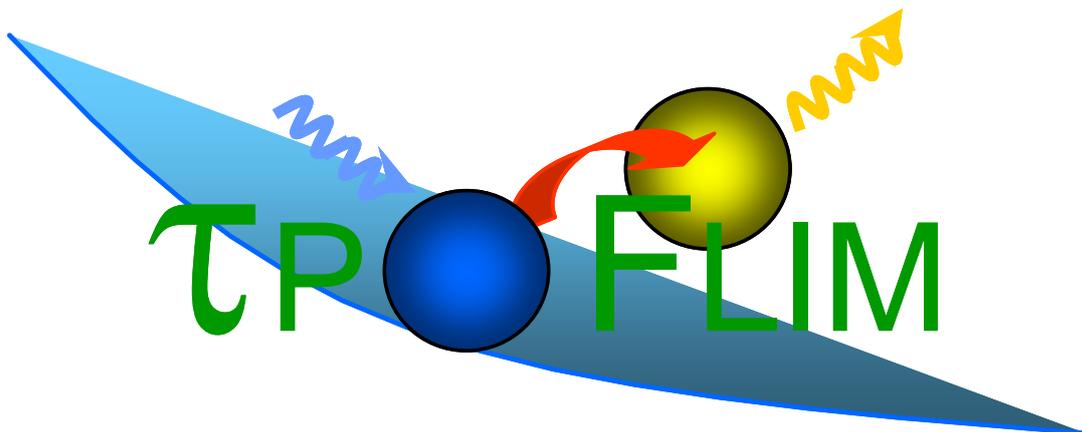


## TAU-POGRAPHY-FLIM Software



VERSION 3.1

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Seules les inscriptions de type S et C permettent un éventuel accès au programme source.

## Taupographie-FLIM software installation

Download the software

Create a folder that will contain the software (C:\Program Files\Flim)

Copy the content of the archive flim\_install.zip to this folder

Create a desktop shortcut to the executable file

The software uses the following libraries and files:

Flim.exe	Application
QtCore4.dll	Library Qt
QtGui4.dll	Library Qt
qwt.dll	Graphics library under Qt
cminpack.dll	Calculation Library: Levenberg-Marquardt algorithm
libtiff	Reading/writing library /image files in TIFF format
flim.ini*	Gives the convergence parameters for the Levenberg-Marquardt algorithm used in the least squares method
flimcolortable.txt*	Gives the pseudo-color table (3x8 bits) for the intensity and taumap display

\* If these files are absent the software uses default values.

NB : Flim.ini is searched in the 'documents' folder; flimcolortable.txt is searched in the 'documents' folder and then in the folder containing the program.

### Flim.ini Default contents

1.0e-3            relative error on the sum of squares (ftol)  
1.0e-3            relative error on X (xtol)  
1.0e-3            relative error on the gradient (gtol)  
400                maximum number of evaluation of the function in an iteration  
                  (maxfev)  
1.0e-3            relative function error (epsfcn)

If this file is present in the folder 'documents', the default values of parameters are replaced by the values read from this file.

NB: the maximum likelihood method uses the gradient conjugated algorithm (library GSL). The convergence are fixed (ftol=0.001, xtol=0.01, gtol=0.001, maxiter=100)

### Table of colors

In the absence of the flim colortable.txt file in the folder 'Documents' or in the folder of the program, a grey scale is used.

## Presentation of the Taupographie-FLIM software

The software  $\tau$ poFLIM can be used to analyse fluorescence decay data obtained using the Hamamatsu STREAK-FLIM system (NAF format files) or by using another system if the data are stored as a stack of TIF format XxT images (the current version does not support multi-TIF files). It is also possible to import saved decay values into a text file.

Starting with version 2.0, in the case of a NAF file containing time-lapse data, it is possible to analyse the data from time-to-time.

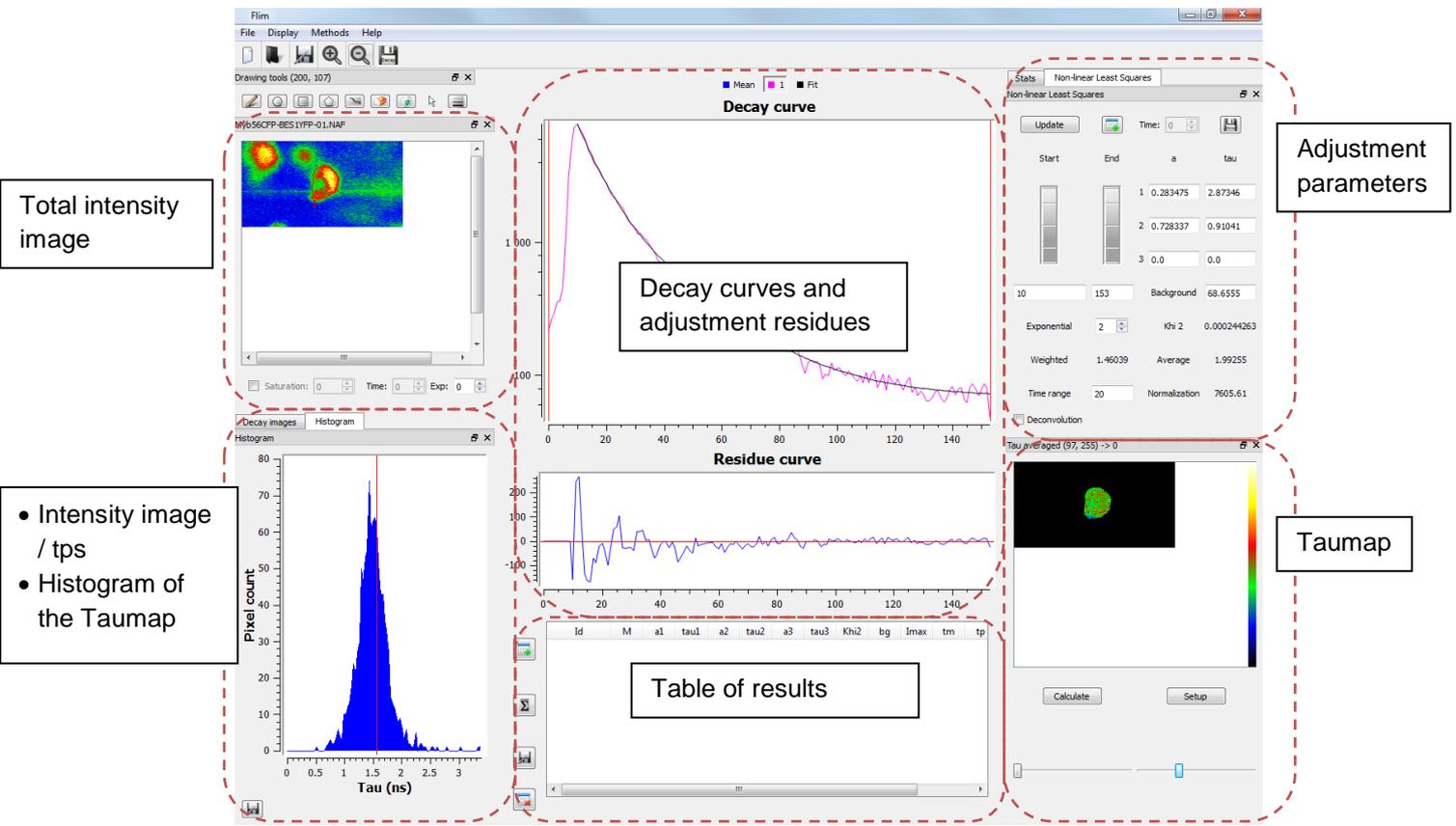
In version 3 it is possible to manage datasets (NAF or TXT files).

