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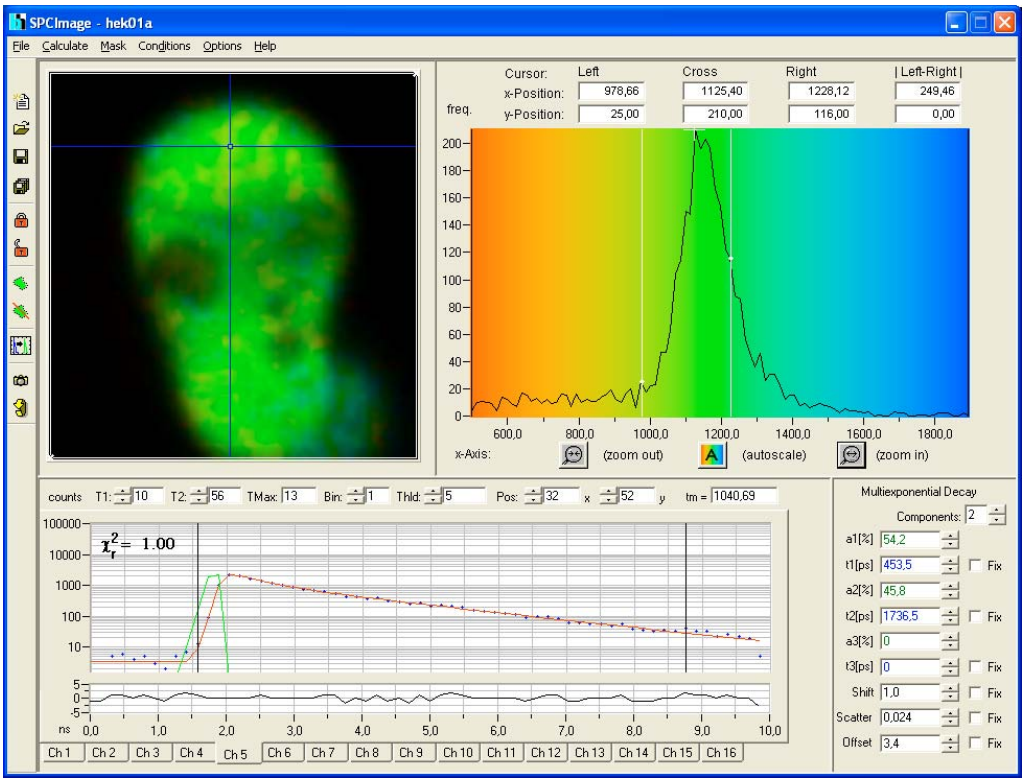
SPCImage 2.9

Data Analysis Software for Fluorescence Lifetime Imaging Microscopy



System requirements*:

- **Recommended Software Platforms: Windows™ 2000 or XP**
- **CPU: Intel Pentium™ or AMD-Athlon™ with at least 1 GHz*)**
- **Memory: 512 Mb or more**
- **Harddisk: 5 Gb free space**
- **Graphics Card: Resolution of 1024x768 or better (+ dual monitor option **)**
- **Monitor: 17´´ or larger.**

*) recommend for analysis of high-resolution images – poorer specs may work but can cause long calculation times
**) when running acquisition software and analysis software simultaneously

The fluorescence decaytime of *a* specific molecule located in a *defined* microenvironment is a physical constant. Unfortunately the situation for fluorescence decay data which were aquired from complex biological samples turned out to be not so simple.

Taking in mind that different molecules contribute to the fluorescence within each pixel of an image a heterogeneity of the lifetime-data gets unavoidable. Moreover the fluorescence decay is measured with a system providing a fixed time-resolution. Further complications are intruduced by the signal-to-noise of the data which is given by the the fluorescence intensity of the sample, the quantum yield of the detector, and the measurement time.

The approach of the analysis tool "SPCImage" is very general and it is finally up to the user to decide which result he is going to trust under a certain experimental condition. We tend away to make the software more restrictive to avoid unnecessary limitations when analysing complex experimental data. However, the mathematical concept of the analysis has to be clarified in order to enable a scientiest to make a sound decision. Fluorescence time-domain imaging belongs to a class of measuring techniques in which the information of interest is extracted by post-processing the experimental data. In general three tasks have to be performed:

i) Definition of a model function which is suitable for the description of the measurement data

ii) Invoking the algorithm to fit the model parameters to the data set.

iii) Selecting the parameter(s) of interest from which the (color-coded) image is created.

After the first phase in which the SPCImage software was applied to "real-life" measurements the manual was extended to provide more background information for enabling the user to interpret the calculated data in a reliable way. We would like to encourage the user to take some time to read this documentation – all further suggestions and comments will be highly appreciated.

1. Introduction

The SPCImage software was designed to analyse and present the data of fluorescence lifetime imaging (FLIM) measurements by fitting it to a "model". The model´s parameters come from an underlying theory that the fluorescence decay of the investigated sample is supposed to satisfy. The basic approach in all cases is the same: The software minimizes the chi-square value between the data and the model function during the fit process. The result is a set of parameters (e.g. lifetimes) for each individual pixel of the image. In a subsequent step the user selects one of the parameters or a mathematical combination of different parameters to create a "lifetime image".

The time slope of the fluorescence obeys an exponential behaviour if the transfer rate which deplets the excited state is a constant in time. This holds true for all experiments which use moderately low intensities far away from the threshold of stimulated emission. However, there are several effects which make the definition of the model function for a typical sample fluorescence more complicated. Two of them are:

i) The fluorescence which originates from one pixel is an overlay of the emissions of different chromophores.

ii) The chromophores within a single pixel of the FLIM image are in different photophysical states (e.g. due to changes in the microenvironment or energy transfer processes).

Therefore the time slope of the fluorescence decay usually is a *sum* of different exponential decays. With this in mind and the limitation of up to three different components the model

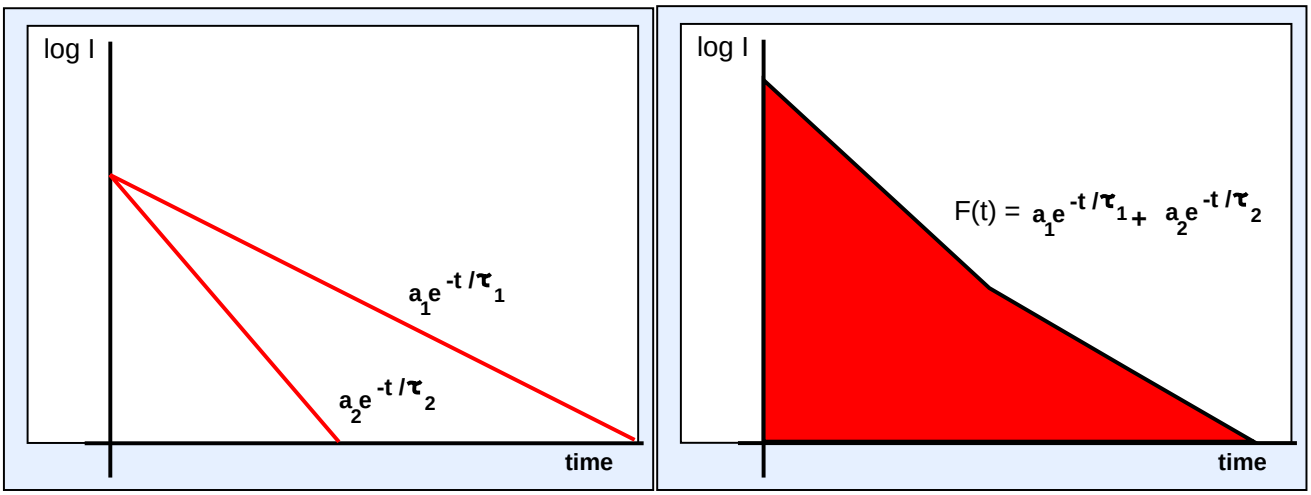


Figure 1: Sum of two exponential decays showing different lifetimes

function for each individual pixel runs as:

It is up to the user how many exponential components are select for an image. Measurements with quite poor signal-to-noise ratios (SNR), i.e. a low number of photons per pixel might allow a single exponential model. In this case the amplitude coefficients a_2 and a_3 are set to zero. However, in cases where the fluorescence was measured with a good SNR the user will have to switch to a double exponential decay or even go up to three exponential components.

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3} \qquad (1)$$

Equation (1) is valid if the width of the instrumental response function (IRF) of the measurement system is extremely small compared to the width of the time channels of the histogram in which the photons are stored. The IRF defines the overal time-resolution of the measurement system. It is built up by the pulse-width of the laser (which is negligible small for a femtosecond laser system), the electrical resolution of the TCSPC card and (most important) the transit-time-spread of the detector. In a typical experiment (fast lifetimes, small channel-width) the measured intensity follows a mathematical concolution of the model function F(t) and the instrumental response function R(t).

$$I(t) = F(t) \otimes R(t - t_S) \qquad (2)$$

Here the calculated function which is fitted to the decay trace is an integral over time. The SPCImage software provides an "estimation" of this response function by calculating the first derivative of the rising part of the fluorescence. However, it is preferable to determine this

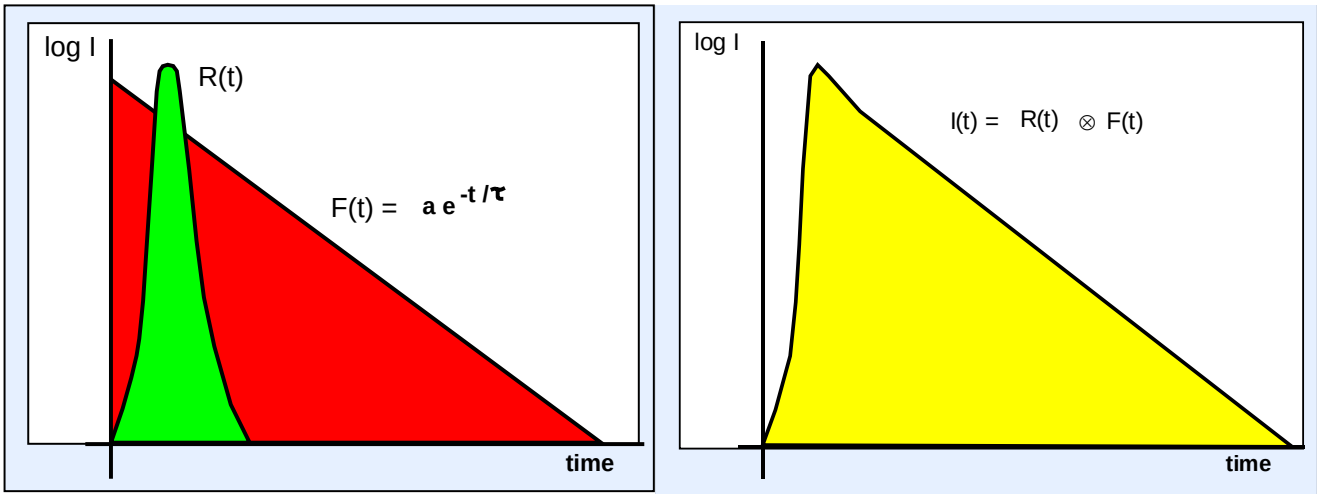


Figure 2: Convolution of the instrumental response function (IRF) with a single exponential decay trace

function by an experiment. The parameter t_S denotes a linear shift between the reponse function and the fluorescence and is determined automatically by the software.

