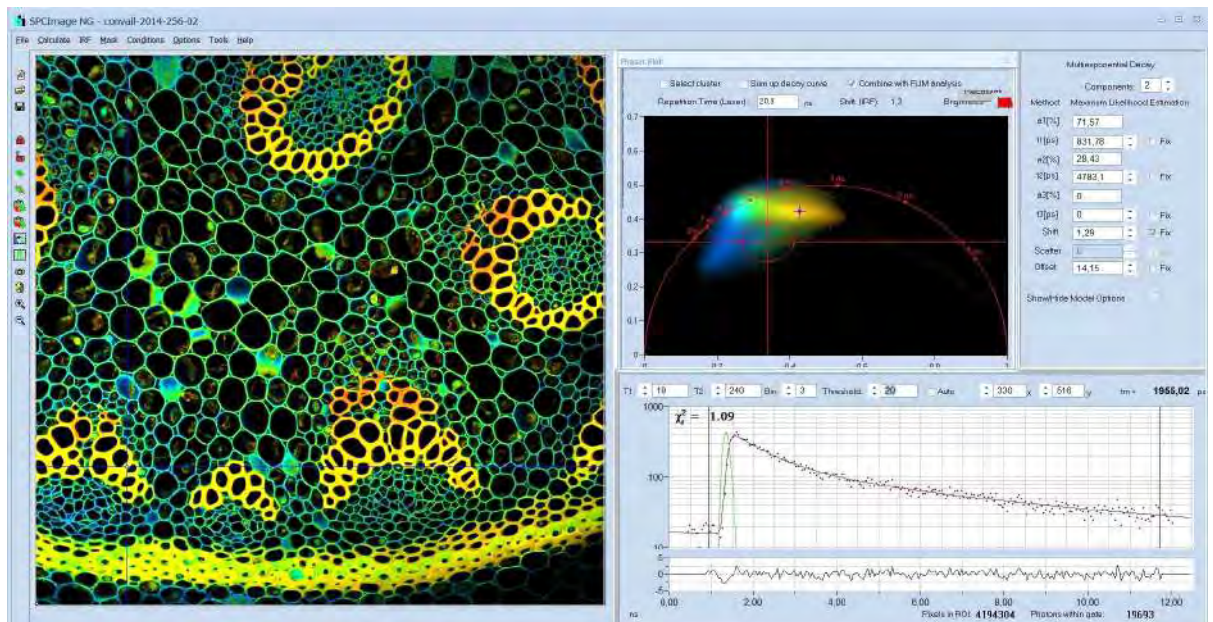


# SPCImage Next Generation

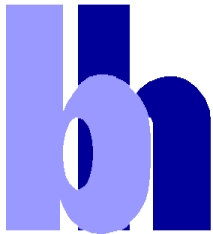
## FLIM Data Analysis Software



**Combination of Phasor and Time-Domain Analysis**  
**Maximum-Likelihood Estimation**  
**GPU Processing**  
**IRF Modelling**

**2022**





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# SPCImage NG FLIM Data Analysis Software

**Abstract:** SPCImage NG is a new generation of bh's TCSPC-FLIM data analysis software. It combines time-domain and frequency-domain analysis, uses a maximum-likelihood algorithm to calculate the parameters of the decay functions in the individual pixels, and accelerates the analysis procedure by GPU processing. 1D and 2D parameter histograms are available to display the distribution of the decay parameters over the pixels of the image or over selectable ROIs. Image segmentation can be performed via the phasor plot and pixels with similar signature be combined for high-accuracy time-domain analysis. SPCImage NG provides decay models with one, two, or three exponential components, incomplete-decay models, and shifted-component models. Another important feature is advanced IRF modelling, making it unnecessary to record IRFs for the individual FLIM data sets.

## Principle

SPCImage NG produces colour-coded images of fluorescence lifetimes and other fluorescence decay parameters from TCSPC FLIM data. It runs an iterative fit and de-convolution procedure on the decay data of the individual pixels of the FLIM images. In the simplest case, the result is the lifetime of the decay functions in the individual pixels. For complex decay functions the fit procedure delivers the lifetimes and amplitudes of the decay components. SPCImage then creates colour-coded images of the amplitude- or intensity-weighted lifetimes in the pixels, images of the lifetimes or amplitudes of the decay components, images of lifetime or amplitude ratios, and images of other combinations of decay parameters, such as FRET intensities, FRET distances, bound-unbound ratios, metabolic indicators, or the fluorescence-lifetime redox ratio, FLIRR. Examples are shown in Fig. 1 through Fig. 6.

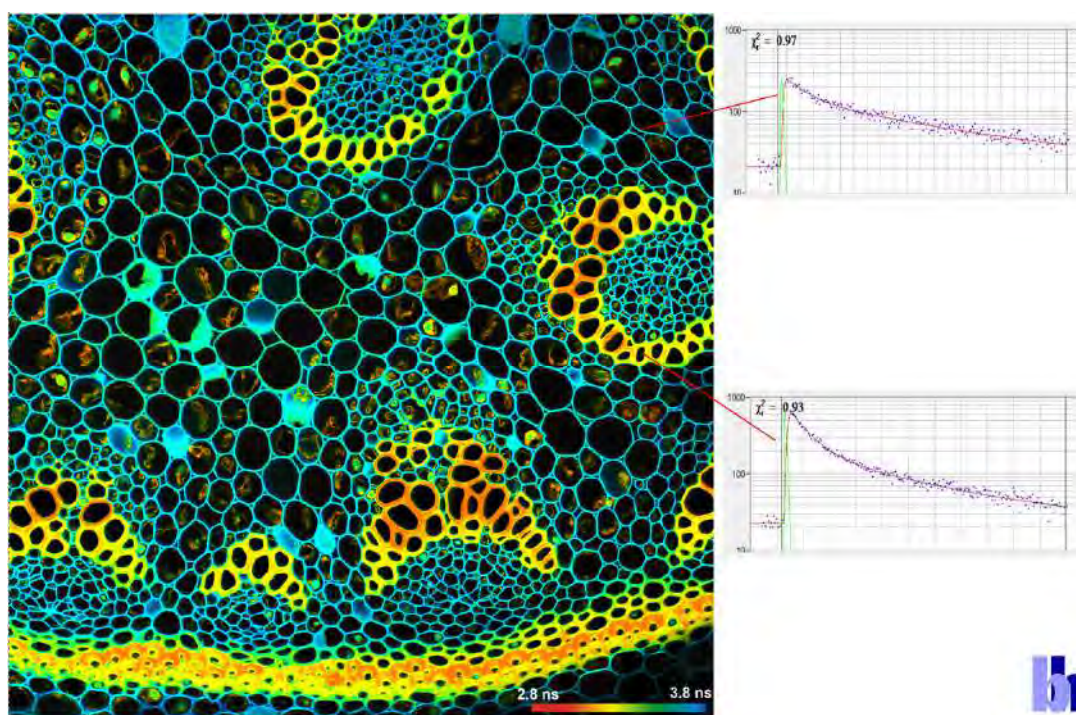


Fig. 1: Image of the amplitude-weighted lifetime,  $t_m$ , of a double-exponential decay. Right: Fluorescence decay curves in selected pixels.



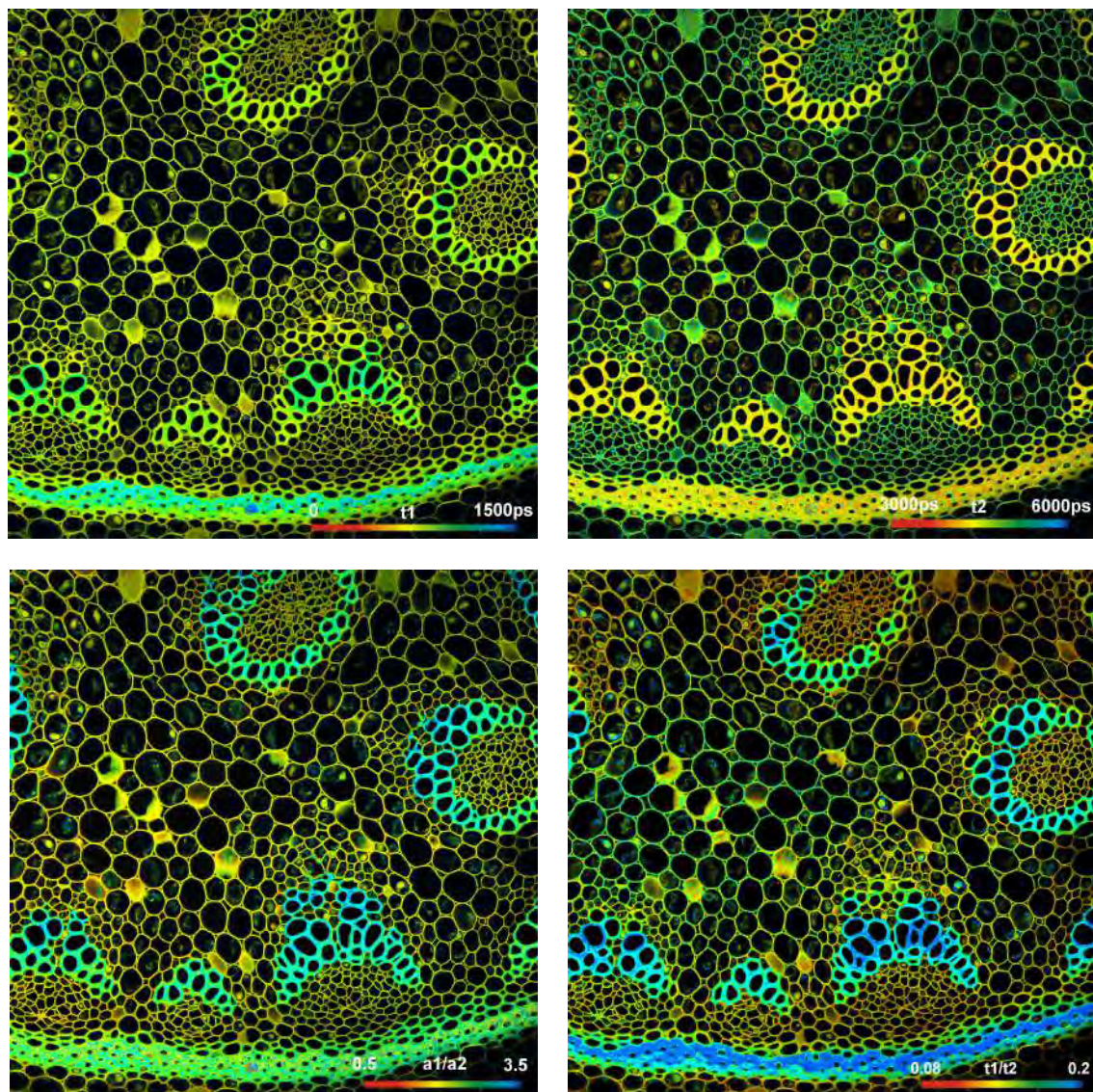


Fig. 2: Upper row: Images of the lifetimes of the fast component,  $t_1$ , and the slow component,  $t_2$ , of a double-exponential decay. Lower Row: Images of the amplitude ratio,  $a_1/a_2$ , and the lifetime ratio,  $t_1/t_2$ , of the fast and the slow decay component.

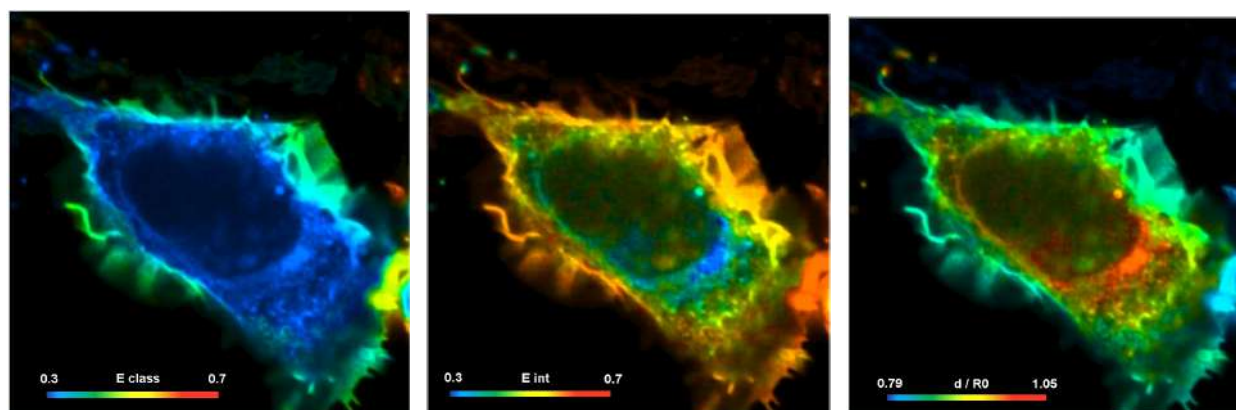


Fig. 3: FRET imaging. Cell with interacting proteins, labelled with a FRET donor and a FRET acceptor. Left to right: Classic FRET efficiency, FRET efficiency of interacting donor fraction, FRET distance



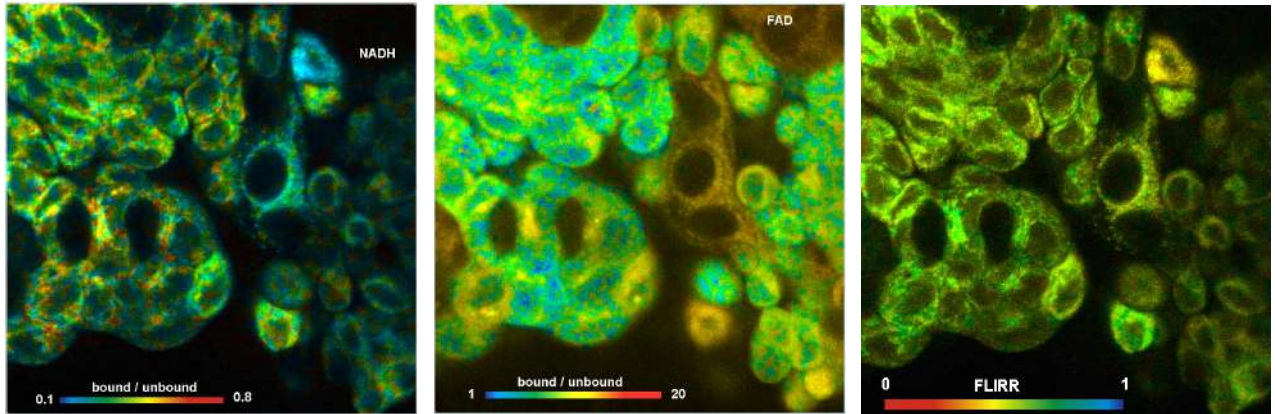


Fig. 4: Metabolic FLIM on the cell level. Bound-unbound ratio of NADH, Bound/unbound ratio of FAD, Fluorescence-Lifetime Redox Ratio, FLIRR.