The analysis can be carried out either on the selected average area decay curves in the object (ROI), or on all the points of one or more ROIs (pixel analysis = calculation of “taumap”).

The adjustment of the decay curve to a multi-exponential model (order 1, 2 or 3) can be carried out using various methods: linear estimation or log-linear regression (for mono-exponential only), non-linear least squares or maximum likelihood (Poisson distribution).

The decay curves can be saved for further analysis. The values of the calculated parameters can be saved in a text file (html), as well as the corresponding decay curves.

It is possible to save the values of a “taumap” as an image TIF file (values encoded in real single precision) and the corresponding histogram in a text file.

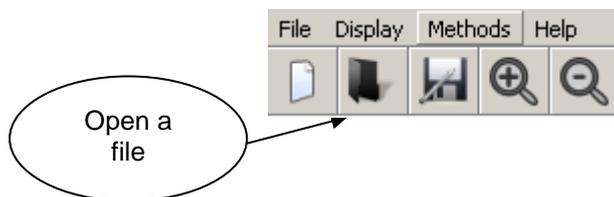


**Screenshot of the working window of the software**

## Quick start

### Load decay data

To open a file, click on 'File' > 'Open' or click on the icon in the Tool Bar.



### Open a NAF file

The .NAF files are specific data format acquired with the Hamamatsu Photonics Streak-FLIM equipment.

Select the file containing the data and click on 'Open'.

The data are loaded and the intensity image is displayed. If the file contained ROIs, they are also added to the image.

## Open a stack of TIFF images

In the case of a stack of TIFF images, the names of the files must be as 'prefix0n.tif' or 'prefix00n.tif' according to the total number of the images in the stack.

Select one of the file and click on 'Open' to load the entire stack.

The image intensity is then displayed.

Indicate in the 'Time range' button (right of the window), that is TIFF format.

## Loading a decay curve (.txt)

Allow you to reload decay curves previously saved by the software or by the Hamamatsu software.

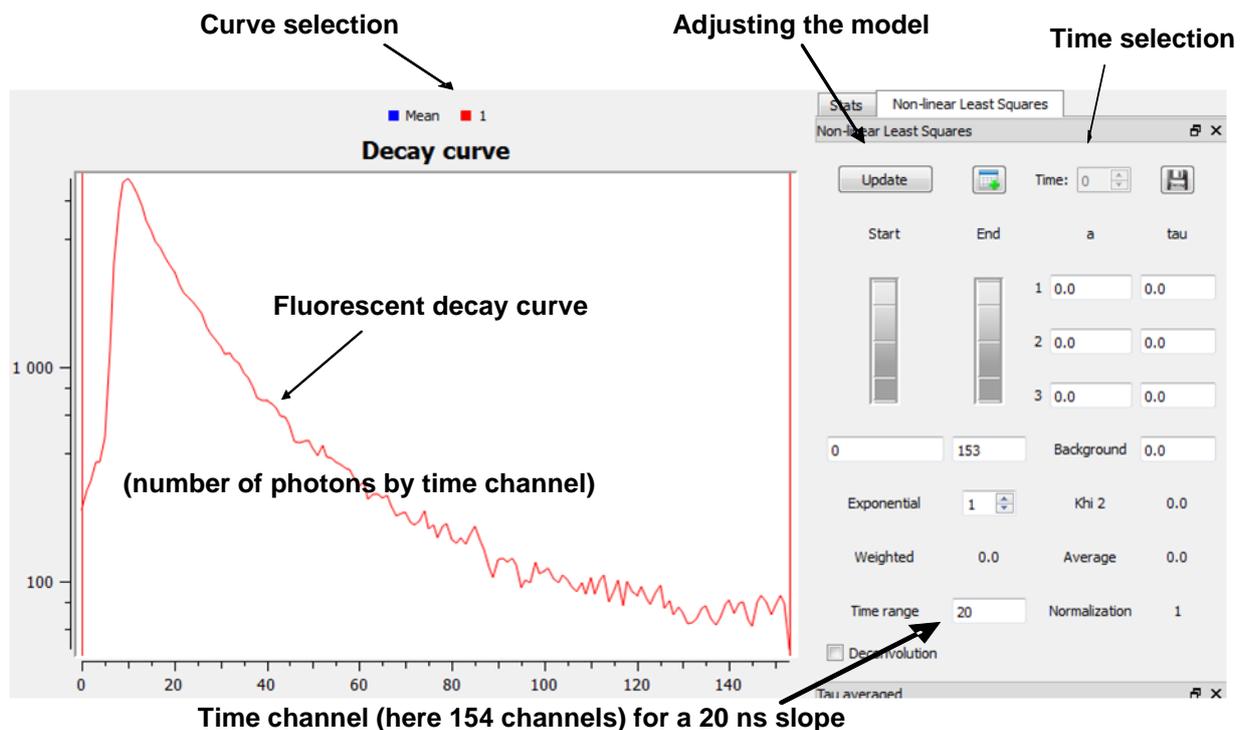
Select the file and click on 'Open'. The decay data are then loaded and the curves are displayed in the central part of the window.

