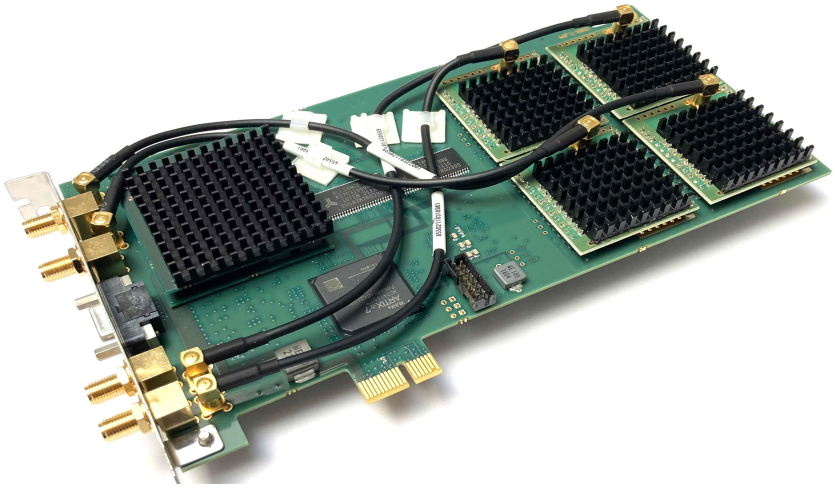
Becker & Hickl GmbH

SPC-QC-104

3-Channel TCSPC / FLIM Module
4-Channel Correlation Module

User Manual



3 Parallel TCSPC / FLIM Channels
1 Timing Reference Channel

or

4 Parallel Absolute-Timing Channels

2023

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Overview

General Features

The SPC-QC-104 TCSPC / FLIM module has three parallel TCSPC / FLIM channels or four absolute time-tagging channels on a single PCI-express board. A reduced function-set module without FLIM, the SPC-QC-004, is available as well. The SPC-QC modules feature high temporal and spatial resolution, high peak count rate, and extraordinarily high timing stability. The electrical IRF width is less than 40 ps FWHM; the timing jitter is about 17 ps RMS. The timing stability over 10 minutes is better than 5 ps RMS, including a bh BDS-SM ps diode laser and a bh HPM-100-06 detector. The peak count rate is on the order of 80 MHz per channel. The module is operated by bh's SPCM data acquisition and control software [1], running under Windows 10 and Windows 11. By using bh's multi-dimensional TCSPC process [1, 2, 3], a multitude of different operation modes is available. There are modes for standard recording of temporal waveforms of optical signals, sequential recording, time- and parameter-tag recording, FLIM, spatial and temporal mosaic FLIM, triggered accumulation of fast time series of curves and images, excitation-wavelength multiplexing, multi-wavelength imaging, and simultaneous FLIM / PLIM [1, 3, 19]. Pixel numbers in the FLIM mode can be as high as 4096 x 4096, pixel rates can be in the MHz range. Data analysis is performed by bh's SPCImage NG software [4, 5].

Basic Functions

The Classic TCSPC Mode

In the 'Single' mode the SPC-QC performs a classic TCSPC measurement [1]. Single photons of optical signals are detected, the times of the photons after the excitation pulse are determined, and histograms of the number of photons over the times of the photons are built up. The histograms resemble the waveform of the optical signal. Three curves can be recorded in parallel by the three channels of the module. The buildup of the histograms occurs in the hardware. Minimum time-channel width is 4 ps, and signals can recorded into up to 65535 time channels. The instrument response width with a fast detector is less than 50 ps, full width at half maximum (FWHM).

Recording of Fluorescence Decay Curves

The typical application of the classic TCSPC mode is recording of fluorescence-decay curves. In the simplest case the SPC-QC records one or several fluorescence decay curves. It can, however, also record other optical waveforms, such as phosphorescence decay curves, time-of-flight distributions of photons diffusing through turbid media, or pulses returned from distant targets in LIDAR applications. An example for fluorescence-decay recording is shown in Fig. 1. Several decay curves of different input channels, different routing channels, and different measurement 'pages' can be kept in the memory and displayed simultaneously.

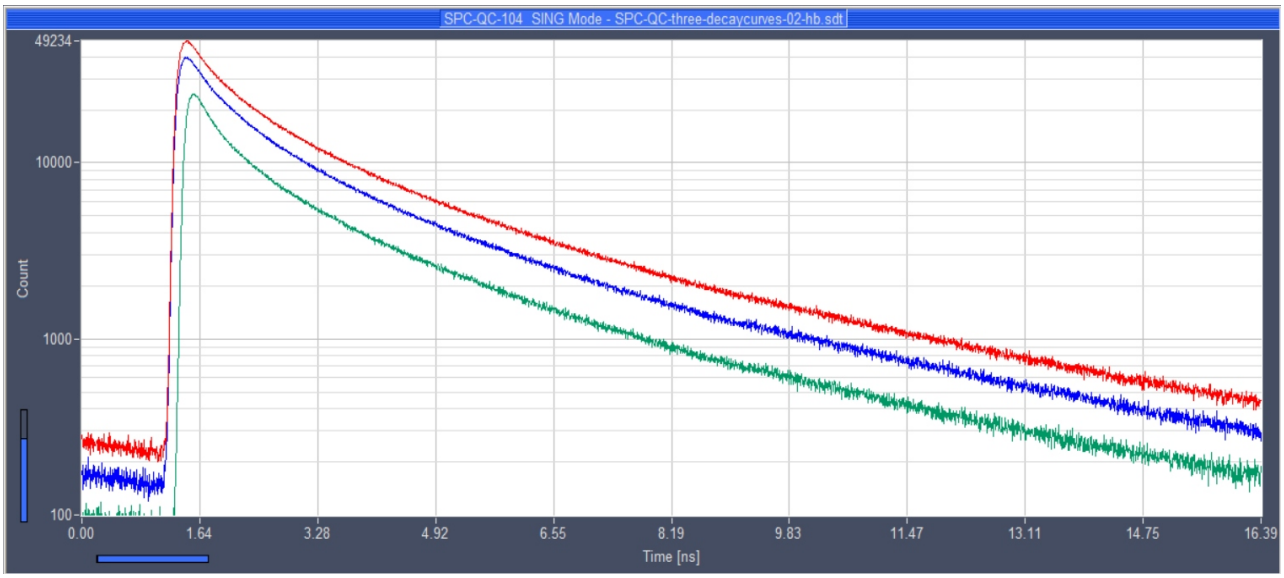


Fig. 1: Three fluorescence decay curves recorded simultaneously in the three channels of an SPC-QC device

Fluorescence Decay Analysis by SPCImage NG

For further analysis, decay curves can be directly transferred to bh's SPCImage NG data analysis software. SPCImage NG [4, 5] fits the curves by a single, double or triple-exponential decay model. For curves which do not completely decay within the pulse period of the excitation light source an 'incomplete decay option' is available. An example of decay analysis is shown in Fig. 2.

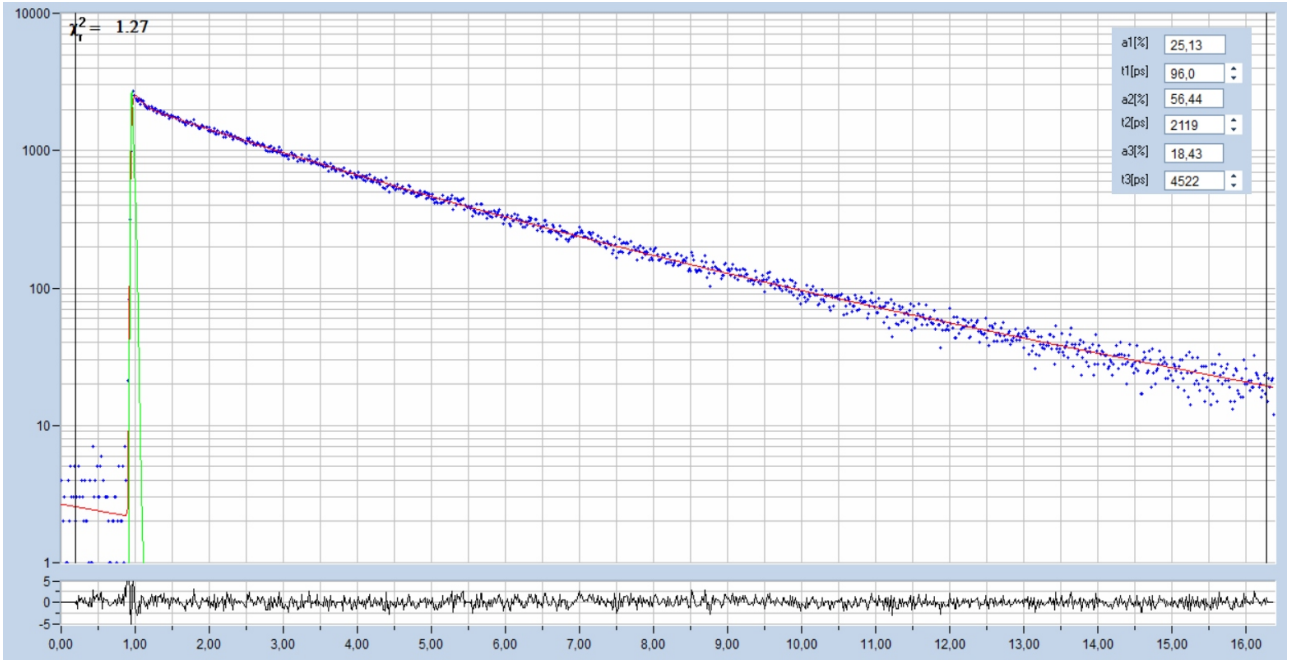


Fig. 2: Fluorescence-decay curve of FAD, 2p excitation at 780 nm, 4096 time channels, 4 ps per channel. Data analysis by SPCImage NG, triple-exponential MLE fit.

LIDAR Experiments

With its long temporal recording range and large number of time channels the SPC-QC is an excellent choice for high-resolution LIDAR applications. An example is shown in Fig. 3.

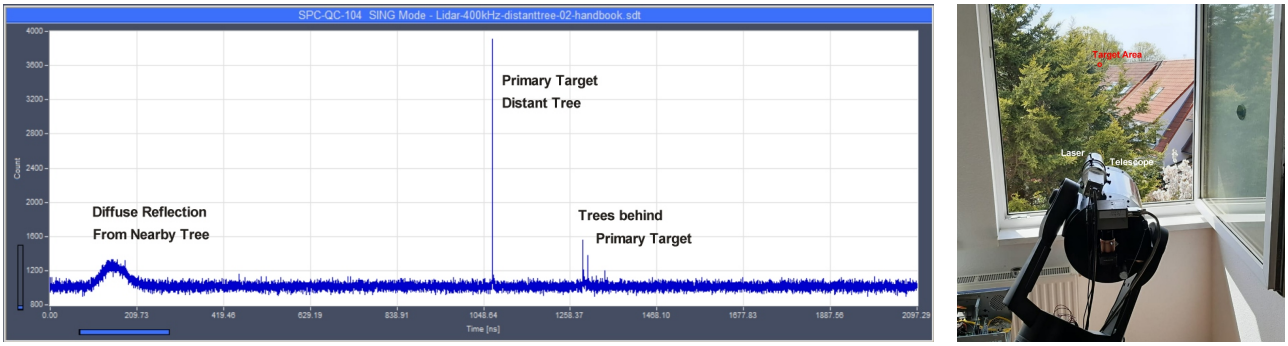


Fig. 3: LIDAR Experiment

A BDS-SM, 640 nm ps diode laser was pointed on a distant tree, the photons returned from it were collected by a 20-cm Meade LX 90 telescope. The laser was pulsed at 250 kHz, the photons were detected by a bh PMC-150 PMT module. Despite the low laser power of the diode laser (average power less than 10 µW, pulse energy <40 pJ) a clean signal was picked up from the target. The bump on the left is from the side-lobes of the laser beam diffusely scattered at a nearby tree, the peaks after the main peak are from trees behind the primary target.

Multi-Dimensional Recording Modes

Multi-Wavelength Recording

The number of curves can be extended by routing the signals of several detectors into one SPC-QC channel, or by connecting a 16 channel detector to one of the SPC-QC channels [1]. The principle is described in Fig. 24, page 22. An example is shown in Fig. 4. Eight signals of different wavelength were recorded by a PML16-GaAsP detector.

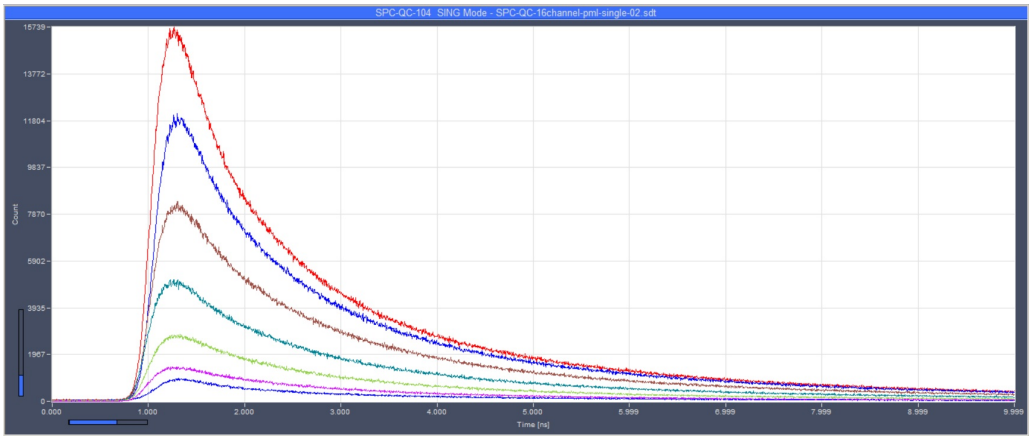


Fig. 4: Multi-wavelength detection. Decay curves at different wavelength, detected simultaneously by PML16-GaAsP detector.

TCSPC data recorded with multi-wavelength detectors can be favourably recorded in the f(t,x,y) mode of the SPCM software. Photon data are built up over one temporal coordinate, (t, the time after the

laser pulse) and a wavelength coordinate. An example for wavelength-resolved fluorescence decay recording is shown in Fig. 5.

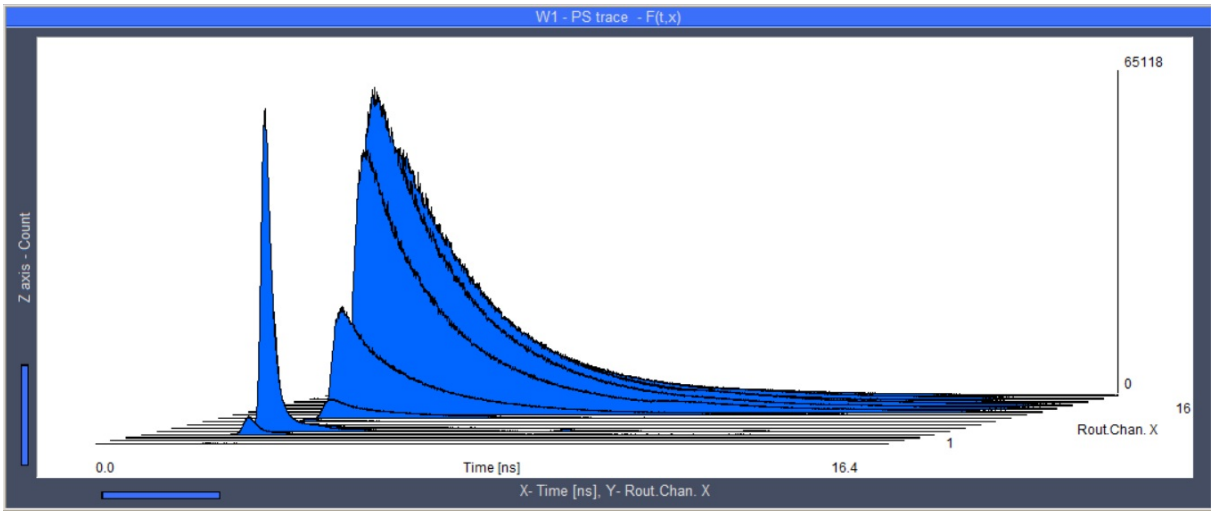


Fig. 5: Fluorescence decay in 16 wavelength channels, simultaneously recorded by PML-SPEC detector.

Excitation-Wavelength Multiplexing

Another way of recording multi-dimensional data is excitation-wavelength multiplexing [1]. Two or more lasers of different wavelength are multiplexed in time. Detected photons are tagged with the number of the laser which was active in the moment when the photon was detected. Combined with several recording channels detecting through different filters, curves of different combinations of excitation and emission wavelengths are obtained. An example is shown in Fig. 6.

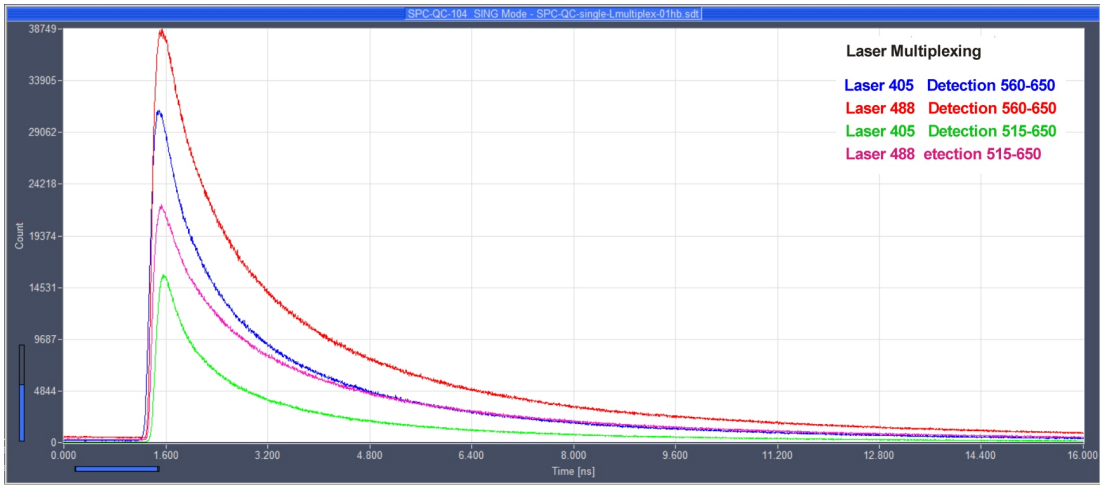


Fig. 6: Laser -wavelength multiplexing. Excitation at 405 and 488 nm, detection at 515-650 nm and 560-650 nm in channel 1 and channel 2 of SPC-QC module.

Time-Series Recording

Sequential (or time-series) Recording delivers a temporal sequence of fluorescence decay curves or other optical waveforms. The operation mode is f(t,T), but the Single Mode with page stepping (see 'Measurement-Control Functions') can be used as well. An example is shown in Fig. 7.

