

# Help for Time-Resolved Analysis

## TRI2 version 2.4

P Barber, 22.01.10

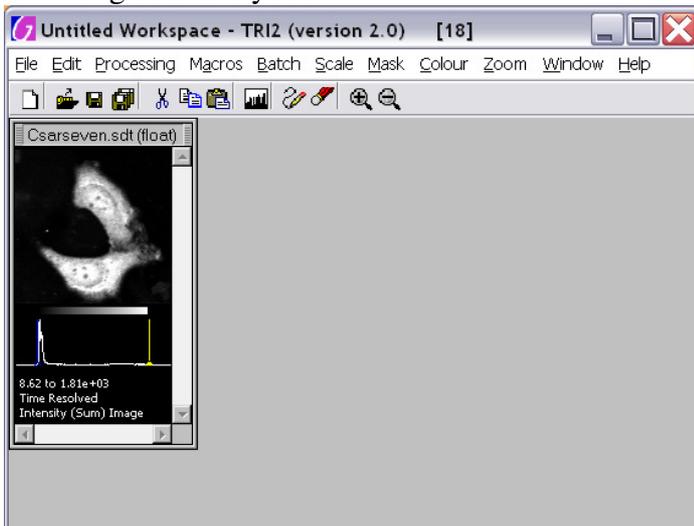
## Introduction

“Time-resolved Analysis” (TRA) becomes available under the processing menu once you have loaded and selected an image that contains suitable multi-dimensional data. This is typically an “ics” file.

When such an image is loaded the intensity or sum image is shown in the usual image window. If the image has been identified as a time-resolved image then the words “Time Resolved” appear in the extra text below the intensity image.

There are many processing options available, please be aware that many of them may not be relevant to TRA and many only work on the 2D intensity image. One process that does work on 3D data is the arithmetic function.

TRA allows you to fit functions to single pixel or binned data and also to perform functions to whole images. Such as performing a fit at every pixel, or bin of pixels, or using all pixels together in some form of global analysis.



More detail can be found in the following document, but for details about the fitting functions please refer to: [Time Resolved Fitting \(Detail\).pdf](#)

### **NEW**

As of version 2.4 we now have a Bayesian method for mono-exponential fitting and we hope bi-exponential will follow soon. This analysis accounts for the Poissonian nature of low photon count data and is more accurate and robust in these situations. Another benefit of this technique is that the errors reported for the fitted parameters are now realistic error intervals, giving you directly the confidence in the values. Unfortunately nothing is free, and the Bayesian algorithm is much slower to run than the Marquardt methods. Also parameter fixing and global methods are not available with the Bayes method.

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# Time-Resolved Analysis

## Inputs

Select an image that has time resolved data in any workspace to make it the subject of TRA. Choose **Processing->Time-Resolved Analysis** from the workspace if the TRA window is not visible.

**Image**  
Chosen via workspace  
Has Pre-Processing options

**Instrument response/Excitation/Prompt**  
Chosen via menu  
Has cursor options

**Data:** Csarseven.ics

Click on image to select pixel to analyse: (68, 43)

X: 68  
Y: 43

**Type of Fit:** Bi-exponential (Marquardt)

Fit Function:  $f(t) = Z + A1 \exp(-t / \tau1) + A2 \exp(-t / \tau2)$

Estimate Initial Values | Estimate Cursors

Pre-fit estimation: Integral Fit Estimate | Target ChiSq: Repeat fit until ChiSq < 1.5

**Fitted Parameters:**

Parameter	Values	Freedom	Errors
Z	0.31	Free	2.20
A1	22.91	Free	2.87
tau1	2.52E+0	Free	9.35E-1
A2	11.44	Free	8.39
tau2	2.43E-1	Free	3.01E-1