## Adjusting a decay curve

1. Draw an ROI (Region of Interest) on the intensity image using the drawing tool.



2. Select a decay curve using the button at the top of the center window.



3. Select the calculation algorithm (by default: non-linear least squares)



4. Select (in the right part of the window) the number of exponential for the selected adjustment model and click on 'Update'.

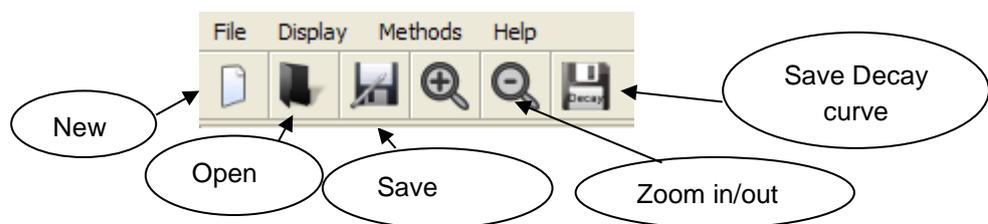
The decay curve is then adjusted and the parameters are presented in the right part of the window.

NB: The values of the pre-exponential coefficient are normalized with respect of the maximum of the curve.

The adjustment window can be changed using the two knobs on the right side. By default, the adjustment is made between the time corresponding to the maximum of the decay curve and the end of the curve.

## Description of the various elements of the main window

### Main tool bar



### Menu File :

File>New  : clear the data buffer and reset all the parameters

File>Open  : open the data file

File>Add : add a new data set. The 'Exp' value is incremented by '1'.

File>Save as  : save the table of results

File>Print: print the table of results.

File>Load Intensity Color table: load the color table for displaying the intensity of images (default=greyscale). File of the same format as the file Flimcolortable.txt

File>Load Taumap Color table: load the color table for displaying lifetime images (default=greyscale)

File>Save decay curve  : save in a text file the selected decay curve values (choose the name file).

Zoom   : zoom + or – on the intensity images and on the lifetime images. (Taumap)

**Menu Display** : display/hide various elements of the window.

**Menu Methods** : select the calculation algorithm to make adjustments

- Linear estimation : RLD (Rapid Lifetime Determination), only for mono-exponential case
- Log-linear regression: linear regression after log transformation of the data. (assume a background equal to 0). Only for mono-exponential case
- Non-linear least squares: default choice. Adjustment no-linear least squares method using the Levenberg-Marquardt (library CMINPACK) algorithm.
- Maximum likelihood: maximum likelihood method. Assume that the background is distributed as a Poisson law. To be used with very noisy data (calculation at the pixel level). Use the conjugated gradient algorithm (library GSL).
- *Instrumental response* : *load a measured instrumental value. Functionality not taken into account in this version.*

## Left part of the window = visualization of the data

The left part of the window allows the visualization of the data. The upper panel shows the total intensity image, integrated on the whole time channels.

**Name of the open file** → Image08.NAF

**Intensity image (x,y)**  

$$I(x, y) = \sum_{t=1}^{t_{\max}} I(x, y, t)$$
 (projection of the intensities for the whole time channel)  
 The ROI or existing ROI are imported

**Adjusting the saturation for the display** → Saturation: 0

**Time index in the case of the time series** → Time: 0

**N° of the whole time series** → Exp: 1

**Intensity image  $I(x,y)$  by time channel  $t$**

**Scrolling slider for the images  $I(x,y)$  depending to the channel number**

**Number of the time channel displayed** → 20

**Saturation value for the displayed image** → Saturation: 0

In the lower panel, the 'Decay image' tab displays the intensity images per time channel.

The 'Histogram' tab displays the histogram of the 'taumap' on the right panel

## Drawing tools bar: defining one or more ROIs (Region Of Interest)

You can create or suppress ROIs using the drawing tools.



When this button is selected the hover with the pointer automatically displays the decay curve associated with the pixel. Select the pixel by clicking on the image and display in the 'Decay Curve' window the decay curve as a function of time.



These buttons allow drawing spherical, rectangular and polygonal ROIs. Each point of the curve corresponds to the average intensity as a function of time (sum of the intensity of the pixels included in the ROIs divided by the number of pixels in the ROIs).



This button allows you to draw a line as a ROI (this tool is important when linear structure like membranes are selected). The thickness (pixel) of the line is adjustable by clicking on this button