Another complication can arise due to scattered light or a second harmonic generation inside the sample (in case of 2p exitation) which bleeds into the detection channel causing a pronounced peak in the first part of the curve. Although – in principle – this part could be

$$\tilde{I}(t) = I(t) + s \cdot R(t) \qquad (3)$$

considered by a very fast lifetime component which is beyond the FWHM of the detector

Since the scattering and/or second harmonic generation processes R(t) are extremely fast compared to the response time of the system it adds linearly to the decay trace I(t) with a

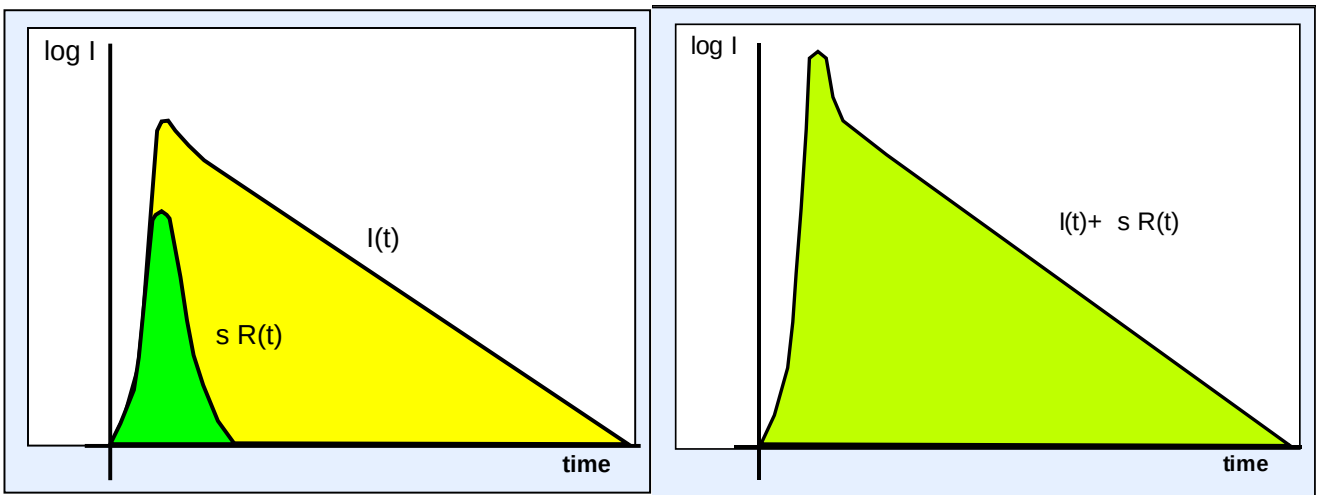


Figure 3: Effect of scattering or fluorescence bleedthrough

factor s ="scatter" which itself is defined as a fitting parameter.

In addition almost all detection systems pick up some ambient (room) light which together with the noise of the detector produces a constant baseline or "offset". This number has to be taken into account to avoid the artificial generation of a long-lifetime component by the fitting process. The "offset" a_0 can either be measured by an independent dark experiment or determined automatically by means of the photons which are in the time channels in front of the rising part of the fluorescence decay trace. However, next to the dark noise of the detector also an effect called "afterpulsing" contributes to the noise. Since this effect depends on the countrate of the detector it is not possible to derive it from a dark experiment.

Please make sure the measurement control parameters and cable-length are selected accordingly to get enough time channels into this "pre-curve" part.

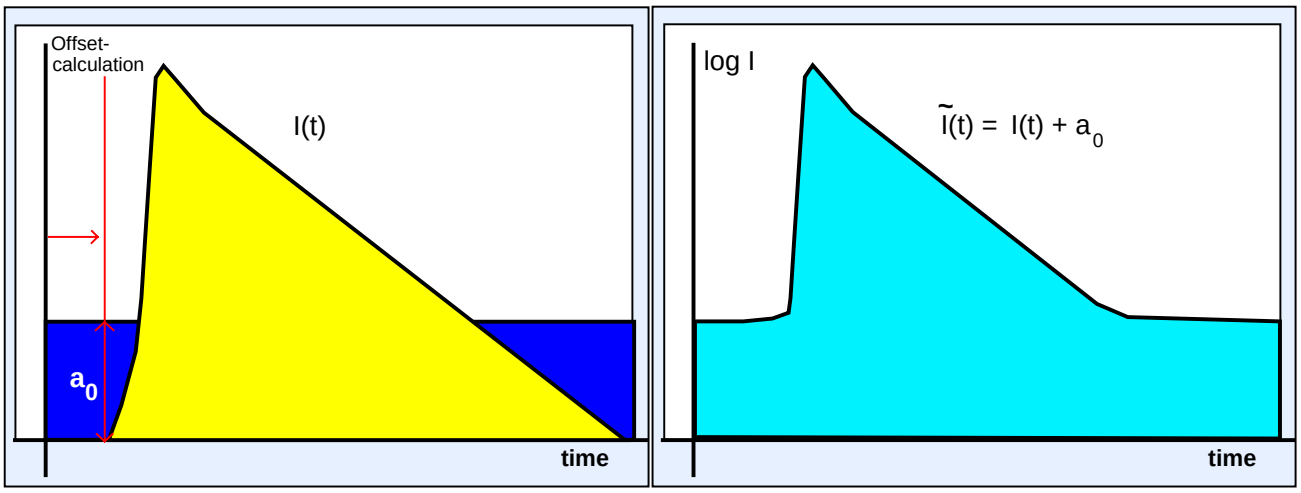


Figure 4: Effect of ambient light and detector dark noise

The "Incomplete Model" option should be used if the fluorescence decay is slow compared to the time window which is defined by the repetition rate of the laser system . If the time between the exciting laser pulses is given as an input parameter the software calculates the amount of fluorescence which arises from all previous laser-pulses. The most significant contribution is of course from the laser period which is located directly before the current one

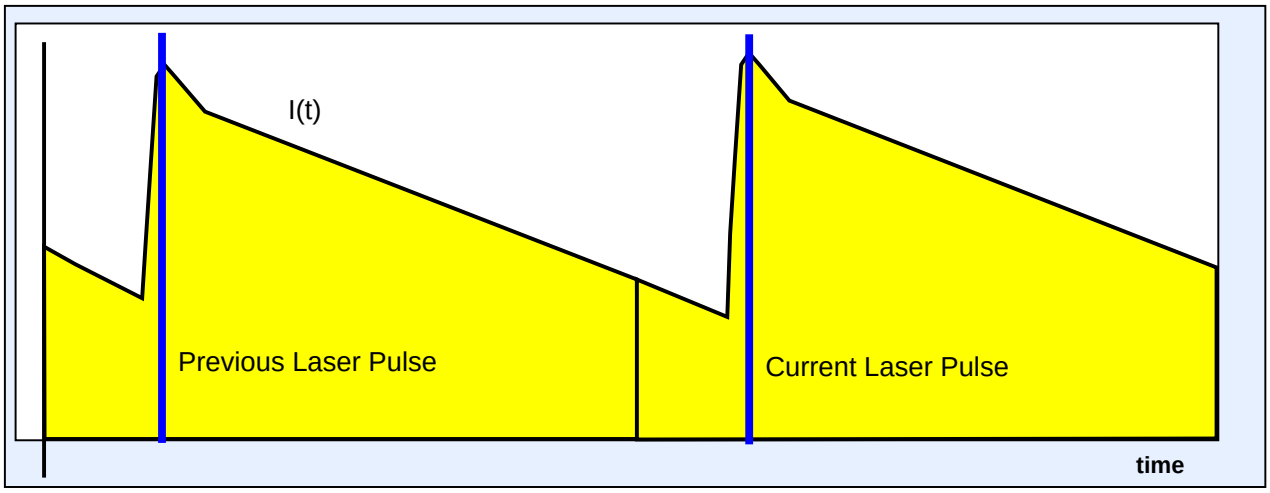


Figure 5: Effect of "incomplete decay" on the fluorscence decay

as shown in fig. 5.

The situation gets more complicated if there is a constant offset due to a considerable amount of ambient light in addition to the incomplete decay. In this case the offset calculation from the datapoints in front of the rising part of the fluorescence fails for version 2.7 and lower. The

newer versions are able to handle offset correction and incomplete decay simultaneously by substracting the amount of fluorescence from the offset value.

2. Analyzing fluorescence lifetime images

The *SPCImage* software provides access to the "lifetime information" of a time- and spatialy resolved dataset which was measured with one of the SPC-730/830/144 imaging boards. There are two possiblilties to tranfser the data from the measurement software *SPCM* to *SPCImage*: DDE-transfer or file import. The DDE transfer is convenient if the user wants to perform fast checks on the quality of the data. Some of the parameters contained in the .sdt file which is generated by the measurement software are not stored in the file format of *SPCImage* (.img). In case you only use DDE-Transfer these parameters are lost and can not be reproduced or reloaded.

The setup CD for *SPCImage* contains several sample data files which can be found in the \Datafiles directory. After successfully importing the "cells.sdt" file (please check the User-Dialogs ***File>Import*** section for instructions) an intensity image is displayed.

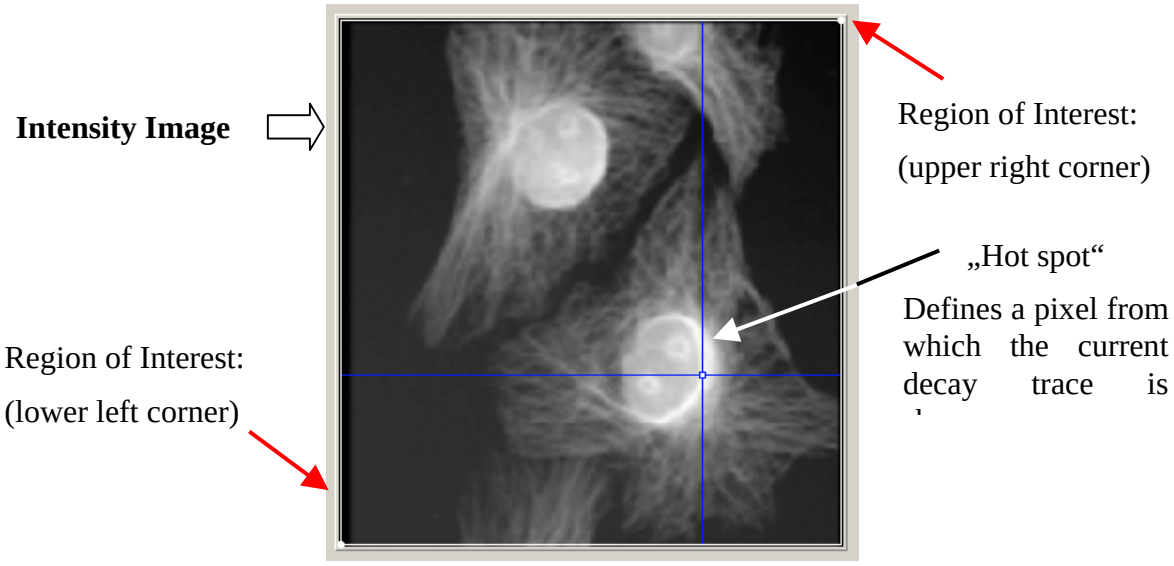


Figure 5: Image which is shown after importing the cell.sdt file

SPCImage uses an autoscaling mechanism to select the intensity scale of the image. In cases where this method produces unsatisfactory results this scale can be changed by the ***Options > Intensity*** dialog (see section 3). It is also possible to choose the relative orientation in this dialog (mirroring of axes).

After loading the data the software will choose the brightest pixel of the image as a “Hot spot”. The location is indicated by the blue crosshair and is also given as a numerical postion (x,y) in the decay window which is described below. The IRF is calculated from the data trace which belongs to the selected pixel by an estimation method described in the introduction.

The pixel selection can be changed by moving the blue crosshair in case there are doubts if an automatically selected pixel contains distortions. Please invoke the Calculate>System Response (F6) command to repeat the estimation of the IRF for the currently selected pixel.

Two white crosshairs are located in the upper right and lower left corner of the image. They define a region of interest (ROI) which will be used during the data analysis. They can be changed by clicking on the white dots and moving them to a different location. Thus, object(s) which fill the image only partly can be selected by defining a “box” around them. Please note: Defining ROIs may save a lot of computation time since only the area inside the crosshairs is taken into account.

