exponential Fits

Exponential fits have already been used as a training example in section 4. This section deals with an example containing five exponential decay components. The decay model is given by:

$$F(t) = A + B_1 \exp\left(-t/T_1\right) + B_2 \exp\left(-t/T_2\right) + B_3 \exp\left(-t/T_3\right) + B_4 \exp\left(-t/T_4\right) + B_5 \exp\left(-t/T_5\right)$$

The example file is “Exp5”, and the opening screen is shown below.



The data in this file contains five exponential decay components with equal B vales. The lifetimes are 2.0, 4.0, 8.0, 16.0 and 32.0 nanoseconds respectively. It should be noted that the data is collected to 50,000 counts in the peak, which is significantly higher than the 10,000 counts used in previous examples. Also note that the data are collected into 4095 channels (time window of 450 ns) compared to the previous examples which were collected into 1024 channels (time window of 112 nanoseconds).

In practice this means that it will have taken 20 times longer to perform the measurement. The reason that this extra information is needed will become evident from the data analysed below. However, it is intuitively obvious that more complicated models require more data (i.e. more photons/counts) than simple models.

The example file contains five fits. In all five cases, separate prompt and decay ranges were used. For the prompt the range used was 150 – 200 channels and for the decay the range was 140 – 3500 channels.

1-exp

Clearly, a one exponential decay model is incorrect.

2-exp

Clearly, a two exponential decay model is incorrect and the residuals show that more information is available.

3-exp

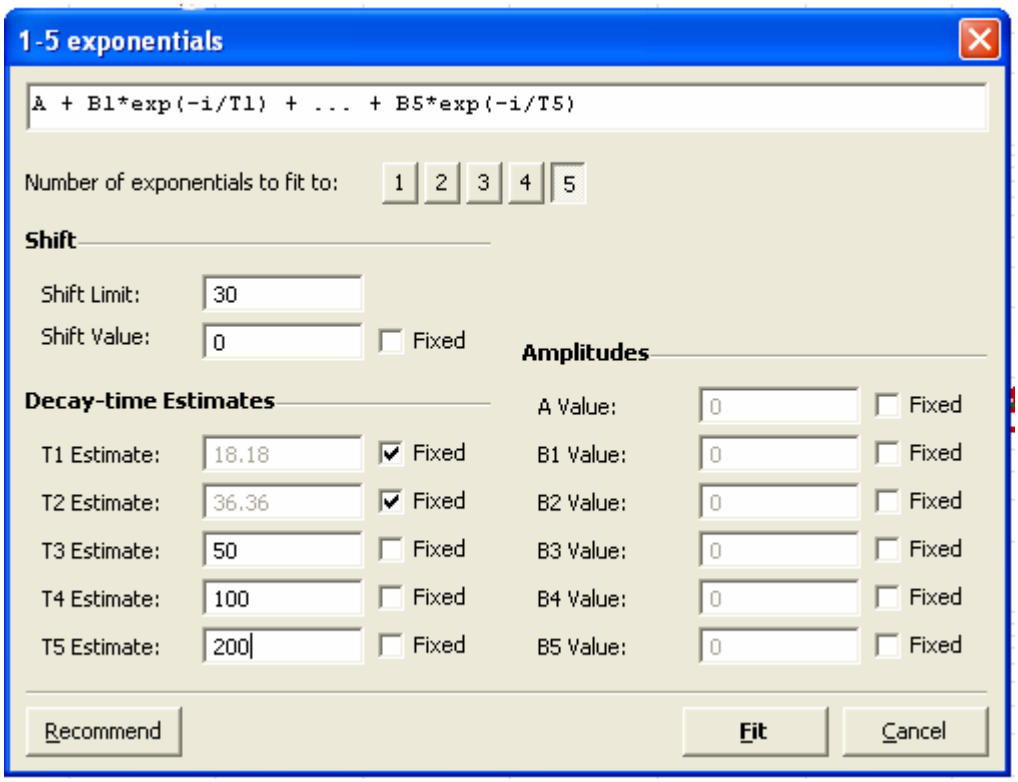
This is really quite a good fit and, looking at the residuals, there is little evidence for trying anything more complicated. However, we know that this data contains five decay components. So, however good the fit, the lifetime values are meaningless. This illustrates two very important points:

- Just because the model gives a good fit, it does not prove it is the correct model. There has to be an independent reason to believe the model is appropriate. This generally comes from careful planning of a series of measurements to test a hypothesis. Don't be tempted to measure today and interpret tomorrow!
- With three exponentials, you can fit just about any TCSPC data!

It is tempting, from the above, to conclude that more complicated models cannot be studied by this technique. Fortunately that is not the case. The fit above allowed all values to be varied. However, if there is prior knowledge of some of the decay components either from a different measurement or even a different technique, then some lifetime values can be fixed.

5-exp (2 fixed)

This fit example illustrates the point made above. Two of the values are “fixed” and reasonable starting guesses have been made for the other three lifetimes. The parameter input screen for this is shown below.



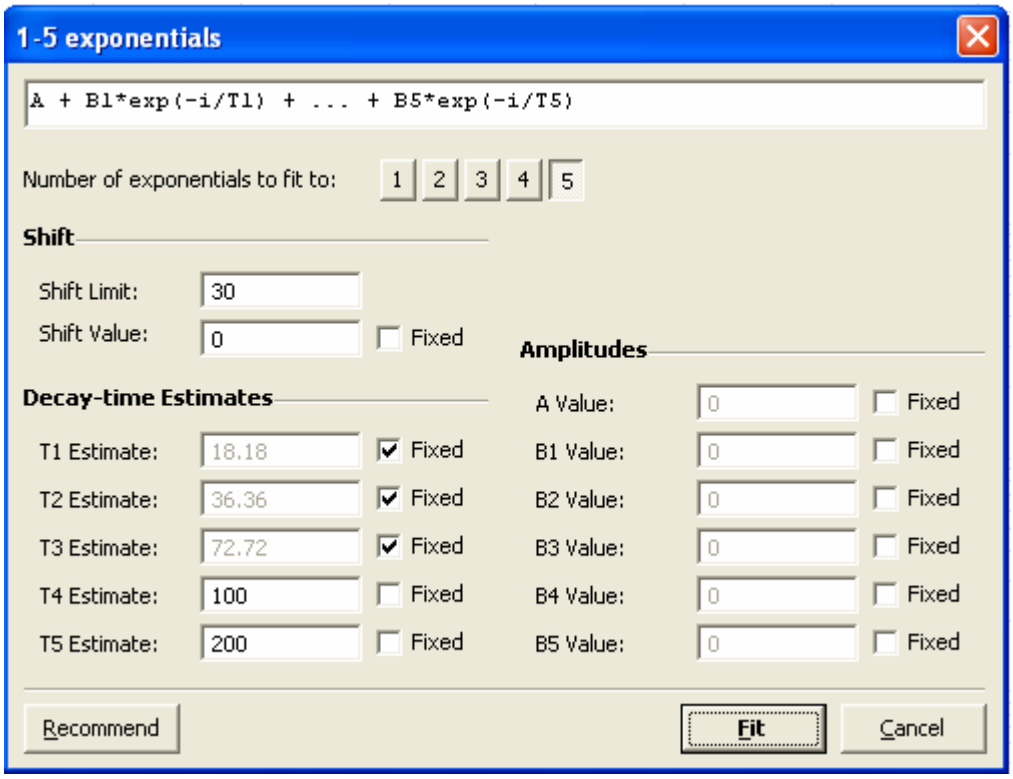
You can see that T1 and T2 have all been “Fixed”. If you check the fit results you will see that the fit has found the following values:

T1 = 2.0 ns (fixed)
T2 = 4.0 ns (fixed)
T3 = 8.6 ns (expected value of 8.0 ns)
T4 = 17.1 ns (expected value of 16.0 ns)
T5 = 32.1 ns (expected value of 32.0 ns)

The agreement, which is reasonable, is an indication that fixing parameters can yield some useful results.

5-exp (3 fixed)

This example takes the fixing of lifetime values on step further. In this case it is assumed that three of the five lifetimes are known. The parameter input screen is shown below.



You can see that T1, T2 and T3 have all been “Fixed” and reasonable estimates have been made for the unknown lifetime values. If you check the fit results you will see that the fit has found the following values:

T1 = 2.0 ns (fixed)
T2 = 4.0 ns (fixed)
T3 = 8.0 ns (fixed)
T4 = 16.3 ns (expected value of 16.0 ns)
T5 = 32.0 ns (expected value of 32.0 ns)

As expected, the agreement is better than in the previous example.

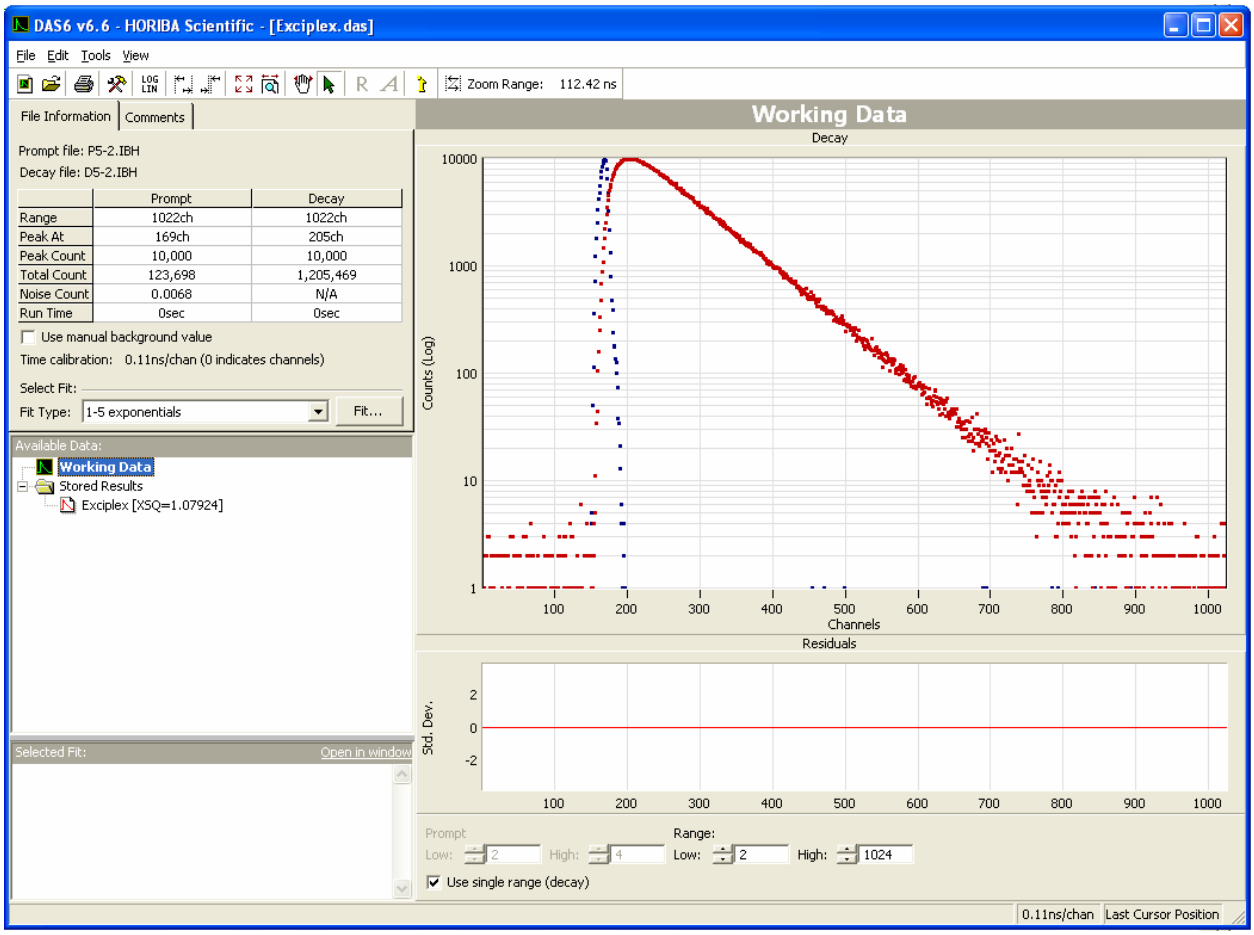
It is recommended that you experiment with this dataset to gain experience.

5.2 Exciplex

The exciplex decay model is given by:

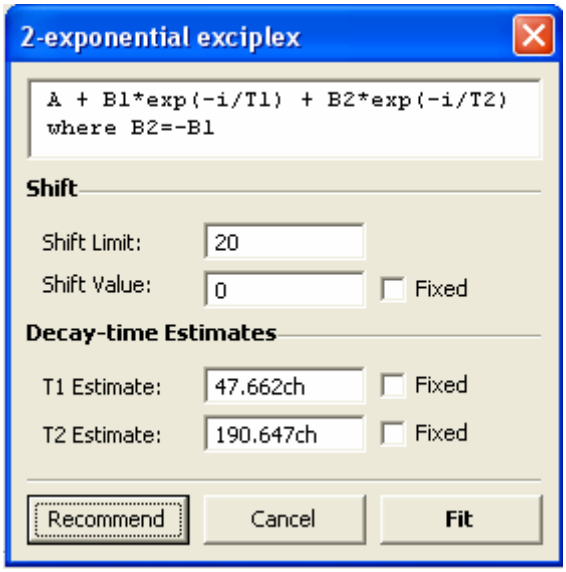
$$F(t) = A + B\left(\exp\left(-t/T_1\right) - \exp\left(-t/T_2\right)\right)$$

The example file is “Exciplex”, and the opening screen is shown below.



This is an example with lifetime values of 8.5 ns and 2.0 ns for T_1 and T_2 respectively. It is interesting to note the distinct rise time on the data. Also, it is mathematically evident that in this case T_1 must always be greater than T_2 (the lifetime associated with the negative pre-exponential –“rise time”).

The example file contains only one “Stored Result” and the parameter input screen, obtained by selecting “Exciplex” as the fit type, is shown below.



Examination of the fit results shows lifetime values of 8.4 ns and 2.0 ns, which are in good agreement with the values expected.

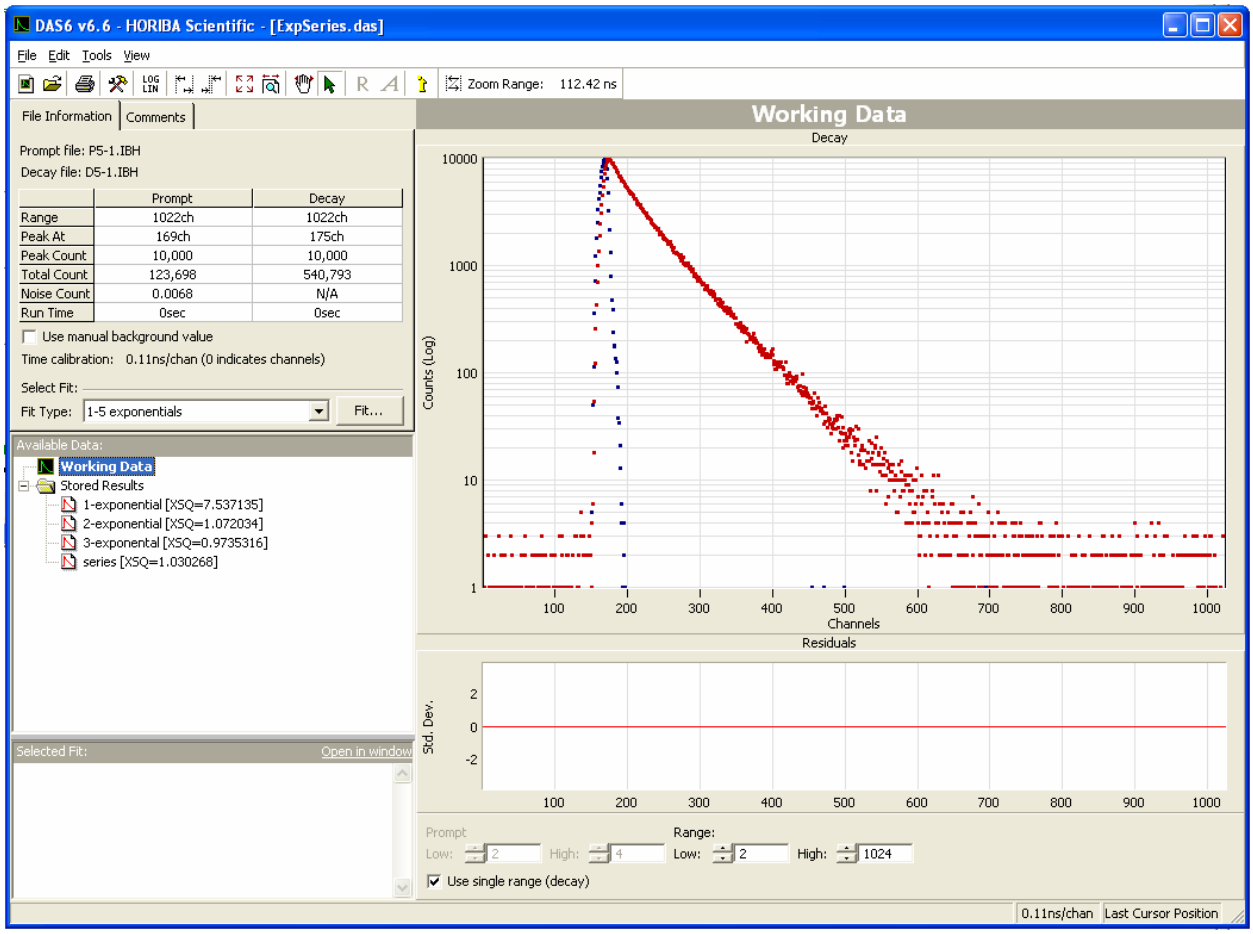
5.3 Fit to Exponential Series

This module simply allows fits to be performed for up to 30 fixed lifetimes. Reconvolution is performed, but no shift. The fitted function is of the form:

$$F(t) = A + B_1 \exp\left(-i/T_1\right) + B_2 \exp\left(-i/T_2\right) + \ldots + B_{30} \exp\left(-i/T_{30}\right).$$

The main purpose of this module is to parameterise otherwise difficult data so that an impulse response function can be calculated (i.e. so that a smooth curve representing the deconvoluted data can be extracted).

The example file is “ExpSeries”, and the opening screen is shown below



The example data is a mixture of seven decays of equal initial intensity (i.e. equal B values) with lifetime values of 10, 20, 30, 40, 50, 60, and 70 channels. (Note that this example is working in channels rather than nanoseconds).

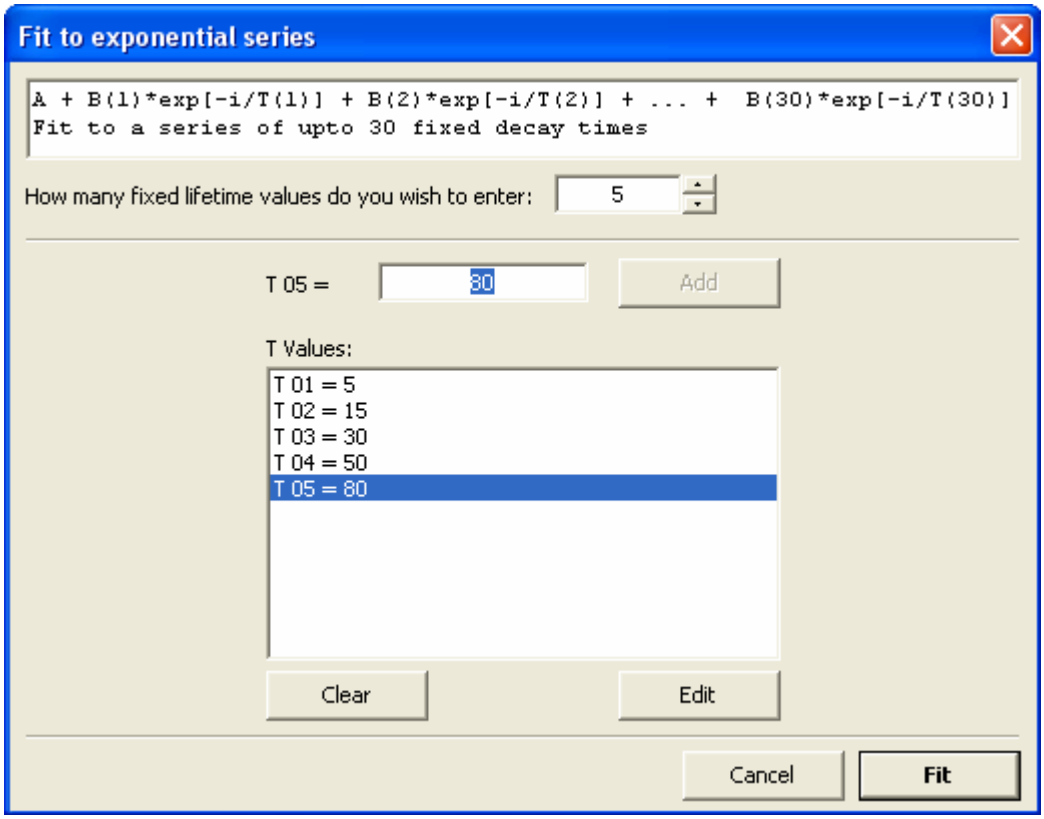
There are four fits in the “Stored Results” tree.

1-exponential
2-exponential
3-exponential

These are included for general interest and to illustrate the point that most data can be fitted to a three exponential model (see section 5.1).

Series

This example uses the “Fit to exponential series” module to parameterise the data. A data range of channels 140 to 800 has been selected and the parameter input screen is shown below.



In this example we have fitted to a series of 5 exponentials and the fixed lifetime values can be seen on the parameter input screen above. The fit results can be viewed in the results window by clicking on the ‘series’ entry in the tree view.

5.4 Förster Quenching

This is an energy transfer model based on dipole-dipole interaction and is described extensively in the scientific literature (see, for example, J.B. Birks, *Photophysics of Aromatic Molecules,* 567-576*,* Wiley, 1970). The Förster approximation assumes the sample under test has sufficiently high solvent viscosity, that the diffusion length is small compared with the critical transfer distance. Molecules therefore effectively remain stationary during the energy transfer process. Rapid Brownian rotation is assumed, so that the orientation factor is replaced by its average value. The decay function is:

$$F(t) = A + B_1 \exp\left[\left(-t/T_1\right) - 2\gamma\left(-t/T_1\right)^{1/2}\right] + B_2 \exp\left(-t/T_2\right)$$

Where

$T_1 = \text{Unquenched donor lifetime}$

$T_2 = \text{Decay time of (optional) additional component}$

$a = \text{Acceptor concentration (mole/litre)}$

$a_0 = \text{Critical acceptor concentration (mole/litre)}$

$\gamma = a/a_0$, with $10^{-3} \le \gamma \le 10^2$

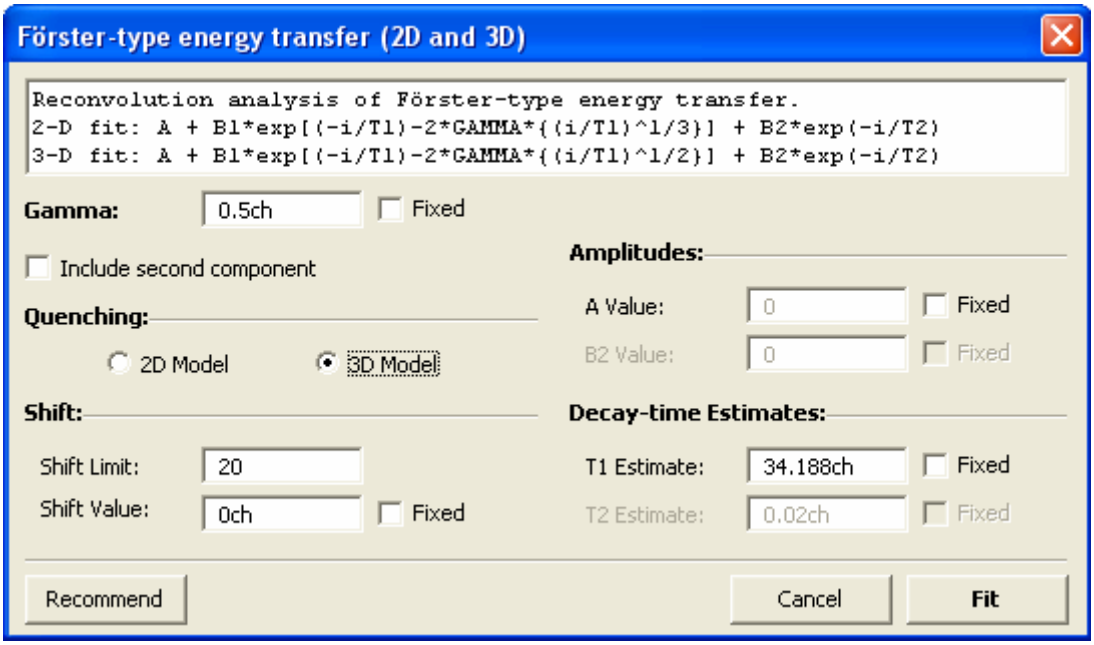
This means that in an experiment varying acceptor concentration, we can use a plot of γ *vs* a to determine Ro, the distance at which energy transfer is 50% efficient.