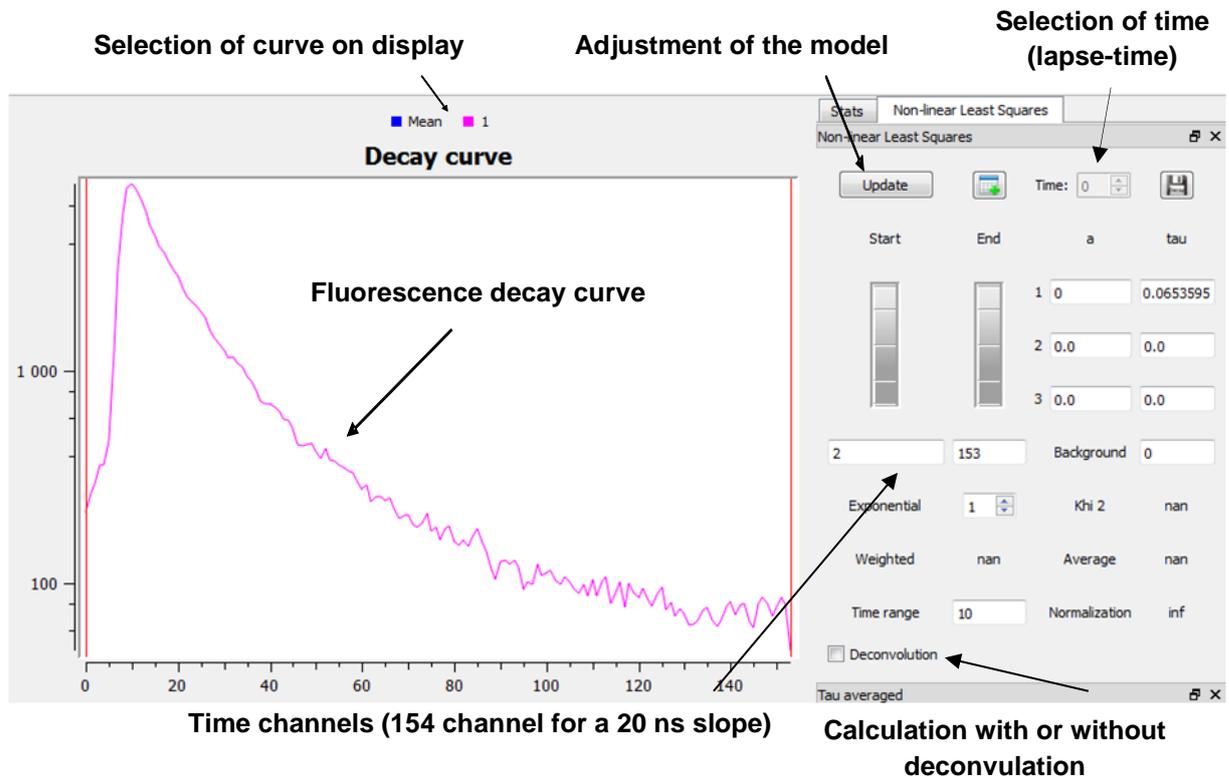
delete the selected ROI



delete all ROIs.

The arrow  activates the ROI selection mode ROIs

## Middle part of the window = display decay curves and parameters (mono- or multi-exponential models)

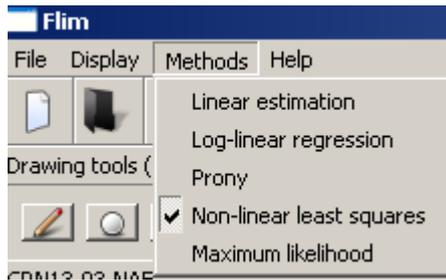


The fluorescence decay curve  $I=f(t)$  is automatically displayed for each ROI. The abscissa scale represents the time channel number and the ordinate scale represents the intensity (logarithmic scale in number of counts). The  $I$  value for each time channel corresponds to the mean of the  $I$  values for the pixels included in the ROI. You can display several curves by open image. Each curve corresponds to distinct ROI.

The 'Mean' curve displays by default the average decay curve calculated for the entire image. It is also used to display the average curve stored in the result table (see the backup paragraph of the tutorial)

The selected curves can be deleted by clicking the 'Delete' key of your keyboard. In that case the corresponding ROIs are also deleted.

On the right part, by clicking on the 'Update' button, the selection is adjusted as a mono or multi-exponential model according to the method selected in the 'method' menu (by default, the applied method corresponds to a 'non-linear least square adjustment method').



The non-linear least squares method is well adapted to data being noisy by a variable fitting a normal distribution. This is approximately correct when the mean of the decay curves are adjusted on a ROI.

In the following example, the adjustment type is the secondary order (exponential decay of 2<sup>nd</sup> order):

$$I = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} + y_0$$

**Decay curve**

■ Total curve ■ Curve 1 ■ Fitted curve

Experimental curve

Adjusted curve

**Residue curve**

Stats Non-linear Least Squares

Non-linear Least Squares

Update

Time: 0

Start	End	a	tau
1	0.683378	2.66926	
2	0.278085	0.622491	
3	0.0	0.0	

12 153 Background 114.11

Exponential 2

Weighted 2.07726 Average 2.49186

Time range 10 Normalization 5292.91

Tau averaged (82, 12) -> 0

Value of each point of the curve (= subtraction of the experimental and the adjusted values)

Id	M	a1	tau1	a2	tau2	a3	tau3	Khi2	bg	Imax	tm	
1	Image08:1:0	NL	0.683	2.669	0.278	0.622	0.000	0.000	0.00...	114.1	5292.9	2.492

Table of the estimated parameters  
This table can be saved

Applied adjustment model

To apply the adjustment model to the curve

To update the time window

Number of the time channel corresponding to the left part of the adjustment window (here adjustment from the 12<sup>th</sup> channel)

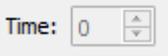
Number of the time channel corresponding to the right part of the adjustment window (here adjustment to 154<sup>th</sup> channel)

The model is fit to the raw data (not normalized). In contrast, the pre-exponential coefficients display in the right part of the window are normalized in the respect to the

maximum value of the curve and represent approximately the part of each exponential term in the final model.

When least square or maximum likelihood adjustment method are used, it is possible to fix the value of one or more parameters ( $y_0$ ,  $a_i$ ,  $\tau_{i_j}$ ) :

- Indicate the values in the corresponding box
- double click on the box which becomes red.

When using a time serie, incrementing the time index with the button  causes an automatic update of the decay curves and the adjustment of the current model.

## Deconvolution :

By default, calculation is made without deconvolution of the measured data. To activate the calculation with deconvolution mode, click on the button in the left part of the parameter panel. The method does not use a previous measured response, but is based on a Gaussian model of the instrumental response.

When activated, the deconvolution is also applied to the Taumap calculation.

NB : Deconvolution method is available only with the least squares method.

## Middle and right part of the window = adjustment results

Results are indicated in the parameter panel.

The pre-exponential coefficient are normalized (after estimation) with respect to the maximum observed value, therefore the sum is close to 1 (the meaning of 'a' is: the component of a lifetime  $\tau_1$  contributes to  $100\alpha_1$  % of the intensity I, and the component of a lifetime  $\tau_2$  contributes to  $100\alpha_2$  % of the total intensity I).

