SPCImage Next Generation

FLIM Data Analysis Software
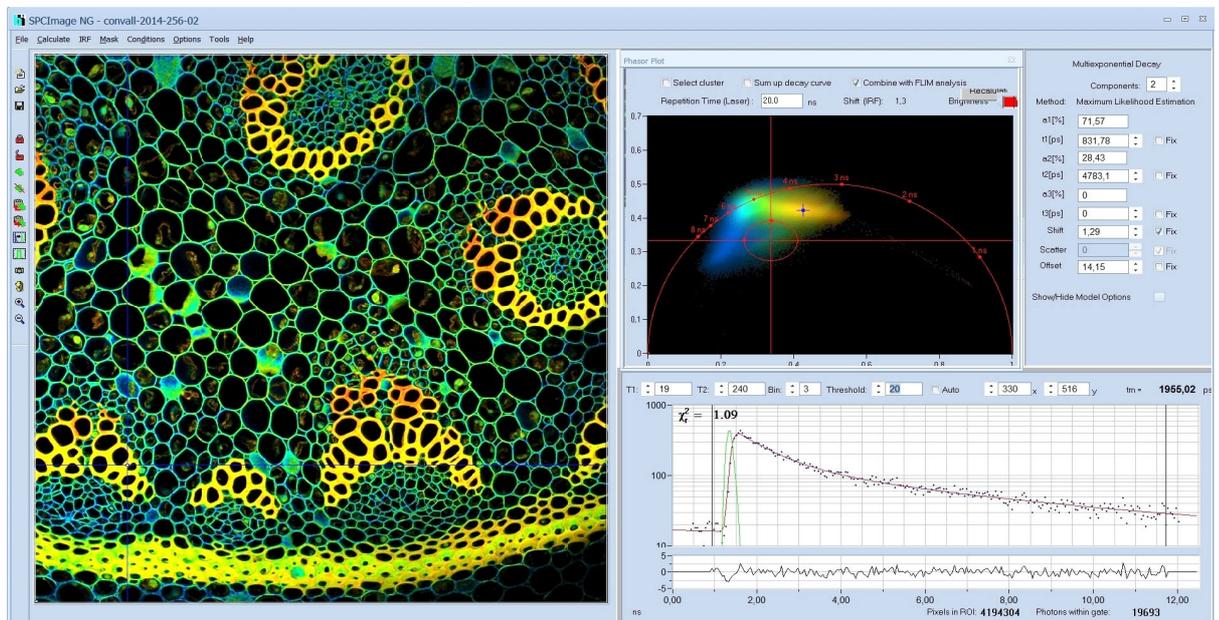
Combination of Phasor and Time-Domain Analysis

Maximum-Likelihood Estimation

GPU Processing

2019
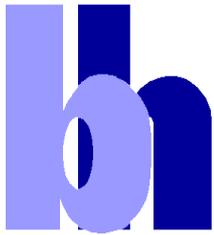
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SPCImage NG FLIM Data Analysis Software

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 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, or the fluorescence-lifetime redox ratio, FLIRR. A few examples are shown in Fig. 1 through Fig. 4.
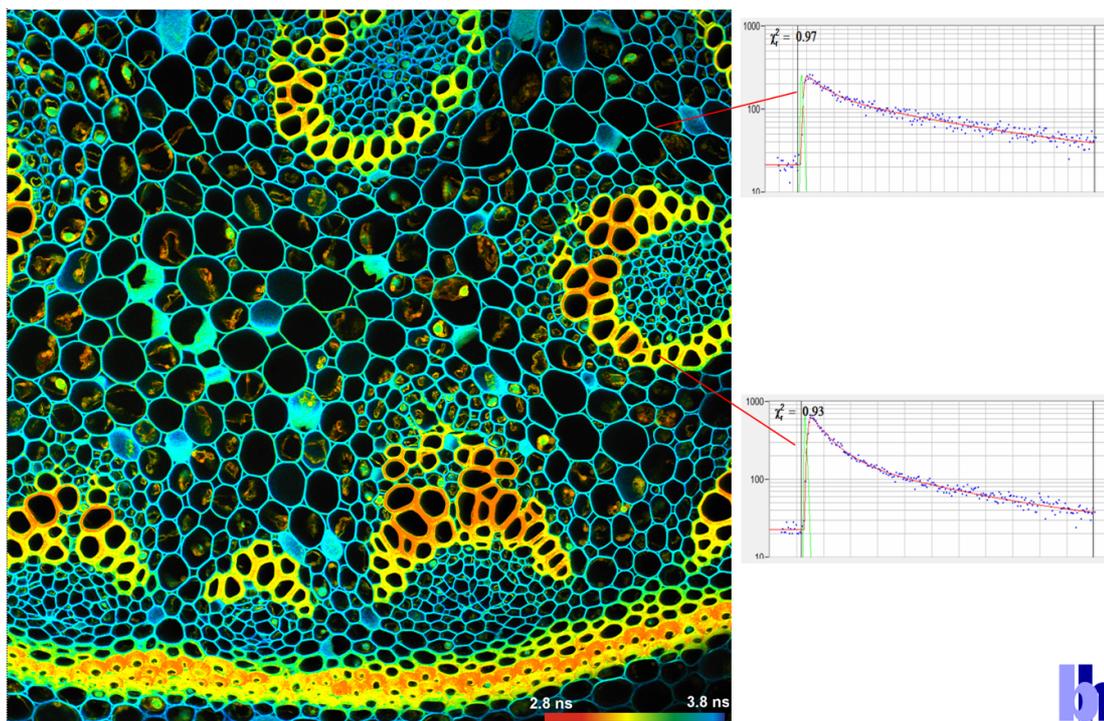
Fig. 1: Image of the amplitude-weighted lifetime, tm, of a double-exponential decay. Right: Fluorescence decay curves in selected pixels.
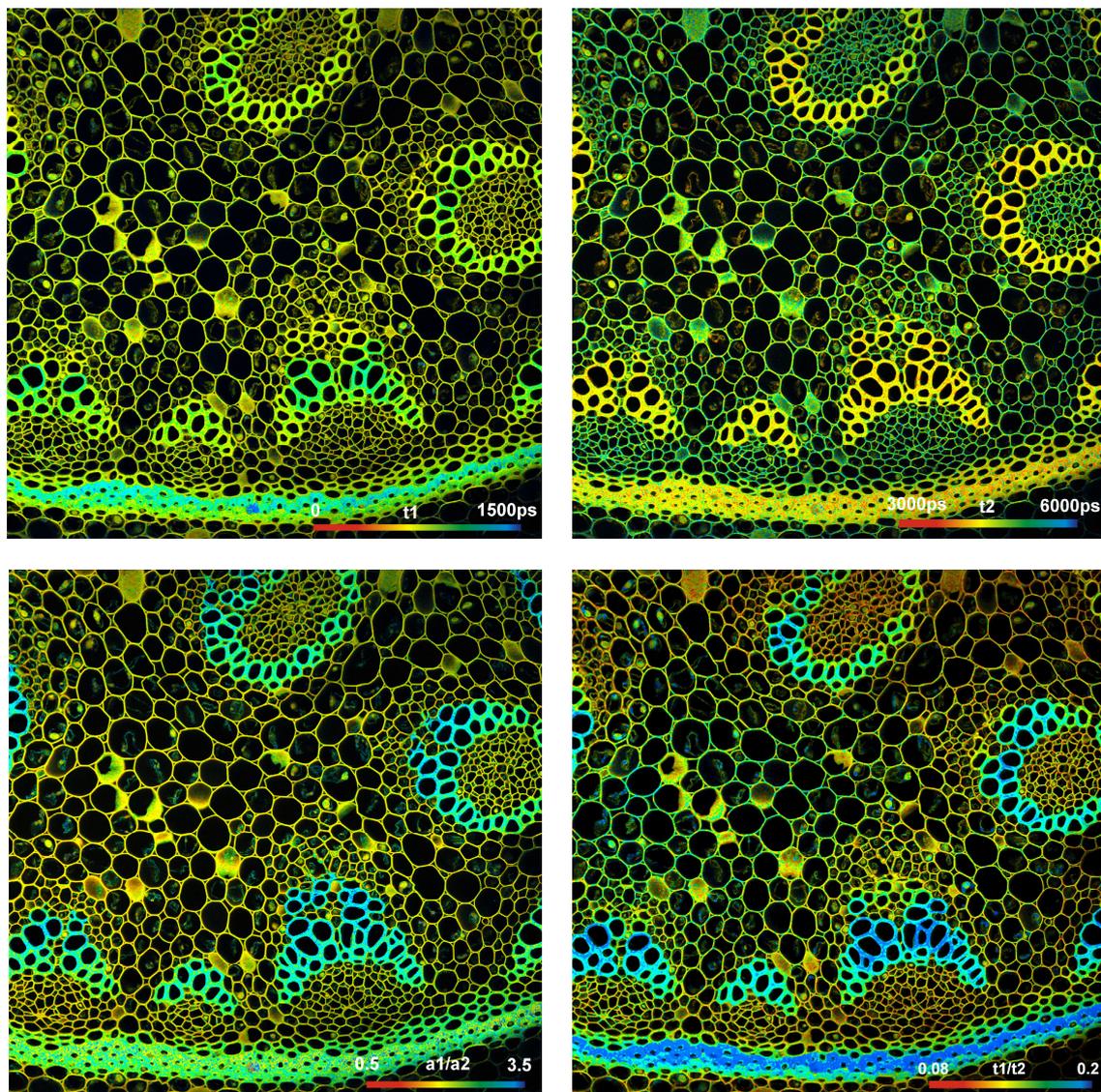
Fig. 2: Upper row: Images of the lifetimes of the fast component, t1, and the slow component, t2, of a double-exponential decay. Lower Row: Images of the amplitude ratio, a1/a2, and the lifetime ratio, t1/t2, of the fast and the slow decay component.
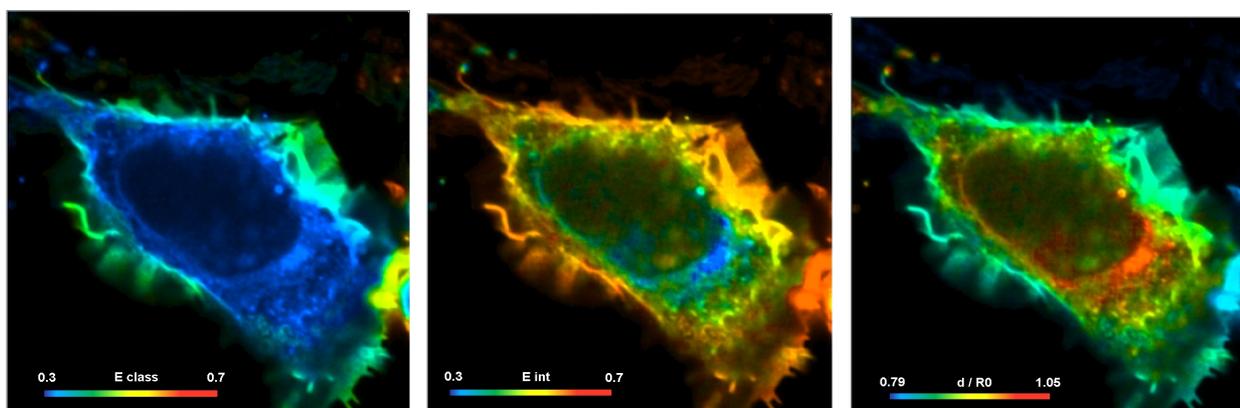
Fig. 3: Cell with interacting proteins, labelled with a FRET donor and a FRET acceptor. Left to right: Classic FRET efficiency, FRET efficiency of interacting donor fraction, FRET distance
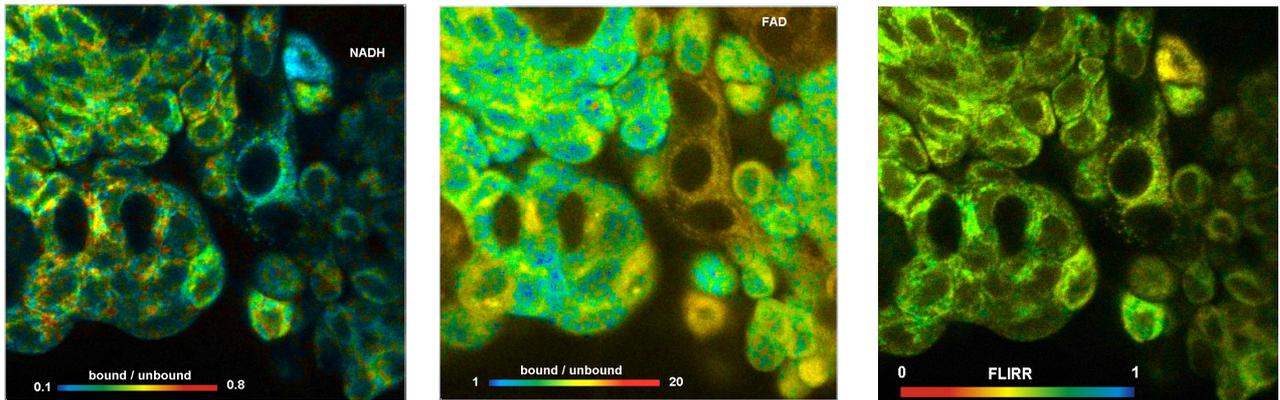
Fig. 4: Metabolic FLIM. Bound-unbound ratio of NADH, Bound/unbound ratio of FAD, Fluorescence-Lifetime Redox Ratio, FLIRR.