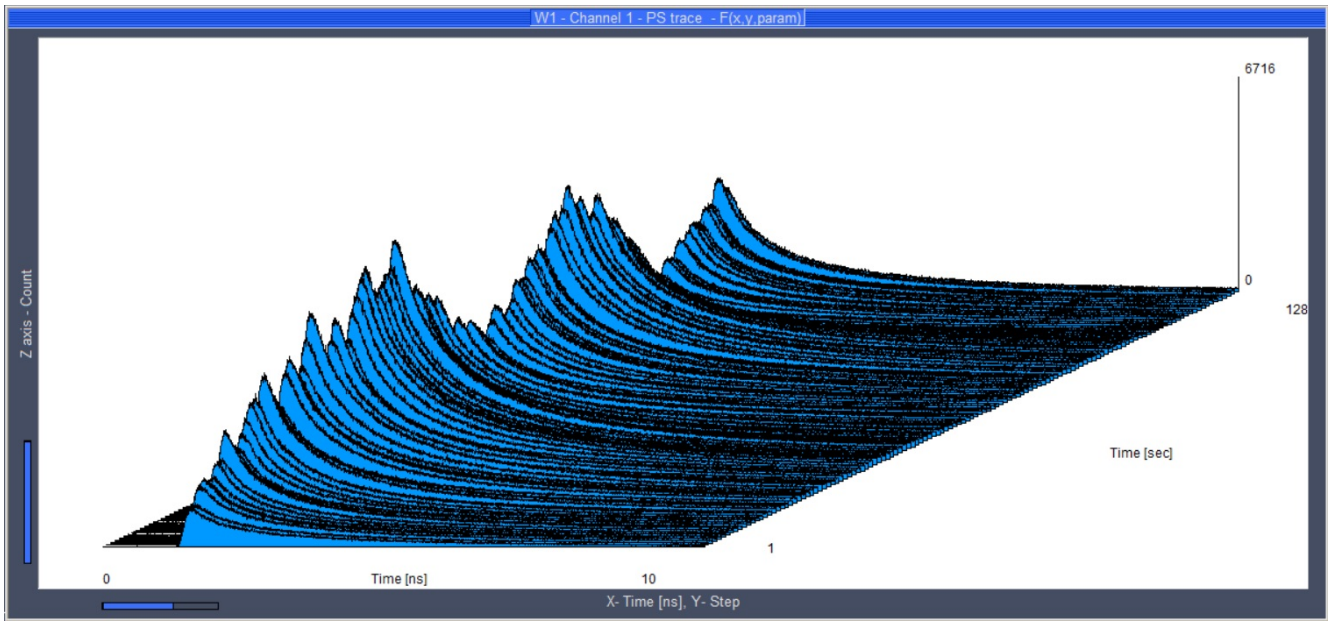


Fig. 7: Sequential recording of decay curves, 16 curves, Acquisition time 5 seconds per curve, 4096 time channels, 4 ps per channel. 3D Display function of SPCM.

Recording and Processing of Time- and Parameter-Tagged Photons

The SPC-QC module records photons in three of its four input channels and reference pulses from a pulsed laser in the fourth channel. The operation mode is 'FIFO'. Each photon is characterised by its time in the fluorescence decay curve, its time from the start of the experiment, and, if needed, by a number of other parameters which are encoded in the routing information of the photon. The data are first buffered in a FIFO (first-in-first-out) buffer. From the FIFO data, the SPC-QC creates a data stream of time- and parameter-tagged single photon data and sends them to the SPCM data acquisition software. SPCM stores these data for further analysis, and/or immediately calculates decay curves, auto- and cross-correlation functions, photon counting histograms, or time traces (or Multichannel Scaler, MCS curves) from them. Most applications are in single-particle detection, single-molecule spectroscopy, and fluorescence-correlation spectroscopy.

Fig. 8 shows the intensity trace of fluorescent nanoparticles drifting through a laser focus. When a particle moves through the focus it emits a burst of photons, as can be seen in Fig. 8.

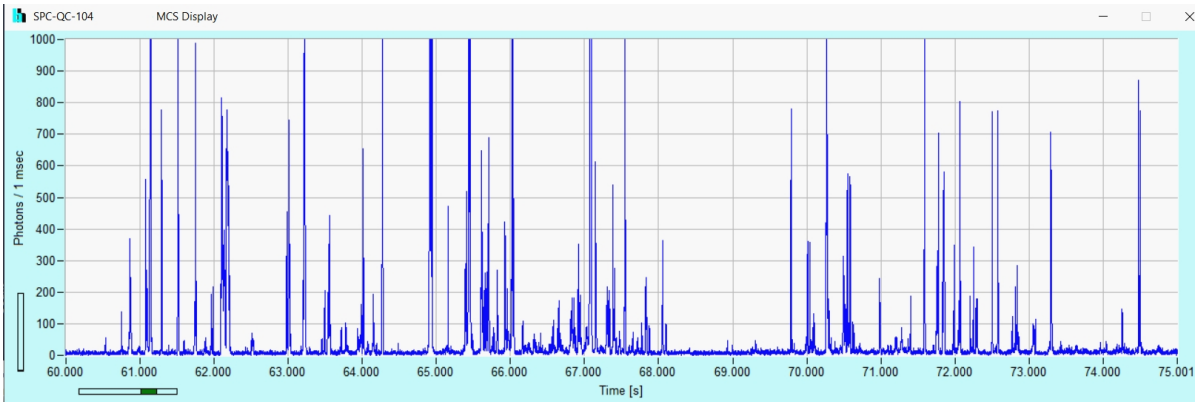


Fig. 8: Intensity trace, fluorescent nanoparticles drifting through a laser focus

The individual photon bursts can be further analysed by 'SPCDynamics' software, see Fig. 9. SPCDynamics displays fluorescence decay curves integrated over the bursts in a selected time interval, a phasor plot of the decay data of the bursts within a selected time interval, and decay curves and fluorescence lifetimes of individually selected bursts.

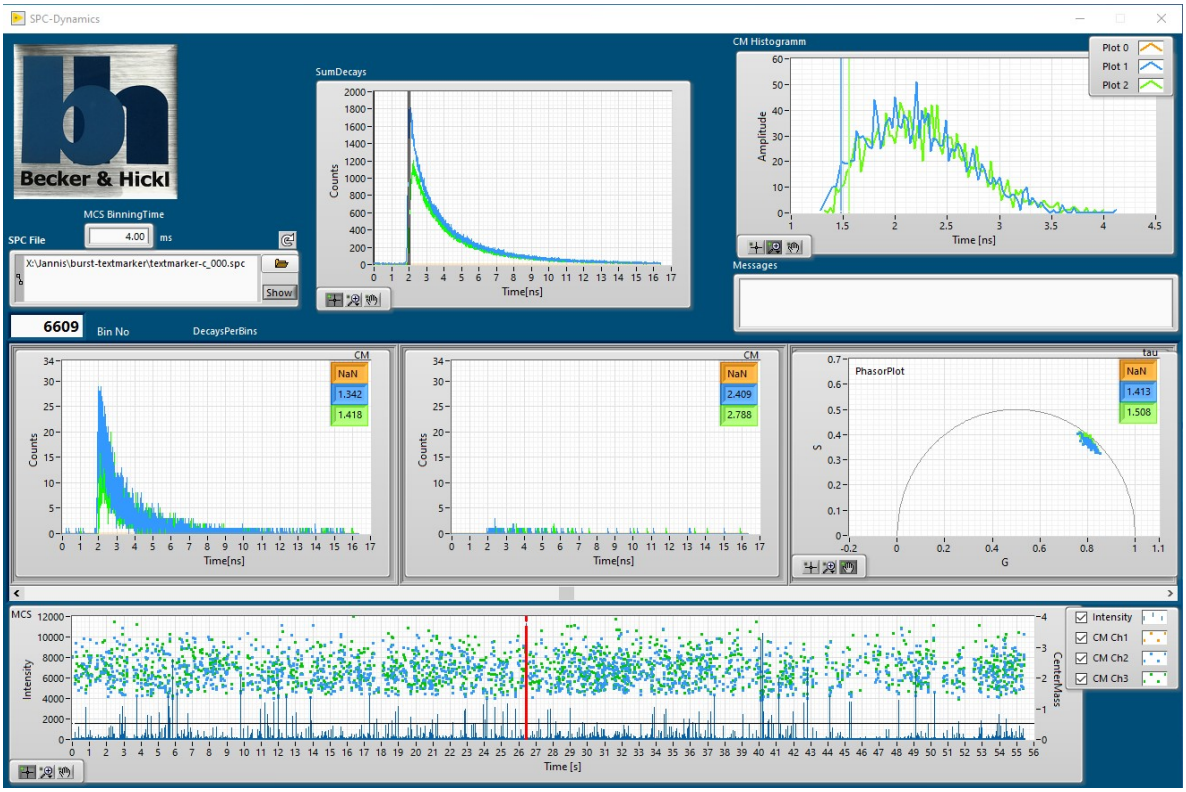


Fig. 9: Analysing photon bursts from single particles or single molecules by bh 'SPCDynamics' software

Fluorescence Correlation Spectroscopy (FCS)

An application of the SPC-QC FIFO Mode to Fluorescence-Correlation Spectroscopy (FCS) is shown in Fig. 10. A limited number of fluorophore molecules is diffusing through the laser focus. The FCS function is the autocorrelation of the intensity fluctuations over the experiment time. It contains information about the diffusion speed of the molecules, the average number of molecules in the focus, and the intrinsic brightness of the molecules [1]. FCS is especially interesting when the fluorophore molecules are attached to biomolecules. In that case, the FCS curve tells the user about the diffusion speed and thus the average size of the biomolecules. It is also possible to attach fluorophores of different emission wavelength to different biomolecules. The cross correlation between the fluctuations in the two spectral channels then tells whether the biomolecules are separate entities or chemically linked to each other.

A typical FCS result is shown in Fig. 10. A decay curve is shown upper left, an FCS curve upper right. The curve at the bottom is the intensity trace. It shows the fluctuation of the fluorescence intensity caused by random number of molecules in the laser focus.

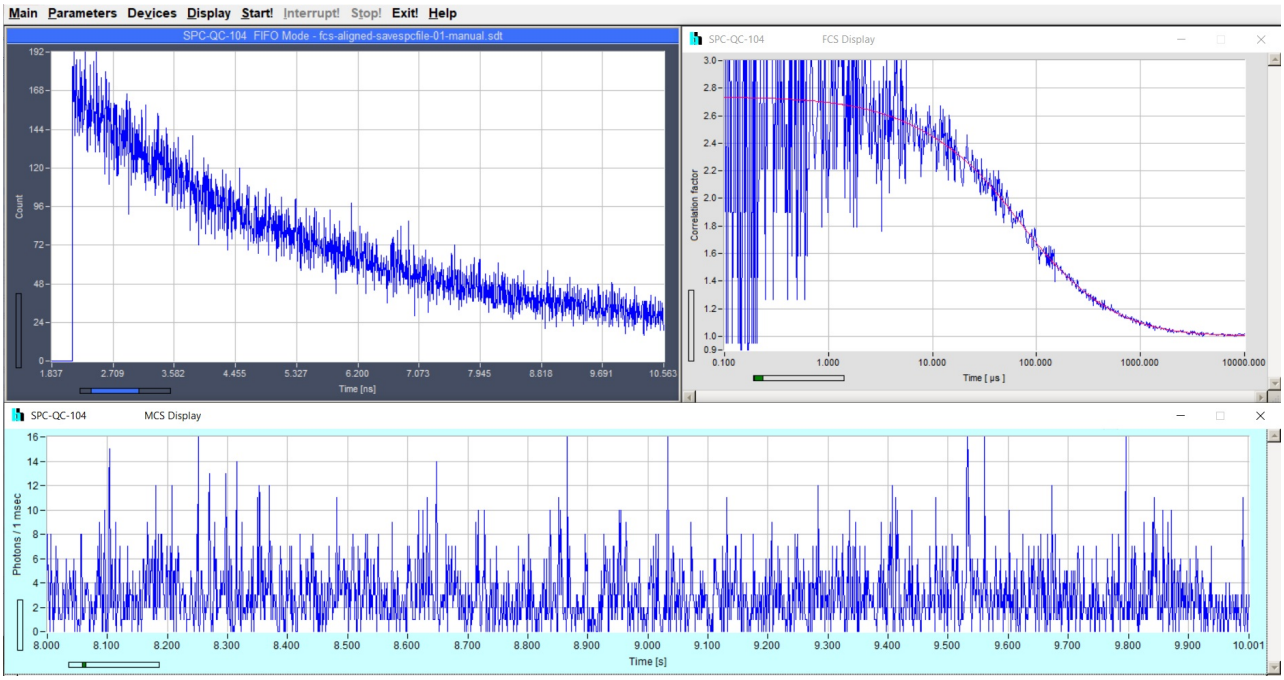


Fig. 10: Single molecules diffusing through laser focus. Decay curve, FCS curve, intensity trace. Raman light suppressed by time-gating.

Simultaneous Recording of Fluorescence and Phosphorescence Decay

A special feature of the FIFO mode is Triggered MCS recording. Triggered MCS is used for simultaneous fluorescence and phosphorescence decay measurement by laser on/off modulation [1, 19]. Three channels can be recorded simultaneously, or one channel can be used with a multi-wavelength detector. An example is shown in Fig. 11.

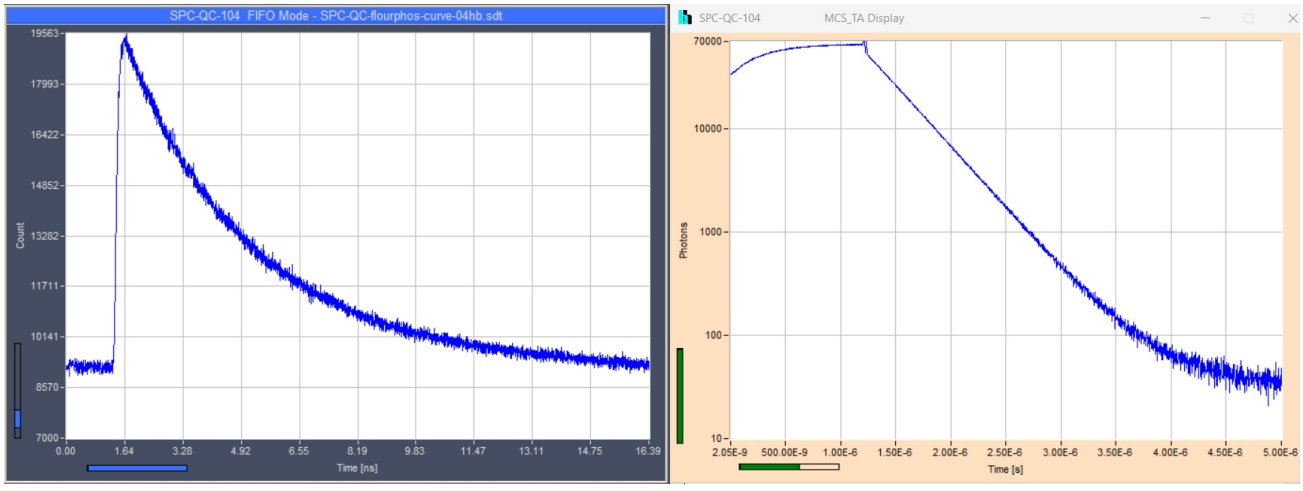


Fig. 11: Fluorescence and phosphorescence decay recorded simultaneously. Fluorescence decay curve shown left, phosphorescence decay curve shown right.

TCSPC FLIM

The SPC-QC-104 records FLIM images in combination with an optical scanner. The operation mode is 'FIFO Imaging'. The images are built up from a stream of time- and parameter-tagged single-photon data and pixel, line, and frame markers. The recording process is illustrated in Fig. 25, page 23. The result is an array of pixels, each containing a fully resolved decay curve in a large number of time channels. Three images can be recorded in parallel by the three SPC-QC channels, or one channel can be used with a multi-wavelength detector. Typical FLIM data formats are 512 x 512 to 2048 x 2048 pixels, with 256 to 1024 time channels per pixel. The principle can be extended for simultaneous FLIM / PLIM recording. Examples are shown in the figures below.

FLIM in Three Parallel Channels

Fig. 12 shows FLIM images recorded in three parallel wavelength channels of the SPC-QC-104 module in combination with a bh DCS-120 FLIM scanner. The image resolution is 1024 by 1024 pixels, the decay data were recorded in 256 time channels.

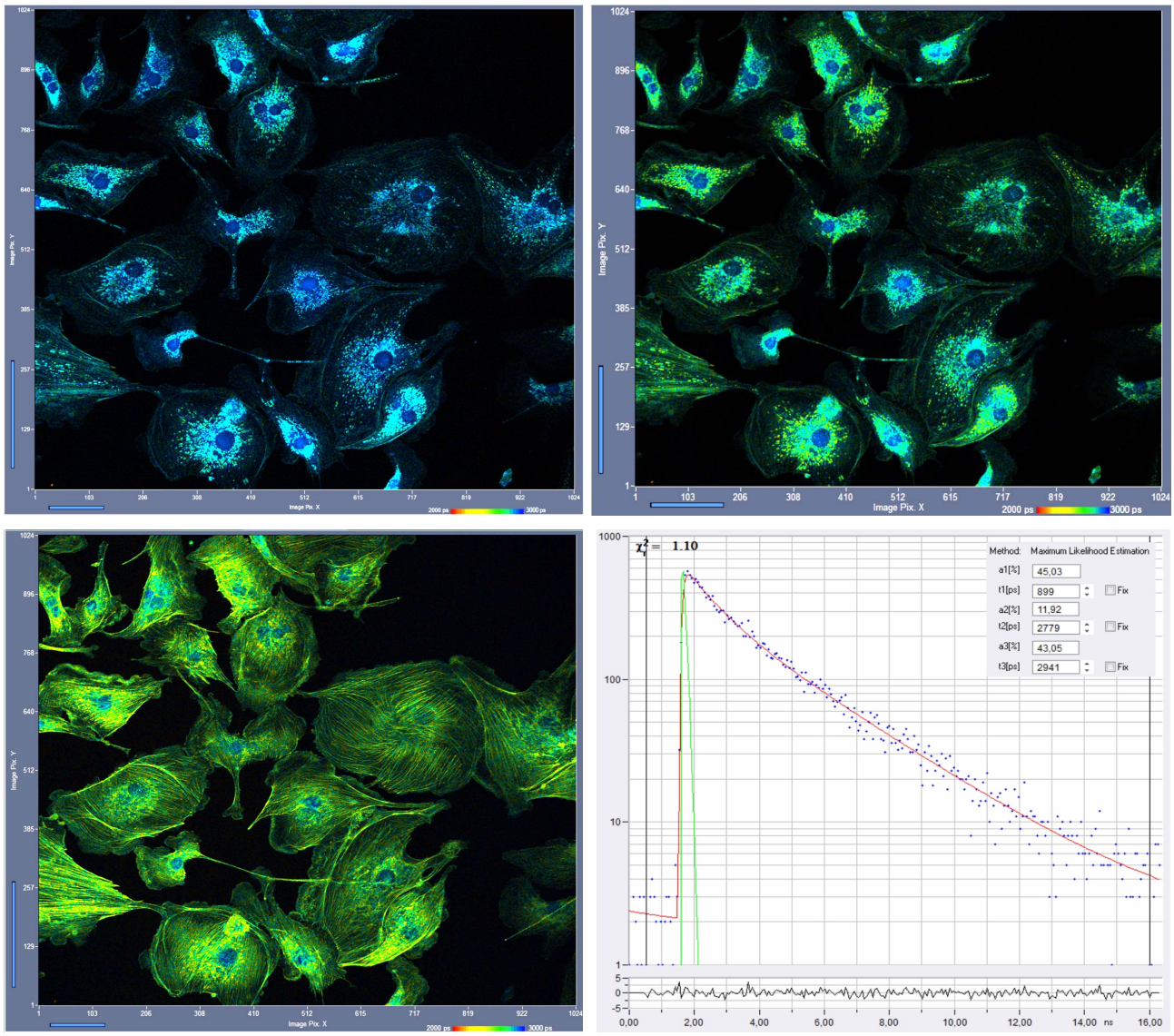


Fig. 12: SPC-QC-104, 3-channel FLIM of BPAE cells, bh DCS-120 confocal FLIM system, 1024 x 1024 pixels. Decay curve in selected spot of third image shown lower right.