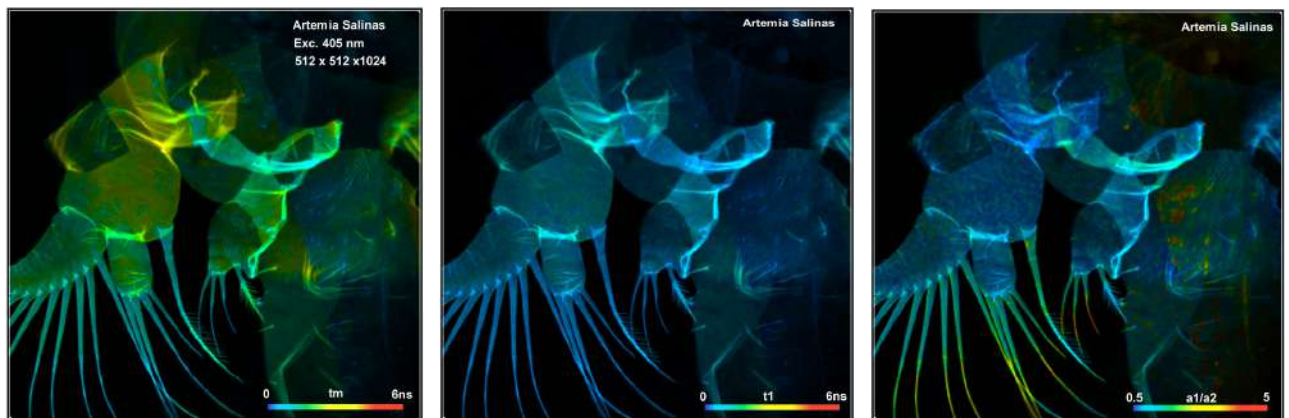


Fig. 5: Autofluorescence FLIM of small organisms. *Artemia salinas*, amplitude-weighted lifetime, tm, lifetime of fast decay component, t1, amplitude ratio of fast and slow component (metabolic ratio), a1/a2.

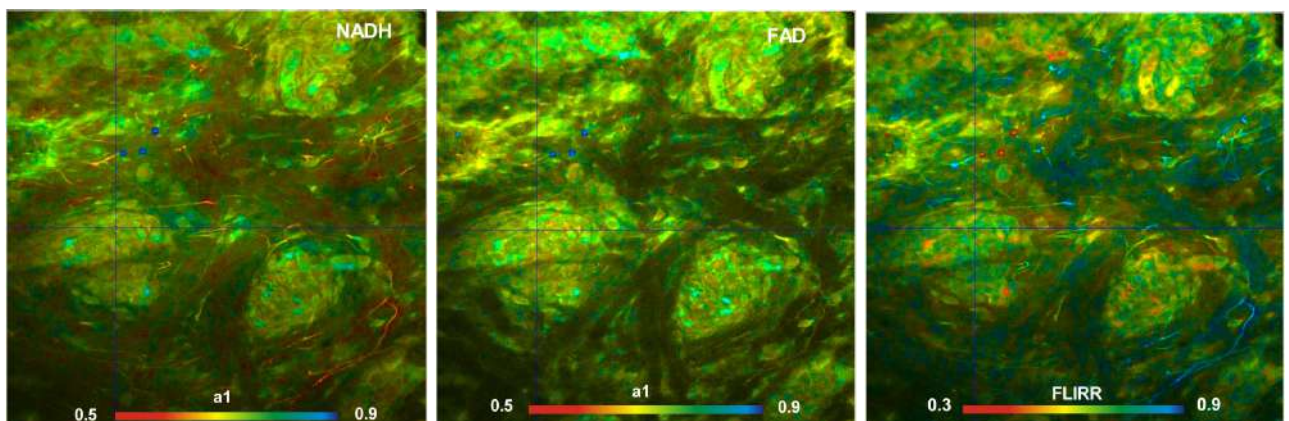


Fig. 6: Two-photon deep-tissue metabolic FLIM. Mammalian skin, NADH and FAD images, metabolic indicator a1 and FLIRR image.

## GPU Processing

bh FLIM data can contain an enormous number of pixels and time channels. Images with 1024 x 1024 or even 2048 x 2048 pixels are not uncommon, and time-channel numbers of 1024 are routinely used in combination with the bh HPM-100-40 detectors [2]. Such data sets are equivalent to a stack of 1024 One-Megapixel images. Processing such amounts of data by the CPU of even a fast computer can take tens of minutes. SPCImage NG therefore uses GPU (Graphics Processor Unit) processing. The TCSPC data are transferred into the GPU, which then runs the de-convolution and fit procedure for a large number of pixels in parallel. This way, data processing times are reduced from formerly several minutes to a few seconds. The short processing time not only simplifies the processing of larger series of data set from a given experiment, it also makes it easy to select the best data sets from a given experiment, or run the analysis with different decay models or different IRF models to find the best way to process them. GPU processing is running on all NVIDIA cards and a number of other NVIDIA-compatible display devices. When SPCImage NG finds a suitable device in the computer it automatically runs the data analysis on it.

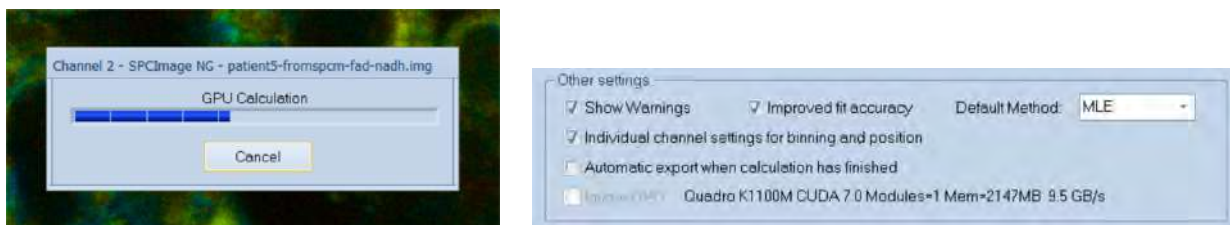


Fig. 7: Left: Progress panel during lifetime calculation, showing that GPU processing is used. Right: Part of 'Preferences' panel, indicating that a Quadro K1100M CUDA 7.0 device was found.

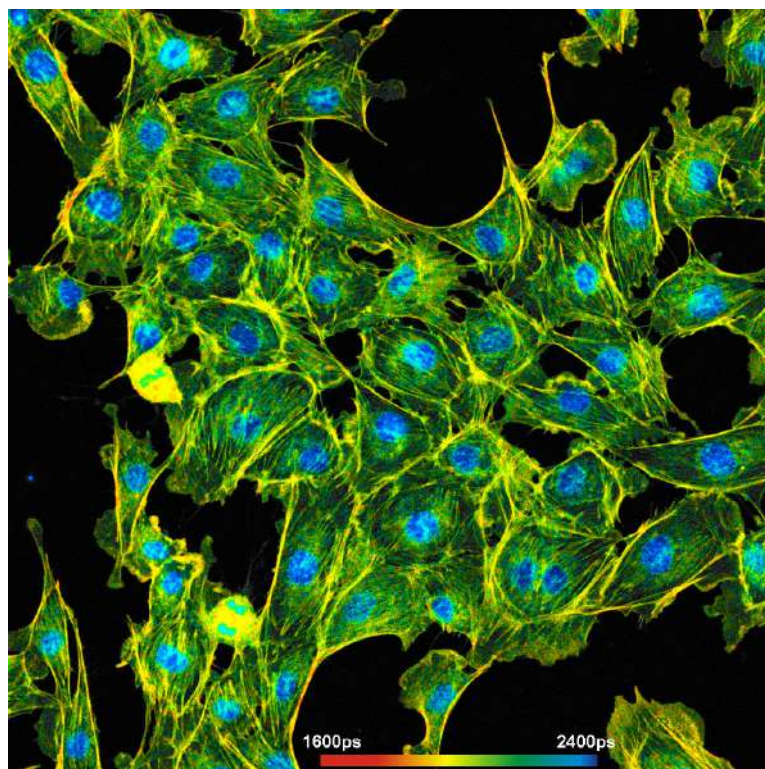


Fig. 8: A lifetime image with 1024 x 1024 pixels and 1024 time channel per pixel. The image was calculated on an NVIDIA GPU in 10 seconds. Recorded by bh FASTAC FLIM system on a Zeiss LSM 880 NLO.



## Phasor Plot

SPCImage FLIM analysis software combines time-domain multi-exponential decay analysis with phasor analysis [15]. Phasor analysis expresses the decay data in the individual pixels as phase and amplitude values in a polar diagram, the 'Phasor Plot'. Pixels with similar decay signature form distinct clusters in the phasor plot. Clusters of interest can be selected and back-annotated in the lifetime image for further processing or for combination of pixel data. An example is shown in Fig. 9.

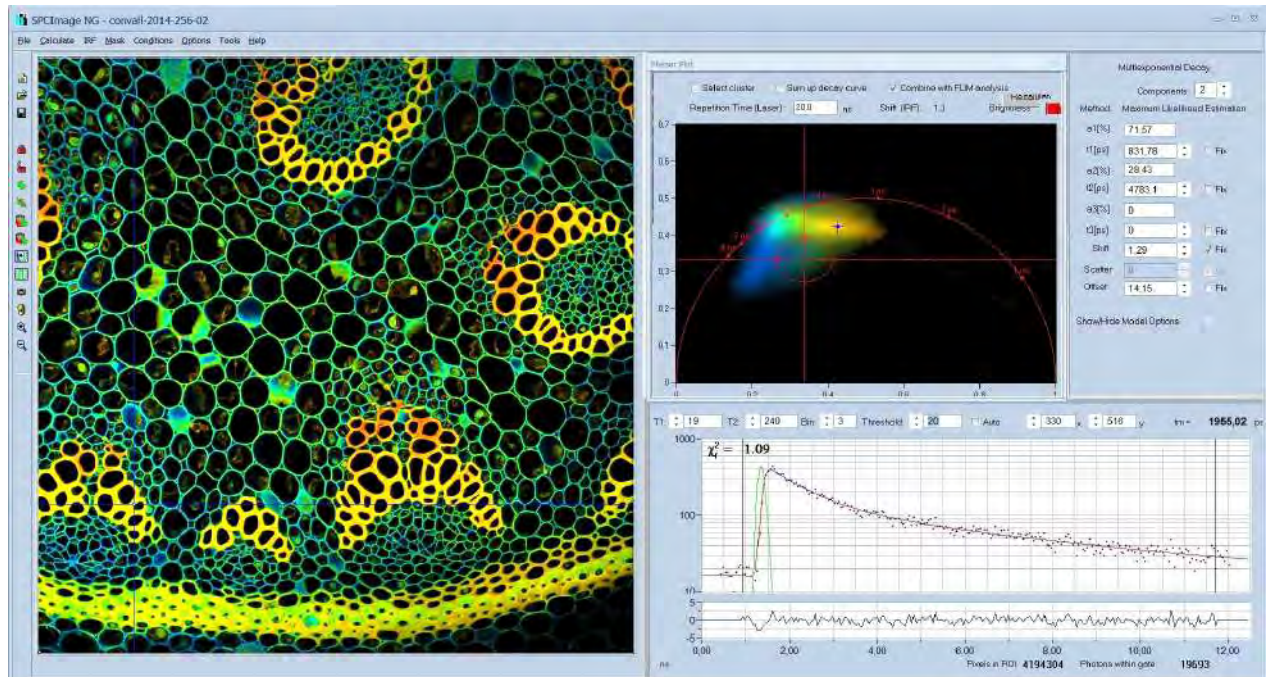


Fig. 9: Combination of time-domain analysis (left and lower right) and Phasor Plot (upper right)

## Image Segmentation by Phasor Plot

Selection of pixels with an interesting phasor signature is demonstrated in Fig. 10. It shows a lifetime image of a tumor in a mouse, the corresponding phasor plot with selection of the phasor range of the tumor, and back-annotation of the pixels within the selected phasor range in the image.

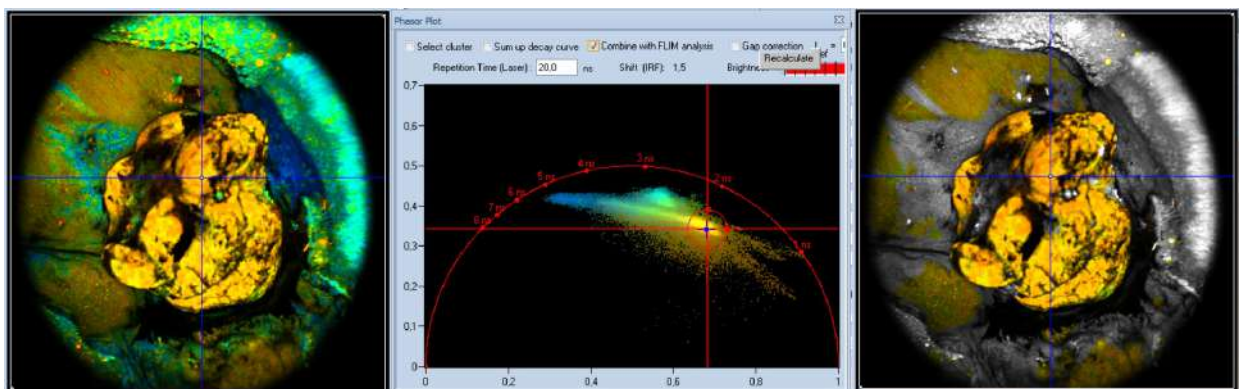


Fig. 10: Left to right: Lifetime image of a mouse tumor, phasor plot with selection of the phasor range of the tumor, and back-annotation of the pixels with the selected phasor signature in the image.

The procedure described above can be used in images containing a large number of cells in the same field of view, as shown in Fig. 11. Cell compartments with different decay signature form separate clusters in the phasor plot. Interesting clusters are selected by the 'Select Cluster' function and back-annotated in the images as shown in Fig. 12 and Fig. 13. A single decay curve can be built up for the combined pixels inside the phasor cluster selected. This curve contains a high number of photons and can be used for precision multi-exponential decay analysis.

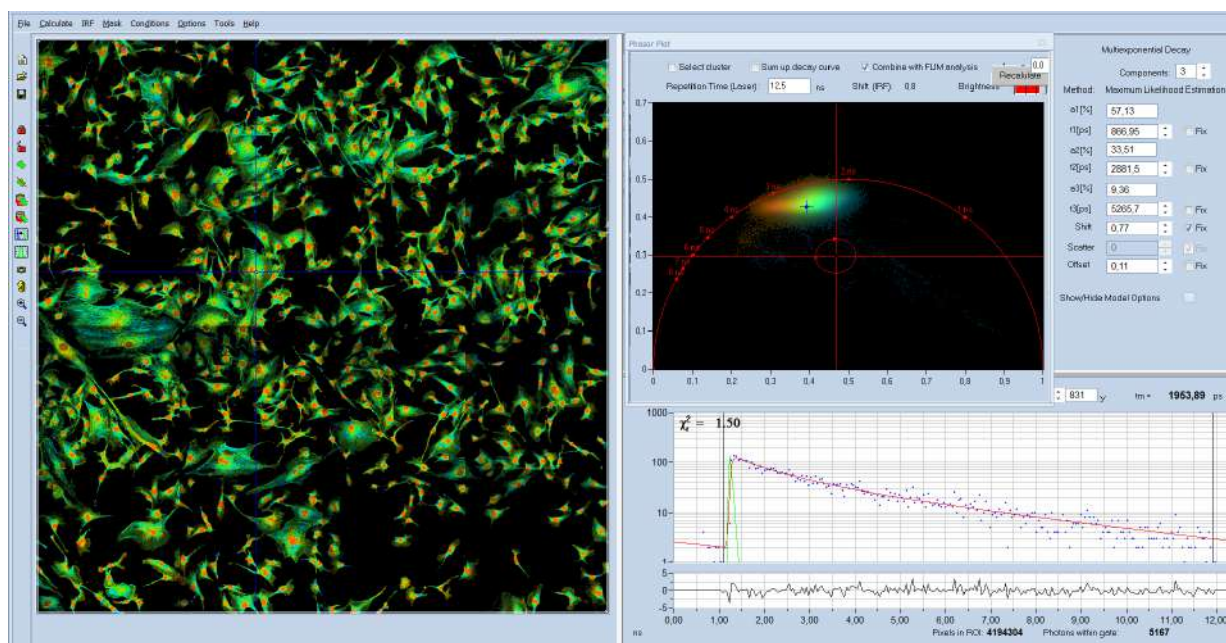


Fig. 11: Spatial Mosaic FLIM image showing a large number of cells. The phasor plot (upper right) displays distinct clusters for the nuclei and the cytoplasm.