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 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. 5.
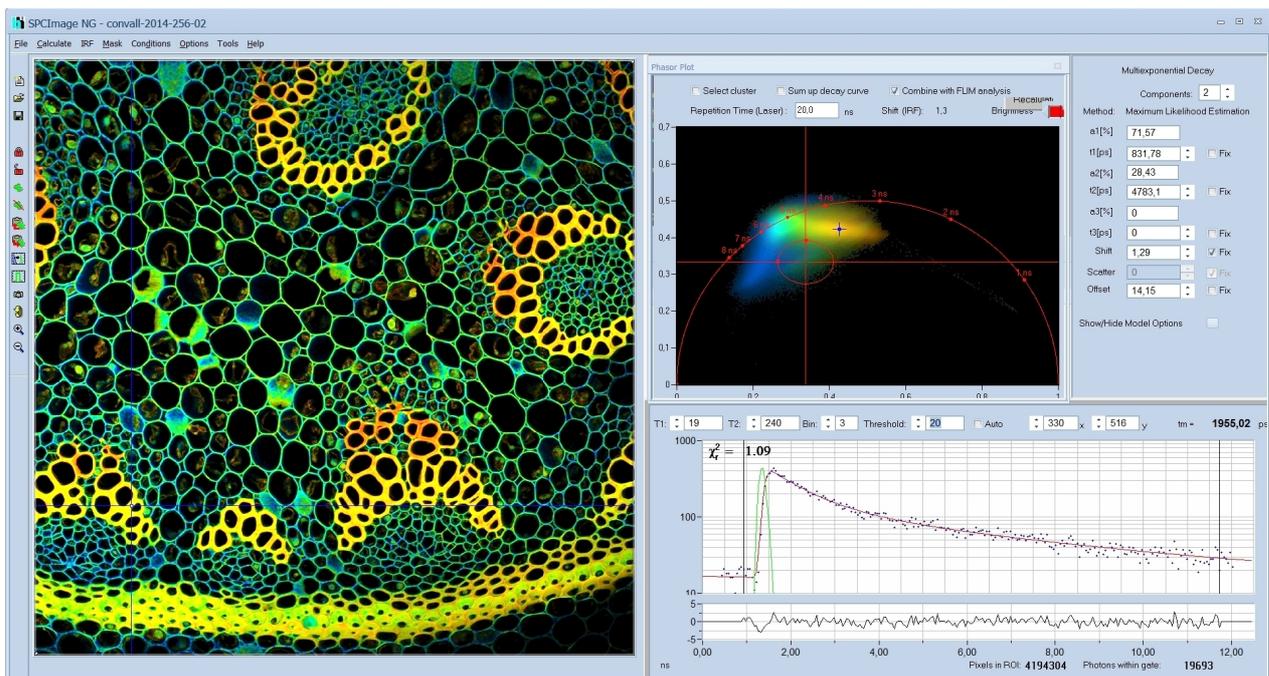
Fig. 5: Combination of time-domain analysis (left and lower right) and Phasor Plot (upper right)

In addition to the functions described above SPCImage can analyse single fluorescence decay curves, multi-wavelength FLIM data, spatial and temporal mosaic FLIM data, and simultaneously recorded FLIM/PLIM data [1]. It can extract phosphorescence intensities from normal FLIM data, extract SHG signals, and distinguish regions with single-exponential decay from regions where the decay is multi-exponential. Moreover, SPCImage is able to display time-gated images, e.g. to enhance SHG signals in FLIM images, or to reject un-depleted fluorescence in STED-FLIM images [3]. A batch-processing function and a batch export function are available for analysing a large

number of FLIM data sets automatically and to convert them into bmp or tiff images. Please see [1] 'The bh TCSPC Handbook', chapter 'SPCImage Data Analysis'.