The above equation is, in fact, a 3-dimensional Förster model. DAS6 also allows fitting to a 2-dimensional model given by:

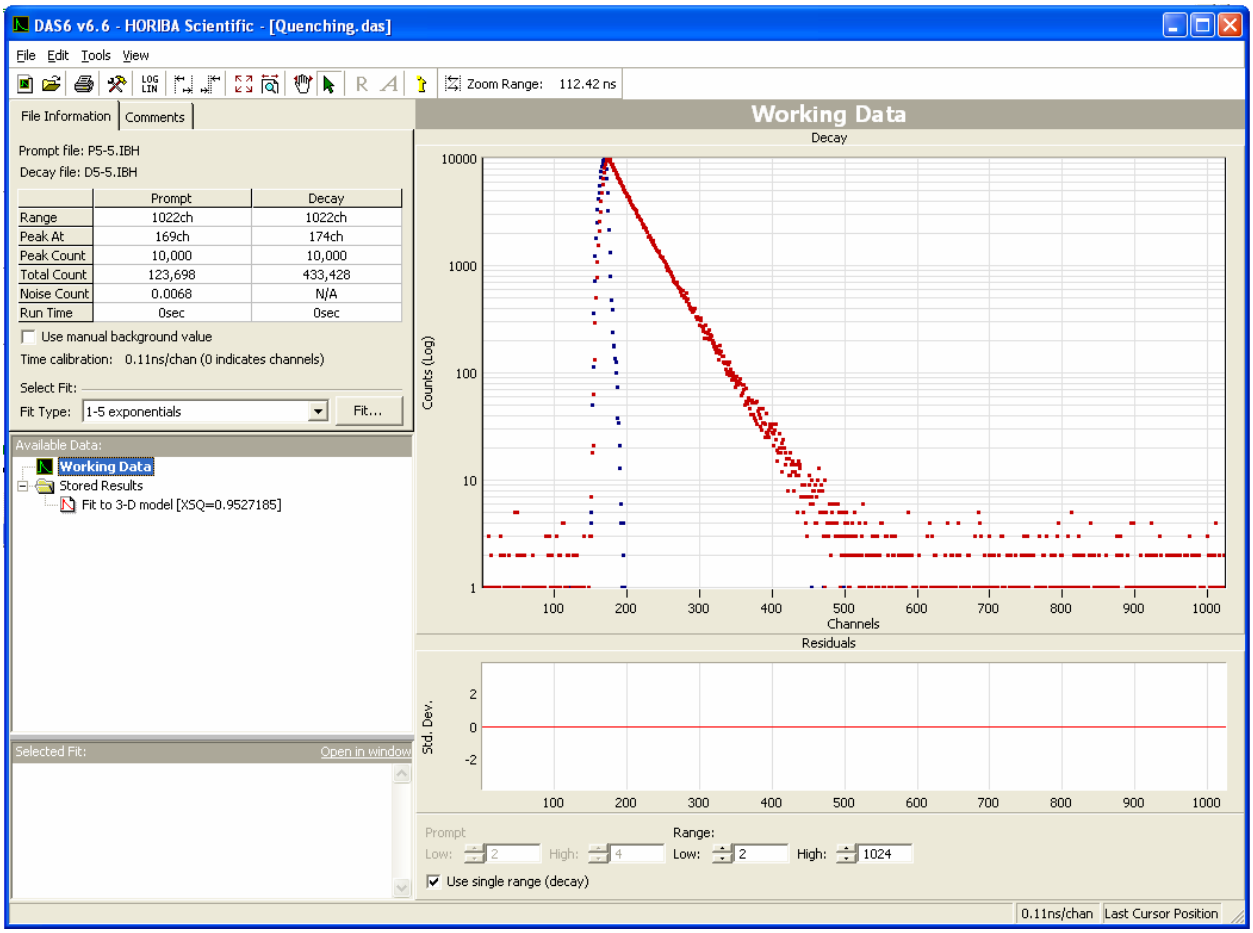
$$A + B_1 \exp\left[\left(-t/T_1\right) - 2\gamma\left(-t/T_1\right)^{1/3}\right] + B_2 \exp\left(-t/T_2\right)$$

DAS6 can adjust all of the parameters of the model, or accept constraints. One possible constraint is T_1, since it should be known in many instances. Remember that sensible constraints lead to greater stability in the fitted parameters, often making the difference between *curve fitting* and kinetic sense.

The example file for this module is “Quenching”, which uses a donor lifetime of 5 ns and a γ value of 0.3. The example does not include an optional decay component. Data channels from 140 to 600 were included in the analysis and the parameter input screen is shown below.



The resulting fit is shown below.



5.5 Yokota-Tanimoto Quenching

The Yokota-Tanimoto theory extends the Förster model by taking into account molecular diffusion during the energy transfer process (see M Yokota and O Tanimoto, J. Phys. Soc. Japan, 22, 779–784, (1967); J B Birks, "*Photophysics of Aromatic Molecules"*, Wiley Interscience, 1970, 576 – 580). The decay law is:

$$F(t) = A + B_1 \exp\left[\left(-t/T_1\right) - 2\gamma C(t)\left(t/T_1\right)^{1/2}\right] + B_2 \exp\left(-t/T_2\right)$$

Where:

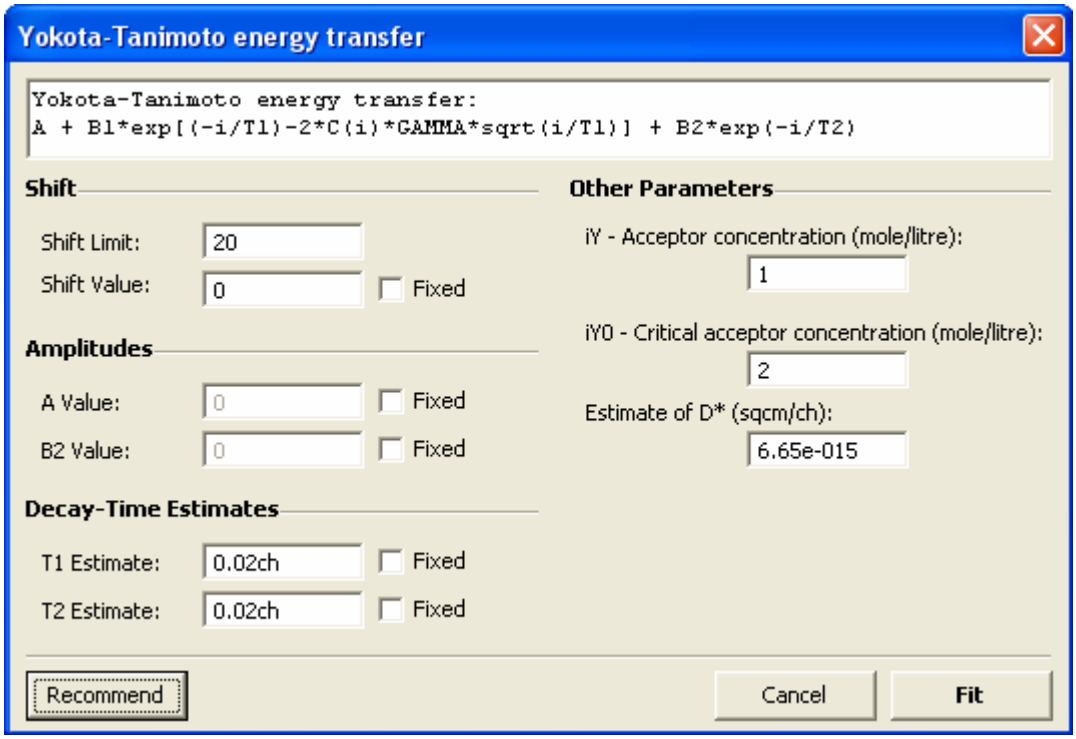
T_1	= Unquenched lifetime
T_2	= decay time of (optional) additional component
A	= Acceptor concentration (mole/litre)
A_0	= Critical acceptor concentration (mole/litre)
γ	= A / A_0
$C(x)$	= $[(1 + 10.87x + 15.5x^2) / (1 + 8.734x)]^{3/4}$ with $x = (K_m.R_0^6)^{-1/3} .t^{2/3} .\ D^*$
D*	= Effective diffusion coefficient (cm^2/ch)

$10^{-28} <= D^* <= 1$; $0 <= [^iY] <= 10$ and $0 <= [^iY]_0 <= 10$

Note that the program requires times and time related parameters to be in terms of channels. In particular, the effective diffusion coefficient is required in units of cm^2/ch. This is calculated from the more usual units of cm^2/sec as follows:

Take	D*	= $1.9 * 10^{-5}$	cm^2/sec, say
and	CAL	= 0.35	ns/ch, say
then	D*	= $(1.9 * 10^{-5}) * (0.35 * 10^{-9})$ cm^2/ch	
ie	D*	= $6.65 * 10^{-15}$ cm^2/ch	

The parameter input screen is shown below.


Yokota-Tanimoto energy transfer
Yokota-Tanimoto energy transfer:
A + B1*exp[(-i/T1)-2*C(i)*GAMMA*sqrt(i/T1)] + B2*exp(-i/T2)
Shift
Shift Limit:
20
Shift Value:
0
Fixed
Amplitudes
A Value:
0
Fixed
B2 Value:
0
Fixed
Decay-Time Estimates
T1 Estimate:
0.02ch
Fixed
T2 Estimate:
0.02ch
Fixed
Other Parameters
iY - Acceptor concentration (mole/litre):
1
iY0 - Critical acceptor concentration (mole/litre):
2
Estimate of D* (sqcm/ch):
6.65e-015
Recommend
Cancel
Fit

5.6 Micellar Quenching

This model deals with quenching in systems with micelles. The decay function is given by the equation:

$$F(t) = A + B\exp\left[\left(-t/T_1\right) - C\left\{1 - \exp\left(-t/T_2\right)\right\}\right]$$

Where the above symbols can be approximated in their simplest form to (see Reekmans et al. for details)

T_1 = unquenched lifetime = $1/k_0$

T_2 = quenched lifetime = $1/k_q$

B = intensity at time zero = F_0

C = number of quenchers per micelle = n

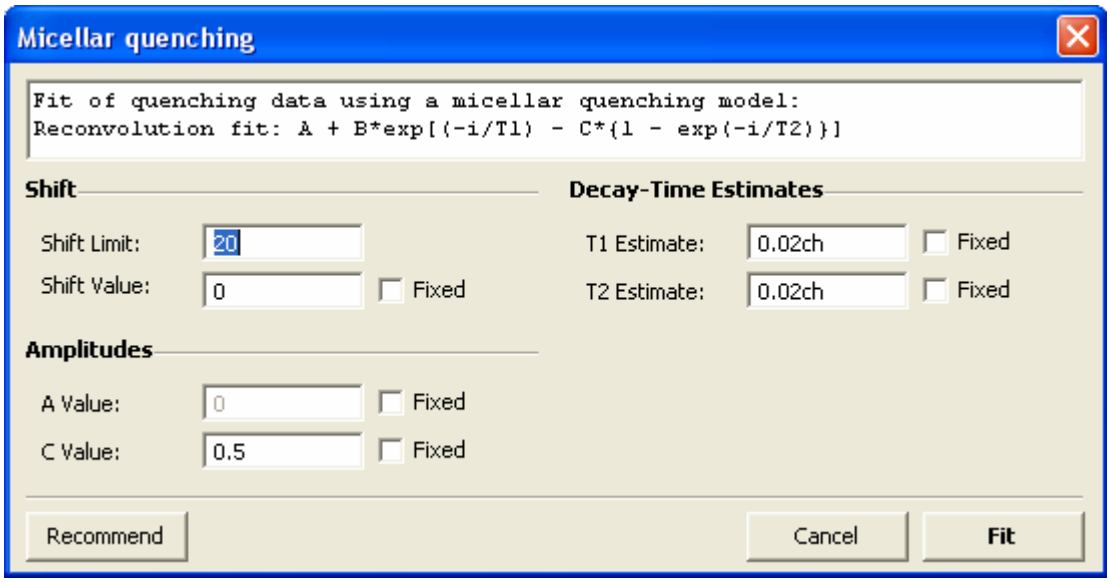
The aggregation number <a> can be obtained

$<a> = nS_m / Q_m$

where S_m is the amount of micellized surfactant and Q_m is the amount of micelle bound quencher.

For a useful references on micellar quenching see for example P.P Infelta et al., J. Phys. Chem. *78*, 190-195, (1974), S. Reekmans et al., Langmuir, *9*, 2289-2296, (1993). R.G. Alargova et al., Langmuir, *14*, 5412-5418, (1998).

The parameter input screen is shown below.



5.7 Lifetime Distribution

In many physical situations, the fluorophores under investigation can be in diverse local environments, each affecting the fluorescence lifetime to a slightly different degree. Rather than characterising the resulting complex decay with more and more exponential components, it can be argued that it is more realistic to describe such kinetics in terms of a distribution of decay components, whose decay times are distributed according to an assumed distribution function. The fitted parameters then reduce to two: a mean decay time and a measure of the width of the associated distribution function.

Two methods are available for performing lifetime distribution analysis. The first method employs a Top Hat distribution function which allows only a single lifetime distribution to be fitted and the second is the Non-Extensive Decay Distribution which allows up to five lifetime distributions to be modelled.

5.7.1 Top hat distribution

It is a point of academic discussion whether it is physically more realistic to consider distributed decay times or distributed decay rates. The arguments tend to favour decay rates. This is fortunate, since the integrals involved in deriving the decay laws cannot be solved in closed form with the decay time formulation. The distributed decay law in terms of decay rates can be written as:

$$F(k_m, \sigma_m, t) = \int_0^\infty D(k_m, \sigma_m) \exp(-kt)\, dk$$

where

$F(k_m, \sigma_m, t)$ is the decay law
$D(k_m, \sigma_m)$ is the distribution function
$k =$ decay rate
$k_m =$ mean decay rate
$\sigma_m =$ standard deviation of the distribution function

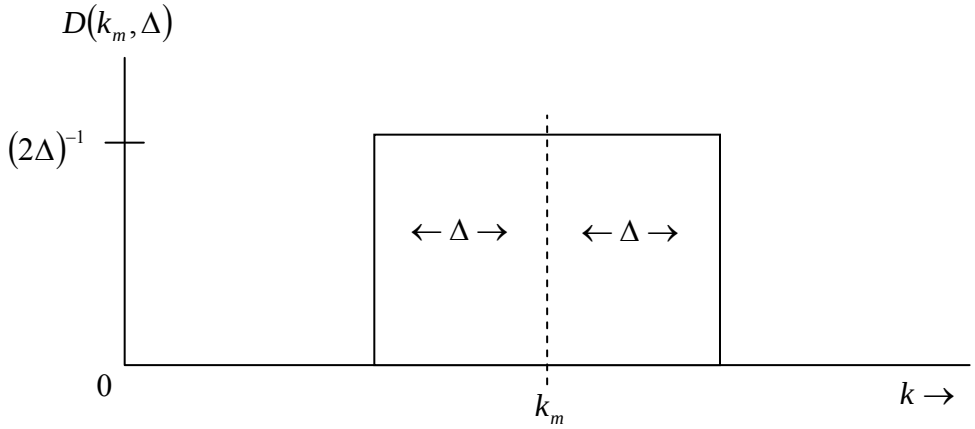
Ideally, one would like to use a distribution function derived *ab initio* from the physical mechanisms responsible for the spread of decay rates. In the absence of such detailed analysis, one might chose a reasonably behaved function, such as top-hat (rectangular), triangular, Gaussian, Lorentzian, gamma, etc.

It is important to avoid physically meaningless negative values of k in the distribution integral above. Distribution functions which fall asymptotically to zero with decreasing (k-k_m) therefore present a cut-off problem. This is most serious with the Lorentzian distribution function, because it tends to zero very slowly, as $(|k\text{-}k_m|)^{-1}$. The cut-off problem is avoided with the gamma function, since this is defined for

positive arguments only. The geometric distribution functions (top-hat, triangular) also avoid the problem, since they go to zero in a well defined manner.

In practice, the form of the resultant decay law does not depend strongly upon the form of the distribution function. Unimolecular kinetics in a homogenous environment gives a single exponential decay; a straight line in a semi-logarithmic display. A distribution of decay rates causes this straight line to *bend*, with a gradient that decreases monotonically with time. A distribution which tends towards zero slowly, such as the Lorentzian, will yield a lower fitted width, σ_m, for a given amount of *bend* than one with a sharp cut-off, such as the top-hat. However, both will bend and if one fits well, then so, in general, will the other (provided that cut-off problems at $k=0$ can be avoided). In our experience, the detailed forms of the decay functions are insufficiently different to discriminate between them on the basis of measurements and curve fitting.

For these reasons, we have selected the top-hat distribution function as the basis for our fitting model. In this case, if the full width of the top-hat function is 2Δ, then,



$$D(k_m,\Delta)=(2\Delta)^{-1} \qquad (k_m-\Delta)<k<(k_m+\Delta)$$
$$=0 \qquad \textit{otherwise}$$

then

$$f(k_m,\Delta,t)=\frac{1}{2\Delta}\int_{k_m-\Delta}^{k_m+\Delta}\exp(-kt)\,dk$$

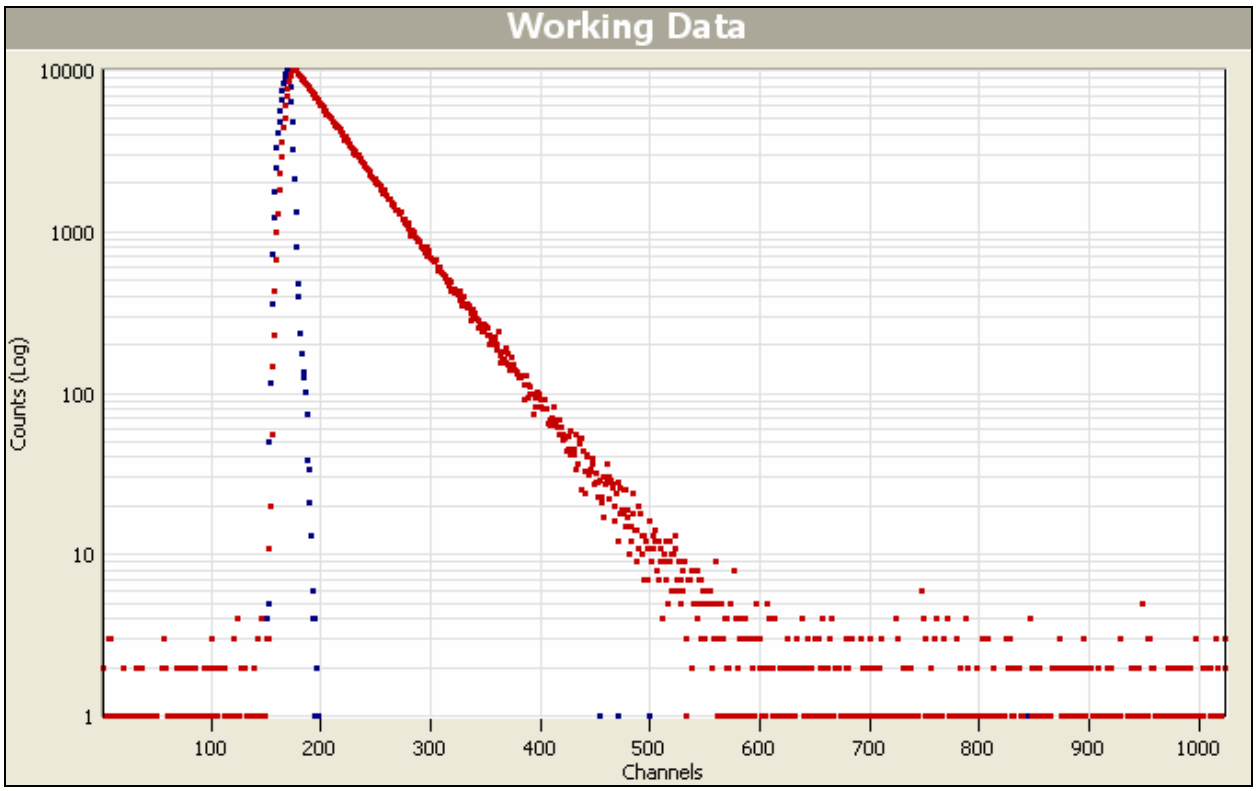
$$\therefore \quad f(k_m,\Delta,t)=\frac{\exp(-k_m t)}{k_m t^2\Delta}\left[(k_m t+1)\sinh(t\Delta)-t\Delta\cosh(t\Delta)\right]$$

The parameters used in the fit model are the mean decay time, T_m, and fractional spread, dT_m/T_m, rather than k_m, Δ. The use of time, rather than rate variables was decided on the basis of convenience and consistency with the other FIT programs. The internal working of the module uses the rate variables defined above. Input and output parameters are converted as follows:

$$Tm = 1/k_m$$
$$dTm/Tm = \Delta/k_m$$

Note also that the width parameter used is Δ, the half width at half height, because we thought it easier to visualise than σ_m, the standard deviation. The relationship between the two quantities is $\Delta = \sqrt{3 * \sigma_m}$. The limiting width, where negative decay rates begin to be included in the distribution is therefore dTm/Tm = 1. The fitting procedure stops, with an appropriate error message, if this condition is encountered.

The first example file is “DistExp1”, and the mean lifetime is 5.0 ns with a spread of 1.0 ns (i.e. 20%). The decay in the opening screen is shown below.

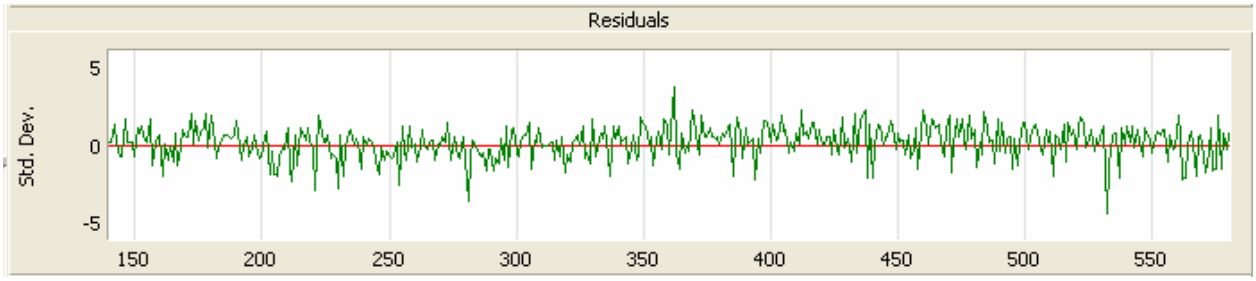


The “Stored Results” shows two examples of fits.

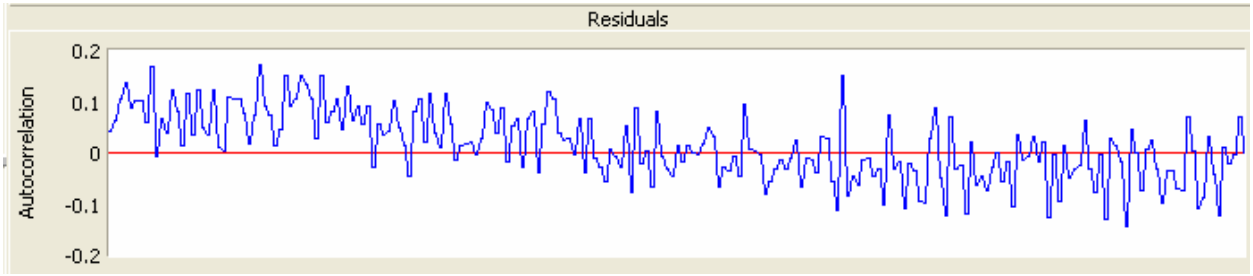
1-exponential

The analysis to a single exponential decay model was performed over channels 140 to 600 and the fit to the model is really quite a good fit.

- The lifetime value of 5.06 ns is reasonable.
- The value χ^2 of 1.15 is good.
- At first glance, the residuals appear to be reasonably random.