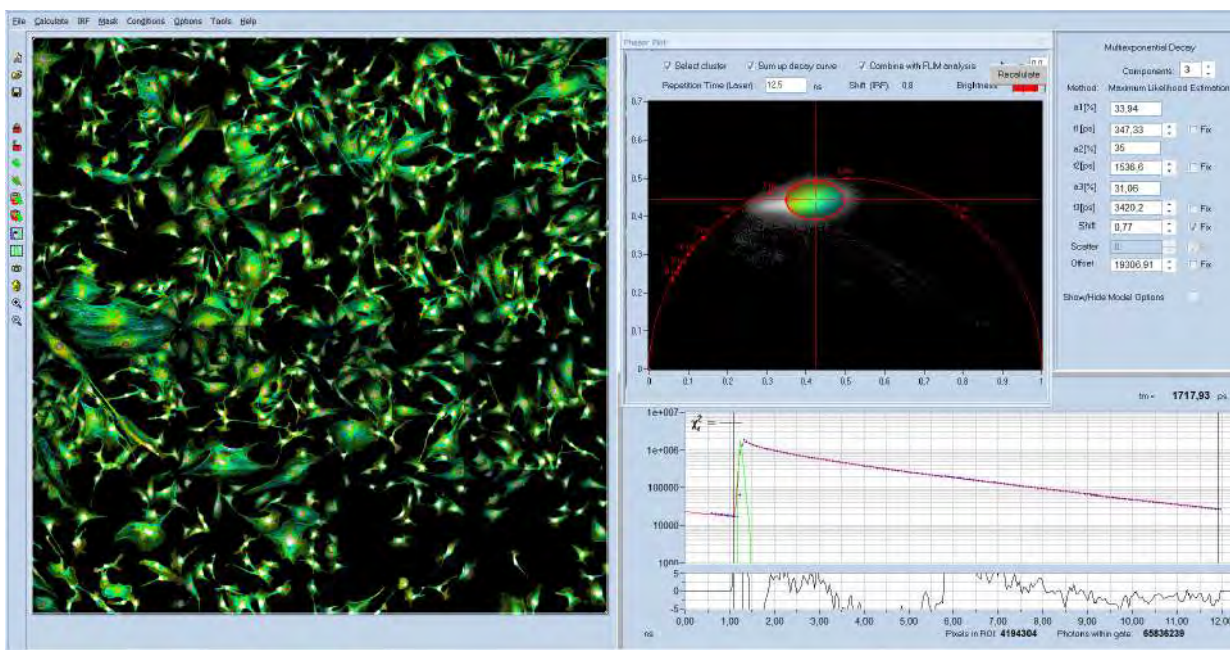


Fig. 12: The cytoplasm of the cells has been selected by the 'Select Cluster' Function. A combined decay curve for the selected pixels is displayed by the 'Sum up decay curves' function.



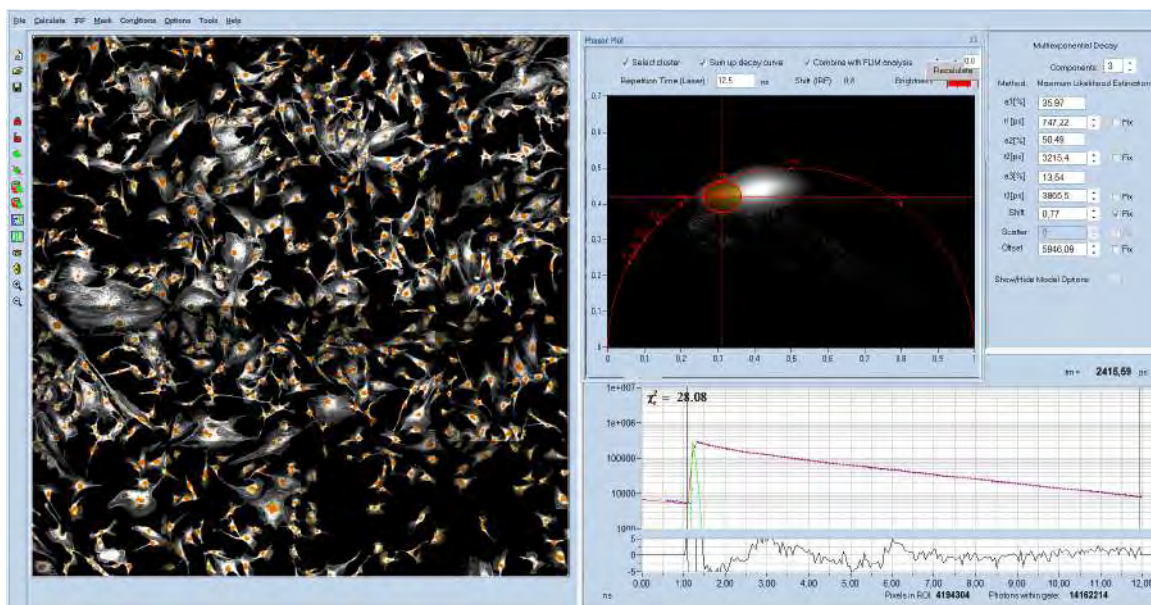


Fig. 13: The nuclei of the cells have been selected by the 'Select Cluster' Function. The histogram (shown right) refers to the selected pixels. A combined decay curve for the selected pixels is displayed by the 'Sum up decay curves' function.

## Precision Decay Analysis of Moving Objects

Precision lifetime imaging of live objects is often hampered by motion in the sample. To avoid that details are smeared out it is often attempted to record the FLIM data in a single, fast scan. This approach is not very successful. It usually results in low photon numbers in the pixels, making precision multi-exponential analysis impossible. The bh TCSPC systems solve the problem by recording a 'temporal mosaic'. That means a series of fast scans is performed and the data are written in subsequent elements of a data mosaic [1, 8]. The entire mosaic is then loaded into SPCImage NG, and a preliminary data analysis is performed. The data are loaded into the phasor plot, the phasor signature of the detail of interest is selected, and the selection is back-annotated in the mosaic image. Combination of the pixels yields a single decay curve with a large number of photons. Fig. 14 demonstrates the procedure at the example of a live water flea. The decay curve of the combined pixels is shown lower right, the decay parameters upper right. Decay analysis shows decay parameters typical of FAD, with a small contribution of FMN. That means the procedure is able to perform metabolic FLIM at a moving leg of a water flea!

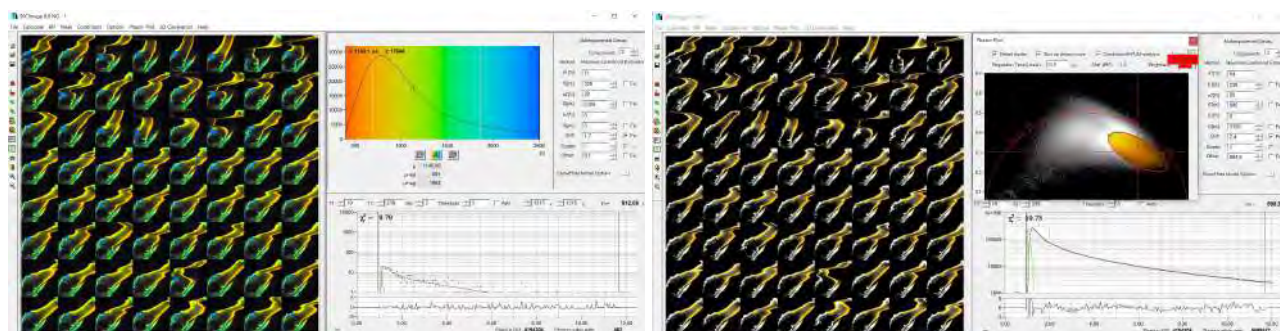


Fig. 14: Temporal-Mosaic FLIM data showing the leg of a live water flea. Left: The photon number in a single pixel is too low for precision decay analysis. Right: Selection of the phasor range of interest and combination of the pixels yields a high accuracy decay curve.

## Trajectory of Dynamic Systems in the Phasor Space

Another feature of the phasor plot is that it displays dynamic changes in the fluorescence-decay behaviour of a sample. An example is shown in Fig. 15. It shows a temporal-mosaic recording of chloroplasts in a leaf [1]. The image mosaic (shown left) shows how the chloroplasts change their fluorescence decay time with the time of exposure. The phasor plot (shown right) displays the trajectory the system is taking in the phasor plot. In the present case, the phasor trajectory shows that the fluorescence decay functions of the chlorophyll for a given time after the start of the exposure are close to single exponential. This contradicts common opinion. Obviously, strongly multi-exponential decay reported for chlorophyll in live plants comes from the dynamic change of the lifetime during the acquisition time period rather than from intrinsic multi-exponential behaviour.

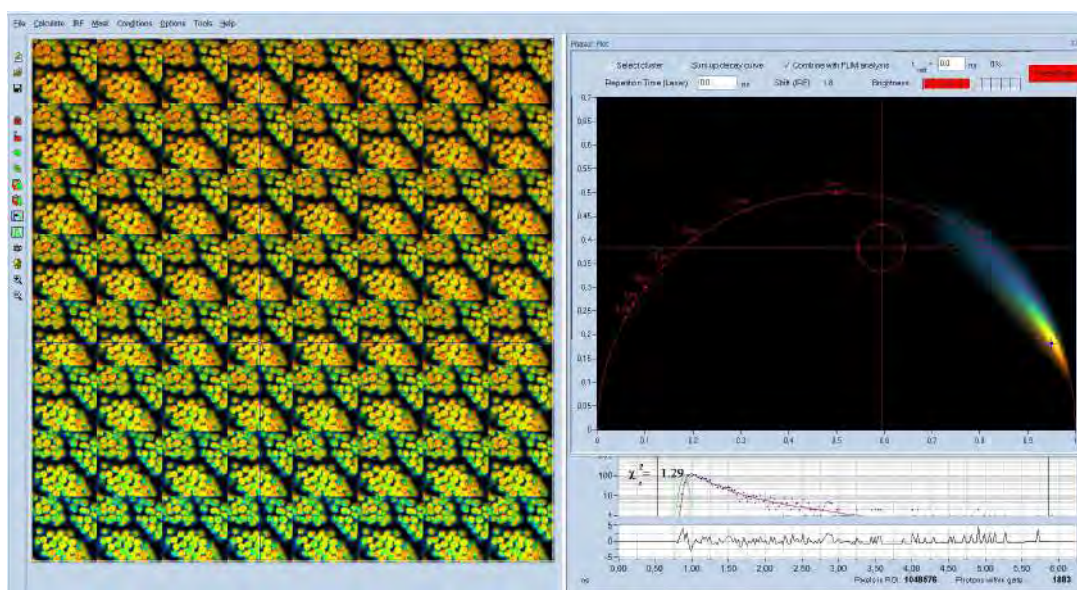


Fig. 15: Temporal-mosaic FLIM of a plant leaf. The Phasor Plot shows the trajectory the system is taking in the phasor space.

## Analysis of Special Data Types

### Ultra-Fast FLIM

For many years, TCSPC FLIM has been performed with 256 time channels and a time-channel width of about 50 ps. With the introduction of the HPM-100-40 hybrid detectors [2] bh moved to a standard FLIM format of 1024 time-channels, and a channel width of 10 ps. Recently, bh have introduced FLIM systems with HPM-40-06 detectors [1, 3]. The systems have an IRF width of <20 ps, fwhm [5, 6, 7]. To fully exploit an IRF this fast, the data must be recorded with 4096 time channels and a time-channel width of 1 ps or shorter. For the determination of component lifetimes in the range of 20 ps and below even shorter time-channel width is appropriate. Recent versions of SPCImage NG therefore account for analysis of decay data with femtosecond time-channel width and 4096 time channels [1, 4]. An example is shown in Fig. 16. The time-channel width is 300 femtoseconds. With a channel width this small the IRF (green curve) is sampled with about 70 data points. Together with a large number of photons and triple-exponential analysis, this allows decay components down to less than 10 ps to be determined, see histogram of  $t_1$ , Fig. 16, right.



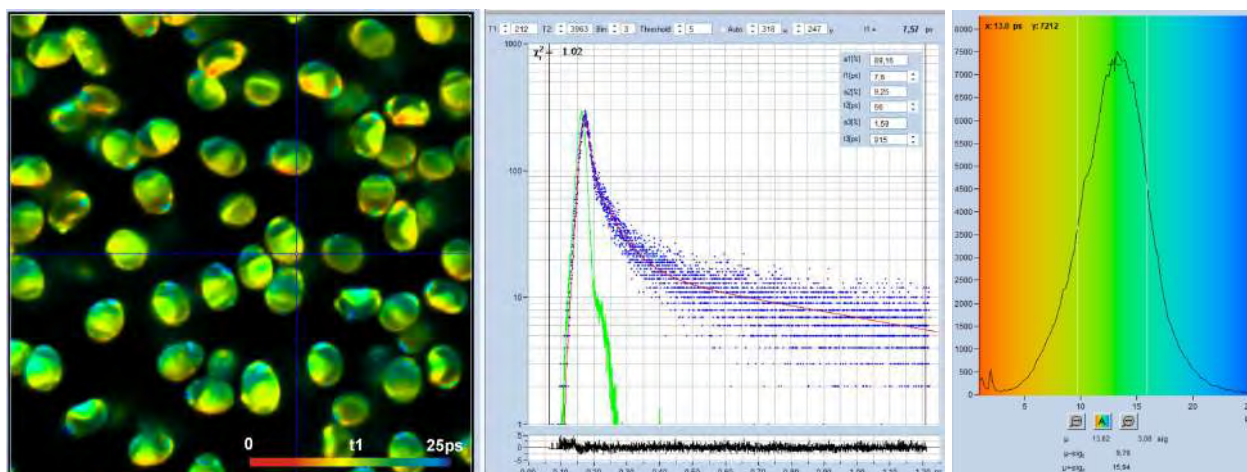


Fig. 16: Ultra-high resolution FLIM of *Paxillus involutus* spores [5]. Left to right: Lifetime image of fastest decay component,  $t_1$ , decay curve at cursor position, histogram of  $t_1$  over the pixels of the image. Time-channel width 300 femtoseconds, 4096 time channels, IRF width 23 ps, observation-time interval 1.25 ns. Analysed with real IRF, recorded from powdered sugar. Triple-exponential decay analysis shows that the fast decay components is in the range of 13 picoseconds.