FLIM Analysis by SPCImage NG

FLIM data taken with the SPC-QC can be analysed by SPCImage NG [4, 5] the usual way. SPCImage performs an MLE (Maximum-Likelihood Estimation) on the pixel data. Single-double, and triple-exponential models are available. IRF modelling creates an IRF from the FLIM data themselves. Frequent measurement of the system IRF is thus avoided. IRF Processing runs on a Graphics-Processing Unit (GPU). Processing times of formerly 5 to 20 minutes are reduced to a few seconds. Please see section 'SPCImage NG Data Analysis Software', page 67 for details.

An example is shown in Fig. 13. The image has 1024 x 1024 pixels, and 1024 time-channels per pixel. It was analysed using a triple-exponential decay model and MLE fitting. A colour-coded lifetime image of the amplitude-weighted lifetime, tm, is shown on the left. A histogram of the lifetime over the pixels of the image, and the decay parameters at the cursor position are shown upper right. The decay curve at the cursor position is shown lower right.

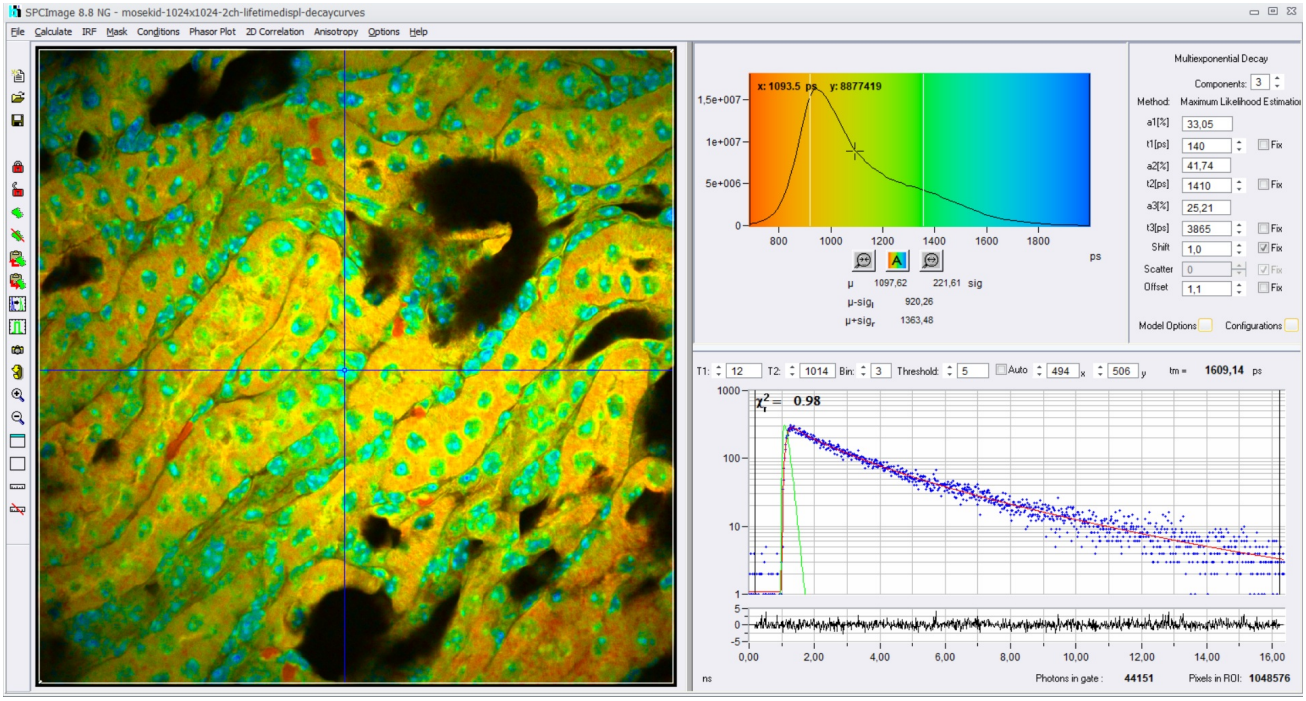


Fig. 13: FLIM analysis with SPCImage NG. High-resolution multiphoton FLIM, Invitrogen mouse-kidney sample, 1024 x 1024 pixels, 1024 time channels. Recorded with SPC-QC and DCS-120 MP system.

Multi-Wavelength FLIM

With the bh MW-FLIM or MW-FLIM GaAsP detector assemblies [1] the SPC-QC module is able to record multi-wavelength FLIM. Images in 16 wavelength intervals are recorded simultaneously. There is no wavelength-scanning or time-gating process. Any photon that arrives at the detector is marked with its wavelength, its time in the excitation pulse period, and its position in the image area. The data analysis software then builds a photon distribution over these parameters. The photon distribution is equivalent to a stack of 16 FLIM images for different wavelength. An example is shown in Fig. 14.

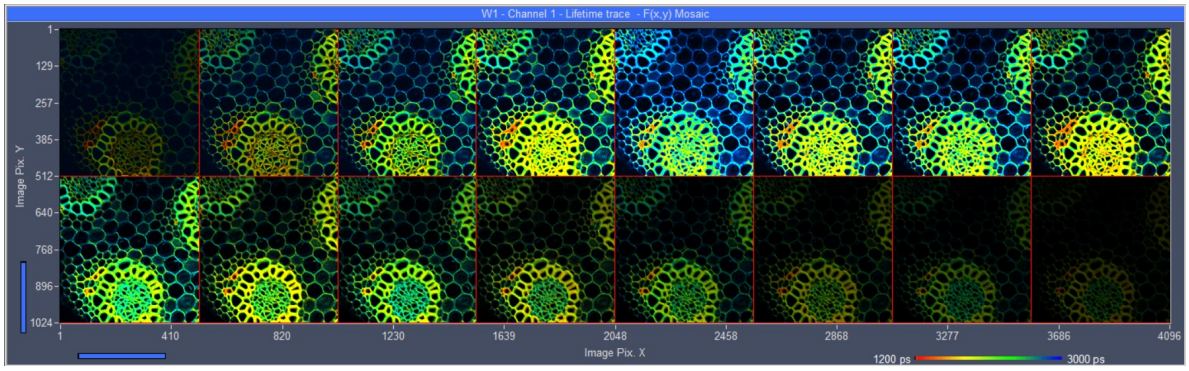


Fig. 14: Multi-wavelength FLIM. MW-FLIM GaAsP 16-channel detector, connected to one input channel of SPC-QC. Mosaic-display function of SPCM with online-lifetime calculation. Each mosaic element represents one wavelength channel and was recorded with 512 x 512 pixels and 256 time channels per pixel.

Laser-Wavelength Multiplexing

Laser wavelength multiplexing is used when the sample contains different fluorophores which cannot be excited at a single wavelength or the fluorescence which cannot be spectrally separated on the detection side. Laser-wavelength multiplexing is integrated in the scanner/laser control module of DCS-120 confocal FLIM systems [6]. The function can be used with the SPC-QC-104 the same way as with the SPC-150N or SPC-180N modules. An example is shown in Fig. 15.

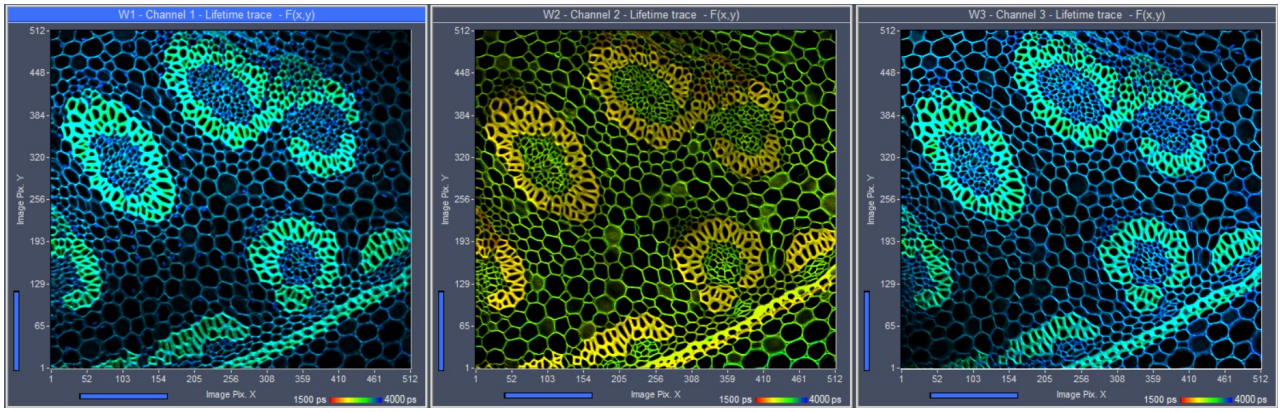


Fig. 15: Laser-wavelength multiplexing. Left: Laser 405 nm, detection 560-650 nm. Middle: Laser 488 nm, detection 435 to 540 nm, Right: Laser 488 nm, detection 560-650 nm. Online-Lifetime image display of SPCM data acquisition software.

Simultaneous Fluorescence and Phosphorescence Lifetime Imaging

Simultaneous Fluorescence and Phosphorescence Lifetime Imaging (FLIM / PLIM) is based on fast on/off modulation of the laser and building up separate photon distributions for the photons detected in the laser-on phases and the laser-off phases. The laser-on photons deliver the FLIM image, the laser-off photons the PLIM image. The technique is integrated in the scanner control of the DCS-120 confocal and multiphoton FLIM systems [6]. It is also available for the bh FLIM systems for the Zeiss LSM laser scanning microscopes [7, 8]. Details of the technique are described under 'Simultaneous FLIM/PLIM', page 25. Please see [1] for applications. An example is shown in Fig. 16. The image upper left is a colour-coded FLIM image, the image on the right is the PLIM image. The windows at the bottom show FLIM and PLIM decay curves for selected stripes over the images.

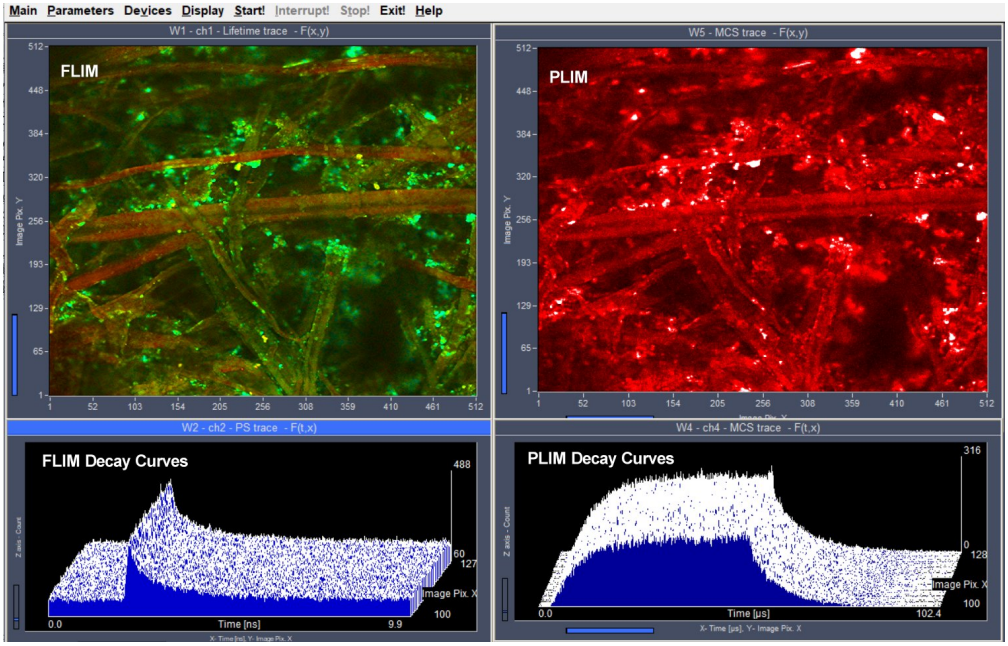


Fig. 16: Simultaneous FLIM / PLIM. DCS-120 confocal FLIM system with SPC-QC-104, Pixel time 8.8 µs, laser-on time 4.4 µs. Image and curve display of SPCM.

Integration in DCS-120 Confocal and Multiphoton FLIM Systems

The SPC-QC integrates seamlessly in the bh DSC-120 Confocal and Multiphoton FLIM systems [6]. With the two DCS-120 channels, the system records up to two images of different wavelength or different polarisation in parallel. Laser and scanner control is fully integrated in the operating software. Pixel numbers are up to 2048 x 2048, time-channel numbers up to 4096. The system scans at a speed of about four frames per second, images of small size and small pixel number are scanned at a speed of up to 10 frames per second. A typical user interface is shown in Fig. 17.

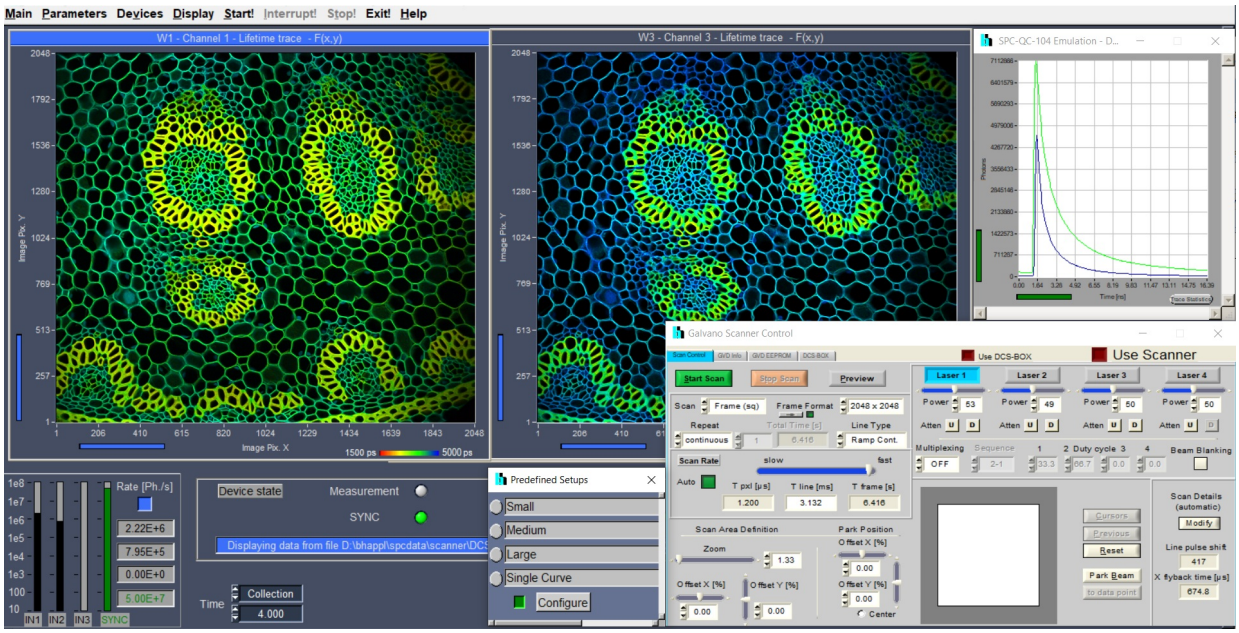


Fig. 17: DCS-120 FLIM system with SPC-QC. Two parallel recording channels. User interface with two FLIM images, online lifetime display, predefined setup panel, laser and scanner control, and decay-curves in regions of interest.

Absolute-Time / Coincidence Measurements

Different than the FIFO mode, the Absolute-Time Mode the SPC-QC module records photons in all four input channels. Laser pulses are not recorded. Photon times in one channel are determined with reference to photon times in another channel, not with reference to laser pulses. Each photon is characterised by its time from the start of the experiment, and, if necessary, by a number of other parameters which are encoded in the routing information of the photon. The SPC-QC creates a data stream of time- and parameter-tagged single photon data and sends them to SPCM. SPCM stores these data for further analysis, and/or builds up coincidence curves, auto- and cross-correlation curves, or time traces (or Multichannel Scaler, MCS curves) from them. An example is shown in Fig. 18.

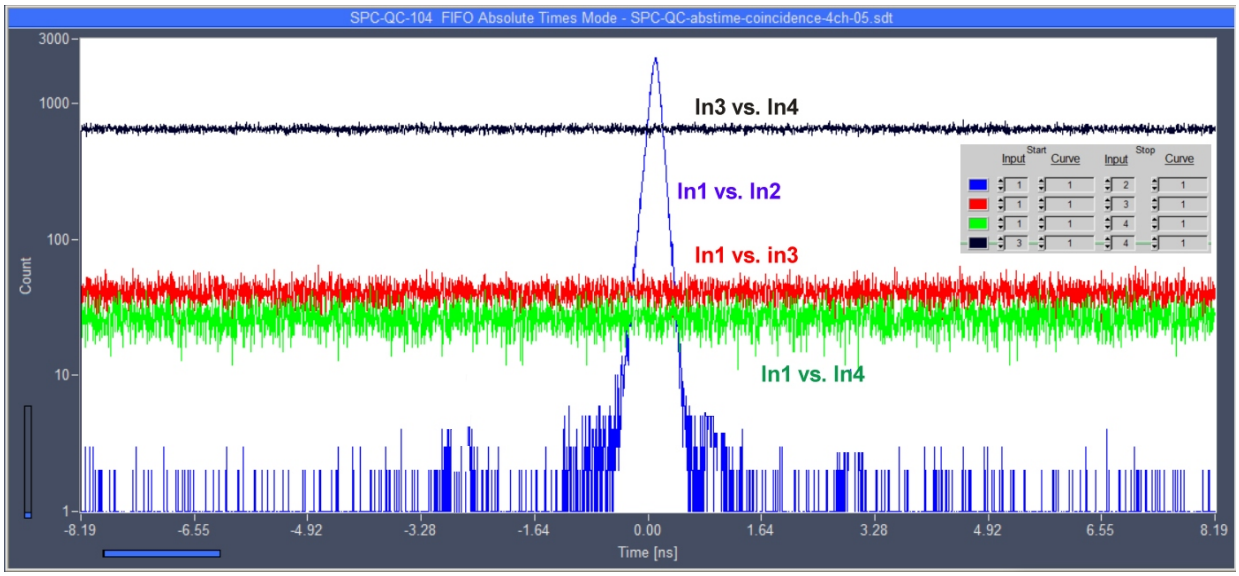


Fig. 18: Coincidence curves. Blue input 1 versus input 2, red input 1 versus input 3, green input 1 versus input 4, black input 3 versus input 4. There is temporal coincidence of the events at input 1. Events at input 1 and inputs 2, 3, and 4 display only random coincidences. Also the events at input 3 and 4 do not coincide.