The “Decay-Graph” is located beneath the intensity image and contains several numerical parameters. The following items are shown: The photon decay data (blue), trace of the fit

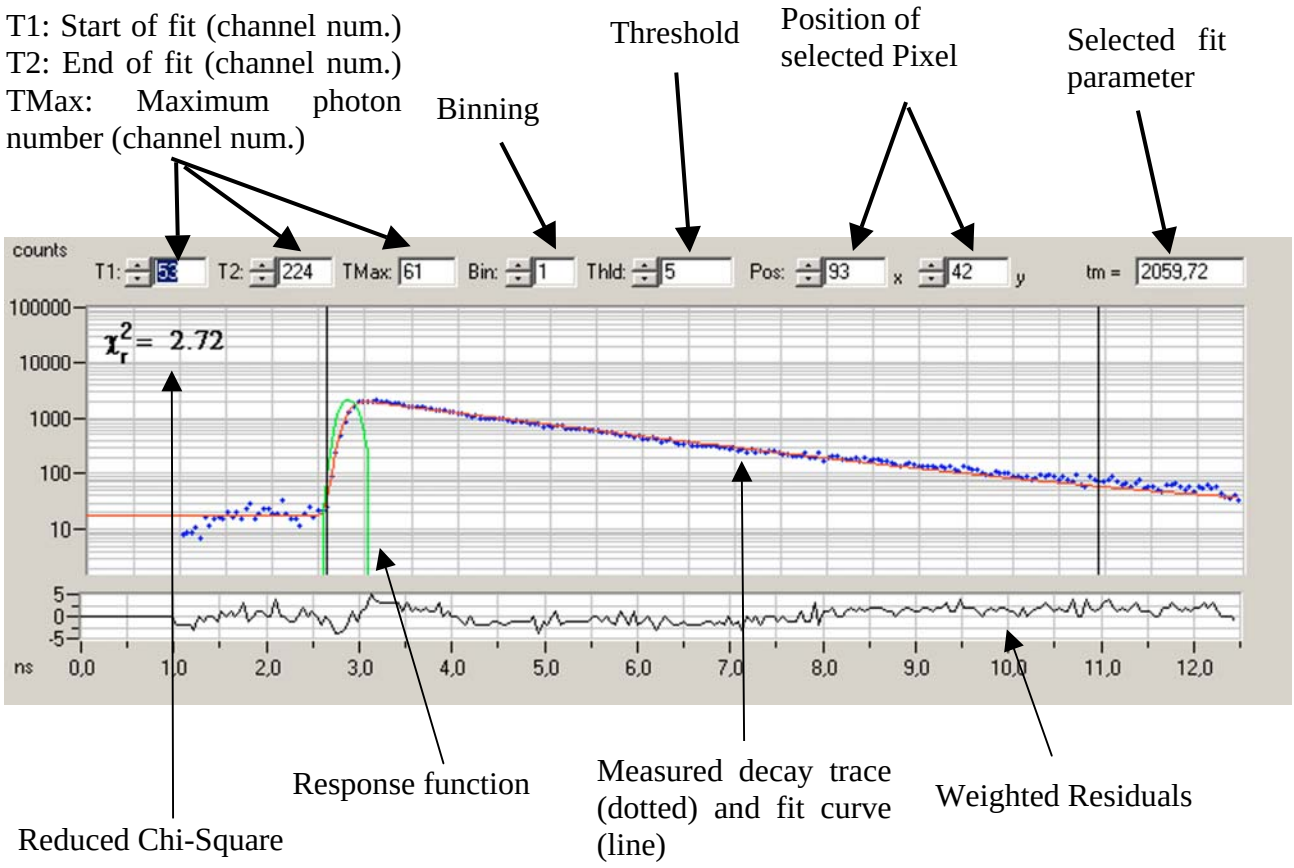


Figure 6: Decay graph which is shown after importing the cell.sdt file

(red) and the response function (green). Deviations between photon data and fit-trace are represented graphically by the weighted residuals at the bottom.

It is important to define a range of time channels to improve the quality of the fit. Only data-points between the two vertical black cursor lines are used for the fit process. In addition the start and end position of this range are given numerically by the ***T1*** and ***T2*** values (time channels).

By default all time channels in front of the first cursor line (i.e channels 0 to T1-1) are used for calculating the baseline or "offset" as indicated in the introduction. Since the calculation of the offset is very important in order to receive correct lifetimes we recommend to shift the rising part of the fluorescence to about $1/10^{th}$ of the complete range to have enhough data points in front of fluorescence decay . The ***TMax*** value indicates the maximum of the fluorescence decay given as channel number. This value is for reference only and can not be changed manually.

The ***Threshold***-parameter defines the minimum number of photons in the peak of a fluorescence curve which are necessary in order to take the fluorescence decay curve into account. The feature can be used to suppress "dark" pixels which are skipped during the calculation process. This will not only accelarate the calculation process but also improve the quality of the parameter histogram (see below) since outliers due to a bad signal-to-noise ratio will be suppressed.

By default the ***Selected fit parameter*** shows the weighted average of the different lifetime components in each pixel (t_m).

$$\tau_m = \sum_{i=1}^{N} a_i \tau_i \Big/ \sum_{i=1}^{N} a_i \qquad \textbf{(4)}$$

The ***Binning*** factor denotes the number of surrounding pixels which are summed into each decay trace:

The number of photons in each decay trace will increase effectively if the binning value is incremented. The spatial resolution will decrease since the binning strategy is used for all the pixels within the region of interest during the fit process. Please adjust the binning factor in accordance to the complexity of the fit model and the signal-to-noise ratio of your measurement.

As a rule of thumb the peak value of the curve should be at least 100 for a single exponential fit, about 1000 for a double-exponential decay and 10000 for three exponentials. This holds true for situations where all lifetimes are unkown. The required number of photons will be decreased if there is any a priori knowledge of a lifetime which can be use to "fix" one or more values

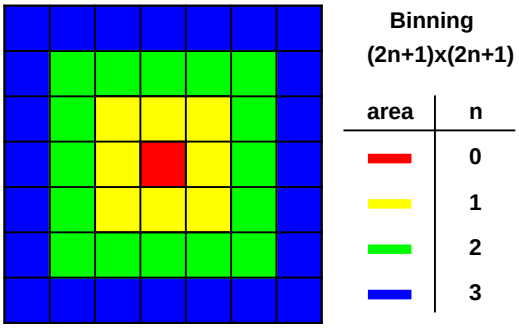


Figure 7: Binning strategy and dependency on the binning-factor "n"

To start the image analysis within a selected ROI the ***Calculate>Decay Matrix*** command is used. During the process of calculating the decay matrix the region of interest is analysed pixel by pixel and line by line. The following figure shows the situation if the binning-factor is set to 1. In this case photons of all surrounding pixels are added into the decay trace of the pixel for which the parameters are currently calculated.

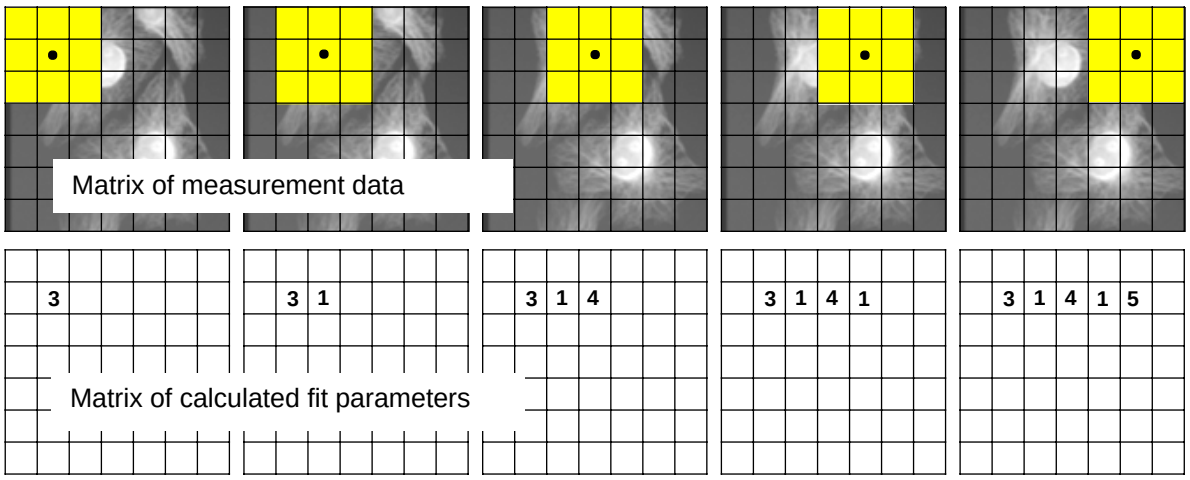


Figure 8: Sequence of calculation of the fit parameters

After the calculation has been finished a color coded image will be shown. This image derives the intensity information from the number of photons in each pixel and the color information from a selected fit parameter which value is coded by a continuous color scale (here running from red to blue) .

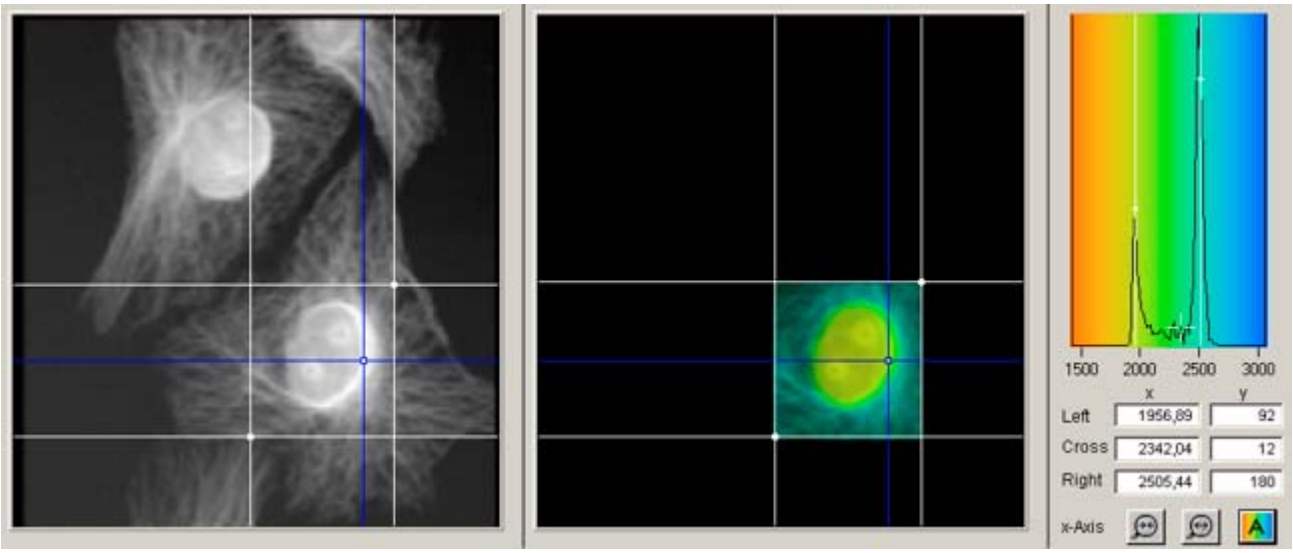


Figure 9: Result of performing the Calculate>Decaymatrix command wihtin a defined ROI

Next to the lifetime image a histogram shows the distribution of the parameter which is currently selected (e.g. tm). The result is a histogram which shows the "relative frequencies" of the fit parameter. If the intensity window is hidden by disabling the ***Options>Prefrences: Show Intensity Window***- checkbox the histogram window will be displayed enlarged (Figure 10).