## Dual-Channel Metabolic FLIM

Metabolic FLIM is increasingly using wavelength multiplexing in combination with dual-channel detection [1, 9, 10]. This way, NADH and FAD images are obtained with virtually no spectral crosstalk. Since it is desirable to cross-calculate ratios of decay parameters between the two channels SPCImage NG has been extended to hold two FLIM data channels in the memory, and to display two images simultaneously. For colour coding of the images, ratios of decay parameters from different channels can be selected. An example is shown in Fig. 17.

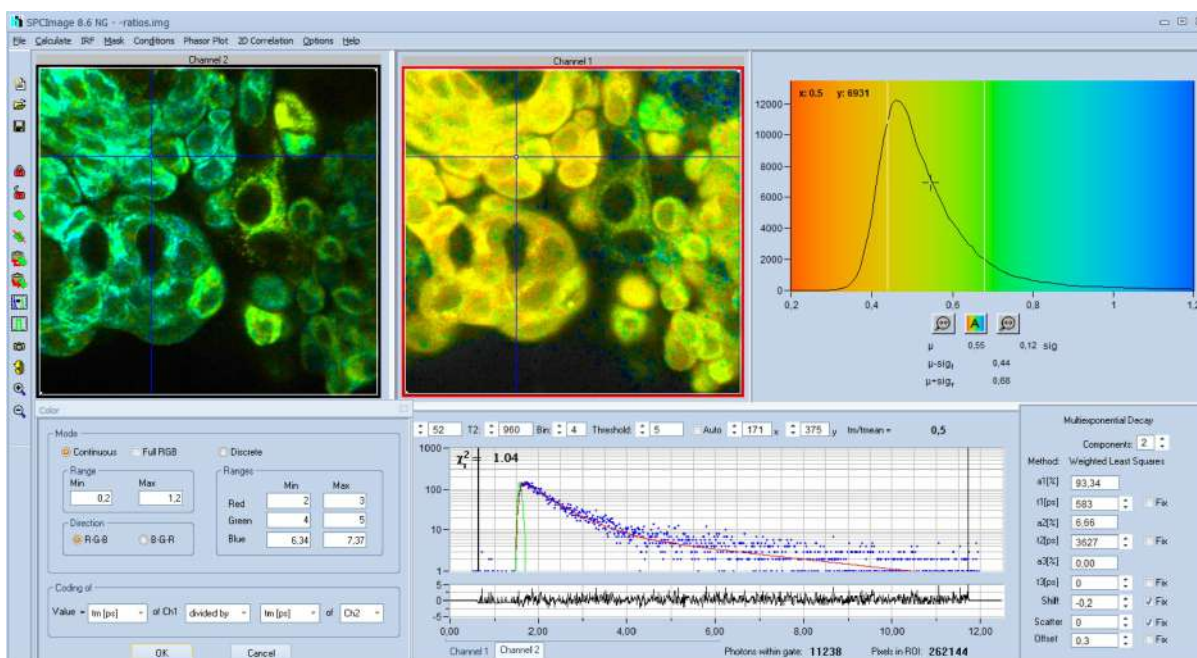


Fig. 17: Dual-channel metabolic FLIM, recorded by wavelength-multiplexed excitation and recording in separate wavelength channels. NADH image shown left, FAD image shown right. Both images colour-coded with the ratio of the amplitude-weighted lifetimes of NADH and FAD.

## INT FLIM Data

INT FLIM (Intensity-linear FLIM) is a new recording mode of the bh SPC-180N family modules. For every pixel, the data contain the usual decay function with the selected number of time channels together with the total photon number [1, 11]. The photon number is determined by an additional fast counter channel in parallel with the timing electronics. The technique delivers high-resolution decay data with a linear intensity scale. SPCImage NG automatically recognises such data and handles them accordingly. That means the fit procedure is performed on the decay data as usual, but the image intensity comes from the photon number in the fast counter channel. An example is shown in Fig. 18.

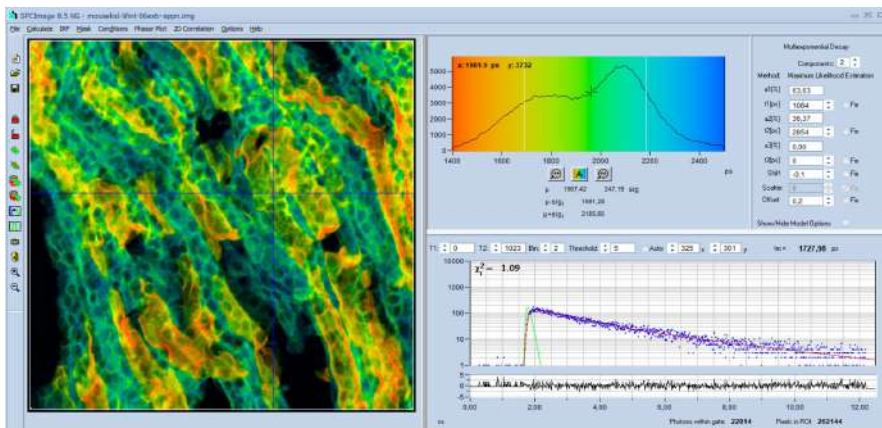


Fig. 18: Data from INT-FLIM recording. INT FLIM data contain decay data from the TCSPC timing electronics in combination with intensity data from a fast counter. The technique is used to obtain high-resolution decay data with a linear intensity scale. SPCImage NG automatically recognises such data and processes and displays them with the correct time scale and intensity scale.

## Simultaneous FLIM / PLIM

The bh FLIM systems are able to record FLIM and PLIM simultaneously [1, 13]. SPCImage NG loads the entire FLIM/PLIM data set, and displays both images simultaneously. Please see Fig. 19.

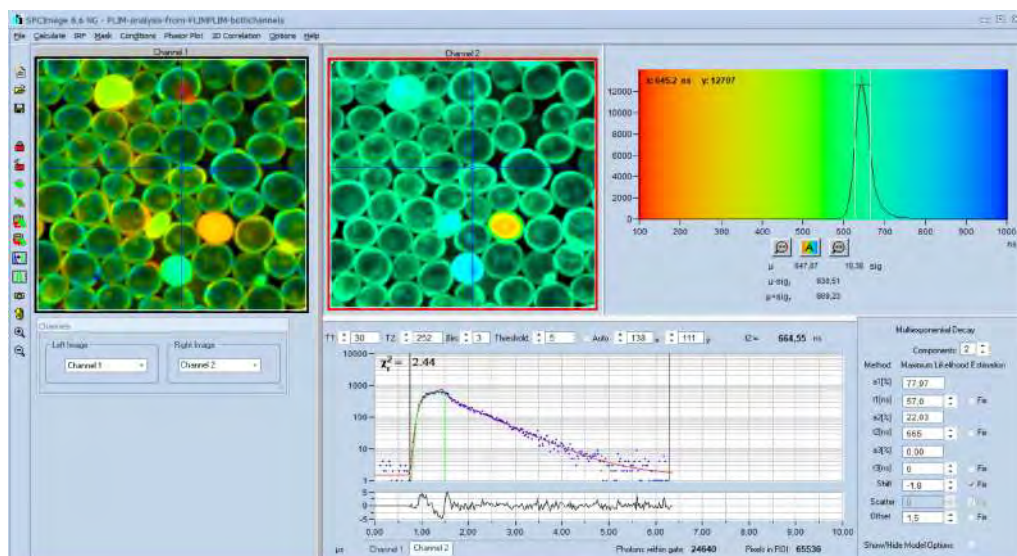


Fig. 19: Analysis of data from simultaneous FLIM / PLIM experiment. Left FLIM, right: PLIM. Decay curve and histogram shown for PLIM.



## Single-Curve Analysis

Recent versions of SPCImage NG simplify the analysis of single-curve fluorescence decay data. The data can either be recorded by scanning a sample solution with a bh FLIM system or by recording a single curve in a cuvette setup. Analysis is possible with all decay models implemented in SPCImage, and with a synthetic or a measured IRF. Fig. 20 shows the decay function recorded from an NADH solution with a fast FLIM system of 20 ps IRF width.

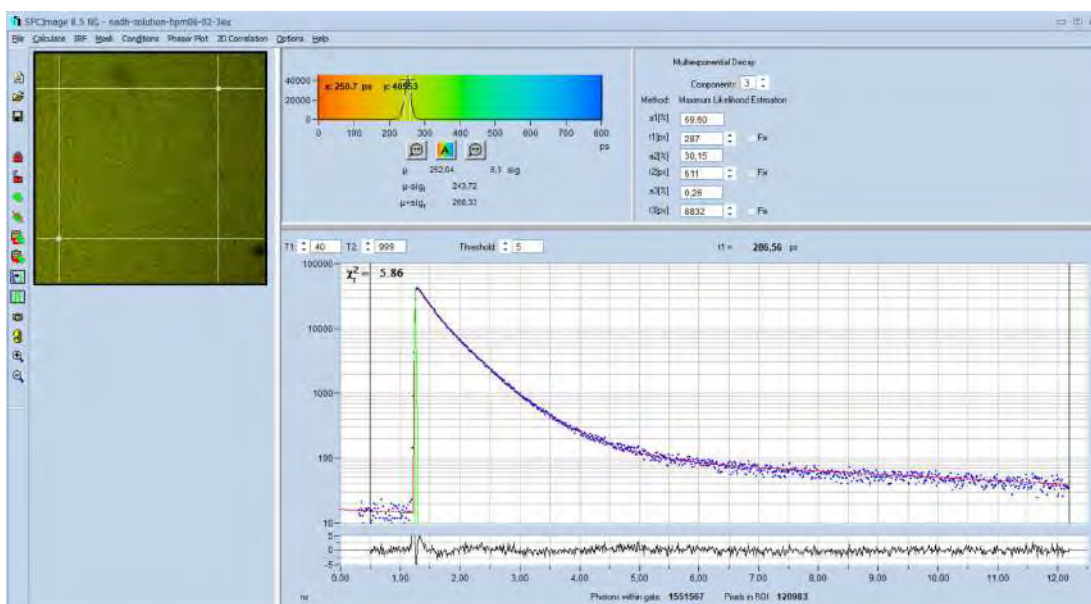


Fig. 20: Fluorescence decay of an NADH solution. Data recorded by FLIM, pixels within cursor area combined into single decay curve. Triple-exponential decay analysis with synthetic IRF.

## Batch Processing

A batch-processing function is available for analysing a large number of FLIM data sets automatically. Please see [1], 'The bh TCSPC Handbook', chapter 'SPCImage NG Data Analysis'.

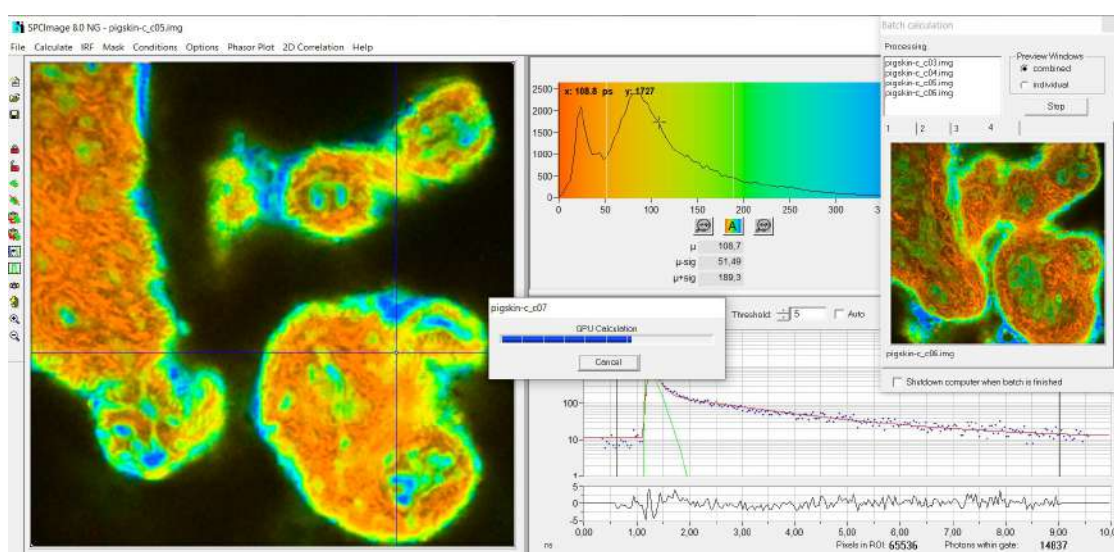


Fig. 21: Batch Processing in progress. Subsequent files are loaded, analysed, and saved.

## Histograms

### Decay Parameters

SPCImage has histogram functions for the decay parameters. The histogram shows how often pixels of a given parameter value occur in the lifetime image. The parameter histogram is thus an efficient tool to determine decay parameters in selected regions of interest, get an estimate of the variance of parameters, and to compare decay parameters of different samples [5, 12].

The histogram refers either to a selected region of interest or, if no ROI was defined, to the entire lifetime image. Together with the various options to select decay parameters and combinations of decay parameters a wide variety of different histograms can be obtained. A few examples are shown in Fig. 22 through Fig. 24.

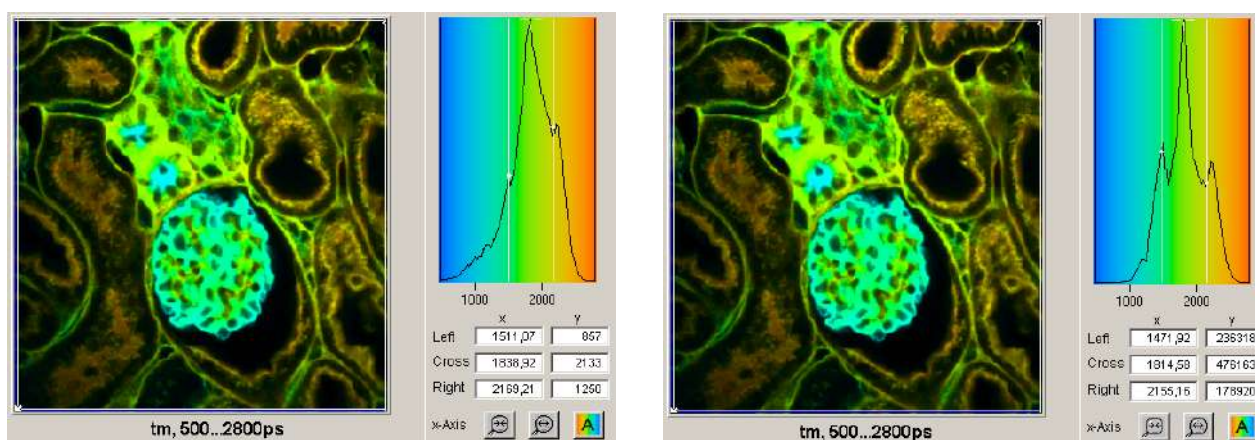


Fig. 22: Histograms of the mean (amplitude weighted) lifetime of double-exponential fit. Left: Un-weighted frequency of pixels. Right: Intensity-weighted frequency of pixels. Note that the peak at 1500ps is enhanced due to high intensities of the pixels, and peak at 2100 ps is reduced due low intensities.