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 fast HPM detectors [2]. Such data sets are equivalent to a stack of 1024 1-Megapixel images. Processing such amounts data by the CPU of even a fast computer can take tens of minutes. SPCImage NG therefore uses GPU (Graphics Processor Unit) processing. The image data are transferred into the GPU, which then runs the de-convolution and fit procedure for a large number of pixels in parallel. Data processing times are thus massively reduced. GPU processing is running an 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.
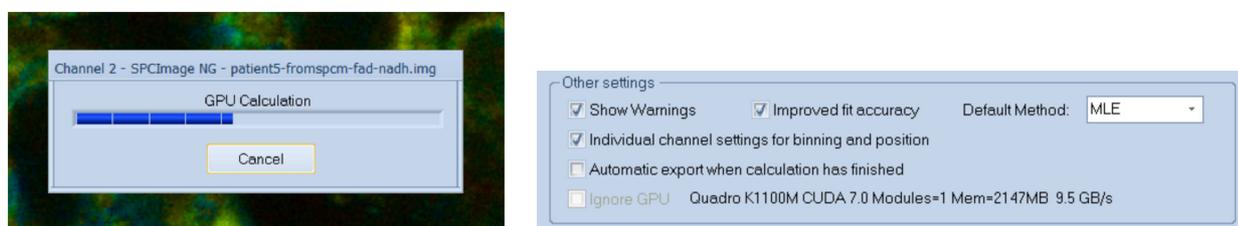
Fig. 6: Left: Progress panel during lifetime calculation, showing that GPU processing is used. Right: Part of 'Preferences' panel, indicating that a Quadro K1100M CUDA 7.0 device was found.
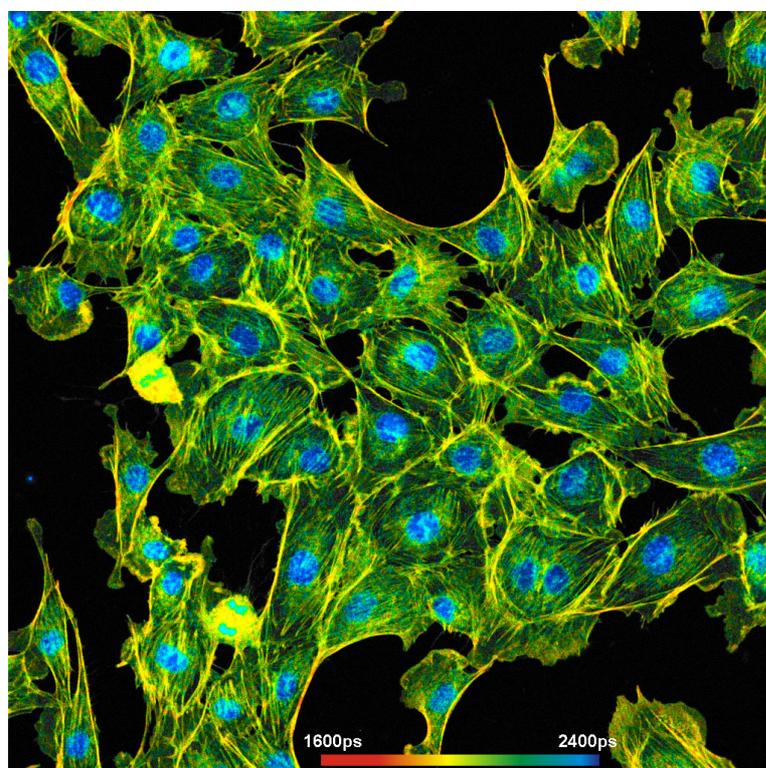
Fig. 7: A lifetime image with 1024 x 1024 pixels and 1024 time channel per pixel. The image was calculated on an NVIDIA GPU in 10 seconds. Recorded by bh FASTAC FLIM system on a Zeiss LSM 880 NLO.

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 resulting function is the 'Instrument Response Function', or IRF. The measured waveform, $f_m(t)$, is the convolution integral of the real fluorescence decay function, $f(t)$, with the IRF. 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]. There is also another complication: The measured data contain noise from the statistics of the photons, i.e. $f_m(t)$ itself is not accurately known.

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 with 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 if the image.

Maximum-Likelihood Algorithm

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 often observed for the least-square fit. Most importantly, the maximum-likelihood algorithm delivers a massively increased accuracy for the component lifetimes and amplitudes of multi-exponential decay functions. A comparison between the two algorithms is given in Fig. 8.
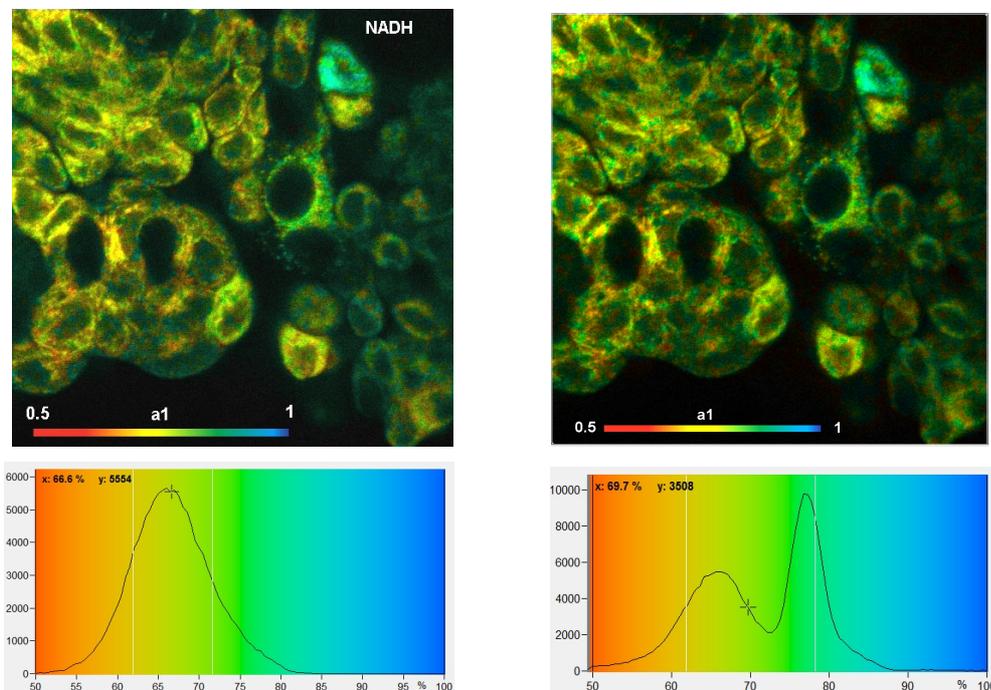
Fig. 8: Image of the amplitude, a1, of the fast decay component of an NADH FLIM image, and histograms of a1 over the pixels. Left: Least-square fit. Right: Maximum-likelihood fit. The least-square fit shows a single, broad distribution, the maximum-likelihood fit reveals two populations of different a1.

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. 9. MLE can be declared the default under 'Preferences', Other Settings'. The least-square fit and the first-moment lifetime calculation are not running GPU processing.
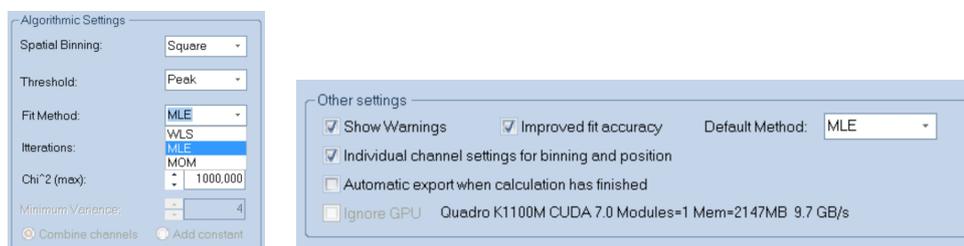
Fig. 9: Selecting MLE under Model, Algorithmic Setting (left) and declaring MLE the default method under Preferences, Other Settings.