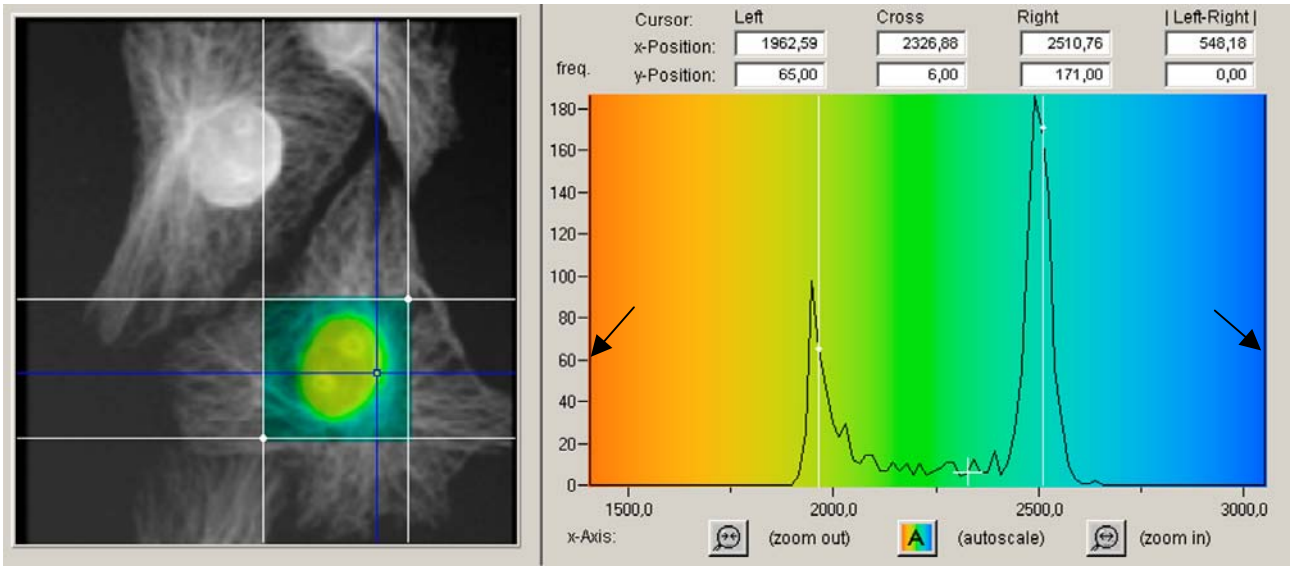


Figure 10: Combined presentation of lifetime & intensity image and detailed distribution histogram

If – in additon – the ***Options>Prefrences: Show Intensity in Lifetime Window*** checkbox is selected the images of both windows are merged. Please note: Black and white regions are denoting the status were no analysis was performed.

Three white cursors (two vertical lines + one crosshair) are provided in order to enable the user to quantify the values given in the histrogram graph. The software first determines the mean value of the distribution and marks it by the small white crosshair. In addition the two white vertical cursor lines include 66% of all values which were found within the region of interest. This is done by collecting 33% on each side of the mean value.

The numerical values of the cursor positions are given above or below the distribution graph depending on the display type (large or small). The x-Position of the "Cross" marks the average value of the lifetime within the selected region of the image whereas the x-position of the "Left"- and "Right"-cursor denote the range of the single sigma standard deviation.

In case of two distinct lifetimes as for the example given above it is not very reasonable to determine an average value of both. In order to select a certain sub-range of the histogram the black border of the histogram graph can be moved as shown in figure 11.

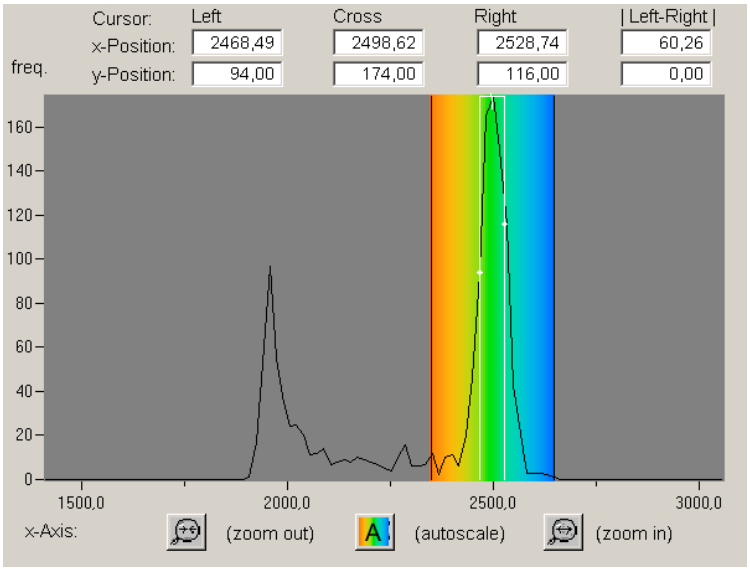


Figure 11: Manual selection of a peak in order to determine width and position

As visible from figure 11 the color range now runs within a much smaller part of the histogram. The appropriate numercial values can be found in the ***Options>Color*** dialog as ***Minimum*** and ***Maximum*** of the ***Color Range***. In this case, only the values between the black lines are taken into account for calculating the mean value and the 66% range. This holds true if the option ***Analyse within color range*** is switched-on in ***Options>Preferences: Distribution Window*** (default setting).

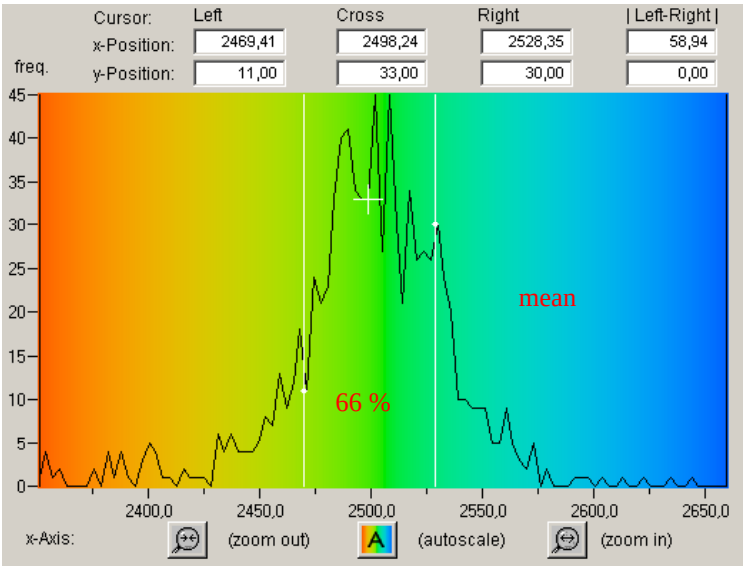


Figure 12: Zooming into a selected peak

With this method it is possible to pick out any peak within the histogram. The "zoom in" button enables the user to zoom the selected peak to the full scale in order to receive more details of the curve structure.

Another method to find the width of a peak is to move the white cursors manually to the maximum value and the FWHM of a certain peak. Please note that these manual selections are overwritten any time the ***Color Range*** was changed or the distribution graph was changed due to a recalculation of the parameters.

The default setting for the fit model is a single exponential decay. This is the simplest model to analyse a FLIM image (in this case the t_m value is the same as t_1). To check if this model is appropriate the reduced χ^2 should be displayed. The χ^2 is defined as

$$\chi_r^2 = \frac{1}{N-p}\sum_{i=1}^{N}\frac{(d_i - f_i)^2}{d_i} \qquad (5)$$

In equation 5 the data points are denoted by d_i whereas the corresponing values of the fit-model are given by f_i . Numbers of fit parameters and datapoints are p and N, respectively. The figure below shows the reduced χ^2 pattern after selecting ***Options>Color: Coding of Value = Chi*** with ***Options>Color: Mode: Discrete.***

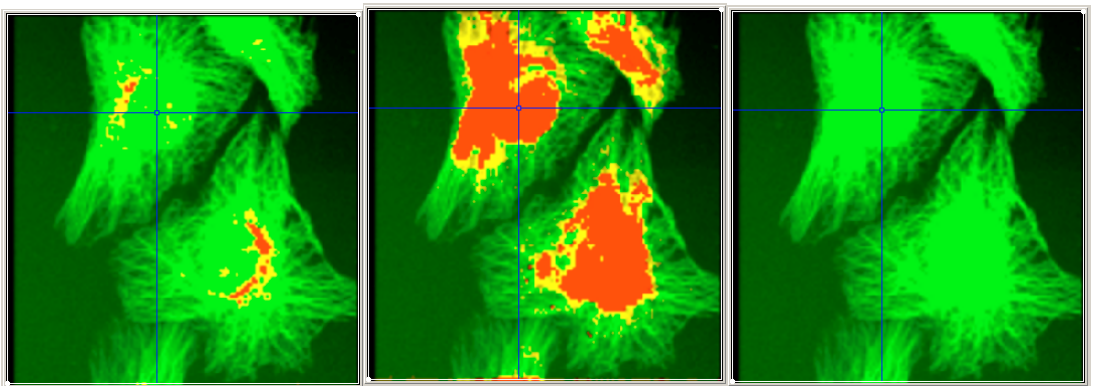


Figure 13: Checking the χ^2 values of a fit

The green color is running from 1 to 2, yellow from 2 to 2.5 and red for value 2.5 and larger. If one decides to reject all fits with χ^2 values > 2.5 the red color shows all areas where a single exponential fit is not appropriate. On the left side the situation is shown for a binning-factor of 1. The small red areas in the left image of figure 13 are partly due to a mixing of two different lifetimes due to the summing procedure of the binning process. If the binning is switched off like for the right image of figure 13 the mixing does not occur and the single exponential model holds true for the whole image. However, another consequence of the binning is an improved signal-to-noise ratio in each curve. The image in the middle shows the situation for a binning = 2. In this case the whole inner part has to be rejected since it deviates clearly from a single exponential decay. Consequently it is necessary to switch to a double exponential decay model. The left image of figure 14 shows the result after increasing the number of components in the "Multiexponential Decay"-window to binning =2 . After recalculating the data there are still spurious pixels with a

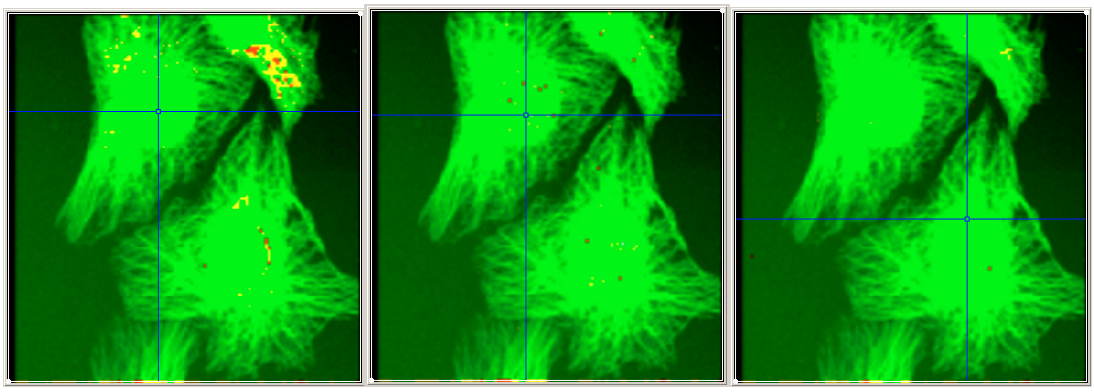


Figure 14: Checking the chi^2 values

high χ^2. Often the "pixelised" outliers are due a problem at the front part of the fluorescence including the rising edge. Most of the problems can be removed by invoking the scattering parameter . The fit algortihm will add a certain fraction of the IRF to the fluorescence in order to improve the fit of the datapoints near the rising edge as shown in figure 3. The center image of figure 14 shows the effect after invoking ***Calculate>Decay*** parameters again.

A few outliers remain in which the optimal chi was not found. To enhance the quality of the fit the ***Calculate>Improve Matrix*** command can be used. This method uses an extensive search of the shift parameter to find the global minimum of the χ^2 value. Since this procedure is more time consuming than the normal procedure the calculation of the entire image will take longer. The result of the recalculation can be seen on the right of figure 14. If there are still pixels in which the fit seems to have failed it is possible to increase the number of itterations and the ***Delta Chi^2*** value. By setting this value at ***Options>Numerical Settings:Algorithmic Setting*** it can be determined at which step of the itteration the optimization of the χ^2 will stop**.** After these changes the "Calculate>Improve Matrix" command has to be invoked again.