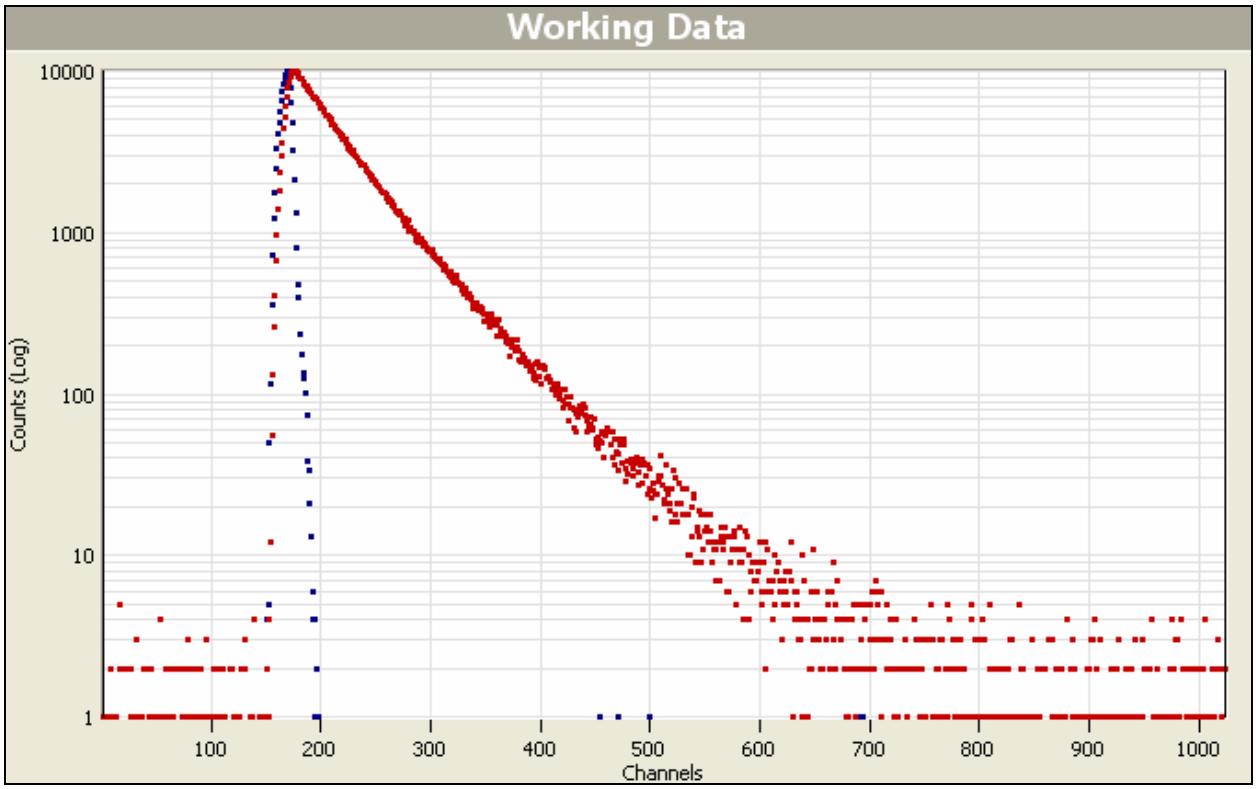
However, the residuals are not truly random. On closer inspection a slight oscillation can be seen. This can also be seen when the autocorrelation is viewed by selecting the $\mathcal{A}$ tool.



distribution

In this case, the distribution decay model has been selected. The data has a 5 ns decay with a spread of 20%, and the program finds a decay of 5.02 with a spread of 17.3%. Note also that the autocorrelation is random.

The second example file is “DistExpTopHat”, and the mean lifetime is 5 ns with a spread of 2.5 ns. The decay in the opening screen is shown below. You should immediately notice that this decay shows more curvature than the data in the previous example.



Again, the “Stored Results” shows two examples of fits.

1-exponential

In this case, the fit is clearly poor.

distribution

In this case, the distribution decay model has been selected. The data has a 5 ns decay with a spread of 50%, and the program finds a decay of 5.02 with a spread of 48.7%.

The first example illustrates that it really needs a significant spread in lifetimes for the effect of the distribution to be observed. For 1024 channels of data, with 10,000 counts in the peak, the lower detection limit is likely to be around 20%. The second example demonstrates that, for wider distributions, the reliability of the fit is really very good. Of course, the detection limit can be pushed lower by using more data channels and counting to a higher peak (i.e. by counting more photons).

5.7.2 Non-Extensive Decay Distribution

The non-extensive decay distribution module in the DAS6 data analysis software allows decay data to be fitted using techniques based on Tsallis non-extensive statistics [C. Tallis, Braz. J. Phys. 39 (2009) 337]. This approach was initially applied to interpret time-resolved fluorescence decays by Włodarczyk [J.

Włodarczyk, B. Kierdazuk, , Biophys. J. 85 (2003) 58], with a recent application to model time-resolved decays given by Rolinski and Birch [O.J. Rolinski, D.J.S. Birch. J. Chem. Phys. 129 (2008) 144507].

This method allows more than one lifetime distribution to be modelled and the results can be displayed graphically.

The following power-like model is used to extract the lifetime distributions from the data

$$F(t) = A + \sum_{k=1}^{5} B_k \left[1 - \left(1 - q_k\right) t/\tau_k\right]^{1/\left(1-q_k\right)},$$

where τ_k is the mean value of the lifetime distribution and q is a parameter of heterogeneity defined by

$$q = 1 + \frac{2}{N} = 1 + \frac{\left\langle \left(\gamma - \langle\gamma\rangle\right)^2 \right\rangle}{\langle\gamma\rangle^2},$$

q describes the fluctuation according to the mean value of the decay rate $\langle\gamma\rangle = \langle 1/\tau \rangle$, which is also indicative of the width of the distribution and the number of decay channels (N). It is worth noting that the components of F(t) become exponentials as q_k tends towards one

$$\left[1 - \left(1 - q_k\right) t/\tau_k\right]^{1/\left(1-q_k\right)} \xrightarrow{q_k \to 1} e^{-\frac{1}{\tau_k}}.$$

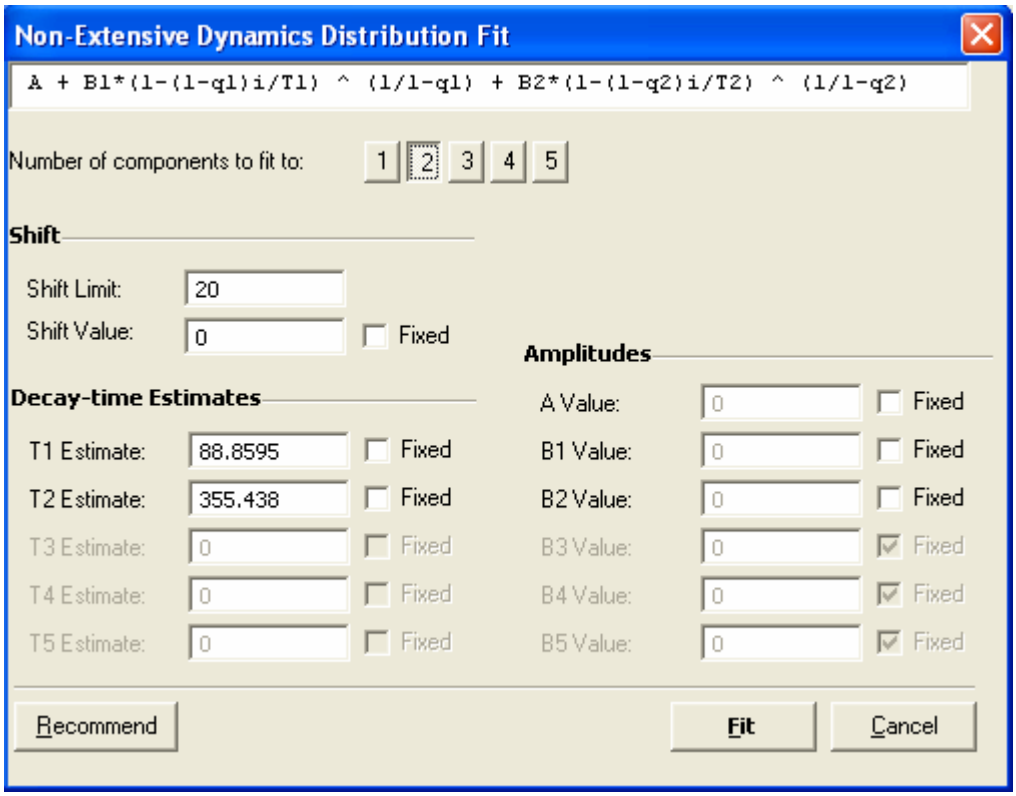
The analysis software places limits on the values of q $(1.01 \le q \le 1.3)$, the lower limit representing $q \to 1$ and the upper limit allows the mean value of F(t) to be well defined. When q =1.01 then the lifetime is tending towards a simple exponential term. When q=1.3 then the distribution of lifetimes is significantly large.

The results of this analysis are shown graphically with the lifetime distribution for each component plotted using the gamma function

$$g(\tau; q, \tau_\infty) = \frac{1}{\Gamma\left(\frac{1}{q-1}\right)} \frac{\tau_\infty}{q-1} \left(\frac{1}{q-1}\frac{\tau_\infty}{\tau}\right)^{-\frac{q-2}{q-1}} \exp\left[-\frac{1}{q-1}\frac{\tau_\infty}{\tau}\right],$$

with the area under the distribution normalised by the pre-exponential (B value) of the particular distribution component.

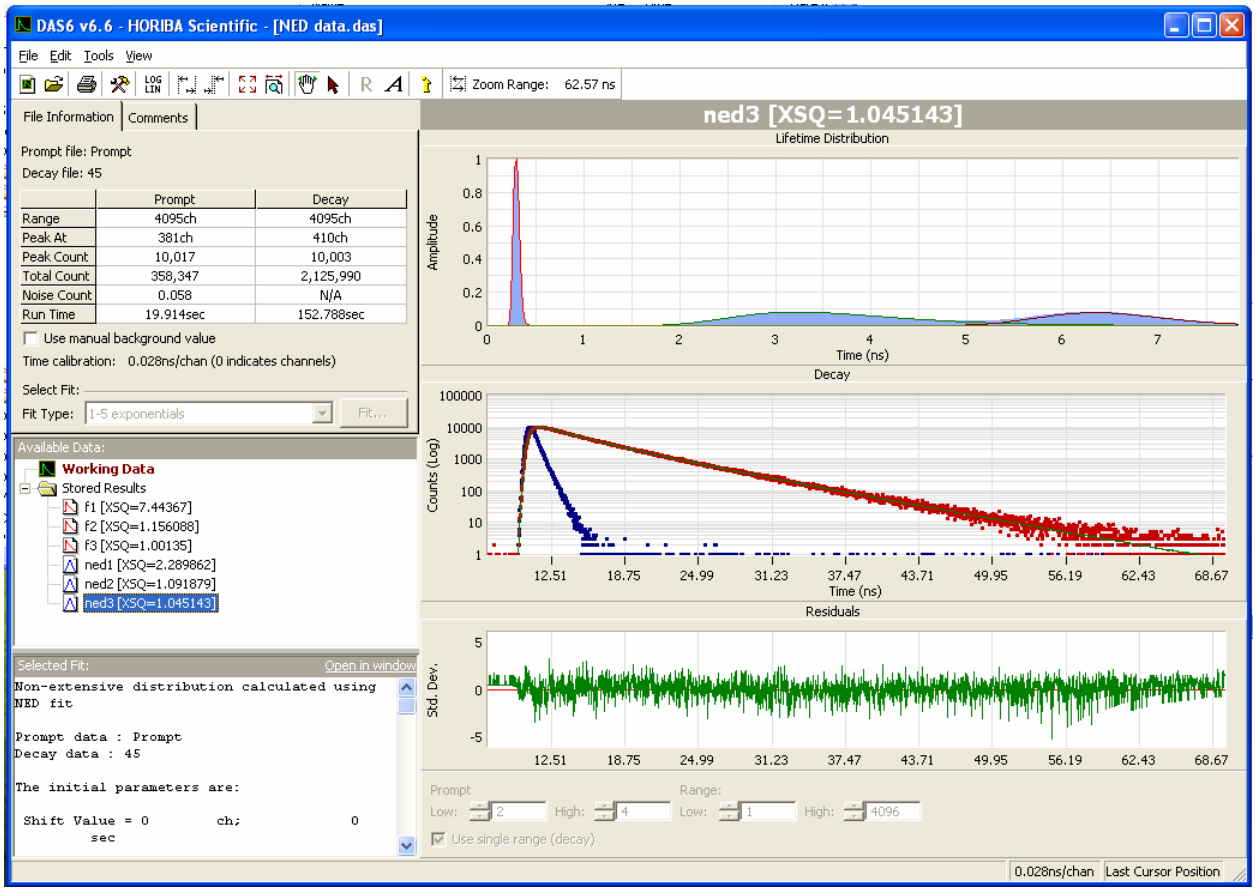
The parameter input screen has the same format as the exponential fit and is shown below



From here it is possible to select the number of lifetime components to fit. Decay time estimates are automatically calculated, but these can be set by the user. Pressing the “Recommend” button will reset the lifetime estimates back to the automatically calculated values. It is possible to fix the amplitude and decay time estimates preventing them being varied during fitting. When the initial estimates have been selected the “Fit” button should be selected to perform the fit.

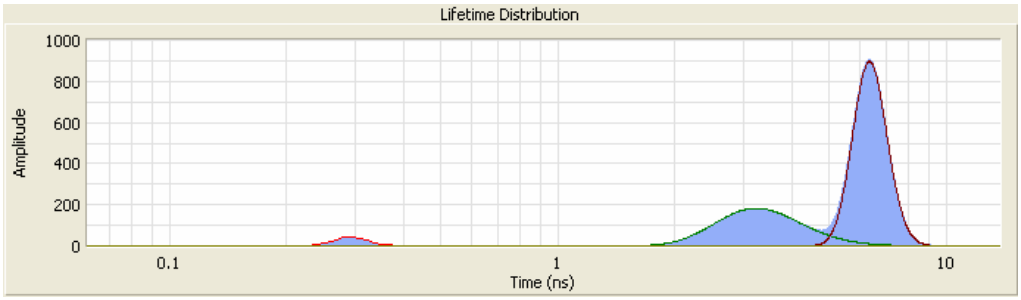
The distribution fit first calculates an estimate of the lifetimes (T) and amplitudes (B) without the q parameter, it then fine tunes these values and allows *q* to vary in order to achieve the minimum chi-squared value and optimum fit.

In the following example a three component Non-Extensive Dynamics Distribution is fitted to the decay curve. The data can be found in example file NED data.das. (example is for hsa)



The top plot shows the lifetime distributions, shown in red, green and purple with the blue graph representing the sum of the three liftetime distributions. Note the area under the lifetimes is scaled with respect to the normalised B value. This is chosen from the View menu: Scale Distribution to B's. This scaling gives an area which is a representation of the relative amount of each of the components. It is possible to remove the scaling to allow comparison with other models as discussed below.

In order to allow comparison with other models it is possible to change the x-axis to a log scale and



display the lifetime distributions without scaling to the B values. This can be done using the View menu and the result for the above example is show here.

The middle graph gives the measured data (red dots) with the fitted curve (green line) and the bottom plot shows the standard deviation between the actual and fitted model.

It is possible to view the detailed analysis in the results window or view in a separate window by selecting 'Open in Window'. The results for the above fit are as follows

```
-----------------------------------------------
The Non-Extensive Parameters are::

 SHIFT = -3.337766      ch;  -9.382007E-11 sec    S.Dev = 1.618703E-12  sec
 T1    = 10.77276       ch;  3.028076E-10  sec    S.Dev = 5.504151E-11  sec
 T2    = 108.7003       ch;  3.055419E-09  sec    S.Dev = 4.071503E-11  sec
 T3    = 223.6656       ch;  6.286936E-09  sec    S.Dev = 1.312063E-11  sec

 Q1    = 1.010              S.Dev = 0
 Q2    = 1.059              S.Dev = 0
 Q3    = 1.010              S.Dev = 0

 A     = -0.4618748
 B1    = 9.345834E-03       [1.74 Rel.Ampl]
 B2    = 0.0213441          [40.21 Rel.Ampl]
 B3    = 0.014975           [58.05 Rel.Ampl]


The Lifetime distribution plot parameters are:

 FWHM 1 = 2.61          ch; 7.346889E-11  sec
 FWHM 2 = 70.56         ch; 1.983296E-09  sec
 FWHM 3 = 53.74         ch; 1.510593E-09  sec

 Modal Lifetime 1 = 11     ch;     3.091951E-10  sec
 Modal Lifetime 2 = 116    ch;     3.260602E-09  sec
 Modal Lifetime 3 = 226    ch;     6.352553E-09  sec
-----------------------------------------------

 CHISQ = 1.045143
 [ 2226 degrees of freedom ]
```

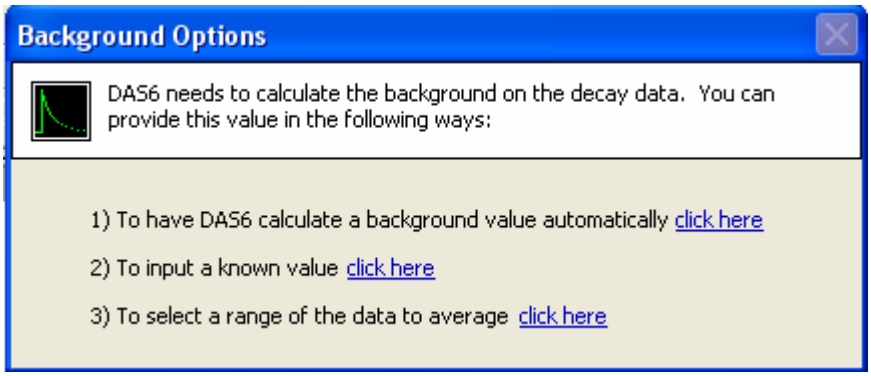
In this case the Chi-squared value appears to give a good fit and the standard deviation graph appears to provide random residuals. The relative amplitudes of the three components are given which gives an indication of the contribution of each component to the model. The lifetimes and Q values are displayed along with an estimate of their standard deviation.

The full width half maximum (FWHM) value for each of the lifetime distributions is also given this gives a measure of the spread of the distribution. As the gamma function is asymmetric the calculated lifetimes may not appear at the peak of the distribution curve thus, the Modal Lifetime, which corresponds to the peak of the distribution is also displayed.

6. Anisotropy Analysis Module

6.1 Starting an Anisotropy Analysis

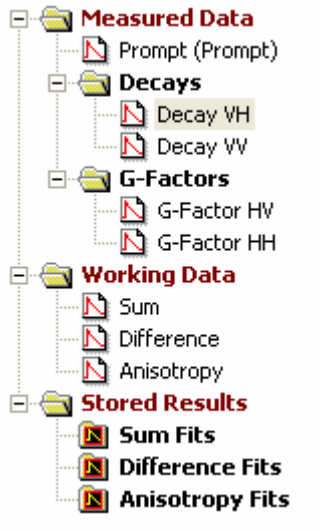
This section describes how to get started using an anisotropy file produced by DataStation. For files created using the “New/Anisotropy” template, see section 10. On first opening an anisotropy file, the following window is displayed.



Generation of the sum, difference and anisotropy intermediate data (see Appendix B) requires the measured data $Y_{vv}(i)$ and $Y_{vh}(i)$ to be combined. However, if there is background noise, this needs to be subtracted before the curves are combined. To obtain a good analysis of anisotropy data, it is important to set the background values correctly since this affects all subsequent operation on the data.

As can be seen from the screenshot above, there are three ways to set the background. The first way is easy, automatic and is adequate in most circumstances. The other options require more user intervention (and thought) but allow more flexibility. The example file “Anisotropy.DAS” has already been loaded, so the above message does not appear: it can be viewed using the “Edit -> Change Background” menu selection.

The figure below shows the layout of typical anisotropy data. The data in this file contains measured prompt and g-factor decay data, in addition to the “VH” and ”VV” decays. Note that prompt and g-factor decay data are optional.



There are three main sections (coloured red):

- Measured Data – this is the actual acquired data, it shows the prompt, decays and g-factor correction decays.
- Working Data – this is where the intermediate data is displayed.
    - Stored Results – this is where the final fitted data is displayed.

Under “Working Data” you will see sections for “Sum”, “Difference” and “Anisotropy”. The “Anisotropy” is used in the “Impulse Anisotropy Analysis”, as described in section 6.2. The “Sum” and “Difference” data are used in “Reconvolution Anisotropy Analysis”, as described in section 6.

A general description of anisotropy analysis is given in Appendix B.

The example data file “Anisotropy.DAS” is a DataStation file that can be used for practice. It is real measured data.

6.2 Impulse Anisotropy Analysis

The anisotropy intermediate data can be analysed using the exponential fitting module, to fit to the following function:

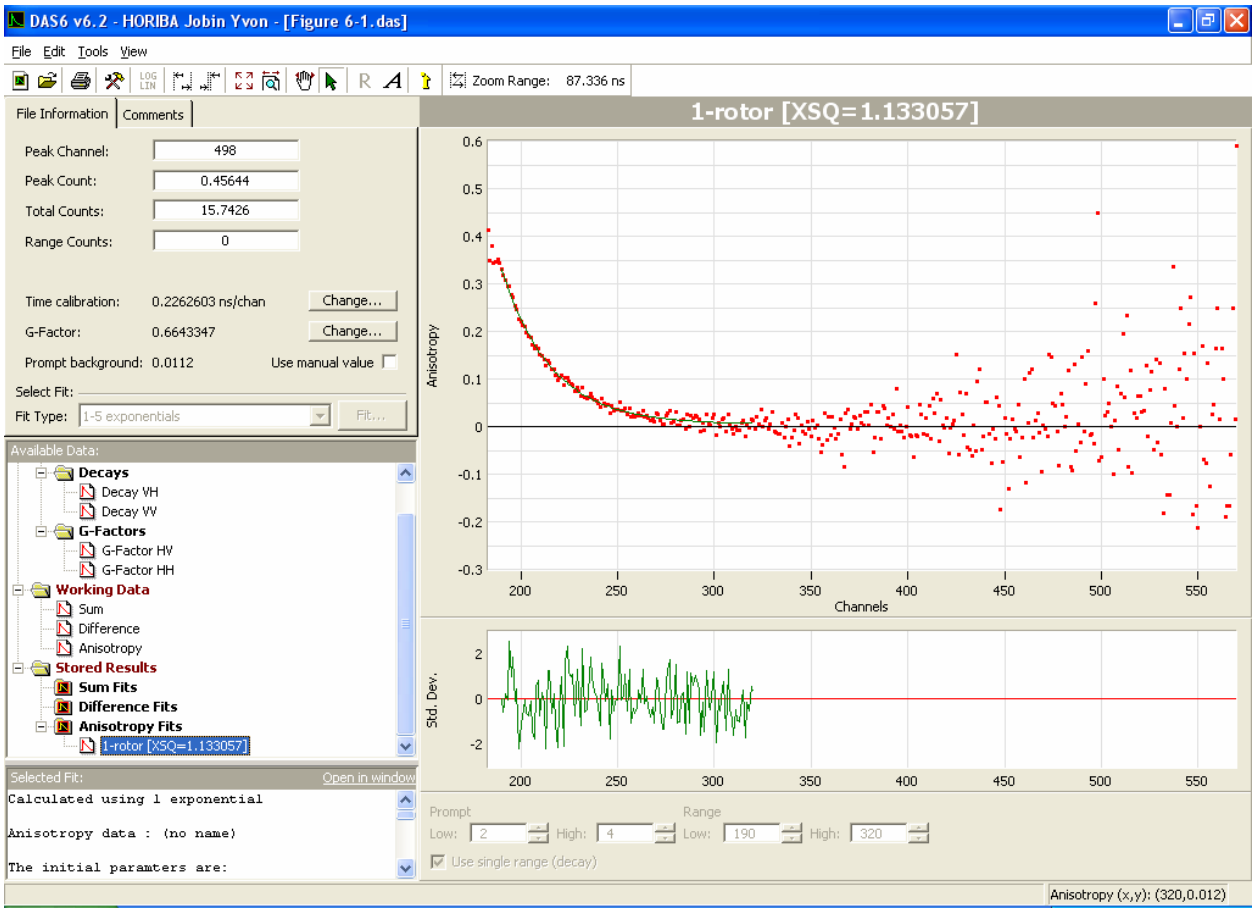
$$F(t) = R_\infty + B_1 \exp\left(-t/Tr_1\right) + B_2 \exp\left(-t/Tr_2\right)$$

The example data file “Anisotropy” contains sample fits for both impulse and reconvolution models.

To reproduce the impulse anisotropy analysis proceed as follows:

1. Click on Working Data/Anisotropy.
2. Move the low and high cursors to 190 and 320 respectively.
3. Click “Fit”. Note that only the “1-5 exponentials” can be selected.
4. Click “Recommend”, click “Fit” and click “Keep”.
5. Enter a name for the fit, then click “OK”.

You will see that a fit has been added to the “Stored Results” tree in the “Anisotropy Fits” section. The fit result viewed, by clicking on the fit and then clicking the “zoom to full view” tool, is shown below.



Note the following:

- The initial shape (early channels) is distorted by the instrumental response.
- The final shape (late channels) is very noisy, but evenly distributed about zero.

- The initial anisotropy (R_0) is difficult to estimate visually, but looks to be between 0.3 and 0.4. It can, in fact be obtained from the values A + B.

  Note that the maximum theoretical value of R_0 for a perfect rotor is 0.4 (for one photon excitation).

On examination of the fit results, you will see a rotational correlation time (T1) of 5.8 ns and a residual anisotropy (A) of 4.5×10^{-3}, a value that can be considered to be zero (values <0.01 can effectively be considered to be zero).