Phasor Plot

SPCImage FLIM analysis software combines time-domain multi-exponential decay analysis with a phasor plot. In the phasor plot, the decay data in the individual pixels are expressed as phase and amplitude values in a polar diagram [6]. The principle is illustrated in Fig. 10.
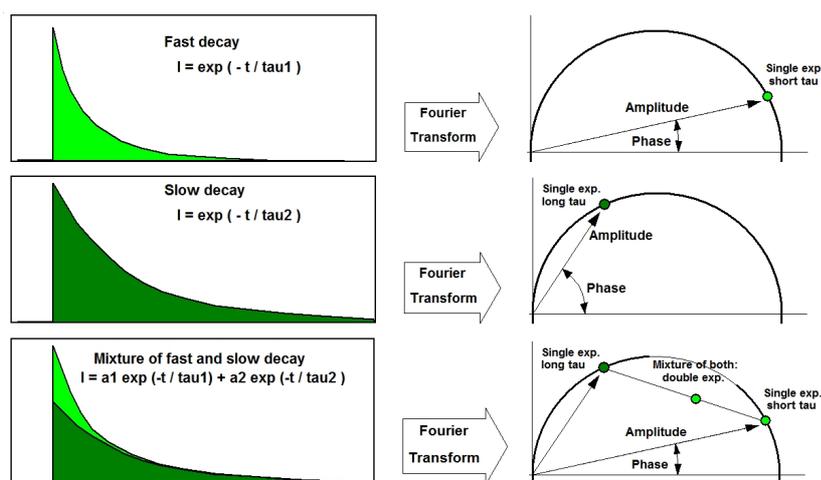
Fig. 10: Principle of phasor analysis

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. 11 and Fig. 12. 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. 12. Please see [1], 'The bh TCSPC Handbook', chapter 'SPCImage Data Analysis'.
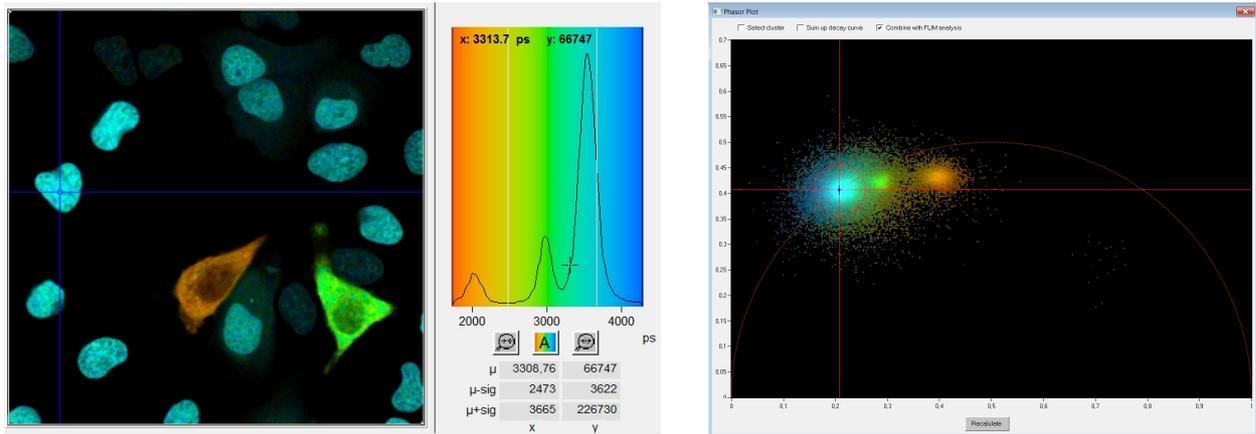
Fig. 11: Left: Lifetime image and lifetime histogram. Right: Phasor plot. The clusters in the phasor plot represent pixels of different lifetime in the lifetime image. Recorded by bh Simple-Tau 152 FLIM system with Zeiss LSM 880.
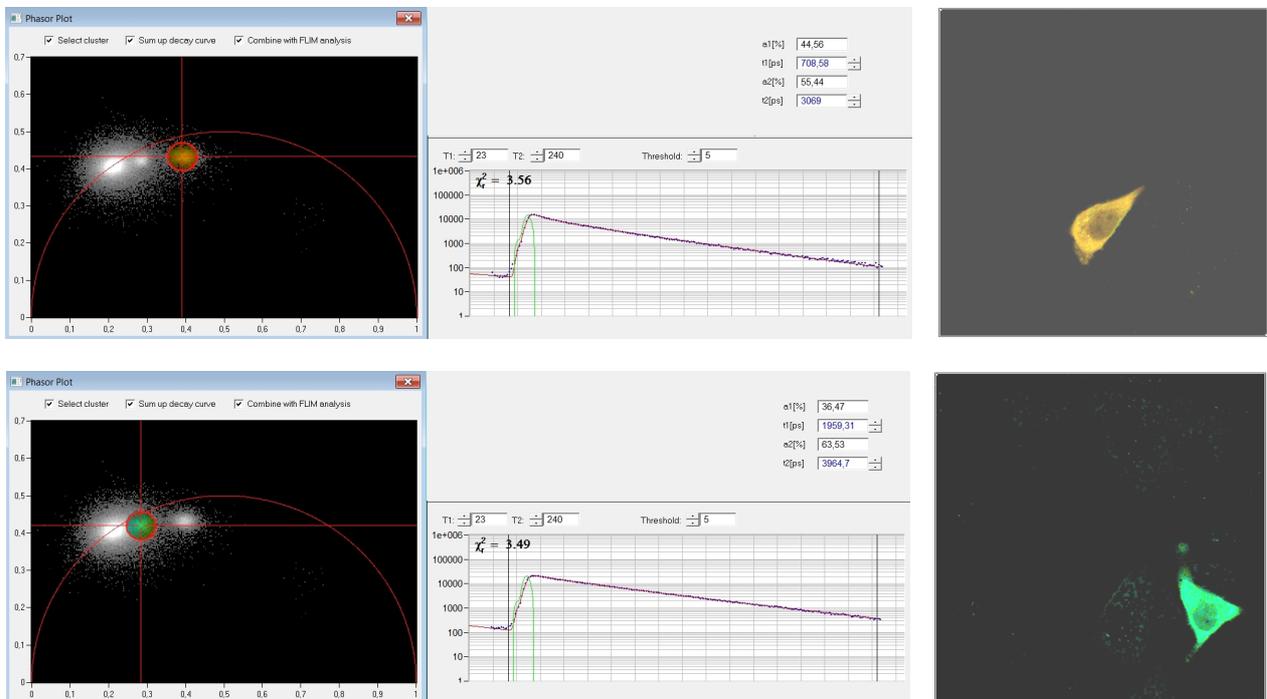
Fig. 12: Left: Selecting two different clusters in the phasor plot. Middle: Combination of the decay data of the corresponding pixels in a single decay curve. Right: Display of the pixels corresponding to the selected cluster in the phasor plot.

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. 13. 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.
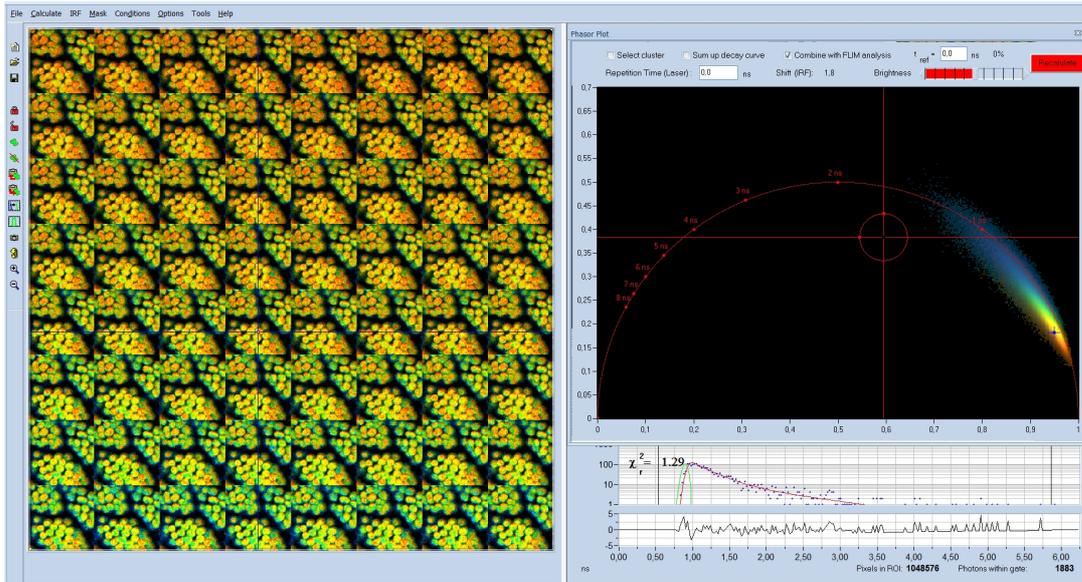
Fig. 13: Temporal-mosaic FLIM of a plant leaf. The Phasor Plot shows the trajectory the system is taking in the phasor space.

Parameter Histograms

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.