The resulting average lifetime image (t_m) of the double exponential analysis is shown in figure 15. The positions of the peaks are shifted somewhat to shorter lifetimes. The peak of the faster lifetime is smaller since the region of interest has more pixels containing the tubulin structure rather than pixels showing the nucleus

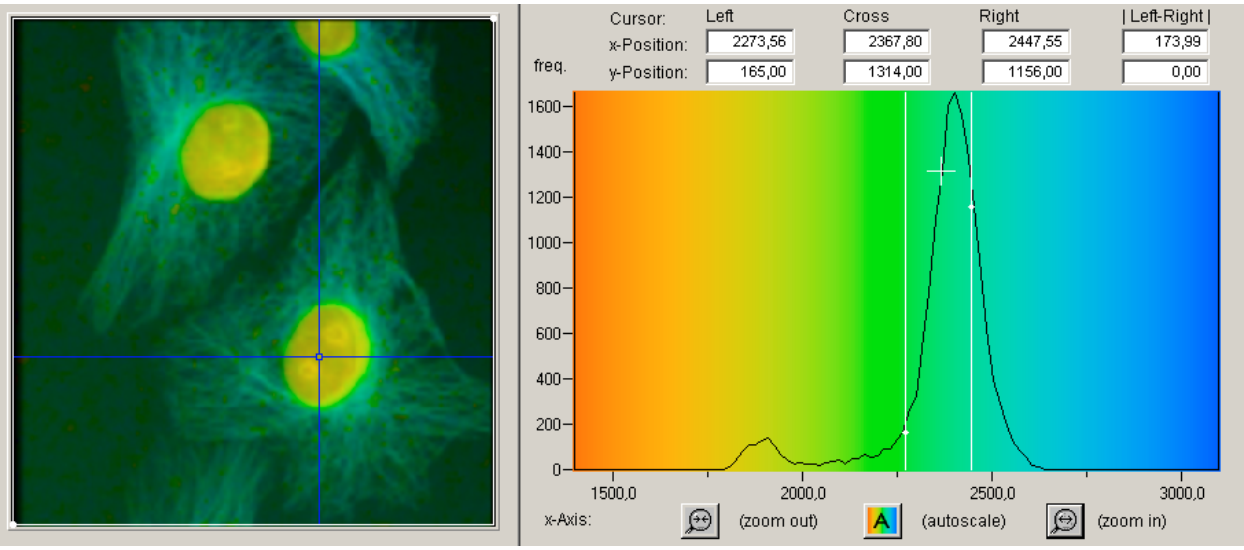


Figure 15: Double exponential decay analysis

When looking at the two components seperately it is readily visible that the distribution of t_1 is relatively broad. Although there are obviously two peaks included it is almost impossible to seperate them at the give signal-to-noise ratio. It is, therefore, a reasonable appraoch to take the average value as a fixed component and to consider the (66%) values as an error range. After recalculating the image and looking at the t_2 distribution the situation is similar – there is a certain tendency for the lifetime however the peaks are not seperated cleary.

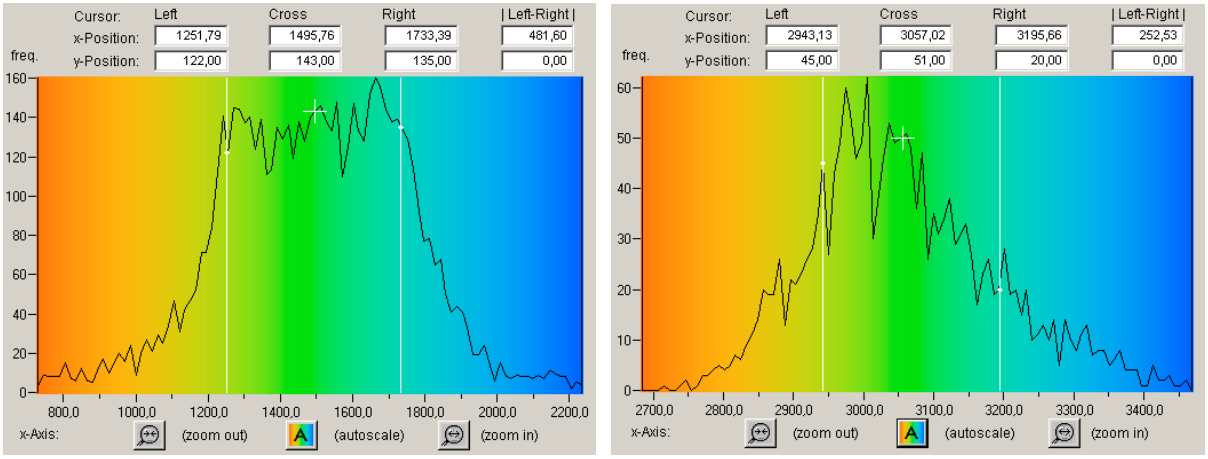


Figure 16: Distribution of the two different lifetimes

Together with the discrete color coding it is also possible to define a range for the amplitude parameter which is coded with the same color (figure 17). The green parts of the image denote 1,50 ns +/- 0,25 ns with relative amplitude of 76 +/- 10%. The other lifetime component is 3,06 ns +/- 0,14 ns with a relative amplitude of 53 +/- 16 % in the tubulin area (not shown).

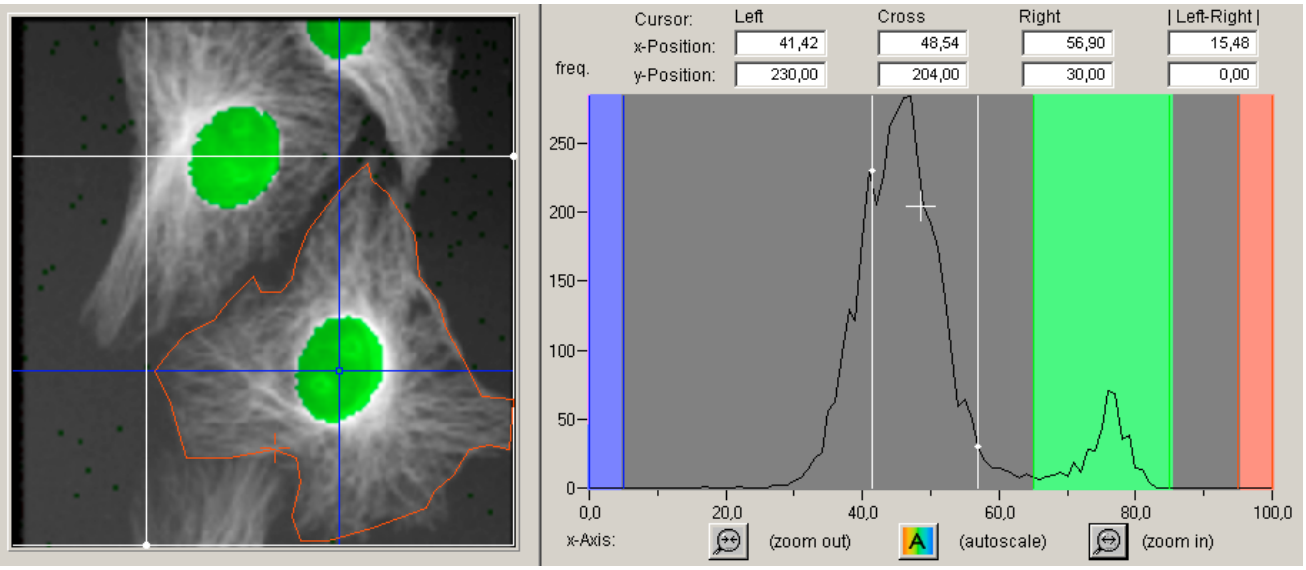


Figure 17: Color coding of the relative amplitude a_1 of the fast lifetime component (t_1=1,50 ns ±0,25 ns)

In figure 17 the mask tool was used to select the outline of a selected cell. In the following it is described how to define this region of interest.

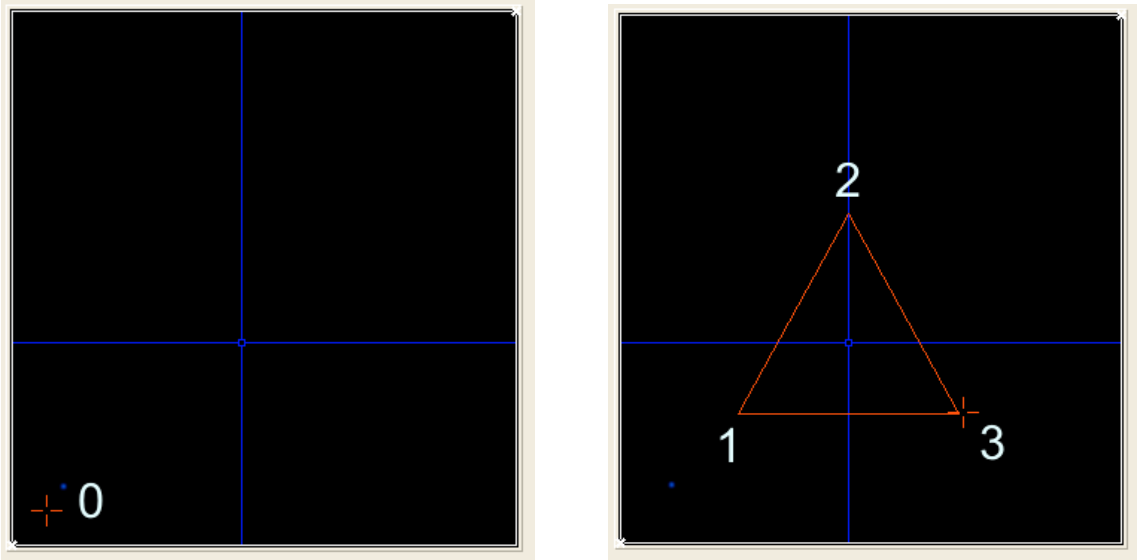


Figure 18: Basic mask operation

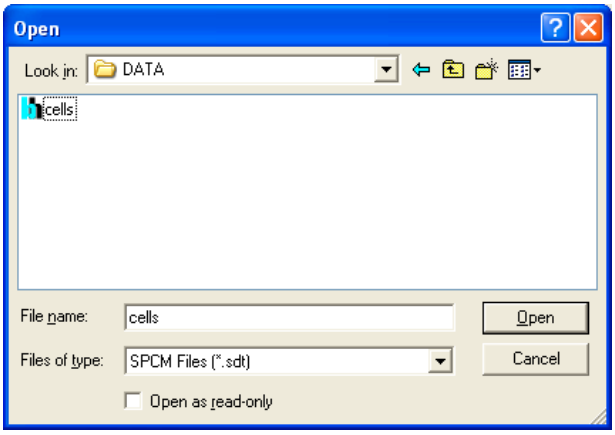
After invoking the ***Mask>Define*** command in the menu a red cross will appear somewhere on top of the image as shown in figure 18 on the left (the "0" is just a hint inside this documentation that this point will not be included in the polygon trace – it is not shown when running the software). After this please move the cross to the first point which should be included in the polygon (denoted as "1" in this documentation). When moving to the next point ("2") a line between "1" and "2" shows the first border of the region-of-interest. For the next point "3" and all further points will then mark the complete region.