Chi sq. / d.f.: 1.066676  
# iters: 9  
Error: 0

Freedom Key: Free | Restrained | Fixed

Pixel Fits | Make Images

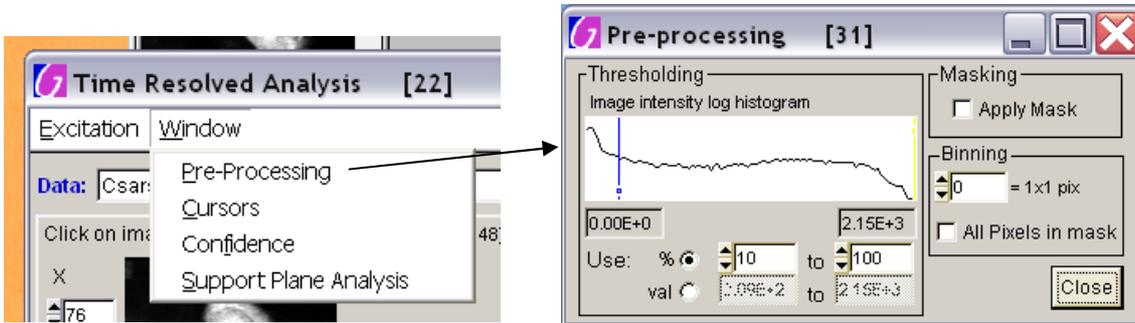
Status: OK | Close

**Fit Type**  
Chosen on this panel  
Has options on this panel

**Parameter Freedom**  
Chosen on this panel  
Has free/fixe/restrained options

**Selected Pixel & Data**  
Chosen by clicking on this image or using the 'X' and 'Y' controls.

## Pre-Processing

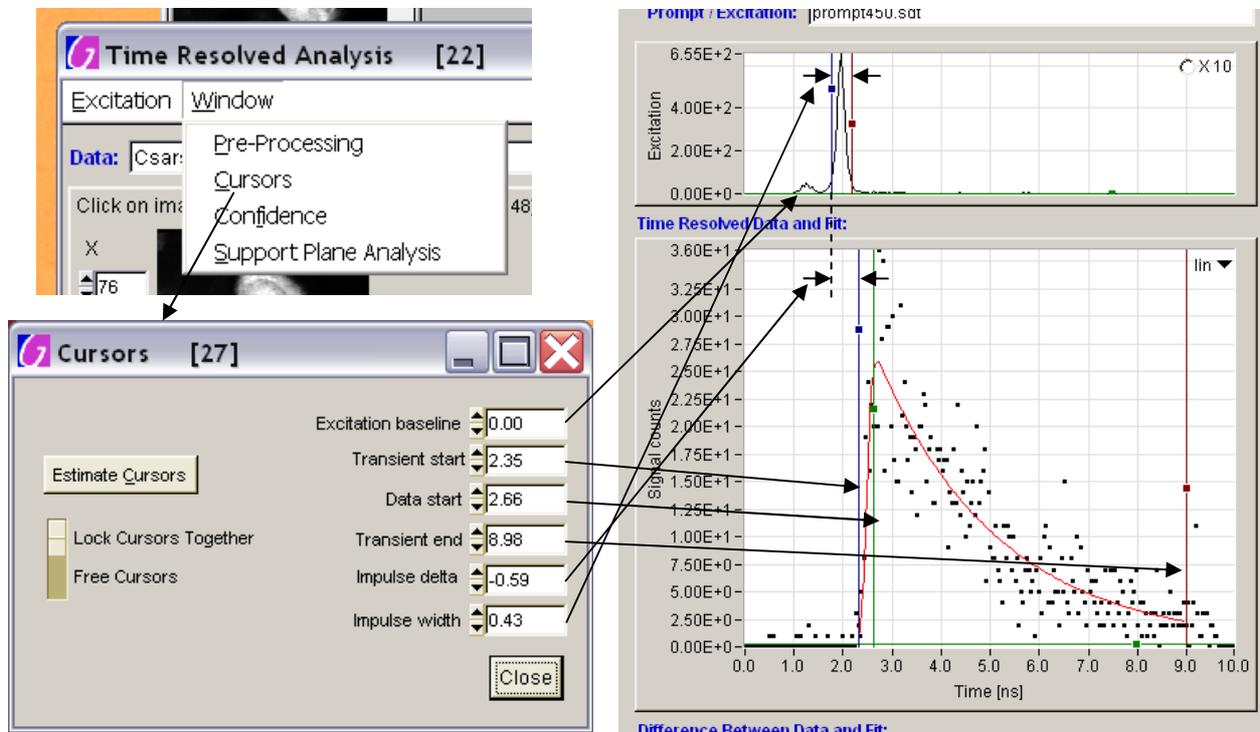


**Thresholding:** This allows you to excluded pixels with a low and/or a high intensity. You can choose to use a threshold based on a % of the intensity or on a fixed value. A fixed value is useful for batch processing when you want to exclude a background below a fixed intensity. The image demonstrates excluding all pixels with an intensity in the bottom 10% of all pixels.

**Masking:** If there is a mask associated with the image in the workspace it can be applied to TRA by checking the “**Apply Mask**” box.

**Binning:** The time-resolved data of the pixels in the neighbourhood of the selected pixel can be added together. Use “Binning” to select a 3x3 or 5x5 pixel area, for example. Checking the “**All Pixels**” box adds all the data of all the pixels that have not been excluded via thresholding or masking (N.B. if this box is checked that selected pixel become irrelevant).

## Cursors

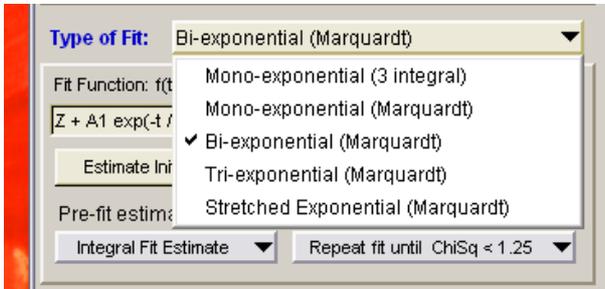


The cursors panel allows you to manually change to cursor positions. It also allows you to **lock the cursors together** so that: the impulse delta (time difference between the start of the prompt

(excitation) and the start of the transient (time-resolved data) and the impulse width are maintained if the transient start is moved, or the impulse delta is maintained if the prompt start is moved.

**Estimate Cursors:** Places all the cursors (prompt and data cursors, except prompt baseline) in sensible positions.

## Type of Fit



Choose the model function with the “**Type of Fit**” control and the mathematical function associated with it will be displayed.

**Estimate Initial Values:** Uses a three integral fit to the data to estimate starting parameters for the current model. The values calculated are placed in the value boxes of the “Fitted Parameters”.

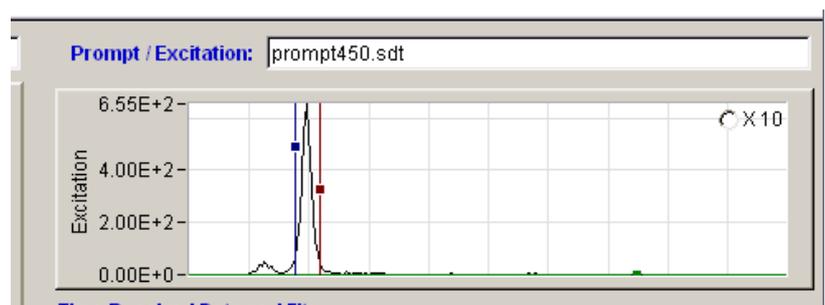
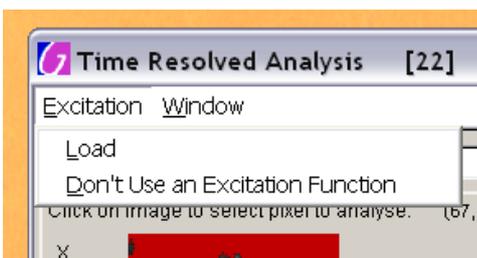
**Estimate Cursors:** Places all the cursors (prompt and data cursors, except prompt baseline) in sensible positions.

**Pre-fit Estimation:** This control determines where the starting parameters arise from. “**Integral Fit Estimate**” performs the “Estimate Initial values” before each fit. “**Estimate from last fit**” uses the fitted parameters from the last fit as starting values for the next. “**Estimate from panel values**” always uses the values that are in the Fitted Parameters value boxes as the starting values for a new fit. N.B. for single fits the last two options will be identical because the values from the last fit will always be displayed in the Fitted Parameters value boxes. This is not the case, however, for making images based on pixel fits.