Module Architecture

Detector / Laser Inputs

The architecture of the SPC-QC-104 is shown in Fig. 19. The module has four inputs for detector and/or laser pulses and 10 inputs for digital control signals. The input signals from the detectors and the laser are received by four identical constant-fraction discriminators, CFD1 through CFD4. The CFDs provide defined trigger pulses the temporal position of which is independent of the amplitude of the input pulses. The CFDs are identical with those of the SPC-150N and -180N series. The feature extraordinarily high input bandwidth and unprecedented timing stability. For principle of the CFDs and parameter setup please see section 'Discriminators' of this manual or bh TCSPC Handbook [1], section 'Constant-Fraction Discriminators' and 'System Optimisation'. CFD1, CFD2, and CFD3 are intended to receive detector pulses. Depending on the operation mode, CFD4 can either receive a fourth detector signal or a reference pulse from the excitation source.

Control Inputs

The SPC-QC module has four 'Routing' inputs, a 'Count Enable' input, four 'Marker' inputs, and a 'Trigger' input. All control inputs are designed to receive TTL or CMOS compatible signals.

The function of the *Routing Signals* is to direct photons into different memory blocks depending on the logical state of the signals. With the four routing bits 16 separate memory blocks can be addressed. The routing bits can come from a multi-wavelength detector, from a laser multiplexing device - usually a bh GVD-120 or GVD-140 scan controller - or another device which controls a parameter in the external experiment setup. Please note that the routing bits are common for all four timing channels. That means that laser wavelength multiplexing or experiment-parameter multiplexing automatically acts on all SPC-QC recording channels. The tradeoff is, however, that only one multi-wavelength detector (bh PLM-16, PML-16 GaAsP, or MW FLIM detector) can be used on one SPC-QC module. The limitation to one multi-wavelength detector is due to the limited number of control-input lines. Since rarely several multi-wavelength detectors are used in one TCSPC system it is not normally a problem to system design.

The *Count-Enable* signal enables or prevents the recording of photon pulses received by the detector inputs. It is used in combination with multi-wavelength detection to prevent the recording of photons for which no valid routing information could be generated. It is also used to enable or disable the recording of photons depending on special conditions in the external experiment setup.

The *Marker Inputs* are used to feed external events - usually from activities in an external experiment setup - into the SPC-QC module and insert them into the data stream. The typical use of the markers is to record synchronisation pulses from optical scanners. A pixel clock (M0) indicates the transition of the scanner to the next pixel, a line clock (M1) indicates the transition to the next line, and a frame clock (M2) the beginning of a new frame. A fourth marker is reserved for the marking of other external events, usually from a periodic stimulation of the sample.

The *Trigger* input is used to start the recording of data on command, or to initiate a new step or cycle of a sequential recording procedure.

Timing and Signal-Processing Logics

The timing of the detector pulses and a large part of the subsequent data processing is performed in an FPGA (Field-Programmable Gate Array). Inside the FPGA, the CFD output pulses are fed into four TDCs (Time-to-Digital Converters), please see section 'Time-Conversion Principle' in this manual. The TDCs determine absolute times of the pulses with reference to the system clock sequence.

Because all TDCs are operating on the same system clock and are implemented in the same FPGA they deliver photon times which are exactly comparable. The TDC results are fed into the signal-processing block of the FPGA. In addition, the signal-processing block receives the routing signals, the 'Count Enable' signal, and, if enabled, the 'Experiment Trigger' signal. Moreover, state-transition of the markers, M0...M3 can be received and included in the data stream to mark external events in the experiment setup.

Depending on the operation mode, the signal-processing logics can perform the following operations:

- In the internal-histogram modes (Single, Oscilloscope, f(t,T)) it calculates the differences between the times delivered by TDC 1, 2 and 3 against the times delivered by TDC 4. The resulting values are the times of the photons after the previous laser pulse. The processing logics builds up the photon distributions over these times in the on-board memory. The distribution is the waveform of the optical signal, typically a fluorescence-decay curve, see Fig. 23, page 22. In addition to the photon times the procedure can use the state of the routing signals to create separate photon distributions for different states of the routing signals. A typical application is multi-wavelength detection, please see Fig. 24.
- In the FIFO (Parameter-Tag) mode the signal processing logics calculates the differences between the times delivered by TDC 1, 2 and 3 against the times delivered by TDC 4. Photon by photon, it sends the results to the bus interface. From there, the values are read by the SPCM software, which then builds up the photon distributions. The on-board memory is used as a FIFO (first-in-first-out) buffer to bridge time intervals in which the software is not able to read the data. In addition to the photon times the processing logics sends the state of the routing signals for the individual photons. Using these data, SPCM routes the photons into separate photon distributions for different routing vectors, please see Fig. 24. Moreover, external events, such as scan clocks from a scanning device, or other reference pulses from the experiment setup are included in the data stream. The software builds up decay curves, FCS curves, intensity traces, lifetime images, or FLIM / PLIM images from such data. In addition to building up photon distributions, SPCM can save the parameter-tagged data into a file. For every photon, the parameter-tag data contain the time after the laser pulse, the absolute time from the start of the experiment, and the state of the routing signals. The data also contain entries for the marker events. Please see SPCM description in the bh TCSPC Handbook [1].
- The FIFO Imaging mode builds up fluorescence-lifetime images (FLIM). The general principle is similar to the FIFO mode. The marker inputs, M0 to M2, are used to record the pixel clock, line clock, and frame clock pulses from an optical scanner. A data stream of photon pulses and marker pulses send to the software which builds up a photon distribution over the coordinates of the scan and the photon times after the reference pulses from the laser. The result is an array of pixels, each containing a complete fluorescence decay curve in the form of photon numbers in consecutive time channels, see Fig. 25, page 23. Three images can be recorded simultaneously in the three photon channels of the SPC-QC. Using one or two of the routing bits, the recording can be combined with laser multiplexing, see Fig. 27, page 24. Alternatively, a multi-wavelength image can be recorded by using the routing information of a multi-wavelength detector. The result is a lifetime image that contains 16 decay curves for different wavelength in each pixel, see Fig. 26, page 24. Moreover, the FIFO Imaging Mode is able to record FLIM and PLIM images simultaneously. The laser is on/off modulated synchronously with the pixels of the scan. The software builds up two photon distributions. One is over the pixels and the photon times in the laser pulse period, the other over the pixels and the photon times in the laser on/off period. The first one is the FLIM image, the second one the PLIM image. Please see 'Simultaneous FLIM/PLIM', page 25.

- The Absolute-Time mode is similar to the FIFO mode in that it creates a data stream of time- and parameter-tagged single-photon data and sends them to SPCM. However, the absolute time mode does not determine photon times in a laser pulse period. Instead, for each photon and each detector it determines the times of the photons from the start of the recording and the status of the routing bits. 'Markers' can be inserted in the data stream to tag the times of external events and associate them to the photons. The SYNC input is used to record photons from a fourth detector. SPCM stores the single-photon data for further analysis, and/or immediately calculates coincidence curves, auto- and cross-correlation functions, photon counting histograms, or time traces (or Multichannel Scaler, MCS curves) from them.

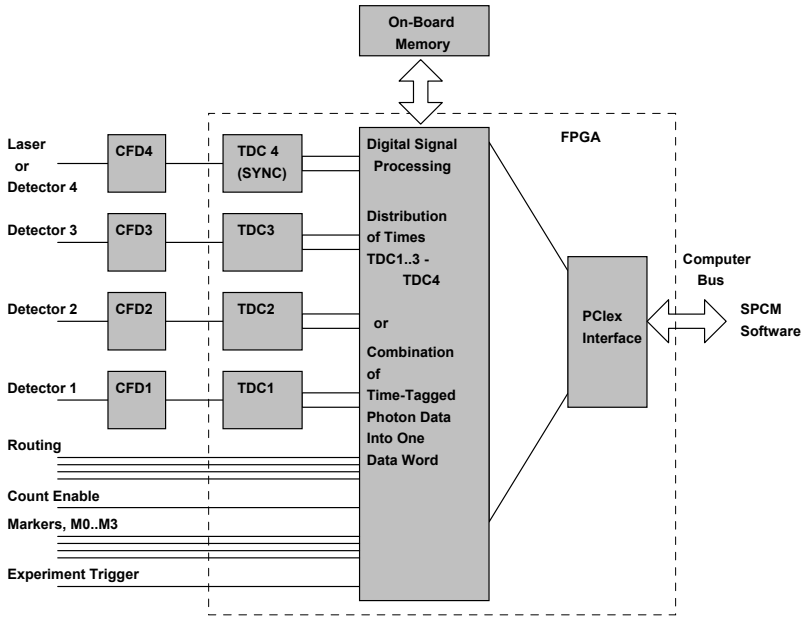


Fig. 19: Architecture of the SPC-QC-104 module

Discriminators

Single-photon pulses from a PMT have a considerable amplitude jitter. If the pulses were sent through a normal discriminator the amplitude jitter would cause a timing jitter, Δt, on the order of the duration of the leading edge of the photon pulses, see Fig. 20, right. For normal (high-speed) PMTs this would be about 1 ns - far too much for the standards of TCSPC.

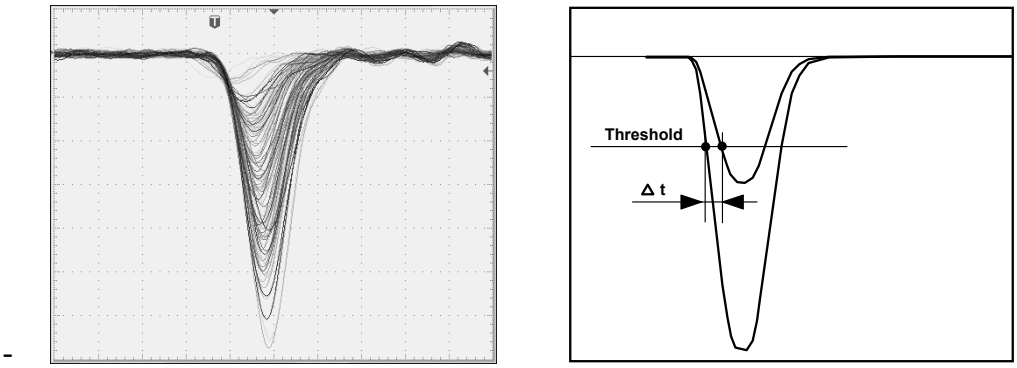


Fig. 20: Left: Single-photon pulses from a normal PMT. Right: When the pulses are received by a normal discriminator the amplitude jitter would induce a timing jitter, Δt.

Obviously, a TCSPC device needs a discriminator which triggers at a constant fraction of the pulse amplitude, see Fig. 21, left. If the discriminator threshold is varied in a way that it is a constant fraction of the pulse amplitude the timing variation, Δt, vanishes. Please see Fig. 21, left.

The principle of a CFD is usually described as shown in Fig. 21, left. However, the principle has a flaw: The electronical implementation is impossible. When the leading edge of the pulse crosses the threshold the final amplitude of the pulse and thus the correct threshold are not known yet. The practical implementation is therefore different. The detector pulse is re-shaped by an electrical network (usually a system of short delay lines, see [1]) so that a bipolar pulse is obtained, see Fig. 21, right. The temporal position of the zero-cross point is independent of the amplitude. Therefore a discriminator picks off the baseline transition of the bipolar pulse. In practice, the optimum 'zero-cross level' may be not exactly at the zero line but slightly above or below. The zero-cross level is therefore adjustable to optimise the timing performance for a given detector. Moreover, there is a second discriminator which looks at the original detector pulse. Only if the pulse exceeds the threshold of this discriminator the zero-cross discriminator is enabled. The complete CFD therefore has two control voltages - a threshold voltage and a zero-cross voltage. The timing performance is optimised by the Zero-Cross-Level, the amplitude range by the Threshold voltage.

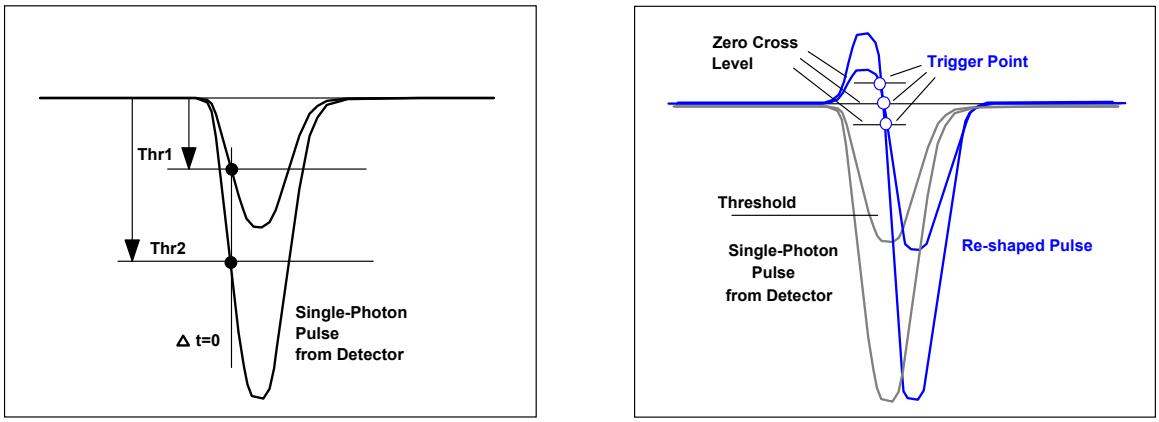


Fig. 21: Commonly described Principle of a CFD (left) and practical implementation (right)

Time-Conversion Principle

TCSPC is based on the detection of single photons, the determination of their detection times, and the buildup of photon distributions or correlations of the times of the photons. The core of any TCSPC device is therefore a time-measurement circuit which converts the photon times - either absolute or relative ones - into digital data words.

There are two generally different time-conversion principles. The bh SPC-130, -150, -160 and -180 TCSPC modules use a TAC/ADC principle; the SPC-QC-104 uses direct time-to-digital (TDC) conversion.

The two principles are illustrated in the figure below. The TAC / ADC principle is shown left. It uses a linear voltage ramp between a start pulse (usually the photon) and a stop pulse (usually a reference from the laser). The voltage is converted in a digital data word which expresses the time of the photon in the laser pulse sequence [1, 2]. The advantage of the TAC / ADC principle is the extremely high time resolution. The IRF width of the SPC-150 NXX and -180 NXX modules is 2.8 ps fwhm (full width at half maximum), the effective timing jitter is about 1.5 ps (rms) [1, 3]. The time-channel width

can be as small as 200 femtoseconds. A resolution this high is not reached by any other TCSPC device. It is more than 13 times higher than for the TDC principle.

The TDC principle is shown on the right. The photon pulses from the detector(s) and the reference pulses from the laser are sent through chains of delay elements. The timing logics looks at the data in the delay chains, identifies start-stop pairs of photons and laser pulses, and this way determines the temporal positions of the photons in the laser pulse sequence. The time resolution of the TDC principle is significantly lower than that of the TAC/ADC principle. The IRF width of the SPC-QC-104 is about 37 ps (fwhm, including synchronisation), the effective timing jitter about 17 ps (rms), please see Fig. 30, page 26. The minimum time-channel width is 4 picoseconds. The advantage of the TDC principle is that it works up to extremely high photon rates, and that several timing channels can be implemented on a single PC board.

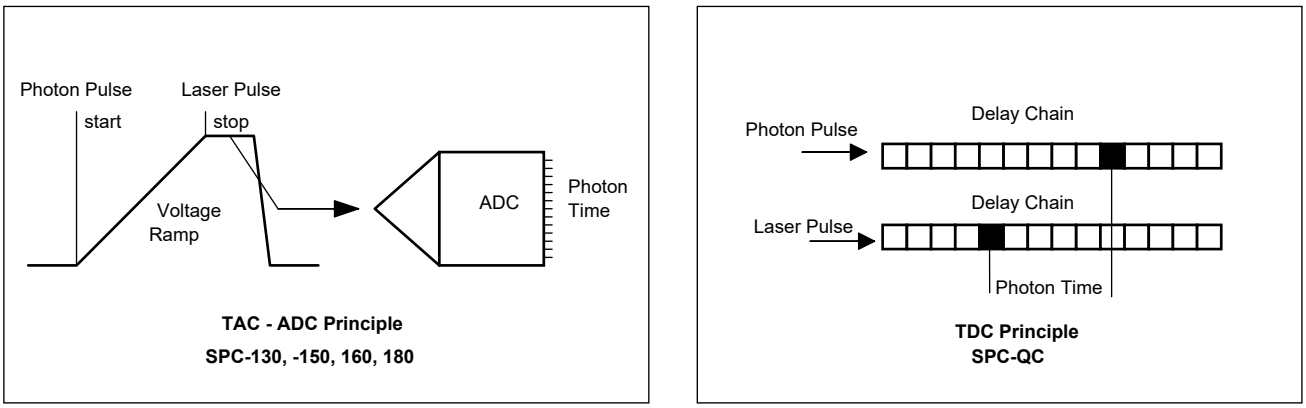


Fig. 22: Time-conversion principles. Left: TAC/ADC principle. Right: TDC principle

Buildup of Fluorescence-Decay Data

As all bh TCSPC devices, the SPC-QC records time-resolved optical data by detecting single photons of a repetitive light signal and building up photon distributions over the times of the photons after a timing-reference pulse. In the simplest case the photon distribution can just be built up over these times, in other cases it includes also other parameters, such as the wavelength, the time from a stimulation of the sample, or the position of the excitation beam within a scanning area [2]. The buildup of the photon distribution in the SPC-QC does not differ substantially from the procedure used in the bh SPC modules [1]. The only difference is in the method of the time measurement and in the fact that the SPC-QC measures the time from the laser pulse to the photon, whereas the SPC modules measure it from the photon to the next laser pulse [1, 2, 3].

Classic TCSPC Mode

Classic TCSPC builds up a photon distribution over the times of the photons in the excitation pulse period. The principle is illustrated in Fig. 23.

When a photon is detected, the arrival time of the corresponding detector pulse in the signal period is measured. The detection events are collected in a memory by adding a '1' at an address proportional to the detection time. After many signal periods a large number of photons has been detected, and the distribution of the photons over the time in the signal period has been built up. The result represents the waveform of the optical pulse.