Please note: In the current verson it is not possible to change the points of the It is not possible to chanegf

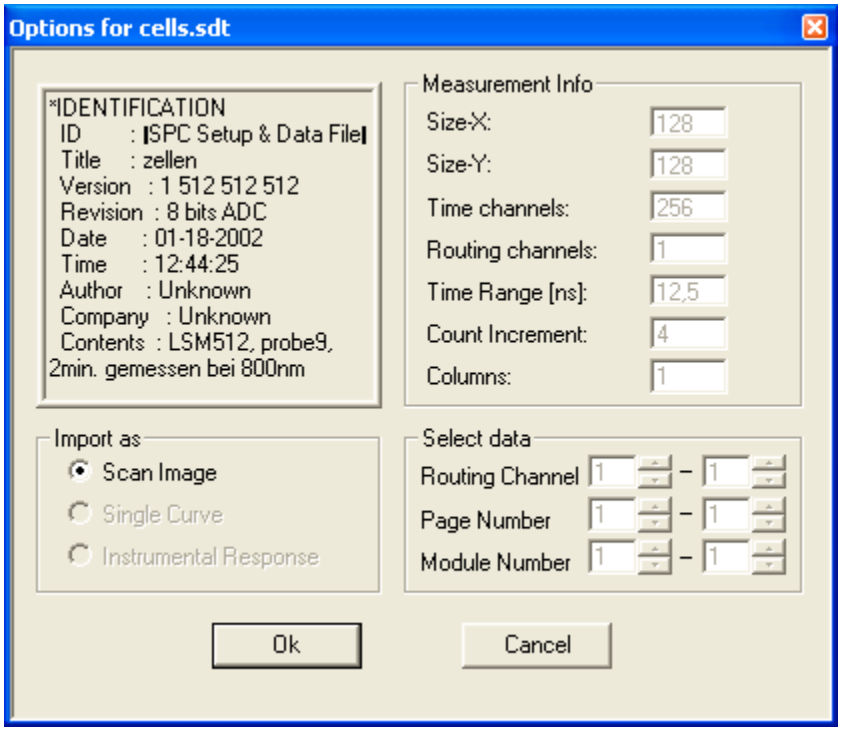
The defined mask will effect the result in the histogram as only values which are inside this mask are taken into account. In figure 17 the mask was used to get the lifetime distribution of a single cell inside the image. To delete the mask the ***Mask>Undefine***

3. User-Dialogs

Usually the *SPCImage* software gets the fluorescence lifetime data from the measurement software *SPCM*. Therefore it is necessary to transfer the data between the two applications. The dataset from the SPC module can be loaded into the application by importing an .sdt file which was previously created by the measurement software. In addition it is possible to load-in ASCII datafiles from a third party measurement device or invoke a direct data transfer from the measurement software.



On successfully loading an ".sdt"-File a dialog appears which shows a brief summary of the measurement information which is stored in the .sdt file. The identification part is shown on the left. It contains information about the version of the acquisition software, the time and date of the measurement and a comment line eventually contained in the data file. The size of the

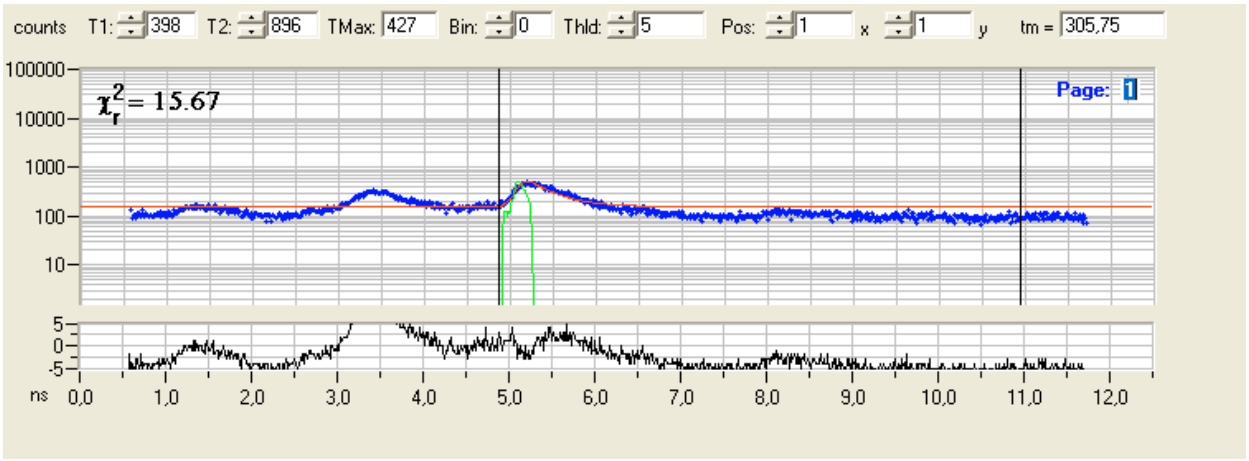


image, number of time channels and other measurement parameters are given at the right side of the dialog.

The *Count Increment* denotes how the histogram value was incremented on the detection of one photon. This count increment parameter is determined during the measuement to increase the amplitude for measurements with low count rates. This procedure does not enhance the signal to noise ratio and the SPCImage software always normalizes the count increment to unity when importing the data**.**

Another important parameter is denoted by the number of routing channels. If this number is greater than 1 the dataset actually consists out of more than one images. With version 2.8 and higher it is possible to import more than one image simultaneously. As consequence it is necessary to select the different images by using the tab-control at the bottom of the decay graph as shown in the first page of this manual. Also it is possible to import all imgages from mutli-board configurations at the same time In this case the tab-control will appear on the right side of the decay graph.

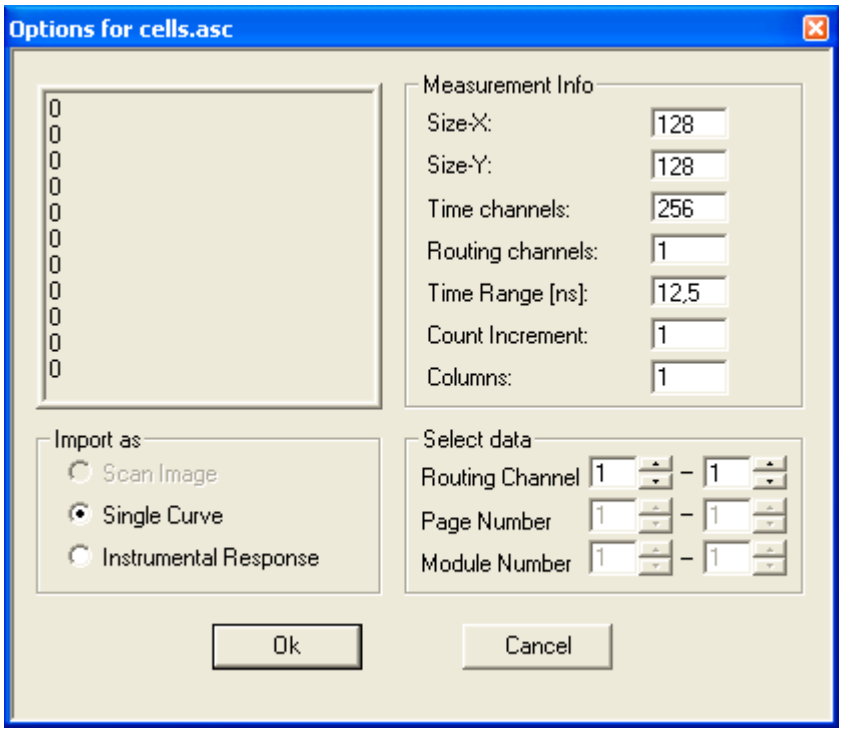
The simultaneous import of more than one page is now supported. Different pages are created if the user performs subsequent measurements which are all stored in the memory of the board. This will happen if the "Step"-parameter is greater than one for example. The data of all steps are stored to different pages which can be selected by changing the number in the upper right of the decay graph.



Please note that all settings which corrsespond to the fit-model, intensity, and the color selections can be different for the seperate images. A convenient way to copy the settings between the individual images is to use the Conditions>Store and Conditions>Load command as described in chapter 4.

Alternatively it is possible to import files in ASCII format. In this case the import dialog comes up with default values for the measurement parameters since the current version of the software does not analyse a header eventually contained in the ASCII data.

By switching to "Instrumental Response" the program will try to import single curve data which contain the temporal behaviour of the measurement system (optics + electronics). Before importing the ASCII data the parameters on the right have to be set manually by the user. Please note that the instrumental response function has to have the same number of time channels than the measurement data which was loaded-in previously. If the ASCII file contains two colums – one for the time axis and another for the number of photons please use *Select->Columns = 2* . Please note that you have to select the *Time Range* according to the measurement settings. If the ASCII file was generated by the SPCM software it is also necessary to choose the *Count Increment* correctly. This will normalize the data if the measurement was performed with a count increment > 1.



>Columns = 2 . Please note that you have to select the *Time Range* according to the measurement settings. If the ASCII file was generated by the SPCM software it is also necessary to choose the *Count Increment* correctly. This will normalize the data if the measurement was performed with a count increment > 1.

Options → Intensity

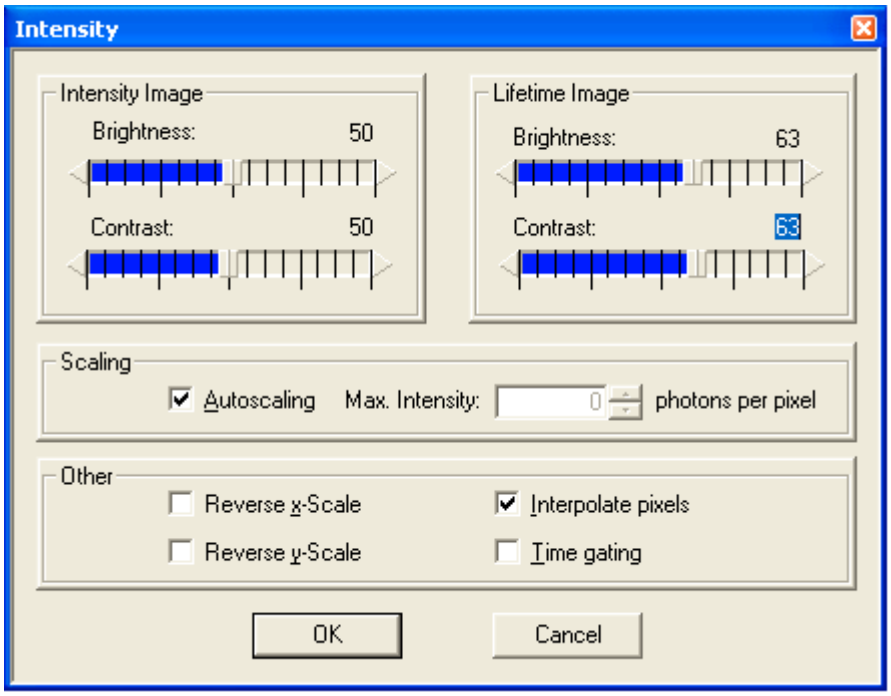
An intensity image of the data is displayed after the data was successfully imported. This image is calculated from the time integrated number of counts for each pixel (pixel sum). An autoscaling of the intensity is performed by default which selects a range from 0 (black) to the maximum pixel sum within the image (white). There are two possible ways to change the intensity of the image:

i) When the "autoscale" checkbox is disabled a user defined maximum can be inserted. This feature enables an "absolute scaling" for comparing different measurements (or routing channels within one measurement).

ii) The brightness of the image can be controlled by the *Intensity Settings* Dialog. Both sliders can be used to change the image appearance.

The *Reverse: x-Scale* and *y-Scale* checkboxes must be selected in the same way as in the measurement software to achieve the same orientation of the image.

When switching off the "Interpolate pixels" option updating the image will be considerably faster. This option is especially recommended when the resolution of the image is better than 256x256 pixel. ***Please note that this option will work in 32-bit color mode ("True" Color) only.***



If the *time gating* option is selected only the photons located inside the range between the two vertical cursor lines are taken into account. With this option it is possible to create "time-gated" intensity images.

Calculate → System Response (F6)

After importing the measurement data a fit will appear in the "single curve"-diagram at the bottom of the window. It belongs to the brightest pixel of the image which was selected automatically and which is denoted by the small blue cursor within the intensity image. The model function of the fit curve is a "Multiexponential Decay" with offset-correction (a_0) for taking ambient light and/or dark noise into account:

$$F(t) = a_0 + a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$$

The number of exponential decays that are used is given by the *Components* parameter in the lower right of the application window: The constant a_0 is denoted as "Offset" and can be fixed, i.e. held at a user given value, by activating the check box next to the value field. If the offset is not fixed the program will calculate this parameter by averaging the number of photons in front of the first vertical cursor! Please note that the intensity coefficients a_1 through a_3 are given as *relative* amplitudes which can not be changed by the user. The decay-times τ_1 through τ_3 are denoted in picoseconds (ps) and are "fixable". If the *Fix* - box is checked the corresponding value must be inserted by the user and is not changed by the program during the fitting process.