**Target ChiSq:** The Target Chi Square control can be used to determine how stringent the fitting engine should be. This value is used to determine how many tries (re-fits) are made to try and improve the fit (the lower the ChiSq, the better the fit)

## Instrument Response/Excitation/Prompt

You are able to load a file that describes the response of the instrument used to acquire the image. This is also often called the excitation function or the prompt function. This file can be in ics or sdt format and contains a single time-resolved signal. This must have been captured with the same instrument settings as the image and so has the same number of time-resolved points (number of channels) over the same time period.



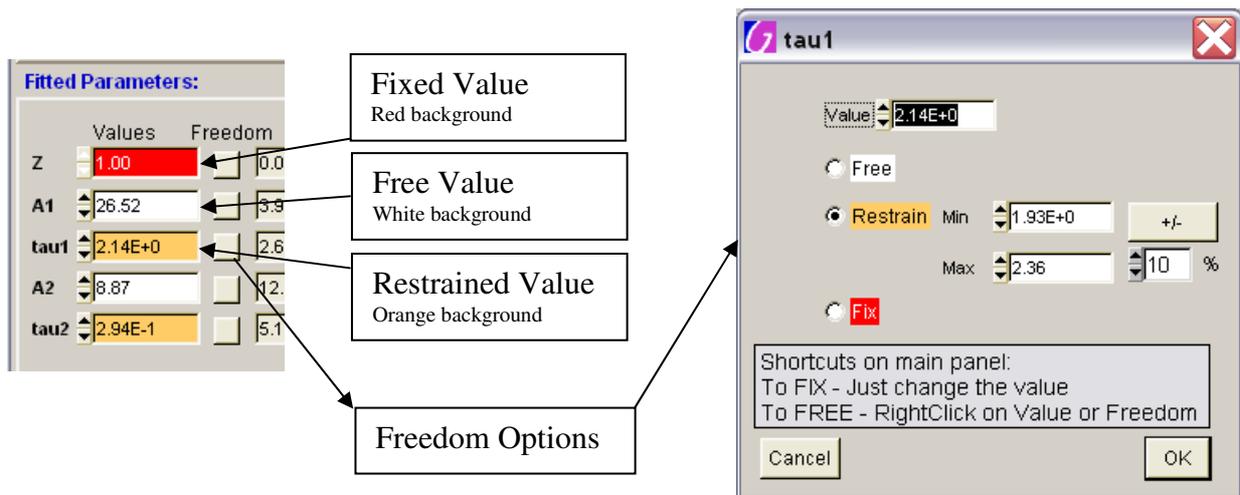
The excitation function will be convolved with the pure fit function before comparing the result to the raw data when determining the goodness of fit. A better estimate of the model parameters can thus be achieved.

You can choose to not use an excitation function by selecting this menu option.

“X10” expands the y-axis of the excitation graph for better setting of the “Excitation Baseline” cursor.

## Parameter Freedom

The freedom of each model parameter can be set. In other words constraints can be applied to fix or restrain the value of certain parameters.



Constraints can be applied by clicking the freedom button to call up the freedom options panel for each parameter. Here you can free the parameter, fix it to any value by changing the “Value” box and checking the “Fix” button, or restrain the parameter between minimum and maximum values. The “+/-” button and “%” box allow you to quickly set min and max limits based on the current parameter value (i.e. Clicking “+/-” with “10 %” sets the min at value-10% and the max at value+10%). There are shortcuts to free and fix parameters from the main TRA panel. Just type a new value in the parameter value box to fix that parameter. To free a parameter, right-click on the value box or the freedom button.

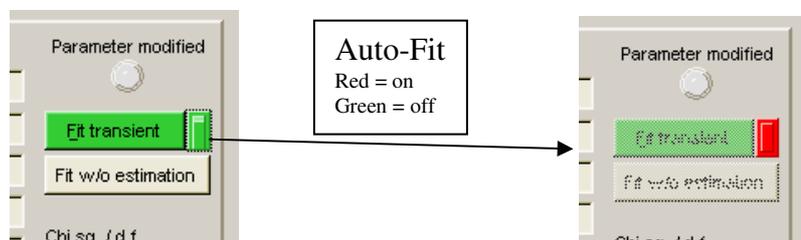
## Fitting

The “Fit transient” button will perform a fit based on all the inputs provided and will perform an initial pre-fit estimation of the parameters for Marquardt fitting.

The “Fit w/o estimation”