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. 14 through Fig. 16.
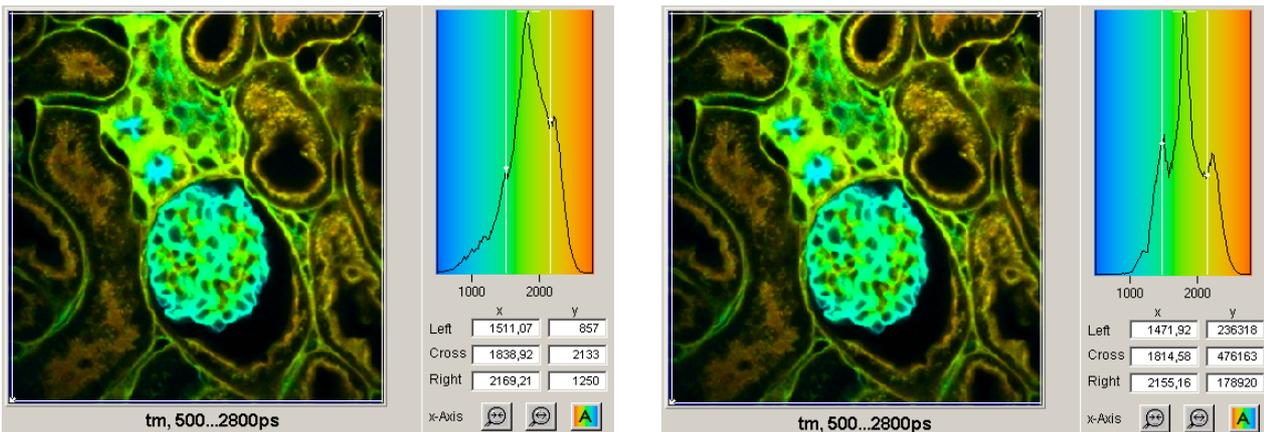
Fig. 14: Histograms of the mean (amplitude weighted) lifetime of double-exponential fit. Left: Un-weighted frequency of pixels. Right: Intensity-weighted frequency of pixels. Note that the peak at 1500ps is enhanced due to high intensities of the pixels, and peak at 2100 ps is reduced due low intensities.
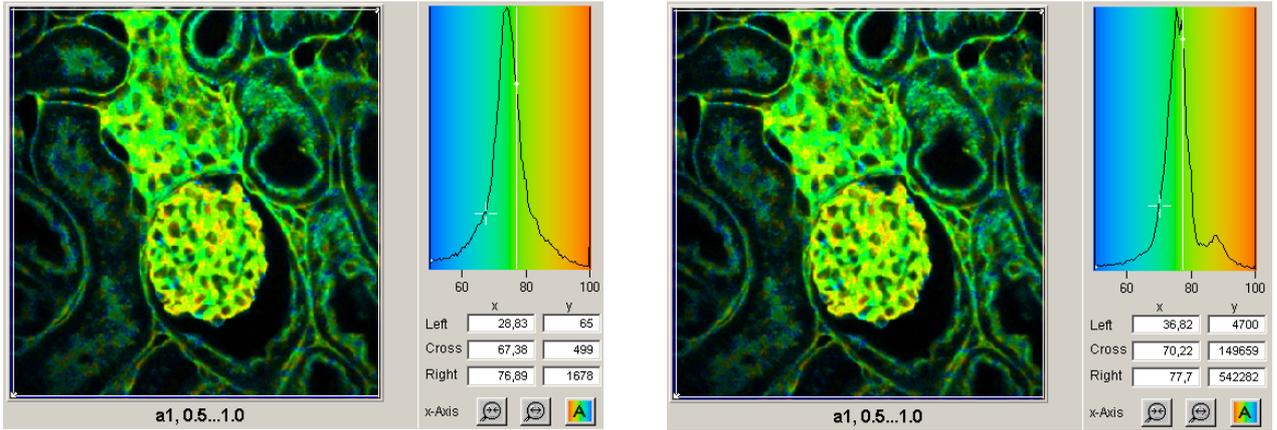
Fig. 15: Histograms of the amplitude, a1, of the fast decay component. Left: Un-weighted frequency of pixels. Right: Intensity-weighted frequency of pixels. The peak at 0.85 (85%) is only visible in the intensity-weighted histogram because it is caused by a small number of pixels that have high intensity.

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. 16.
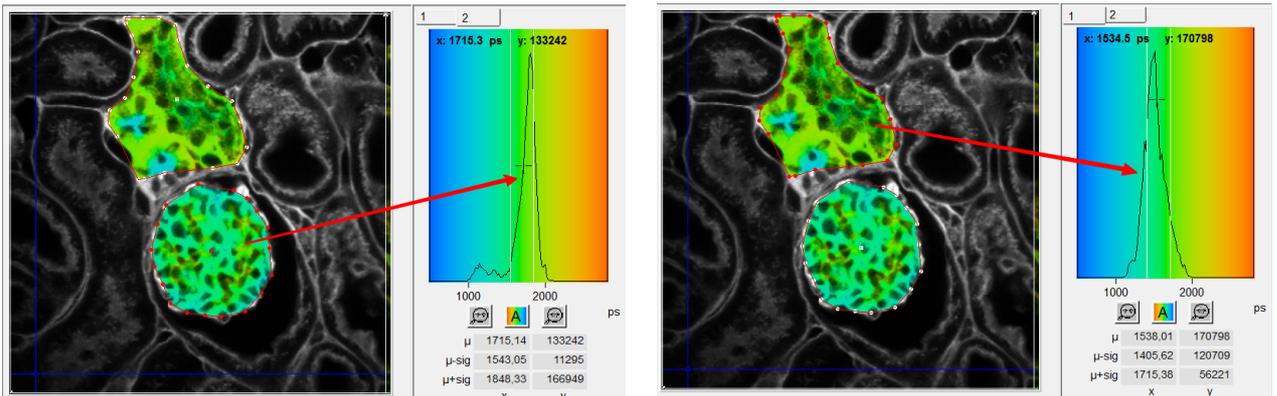
Fig. 16: Multiple ROIs, selection via the buttons on top of the histograms

2-D Histograms

2D histograms present density plots of the pixels over two selectable decay parameters. The decay parameters can be lifetimes, t1, t2, t3, or amplitudes, a1, a2, a3, of decay components, amplitude or intensity-weighted lifetimes, tm or ti, or arithmetic conjunctions of these parameters. An example is shown in Fig. 17. A histogram of the amplitude, a_1, of the fast decay component versus the amplitude-weighted lifetime, tm, has been selected. Cursors in the histogram are available to select special parameter combinations and highlight the corresponding pixels in the lifetime image.
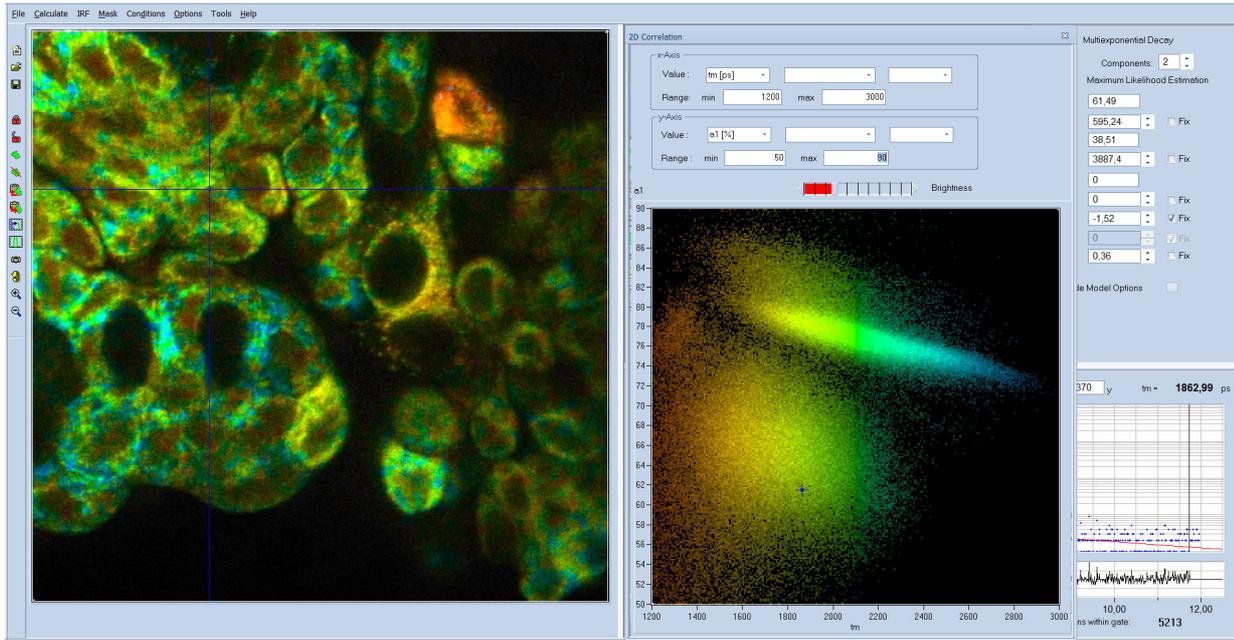
Fig. 17: 2-D histogram showing density plot of pixels over amplitude-weighted lifetime, tm, and amplitude of fast component, a1.