The analytical function is convoluted with an "instrumental response" function before it is fitted to the data. By default the fitting algorithm uses a response function which is calculated from the first derivative of the rising part of the fluorescence. However, the *true* shape of the instrumental response is determined by the detector and is also influenced by the excitation source and the optical pathway of your system. Therefore the shape of the system response calculated by this procedure is only a rough approximation and may cause deviations especially in the first part of the curve. Please note that only the region *between* the two vertical lines is taken into account by the fitting procedure. Therefore it may appear that the fit outside these lines is not correct and the cursors have to be moved to the region of interest.

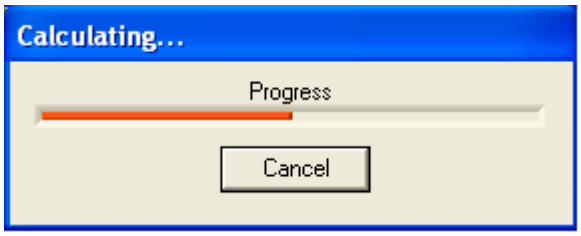
The first cursor line should by placed directly in front of the rising edge of the fluorescence decay and the second near the end of the curve. This is done by default after importing the measurement data.

The *Shift* parameter determines how the instrumental response function is located relative to the rising edge of the fluorescence (given in channels). The *Scattering* factor takes into account how much of the exciation light is directly scattered instead of being absorbed and emitted as fluorescence.

If the curve appears to have very few counts it is a good idea to increase the *Binning* factor. This factor defines how many pixels are combined before the decay time is calculated. The preselected value of 1 means that (2 n + 1) x (2 n +1) = 9 pixels are summed up to produce one decay curve. Very large values are not recommended since a higher factor will decrease the spatial resolution. In addition you can select a *Threshold*. This value defines the lower limit of photons that has to be in the maximum of the curve - otherwise fitting for this pixel is skipped.

Calculate → Decay Matrix (F2) / Calculate → Improve Matrix (F3)

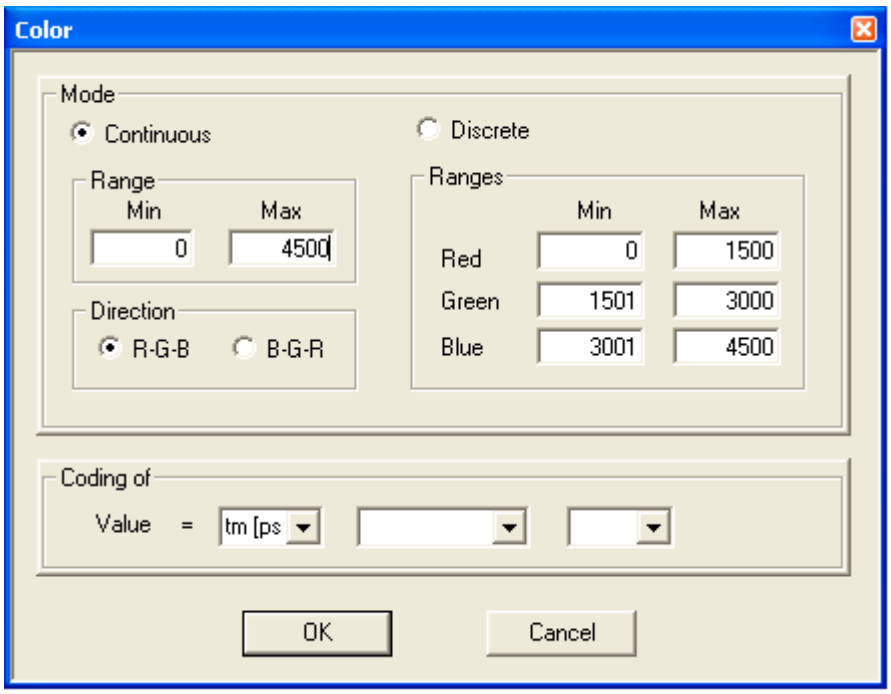
Now you can create a lifetime image by using the function to calculate the "Decay Matrix" which contains the calculated fit parameters for every pixel. This procedure needs most of the computational resources and takes several seconds to some minutes depending on model complexity, image size and computer speed.



The algorithm may yield only a local minimum of the χ^2-value in some pixel might be far from ideal depending on the *Itterations* parameter and *Delta Chi^2* parameter selected in the *Options->Numerical Settings* dialog box. The *Calculate->Improve Matrix* command invokes a more extensive search procedure which delivers the global χ^2- minimum with a higher probability. However, this calculation takes longer due to the higher computational effort.

Options → Color

After calculating the decay matrix for the first time the color images might not reveal the full information. Therefore it is recommended to adjust the color range according to the particular parameter distribution of the individual image. Please use the *Autoscale*-button  for this purpose which is located in the main panel near the parameter distribution graph. After this action the software will try to determine a color-scale automatically which covers the majority of the parameters values found during the fit process. Normally the *Minimum* and *Maximum* values of the *Color Range* (used for continuous colors) and the *Discrete Ranges* (used for discre colors) have to be fine-adjusted manually. Please note that the x-Scale of the export file of the parameter distribution depends on the selected *Color* Range, i.e. a file containing x values running from 1000 to 3000 (ps) would be produced when exporting the example given above.



By default the *Mode* is set to *Continuous*. In this mode the fit parameters are projected into a smooth color range running from *Range Min to the Range Max*. To avoid sharp transitions at the border of this continuous color range values located outside the range are coded with one of the "border-colors". The *Direction* radio button can be used to select if the scale runs from red to blue or vice versa.

If the *Discrete* color mode isselected it is possible to define *Ranges* for *Red, Green* and *Blue.* Unlike for the continuous mode values which are outside the defined area are coded as black, i.e. are not visible.

At the bottom of the dialog three list boxes can be found which belongs to the value which is finally coded by color. As a default the average lifetime "tm" of the decay matrix is taken. This means that for a multi-exponential decay the average lifetime is calculated according to the equation:

$$\tau_m = \frac{\sum_{i=1}^{N} a_i \tau_i}{\sum_{i=1}^{N} a_i}$$

Next to "tm" the *Value* can be choosen as the lifetime components: (t1, t2 , t3) the relative amplitudes (a1, a2, a3) or the relative quantum yields (q1, q2, a3) of the individual components. In this case the color image will present only the selected value. Moreover the color coded value can be the result of a simple arithmetic calculation (sum, difference, product, quotient) selected by the operation selection box. ***In addition the new version allows also to generate a Chi map by selecting Value1 = Chi.*** Please note that in this case the distribution curve in the color scale window (see below) will also change since it always reflects the distribution of the value which is finally used to generate the color image.

The relative quantum yield is defined as:

$$q_i = \frac{a_i \tau_i}{\sum_{j=1}^{N} a_j \tau_j}$$

The Efficiency E is given by:

$$E = 1 - \frac{t_1}{t_2}$$

The intensity weighted mean lifetime τ_i is given as:

$$\tau_i = \sum_{j=1}^{N} a_j \tau_j^2 \Big/ \sum_{j=1}^{N} a_j \tau_j$$

Intensity weighted mean lifetime with pile-up correction:

$$\tau_p = \tau (1 - P/4)$$

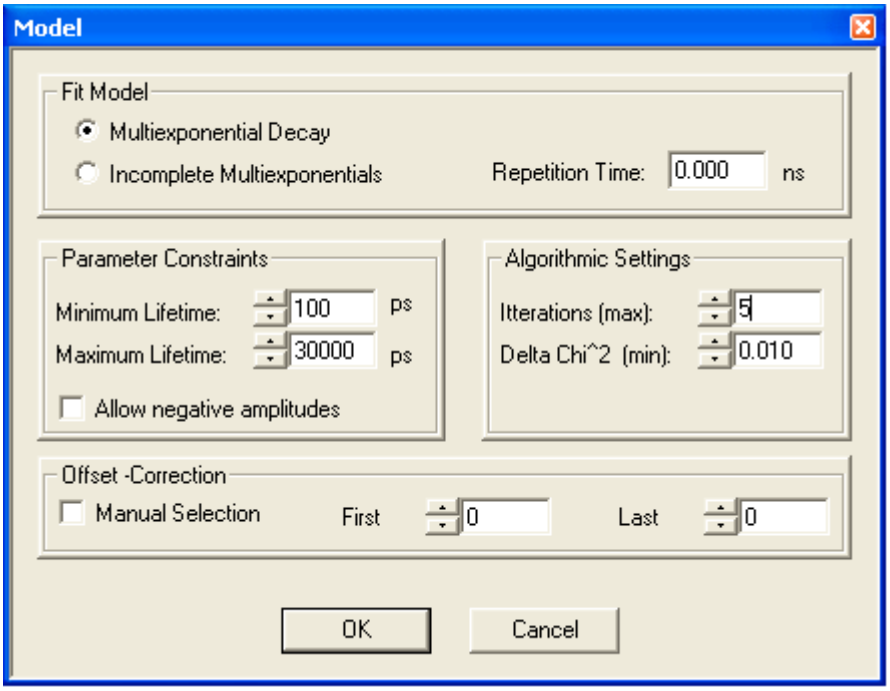
with P being calulated from the total measurement time, the laser repetition rate, and the dead time of the detector.

Options → Model

The *Minimum Lifetime* and *Maximum Lifetime* values define the general limits for the parameters during the fit procedure. The lower limit should be not smaller that the channel width of the measurement to avoid lifetimes which are artificially short. Complementary the upper limit should not exceed the total time range to prevent very long lifetimes which actually reflect a fit to a constant offset.

Please note that fluorescence lifetimes which do not have a complete decay between two excitation pulses can produce distortions in the lifetime values.

In this case the *Fit Model: Incomplete Multiexponentials* can be used and the laser repetition time must be provided by the user.

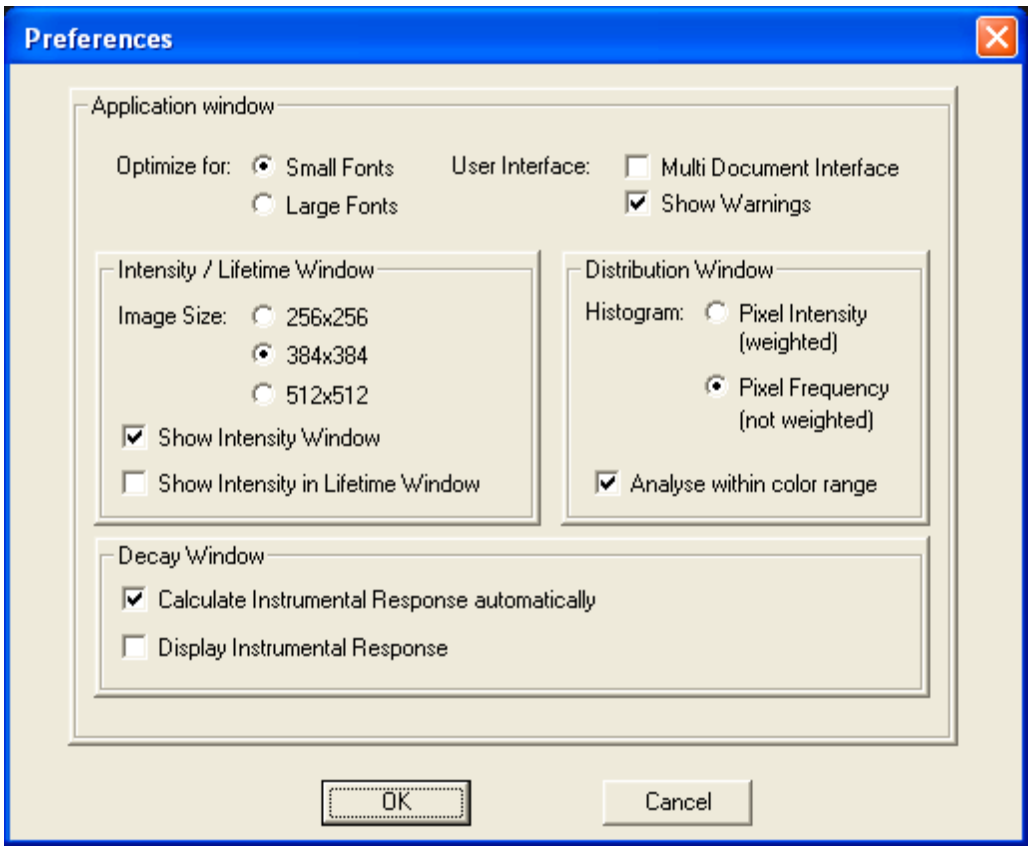


The stop criterion of the itteration process is defined by two algorithmic settings – the maximum number of itterations and the difference between the χ^2 between two successive steps. This values will not only will influence the accuracy of the calculation but also determine the speed of the overall calculation.