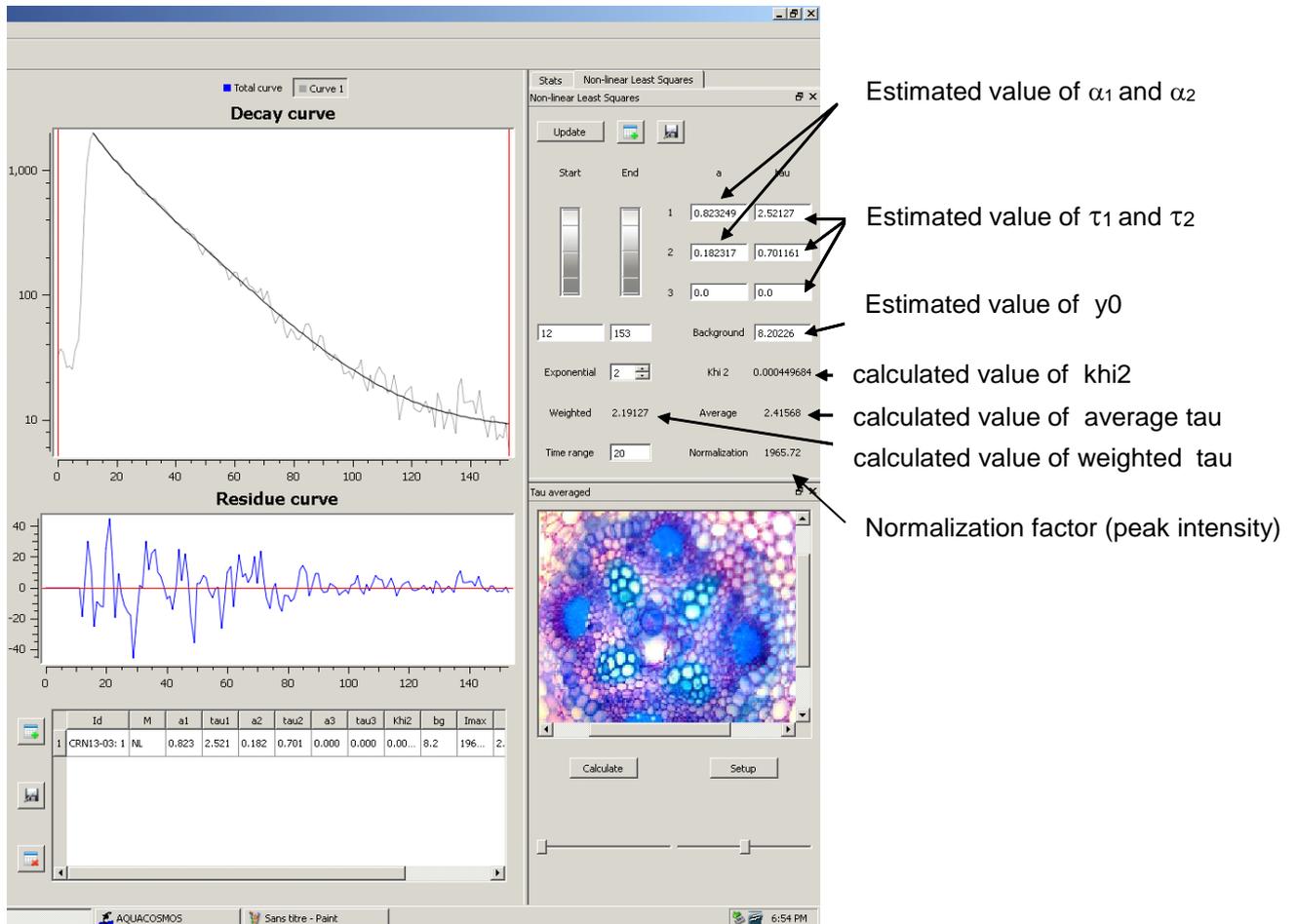
The calculated value corresponding to the mean lifetime at the excited state, mean tau ( $\tau_m$ ) is given by:

$$\tau_m = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$$

And the value of the weighted tau ( $\tau_p$ ), is given by:

$$\tau_p = \frac{\sum \alpha_i \tau_i}{\sum \alpha_i}$$

The value of the normalization of the pre-exponential coefficients corresponds to the intensity of the peak ( $I_{max}$ )



The  $\chi^2$  value corresponds to the sum of the square of the gap between the experimental values and the adjusted values (or the sum of the residues squares displayed in the window 'Residue curve') divided by the number  $n$  of the time channels:

$$\chi^2 = \frac{\sum (I_{\text{mesurée}} - I_{\text{calculée}})^2}{n}$$

A first estimation of the adjustment quality is based on 3 criteria. Thereby check that:

$\sum \alpha_i$  is close to 1, the residues are distributed equivalently to each part of the experimental decay curve and that the khi2 value is minimized.

## Save the data and the results

**Mean curve of the table**

**Save the table**

**Delete the selected rows**

**Add the values and the parameters in the table**

**Save the selected decay curves**

Id	M	a1	tau1	a2	tau2	a3	tau3	khi2	bg	Imax	tm		
1	Myb56CFP-...	ML	0.508	2.210	0.517	0.639	0.000	0.000	0.00...	78.2	7605.6	1.853	1.



This button on the upper part of the parameter panel saves the selected decay curves in a text file. It is similar to 'File>Save Decay Curve' and the button located on the main tool bar, except in that case the name of the file is automatically generated from the name of decay data name and the curves number.



Add the current parameter values to the table located in the lower part of the main window. It has the same effect than the same button located in the left of the

table. To be note that the parameter values are recalculated before to be copied in the table.

## Values of the table

	Id	M	a1	tau1	a2	tau2	a3	tau3	Khi2	bg	Imax	
1	CRN13-03: 1	NL	0.823	2.521	0.182	0.701	0.000	0.000	0.00...	8.2	196...	2.