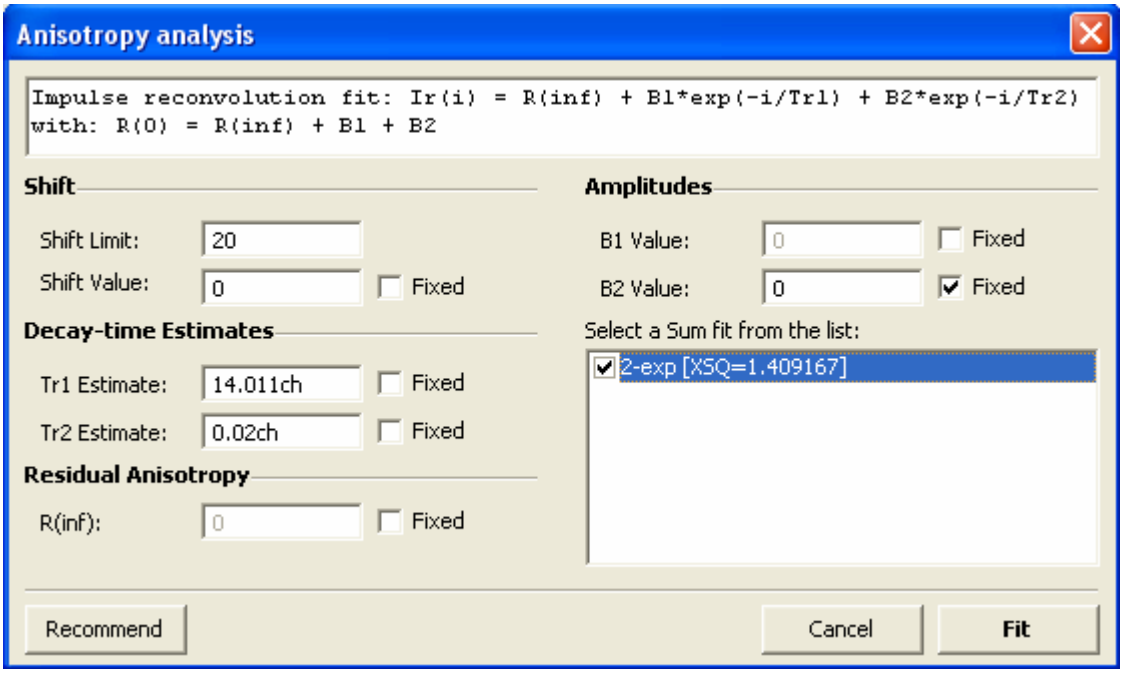
6.3 Reconvolution Anisotropy Analysis

To perform a reconvolution anisotropy analysis of the data, we need to first analyse the sum data. To reproduce the example, proceed as follows:

1. Click on Working Data/Sum.
2. Move the low and high cursors to 170 and 700 respectively.
3. Click “Fit”. Note that only the “1-5 exponentials” can be selected.
4. Click the “2” button to select a 2-exponential fit and then “Recommend” to get initial guesses. By default “A” is fixed at zero because backgrounds have been subtracted already as part of the generation of sum and difference data.
5. Click “Fit” and “Keep”
6. Enter a name for the fit, then click “OK”.

You will see that a fit has been added to the “Stored Results” tree in the “Sum Fits” section. Click on the “Stored Result” to view the fit.

For a simple fluorophore, the sum data should be a single exponential decay and the sum fit should give its lifetime. This example is not a simple fluorophore and required a two exponential fit. Now we can proceed to analyse the difference data. The parameter input screen is shown below.



To perform the difference fit, proceed as follows:

1. Click on Working Data\Difference.
2. Move the low and high cursors to 170 and 400 respectively.
3. Click “Fit”. Note that only the “Anisotropy analysis” can be selected.
4. Ensure that B2 is “fixed” at 0 (this is the default), because we want to fit to a model with a single rotational time.
5. In the “Select a sum fit” box, tick the sum fit. Note that you can perform several sum fits prior to analysis of the difference data. This box allows you to choose which one you want to use for analysis of the difference data.
6. Click “Recommend” then “Fit” and “Keep”.
7. Enter a name for the fit, then click “OK”.

On examining the fit results, you will see that the program has found a rotational time of 5.7 ns, R_0 (the initial anisotropy) is 0.33 and R_∞ (the residual anisotropy) is 3.56 x 10^{-3}. Note the agreement with the values in section 6.2.

7. Global Analysis Module

The “Global Analysis” module allows a fit calculation (up to 5 exponentials) to be performed globally on up to 100 separate decay curves. Global Analysis is useful when you have several decay curves which are expected to conform to the same underlying decay model with shared lifetime parameters. Global analysis helps make best use of the data available and hence can produce a more accurate fit.

When a global dataset is opened in DAS, extra navigation toolbar buttons appear (shown below) allowing browsing of the entire decay curve set. The extra tools are shown below:

The desired curve can be selected using the arrows ◀ ▶ or by typing it numerically in the box.

The Σ button will add all the separate decay curves together and display the result. If the summation button is selected then it is possible to estimate the initial lifetimes based on all the decay curves.

An example DAS data file is included in the examples folder and is named “Global.DAS”. This file contains 9 decays with different ratios of 1.0 ns and 3.4 ns lifetimes, stepping from 10% (1.0 ns) in curve 1 to 90% (1.0 ns) in curve 9.

Example1:

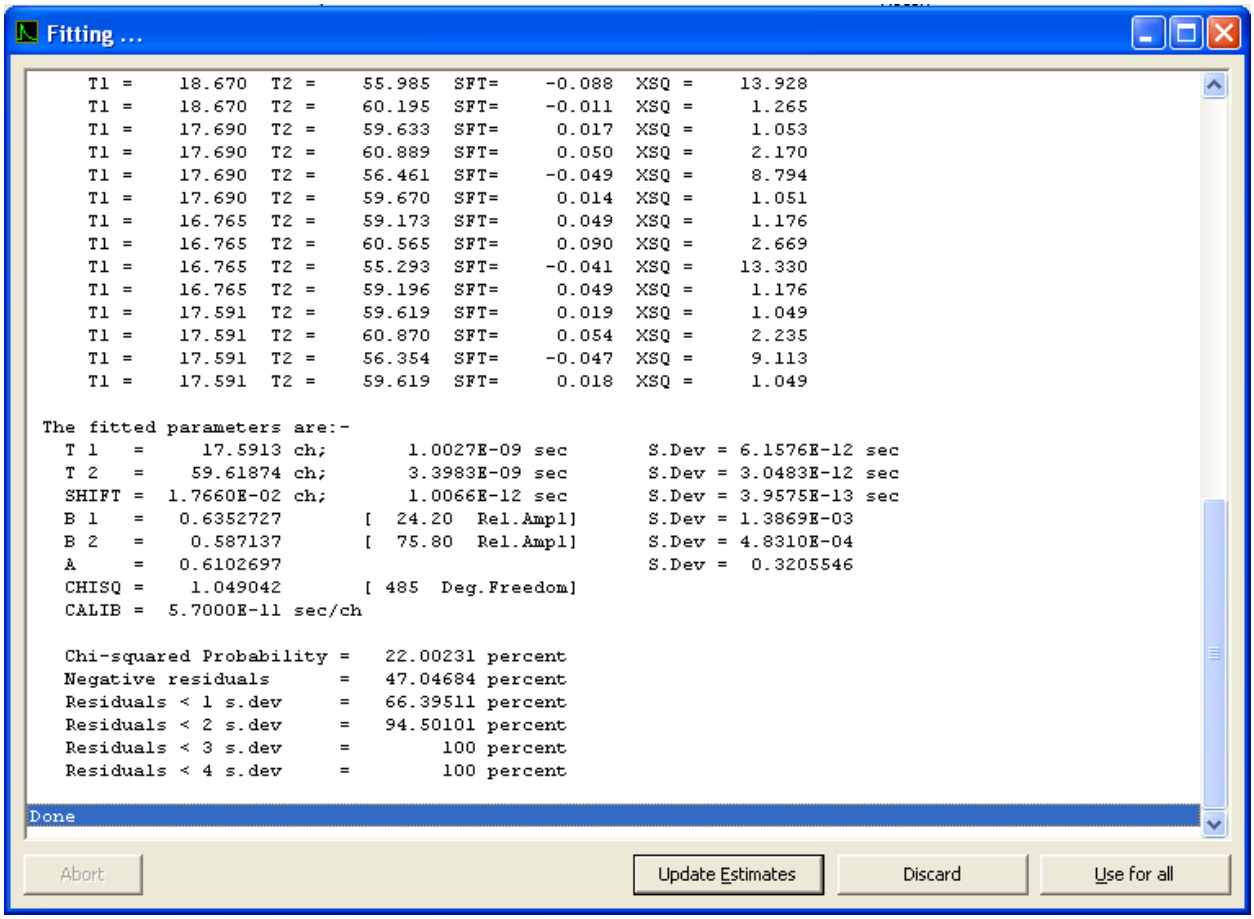
To repeat the stored fit:

1. Ensure the Σ button is not selected.
2. Select the “Working Data” and set the cursors to 190 and 700.
3. Click the “Fit” button. The parameter input screen will appear which is similar to the screen used for the exponential fit module. Select “2” as the number of exponentials, lifetime estimates are automatically calculated. Click “Fit”
4. A preview screen appears, this shows the result of applying the lifetime estimates to the first curve. At this point you can choose to “Discard” the fit or use the initial lifetime estimates and apply for all the curves. Select “Use for all”
5. Enter a name for the fit, then click “OK”.

To view the results for each curve, select the fit under “Stored Results” and step through the each fit in turn using the arrows on the toolbar. “Open in Window” can be used to view the numerical results for each curve.

Example2:

If the steps (1-3) in the previous example are repeated but with the Σ button selected, then the Global analysis module will calculate a preliminary fit of lifetimes based on the summation of all the curves. As all of the decay data has been used in this preliminary fit, the lifetime fitted parameters produced may provide a better estimate for the initial lifetimes.

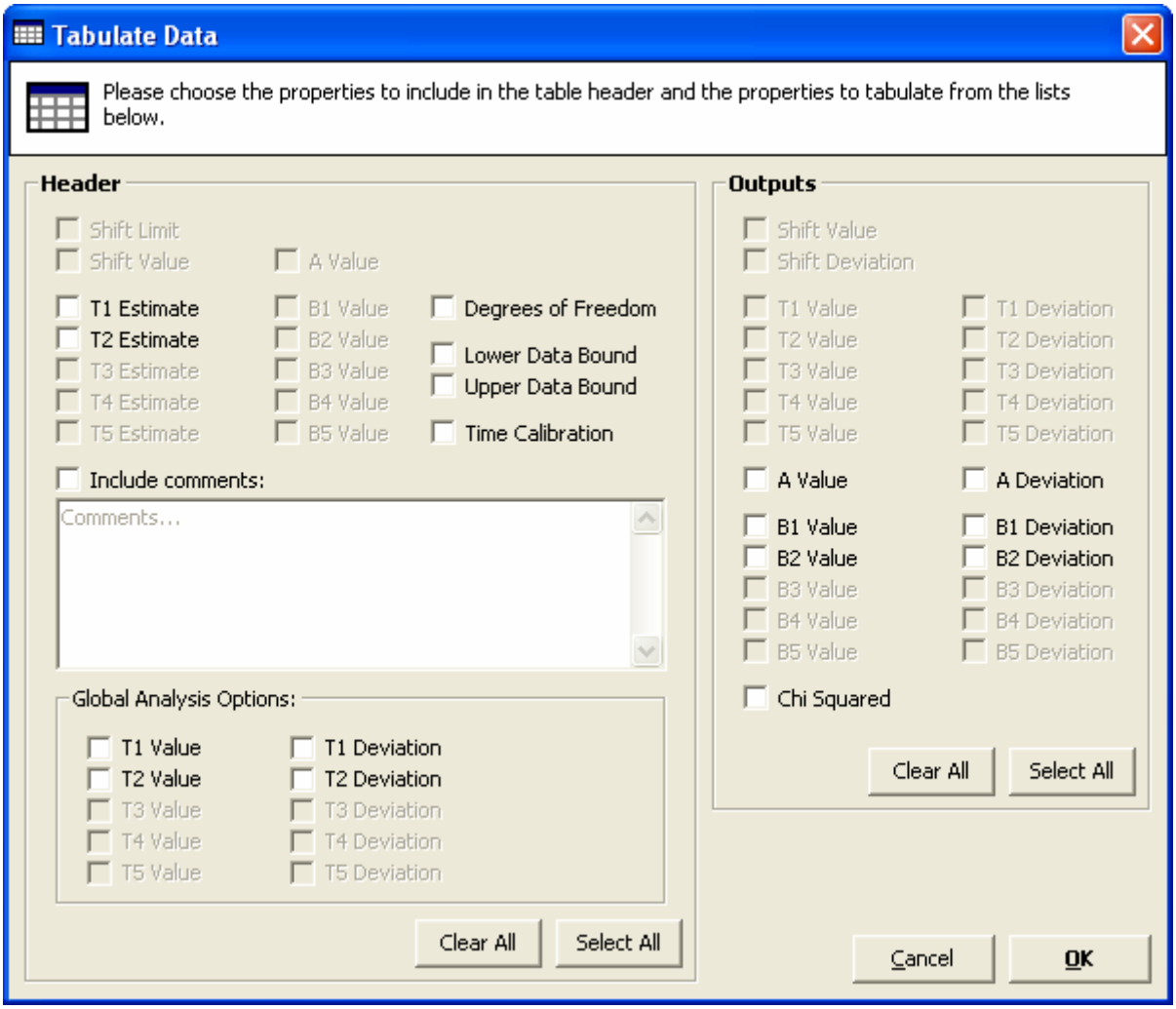


Selecting the “Update Estimates” button will redisplay the lifetime estimate screen but with the new lifetime estimates.

Selecting “Use for all” will continue the global analysis using the initial lifetime estimates.

Tabulated data

Stepping through the results is not convenient when many curves are involved. We have therefore included a table generator for the results. This is activated by clicking the “Tabulate” tool. For the data above, the following window will appear:



To tabulate values, simply tick the appropriate selection boxes then click “OK”. A window will open displaying the tabulated results. If desired, these can be cut and pasted into other applications such as spreadsheets. Note that the tabulated results are split into a “header” section for values which appear only once in the fitted data set and an “outputs” section for data that is individual to each sub-fit in a Global Analysis fit set.

8. Batch Analysis Module

The “Batch Analysis” module allows a fit calculation (up to 5 exponentials) to be performed sequentially on up to 10,000 separate decay curves. Batch analysis is useful when you wish to fit several curves to the same underlying exponential decay model but the curves are expected to have unrelated decay lifetimes.

It can also be used when there are over 100 decays and global analysis cannot be used. It is possible to fix the lifetime values allowing a ‘pseudo global’ analysis to be carried out.

When viewing a batch file, extra navigation toolbar buttons (shown below) allow browsing of the entire decay curve set. The extra navigation tools are shown below:

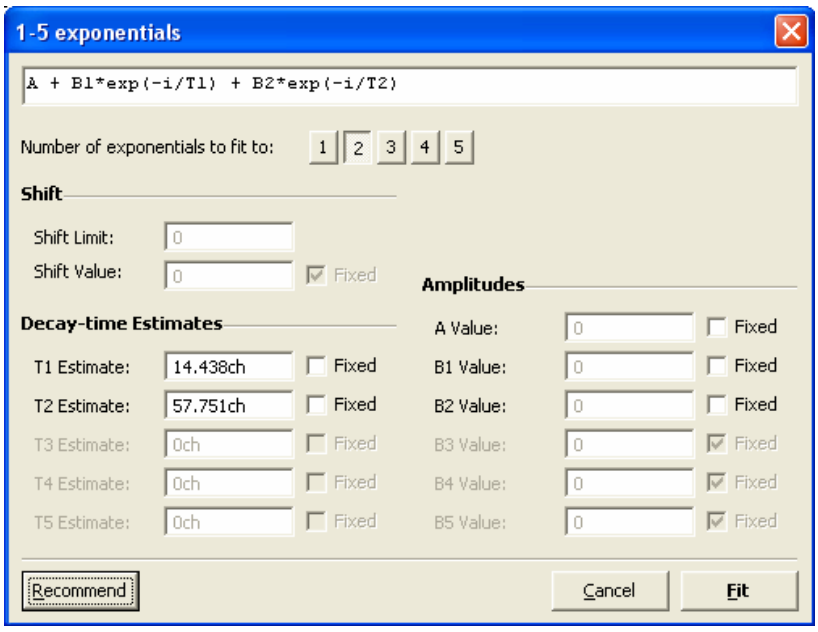
The desired curve can be selected using the arrows or by typing it numerically in the box.

The “Batch Analysis” first allows a test fit to be performed on any curve. If the results of the test fit are adequate then the input parameters can be automatically applied across the entire data set, otherwise the test fit can be discarded. Once a “Batch Analysis” has completed, the fits can be viewed and tabulated in the same manner as shown in the “Global Analysis” module.

An example DAS data file is included in the examples folder and is named “Batch.DAS”. This file contains 9 decays (with no prompt) with different ratios of 1.0 ns and 3.4 ns lifetimes, stepping from 10% (1.0 ns) in curve 1 to 90% (1.0 ns) in curve 9.

1. Select the “Working Data” and set the cursors to 120 and 350.

2. Click the “Fit” button. The parameter input screen will appear.



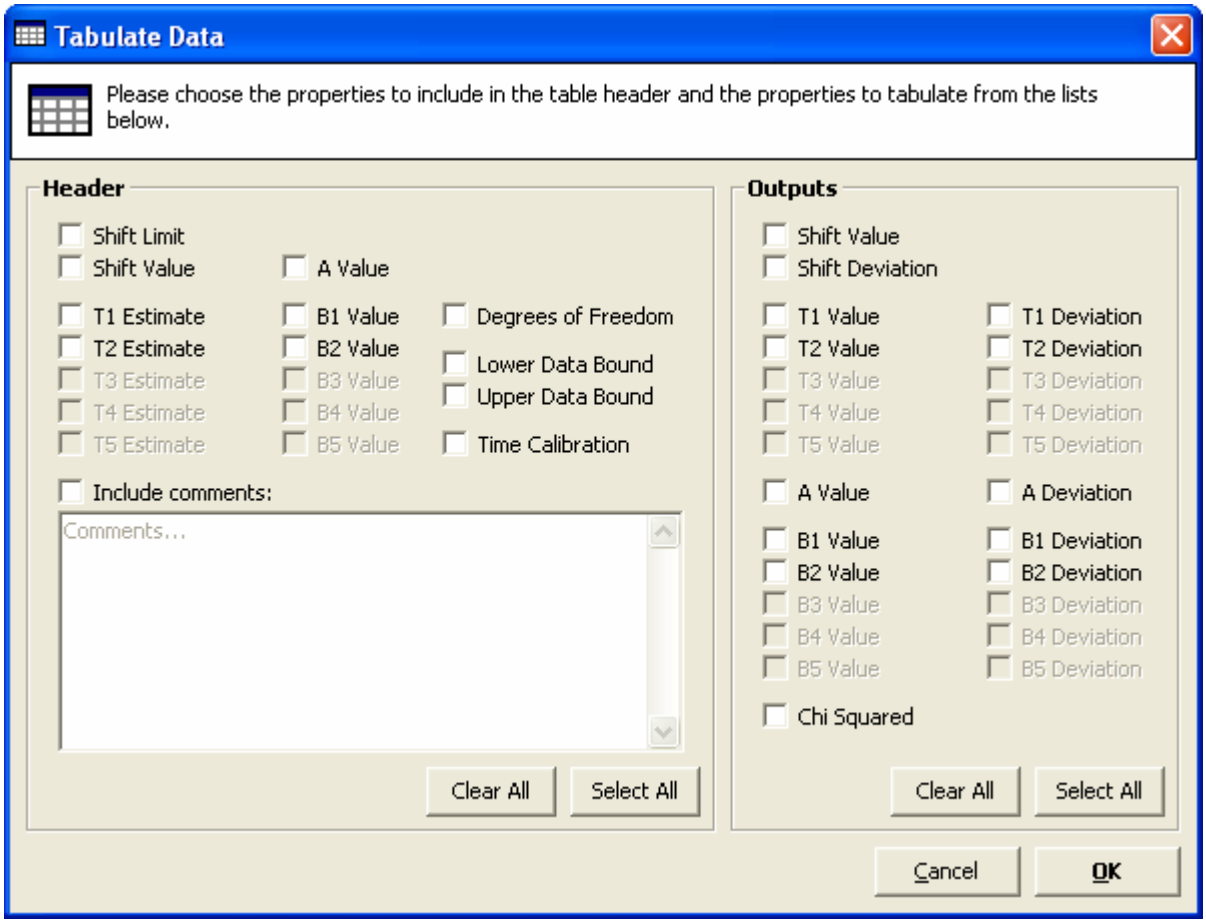
3. Select “2” as the number of exponentials, then click “Recommend” and “Fit”.

4. Click “Use for All” and the fit will run for each curve.

5. Enter a name for the fit, then click “OK”

To view the results for each curve, select the fit in the “Stored Results” and step through the each fit in turn using the arrows on the toolbar. “Open in Window” can be used to view the numerical results for each curve.

However, stepping through the results is not convenient when many curves are involved. We have therefore included a table generator for the results. This is activated by clicking the “Tabulate” tool. For the data above, the following window will appear:



To tabulate the values, simply tick their selection boxes then click “OK”. A window will open displaying the tabulated results. If desired, these can be cut and pasted into other applications such as spreadsheets.

9. Using Microscope Files

DAS has the ability to read and analyse microscope files produced by the HORIBA Scientific **DynaMyc** time-resolved fluorescence microscope and DataStation.

A single *.das* microscope file contains the following data:

- A microscope image
- A collection of X and Y pixel co-ordinates
- A decay dataset for each pixel
- A prompt (optional)

9.1 Analysing a Microscope File

Microscope files can be fitted in one of three ways depending on the fit chosen from the drop-down list at the bottom of the "Information Area" and then clicking the "Fit" button to the right of the list.

1. Batch Fit- This allows a fit calculation (up to 5 exponentials) to be performed sequentially on many separate decay curves. Batch analysis is useful when you wish to fit several curves to the same underlying exponential decay model but the curves are expected to have unrelated decay lifetimes. For more information on this fit technique, see section 8 of this document.

2. Sum Fit- This method first sums all of the decay curves together and the resulting data set is then fitted to determine the lifetimes present. The lifetimes found are then globally applied to all the individual decays allowing the 'B' (amplitude) values to be calculated for each of the curves. This technique allows you to fit the data while using the fact that lifetimes present between decays are linked. To perform a Sum fit, depress the 'Sum' button  on the toolbar then click the "Fit" button at the bottom of the "Information Area"

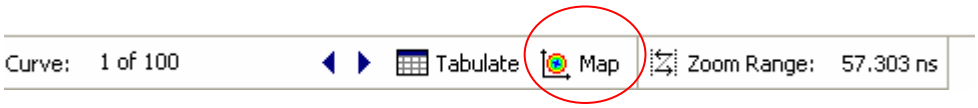
3. Global fit- This fit can only be applied on a file containing less than 100 decays. See section 7 of this manual for more information.

9.2 Viewing the Mapped Data

After a fit has been performed, the resulting fit parameters can be viewed graphically by overlaying them on top of the microscope image. If no fit has been performed yet, the intensity data can still be mapped

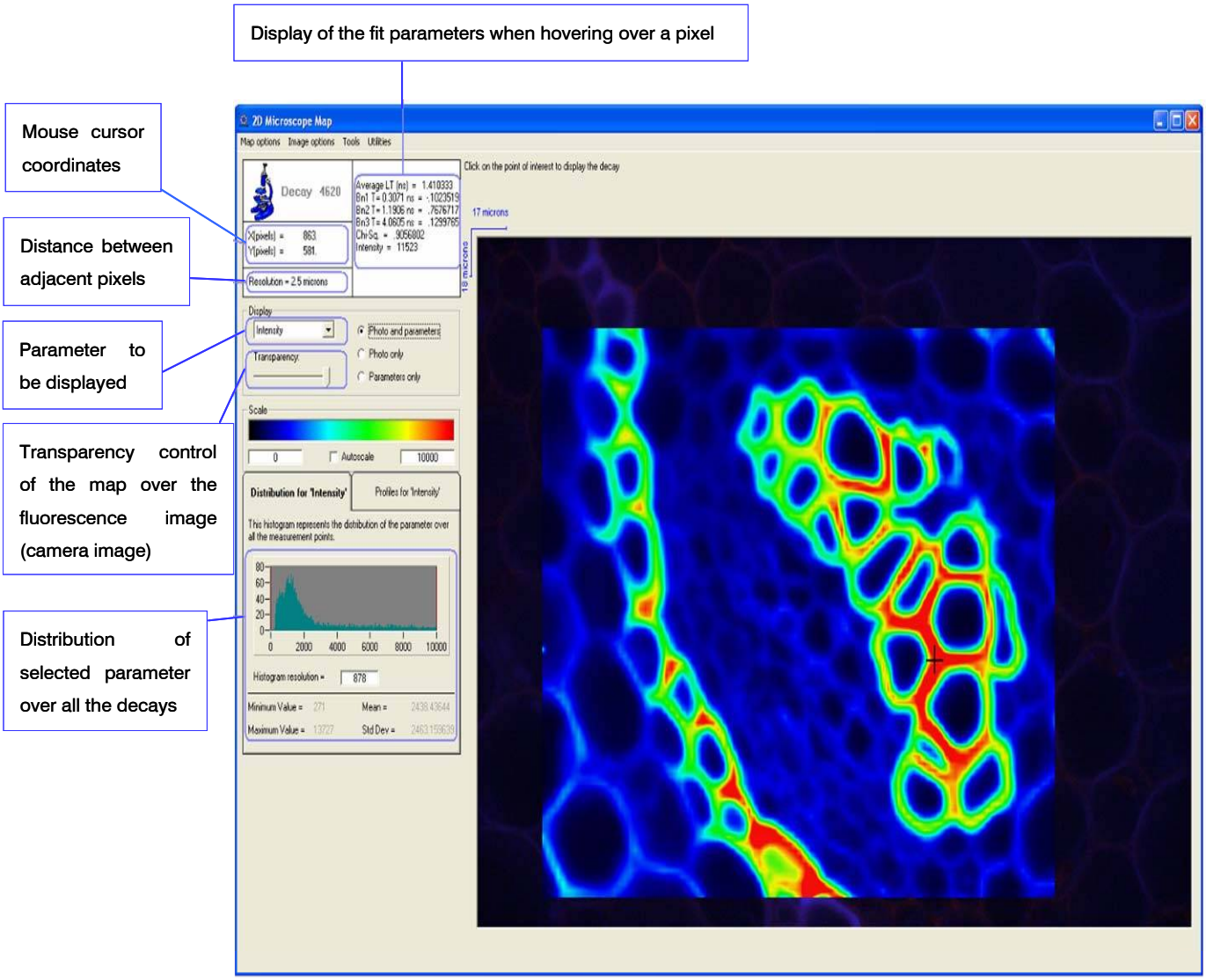
by selecting 'working data' in the tree. Displaying the intensity map can be useful to extract the decays with the highest intensities.