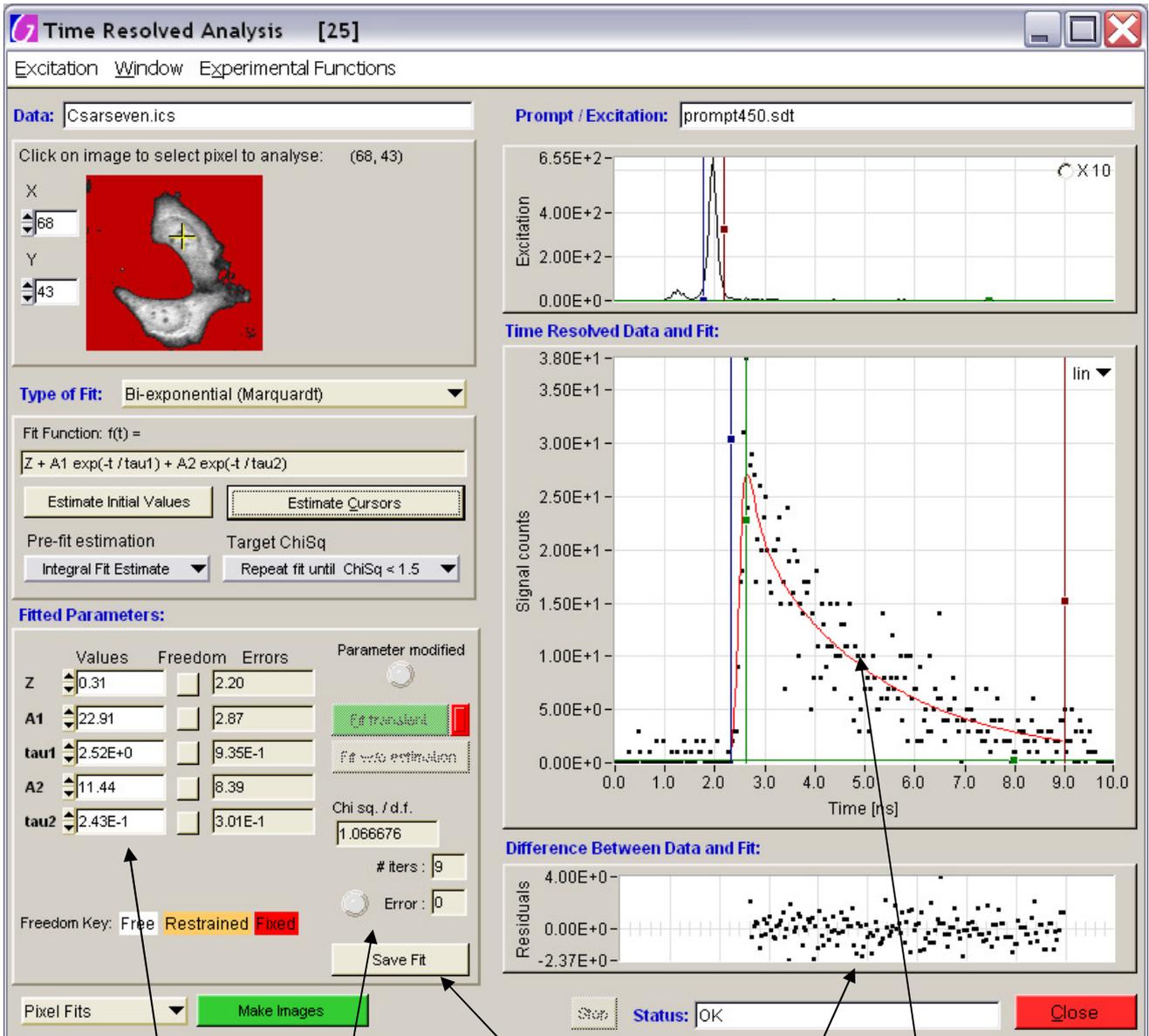
button performs a fit based on all the inputs provided but will not perform an initial pre-fit estimation (i.e. the current panel values will be used as the initial estimate for Marquardt fitting). N.B. Integrals fitting does not require any pre-estimate of the parameters. The “Parameter Modified” indicator informs of when the currently displayed fit and parameter values are no longer valid because something has been manually changed and a new fit should be performed.

If the auto-fit button is on (red) then a new fit will be performed whenever something changes and so the current values should always be valid.



See Appendix A for more detail on the Integral and Marquardt fitting procedures.

## Outputs



Fitted Parameters

Save Fit Data  
Reduced Chi Squared,  
Iterations and Error

Residuals

The Fit

## Errors

Indicates estimated errors in all the determined parameters.

## Reduced Chi Square

The Reduced Chi Square (or "Chi sq. / d.f.") is given by:

$$\chi_r^2 = \frac{\sum_{k=1}^n \frac{[I(t_k) - I_c(t_k)]^2}{I(t_k)}}{n-p} = \chi^2 / (n-p)$$

where:

$I(t_k)$  = the data at time point k

$I_c(t_k)$  = the fit at time point k

$n$  = number of time points

$p$  = number of variable fit parameters

$n - p = d. f.$  (degrees of freedom)

and the lower the Reduced Chi Square the better the fit. In the absence of noise ChiSq should approach zero; for Poisson-limited noise, expect a value around 1.

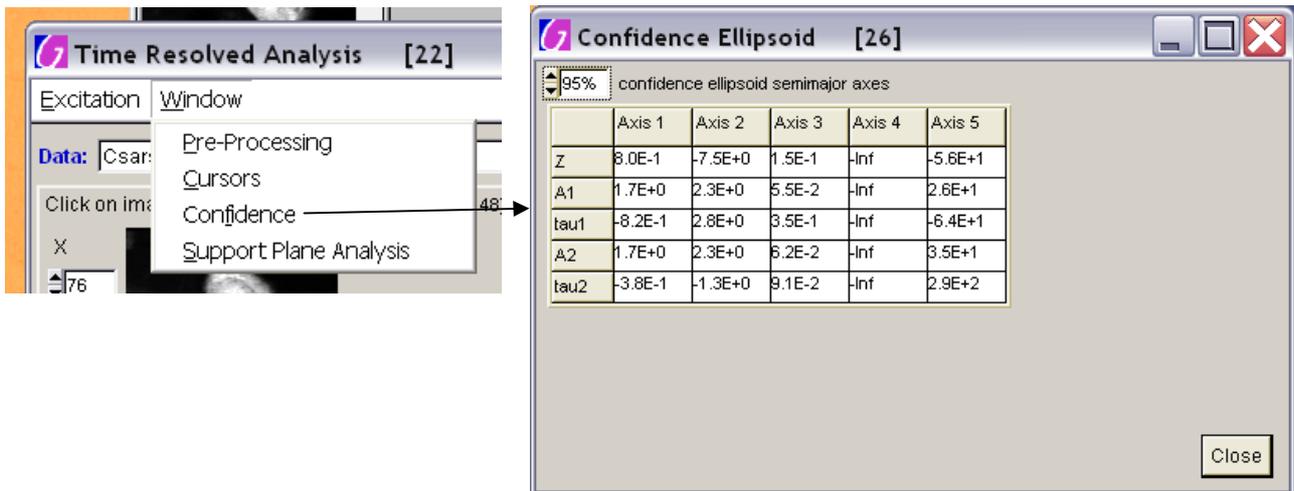
## Iterations

The reported number of iterations (or “# iters”) is, for Marquardt fitting, the total number of iterations of Marquardt fitting. If re-fits occur, the numbers of iterations of each fit are added together. For integral fitting the number of iterations indicates the number of re-fits (See appendix A)

## Error

Indicates if there was an error in the fitting.

## Confidence Ellipsoid



The confidence ellipsoid describes our confidence in the fitted values by indicating the shape of the Chi Square support plane around the minimum (around the fitted solution).