In this table, the following data are saved:

- Id : name of the file (CRN13-03) and the ROI number (1)
- M : adjustment model used (NL for non-linear least squares, ML for maximum likelihood)
- a1, tau1 ; a2 ; tau2..., khi2, bg et Imax.: correspond to the estimated parameters, to the Khi2 value, to the background and the value of normalization.
- tm et tp : correspond to the mean tau and the weighted tau respectively

$$\tau_m = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad \tau_p = \frac{\sum \alpha_i \tau_i}{\sum \alpha_i}$$

- t0 : time channel at the beginning of adjustment (by default the time channel for which I is maximum)
- t1 : time channel at the end of adjustment

NB: these two values are indicated in the boxes located below the adjustment knobs.



Save the data of the table in a html file and save the data of the corresponding curves in a text file with the same prefix and suffix '\_data.txt'.



Calculate the mean curve saved in the table and display the curves instead the curve 'mean'. It is possible to adjust and/or save this curve.

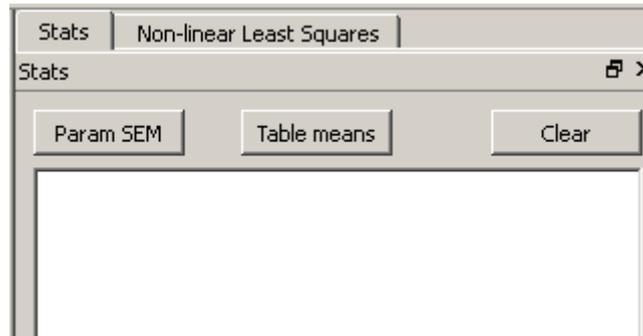


This button has two functions: it allows to delete a selected row or, if no row is selected, the whole table.

NB: During the analysis of a new set of images, it is essential to delete (or reset) all the functions by clicking on the button 'New file' 

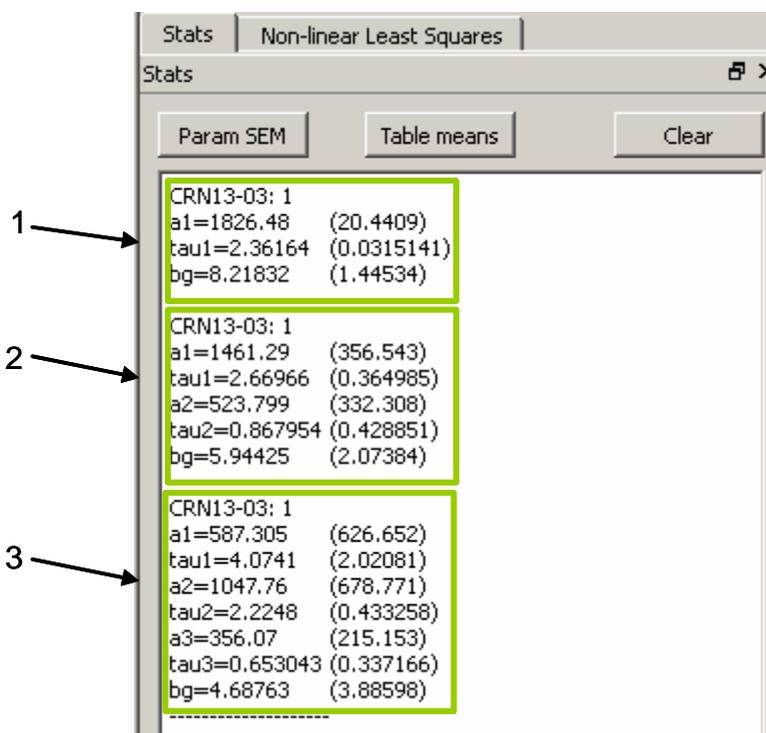
## Estimation of the adjustment quality

When an adjustment is complete, click on the 'Stats' tab of the parameters panel.



NB: The data can be copy and paste in a text document.

**Button 'Param SEM'** : allow to estimate the standard deviation for the various parameters of a curve using a bootstrap method with 30 repetitions



The following values are displayed:

Name of the file: number of the curve

Mean value of the parameters  $\alpha$ ,  $\tau$  et  $bg$  ( $y_0$ ).

In brackets, the value of the standard deviation.

NB: The values of the pre-exponential coefficients are not normalized.

This functionality allows

choosing the better adjustment by minimizing the standard deviation.

As example:

- The frame above (1), (following an adjustment mono-exponential) the mean tau1 value is 2.36 with a standard deviation of 0.031 ns corresponding to a coefficient of variation of 1,3 % (CV = standard deviation / mean).

- In the middle frame (2) (following an adjustment bi-exponential) the mean tau2 value is 2.67 with a standard deviation of 0.36 ns corresponding to a coefficient of variation of 13,6 % (CV = standard deviation / mean).

etc....and for all the parameters.

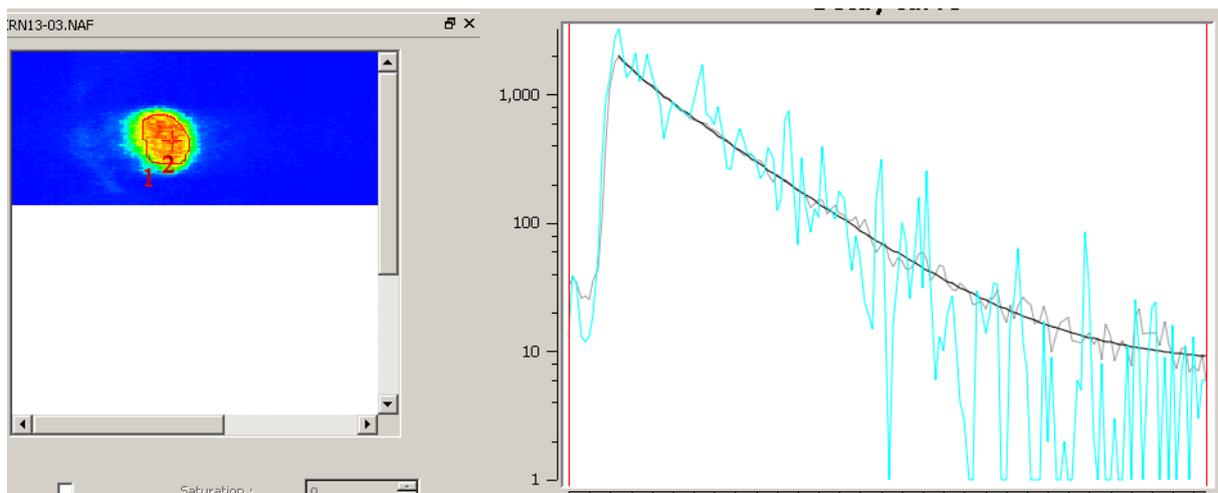
These values of standard deviation or CV have to be compared with those obtained on a population sample (n measures on same type of objects).