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. 18.
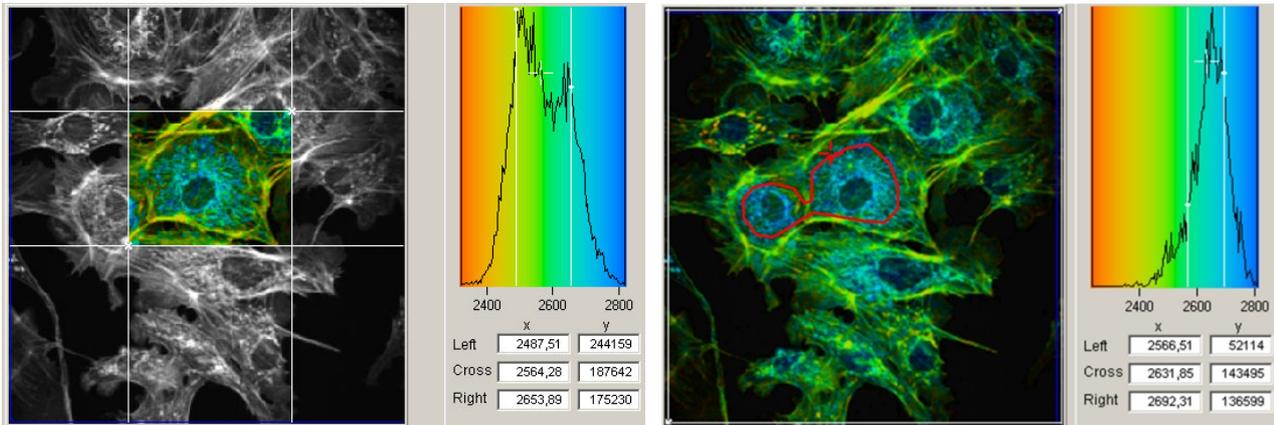
Fig. 18: ROI Definition. Left: Rectangular ROI. Right: Polygonal ROI

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. 19.
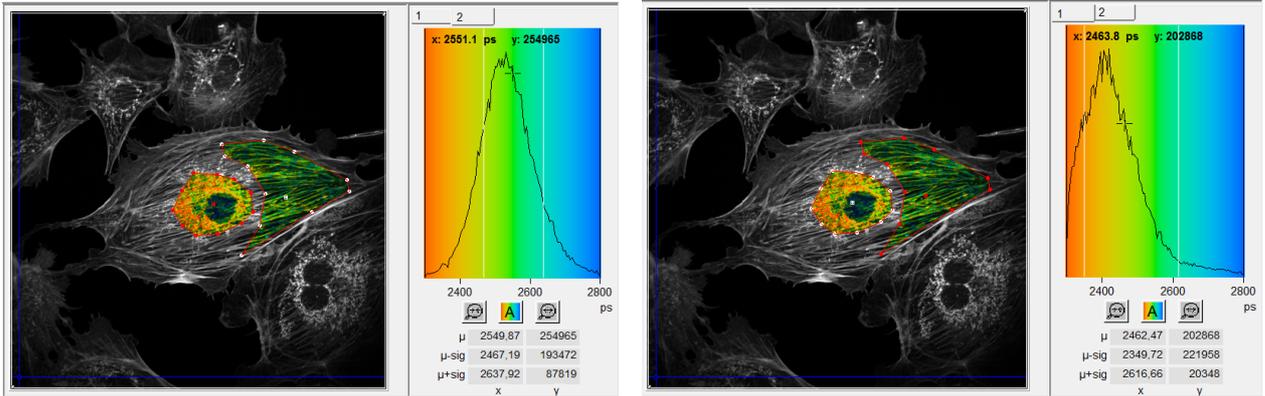
Fig. 19: Multiple ROIs, with selection of parameter histogram.

Image Segmentation from Phasor Plot

Images taken at high pixel numbers and Mosaic FLIM images can contain a large number of cells. In these cases, it is time-consuming, if not impossible, to manually select regions of interest for each of the cells in the image. SPCImage therefore provides an automatic image segmentation function via the phasor plot. Areas with different decay signature form separate clusters in the phasor plot (Fig. 20). Interesting clusters are selected by 'Select Cluster' and back-annotated in the images as shown in Fig. 21 and Fig. 22. The parameter histograms then refer to the selected areas, see Fig. 21 and Fig. 22, right. A decay curve can be calculated for the combined pixels inside the phasor cluster selected.
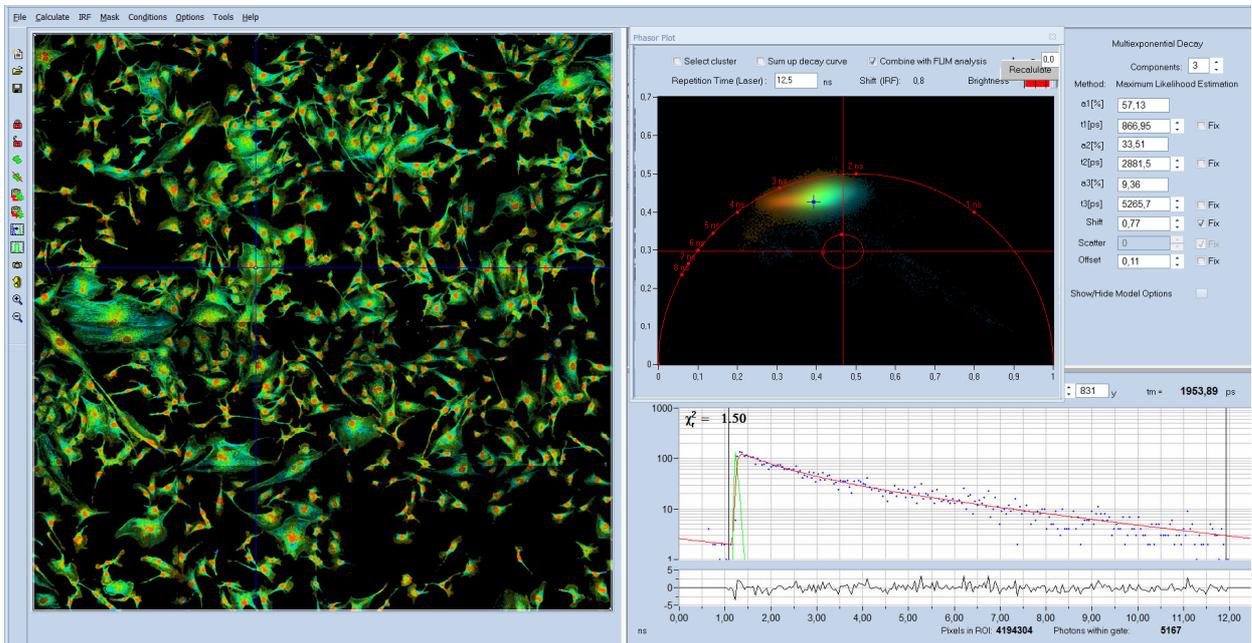
Fig. 20: Mosaic FLIM image showing a large number of cells in the image area. The phasor plot (upper right) displays distinct cluster for the nuclei and the cytoplasm.
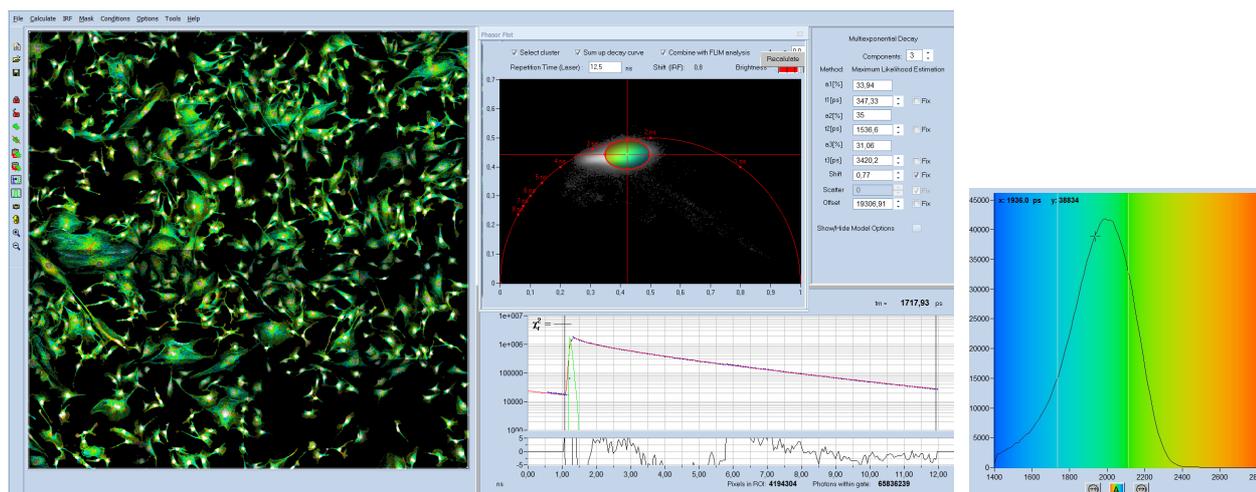
Fig. 21: The cytoplasm of the cells has been selected by the 'Select Cluster' Function. The histogram (shown right) refers to the selected pixels only. A combined decay curve for the selected pixels is displayed by the 'Sum up decay curves' function.
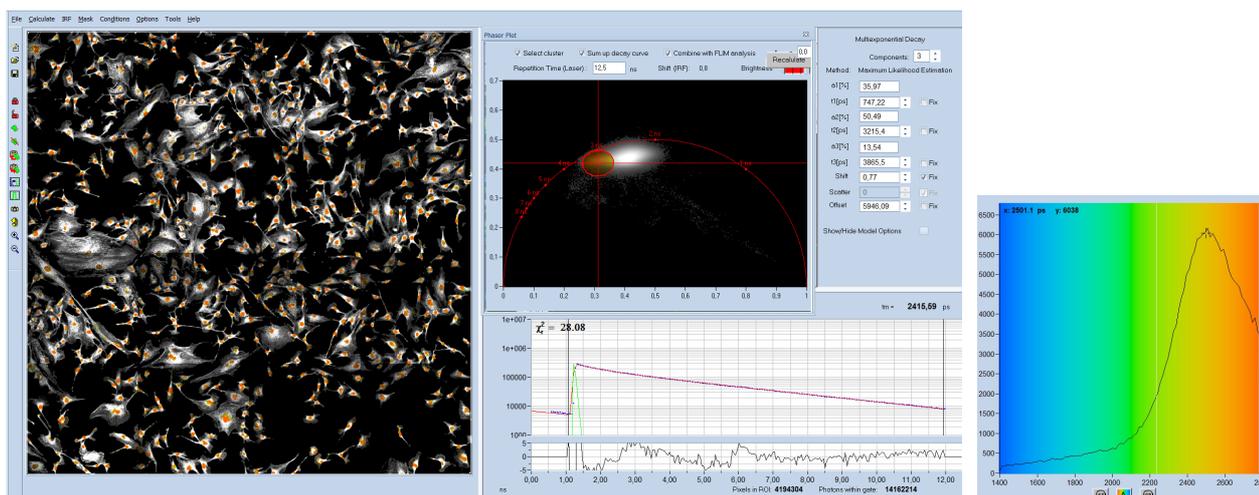
Fig. 22: The nuclei of the cells have been selected by the 'Select Cluster' Function. The histogram (shown right) refers to the selected pixels. A combined decay curve for the selected pixels is displayed by the 'Sum up decay curves' function.