## Image Production



The drop-down menu lets you choose between pixel fits, a global fit or a multi-image global fit.

**Pixel Fits** allow you to use the inputs you have provided and perform a fit on every pixel in the image that has not been excluded through thresholding or masking. Binning also applies to pixel fits. Take care since binning “**All Pixels**” will result in all the pixels being the same! The fitted parameters and the ChiSq values will be used to form images which will appear in a new image workspace window.

**Global Fit** performs a global fit using all the pixels that have not been excluded through thresholding or masking. A **Multi-Image Global Fit** will use all the time-resolved images in the current workspace

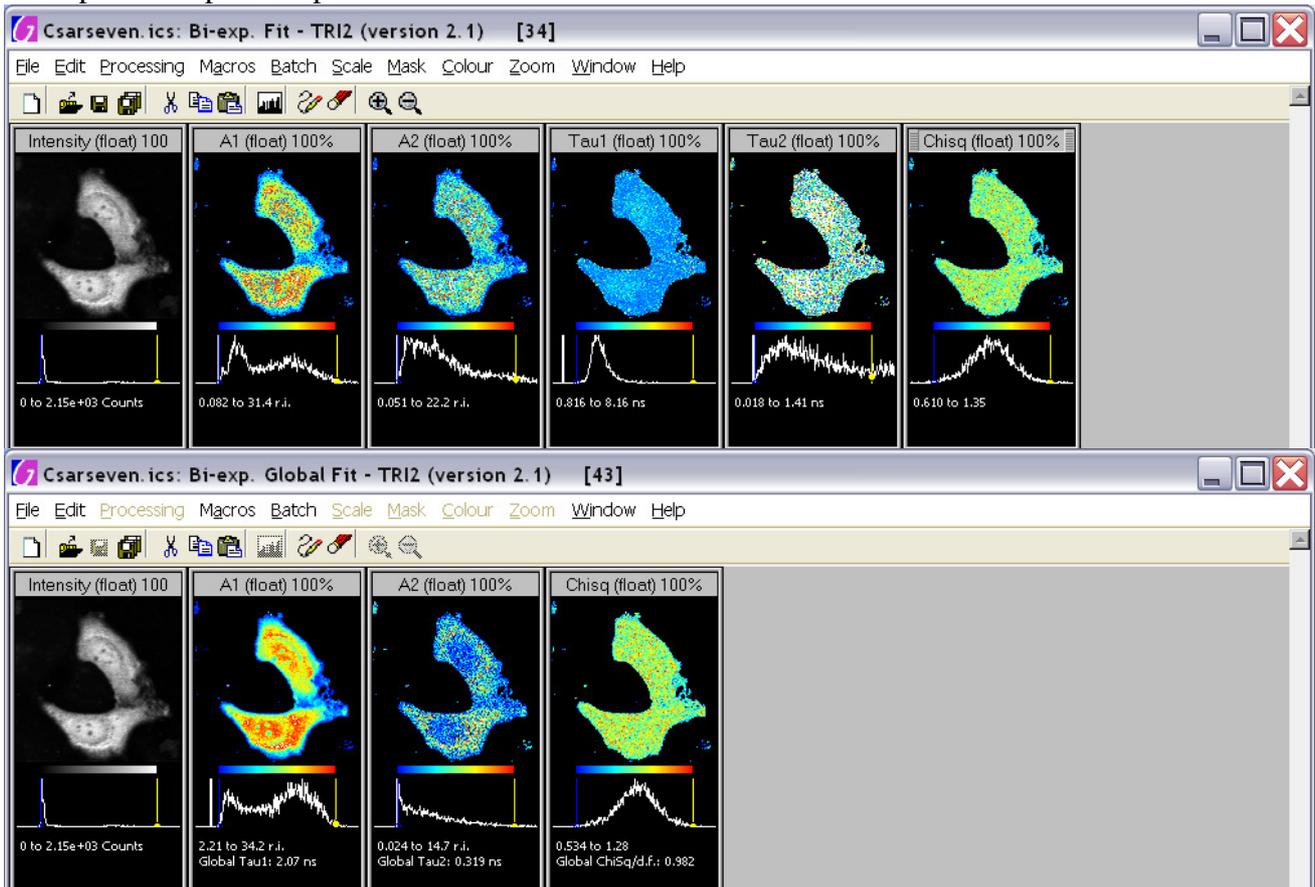
and fit them all simultaneously. In short, global analysis is a powerful technique in which some parameters of the fitting function are restrained to be the same for every pixel (globally fitted parameters) and others are free to be different for each pixel (locally fitted parameters). All the time-resolved image data are considered simultaneously when the best fit is determined (see appendix B for a more detailed description of how this global fitting is performed).

The table below details which parameters are considered global and local for the current software:

Parameter	Mono-exponential	Bi-exponential	Tri-exponential	Stretched Exponential
Z	Local	Local	Local	Local
A <sub>1</sub>	Local	Local	Local	Local
Tau <sub>1</sub>	Global	Global	Global	Global
A <sub>2</sub>		Local	Local	
Tau <sub>2</sub>		Global	Global	
A <sub>3</sub>			Local	
Tau <sub>3</sub>			Global	
h				Global

The locally fitted parameters and the local ChiSq values will be used to form images which will appear in a new image workspace window. The globally fitted values and global ChiSq will appear as extra text on appropriate images in the workspace.

Example workspace outputs:



The global Reduced ChiSq is defined as the sum of all the local ChiSq's divided by the global degrees of freedom:

$$df_{global} = [N.(n - p_{free}^l)] - p_{free}^g,$$

where: N = number of transients (pixels);  $p_{free}^l$  = number of free local parameters;  $p_{free}^g$  = number of free global parameters.