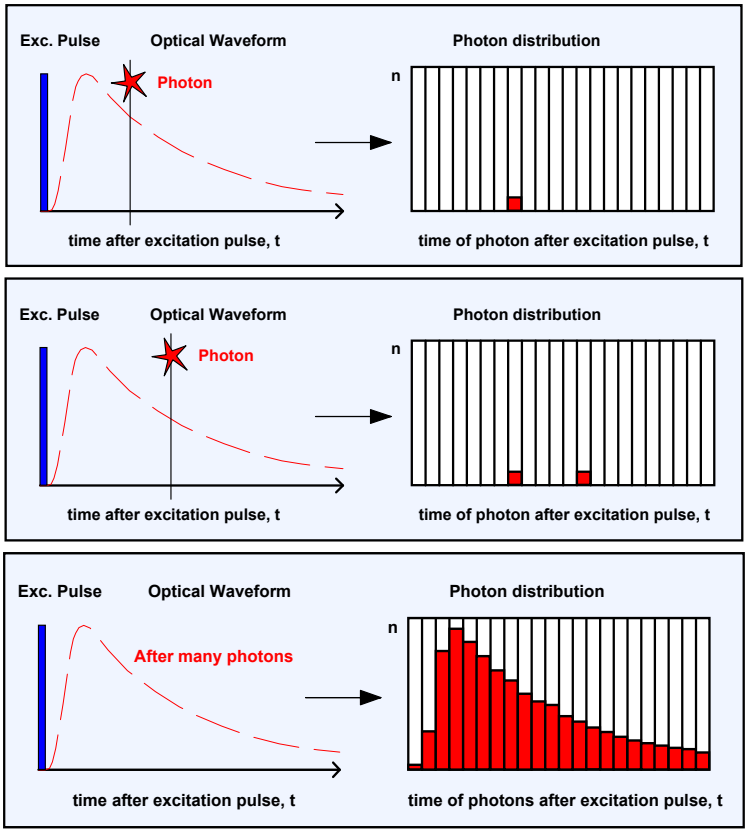


Fig. 23: Classic TCSPC records a distribution over the times of the photons after the excitation pulses.

Multi-Wavelength TCSPC

Multi-wavelength TCSPC is based on splitting the light spectrally into a number of detector channels (or channels of a multi-anode PMT), and using the number of the channel in which the photon arrived as a second coordinate of the photon distribution. The principle is shown in Fig. 24. For each photon, the detector delivers a single-photon pulse which indicates the detection time, and a 'Channel' signal which indicates in which spectral channel the photon arrived. The TCSPC module builds up a photon distribution over the photon time and the channel number. The result is identical with a set of decay curves (in case of the PML-16 and PML-16-GaAsP detector sixteen) for different wavelengths.

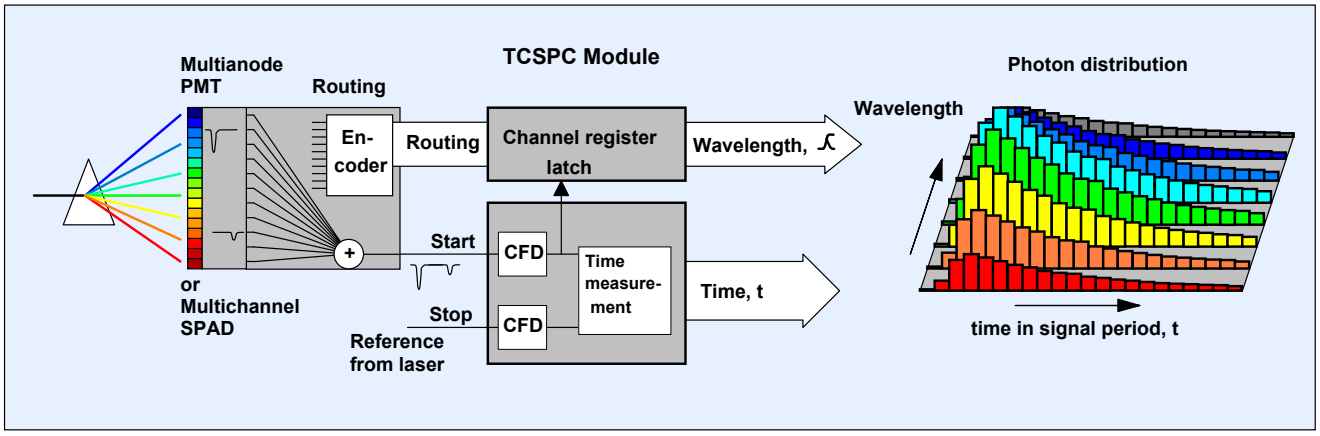


Fig. 24: Principle of multi-wavelength TCSPC

Please note that multi-wavelength TCSPC does not imply any wavelength scanning, detector switching, or other process that rejects a part of the photons. Every photon is put into a place in the photon distribution according to its detection time and its wavelength. Compared to scanning the spectrum with a monochromator and recording individual decay curves, the efficiency is much higher. Multi-wavelength detection, especially in combination with FLIM, has therefore become a commonly used technique of spectroscopy of biological samples [16, 17, 18].

FLIM

FLIM by multi-dimensional TCSPC is based on scanning a sample by the focused beam of a high-repetition rate laser and detecting single photons of the fluorescence signal. The times of the photons are determined by the timing (TDC) electronics, the position of the laser beam in the moment of the photon detection by a scanning interface. The scanning interface counts pixel clock, line clock, and frame clock pulses from the scanner. By counting these pulses, it tracks the position of the beam in the scan area. The recording process builds up a photon distribution over these parameters [1, 2, 11, 12, 14]. The principle is illustrated in Fig. 25.

The result is an array of pixels, each containing a full fluorescence decay curve with a (typically large) number of time channels. The process works at any scan rate, and delivers near-ideal photon efficiency and extremely high time resolution [1, 2, 3].

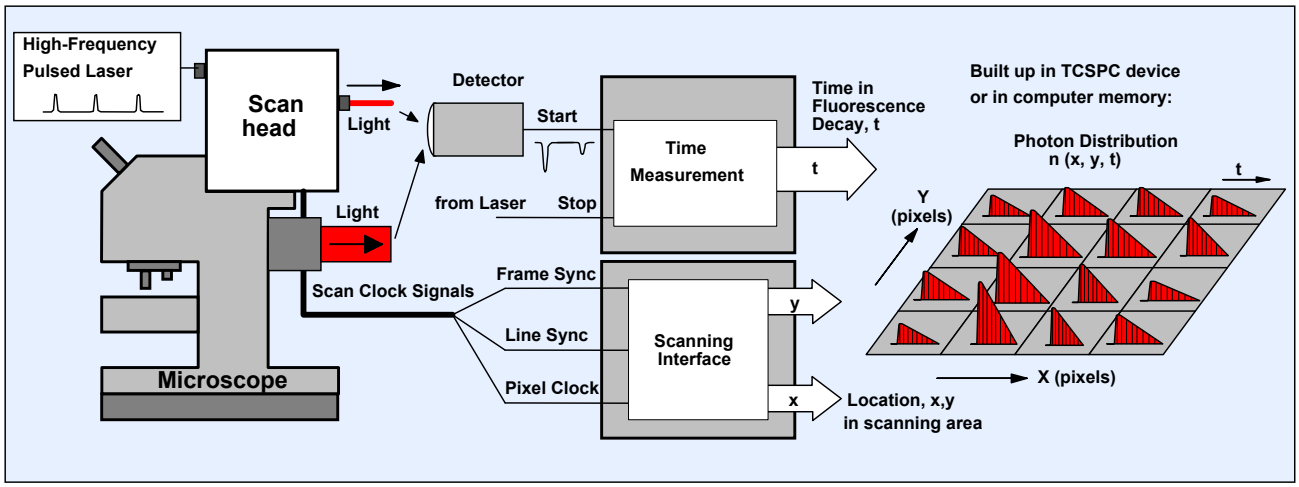


Fig. 25: Fluorescence lifetime imaging. One channel of three.

Multi-Wavelength FLIM

Multi-wavelength (or 'multi-spectral') FLIM uses a combination of the FLIM architecture shown in Fig. 25 with multi-wavelength detection principle described in Fig. 24. In addition to the times of the photons and the positions, x, and y, of the scanner, the TCSPC module determines the (spectral) detector channel that detected the photon. These pieces of information are used to build up a photon distribution over the times of the photons in the fluorescence decay, the wavelength, and the coordinates of the image [1, 2, 13, 14, 15]. The result is an image that contains several decay curves for different wavelength in each pixel. The principle of multi-wavelength FLIM imaging is shown in Fig. 26.

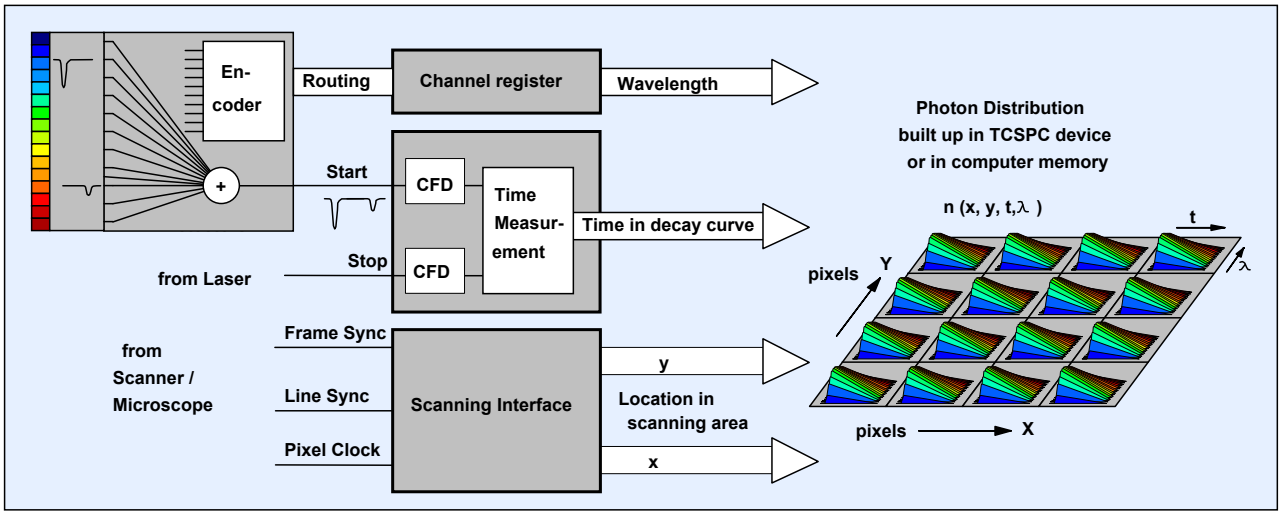


Fig. 26: Multi-wavelength FLIM. The recording process builds up a photon distribution over x,y,t, and λ.

FLIM with Excitation Wavelength Multiplexing

FLIM can be combined with excitation wavelength multiplexing. The principle is shown in Fig. 27. Excitation at different wavelength is achieved by multiplexing (on/off switching) several lasers, or by switching the wavelength of the acousto-optical filter (AOTF) of a super-continuum laser. A multiplexing signal that indicates which laser (or laser wavelength) is active is fed into the routing input of the TCSPC module. The signal represents the excitation wavelength. The TCSPC module is running the normal FLIM acquisition process: It builds up a photon distribution over the coordinates of the scan area, the photon times, and the excitation wavelength. The result is a data set that contains separate images for the individual excitation wavelengths. It can also be interpreted as a single image that has several decay curves for different excitation wavelengths in its pixels.

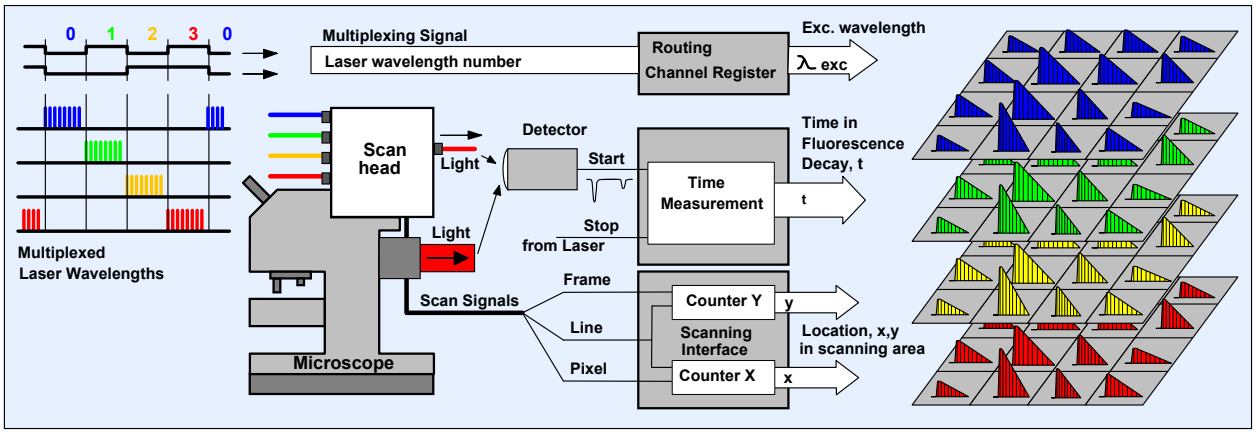


Fig. 27: FLIM with laser wavelength multiplexing. Four lasers multiplexed, four FLIM images recorded. One channel of three.

To avoid interference of the multiplexing frequency with the pixel, line, or frame frequency of the scanner, multiplexing is normally synchronised with the pixels, lines, or frames of the scan. Images for different combinations of excitation and emission wavelengths can be obtained by using several detectors connected to different inputs of the SPC-QC. The most important application of excitation wavelength multiplexing is metabolic imaging with simultaneous detection of NADH and FAD [1, 9, 10].

Simultaneous FLIM/PLIM

A bh TCSPC module can, in principle, record an optical waveform simultaneously on two different time scales. One time scale comes from the TDC times, the other from the system clock. By tagging the photons with these two times, photon distributions on the picosecond and on the microsecond time scale can be build up simultaneously.

The technique is used to record fluorescence and phosphorescence decay data simultaneously [1, 19, 20, 21, 22]. The principle is shown in Fig. 28. A high-frequency pulsed laser is on / off modulated with a period in the microsecond or millisecond range. The TCSPC module determines photon times, t, within the laser pulse period, and photon times, T, within the laser modulation period. Fluorescence decay curves are obtained by building up a photon distribution over t, phosphorescence decay curves by building up a photon distribution over T.

The technique solves a number of problems of phosphorescence decay measurement. The most significant one is that for phosphorescence decay measurement the duration and the period of the excitation must be long. This conflicts with the capabilities of the available lasers, and with the requirement that for fluorescence decay measurement the pulses and the pulse period must be short. With the principle shown in Fig. 28, the effective excitation pulse for the phosphorescence measurement is the laser-on period, but for fluorescence it is the laser pulse period.

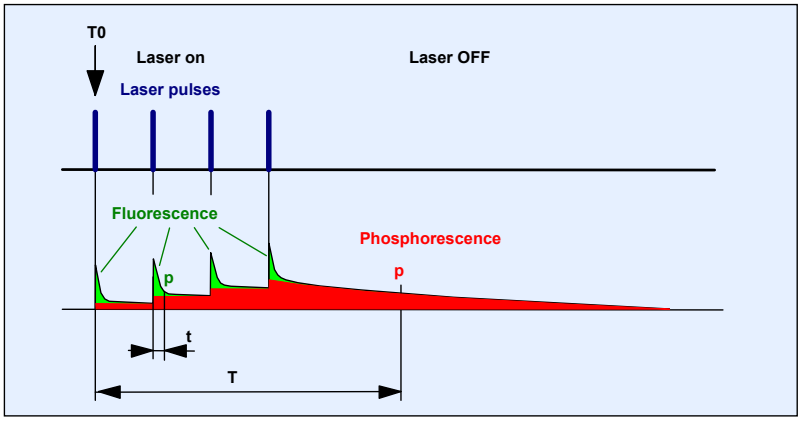


Fig. 28: Build-up of phosphorescence within a burst of laser pulses

To combine the technique shown in Fig. 28 with imaging the scan coordinates must be included in the parameters of the photon distributions. A phosphorescence lifetime image (PLIM) is obtained by recording the photons versus the times, T, in the laser on / off period and the scan coordinates, a fluorescence lifetime image (FLIM) by recording the photons versus the TDC times, t, and the scan coordinates. Both processes can be run simultaneously. The TCSPC system architecture for simultaneous FLIM / PLIM is shown in Fig. 29. Technical details and applications of simultaneous FLIM / PLIM are described in the bh TCSPC Handbook [1].

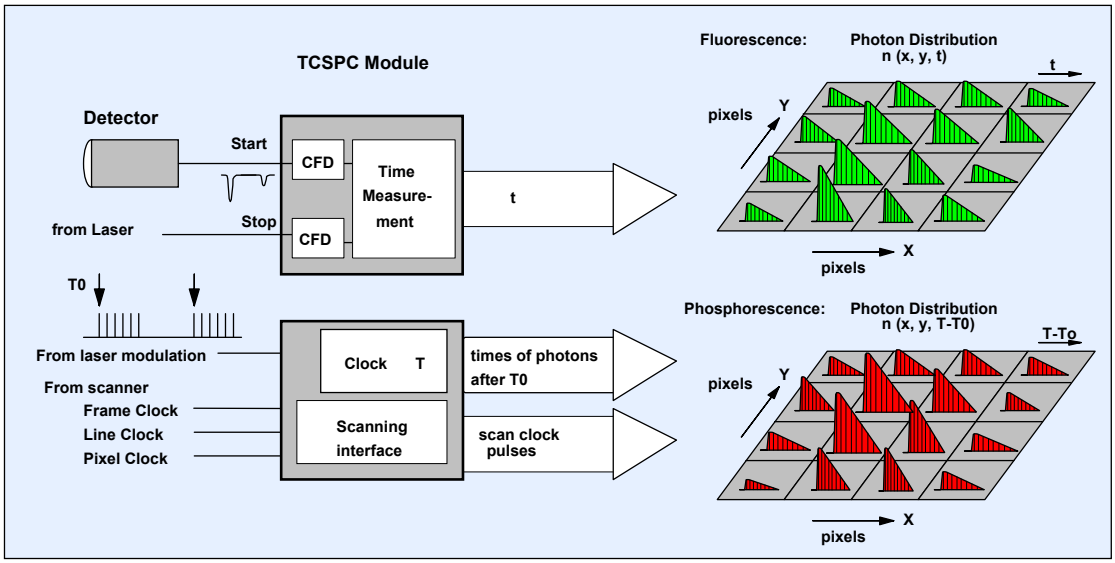


Fig. 29: Simultaneous fluorescence and phosphorescence lifetime imaging in the parameter-tag mode. One channel of three.

The procedure can be refined by using the laser on/off information as a routing signal to better separate the fluorescence in laser-on phases from the phosphorescence in the laser-off phases. Please see bh TCSPC Handbook [1].

Comparison with bh SPC-130 to SPC-180 Series Modules

The advantage of the TDC principle is that the timing electronics can be implemented in an FPGA (Field-Programmable Gate Array). Therefore several recording channels can be implemented on one TCSPC board. Another feature where the TDC is superior to the TAC is that the TDC principle works up to extremely high count rates. In practice, the count rate is limited by pile-up [1, 2], dead time in the detector-discriminator combination [1, 2], degradation of the detector timing performance at high count rate, and, most importantly, by sample degradation due to high excitation power.

On the downside, the time resolution is much lower than for the TAC / ADC principle. A comparison of the electrical IRF of an SPC-180NXX and an SPC-QC-104 is given in the figure below. The IRF width for the SPC-180NXX (left) is 2.8 ps FWHM, for the SPC-QC-104 (right) it is 37 ps FWHM. Although an IRF width of 37 ps FWHM is an excellent value for a TDC it does not exploit the full time resolution of ultra-fast detectors, such as SSPDs, MCP-PMTs and ultra-fast hybrid detectors.