Options→Preferences

It is possible to configure the general appearance of the application by this dialog. If the Windows™ operating system uses a "large system font" it is preferable to switch the application window to *Application Window: Optimize for: Large Fonts.*

The *Calculate Instrumental Response automatically* option which is switched-on by default enables a quick estimation of the system response function. In addition the vertical cursors inside the decay graph are set to resonable position, i.e the first is set directly in front of the rising edge and the second cursor is placed at 7/8 of the total time-range. The first cursor has to be placed manually in front of the rising part of the fluorescence trace and the second near the maximum of the trace if the *Calculate response automatically* option is switched off. In this case the the binning factor should be increased to get a trace with a well defined rising edge. Please note: *The Calculate -> System Response (F5)* command takes only the region between the cursor lines into account if the *Calculate response automatically* option is disabled.



With the new version it is now possible to invoke a *Lock* and *Unlock* command from the main menu and the toolbar. In case the fit process is "Locked" the fit values are not recalculated when pointing to a new pixel or changing the conditions of the fit (number of lifetime –

components, fixing of parameters etc.). To inform the user about changing to the "Locked"-mode a warning is shown by default. To simplify the switching process this warning can be suppressed by disabling the *Show Warnings* checkbox .

The application displays resizable and movable windows for the different views if the "Multi-Windows" option is selected. Also the application window can be resized in this case. The option can be used to reduce the total size of the application or to display more than one measurement at a time.

By default the Intensity- and Lifetime Window are shown as seperate windows as denoted by the *Intensity / LifetimeWindow: Show seperately* checkbox. This means that the intensity image is presented next to the lifetime image. To safe space within the main window you may switch this option off. In this case the distribution graph is presented in a larger diagram to display more details.

The application window has to be restarted when changing some of the application window options! This is done automatically after pressing ok – please do not restart the application manually during this process.

For all parts of the lifetime image where no fit parameters could be determined (due to low photon numbers or excluding by the range-of-interest cursors) it is possible to diplay the intensity information instead. Hence, when checking the *Show intensity combined* option you will usually see that a part of the image is gray-scaled.

The group of radio buttons "*Small Size – Large Size*" can be used to switch between the resolutions of the image windows. The "large" option can be helpful to display the details of high resolution images (512 x 512 and higher) whereas the "small" option might fit better for image resolutions (128 x 128 or lower).

iii) Distribution Window

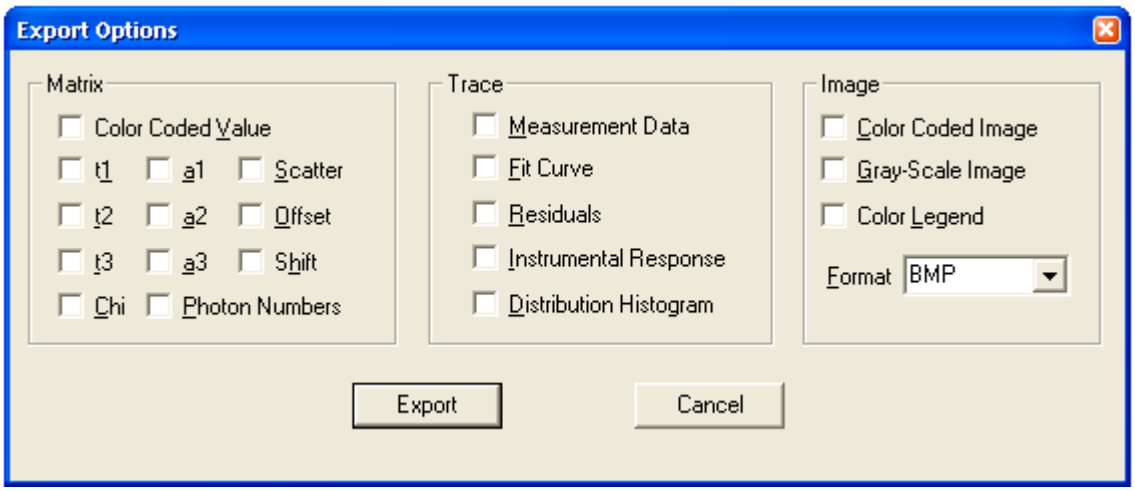
If the calculation of the distribution is switched to *Weighted* the lifetime frequencies are multiplied with the corresponding number of photons in each pixel. Otherwise the "Not weighted"-distribution is calculated which - ignoring pixel intensities – shows the number of

pixels in which the corresponing parameter value was found. If the *Range dependend analysis* is switched-off the program calculates the white vertical cursor lines in the distribution window to include 66% of all values. Otherwise (*Range dependend analysis* = checked) only the values between the black cursor lines in the distribution window are taken into account. With this feature it is possible to "isolate" different peaks within the distribution. The calculation of the "mean value" of the liftme-distribution is affected in the same way.

The *Decay Window: Display Instrumental Response* checkbox allows to show or hide the response function inside the decay diagram.

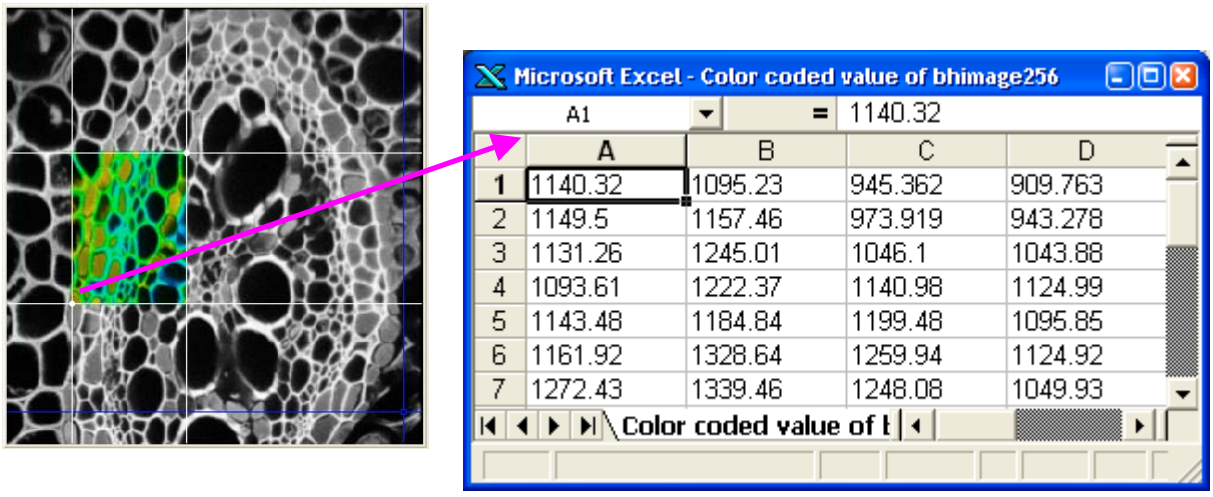
File → Export...

All data generated by the Calculate->Decay Matrix command can be stored into ASCII files with the help of the first group: *Matrix*. After pressing *Export* the program asks for a name of the export file. Please note that the name contained in the dialog box will be only used as "ending" of the complete filename. The first part is automatically added by the program.

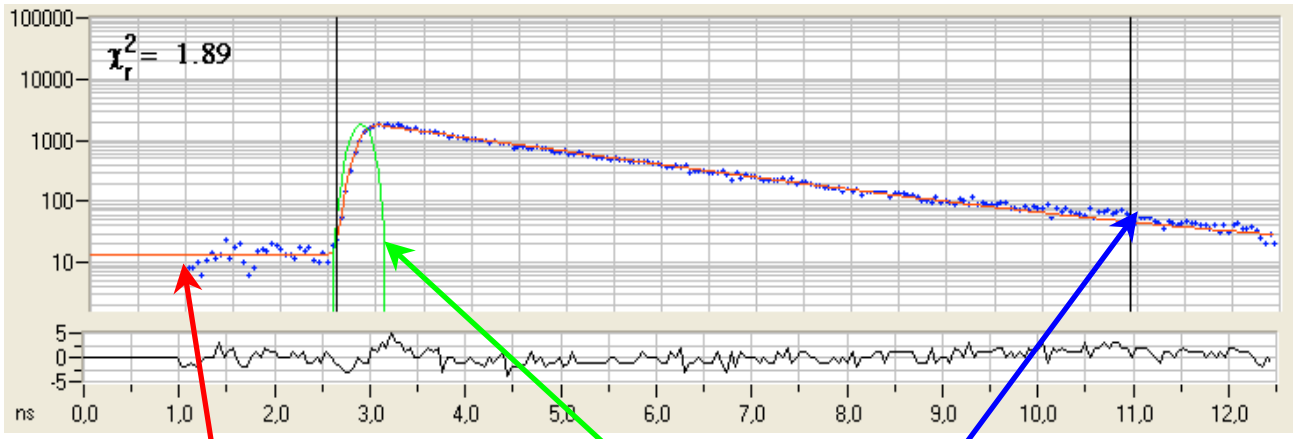


The files created for the *Matrix* contain space seperated values with an end-of-line character for each row. The *Color Coded Value* matrix contains all values from which the current color image was generated (see Options → Color Coding) inside the two white crosshairs.

The example shows the exported file in an excel worksheet. :



Please note that the value inside the white square with the smallest x and y coordinates is exported first. The position of this pixel will change if the x- or y-Axes are reversed (Options -> Intensity Settings -> Orientation). The arrow shows the orientation if both axes are " non-reversed". All other matrix parameters are exported in a similar way. For versions 2.7 and higher it is now also possible to export the intensity of each pixel as the sum of all photons (*Photon Numbers*).



Microsoft Excel - Fit Curve...

A1 = 0

	A	B
1	0	13.625
2	0.0488281	13.625
3	0.0976563	13.625
4	0.146484	13.625
5	0.195313	13.625
6	0.244141	13.625
7	0.292969	13.625
8	0.341797	13.625
9	0.390625	13.625
10	0.439453	13.625

Fit Curve

Microsoft Excel - Response ...

B59 = 15000

	A	B
53	2.53906	92
54	2.58789	428
55	2.63672	1804
56	2.68555	4931
57	2.73438	9297
58	2.7832	13148
59	2.83203	15000
60	2.88086	14205
61	2.92969	10823
62	2.97852	6197

Response

Microsoft Excel - Data Trace...

B256 = 21

	A	B
247	12.0117	42
248	12.0605	45
249	12.1094	36
250	12.1582	36
251	12.207	39
252	12.2559	33
253	12.3047	25
254	12.3535	21
255	12.4023	29
256	12.4512	21

Data Trac

4. Special commands

For more detailed analysis some additional commands were added to the menu which are also available by clicking on the corresponding icon in the task-bar.

Calculate → Lock

The Calculate>Lock command should be used if individual datapoints are analysed after the calculation of the decay matrix. In the "locked" mode no recalculation is performed if the user moves the blue crosshair to different pixels of the image. This guarantees that the color coding from the last calculation of the decay matrix is consistent with the fitting parameters given for the selected pixel.

Calculate → Unlock

After changing any parameter which may effect the result of the fitting process the matrix have to be recalculated. If the calculation was "locked" by a previous action please use the "unclock" command in order to take the changes into effect.

Conditions → Store

After the selection of the fit-model, the time-range for the fitting procedure, the region of interest in the image etc. it is possible to backup all these settings by using the Conditions>Store command. This is especially useful if two images or traces (i.e. aquired in different routing channels) have to be analysed with exactly the same settings.

Conditions → Load

Loads-in all settings which were backuped with the Conditions-Store command.

Mask → Define

The function allows you to define a polygon in which the parameter distribution is calculated. Switch on ‘Show Mask Polygon’ in ‘Preferences’ when you use the function.

Defines the curve currently displayed in the decay window as an IRF.