## Support Plane Analysis

Once we have a fit, with resulting fitted parameters and a ChiSq value, we can investigate the local map of ChiSq and ask questions like (for example):

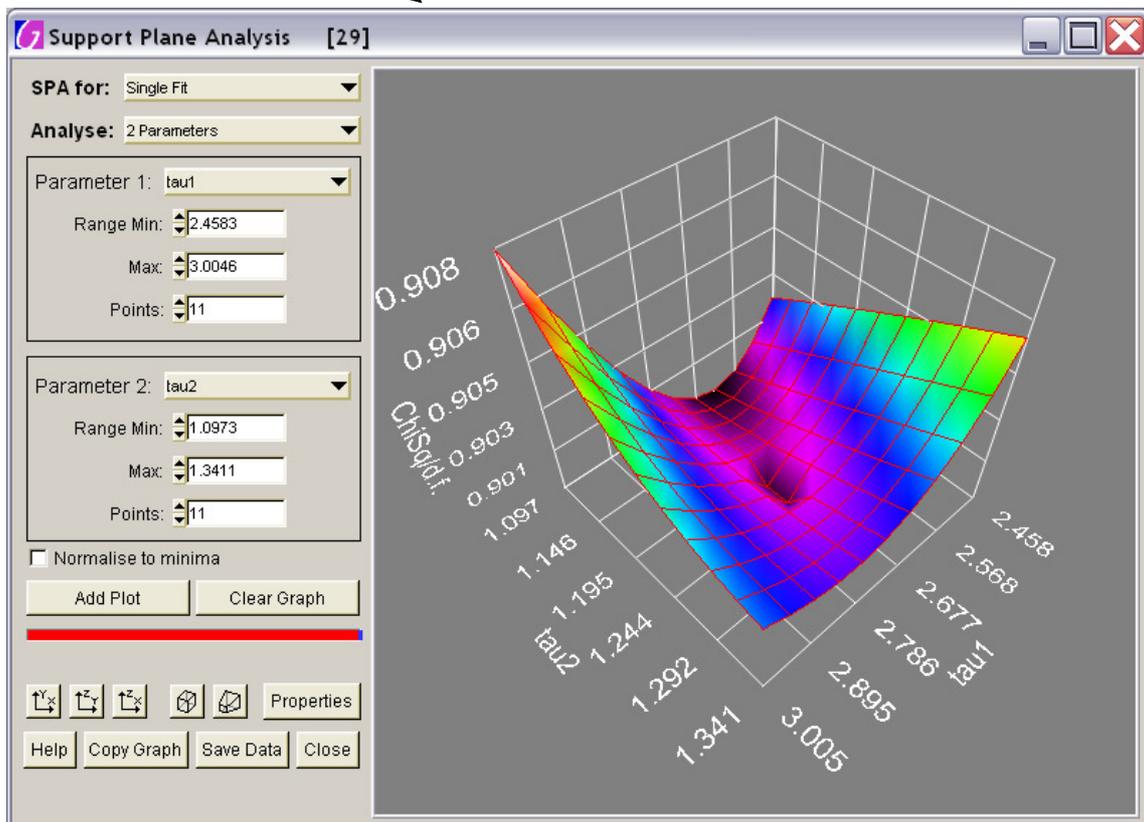
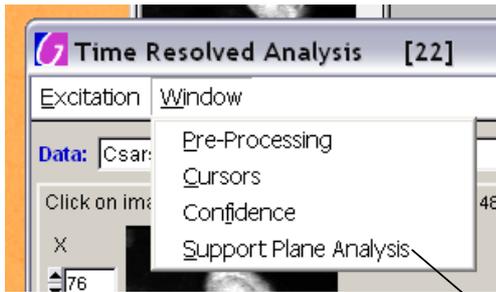
What is the ChiSq if tau was forced to be 1.9 rather than 1.8?

What is the likely error in tau?

How likely is it that tau is 1.9 rather than 1.8?

This is where support plane analysis (SPA) is useful.

Support plane analysis is available from the window menu of TRA.



**SPA for:** allows you to choose between SPA for the single transient on the TRA panel or global fitting on the whole image. Global SPA may take some time since a global fit must be performed at every point on the graph.

**Analyse:** selects one (1D support plane) or two (2D support plane) parameters to vary.

**Parameter 1** and **Parameter 2:** allow you to choose which parameters to vary, over what range and in how many steps.

**Normalise to minima:** divides every point on the graph by the minimum ChiSq calculated.

**Add Plot:** Performs the calculations and add a plot to the graph. Many plots can be added each with the same or different range, or from different pixels (select a different pixel on the main TRA panel

and then click “Add Plot”. If you change the parameters to vary in between plots you may get some strange results. You can change each plot’s appearance by clicking “Properties” to access the properties panel.

**Clear Graph:** Clears all plots from the graph.



: Orientate the graph

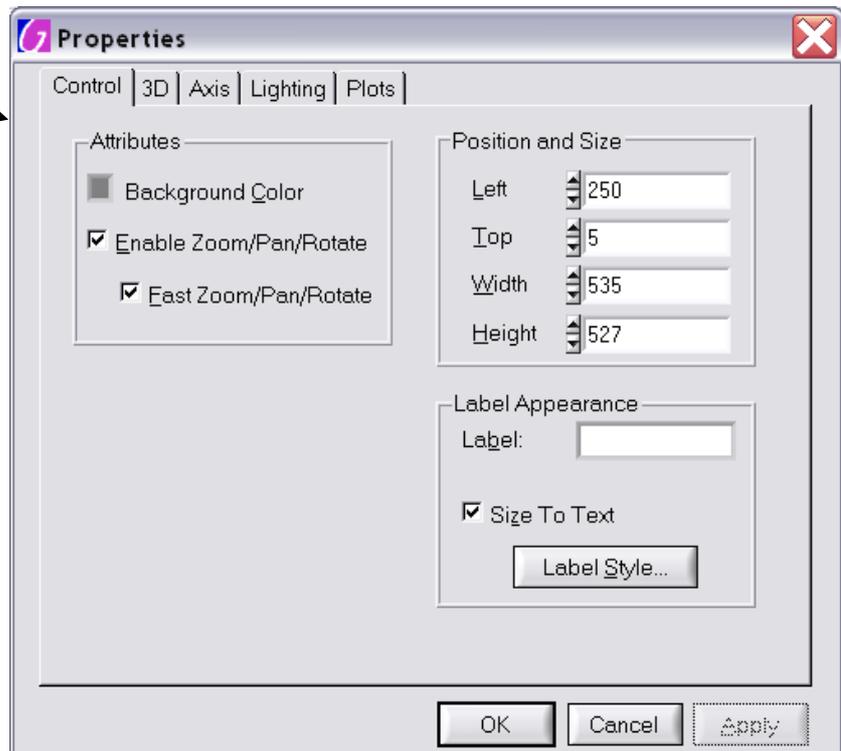


: Change the view of the graph to orthographic or to perspective, respectively.