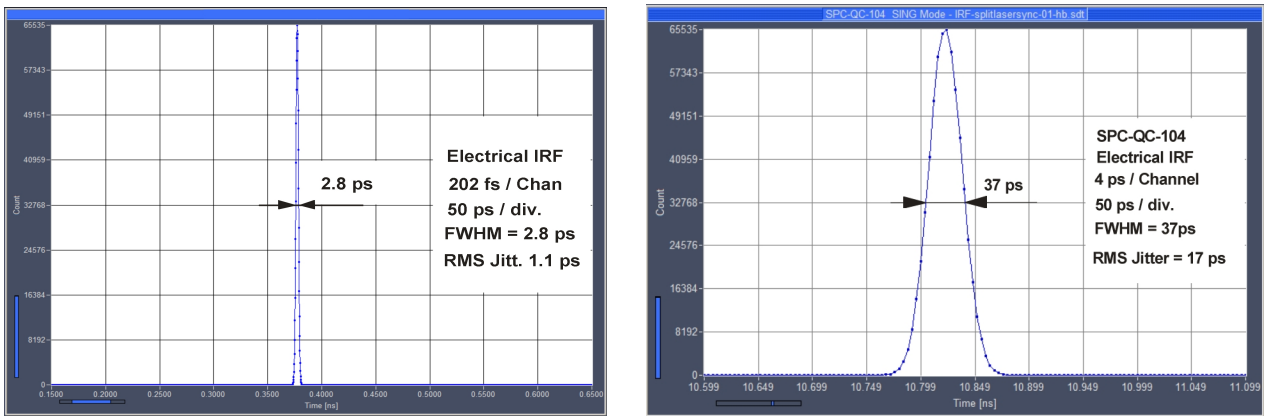


Fig. 30: Electrical IRF for SPC-180NXX (left) and SPC-QC-104 (right). Time scale, 50 ps /division

Another critical feature is timing stability. For many years stability was a problem for the TDC. In the SPC-QC-104 the stability problem has largely been overcome by a new TDC-logics structure. A comparison of the timing stability of an SPC-180NXX and an SPC-QC-104 is shown in the figure below. For the SPC-180NXX the stability of the first moment of the IRF is better than 0.4 ps RMS, for the SPC-QC-104 it is better than 5 ps RMS (note different time scales). Although the SPC-QC does not reach the stability of the SPC-180NXX possible timing drift remains far below the IRF width and is thus barely a problem in practical application.

Fig. 31: Timing stability of an SPC-180NXX (left) and of an SPC-QC-104 (right). Time-series of measurements, display in Colour-Intensity mode. Note different time scale.

System Setup

Software Installation

We recommend to install the software from https://www.becker-hickl.com. You are then sure that you get the latest software version. Please open www.becker-hickl.com (Fig. 32, left). Click on the ‘Products’ button. In the Products panel, click on ‘Software’ (Fig. 32, right).

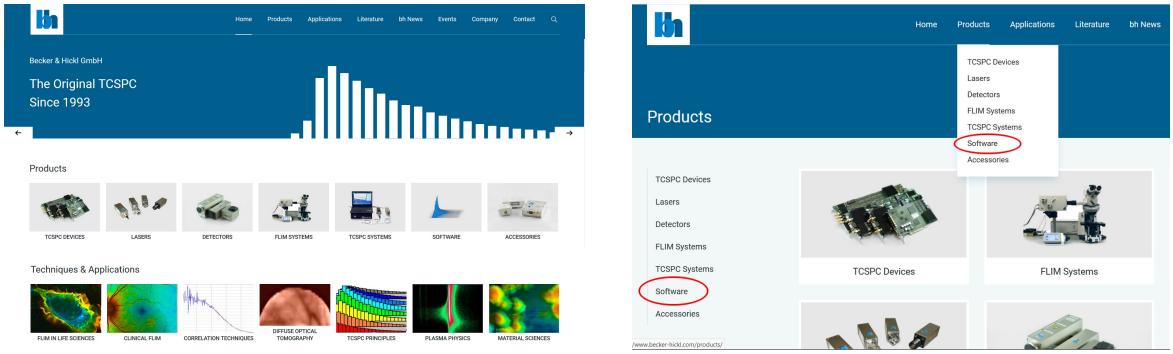


Fig. 32: For TCSPC software installation open www.becker-hickl.com, and click the ‘Products’ button’. Then select ‘Software’

This opens the panel shown in Fig. 33, left. Select ‘tcspc-setup_64.exe’ or (for older 32-bit computers) ‘tcspc-setup_32.exe’. You can start the installation right away or first save tcspc_setup.exe on the hard disc and run it from there. The start of the setup program opens the usual Windows installer, see Fig. 33, right.

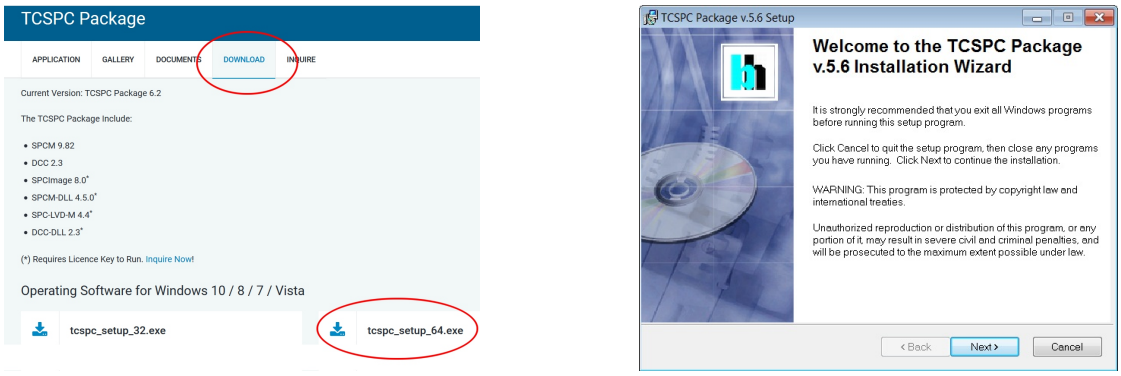


Fig. 33: Selection of the TCSPC software (left) starts the Windows installer (right)

In the next step you can select what exactly you want to install, see Fig. 34, left. Non-imaging applications may need only the SPCM and the DCC application. The installation of these components is free. For fluorescence-decay analysis and FLIM data analysis you need SPCImage NG. SPCImage NG requires a license number - please type it in as shown in Fig. 34, right. If you purchased SPCImage NG with the TCSPC software package the number has been delivered with your system. If you don’t find it please request one from bh.

The installation procedure lets you also install DLLs for the bh TCSPC systems. The DLLs are only needed if you plan to develop your own instrument software.

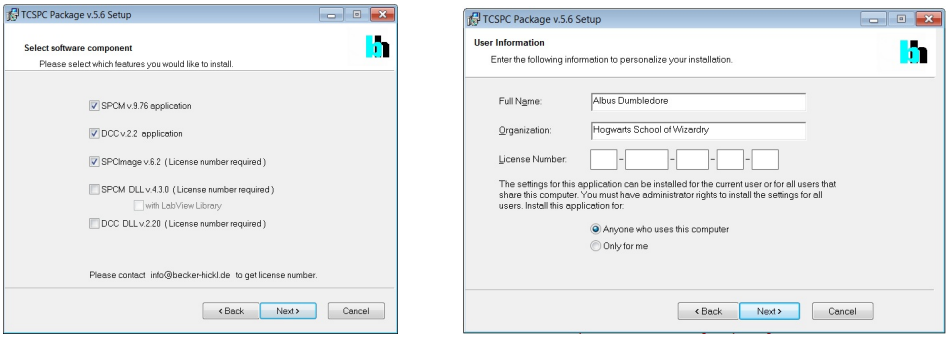


Fig. 34: Selection of software components (left) and license number input for SPCImage NG

After that, please follow the instructions of Windows and finish the installation. Problems can occur if Windows on your computer has not been updated for a longer period of time. The TCSPC package may then not be compatible with the Windows installer. Therefore, please run pending Windows updates before you start the installation.

Hardware Installation

The SPC-QC-104 and the SPC-QC-004 are PCI-express modules for installation in a standard PC. To exploit the full capability of the SPC-QC-104 the computer should have 32 GB of memory.

The SPC-QC module occupies a single PCIe slot. Both slots with short PCIex connectors and with long PCIe connectors can be used. To install the module, turn off the computer, remove the slot bracket of a free slot, insert the module, and fix its slot bracket to the computer frame with the screw that previously held the blind plate. Please see Fig. 35. Some computers have other mechanisms to hold the module in place. In any case it is important that the module is securely fixed. If it is not, you almost certainly get contact problems in the bus connector and EMC (electromagnetic compatibility) problems. If additional experiment-control modules, such as a DCC-100, a GVD-120 or a GVD-140 are to be used, slots have to be provided also for these. PCIex versions of these modules may have additional inputs for power supply. If so, please connect one of the internal power supply lines to the corresponding connector on the board. Please see bh TCSPC Handbook [1], chapter 'Installation of the bh TCSPC Modules'.

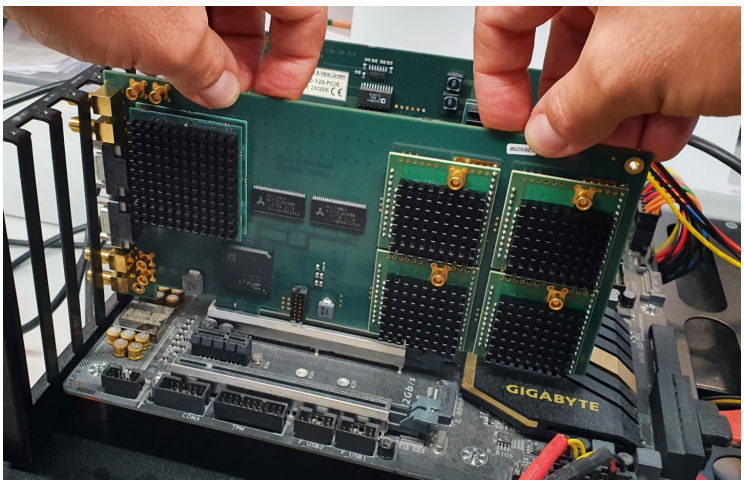


Fig. 35: Insertion of a SPC-QC-104 in a PC. The module can be inserted both in long and in short PCIex connectors.

Software Start

When the module has been inserted in the computer switch on, wait until Windows has started, and start the SPCM Software. If SPCM has not been installed yet, install it now. After starting SPCM the initialisation panel shown in Fig. 36, left should appear. The installed modules are marked as ‘In use’. The modules are shown with their serial number, PCI address and slot number.

If the SPC module is not found at this stage for whatever reasons, the software starts in an emulation mode (see below, ‘Starting the SPC software without an SPC module). What you see in this mode is generated by the software or loaded from a file - it is not the data recorded by the SPC module.

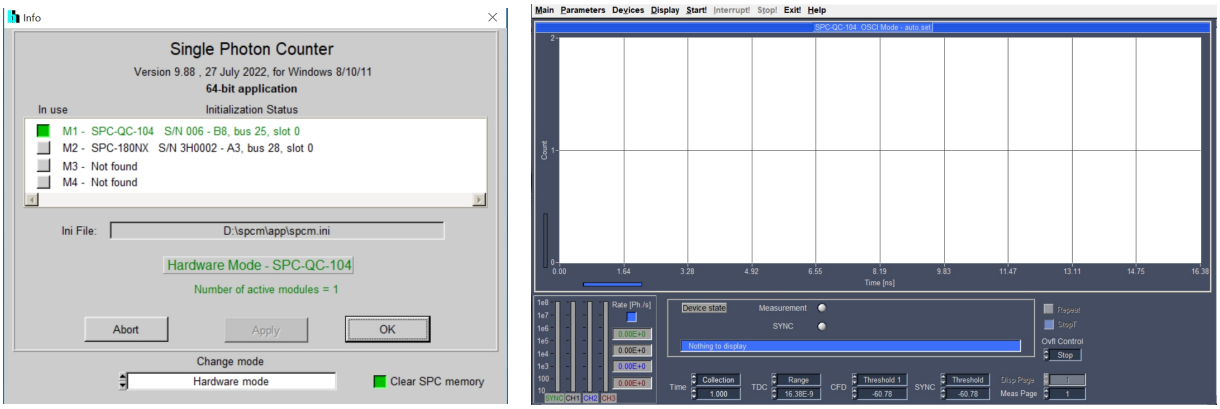


Fig. 36: Starting the SPCM software. Initialisation panel (left) and main panel of SPCM (right).

The software runs a hardware test when it initialises the modules. If an error is found, a message ‘Hardware Errors Found’ is given and the corresponding module is marked red. In case of non-fatal hardware errors you can start the hardware mode of SPCM by selecting ‘Hardware Mode’ in the ‘Change Mode’ panel. However, please note that this feature is intended for trouble shooting and repair rather than for normal use.

When the initialisation window appears, click on ‘OK’ to open the main window of the SPCM software (Fig. 36, right). At the first start the software comes up with default parameters which may not be appropriate for your measurement problem. Therefore, changes may be required for your particular application. Please see section 'SPCM Software'.

Firmware Update

It can happen that the SPCM version you are running is not compatible with the firmware of the SPC-QC module. In this case the initialisation panel display a warning as shown in Fig. 37.

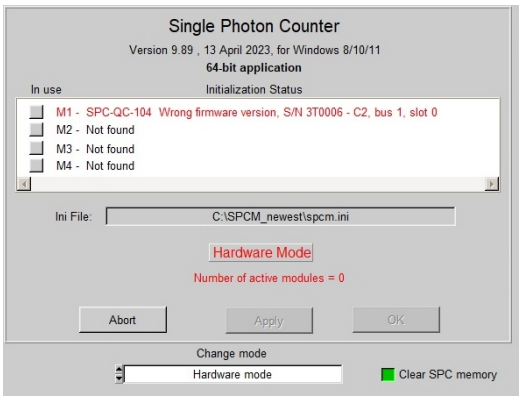


Fig. 37: Warning: Firmware not compatible with SPCM software version

When the warning appears you either have an old software version or an old hardware version. A software update is performed as shown under 'Software Installation'. For a hardware update, please proceed as shown in Fig. 38. The correct firmware is then transferred from the TCSPC Installation package into the SPC-QC module (Fig. 39).

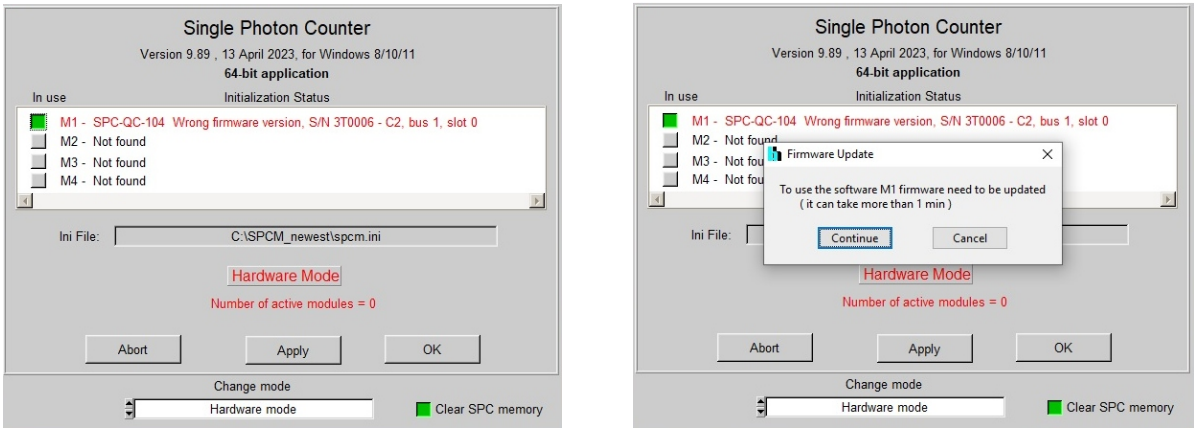


Fig. 38: Left: To update the firmware click the 'In Use' Button and the OK button. Right: Follow the instructions of SPCM

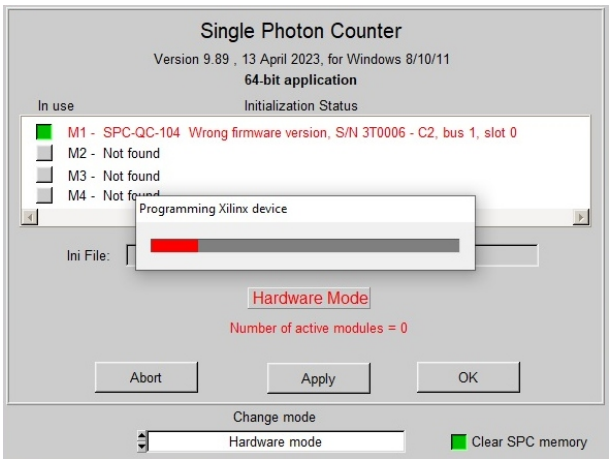


Fig. 39: The correct firmware is transferred from the TCSPC installation package into to SPC-QC module.

Starting the SPCM Software without a SPC-QC Module

You can use the SPCM Software without a SPC-QC module. In its start window the software will display a warning that the module is not present, see Fig. 40.

To start the software for the desired module type, click into the ‘Change Mode’ field and select the desired module type from the list. Then click on ‘Apply’, ‘OK’. The software will start in a special mode and emulate the SPC-QC device memory in the computer memory. You can set the TCSPC system parameters, load, save, process, convert and display data, i.e. do everything except for a real measurement.

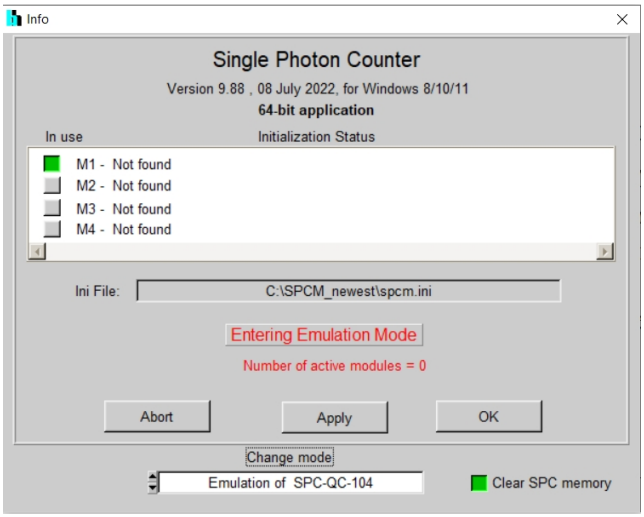


Fig. 40: Startup panel in the emulation mode (without a TCSPC module)

First Light

This section describes the general procedure for the initial setup of a measurement system with the SPC-QC4. For simplicity, the procedure is described at the example of a simple fluorescence-decay experiment. Complex systems, such as FLIM systems with scanners, dual or triple channel systems, systems with multi-wavelength detection, or systems with laser multiplexing are based on the same general principles, and the initial setup can be done in a similar way.