**Button 'Table Means'** : displays the mean values and the standard deviation of the data stored in the table of result.

**Button 'Clear'** closes the window

## Carry out a mapping of the lifetime

The problem is not to give an average lifetime within the object (or a ROI) but to establish a mapping of the duration within the object. This corresponds to the evaluation of lifetime at each pixel and therefore to perform as many adjustment as there are pixel in the image.

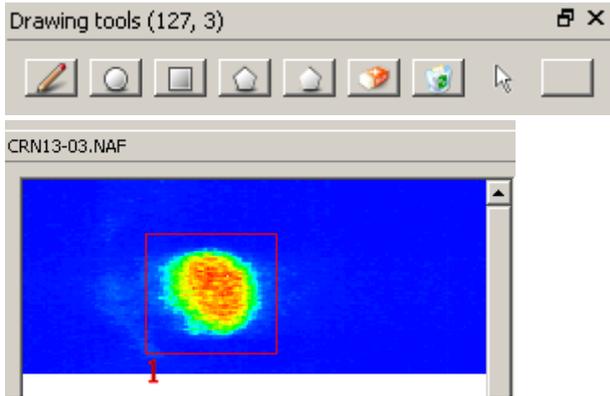


In the above example, the grey curve corresponds to the experimental decay curve (mean of unit curves of the pixels contained in the polygonal ROI, denoted 1 in the image) and the black curve to the adjusted curve.

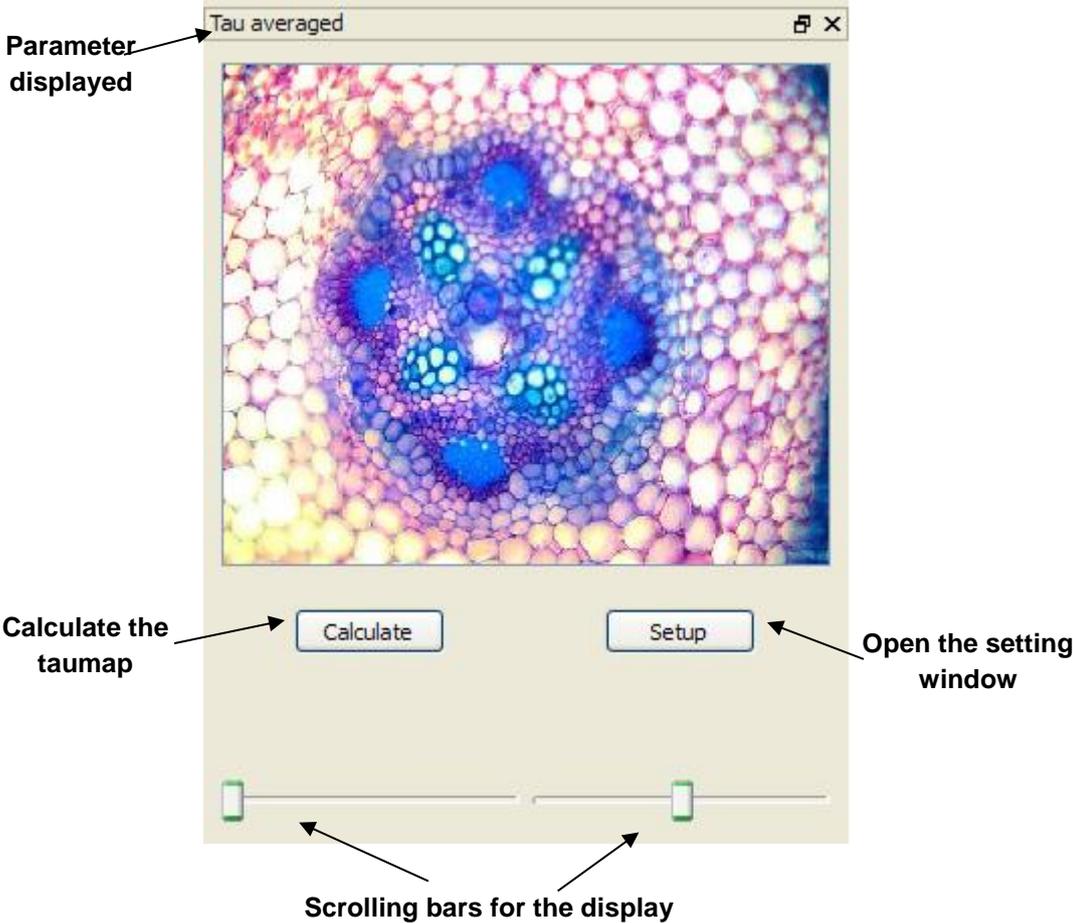
The blue curve illustrates an example of decay measured for one pixel labelled by a red cross on the image.