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 illumination path. 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 SPCImage in 2000.
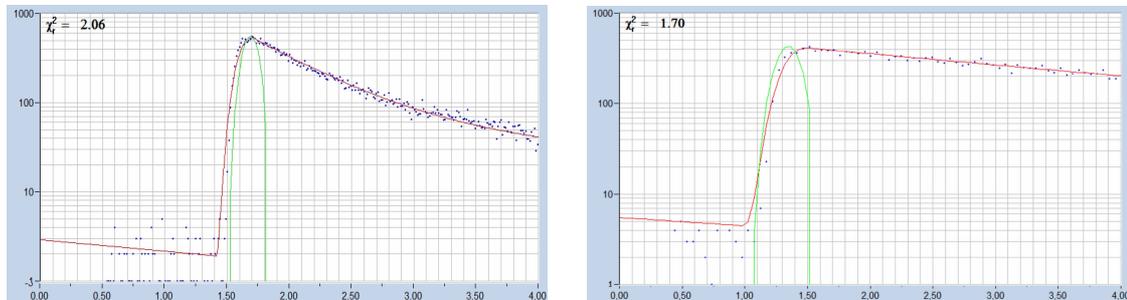
Fig. 23: Auto IRFs (green curves) calculated from FLIM data

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 [4].

IRFs of the type x·e^{-x}

A function of the type $x \cdot e^{-x}$ closely resembles the IRF of hybrid detectors with GaAsP cathodes. It also fits reasonably well to the response of 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 laser pulse of selectable width. The definitions are shown in Fig. 24, two examples for IRFs of the $x \cdot e^{-x}$ type are shown in Fig. 25.

Fig. 24: Definitions for IRF of the type $x \cdot e^{-x}$
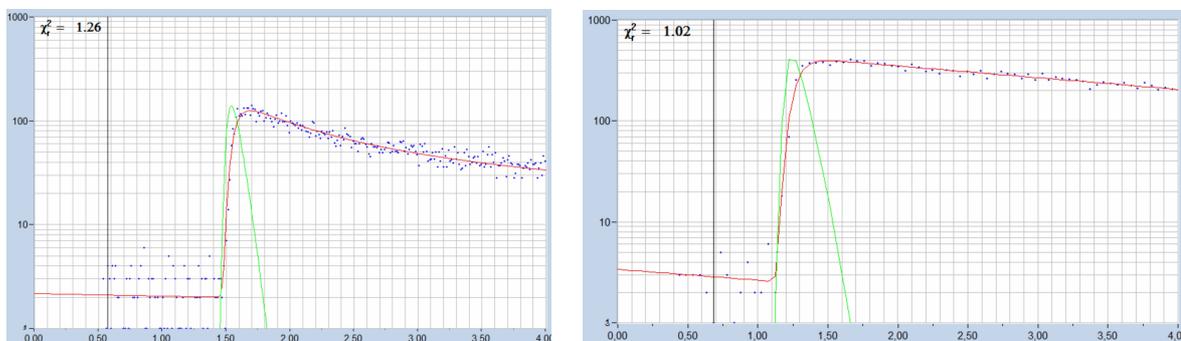
Fig. 25: IRF of the type xe^{-x}. Left: Modelled for ps diode laser plus HPM-100-06 hybrid detector. Right: IRF modelled for HPM-100-40 hybrid detector and ps diode laser.

IRF from recorded data

Although the use of recorded IRFs is not recommended for reasons described above SPCImage is able to run the FLIM analysis with real IRF data. The IRF can either be derived from an independent FLIM data file or from the FLIM data themselves. The latter option is convenient for multiphoton tissue FLIM data. These often contain a substantial amount of SHG signal. Select a region which is dominated by SHG (Fig. 26, left), declare the waveform of this area an IRF (Fig. 26, middle), and run the data analysis with this IRF (Fig. 26, right).
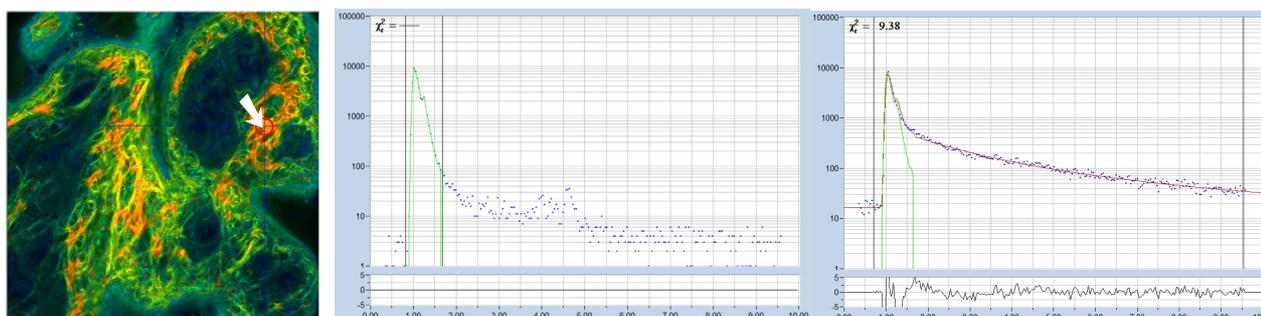
Fig. 26: IRF from multiphoton tissue FLIM data. Find a region dominated by SHG, declare the signal of this region an IRF, and analyse the image with it.

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, τ, 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. 27. 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.
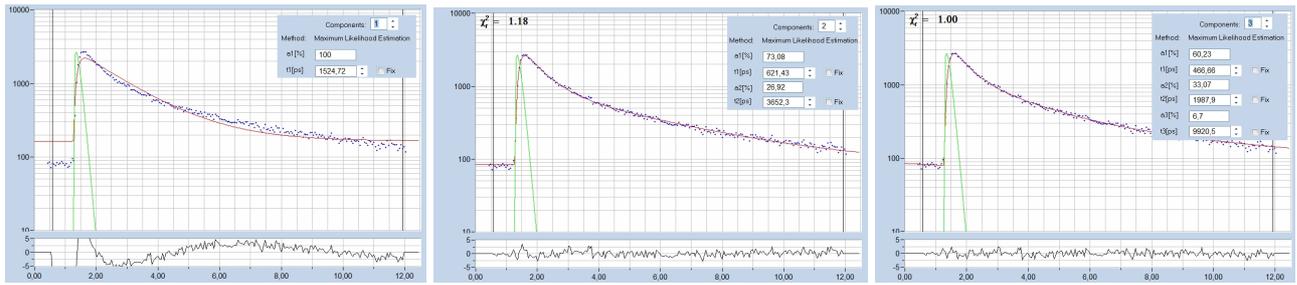
Fig. 27: Fit of decay data with a single, double, and triple-exponential model.