A basic system is outlined in Fig. 41. A pulsed light source - usually a ps diode laser - is pointed into a fluorescent sample, and the fluorescence light is detected by a single-photon detector. A filter or a package of filters may be used in front of the detector to make the setup daylight-compatible and to suppress scattered excitation light. Additional optical elements are not required for basic system setup, although they are certainly present in more complex experiments.

The synchronisation signal from the laser is connected to the SYNC input of the SPC-QC module, the detector signal to one of the CFD inputs. Systems with PMCS-150 detectors, id-100 SPAD detectors or other SPADs work as indicated in Fig. 41, left. The id-100 requires a pulse inverter as indicated in the figure. For HPM-100 detectors or PMC-150 detectors a DCC-100 detector controller is necessary, see Fig. 41, right.

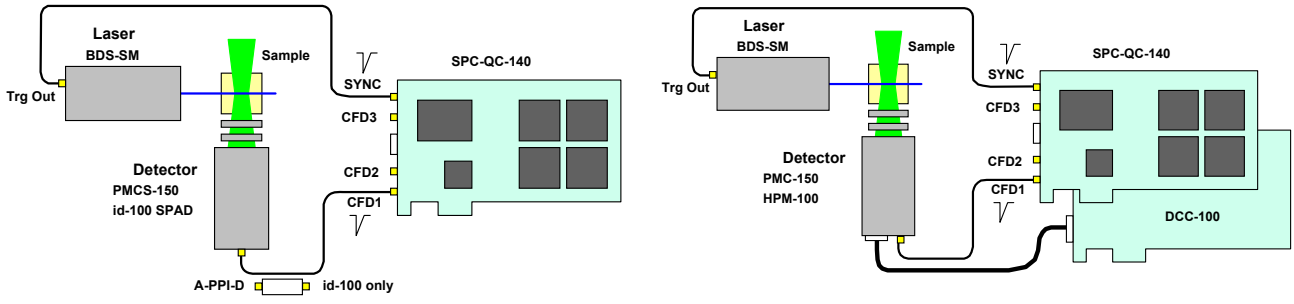


Fig. 41: Basic fluorescence-decay measurement system

When all system connections are in place turn on the system computer, and start SPCM. For the initial setup, we recommend to use system parameters as shown in Fig. 42.

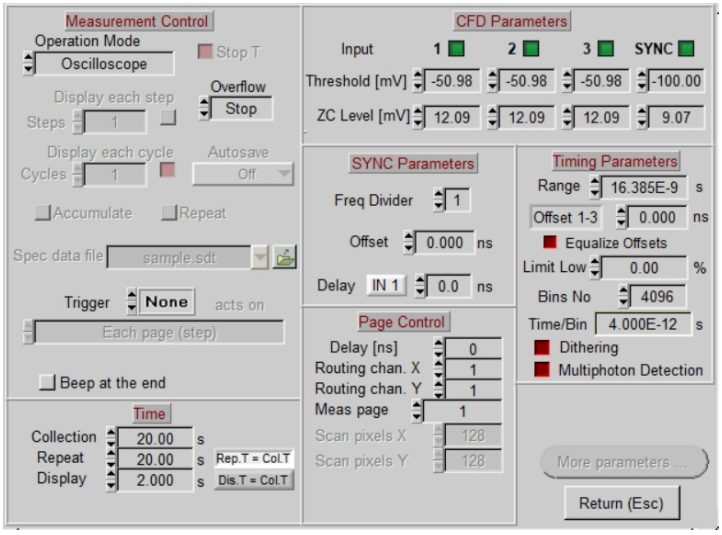


Fig. 42: System parameters for initial system setup

Operation mode is 'Oscilloscope', collection time is 1 second. Trigger is 'None'. All detector inputs and the Sync input are enabled. The thresholds of the detector and Sync discriminators are -60 mV, the zero-cross levels are +12 mV. The Timing (TDC) Parameters are: Range = 16 ns, Offset = 0, Limit Low = 0, Channel Number 4096. This gives a time-Channel width of 4 ps. Memory Offset is zero. The page control parameters are: Delay = 0 ns, Routing X and Y = 1, Measurement Page =1. With these settings, the TDC-QC should record at least some photons for all conceivable detectors and under all conceivable circumstances.

The display of curves in SPCM is controlled by the Trace Parameters in combination with the Display Parameters. To display the desired data these parameters must be appropriately set. The recommended Trace Parameter setup is shown in Fig. 43.

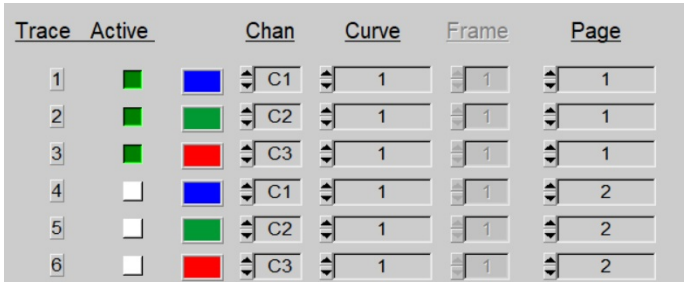


Fig. 43: Trace Parameters for system setup

With the parameters shown, SPCM displays three individual curves built up from the photons in the specified channels. The colours for curve 1, 2, and 3 are blue, green, and red, respectively. With the detector connected to CFD 1 (see Fig. 41) a blue curve should show up. Traces (4 to 6) for a second memory page are reserved but not turned on.

Curve style, vertical range, and background / grid colours are defined by the 'Display Parameters'. For system setup we recommend the settings shown in Fig. 44.

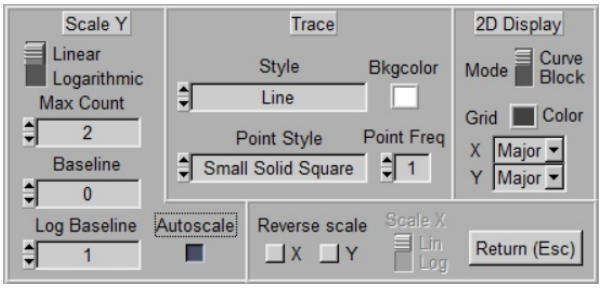


Fig. 44: Display Parameters for system setup

With the settings shown above the user interface should be as shown in Fig. 45. The curve window is resizable - we recommend to pull it up to the full size as shown in the figure.

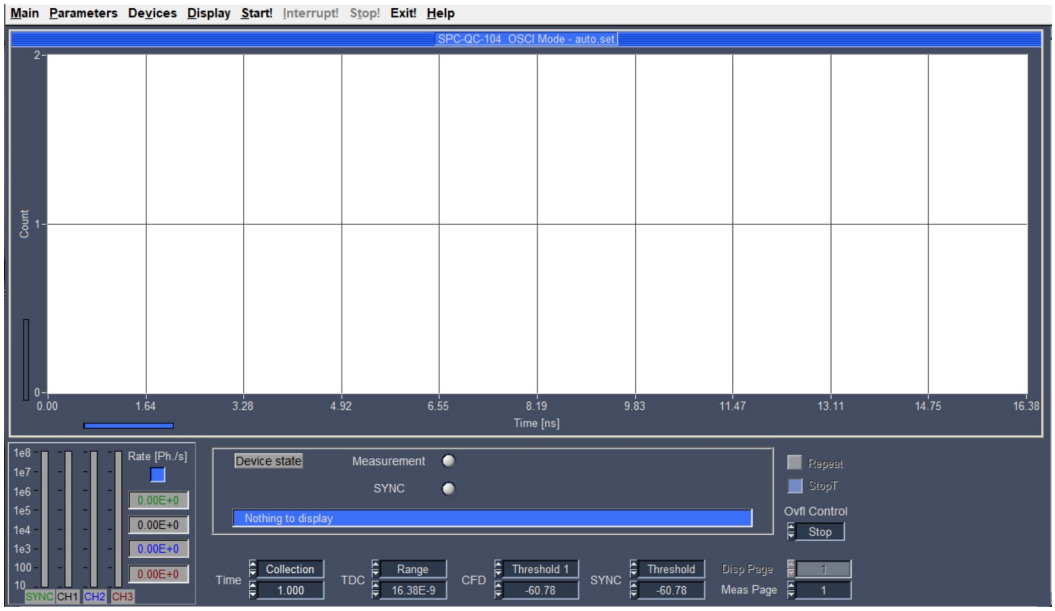


Fig. 45: SPCM user interface for system setup

When the main panel is as in Fig. 45, turn on the laser and the detector. If the laser has selectable repetition rate, use a frequency of 50 MHz to 80 MHz. The 'SYNC' rate bar in the lower left should show the laser repetition rate. The rate bar for Channel 1 should show a photon rate. For detectors which are controlled via a DCC card, enable the DCC output to which the detector is connected, and increase the 'Gain' until you see a count rate in Channel 1. Details of detector setup are described in the bh TCSPC Handbook [1], chapter 'System Optimisation'.

When you click the Start button a decay curve should show up in the curve window. If you are lucky it looks as shown in Fig. 46, page 35: The decay curve is perfectly placed in the observation-time interval and it has been recorded at a reasonable time scale. No further refinements are needed in this case.

In most cases, however, the decay curve will be badly shifted, or there will be no decay curve visible at all. If no decay curve shows up the reason is almost certainly a mistake in the optical setup or in the detector setup. Make sure that the detector is turned on, that the detector has not been driven in an overload shutdown, and that it receives a reasonable amount of fluorescence light. If necessary, try with more or less filters. If the detector is operated via a DCC card make sure that a reasonable detector gain is set. Please refer to the detector-setup instructions in the bh TCSPC Handbook [1], chapter 'System Optimisation'.

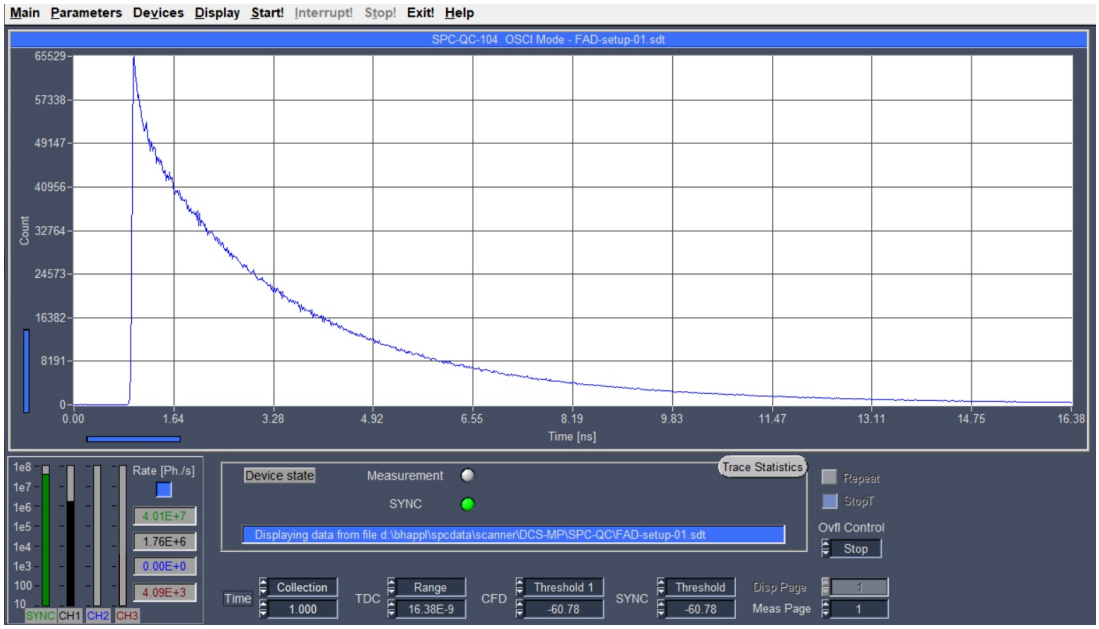


Fig. 46: Laser running, detector receiving photons, Measurement started, decay curve displayed in curve window

A shift in the curve results from different optical and electrical signal transit time in the Sync and detection channel. To correct it, change the Offset in the TDC timing parameters, or the Offset in the SYNC parameters, see System Parameters, Fig. 42. To conveniently adjust the offset during the measurement, the Offsets are also accessible directly from the main panel, see Fig. 47. Increasing the TDC offset shifts the curve to the right, increasing the SYNC offset shifts the curve to the left, see Fig. 48.

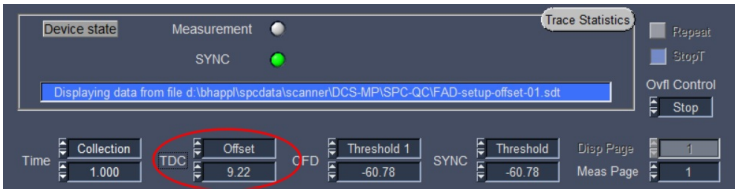


Fig. 47: Changing the TDC Offset from the main panel

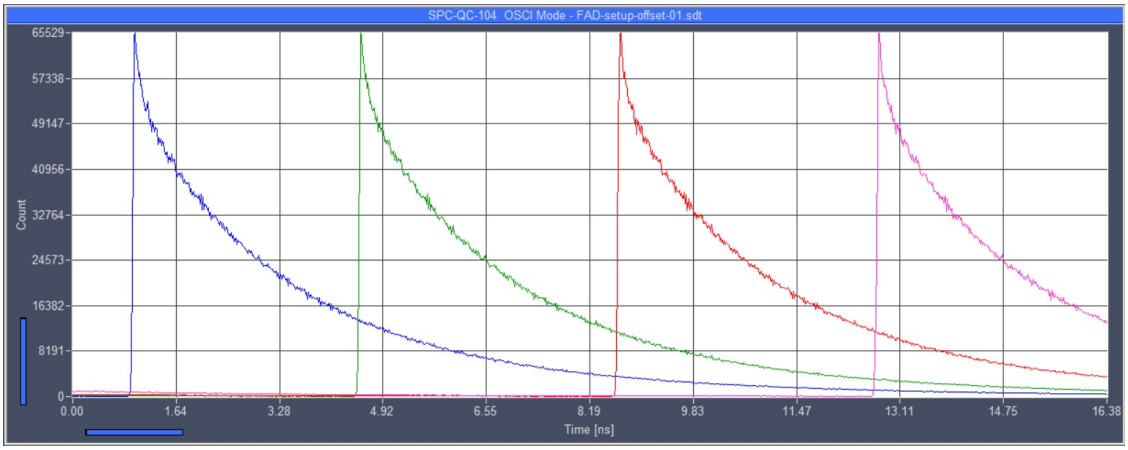


Fig. 48: Shifting the curve by the TDC Offset parameter. From left to right, the curves were recorded with increasing Offset.

The parameters used above are appropriate for fluorescence decay times on the order of a few nanoseconds. For longer decay times a different recording time interval may be desirable. The interval

is defined by the TDC Range parameter, see 'System Parameters', Fig. 42. Recordings with different TDC ranges are shown in Fig. 49. With decreasing range the curves are stretched to the right, as can be seen in Fig. 49. Please note that very long recording time intervals may require lower laser repetition rate.

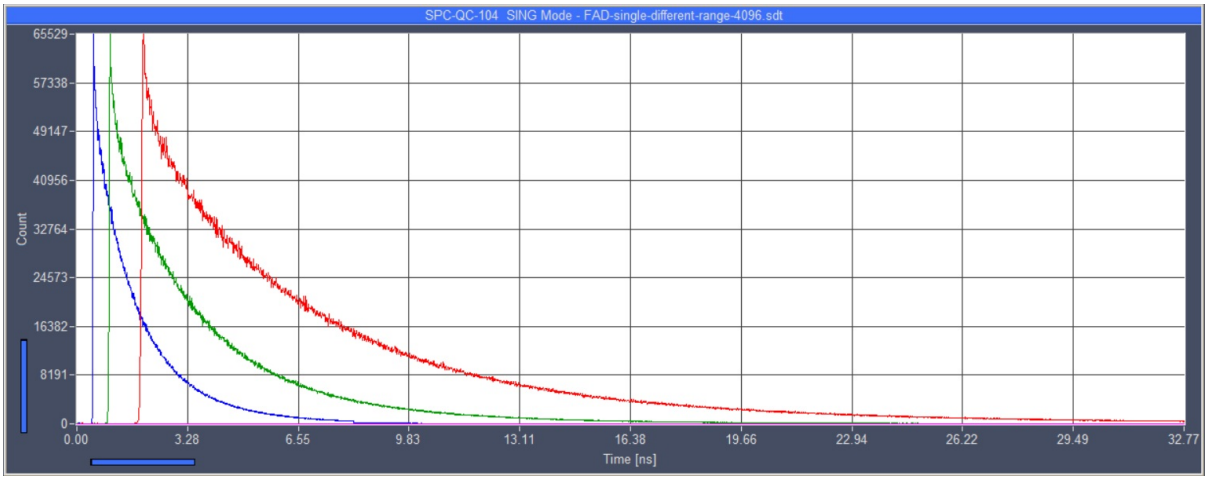


Fig. 49: Fluorescence decay recorded with different TDC Range

SPCM Software

Overview

All bh TCSPC modules come with bh's SPCM data acquisition software. SPCM runs the data acquisition in the various operation modes of the SPC modules while controlling peripheral devices, such as detectors, lasers, scanners, or motor stages [1]. Operation modes are available for almost any conceivable TCSPC application. There are modes for fluorescence and phosphorescence decay recording, multi-wavelength decay recording, laser-wavelength multiplexing, recording of time series, FCS and photon counting histograms, and there are modes for FLIM, multi-wavelength FLIM, Mosaic FLIM, time-series FLIM, Z stack FLIM, and simultaneous FLIM/PLIM. Since July 2019 SPCM comes with extended multi-threading capabilities, greatly improving the throughput rate even in case of complex online data and display operations.