The mapping window is displayed by clicking the 'Map' button in the toolbar at the top of the screen, as shown below.



This displays a window containing the microscope image and a matrix of 'pixels' on top of this. An image of this is shown in below. Features that should be noted in the mapping window:-

- The fit parameter that you wish to analyse should be selected using the drop down box at the top left of the Map window
- The pixels superimposed on the image may vary in colour; this variation represents the relative magnitude of a particular fit parameter.
- Hovering the cursor over a particular individual pixel allows you to view the value of its associated fit parameter at the top of the screen. Also, when clicking on a particular pixel, the fitted lifetime data of the selected pixel is displayed in the Main DAS window behind the Map window.
- Graphs of a selected fit parameter are displayed at the bottom left of the Map window. By default, a graph of the distribution of the values of the parameter is shown. This distribution applies to the values found across all of the pixels plotted on the image.
- Alternatively, by clicking a tab, the distribution graph can be replaced by a graph of the profile of a 'slice' across the image. This graph can display the fit parameter values as a function of either X or Y coordinates across the image.
- Several options are available to customise the view. These options are described in the figure below.



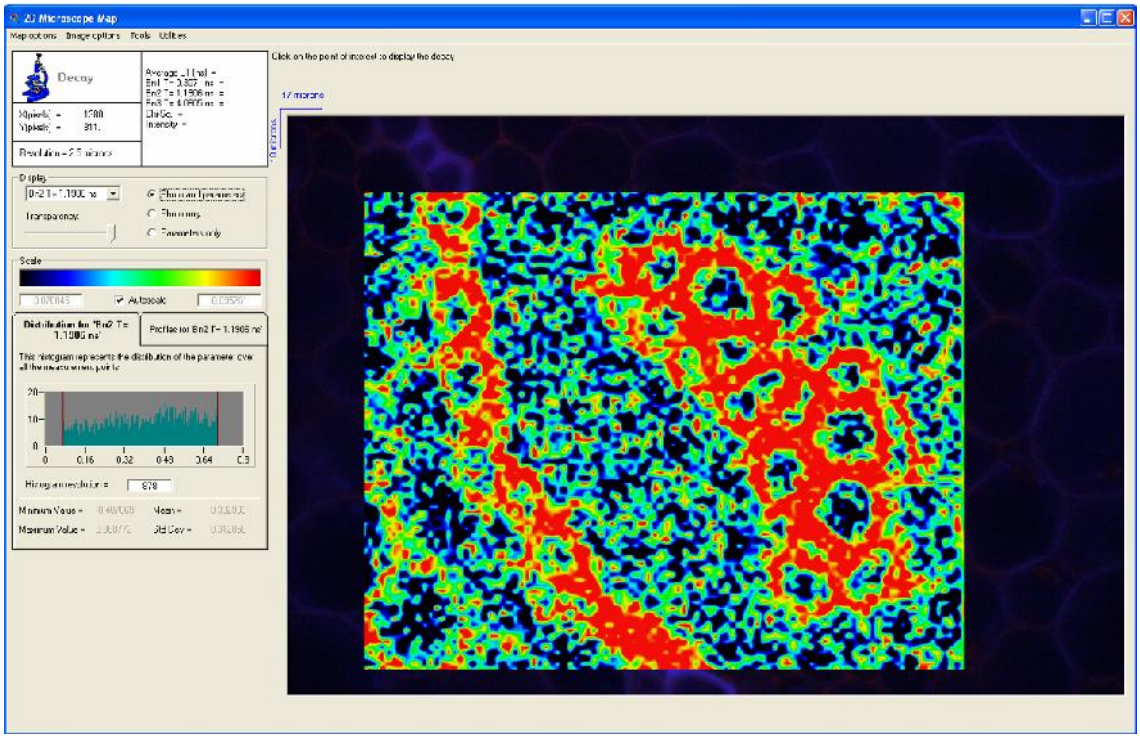
9.3 Menu description

Map options:

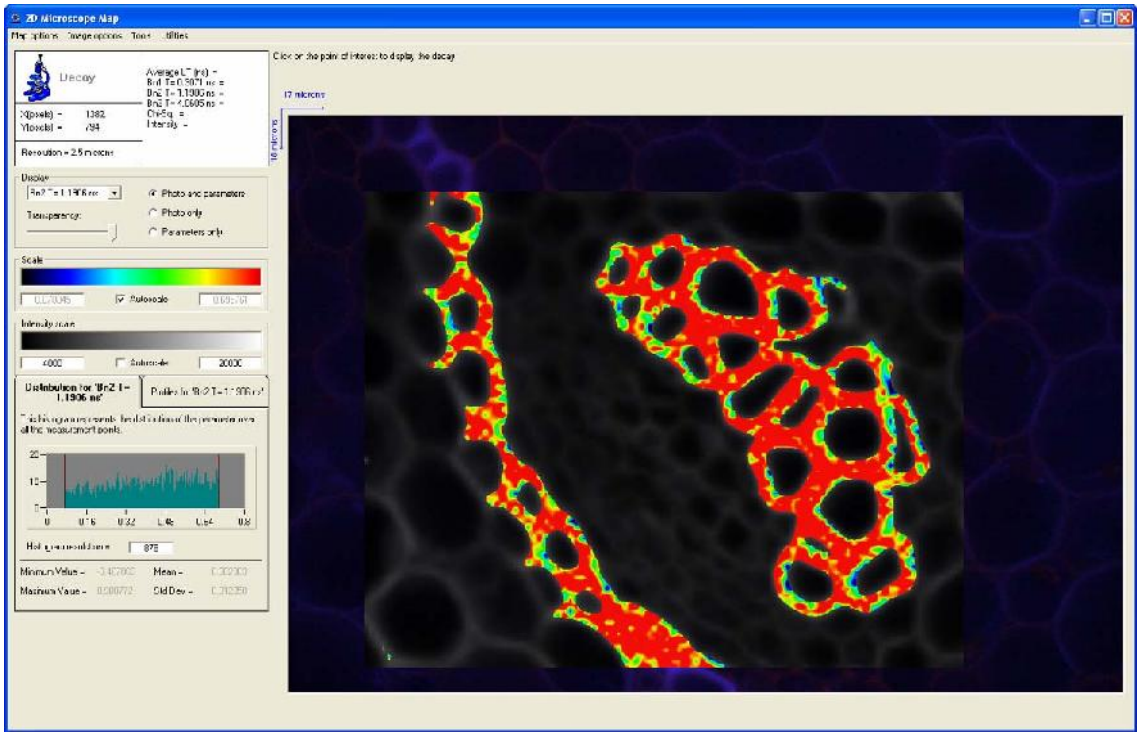
- Colour scale: displays the fit parameter map in false colours

- Greyscale: displays the fit parameter map in grey scale tones

- Intensity threshold: when selecting this option, an intensity scale bar is shown below the main scalebar. The user can select the intensity range of the pixels to be displayed. Any pixels with an intensity outside this range are displayed on a greyscale. This option allows users to 'filter out' low intensity decays, to enhance contrast on the map. See figure below:



(a)



(b)

Figure: Mapping B2 values – (a) No intensity threshold, (b) Intensity threshold only displays the pixels with intensity between 4000 and 20000 photons. For the other pixels, the intensity is displayed in grey tones.

- Pixel shape – squares: displays the decay locations as individual square pixels.
- Pixel shape – disks: displays the decay locations as individual circular pixels.
- Pixel shape – smooth map: the pixels values are interpolated to generate a smoother map.

Image options:

- R, G, B channels: allows users to select which image channels (camera image) are displayed. This option allows unwanted wavelengths to be filtered out which may have been imaged due to poor selection or unavailability of suitable filter cubes for imaging.

Tools:

- Measure distances: displays the actual distance in microns between two points selected by clicking on the image/map.
- Zoom: opens a new map window corresponding to a rectangular area selected by the user on the current image/map.

- FRET: this option allows the user to enter the lifetime of the FRET donor in absence of the FRET acceptor. It is then possible to display maps for the FRET efficiency and the FRET ratio R/Ro. These parameters are defined as:

  FRET efficiency: $E = 1 - (\tau_{D\text{-}A} / \tau_D)$, where $\tau_{D\text{-}A}$ is the donor fluorescence lifetime in presence of the acceptor and τ_D is the donor fluorescence lifetime in the absence of the acceptor.

  $E = 1 / (1 + r/R_o)^6$ where r is the donor-to-acceptor separation distance and R_o is the Förster distance of the donor-acceptor pair, i.e. the distance at which the energy transfer efficiency is 50%.

Utilities:

- Print map: prints the current map display. The user can select to print the map, the statistical distribution and/or the detailed raw data of the map.

- Save map: saves the map as an image file, in bmp, jpeg or gif format.

- Export data: exports the map raw data as a text file.

10. Creating DAS Files

The DAS6 Decay Analysis Software uses a custom file type: ".das". This data set (commonly called a DAS file) allows you to store the results of your fits, alongside the data used to create them, and to access all of the data at any time using HORIBA Scientific software.

There are a variety of ways to produce .das files that can be read by the DAS software. These include:

- Using the DataStation software from HORIBA Scientific (HORIBA Jobin Yvon IBH)
- Importing raw text or IBH binary files into the DAS software using the *File -> New* menu command
- Using the supplied DAS File Conversion Utility (DFCU) to read .das, IBH and raw text files

10.1 Types of DAS File

DAS files can be used to store 5 types of data. These types are listed in Table 1 along with the details of their contents, possible fit types and how to create them.

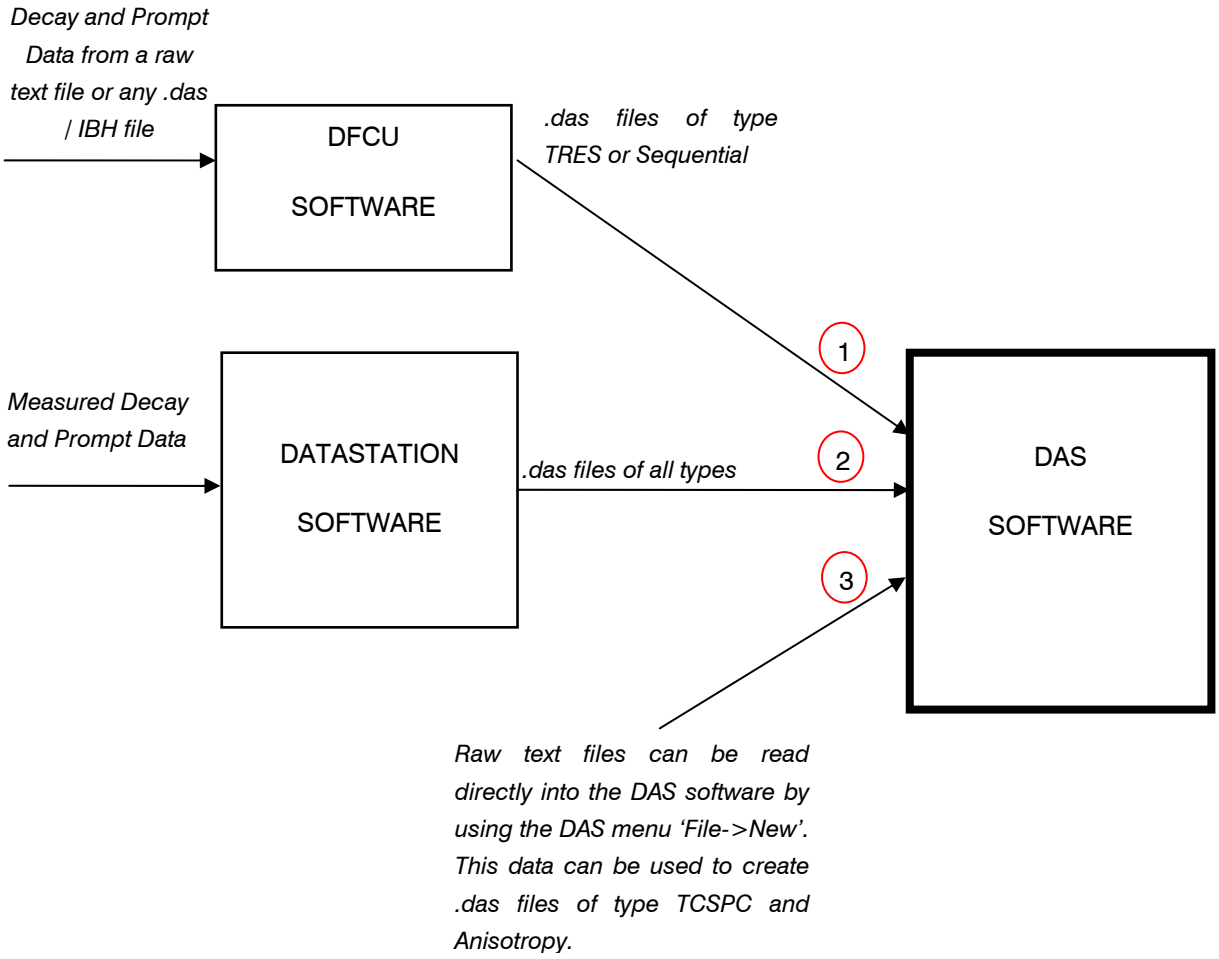
Table 1: The five .das File Types

.das File Type	Contents Of File	How To Create This .das File Type	Fit Modules That Can Be Used With This File Type
TCSPC	Contains data for a single decay and prompt data	Use DataStation or create from within the DAS Software by using raw text files.	Foundation Exponential, Exciplex kinetics, Lifetime distribution, Exponential series, Förster energy transfer, Yokota-Tanimoto energy transfer, Micellar quenching
TRES Data (Time-resolved emission spectra)	Contains data for multiple decays and prompt data	Use DataStation or DFCU utility	Global exponential analysis
Sequential TSPC	Contains data for multiple decays and prompt data	Use DataStation or DFCU utility	Batch exponential analysis
Microscope	Contains data for multiple decays and prompt data as well as images	Use DataStation	Batch exponential analysis, Global exponential analysis
Anisotropy data	Contains decays and prompt data for anisotropy analysis	Use DataStation or create from within the DAS Software by using raw text files	Anisotropy analysis

10.2 Importing Data into DAS

There are a several ways to get data into the DAS software depending on which of the file types you wish to create. The figure below illustrates the possible ways to import data:

Figure a: Methods of importing Data into DAS Software



DAS files are created by default when fluorescence lifetime measurements are made using the Datastation software (method 2). For more information on using Datastation to produce DAS files, please see the Datastation user manual. The remainder of this chapter deals with how to use methods 1 and 3 to import data into the DAS software.

10.3 Creating a Decay Analysis Data Set Using DAS

The creation of a decay analysis data set is described below using the example files “DasPrompt.IBH”, “DasDecay.IBH” and “DasFile.DAS”. These are respectively a prompt, decay and the resulting DAS file.

1. Start DAS and click on the “New” tool.

2. In the pop-up window select “TCSPC” and then click “New”.

3. Click “Browse” to open a file explorer window. Note that IBH is the default file type listed in the “Files of Type” box. However, opening the drop down menu for this box you will see that various other file types are supported (including simple text files).

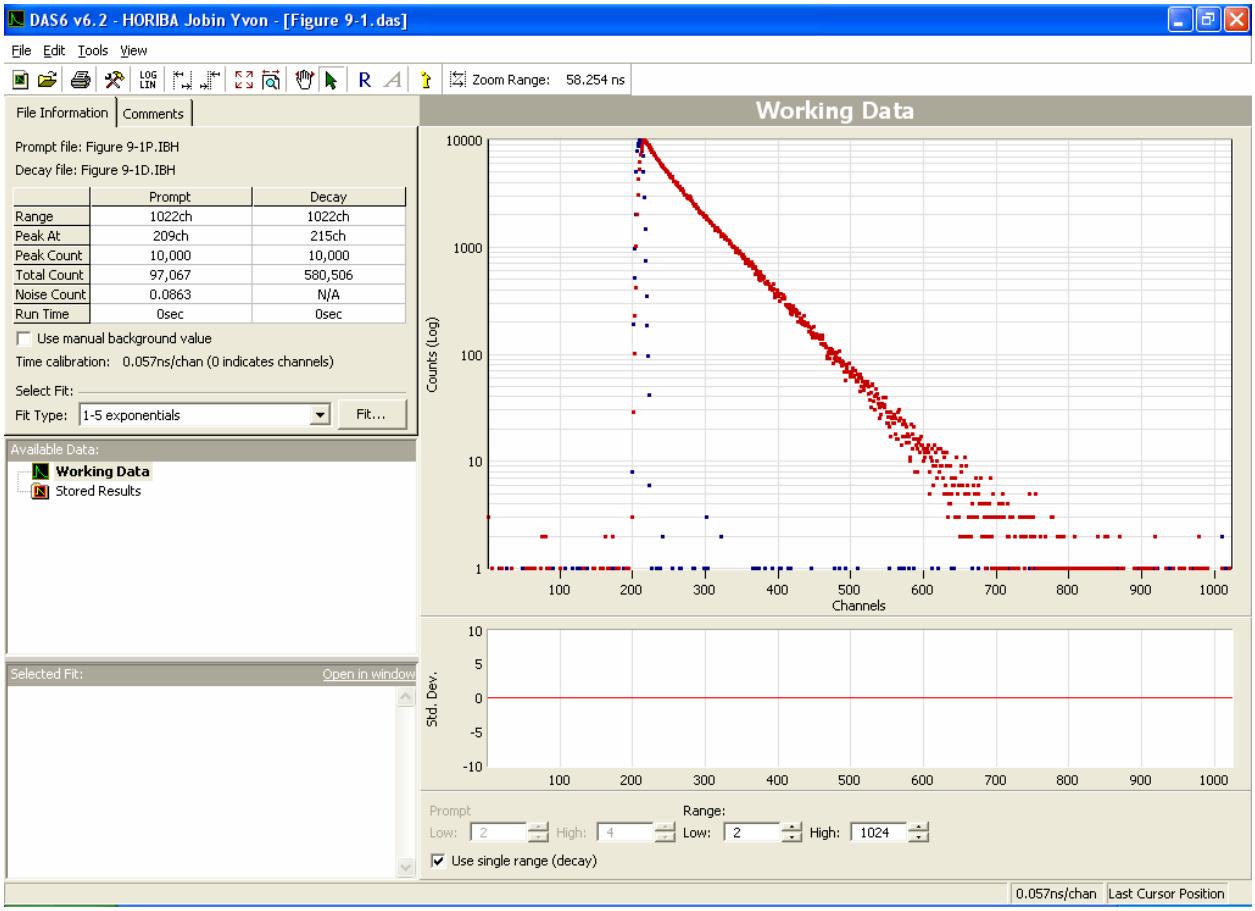
4. Use the file explorer to find and select the file “DasPrompt.IBH”.

5. You have just selected the prompt file. In the pop-up window, click “Next” then repeat the process to select file “DasDecay” to select the prompt.

6. Click “Next” and then, in the pop-up window enter a name for the DAS data set. In this example type “DasFile”. The resultant .DAS file will be saved in a location that can be set by the user in the Tools->Settings->Folder window. The default location is to save the newly created file in the same folder as the imported decay data.

7. Click the “Finish” button to complete the process.

The screen will appear as shown below, and the new data set will be ready for use.



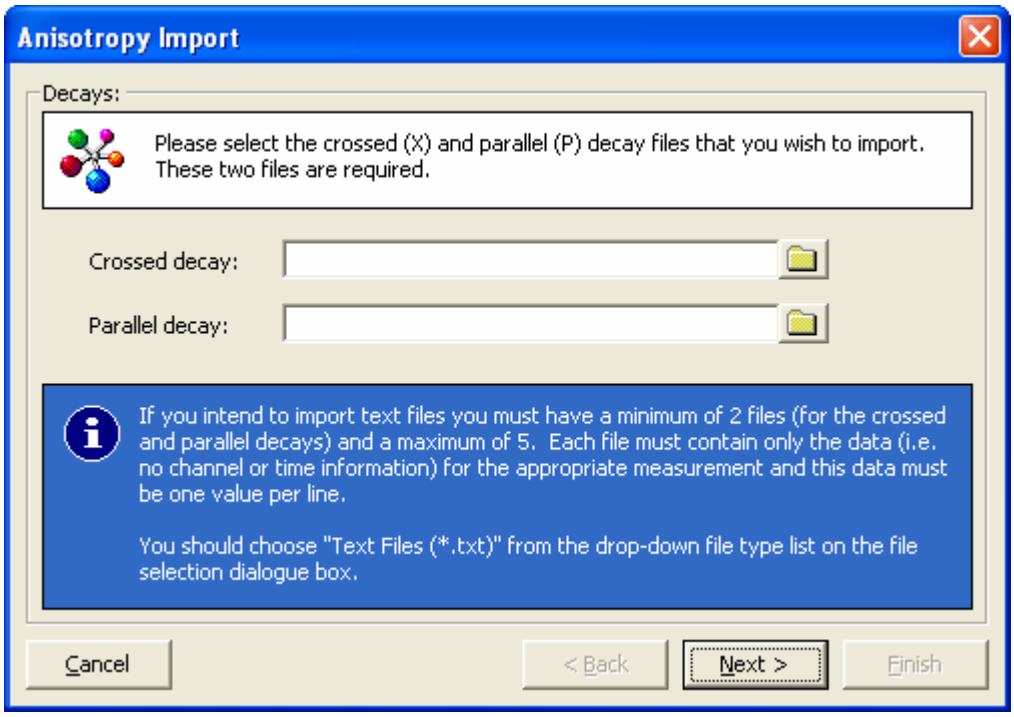
Note that, because one of the IBH files contains a time calibration, this information was transferred automatically to the new DAS file. When importing some other types of files, an extra step to enter the time calibration may be included. Alternatively the time calibration can be entered (or changed) from the DAS6 “Edit/Time Calibration” menu selection.

Finally, selection of a prompt in steps 4 and 5 above is optional (select ‘skip’), but selection of the decay is mandatory.

10.4 Creating an Anisotropy Data Set Using DAS

Creation of an anisotropy data set is very similar to the process described in section 10.1 for a decay analysis data set. However, in this case these can be between two and five files involved.

1. Start DAS and click on the “New” tool.
2. In the pop-up window select “Anisotropy” and then click “New”. A pop-up window will appear as shown below.
3. Select decay files containing the crossed decay *Yvh(i)* and the parallel decay *Yvv(i)*. Selection of files for these is compulsory.
4. Click “Finish” if no other data needs to be entered, or “Next” to proceed to the next data input screen,
5. Optionally, select a prompt file then click “Finish” or “Next”.
6. Optionally, select files for calculation the g-factor then click “Finish”.

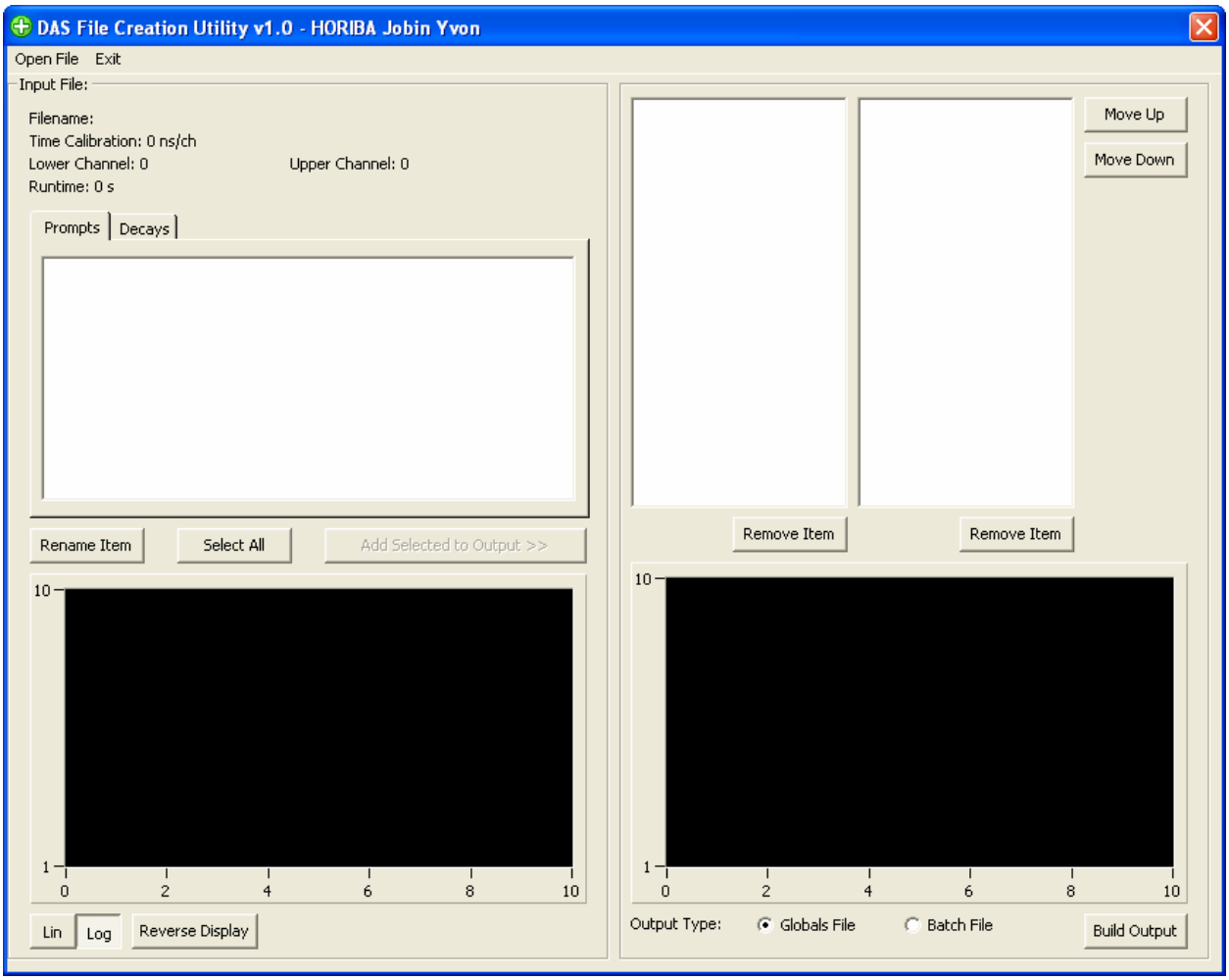


When “Finish” is selected, enter a filename for the data set. The data set will then be ready for use.