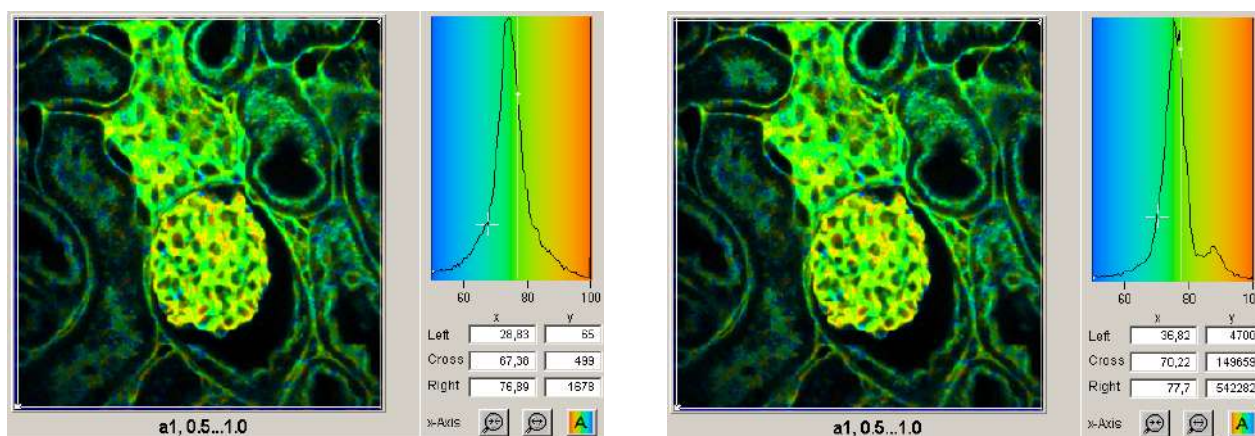


Fig. 23: Histograms of the amplitude,  $a_1$ , of the fast decay component. Left: Un-weighted frequency of pixels. Right: Intensity-weighted frequency of pixels. The peak at 0.85 (85%) is only visible in the intensity-weighted histogram because it is caused by a small number of pixels that have high intensity.

SPCImage NG allows the user to define multiple regions of interest. In that case, every ROI has its own parameter histogram. The desired ROI and the corresponding histogram can be selected by the buttons on top of the histograms, or by clicking on the red dot in the centre of the ROI. An example is shown in Fig. 24.



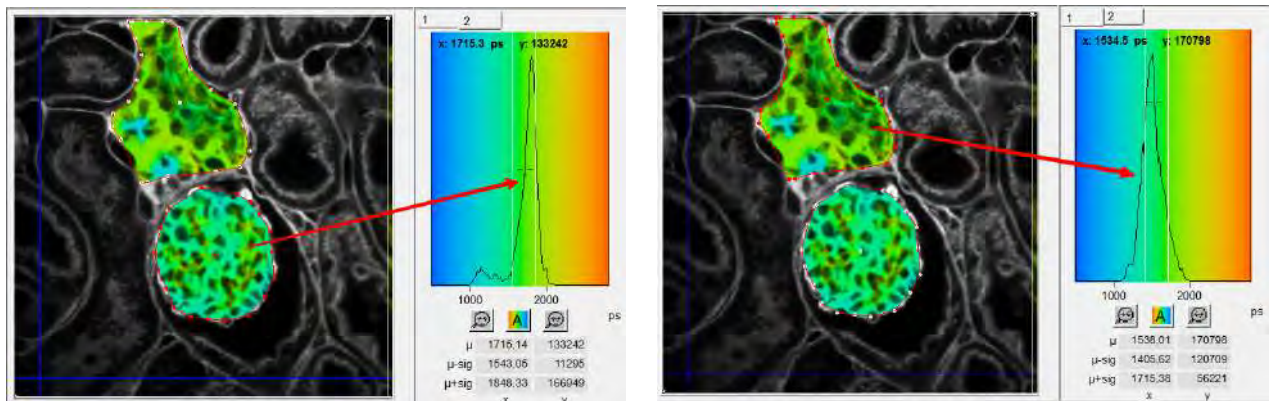


Fig. 24: Multiple ROIs, selection via the buttons on top of the histograms

## 2-D Histograms

2D histograms present density plots of the pixels over two selectable decay parameters. The decay parameters can be lifetimes,  $t_1$ ,  $t_2$ ,  $t_3$ , or amplitudes,  $a_1$ ,  $a_2$ ,  $a_3$ , of decay components, amplitude or intensity-weighted lifetimes,  $t_m$  or  $t_i$ , or arithmetic conjunctions of these parameters. An example is shown in Fig. 25. A histogram of the amplitude,  $a_1$ , of the fast decay component versus the amplitude-weighted lifetime,  $t_m$ , has been selected. Cursors in the histogram are available to select special parameter combinations and highlight the corresponding pixels in the lifetime image.

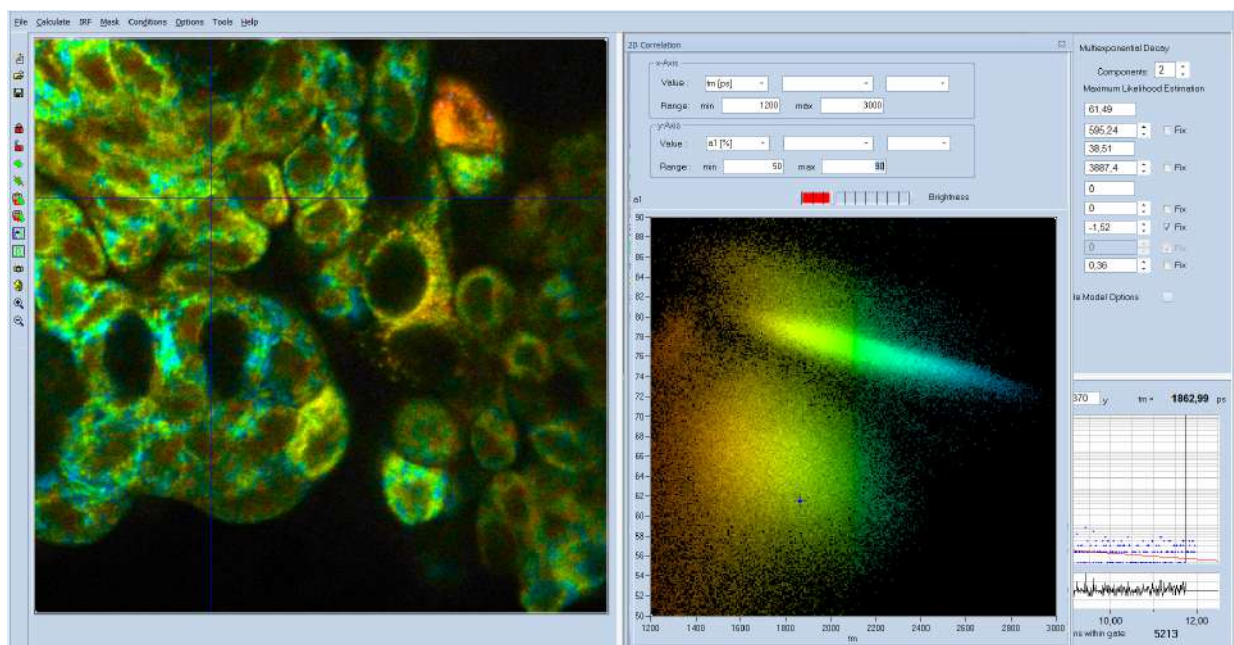


Fig. 25: 2-D histogram showing density plot of pixels over amplitude-weighted lifetime,  $t_m$ , and amplitude of fast component,  $a_1$ .

## Intensity Histogram

Instead of the parameter histogram, SPCImage NG can also display an intensity histogram, see Fig. 26. The cursors in the intensity histogram interact directly with the 'Intensity' Parameters of the image display [1]. An example is shown in Fig. 26, right.

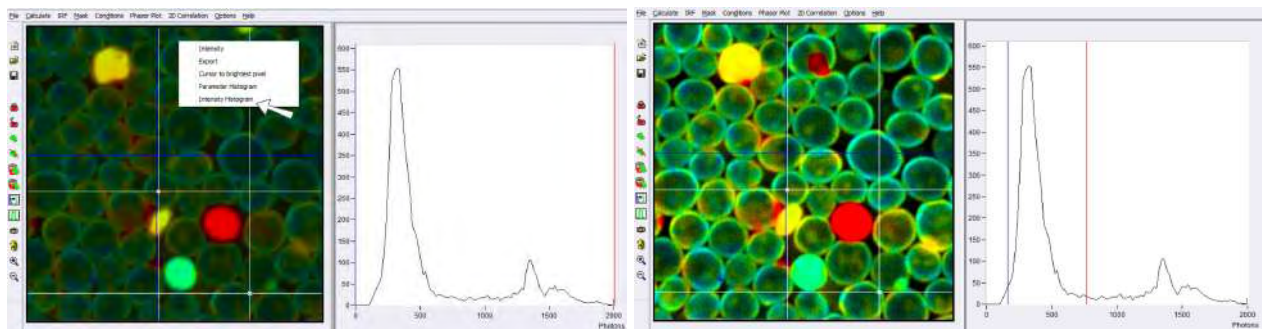


Fig. 26: Intensity Histogram of SPCImage NG. Left: The sample contained a few dead cells with extremely high intensity. Within the entire intensity range of the image, the cells of interest are displayed only dimly. Right: By pulling the cursors to the intensity range of the cells of interest results a perfect image is obtained.

## ROIs

SPCImage NG allows the user to define ROIs in the images. Both rectangular and polygonal ROIs can be defined. Parameter histograms are displayed for the selected ROI, see Fig. 27.

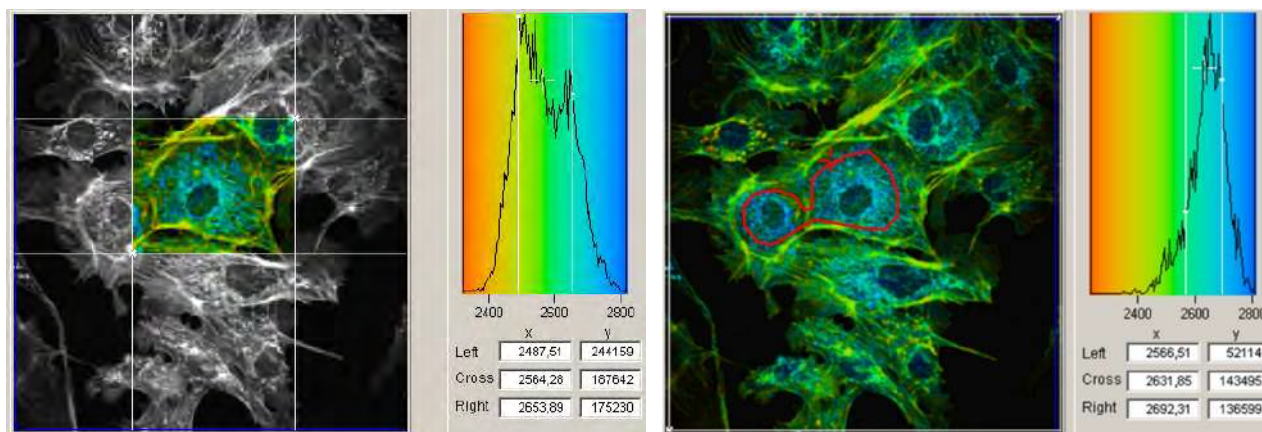


Fig. 27: ROI Definition. Left: Rectangular ROI. Right: Polygonal ROI

Several polygonal ROIs can be defined, and the corresponding parameter histograms be selected via the buttons on top of the histogram window. Please see Fig. 28.

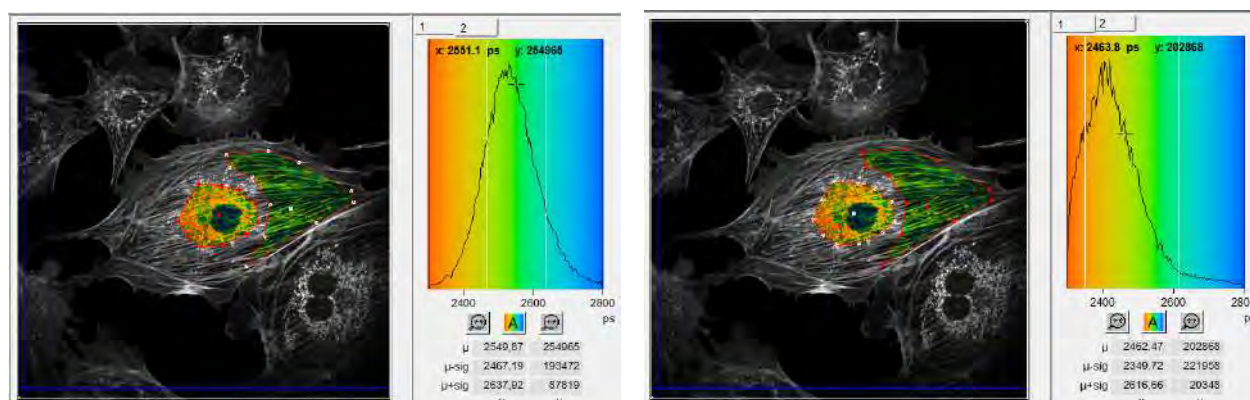


Fig. 28: Multiple ROIs, with selection of parameter histogram.



## Procedures and Algorithms

### Intelligent Binning

Microscopy images are over-sampled to obtain maximum spatial resolution. Oversampling means that the point spread function is sampled by several pixels in x and y. Typical (linear) oversampling factors are around 5, that means the point-spread function is sampled by about 25 pixels. Of course, these pixels contain virtually identical lifetime information. Analysing them individually would result in low photon number per pixel, and in unnecessarily high noise in the decay parameters. To avoid this problem SPCImage NG uses overlapping binning of the decay data. The procedure leaves the number of pixels unchanged but includes decay data from several pixels around the current one into each calculation step [1, 12]. An example is shown in Fig. 29. The image on the left shows a scan of 128 x 128 pixels, analysed by SPCImage NG without binning. The image on the right was recorded with 512 x 512 pixels. Photon rate and acquisition time were the same. To compensate for the lower number of photons per pixel the data were analysed using a binning are of 5 x 5 pixels. This brings the photon number to the same level as in the 128 x 128 image. The effect is striking: The spatial resolution is massively improved, but there is no blur-out of the lifetime information in the binned 512 x 512 pixel image.