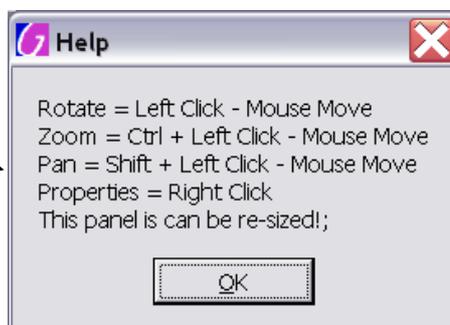
**Hint:** For a more usual view of a 1D support plane, click  and then .

**Hint:** For a contour map of a 2D support plane, click  and then .

**Properties:** Opens the graphs properties panel.



**Help:** Informs about how to control the view of the graph.

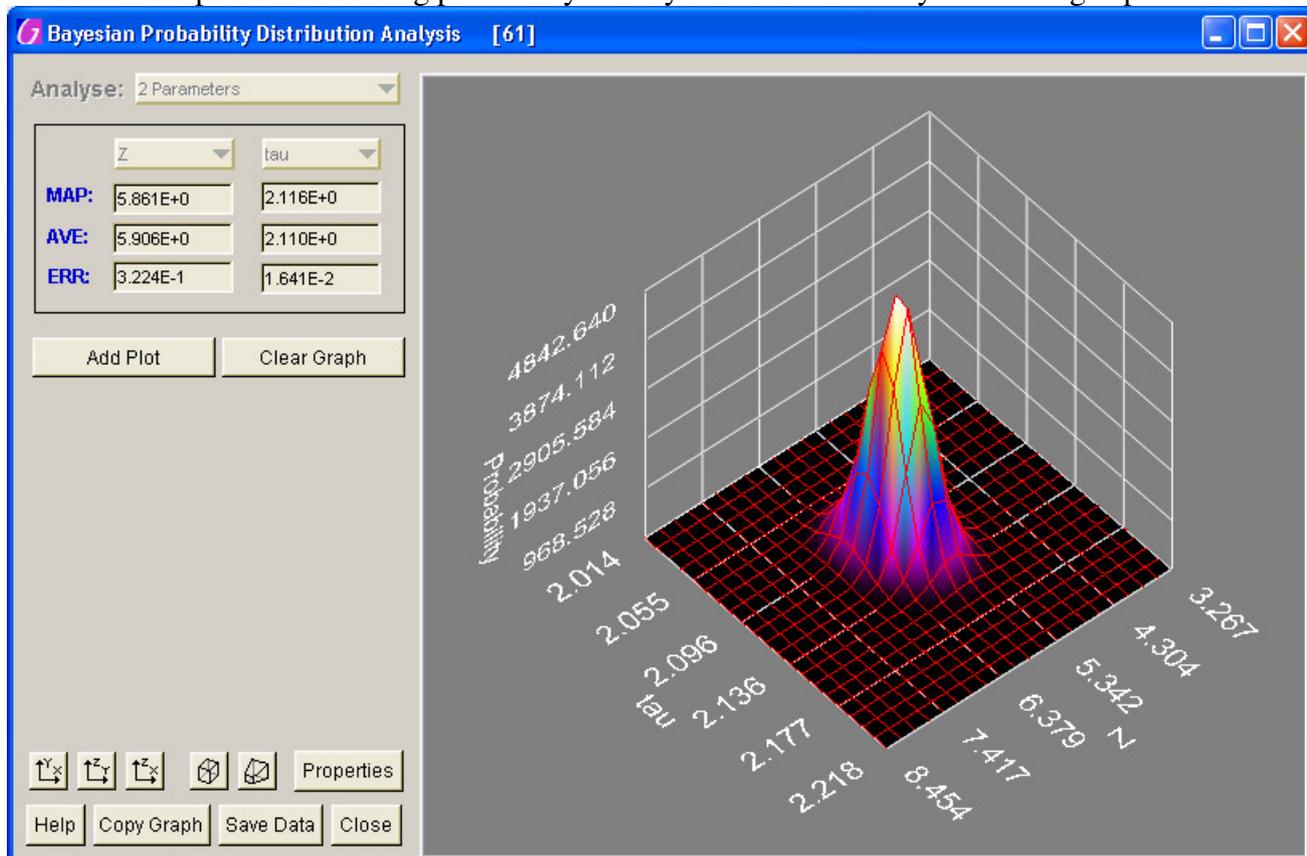


**Copy Graph:** Copies the current view of the graph to the windows clipboard for pasting in other applications (e.g. MS Word or PowerPoint).

**Save Data:** Saves a text file containing the last plot’s data points in a format that can be imported into MS Excel to use it’s 3D graphing functions.

## Bayes Probability Distribution Analysis

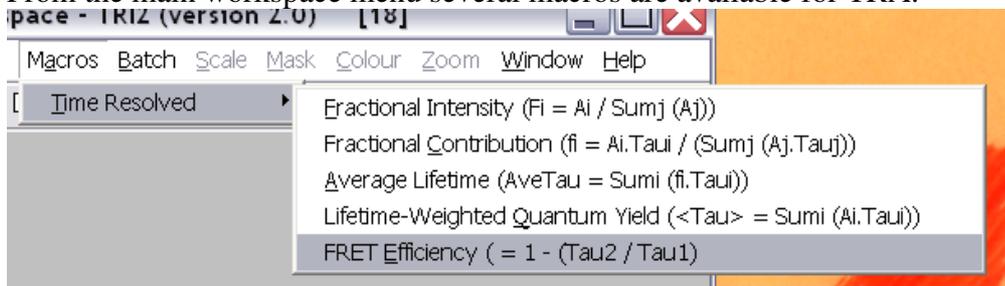
Similar to the Support Plane Analysis, the Bayes Probability Distribution Analysis panel can be used to view and explore the resulting probability density function when Bayesian fitting is performed.