10.5 Creating Batch & Global Data Sets Using the DFCU Utility

Batch and global data sets are generated using the “DAS File Creation Utility” or DFCU for short. The DFCU is a versatile program which opens input files (in the left boxes) and allows movement of the data into an output file. The right boxes show the data that will be output.

The DFCU opening screen is shown below.



Important areas are:

“Open File” click it to open a new file and view its contents in the left boxes. You can open various file formats including DAS, IBH and text.

“Exit” closes the application.

“Prompt” and “Decay” tabs enable selection of prompts and decays in the open input file.

“Add Selected Item” – includes a decay (or prompt) in the output list.

“Output Type” – radio buttons which choose either batch or global format for the output file.

“Build Output” – button which starts the process of building the batch fit (sequential TCSPC) or global fit (TRES) output file. A filename should be input for the resulting DAS file.

The order of items in the output list can be adjusted with the Move Up/Down buttons, items can also be removed from the list.

10.6 Using Text Files with DAS6

DAS6 can import data from text files in addition to the older IBH v4.2 binary format prompt and decay files. The text files must contain only the channel data (without channel numbers or any time information) and be one data value per-line. All text data files are assumed to start from channel number 0. The first two data points will be lost in the conversion, if these channels are important to the data then you should pad the data with two leading zeros as the first two data lines in the file.

To import a text file you should create a new DAS file as normal but select “Text Files (*.txt)” from the file open dialogue box. If the imported file does not conform to the above specification an error message will be displayed. When loading decay data from a text file you will be prompted as to whether you want Poissonian (for photon counting data) or Gaussian variances generated from the data. Appended variance data is not supported.

The easiest way to get the data from other file types into DAS6 is to load your raw ASCII data into a spreadsheet (e.g. Microsoft Excel) and then trim any excess text before saving the file. The saved file can then be imported into DAS6.

10.7 Custom Filters

Custom file loading filters can be incorporated into the DAS6 software for ease of use. Please contact HORIBA Scientific IBH if you are interested in using this service.

The current release imports:

- IBH v4.2 files (from the older IBH MS-DOS software)
- Text files (as described above)
- Edinburgh Instruments Ltd. text files (*.DAT) that can be exported from EI software.
- Globals Unlimited files. These contain both prompt and decay information and should be imported as prompt files.
- Ortec Maestro Integer CHN files.

11. When a Prompt cannot be measured

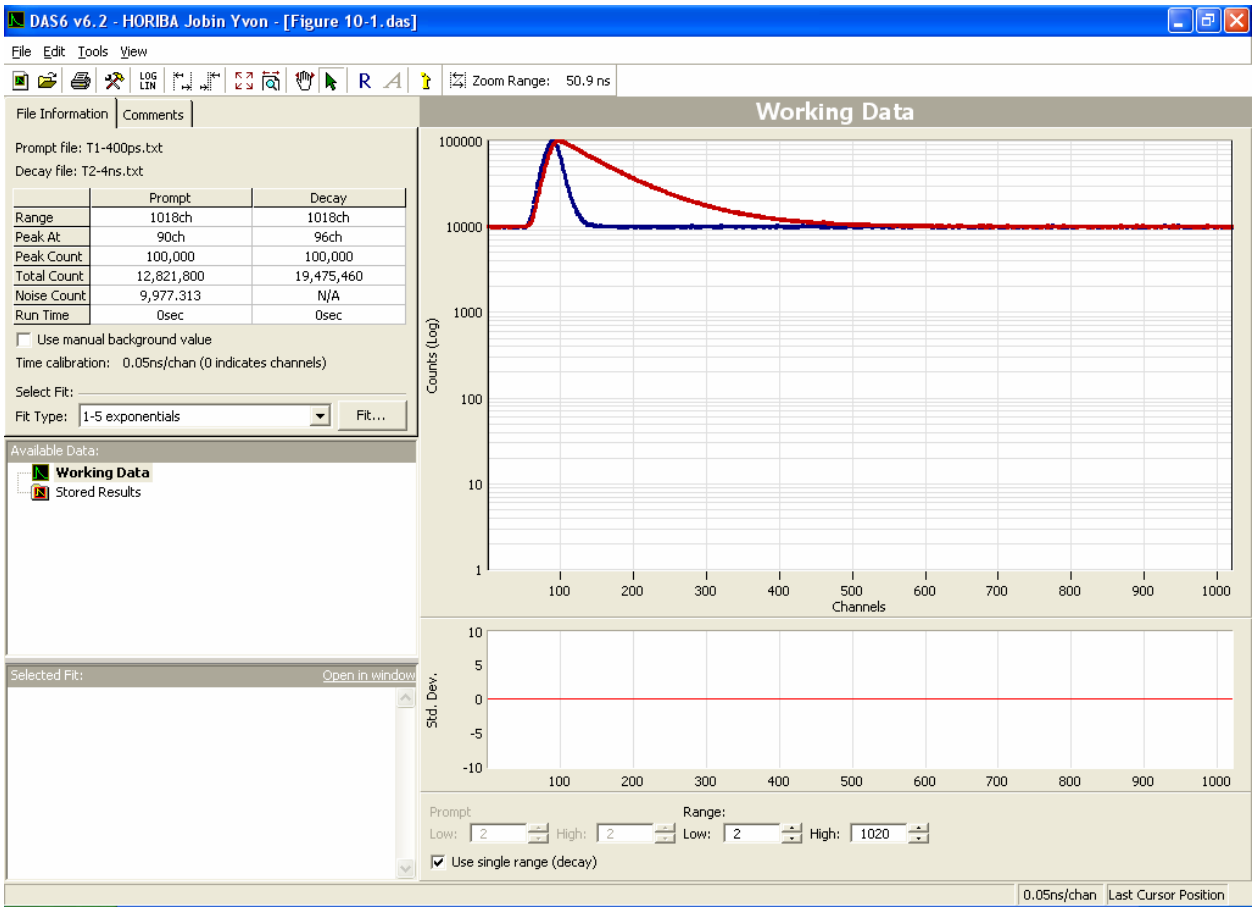
There are situations when it is not physically possible to measure a prompt. Two examples of this are:

- When UV illumination (e.g. from a 260nm nanoled) is used to excite a sample but the detection photomultiplier tube has a glass window and therefore has no sensitivity at the excitation wavelength.

- When visible illumination is used to excite a dye which fluoresces in the NIR and the detection photomultiplier tube has no sensitivity in the visible (e.g. Hamamatsu H10330 which has a cut-off at 950nm).

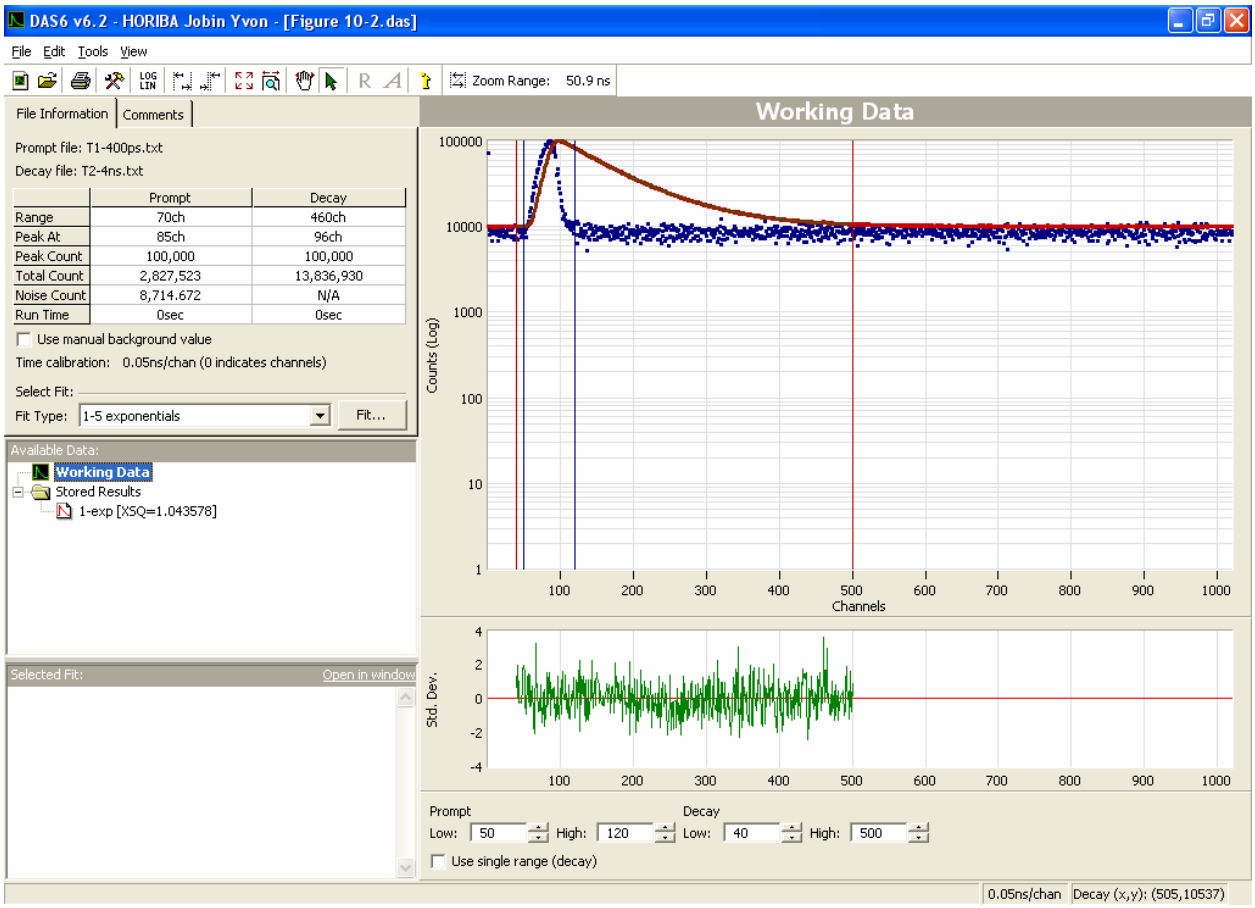
Two options are available to provide an estimated 'prompt'. One option is to take a measurement using a dye with a very short wavelength and use this as a prompt. The other procedure is to measure the fluorescence from a dye with a known lifetime and then recover the prompt using a fast Fourier transform (FFT).

An example dataset is shown below.



Note that this data set has a high background and is typical of the type of noisy data obtained when measuring using NIR photomultiplier tubes. The blue curve is not a prompt but instead represents measurement of a NIR dye with a lifetime of 400ps. The red curve is the normal (unknown lifetime) dye measurement, which in this example has a lifetime of 4ns.

The first stage in the analysis is to use the "Tools → Deconvolute Prompt" menu item to recover a prompt. The function asks for a lifetime value for the dye, which in this case is 0.4ns and it then recovers the prompt as shown below.



The figure above shows an example fit. Note that the blue "prompt" is now narrower than before the FFT procedure and note also that it is noisy. In cases such as this it is always best to use separate ranges for the "prompt" and "decay" (as indicated by the blue and red cursors). It is also advisable to use a manual lifetime estimate rather than the automatic "Recommend" value which can be affected by noise.

The lifetime found in this example is 4.02ns, which is in close agreement with the expected value of 4.00ns.

Finally, because the FFT process, introduces noise, it is recommended to measure the "prompt to higher precision than usual.

Appendix A.
Fundamentals of Decay Curve Analysis

A.1 Introduction

In general, the aims of statistical data analysis are:

a) To reduce lots of data to a few parameters

b) To get maximum useful information from hard-won data

c) To compare theory and experiment

d) To test the appropriateness of competing theories

Statistical methods of data analysis play a key role in time-domain spectroscopy, because the interest is in the parameters of the decay-curves, not in the raw data.

A.2 Decay Curve Measurement

Figure A1.1 shows a typical decay curve. Analysing such data in terms of, for example, an exponential decay law of the form:

$$\begin{aligned} F(t) &= 0 & t < 0 \\ F(t) &= \frac{1}{\tau}\exp\left(-t/\tau\right) & t \geq 0 \end{aligned} \qquad \text{(A.1)}$$

is not straightforward, because several instrumental factors need to be taken into account:

a) The measurement quantises the smooth fluorescence intensity function into discrete data channels

b) There is a noise background beneath the signal

c) The instrument itself distorts the decay curve

d) The location of the "*t=0*" point of equation A.1 is itself unknown. In figure A.1, for example, the "*t*=0" point is situated somewhere around channel number 60

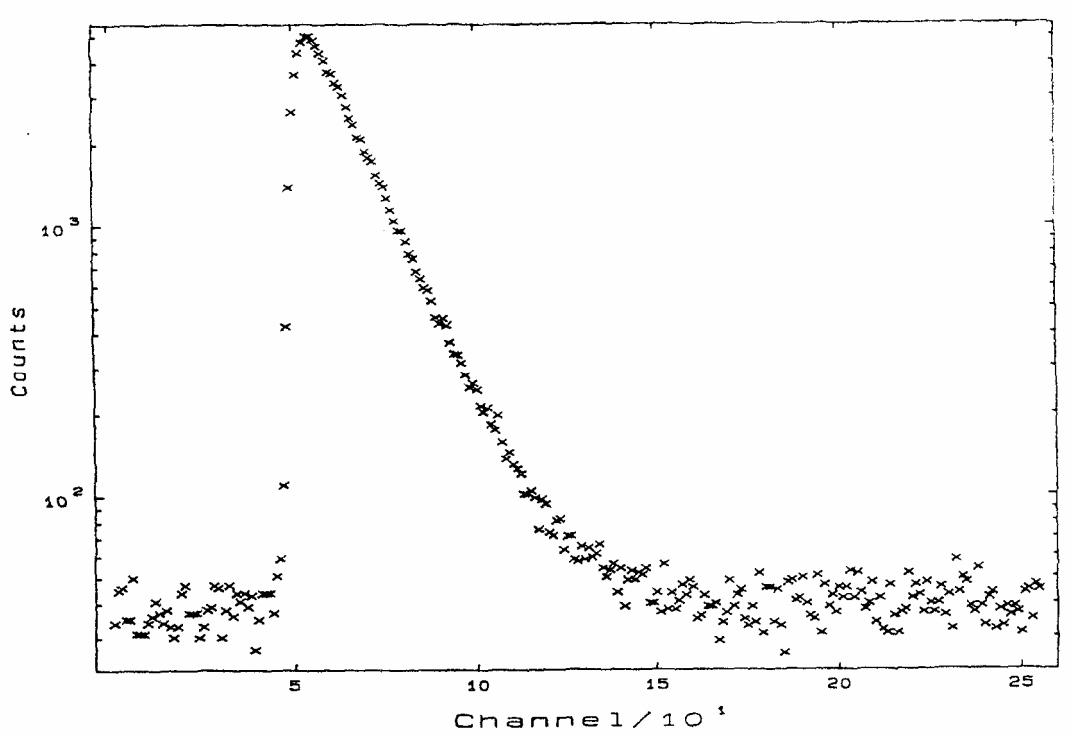


Figure A.1: Fluorescence decay data

Evaluating the function as stepped averages over individual data channels can accommodate point (a). The noise background (b) can be included in the fitting function as an additional unknown parameter. The effect of instrumental distortion (c) in the fluorescence signal is represented mathematically by the convolution integral, e.g.:

$$F(t) = \int_{-\infty}^{\infty} P(t - t')\ f(t')\ dt' \qquad \text{(A.2)}$$

Where *P(t)* is the *prompt* response of the instrument, i.e. the response in the limit $\tau \to 0$. This prompt response needs to be known, if we are to use equation A.2 in the analysis of fluorescence decays. In practice, we normally replace the sample with a scatterer, re-tune the monochromator and measure an *instrumental profile* (also known as the *prompt*), as illustrated in figure A..2.

This, of course, is an imperfect measure of *P(t)*, because it is measured at the *wrong* wavelength, it is quantised and noisy, etc. Nevertheless, the use of prompts in decay curve analysis does bring very substantial benefits in terms of time resolution and the ability to fit meaningfully to complex decay models.

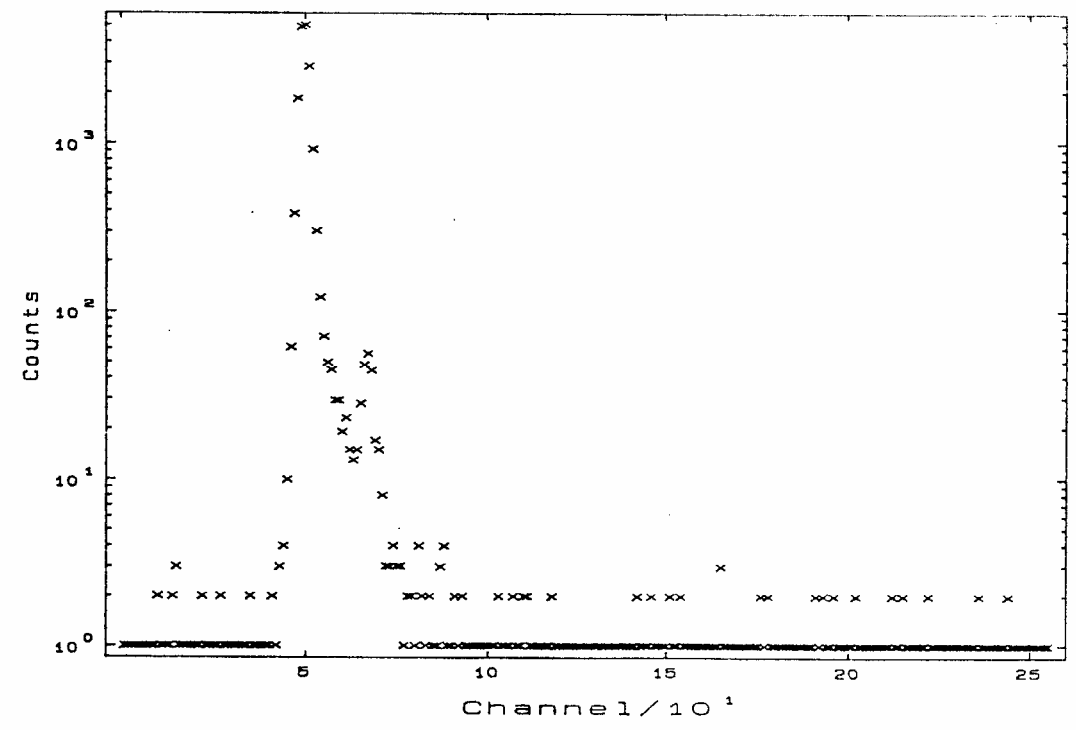


Figure A.2: Lamp profile (prompt)

A.3 Reconvolution Analysis

In reconvolution analysis, the convolution is applied to the theoretical model prior to comparison with the decay data. Of course, sums have to be used (numerical convolution) rather than integrals, because of the quantisation of the data. The procedure is illustrated in figure A.3 below:

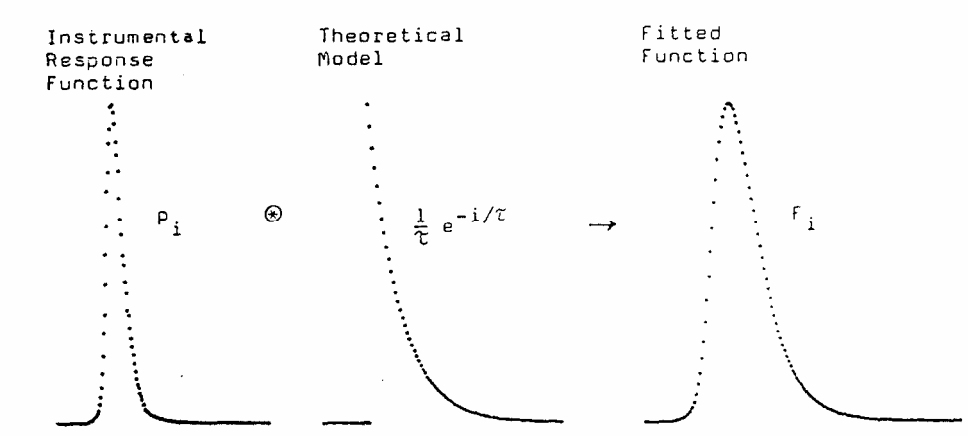


Figure A.1.3: Numerical convolution

Why this way? Why reconvolute rather than deconvolute? If one could deconvolute the data, then comparison with the kinetic model would be very direct. The answer is stability. Deconvolution of experimental data is an ill-posed problem, because the effect of convolution is a smearing out, and therefore a loss of information. You cannot regain the lost information by reversing the action. What you can do is to apply a similar smearing action to the kinetic model, prior to comparison with the experimental data. This is reconvolution.

A.4 Shift

The calculation of the fitted function, as in figure A.3 also determines the position among the data channels of time *t=0* (section A.2, point (d)), since this is directly related to the position of the prompt *P(i)*. However, a small mis-match in positioning is inevitable, because of quantisation, noise and wavelength effects, etc., inherent in the less than perfect representation of the instrumental response by the prompt. Since model functions, such as equation A.1, have their most rapid change at *t=0*, a small positioning error can have a significant effect on the fit.

A.5 The Fitted Function

Finally, what started out as a simple exponential decay, now has the form:

$$F(i) = P(i) \otimes \frac{1}{\tau}\exp\left(-i/\tau\right) \qquad \text{(A.3)}$$

from the numerical convolution (represented by $\otimes$), and

$$F_D(i) = A + B.\,F(i+\Delta) \qquad \text{(A.4)}$$

for fitting the data. The integer "*I*" is used to denote the data channels. The shift parameter Δ is symbolic in this notation since you cannot have non-integral data channels. In practice a spill-over algorithm is used for shifting. The parameter "A" is the noise background and "B" is an amplitude scaling factor.

A.6 The Method of Least Squares

Equation A.4 now needs to be fitted to the fluorescence decay data *Y(i)* of figure A.1. The method used is the *method of least* squares, which uses a quantity χ^2 as a measure of mis-match between data and fitted function. A discussion of the method of least squares can be found in almost any textbook on statistics. In our example:

$$\chi^2 = \chi^2(A, B, \tau, \Delta) \tag{A.5}$$

The method strives to determine the *best fit parameters* A', B', τ', Δ', that will yield the lowest possible value of χ^2.

A.7 The Meaning of χ^2

By definition,

$$\chi^2 = \sum_N \left[\frac{Y(i) - F_D(i)}{\sigma(i)} \right]^2 \tag{A.6}$$

$Y(i)$ = fluorescence decay data (e.g. figure A.1)
$F_D(i)$ = fitting function (e.g. equation A.1)
$\sigma(i)$ = statistical uncertainty of the datum value $Y(i)$, (i.e. its standard deviation)

The sum includes the *N* data channels selected for analysis. For any given value of *i*, the numerator represents the actual deviation between the datum value *Y(i)* and the corresponding fitting function value $F_D(i)$. The denominator represents the deviation expected from statistical (noise) consideration. Equation A.6 can therefore be written as:

$$\chi^2 = \sum_N \left[\frac{\text{actual deviation}}{\text{expected deviation}} \right]^2 \tag{A.7}$$

Now, if the fitting function is appropriate, then the minimum should be characterised by equality between actual and expected deviations. The ratio (equation A.7) is then, on average, unity for each term of the sum and:

$$\chi^2 = N \tag{A.8}$$

This is not quite right for two reasons:

a) The *expected deviation* is a statistical expectation, and therefore the mean value of a statistical distribution. This necessarily makes χ^2 a statistical quantity with an associated distribution function.

b) The number of fitted parameters, *n*, needs to be taken into account. The quantity $\nu = (N-n)$ is called the number of *degrees of freedom*, and should be used in place of *N*.