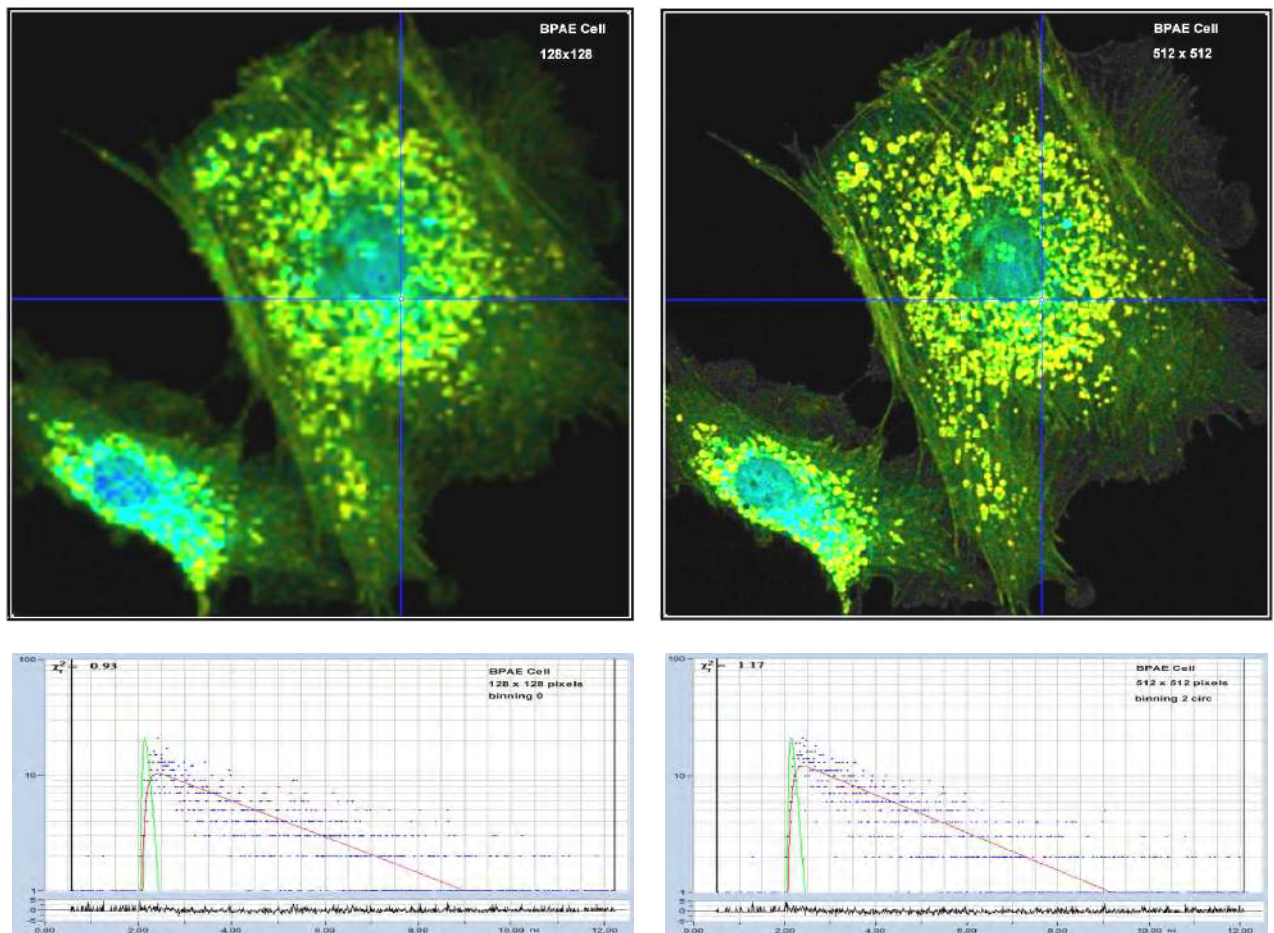


Fig. 29: Effect of spatial binning. Left: 128 x 128 pixels, no binning , Right: 512 x 512 pixels, binning of 5x5 pixels

## De-Convolution

In a real FLIM system the fluorescence is excited by laser pulses of non-zero width, and detected by a detector with a temporal response of non-zero width. The effect on the temporal shape of the recorded signal is shown graphically in Fig. 30. The laser pulse is thought to be broken down into a sequence of (infinitely) narrow pulses of different amplitude (Fig. 30, left). Each of these sub-pulses produces a fluorescence decay of an amplitude proportional to the amplitude of the sub-pulse, and starting at the time of the sub-pulse. The sum of all these decay functions is the real optical waveform of the fluorescence signal, see bottom of Fig. 30, left. The real fluorescence signal is measured by a detector the temporal response of which has non-zero width, see Fig. 30, middle. Again, the detector response can be thought to consist of a sequence of infinitely short pulses. Also here, the measured waveform is the sum of shifted signal components of different amplitude.

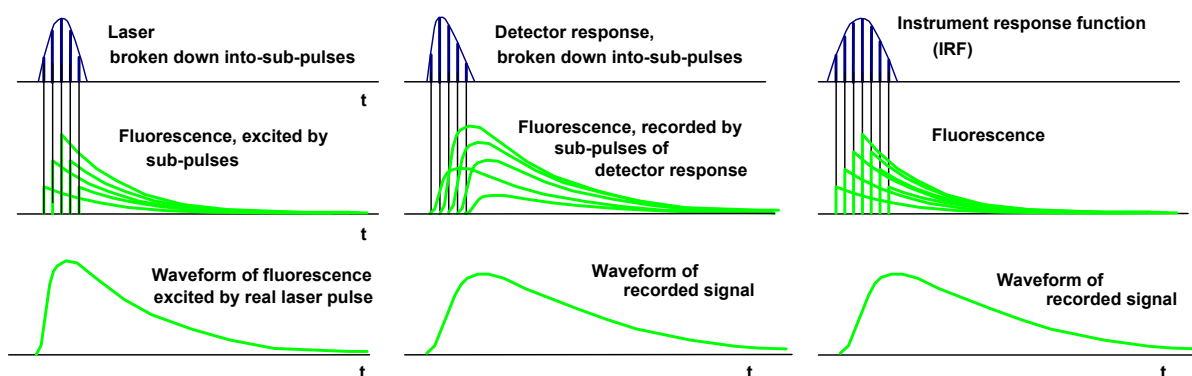


Fig. 30: Left: Convolution of the laser pulse with the fluorescence decay. Middle: Convolution of the real fluorescence waveform with the detector response. Right: Laser pulse and detector response combined into IRF pulse, convolution of fluorescence decay with IRF.

The transformation of the signal waveforms shown above is called 'convolution'. Mathematically, the signal transformation is described by the commonly known 'convolution integral' [1]. The laser pulse shape and the detector response can be combined in a single 'instrument response function', or IRF, see Fig. 30, right. The IRF is the convolution of the laser pulse shape with the detector response, i.e. the pulse shape the system would record if it directly detected the laser. The convolution of the fluorescence decay function with this IRF delivers the same result as the two subsequent convolution steps shown in Fig. 30, left and middle.

The convolution integral cannot be reversed, i.e. there is no analytical expression of  $f(t)$  for a given  $f_m(t)$  and  $IRF(t)$  [1]. The standard approach to solve the de-convolution problem is to use a fit procedure: A model function of the fluorescence decay function is defined, the convolution integral of the model function and the IRF is calculated, and the result is compared to the measured data. Then the parameters of the model function are varied until the best fit with the measured data is obtained. This operation is performed for all pixels of the image.

## Weighted Least-Square Fit

The principle of the traditional weighted least-square (WLS) fit is shown in Fig. 31. The differences,  $\delta(t)$ , between the points of the model function,  $f_{mod}(t)$ , and the data points,  $n(t)$ , are calculated. In principle, the squares of the differences,  $\delta(t)^2$ , could be calculated, summed up, and this sum be used as an optimisation parameter.



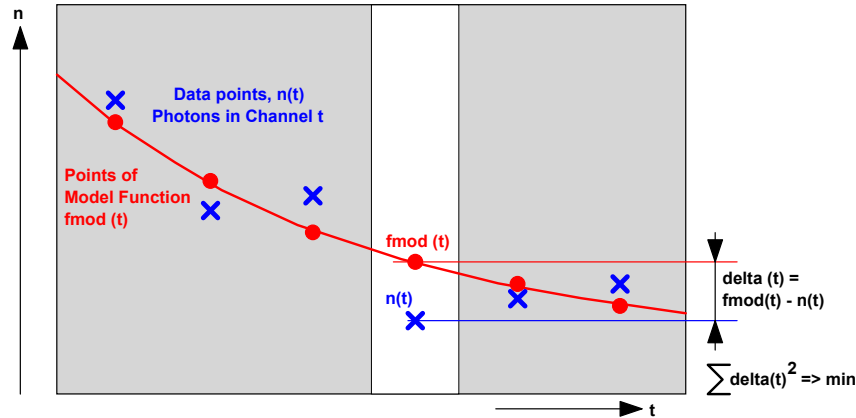


Fig. 31: Least-square fit

For fluorescence decay curves, this procedure has a flaw. The photon numbers,  $n(t)$ , are Poisson-distributed. That means the noise is larger in channels with higher photon number: The noise in  $n(t)$  is  $n(t)^{1/2}$  [1, 12]. Therefore, the squares of the differences must be weighted with the reciprocal of the expected value of the noise.

The required weighting of the delta-squares is the problem of the least-square fit. The correct weight according to the Poisson distribution would be  $1/n(t)$ . This is, of course, not possible because there are time channels with  $n(t)=0$ . The weight of these channels would be infinite, which is a practical impossibility. The commonly used solution is to use a weight of  $1/(n(t)+1)$ :

$$S = \sum \frac{\Delta(t)^2}{n(t)+1}$$

The weighting with  $1/(n(t)+1)$  avoids the singularity problem for  $n(t)=0$ , but, of course, does not weight the deltas in channels with low  $n(t)$  or  $n(t)=0$  correctly. The result is a bias towards shorter lifetimes for decay data of low photon number.

### Maximum-Likelihood Fit

Unlike previous SPCImage versions, SPCImage NG uses a maximum-likelihood algorithm (or maximum-likelihood estimation, MLE) for fitting the data. In contrast to the usual least-square fit, the MLE algorithm takes into account the Poissonian distribution of the photon numbers. Compared to the least-square method, the fit accuracy is improved especially for low photon numbers, and there is no bias toward shorter lifetime as it is observed for the least-square fit. The maximum-likelihood fit is based on calculating the probability that the values of the model function correctly represent the data points of the decay function. The principle is illustrated in Fig. 32. To each point of the model function,  $f_{mod}(t)$ , a Poissonian distribution,

$$p(n) = \frac{E^n e^{-E}}{n!}$$

with an expectation value equal to  $E=f_{mod}(t)$ , is associated, Fig. 32, right. From this distribution, the probability,  $p(n(t))$ , is calculated, that a data point with the photon number,  $n(t)$ , appears in the corresponding time channel of the measurement data. The probability,  $p(n(t))$ , is a measure of how likely it is that the point of the model function is a correct representation of the data.  $p(n(t))$  is calculated for all time channels, i.e. for all pairs of data points and model-function points. The

product of these probabilities is the probability that the model function represents the data. The parameters of the model functions are then optimised until the maximum product of the probabilities is obtained.

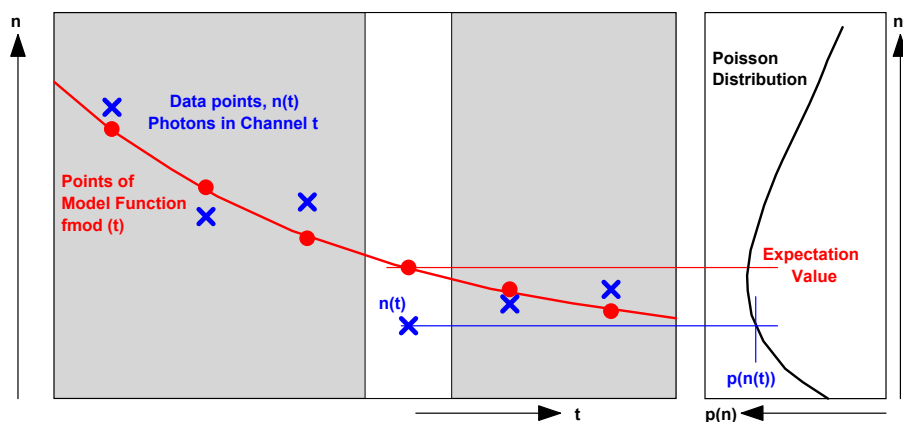


Fig. 32: Principle of MLE fit. For each point of the model function,  $f_{\text{mod}}(t)$ , a Poissonian distribution is associated. The function delivers a probability  $p(n(t))$ , that a given data point,  $n(t)$  fits to the corresponding point of the model function. The product of  $p(n(t))$  over all time channels is used for optimising the parameters of the model function.

The MLE fit has no problem with data points with low photon number or even with a photon number of zero. The Poissonian distribution associates correct probabilities to all these situations, and the product of these probabilities correctly describes the quality of the fit. Consequently, there is no bias toward shorter lifetime, as it occurs in the weighted least square fit.

For comparison with older data sets the weighted least-square fit and the first-moment lifetime calculation are still available in SPCImage NG. The analysis method is selected in the 'Model' panel, see Fig. 33. MLE can (and should) be declared the default under 'Preferences', Other Settings'. The least-square fit and the first-moment lifetime calculation are not running GPU processing.

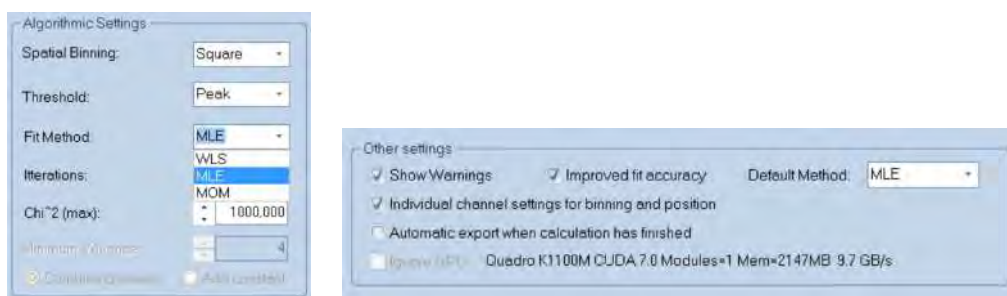


Fig. 33: Selecting MLE under Model, Algorithmic Setting (left) and declaring MLE the default method under Preferences, Other Settings.



## Principle of Phasor Analysis

SPCImage FLIM analysis software combines time-domain multi-exponential decay analysis with phasor analysis. Phasor analysis expresses the decay data in the individual pixels as phase and amplitude values of a periodic waveform in a polar diagram, or the 'Phasor Plot' [15]. The principle is illustrated in Fig. 34.

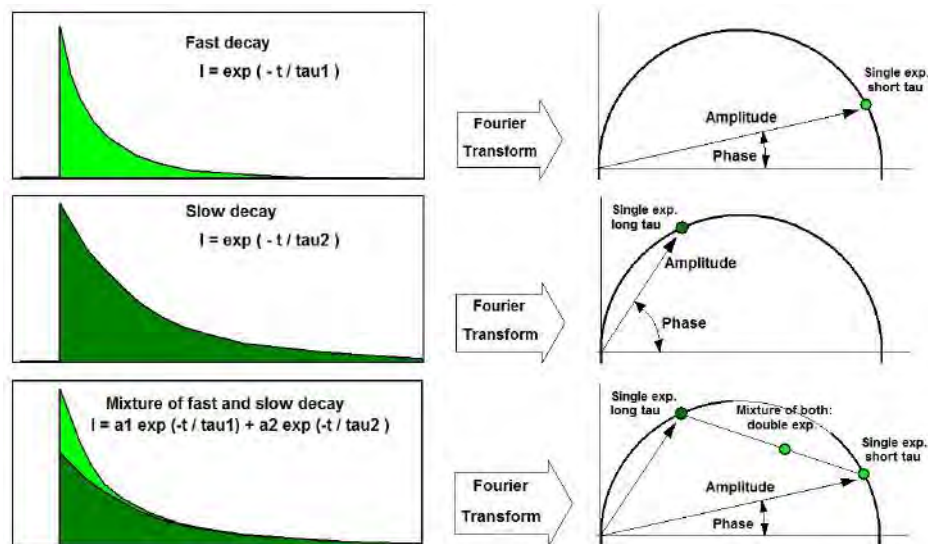


Fig. 34: Principle of phasor analysis

Independently of their location in the image, pixels with similar decay signature form clusters in the phasor plot. Different phasor clusters can be selected, and the corresponding pixels back-annotated in the time-domain FLIM images, see Fig. 35 and Fig. 36. Moreover, the decay functions of the pixels within the selected phasor range can be combined into a single decay curve of high photon number. This curve can be analysed at high accuracy, revealing decay components that are not visible by normal pixel-by-pixel analysis, see Fig. 36. Please see [1], 'The bh TCSPC Handbook', chapter 'SPCImage Data Analysis'.