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. 28 gives a comparison of the ordinary multi-exponential model (left) and the incomplete-decay model. 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.
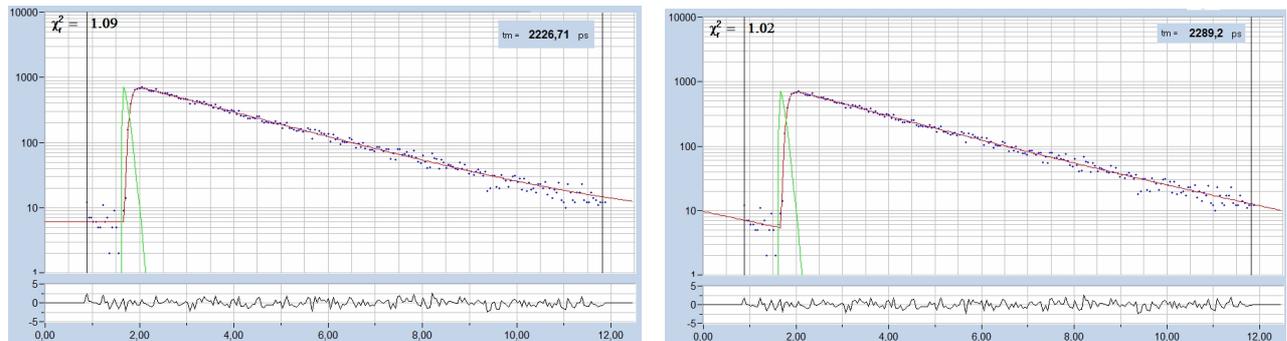
Fig. 28: Fit of the fluorescence decay of a Calcium sensor, lifetime 2.29 ns, excitation with Ti:Sa laser at 80 MHz. Left: ordinary double-exponential model. Right: Incomplete decay model.

The incomplete-decay model makes it possible to record FLIM with fluorophores of more than 10 ns with multiphoton microscopes without the need of reducing the laser repetition rate. An example is shown in Fig. 29.
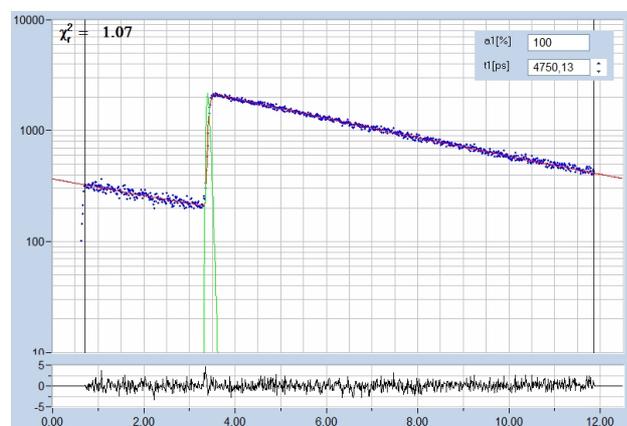
Fig. 29: Fluorescence decay of 5.75 ns dye recorded in a two-photon microscope. Laser repetition rate 80 MHz. The incomplete-decay model fits the data perfectly.

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 [5].

A demonstration is given in Fig. 30 and Fig. 31. A FLIO decay curve together with the model definition is shown in Fig. 30. 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.
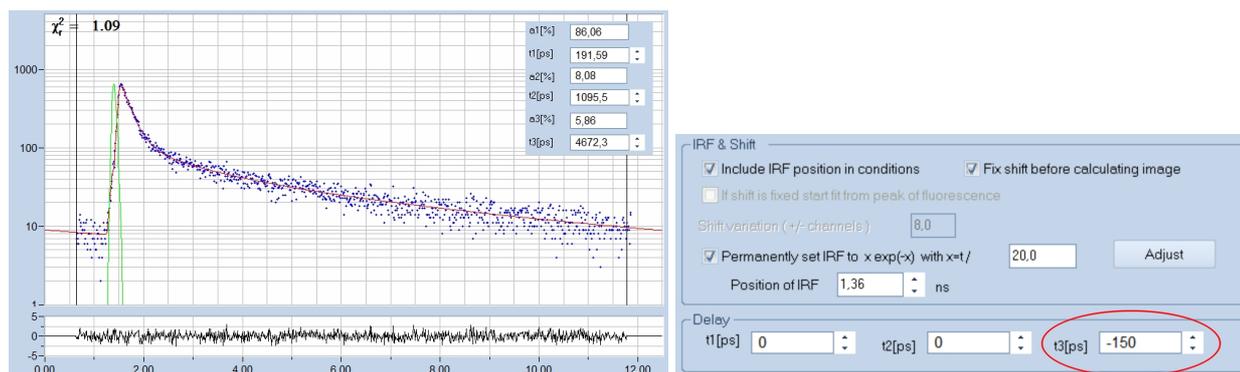
Fig. 30: Decay curve from FLIO data. Fit with shifted-component model, third decay component shifted by 150 ps to earlier time.

FLIO lifetime images obtained by the ordinary multi-exponential model and by the shifted-component model are compared in Fig. 31. For the ordinary model, the lens fluorescence causes a substantial shift of the mean lifetime, tm, to longer values. The shifted-component model is able to deliver an image which contains only the fundus fluorescence, modelled by the components t1 and t2. The corresponding image of the lifetime tm12 is shown in Fig. 31, right. It shows the correct lifetime of the fundus of the eye [5].
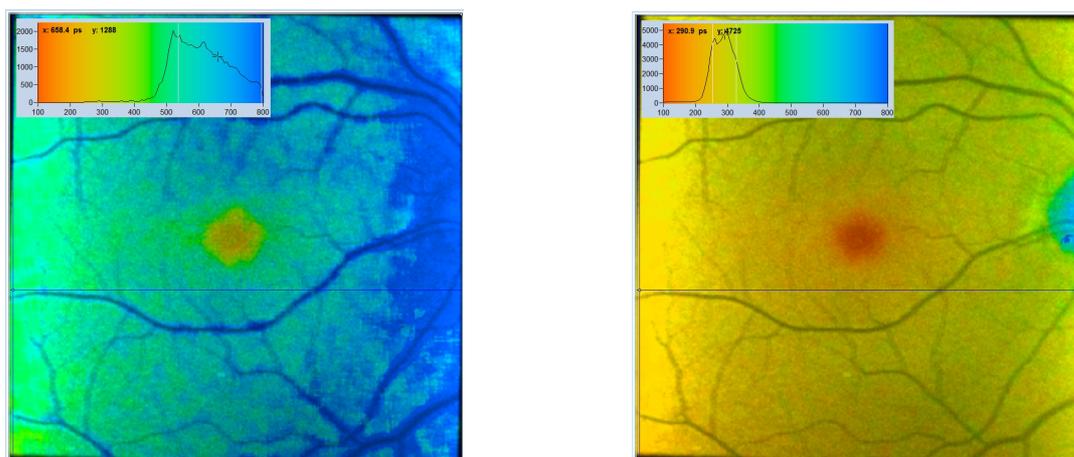
Fig. 31: Comparison of FLIO analysis with the ordinary 3-component model (left) and with the shifted-component model (right). Due to the contribution of the lens fluorescence, the ordinary image is biased towards long lifetime. The delayed-component model delivers an image that does not contain the lens 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 he performance of SPCImage. New applications can be expected especially in the fields of live cell imaging, clinical FLIM, metabolic FLIM, and FLIM of dynamic processes in live systems.

References

1. W. Becker, The bh TCSPC handbook. Becker & Hickl GmbH, available on www.becker-hickl.com
2. Becker & Hickl GmbH, Sub-20ps IRF Width from Hybrid Detectors and MCP-PMTs. Application note, available on www.becker-hickl.com
3. Becker & Hickl GmbH, bh - Abberior Combination Records STED FLIM at Megapixel Resolution. Application note, available on www.becker-hickl.com
4. Becker & Hickl GmbH, Simultaneous Phosphorescence and Fluorescence Lifetime Imaging by Multi-Dimensional TCSPC and Multi-Pulse Excitation. Application note, available on www.becker-hickl.com
5. W. Becker, A. Bergmann, L. Sauer, Shifted-Component Model Improves FLIO Data Analysis. Application note, Becker & Hickl GmbH (2019)
6. M. A. Digman, V. R. Caiolfa, M. Zamai, and E. Gratton, The phasor approach to fluorescence lifetime imaging analysis, Biophys J 94, L14-L16 (2008)

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