A more accurate, though less precise statement of equation A.8 therefore is:

$\chi^2 \approx \nu$ for a good fit

It is customary and convenient to normaliseχ^2, so that its value is independent of ν:

$$\chi^2_\nu = \chi^2 / \nu \approx 1 \text{ for a good fit} \quad \text{(A.9)}$$

In photon counting experiments, the *expected deviation*, $\sigma(i)$, can be estimated from the data, using the Poissonian distribution. Thus:

$$\sigma(i) \approx \{Y(i)\}^{\frac{1}{2}} \quad \text{(A.10)}$$

A.8 Local and Global χ^2

In equation A.6, χ^2 was defined as a sum over the individual data points of a fluorescence decay. It is straightforward to extend this definition to include several such local sums, $\chi^2 L_1$, $\chi^2 L_2$ in a global $\chi^2 G$:

$$\chi^2 G = \chi^2 L_1 + \chi^2 L_2 + \ldots \quad \text{(A.11)}$$

Global, rather than individual χ^2-minimisation makes sense for families of decay curves with family characteristics. Monomer/excimer decays, for example, should show amplitude changes but not decay time changes with emission wavelength. You can therefore fit a whole family of excimer decay curves at different wavelengths using the same decay times for all of them, but allowing for individual amplitudes. Constraining the decay times in this way can often make a difference between *curve representation* and kinetic sense!

The “Global Analysis Module” the HORIBA Scientific IBH library can work with a family of up to 100 decay curves, each with a corresponding instrument response function. Up to five family decay times can be fitted, with individual amplitudes and noise backgrounds.

A.9 The Fitting Procedure

This consists of finding those values of the parameters (e.g. equation A.5) that will yield the lowest possible value of χ^2. In practice the procedures look for a χ^2-minimum, which is not necessarily the same thing.

Two basic procedures exist: liner and non-linear analysis. *Linear analysis* is restricted to linear coefficients of the fitting function (e.g.*A*, *B* of equation A.5). *Non-linear analysis* works with any kind of parameter. Since the Jobin Yvon IBH programs use both methods, it is instructive to go through both, again using the single exponential function as an example.

Linear Analysis

From equations A.4 and A.6:

$$\chi^2 = \sum_N w(i)\left[Y(i) - A - B\,F(i+\Delta)\right]^2 \qquad \text{(A.12)}$$

where $w(i) = \left[\sigma(i)\right]^{-2}$ is the *data weight*.

Since a minimum is characterised by a zero value for the gradient, we can find the best fit values *A'* and *B'* by differentiation of equation A.12:

$$\frac{\partial \chi^2}{\partial A} = -2\sum_N w(i)\left[Y(i) - A - B\,F(i+\Delta)\right] \qquad \text{(A.13)}$$

and

$$\frac{\partial \chi^2}{\partial B} = -2\sum_N w(i)\,F(i+\Delta)\left[Y(i) - A - B\,F(i+\Delta)\right] \qquad \text{(A.14)}$$

These gradients are zero for *A*=*A'* and *B*=*B'*. Equations A.13 and A.14 can therefore be reduced to the matrix equation:

$$\begin{pmatrix} \sum w(i) & \sum w(i)F(i+\Delta) \\ \sum w(i)F(i+\Delta) & \sum w(i)F^2(i+\Delta) \end{pmatrix} \begin{pmatrix} A' \\ B' \end{pmatrix} = \begin{pmatrix} \sum w(i)Y(i) \\ \sum w(i)Y(i)F(i+\Delta) \end{pmatrix} \qquad \text{(A.15)}$$

All the sums can be evaluated for given values of τ and Δ, thus *A'* and *B'* can be found by matrix inversion.

Non-Linear Analysis

This is a polite phrase for brute force. You simply plug numbers into equation A.12 until you find the χ^2-minimum. The search strategy is a compromise between speed and stability. In simple cases, when the parameters are independent of each other and the χ^2 hypersurface is smooth, then stability is no problem and a search algorithm can be optimised for speed. In the real world, however, parameters may correlate and may need constraining. χ^2 hypersurfaces may be unsymmetrical, bounded by asymptotic flanks, structured and rough! No sensible search algorithm can cope with all situations.

In the HORIBA Scientific IBH algorithm, each parameter is iterated in a hierarchy, such that χ^2 is evaluated for several values of each parameter, as shown for τ in figure A.4 (circles). Once the minimum has been traversed, the last three points are used in a parabolic interpolation to find the best value for the parameter. χ^2 is re-evaluated at this point (square). The statistical uncertainty of the parameter is then determined from the width of the χ^2 parabola. This search strategy is very stable, because only one parameter is altered at any one time, and because care is taken to limit the step length. Careful fine-tuning of the code has resulted in fast execution times – a factor that can become important when fitting complex decay models.

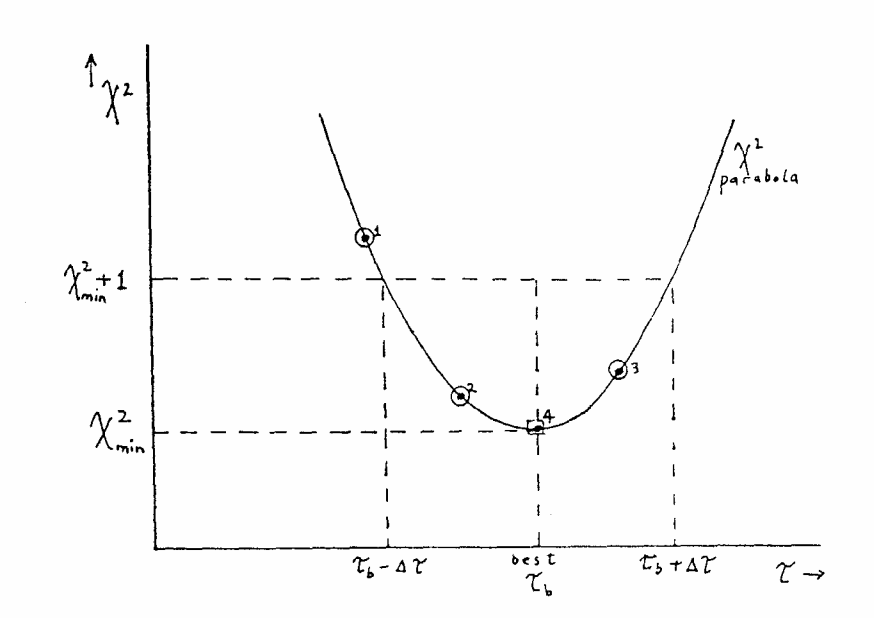


Figure A.4 Non-linear minimisation

The linear parameters are evaluated afresh at each step of the non-linear search, thus ensuring that the χ^2 minimum, when reached, is a true minimum with respect to all the parameters of the fitted function. Since the matrix inversion for the linear parameters is quick, this does not unduly slow down the fitting procedure. Indeed, many fitting programs based on the Marquardt algorithm make no distinction between linear and non-linear parameters. Such programs can therefore be slower to converge with complex decay models, despite the intrinsic speed (and consequently poorer stability) of the algorithm itself.

A.10 The Search Path

The HORIBA Scientific IBH algorithms search for the best parameters using the combined linear and non-linear techniques described in section A.9. The search path for a single exponential component is illustrated below:

```
Successive iterations give:-
   With T= 12.105,  SFT= 0.210 and XSQ= 3.512
   With T= 12.650,  SFT= 0.153 and XSQ= 3.798
   With T= 11.561,  SFT= 0.262 and XSQ= 4.136
   With T= 12.206,  SFT= 0.199 and XSQ= 3.498
```

In each line, a different value of T ($=\tau$) is tried. The XSQ ($=\chi^2_v$) value found for this value of T, with the other parameters (A, B, SFT ($=\Delta$)) at their corresponding optimum values is given on the right. Each line therefore summarises the results of a non-linear search for SFT, with matrix inversions for A and B at each step. The first three lines of the printout define a minimum in XSQ vs T. The fourth line then gives the parabolically interpolated best value of T.

In more complex cases, a similar hierarchy of iterative search loops is used. The sample printout below is for a two-component exponential fit:

```
Successive iterations give:-
   With T1= 2.400,   T2= 13.000;   SFT=  0.015 and XSQ= 0.955
   With T1= 2.400,   T2= 13.762;   SFT= -0.083 and XSQ= 2.016
   With T1= 2.400,   T2= 12.238;   SFT=  0.130 and XSQ= 1.877
   With T1= 2.400,   T2= 12.973;   SFT= -0.005 and XSQ= 0.943

   With T1= 2.493,   T2= 12.973;   SFT= -0.039 and XSQ= 0.950
   With T1= 2.493,   T2= 13.053;   SFT= -0.047 and XSQ= 0.991
   With T1= 2.493,   T2= 12.894;   SFT= -0.032 and XSQ= 0.950
   With T1= 2.493,   T2= 12.814;   SFT= -0.025 and XSQ= 0.965
   With T1= 2.493,   T2= 12.904;   SFT= -0.033 and XSQ= 0.950

   With T1= 2.307,   T2= 13.043;   SFT=  0.036 and XSQ= 0.959
   With T1= 2.307,   T2= 13.119;   SFT=  0.021 and XSQ= 0.969
   With T1= 2.307,   T2= 12.966;   SFT=  0.050 and XSQ= 0.968
   With T1= 2.307,   T2= 13.041;   SFT=  0.036 and XSQ= 0.959

   With T1= 2.418,   T2= 12.960;   SFT= -0.014 and XSQ= 0.941
   With T1= 2.418,   T2= 13.041;   SFT= -0.022 and XSQ= 0.950
   With T1= 2.418,   T2= 12.878;   SFT= -0.004 and XSQ= 0.958
   With T1= 2.418,   T2= 12.972;   SFT= -0.014 and XSQ= 0.941
```

A.11 Weighted Residuals

A typical analysis, a double exponential fit, is shown in figure A.5. The same data analysed using a single exponential model is shown in figure A.6. The crosses are fluorescence data *Y(i)*. The line through them is the best-fit function $F_D(i)$. The lower display box shows the *weighted residuals*, *R(i)*, defined as:

$$R(i)=\left(\frac{Y(i)-F_D(i)}{\sigma(i)}\right)=\left(\frac{\text{actual deviation}}{\text{expected deviation}}\right) \tag{A.16}$$

comparison with equations A.6 and A.7 shows that:

$$\chi^2=\sum_N[R(i)]^2 \tag{A.17}$$

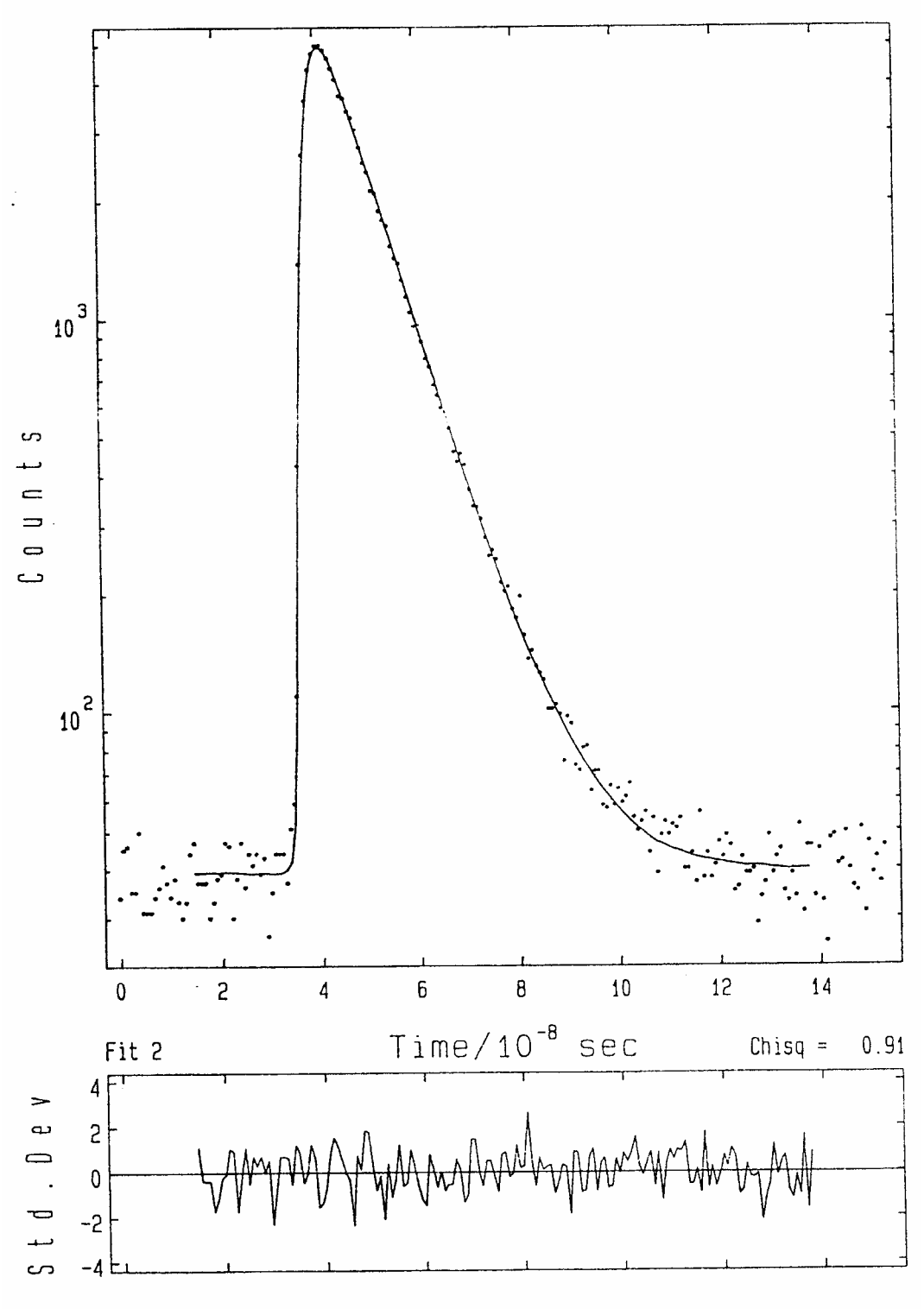


Figure A.5: Analysis with two-exponential components

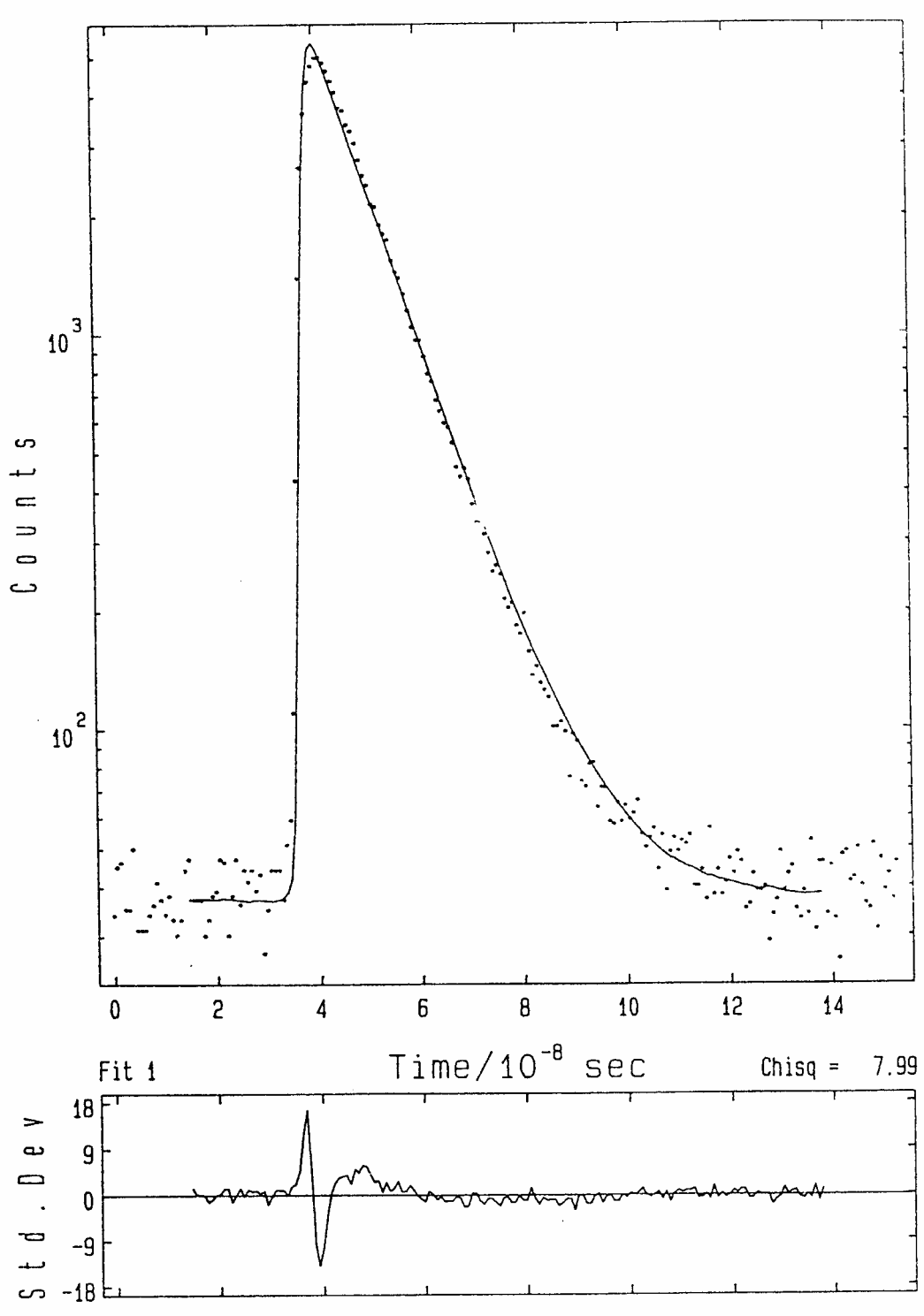


Figure A.6: Analysis with a single exponential component

The weighted residuals are an important means of assessing the goodness of fit because:

a) They show *where* misfits occur.

b) Their normalisation compensates for the varying data precision within the data set and from data set to data set.

c) The deviations are expressed in a statistically meaningful way, in terms of the standard deviations of the associated data noise.

d) The relationship between residuals and χ^2, the fitting criterion, is straightforward.

These points are illustrated in figure A.6. This shows the same data as figure A.5, but analysed with a single exponential model. The misfit is hardly apparent in the main display, but residuals of ≈ 18 standard deviations in the rising edge show clearly that a finite rise-time is present in the data.

A.12 Autocorrelation of Residuals

An alternative method of assessing the goodness of fit is to calculate the residuals autocorrelation function, defined by:

$$A(\Delta t) = \underset{T \to \infty}{\text{Limit}} \int_{t'=0}^{T} R(t')\, R(t'+\Delta t)\, dt' \qquad \text{(A.18)}$$

Of course, we know *R(t)* only in histogram form and only over the region of interest selected for fitting. In practice the integral is replaced by a sum, and the range of decay times Δt is limited to one half the number of channels analysed, because of the necessarily finite range of integration. It is customary to normalise $A(\Delta t)$ so that $A(0) = 1$.

With randomly distributed residuals (a good fit) there should be no systematic correlation and the autocorrelation function should be small and random for $\Delta t > 0$. Systematic features in the residuals lead to systematic structure in the autocorrelation function.

These points are illustrated in figure A.7 below. The two curves were calculated from the residuals of figures A.5 and A.6. It is left as an exercise to the reader to identify the autocorrelation functions corresponding to the one- and two-component fits.

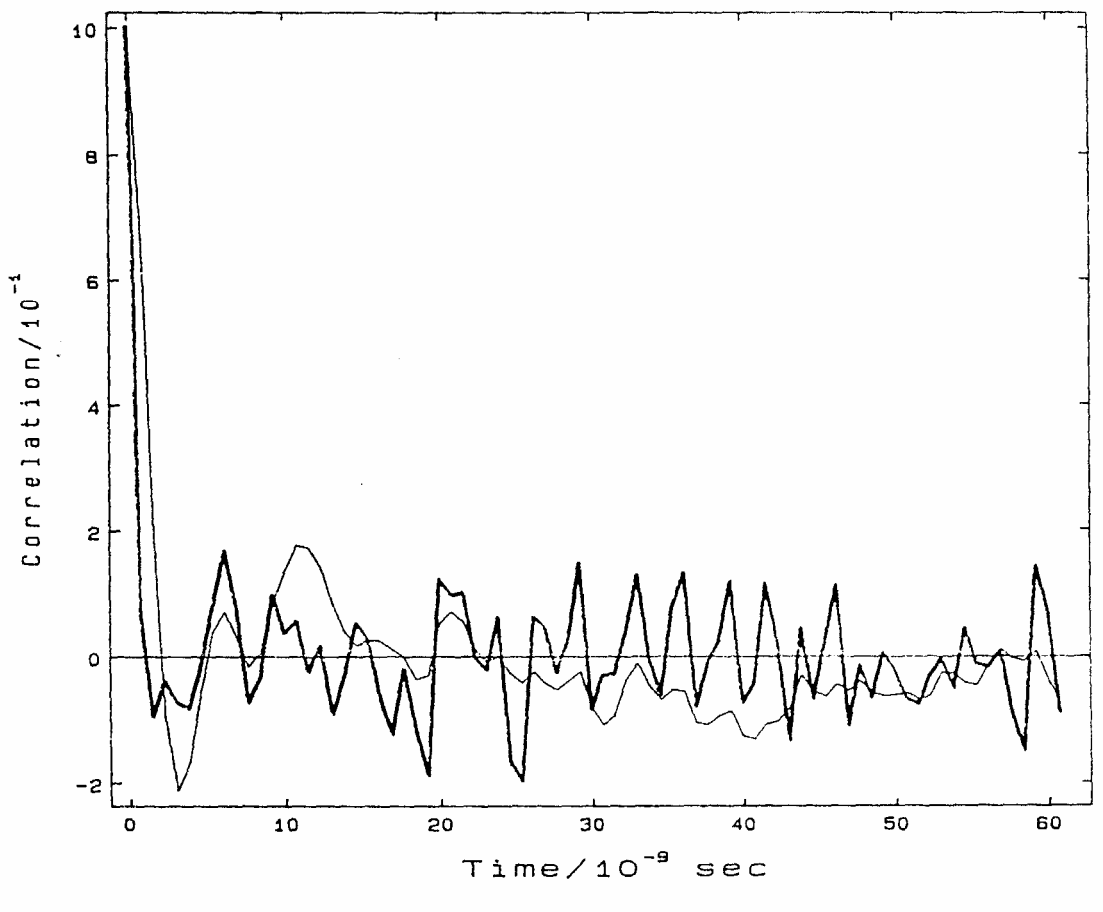


Figure A.7: Autocorrelation of residuals

A.13 Non-Poissonian Data

Fluorescence decays can be measured by many different techniques. In particular, decay curves measured using photomultiplier current sampling with a boxcar integrator or transient digitiser cannot use the Poissonian data weighting scheme of equation A.10.

The DAS6 software was designed to analyse data from a variety of sources. Figure A.8 shows the rising edge of the fluorescence signal from Cr^{3+} ions in a MgO matrix, showing that this state is populated by energy transfer. The data were measured using a transient digitiser interfaced with a data averaging computer. The statistical properties of this signal are more clearly Gaussian than Poissonian. This is noted in the file header, by the signal averaging program. All subsequent analyses and manipulations of these data by the DAS6 software then use and propagate these statistical properties in the appropriate ways – again automatically and without user intervention. An appropriate message keeps the user informed of what is being done.

In figure A.8 the value of XSQ and the normalisation of the residuals are calculated using Gaussian, not Poissonian rules.