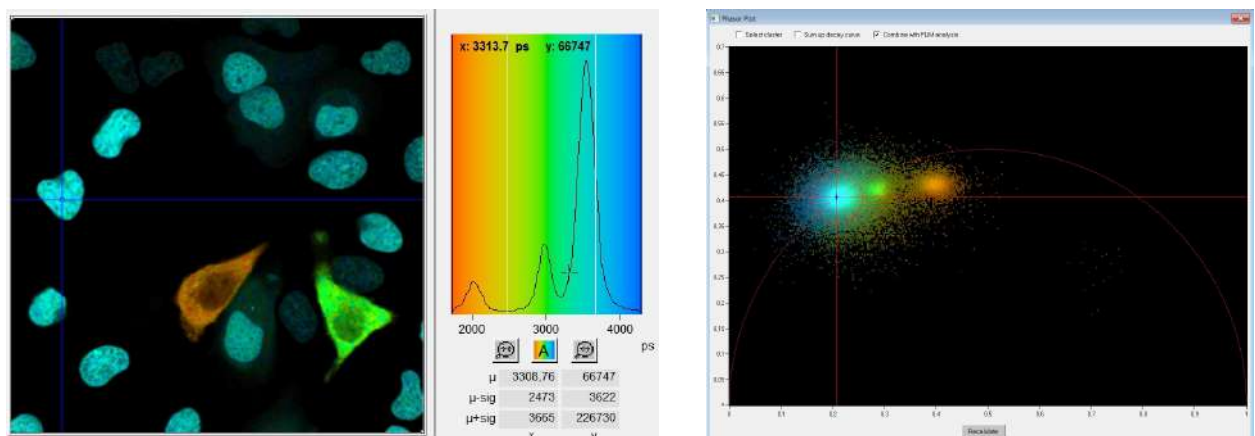


Fig. 35: Left: Lifetime image and lifetime histogram. Right: Phasor plot. The clusters in the phasor plot represent pixels of different lifetime in the lifetime image. Recorded by bh Simple-Tau 152 FLIM system with Zeiss LSM 880.

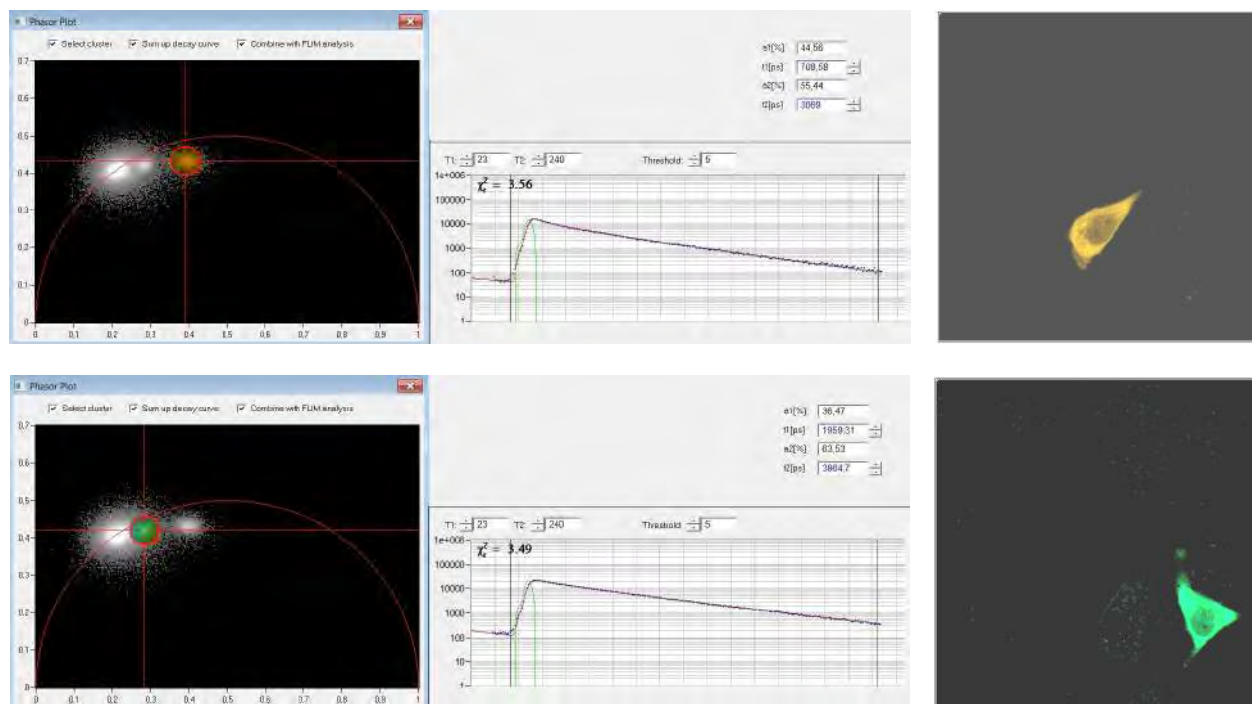


Fig. 36: Left: Selecting two different clusters in the phasor plot. Middle: Combination of the decay data of the corresponding pixels in a single decay curve. Right: Display of the pixels corresponding to the selected cluster in the phasor plot.

## IRF Definition

In a laser scanning system it is difficult, if not impossible, to measure an IRF. The excitation wavelength is usually blocked by filters, and a fluorophore with sufficiently short lifetime is not available. In multiphoton microscopes recording of an SHG signal is an option, but also this is not free of pitfalls. The SHG is emitted in forward direction and only partially scattered back into the detection beam path. This can broaden the recorded signal or distort it by reflections from the condenser lens or other elements in the transmission path of the microscope. SPCImage therefore provides several ways to avoid IRF recording altogether.

### *Auto IRF from FLIM data*

An IRF is calculated from the rising edge of the fluorescence decay curves in the FLIM data. The calculation is run on the combined data of several pixels around the brightest spot in the image. The IRF obtained this way is reasonably correct when the recorded signal is fluorescence with a lifetime several times longer than the width of the IRF. Systematic deviations may occur when SHG or an extremely fast fluorescence decay component are present, or when the rising edge is distorted by laser leakage. Nevertheless, the 'Auto IRF' works well [1], and has been used successfully since the introduction of the first SPCImage version in 2000.



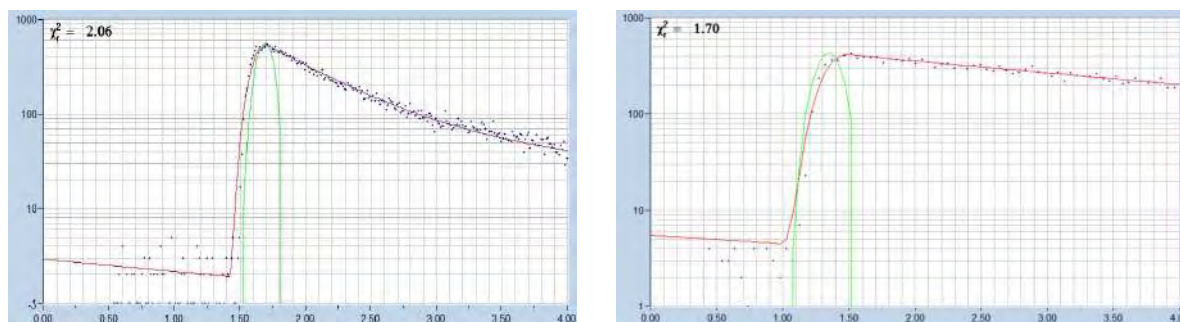


Fig. 37: Auto IRFs (green curves) calculated from FLIM data

### Rectangular IRF

A rectangular IRF can be defined manually. It is used preferentially for PLIM analysis. Excitation of PLIM occurs over the on-time of the laser in the PLIM modulation period. The effective IRF can thus be modelled by a rectangle [13].

### Synthetic IRF of the type $x \cdot e^{-x}$

A function of the type  $x \cdot e^{-x}$  closely resembles the IRF of hybrid detectors with GaAsP cathodes [1]. It also fits reasonably well to the response of many other detectors. SPCImage NG provides a function to automatically adjust the parameter  $x$  to the shape of the effective IRF. For lasers with pulse width above 1 ps the detector IRF can be convoluted with a Gaussian function of selectable width. The definitions are shown in Fig. 38, two examples for IRFs of the  $x \cdot e^{-x}$  type and the fit of fluorescence data with them are shown in Fig. 39.

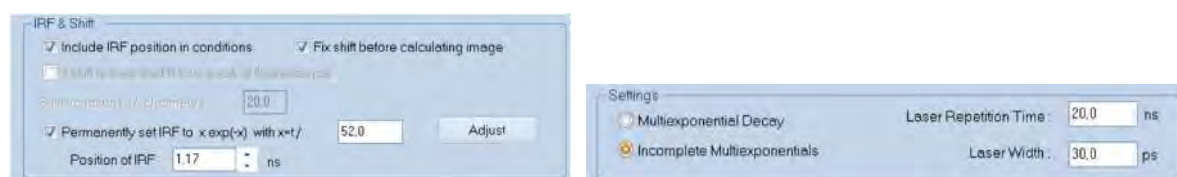


Fig. 38: Definitions for IRF of the type  $x \cdot e^{-x}$

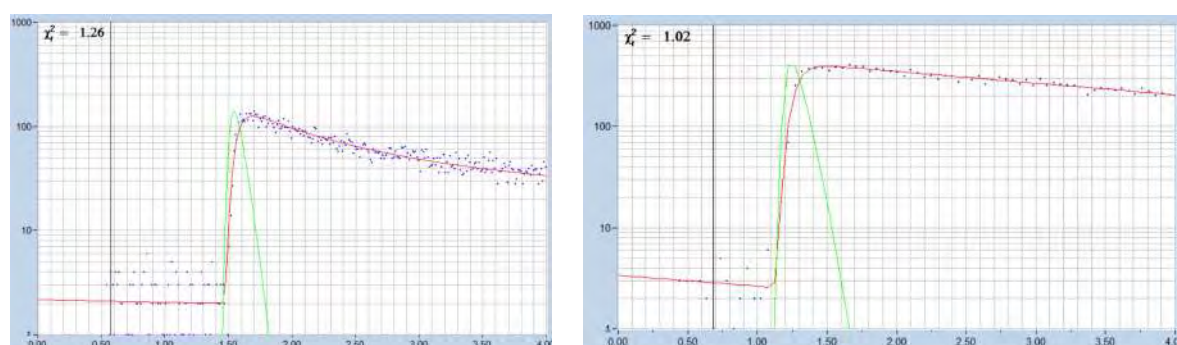


Fig. 39: IRF of the type  $x \cdot e^{-x}$ . Left: Modelled for ps diode laser plus HPM-100-06 hybrid detector. Right: IRF modelled for HPM-100-40 hybrid detector and ps diode laser.

The synthetic IRF works even for FLIM data with an extremely fast decay component. Fig. 40 shows mushroom spores with a dominating decay component on the order of 24 ps. The figure shows that a reasonable fit is obtained even for the fast peak of the decay curve.

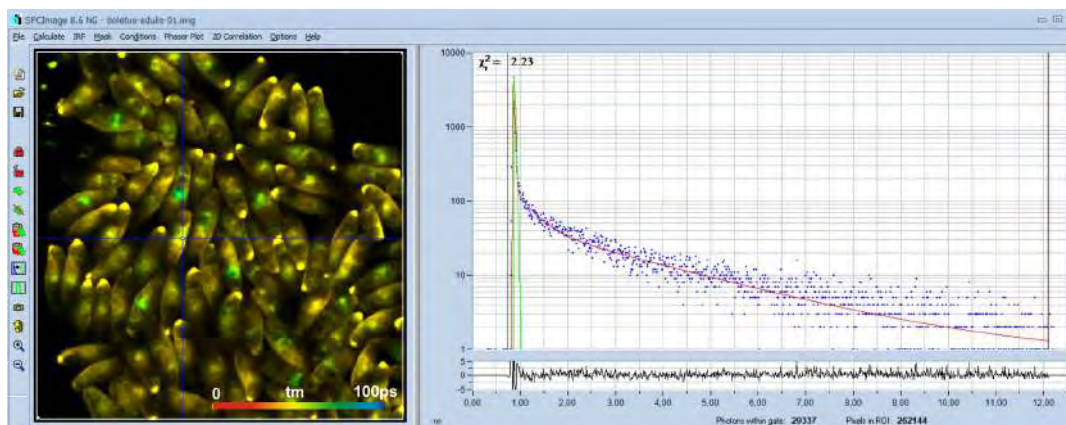


Fig. 40: Spores of *Boletus edulis*, analysed with a synthetic IRF. The amplitude-weighted lifetime is 30 ps, the lifetime of the fast component is about 24 ps. Two-photon FLIM, HPM-100-06 Ultra-fast detector, SPC-150NX TCSPC/FLIM module.

### IRF from recorded data

For standard FLIM analysis the synthetic IRF is fully capable of fitting the decay data correctly. Even for data with extremely fast decay components the synthetic IRF works reasonably well, see above, Fig. 40. Exceptions are high-resolution experiments where ultra-fast decay components with lifetimes in the range of the IRF width or shorter are to be determined. In these cases the synthetic IRF can cause ambiguity, i.e. a slightly shorter IRF width and slightly longer fast-component lifetime and vice versa can yield the same fit quality.

Ultra-fast-decay FLIM is normally performed with femtosecond excitation and two-photon excitation. Under these conditions an IRF is best obtained from SHG data. An image is recorded with the same TCSPC parameters as the FLIM data to be analysed. A suitable area in this image is selected, and the curve within this area is declared an IRF. Please see [1] for details. An IRF recorded from finely ground sugar is shown in Fig. 41. For data analysis with this IRF please see Fig. 16, page 11 of this brochure.

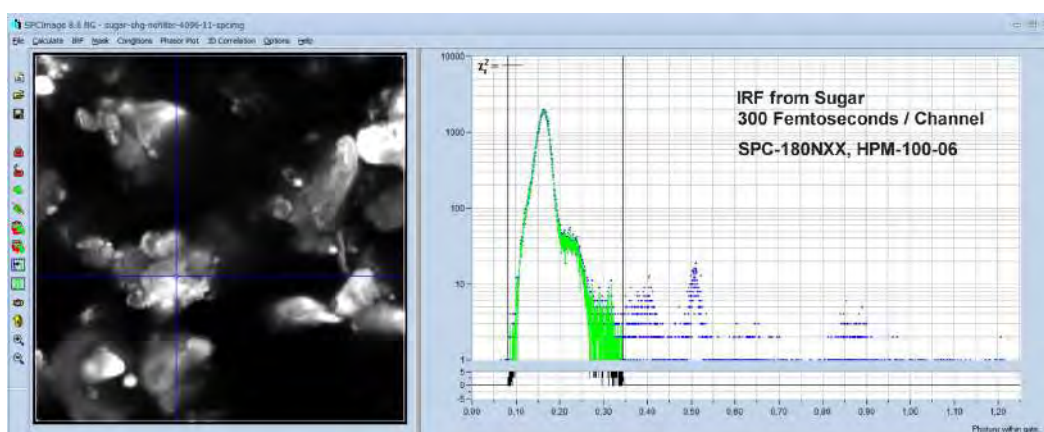


Fig. 41: IRF from SHG of powdered sugar. Ultra-high resolution, 300 femtoseconds / channel

In multiphoton FLIM the IRF can sometimes be obtained from the FLIM data themselves. These often contain a substantial amount of SHG signal. Select a region which is dominated by SHG (Fig. 42, left), declare the waveform of this area an IRF (Fig. 42, middle), and run the data analysis with this IRF (Fig. 42, right).