### Procedure

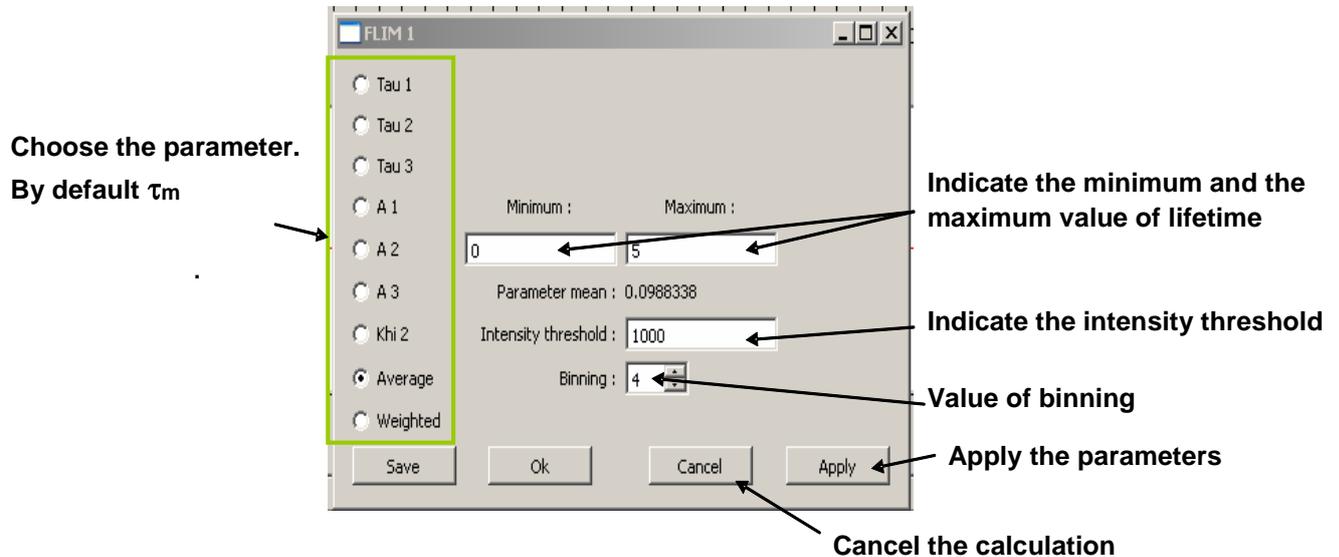
In the image window, you can delete ROIs by clicking on the  button and draw new ROIs around the interesting area in order to minimize the number of pixels.



The setting of the parameters for calculation and the calculation are done in the 'Tau map' window, as presented below:

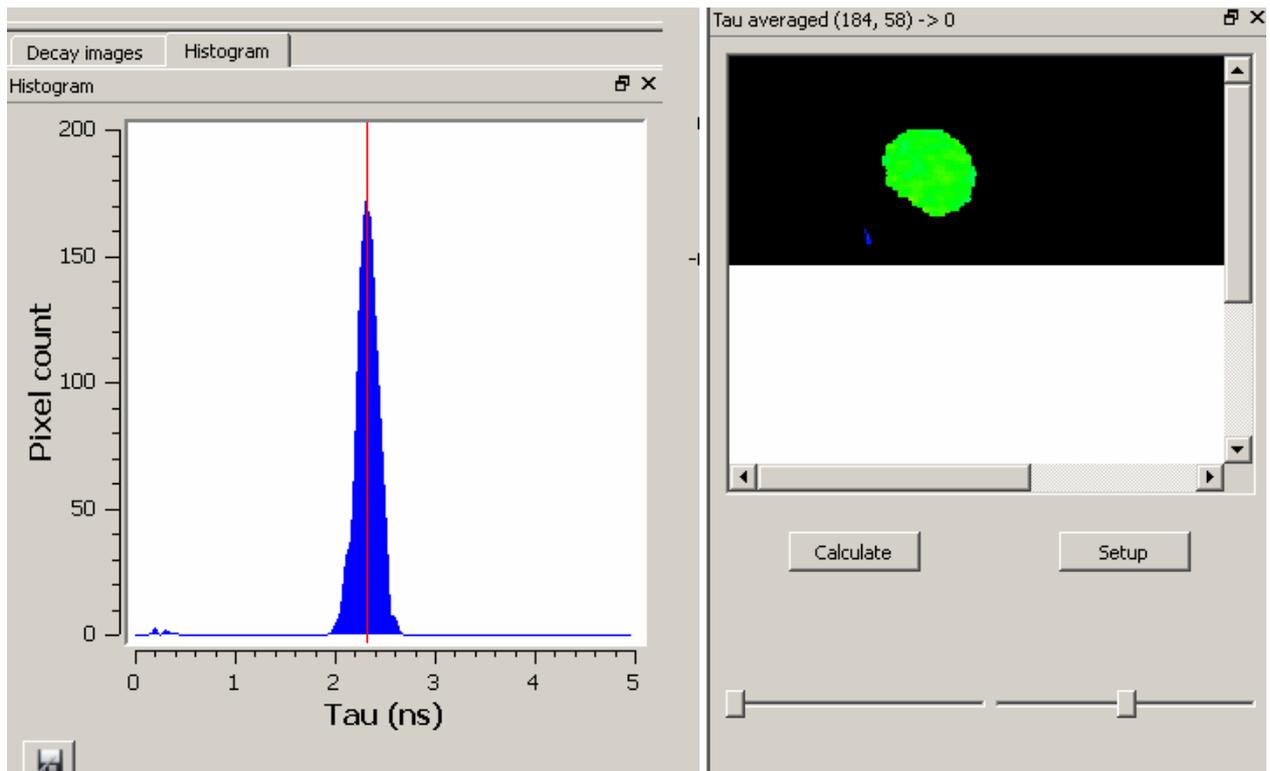


## 'Setup' window:



- **'Intensity Threshold'**: the pixels that display a value under the threshold will be not adjusted (and set to 0 in the mapping image). This allows to greatly reduce the calculation time.
- **'Binning'** allows to choose an average value of the pixel for the calculation grouping them 2x2, 3x3, 4x4, ... This partially avoids the aberrant values and decrease calculation time.
- **'Minimum' and 'Maximum'**: define the min and max values of the calculated parameter to be used for the display. They correspond to the minimum and maximum indicated on the scrolling bar in the 'taumap' window.

The result is presented in two forms: a mapping image of the selected parameters ( $\tau_m$  right part of the figure below) and an histogram corresponding to the distribution of the values of the parameters inside the image or in the selected area (left part of the figure below).



## Backup of the calculated data

- **Mapping image:** open the window 'Setup' and click on 'Save'. The image is stored as a TIFF file.
- **Histogram:** Click on the button located at the bottom left of the histogram. Data are stored in a text file.