Most function on this work in the same way as the SPA panel. The numbers reported are the maximum a posteriori (MAP) value, i.e. the most probable, peak value, of the distribution, the average (AVE) or first moment of the distribution and the error interval (ERR), which is the second moment or standard deviation of the distribution.

## Macros

From the main workspace menu several macros are available for TRA.



These macros will search for suitable images in the current workspace on which to perform the operation (i.e. those named “A1”, “tau1” etc.). New images will be created in the workspace from the result(s). You will be told if a suitable set of images could not be found.

**Fractional Intensity**: Produces a  $F_i$  image for every  $A_i$  found (to a max of 3) according to:

$$F_i = \frac{A_i}{\sum_j A_j}$$

**Fractional Contribution:** Produces a  $f_i$  image for every  $A_i$  and  $\tau_i$  pair found (to a max of 3) according to:

$$f_i = \frac{A_i \tau_i}{\sum_j A_j \tau_j}$$

**Average Lifetime:** Produces one image show the contribution-weighted average lifetime from all  $A_i$  and  $\tau_i$  image pairs found (max of 3) according to:

$$\bar{\tau} = \sum_i f_i \tau_i = \frac{\sum_i A_i \tau_i^2}{\sum_i A_i \tau_i}$$

**Lifetime-Weighted Quantum Yield:** Produces one image from all  $A_i$  and  $\tau_i$  image pairs found (max of 3) according to:

$$\langle \tau \rangle = \sum_j A_j \tau_j$$

**FRET Efficiency:** Produces one image from the  $\tau_1$  and  $\tau_2$  images according to:

$$E = 1 - \frac{\tau_2}{\tau_1}$$

where  $\tau_1$  will be the longer lifetime (i.e. the lifetime of the FRET donor) and  $\tau_2$  the shorter (i.e. the lifetime of the FRET donor in the presence of the acceptor).

All definitions are from: Lakowicz, J. R. *Principles of Fluorescence Spectroscopy* (Plenum Publishers, New York, 1999).

## Batch Processing

Batch processing allows you to form a list of image files and leave the computer to process them one by one. You must first setup all the inputs to your TRA on any image so the computer knows the starting point for each image. This includes any mask but currently only one mask per batch can be specified.

Hopefully, the operation of batch processing panel is obvious so I will not go into it here, except to say...

The output of batch processing will be a number of files. These may be images and/or text files of data. They can be saved into the directory whence the image came or into another specified directory. If your images are read from a CD, the computer may not be able to write the output files to it, so you must specify a different directory in that case.

Different batch operations may appear in future. At the time of writing, there is only one TRA batch operation which performs the following:

- Load the time resolved image
- Copy any mask from the starting image to this image
- Do a fit on the image of the chosen type
- Save all resulting images
- Save raw fit data
- Close/discard the pixel fit workspace created
- Do a single “invariance” fit where all the pixels are binned together
- Save raw fit data
- Close/discard the time resolved image

It is possible to select which parameters to output data on but you must have processed an example image for the system to know what outputs are available to choose from.

## Appendix A – The Fitting Engines

### Triple Integral Fitting Engine

Divides the data in three and does a 3-integral fit.

If the chisq target is not met the fit is repeated until it is

OR tries => 10

WHILE the chisq continues to improve

With each refit the data is divided more (into 4, 5, 6 etc.) and the first three divisions taken

Checks that the last fit was not worse than the previous best

Returns the number of fits performed.

### Marquardt Fitting Engine

Does a fit starting from the initial parameters given

If the chisq target is not met, the fit is repeated until it is

OR tries => 10

WHILE the chisq continues to improve

The previous fit is used as a starting pt for the next one which should never be worse.

Returns the total number of iterations from all the refits added together.

More detail can be found on the fitting functions in the document: [Time Resolved Fitting \(Detail\).pdf](#)

## Appendix B – Global Fitting

The steps involved in global fitting are:

1) Estimate the global parameters.

Sum all the pixels together.

Triple integral estimate of  $Z_{est}$ ,  $A_{est}$ ,  $\tau_{est}$ .

(If this fails  $Z_{est}=0$ ,  $A_{est}=\text{max of summed data}$ ,  $\tau_{est}=2.0$ )

Set suitable values for multi-exp fits, e.g.:

Parameter	Mono-exponential	Bi-exponential	Tri-exponential	Stretched Exponential
Z	$Z_{est}$	$Z_{est}$	$Z_{est}$	$Z_{est}$
$A_1$	$A_{est}$	$\frac{3}{4} \times A_{est}$	$\frac{3}{4} \times A_{est}$	$A_{est}$
$\tau_1$	$\tau_{est}$	$\tau_{est}$	$\tau_{est}$	$\tau_{est}$
$A_2$		$\frac{1}{4} \times A_{est}$	$\frac{1}{6} \times A_{est}$	
$\tau_2$		$\frac{2}{3} \times \tau_{est}$	$\frac{2}{3} \times \tau_{est}$	
$A_3$			$\frac{1}{6} \times A_{est}$	
$\tau_3$			$\frac{1}{3} \times \tau_{est}$	
h				1.5

2) Perform Marquardt fit to get good values for the global parameters.

3) Fix these parameters for every pixel.

4) Estimate initial values for all the individual transients.

Triple integral estimate of  $Z_{est}$ ,  $A_{est}$ ,  $\tau_{est}$ .

(If this fails  $Z_{est}=0$ ,  $A_{est}=\text{max of transient}$ )

5) Calculate the exponential functions and convolve with the instrument response which only needs to be done once. (Local fits are now linear fits if all the taus are global!)

6) For each transient (pixel) call perform a Marquardt fit. One re-fit is tried if chisq is particularly bad.

7) Do the actual global fit. Julian Gilbey says, “It’s basically similar to a single Marquardt fit, except that now we handle the extra intricacies involved in global fitting, in particular, the much larger alpha matrix is handled in a special way.”

8) Calculate the number of degrees of freedom and report the global reduced ChiSq.

More detail can be found on the fitting functions in the document: Time Resolved Fitting (Detail).pdf