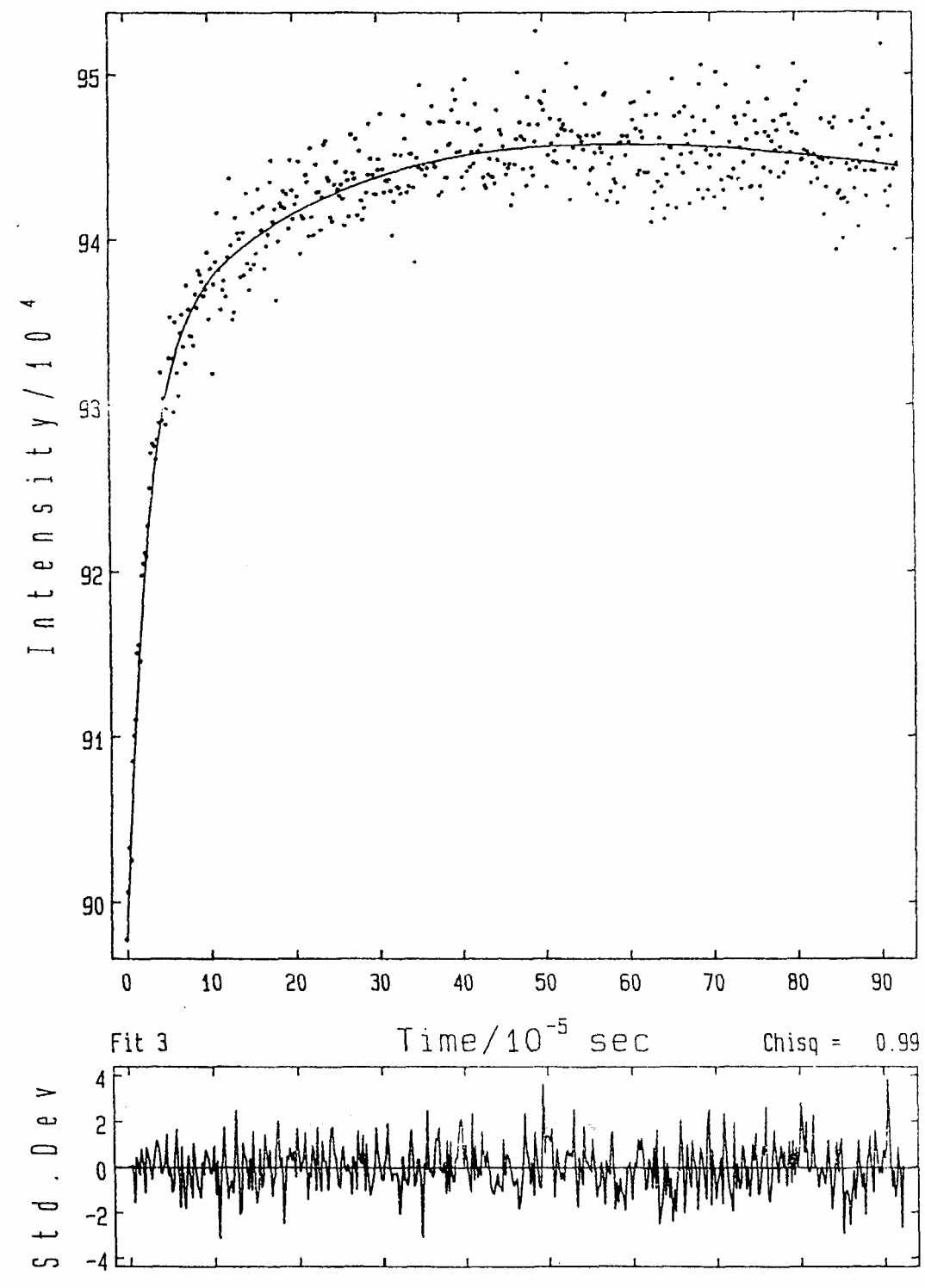


Figure A.8: Fluorescence decay measured with a transient digitiser

(Data by courtesy of Dr. M. O'Neill, measured in the Photophysics Research Laboratory of Strathclyde University)

Appendix B.
Analysis of Anisotropy Decays

B.1 Introduction

The anisotropy decay function, *r(t)* is defined by:

$$r(t) = \frac{I_{vv}(t) - I_{vh}(t)}{I_{vv}(t) + 2I_{vh}(t)} = \frac{I_d(t)}{I_s(t)} \qquad \text{(B.1)}$$

"I" represents idealised (i.e. undistorted) fluorescence intensity. The subscripts "v" (vertical) and "h" (horizontal) refer to the orientation of the polarisers (e.g. I_{vh} refers to a vertical excitation polariser and a horizontal emission polariser). Similarly the subscripts "d" and "s" refer" to "sum" and "difference" respectively. I_{vv} and I_{vh} need to be measured under identical conditions for equation B.1 to make sense. In practice, a number of instrumental effects need to be taken into account when analysing anisotropy data.

1. We observe *Y(i)*, not $I(t)$. Since reconvolution does not apply to the ratio function *r(t)*, we have to be more subtle in accounting for instrumental distortions.

2. If we combine $Y_{vv}(i)$ and $Y_{vh}(i)$ as in equation B.1, the resulting anisotropy decay curve is no longer characterised by Poissonian statistics. Therefore, we have to be more subtle in calculating data weights for analysing anisotropy decay curves.

3. $Y_{vv}(i)$ and $Y_{vh}(i)$ may or may not be measured under identical conditions. Indeed, considerable care in the design of the apparatus and measurement technique is required to obtain truly identical conditions.

B.2 Methods of Measuring Anisotropy Decays

The method(s) of analysis need to take into account the conditions under which $Y_{vv}(i)$ and $Y_{vh}(i)$ were measured. The following conditions commonly apply:

1. **Manual Measurement** - it is often useful to be able to obtain some anisotropy decay information without special apparatus. In such cases it is not possible to obtain identical conditions of observation for parallel and crossed planes of polarisation. A vector reconvolution technique (e.g. see section B.5) can be then used to extract anisotropy parameters in favourable cases.

2. **Toggling** - this method uses a motor-driven polariser to toggle between parallel and crossed orientations, accumulating data into a separate memory segments, typically 100 times during an experiment. Changes in the conditions of observation (e.g. prompt or sample changes) will be shared between the two decay curves, provided these changes are on a comparable or longer timescale to the period of toggling.

3. **T-Geometry** - the T-geometry spectrometer measures truly simultaneous $Y_{vv}(i)$ and $Y_{vh}(i)$, and therefore eliminates mis-match through lamp or sample drift. However, the two detection channels may not be perfectly matched in instrumental response and in time zero placement.

In both methods (b) and (c), the static sensitivities of the instrument in the two polariser orientations may not be the same. An instrument G-factor can be measured using measurements of $Y_{hv}(i)$ and $Y_{hh}(i)$.

B.3 Error Analysis Techniques

A key aspect of anisotropy analysis is error analysis, because anisotropy information is extracted as relatively small differences between large numbers. For this reason, HOROBA Jobin Yvon IBH has designed its software to perform automatic *variance propagation*, to transfer the information on data errors from one processing step to the next. The correct data weighting information is then stored in the DAS, appended to the data. For example, combining parallel and crossed data, $Y_{vv}(i)$ and $Y_{vh}(i)$, to give total fluorescence data $Y_s(i)$, where:

$$Y_s(i) = G\,Y_{vv}(i) + 2\,Y_{vh}(i) \tag{B.2}$$

We must also combine the data variances, $\sigma_{vh}^2(i)$ and $\sigma_{vh}^2(i)$ give $\sigma_s^2(i)$, using the usual rules of variance propagation.

$$\sigma_s^2(i) = G^2\,\sigma_{vv}^2(i) + 4\,\sigma_{vh}^2(i) \tag{B.3}$$

When the intermediate sum data $Y_s(i)$ is saved, in the DAS file, the corresponding $\sigma_s^2(i)$ values are automatically stored also. Any other module in DAS6 will then use the stored variances for data weight calculation, or for further propagation.

B.4 Direct Analysis from Data

The first method makes use of the decay curves $Y_{vv}(i)$ and $Y_{vh}(i)$, together with the G-factor, to calculate the anisotropy decay curve directly, point by point:

$$Y_r(i) = \frac{G\,Y_{vv}(i) - Y_{vh}(i)}{G\,Y_{vv}(i) + 2\,Y_{vh}(i)} = \frac{Y_d(i)}{Y_s(i)} \qquad \text{(B.4)}$$

The method can be used with data from toggling and T-geometry experiments. It does not use reconvolution analysis and therefore does not require measurements of $P_{vv}(i)$ or $P_{vh}(i)$. The $Yr(i)$ curve obtained can then be analysed using the exponential fit module, in terms of a number of exponential decay components, superimposed on a static anisotropy r_∞ (=A in the notation of the fit modules). Figure B.2 shows an example of such an analysis. The method is very popular because it is quick, direct, and gives an excellent visual inspection – important for judging whether interpretation is truly supported by the measurement. The main disadvantage is that instrumental distortions remain unaccounted for – you therefore cannot extract very short anisotropy decay times.

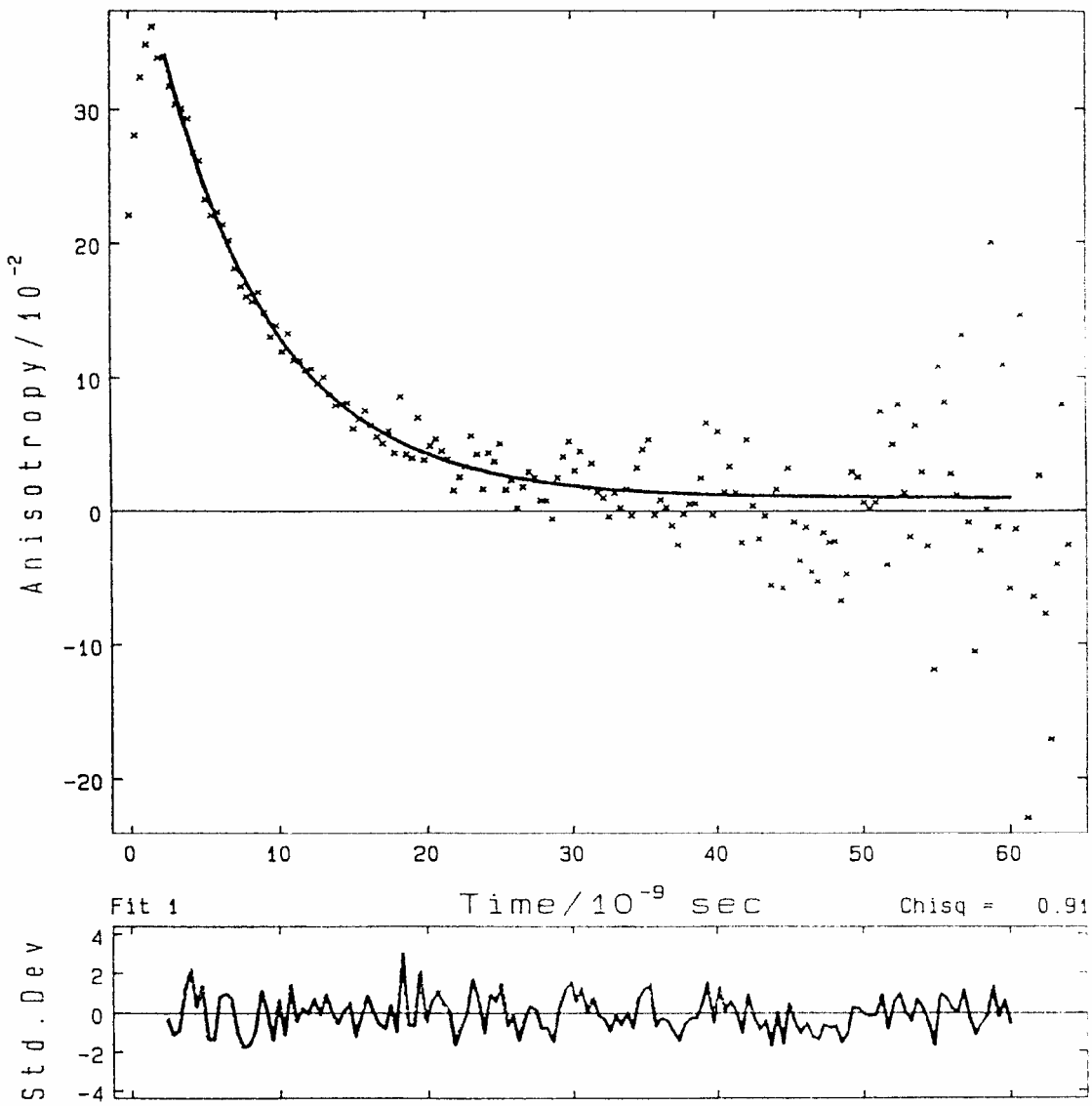


Figure B.2: Direct analysis of anisotropy decay data

Another point to note in figure B.2 is that the anisotropy data are reasonably well defined initially, but become dramatically noisy as time proceeds. It is of the utmost importance to use correct data weights in the analysis of such anisotropy data. If Poissonian rules were applied, the fit would be strongly influenced by the noisy part of the data on the right, leading to spurious parameters, with residuals and χ^2 values that were wrongly scaled.

B.5 Direct Vector Reconvolution

The direct vector reconvolution technique uses $Y_{vv}(i)$ and $Y_{vh}(i)$ for analysis, without calculating sums and differences. For a spherical rotor, characterised by a single anisotropy decay time τ_r and a single fluorescence decay time τ_F, the corresponding fitting functions are of the form:

$$F_{vv}(i) = A_{vv} + B_{vv}\left[1 - r_o \exp\left(-i/\tau_r\right)\right]\exp\left(-i/\tau_F\right)$$
$$F_{vh}(i) = A_{vh} + B_{vh}\left[1 + 2\, r_o \exp\left(-i/\tau_r\right)\right]\exp\left(-i/\tau_F\right) \quad \text{(B.5)}$$

Figure B.3 shows a sample analysis, using the same data as in figure B.2

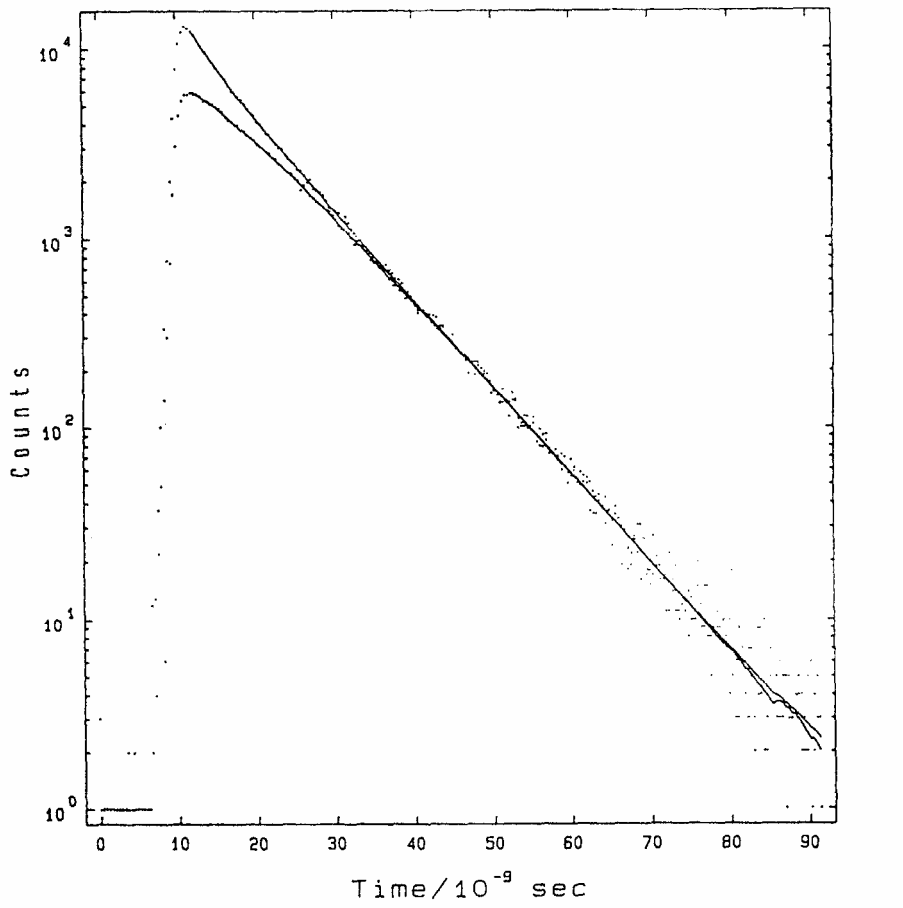


Figure B.3: Direct vector reconvolution of $Y_{vv}(i)$ and $Y_{vh}(i)$

Direct vector reconvolution was designed for use with the manual method of measurements, described in section B.2.a. The results are generally inferior to “Impulse Reconvolution” and the method is not supported in DAS6.

B.6 Impulse Reconvolution

This method is considerably more powerful than the previous one, because the analysis is split into two separate parts: analysis of $Y_s(i)$ followed by analysis of $Y_d(i)$.

The total fluorescence decay, $Y_s(i)$ is free of anisotropy effects and should be straightforward to analyse separately, using 1-4 exponential components.

The difference decay, $Y_d(i)$ is considerably more complex, because it contains fluorescence as well as anisotropy parameters. However, if we freeze the fluorescence parameters, the anisotropy parameters should be much more readily obtainable. In impulse reconvolution this freezing is achieved by accepting the results of the analysis of $Y_s(i)$ as a constraint. Without such a constraint, difference decay data analysis would yield meaningless parameters.

Let $I_s(i)$, $I_d(i)$ and $I_r(i)$ be impulse response functions (IRF) (i.e. decay curve shapes free of instrumental distortion or artefact) for total, difference and anisotropy respectively. Then, from equation B.1:

$$I_s(i).I_r(i) = I_d(i) \tag{B.6}$$

The point about writing equation B.1 in this way is that we can convolute both sides of equation B.6 with $P(i)$ and obtain two equivalent expressions for a fitting function for $Y_d(i)$. $I_s(i)$ is known, from the analysis of $Y_s(i)$: this is the constraint in the analysis of $Y_d(i)$. $I_r(i)$ is not known, but this is what we are after. The impulse reconvolution module uses:

$$I_r(i) = r_{\inf} + B_1 \exp\left(-i/\tau_{r_1}\right) + B_2 \exp\left(-i/\tau_{r_2}\right)$$

with (B.7)

$$r_o = r_{\inf} + B_1 + B_2$$

Thus allowing two anisotropy decay times, together with r_o and r_∞ to be extracted from $Y_d(i)$.

The method is illustrated in figure B.4. Again, the same data as for figures B.2 and B.3 is used to illustrate this technique. The total fluorescence decay was analysed with two exponential components, using the exponential fitting modules. The impulse response function $I_s(i)$ calculated in this way then formed the constraint in the impulse reconvolution, where the difference data were analysed in terms of one anisotropy decay component, together with r_∞.

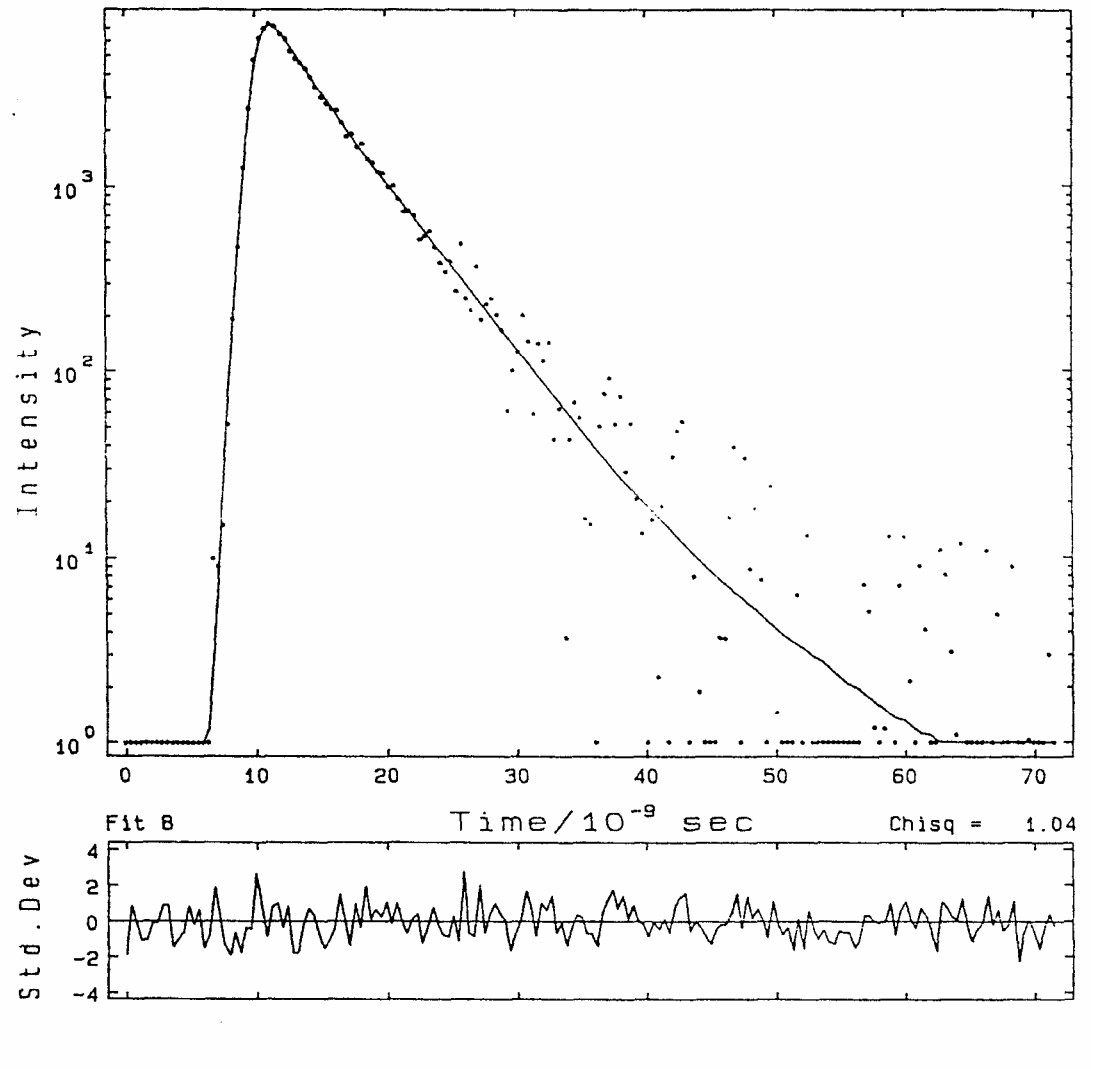


Figure B.4: Impulse reconvolution analysis

B.7 Impulse Response Function Methods

Impulse response functions can be calculated for any fit, using the best-fit parameters. Unlike fitted functions, impulse response functions (IRFs) represent the fitted model without instrumental distortion or background noise. IRFs do not contain any data weights hence there is no basis for judging the statistical validity of fits produced from them.

It can be see from figure B.2, that impulse response methods need more information to be measured than the impulse reconvolution method. Because of this, impulse response function methods are not supported in DAS6.

Appendix C. DAS6 Specifications

Operating systems supported: -

- Microsoft Windows XP (32 bit)
- Microsoft Windows Vista (32 bit)
- Microsoft Windows 7 (32 / 64 bit)

Hardware requirements: -

- 1.5 GHz CPU Speed
- 1GB RAM
- 2GB hard disk space
- Minimum screen resolution 1024 x 768

Over the page, Table 2 lists the specifications of the curve fitting features of DAS.

Table 2: Fitting Features

Feature	Equation	Max number of curves	Max data points per curve	Prompts used	License included in basic DAS software?	Shift parameter option
Foundation Exponential	$F(t) = A + B_1 \exp\left(-t/T_1\right) + \ldots + B_5 \exp\left(-t/T_5\right)$ Up to 5 exponentials	1	16384	0 or 1	Y	Y
Exciplex Kinetics	$F(t) = A + B\left(\exp\left(-t/T_1\right) - \exp\left(-t/T_2\right)\right)$	1	16384	1	Y	Y
Lifetime Distribution	$f\left(k_m, \Delta, t\right) = \frac{\exp\left(-k_m t\right)}{k_m t^2 \Delta}\left[\left(k_m t + 1\right)\sinh\left(t\Delta\right) - t\Delta\cosh\left(t\Delta\right)\right]$	1	16384	1	Y	Y
Exponential Series	$F(t) = A + B_1 \exp\left(-i/T_1\right) + B_2 \exp\left(-i/T_2\right) + \ldots + B_{30} \exp\left(-i/T_{30}\right)$	1	16384	1	Y	N
Förster Energy Transfer	$F(t) = A + B_1 \exp\left[\left(-t/T_1\right) - 2\gamma\left(-t/T_1\right)^{1/2}\right] + B_2 \exp\left(-t/T_2\right)$	1	16384	1	Y	Y

Feature	Equation	Max number of curves	Max data points per curve	Prompts used	License included in basic DAS software?	Shift parameter option
Yokota-Tanimoto Energy Transfer	$F(t) = A + B_1 \exp\left[\left(-t/T_1\right) - 2\gamma C(t)\left(t/T_1\right)^{1/2}\right] + B_2 \exp\left(-t/T_2\right)$	1	16384	1	Y	Y
Micellar Quenching	$F(t) = A + B\exp\left[\left(-t/T_1\right) - C\left\{1 - \exp\left(-t/T_2\right)\right\}\right]$	1	16384	1	Y	Y
Global Exponential Analysis	$F(t) = A + B_1 \exp\left(-t/T_1\right) + \ldots + B_5 \exp\left(-t/T_5\right)$ Up to 5 exponentials fitted globally	100	8192	0 or 1	Y	Y
Batch Exponential Analysis	$F(t) = A + B_1 \exp\left(-t/T_1\right) + \ldots + B_5 \exp\left(-t/T_5\right)$ Up to 5 exponentials fitted sequentially	10,000	16384	0, 1 or same no. of prompts as curves	Y	Y
Anisotropy Analysis	See Appendix B for details	1	16384	0 or 1	Y	Y

Feature	Equation	Max number of curves	Max data points per curve	Prompts used	License included in basic DAS software?	Shift parameter option
Non-Extensive Distribution	$F(t) = A + \sum_{k=1}^{5} B_k \left[1 - (1 - q_k) t / t_k\right]^{1/1-q_k}$	1	16384	0 or 1	N	Y

Appendix D. How to access the DAS log files.

DAS creates a log file each time it runs to record in detail what happens during the session. If anything goes wrong with the application then the log file(s) must be send to HORIBA Scientific support staff so that they can trace the problem. Problems cannot be investigated unless a log file is provided.

The most recent log file is always named DAS6.log. The log files are always created in the application folder, this folder is chosen on installation with the default being:

```
C:\Program Files\HORIBA Scientific\DAS6\
```

If during a session of an application, a problem occurs and the application crashes then, when the application is next started, the previous incomplete log file will be identified and saved. This involves changing the name of the log file from DAS6.log to:

DAS6_yymmddhhmmss.LOG where yymmddhhmmss is the current date and time,

e.g. `DAS_041225161510.log`

HORIBA
Scientific

3880 Park Avenue, Edison, New Jersey 08820-3012, USA
www.horiba.com/scientific

[Design Concept]

The HORIBA Group application images are collaged in the overall design.
Beginning from a nano size element, the scale of the story develops all the way to the Earth with a gentle flow of the water.