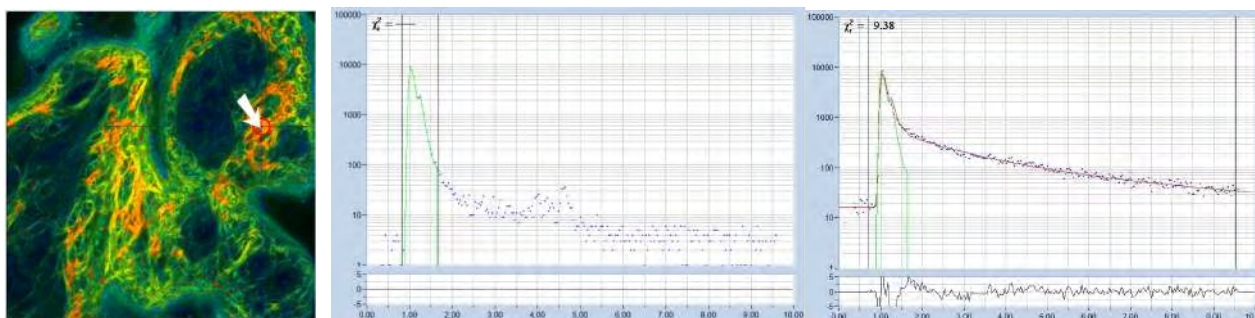


Fig. 42: IRF from multiphoton tissue FLIM data. Find a region dominated by SHG, declare the signal of this region an IRF, and analyse the image with it.

## Model Functions

### *Sum of Exponentials*

The fluorescence decay function obtained from a homogeneous population of molecules in the same environment is a single exponential. Decay functions of mixtures of different molecules or of molecules in inhomogeneous environment are sums of exponential functions of different decay time. The basic model functions used in SPCImage are therefore sums of exponential terms:

Single-exponential model:  $f(t) = e^{-t/\tau}$

Double-exponential model:  $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$

Triple-exponential model:  $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$

The models are characterised by the lifetimes of the exponential components,  $\tau$ , and the amplitudes of the exponential components,  $a$ . In principle, models with any number of exponential components can be defined. However, higher-order models become so similar in curve shape that the amplitudes and lifetimes cannot be obtained at any reasonable certainty. Therefore, FLIM analysis does not use model functions with more than three components. A typical example is shown in Fig. 43. The single exponential model does not fit the data. The double-exponential model fits well, the triple-exponential reveals a weak third component of long lifetime.

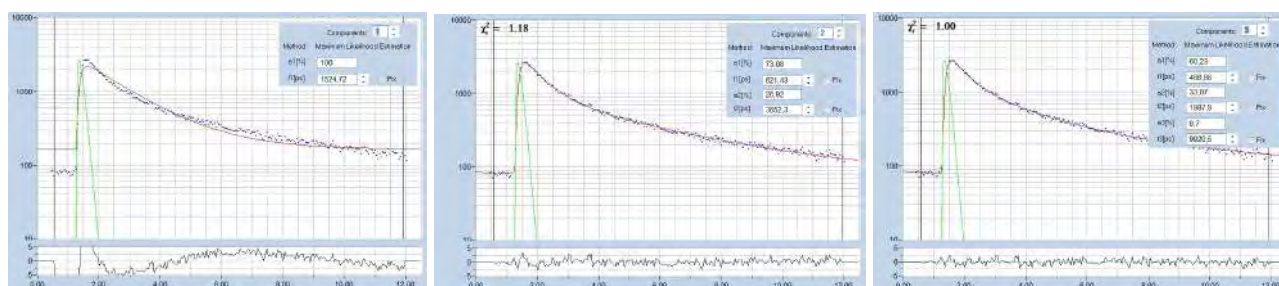


Fig. 43: Fit of decay data with a single, double, and triple-exponential model.

### *Incomplete Decay Model*

At high laser repetition rate the residual fluorescence from the previous laser pulses cannot be ignored. SPCImage has an 'Incomplete Decay' model which takes the residual fluorescence into account. Fig. 44 and Fig. 45 give a comparison of the ordinary multi-exponential model (left) and the incomplete-decay model (right). The ordinary model interprets the intensity left of the rising

edge of the decay curve as offset, the incomplete-decay model fits it correctly with fluorescence from the previous pulses. For a fluorescence decay of 2.3 ns and 80 MHz repetition rate the difference between the two models is still small, see Fig. 44. However, for a lifetime of 4 ns the difference is already 14%, an error which cannot be tolerated. Please see Fig. 45, right.

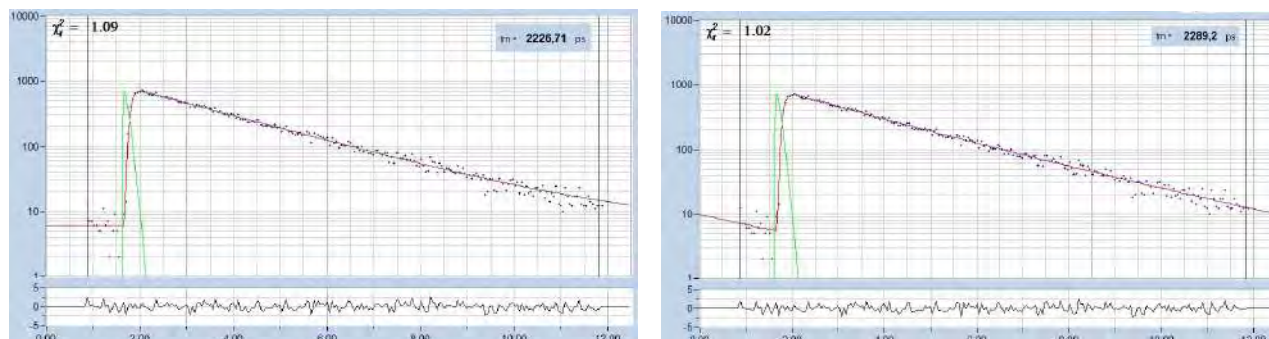


Fig. 44: Fit of the fluorescence decay of a Calcium sensor, lifetime 2.29 ns, excitation with Ti:Sa laser at 80 MHz. Left: ordinary double-exponential model. Right: Incomplete decay model.

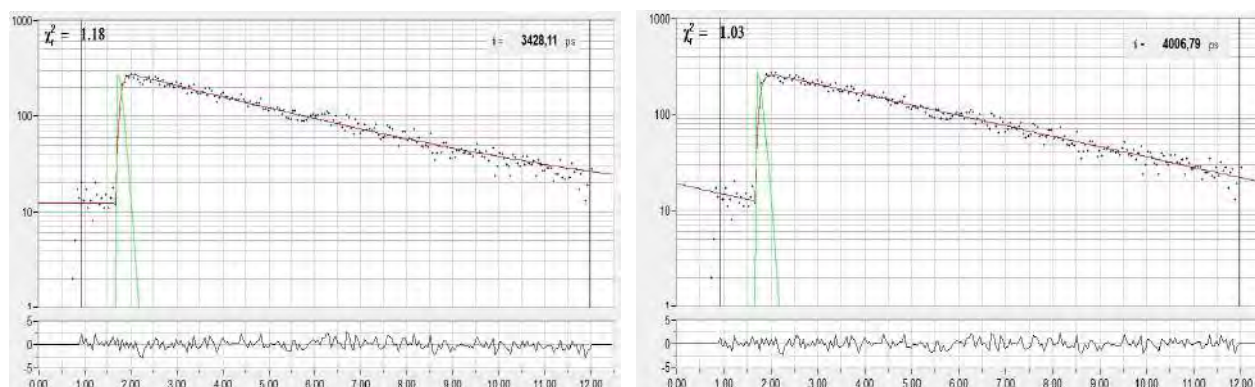


Fig. 45: Fit of the fluorescence decay of fluorescein, lifetime 4.0 ns, excited at 80 MHz. The difference between the models is 14%.

The incomplete-decay model makes it possible to record FLIM with fluorophores of more than 5 ns with multiphoton microscopes without the need of reducing the laser repetition rate. Another example is shown in Fig. 46.

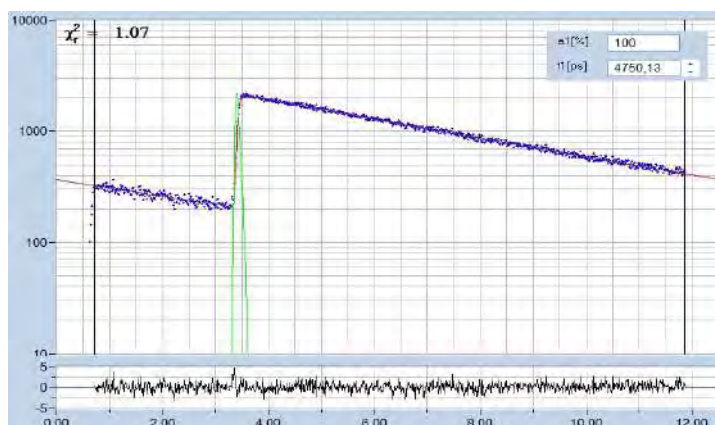


Fig. 46: Fluorescence decay of a 4.75 ns dye recorded in a two-photon microscope. Laser repetition rate 80 MHz.



### Shifted-Component Model

In clinical FLIM it happens that one or several decay components are shifted in time. A typical example is ophthalmic FLIM (FLIO) where fluorescence from the lens of the eye interferes with fluorescence of the fundus. The lens fluorescence appears about 150 ps before the fundus fluorescence. The shifted-component model takes this shift into account [14].

A demonstration is given in Fig. 47 and Fig. 48. A FLIO decay curve together with the model definition is shown in Fig. 47. A triple-exponential model is used; the lens component is modelled by the third decay component and shifted 150 ps towards earlier times. As a result, the model fits the lens component correctly, including the kink in the rising edge caused by the early arrival of the lens fluorescence.

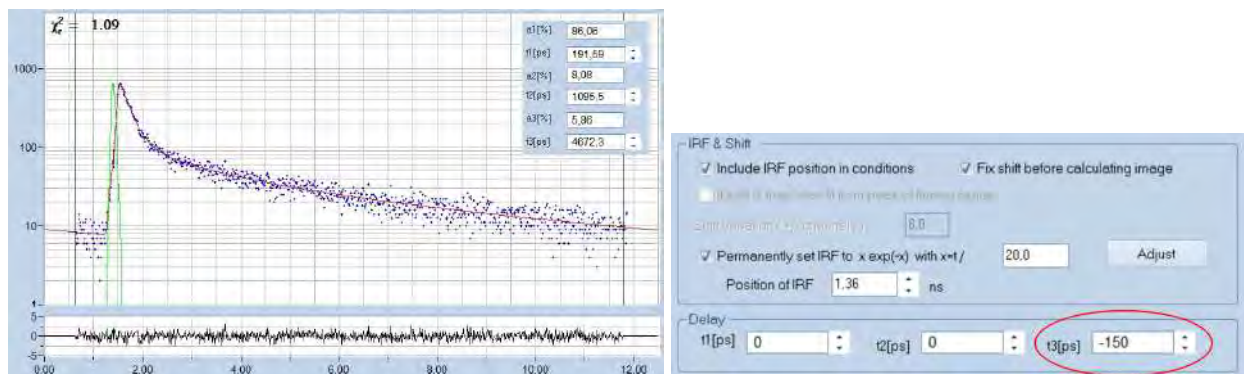


Fig. 47: Decay curve from FLIO data. Fit with shifted-component model, third decay component shifted by 150 ps to earlier time.

FLIO lifetime images obtained by the ordinary multi-exponential model and by the shifted-component model are compared in Fig. 48. For the ordinary model, the lens fluorescence causes a substantial shift of the mean lifetime,  $t_m$ , to longer values. The shifted-component model is able to deliver an image which contains only the fundus fluorescence, modelled by the components  $t_1$  and  $t_2$ . The corresponding image of the lifetime  $t_{m12}$  is shown in Fig. 48, right. It shows the correct lifetime of the fundus of the eye [14].

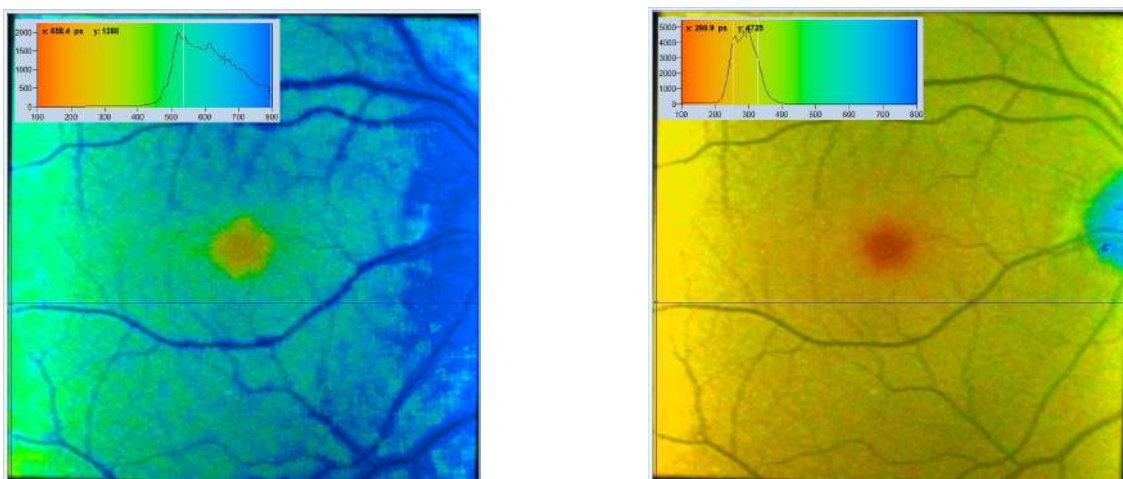


Fig. 48: Comparison of FLIO analysis with the ordinary 3-component model (left) and with the shifted-component model (right). Due to the contribution of the lens fluorescence, the ordinary image is biased towards long lifetime. The delayed-component model delivers an image that does not contain the lens fluorescence, showing the correct lifetime of the fundus of the eye.

## Summary

With the new SPCImage NG version bh's TCSPC FLIM data analysis software obtained a substantial upgrade. Obvious features are a smooth combination of phasor analysis with time-domain analysis, accuracy improvement by MLE analysis, and speed enhancement by GPU processing. Other functions, such as improved modelling of the IRF, improved decay models, and the ability to display additional combinations of multi-exponential-decay parameters further enhance the performance of SPCImage. New applications can be expected especially in the fields of live cell imaging, clinical FLIM, metabolic FLIM, ultra-fast FLIM, and FLIM of dynamic processes in live systems.

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