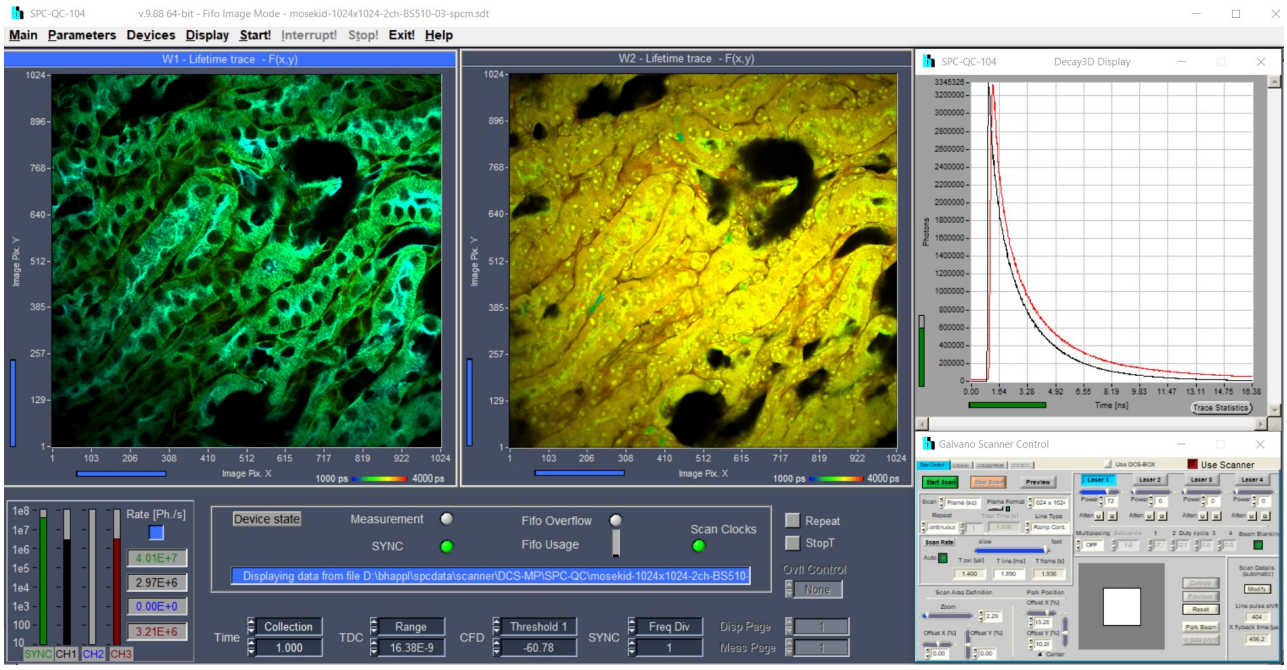


Fig. 50: Example of SPCM Main panel. Dual-channel FLIM, DCS-120 scanner, online-lifetime display, decay curves in ROIs of images

SPCM includes the management of measurement and control parameters, user-interface configuration parameters, and parameters for online data visualisation. Load and save functions are provided to handle measurement and setup data. A large part of SPCM provides display functions for multi-dimensional data. There are display functions for decay curves, series and arrays of decay curves, intensity and lifetime images, and arrays of images. Different projections can be used to create images from selected planes within a multi-dimensional data array. Display functions also contain online calculation and display of decay curves from ROIs within lifetime images. A direct link is provided for communication with SPCImage NG FLIM analysis software.

A 150-page description of the SPCM software is available in the bh TCSPC Handbook [1], chapter 'SPCM Software'. Most of the SPCM functions can be used for the SPC-QC modules as described there. The following sections describe features which are either essential to the function of all TCSPC modules or special for the SPC-QC. For details of functions not described here, please refer to the bh TCSPC Handbook.

System Parameters

The System Parameters define the internal functions of the SPC or SPC-QC module hardware and the data transfer between the hardware and the software. The parameters include the operation mode, control parameters for sequential measurements, page stepping, repeat, accumulation and autosave functions. Furthermore, the system parameters control the settings of the CFD, SYNC, and TDC parameters as well as the routing and scanning parameters. Because not all parameters may be applicable to all operation modes and SPC modules some details of the system parameter panel change with the operation mode and module type. A typical system parameter panel for an SPC-QC-104 system is shown in Fig. 51.

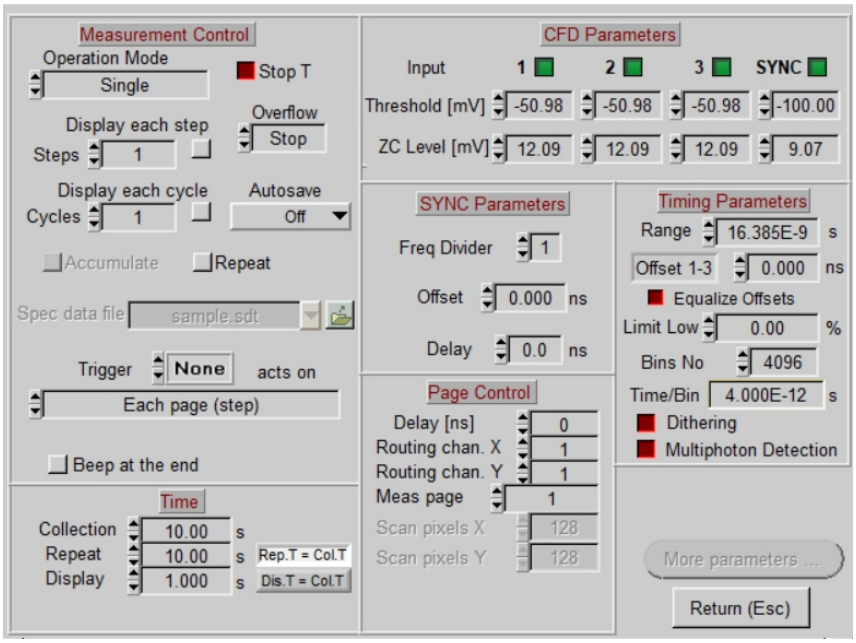


Fig. 51: SPCM system parameter panel, SPC-QC-104, 'Single' mode

Operation Mode

Available operation modes of the SPC-QC-104 are Single, Oscilloscope, F(txy), F(t,T), F(t,EXT), FIFO, FIFO Absolute Time, and FIFO Imaging, see Fig. 52. Deatails of the operation modes are described in the sections below.

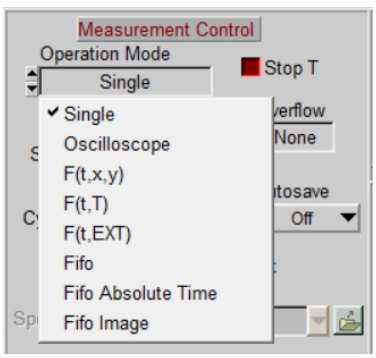


Fig. 52: Operation modes of the SPC-QC-104

Single Mode

In the 'Single' mode the SPC-QC performs a classic TCSPC measurement. In the simplest case it records a fluorescence decay curve or another waveform of an optical signal. An example is shown in Fig. 53. The recording can be run over a predefined collection time ('Stop T'), continued until 65.535 photons are recorded in the maximum of a curve ('Stop Overflow') or continued until the operator manually stops the measurement, see 'Measurement-Control Functions', page 46. With the three recording channels of the SPC-QC three such measurements can be performed simultaneously. The number of time channels per recording channel can be up to 65535. The figure below shows a fluorescence decay curve of quinine sulphate. Please see also Fig. 1, page 6 of this handbook.

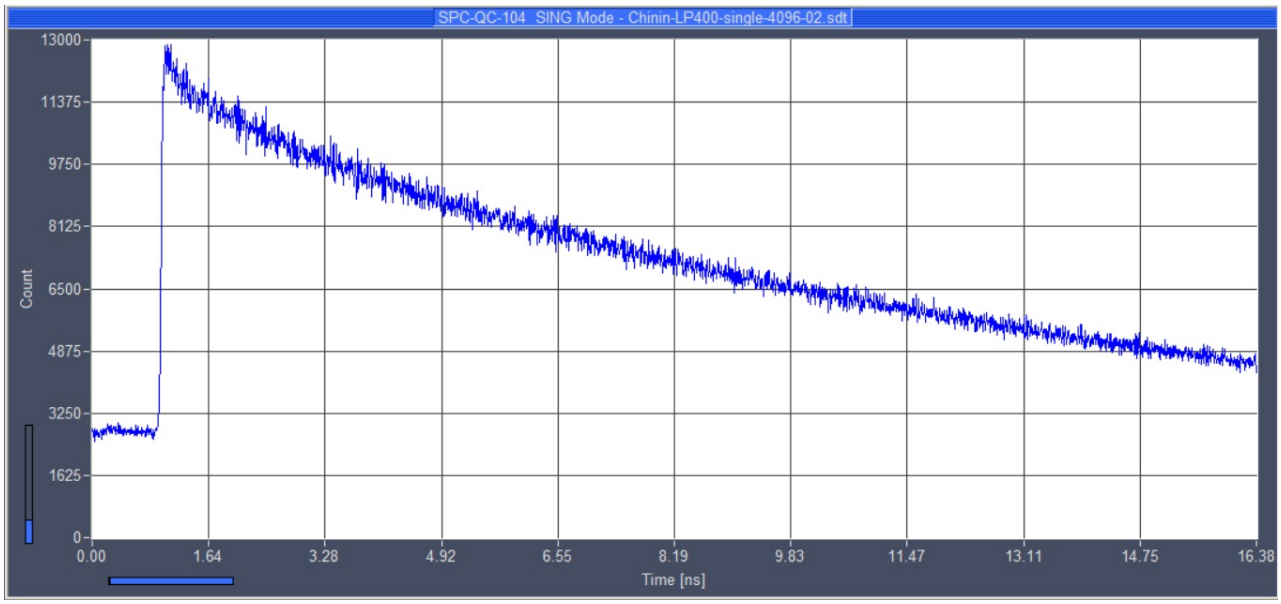


Fig. 53: Fluorescence decay curve of quinine sulphate, recorded in the 'Single' mode

Oscilloscope Mode

In the 'Oscilloscope' mode the SPC-QC performs a fast repetitive measurement in intervals of the selected 'Collection Time'. Parameters and control features are the same as for the Single mode.

F(t,x,y) mode

F(t,x,y) is a one- or two-dimensional routing mode. Typically, signals are recorded by a multi-anode detector. The channels are arranged in one dimension along an x coordinate or in two dimensions along an x coordinate and a y coordinate. The recording procedure builds up a photon distribution over t (the photon time) and x (the distance along x) or a photon distribution over t and the distances along x and y. The main application is multi-wavelength decay recording by the process described in Fig. 24. A typical result is shown in Fig. 54.

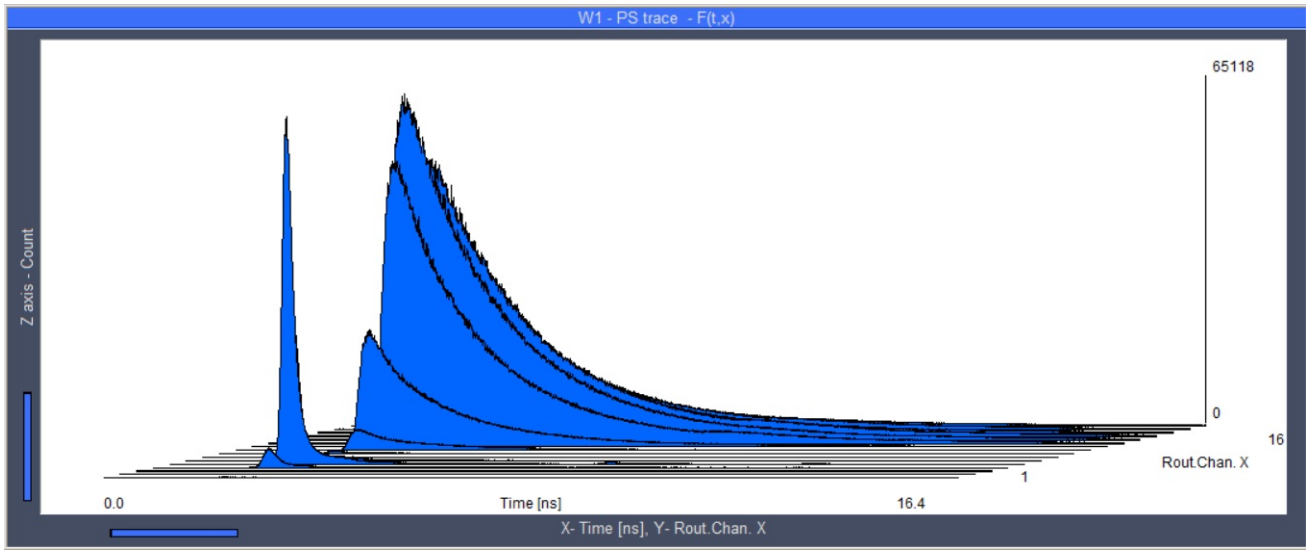


Fig. 54: F(t,x,y) mode. Multi-wavelength fluorescence-decay measurement

F(t,T), F(t,EXT) modes

F(t,T) and F(t,EXT) are sequential recording modes. Sequences of fluorescence decay curves or other optical waveforms are recorded as a function of time or as a function of an external parameter. Three such sequences can be recorded by the thee channels of the SPC-QC. The individual channels can be further extended by laser wavelength multiplexing. The measurement-control parameters are the same as for the SPC modules. Please see [1].

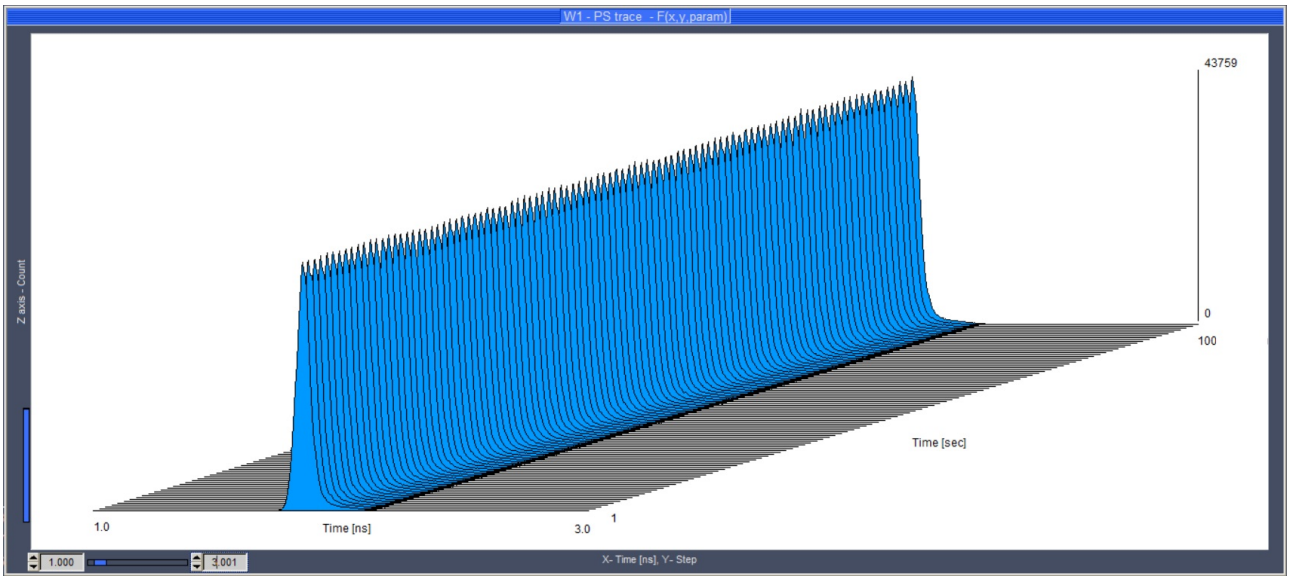


Fig. 55: F(t,T) mode. Sequential recording of an optical signal.

FIFO Mode

In the FIFO mode the SPC-QC creates a stream of time- and parameter-tagged single photon data and sends them to SPCM. For each photon, the module determines the time in the excitation pulse period, the time from the start of the recording, and status of up to four routing bits. Moreover, 'Markers' can be inserted in the data stream to tag the times of external events and associate them to the photons.

SPCM stores these data for further analysis, and/or immediately calculates decay curves, auto- and cross-correlation functions, photon counting histograms, or time traces (or Multichannel Scaler, MCS curves) from them. A special feature is triggered MCS recording, which is used for simultaneous fluorescence and phosphorescence decay recording. Three channels can be recorded simultaneously, each channel can be extended by routing.

User Interface

The user interface of a typical FIFO mode experiment is shown in Fig. 56. Fluorescent nanoparticles are diffusing through the focus of a high-repetition rate laser. When a particle passes the focus it emits burst of photons. The photons are detected by a fast detector, and the corresponding detection events are recorded by the SPC-QC module and transferred to the SPCM data acquisition software. SPCM writes the photon data into a file and, simultaneously, builds up decay curves, FCS curves, and intensity (MCS) traces.

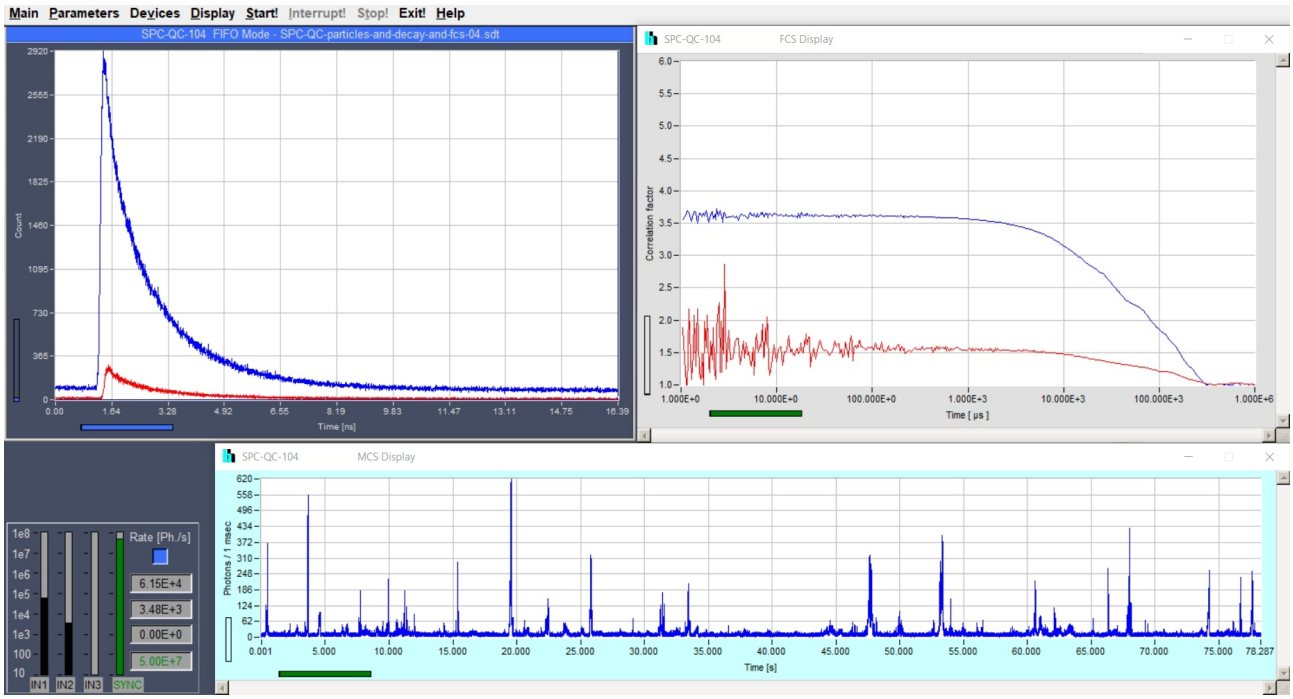


Fig. 56: FIFO Mode. Decay curves, FCS curves, and intensity (MCS) trace of fluorescent particles diffusing through a laser focus

System Parameters

System parameters for a FIFO mode measurement are shown in Fig. 57. Operation Mode is FIFO and the individual photons are saved into a .spc file, see upper left of the system parameters. The runtime display has been configured to calculate and show decay curves, FCS curves, and an MCS (intensity) trace. Details of the runtime configuration are defined under 'Configure'. The corresponding panel is shown on the right. Discriminator and timing parameters are the same as in the other operation modes. Please see 'CFD Parameters' and 'TDC Parameters'.

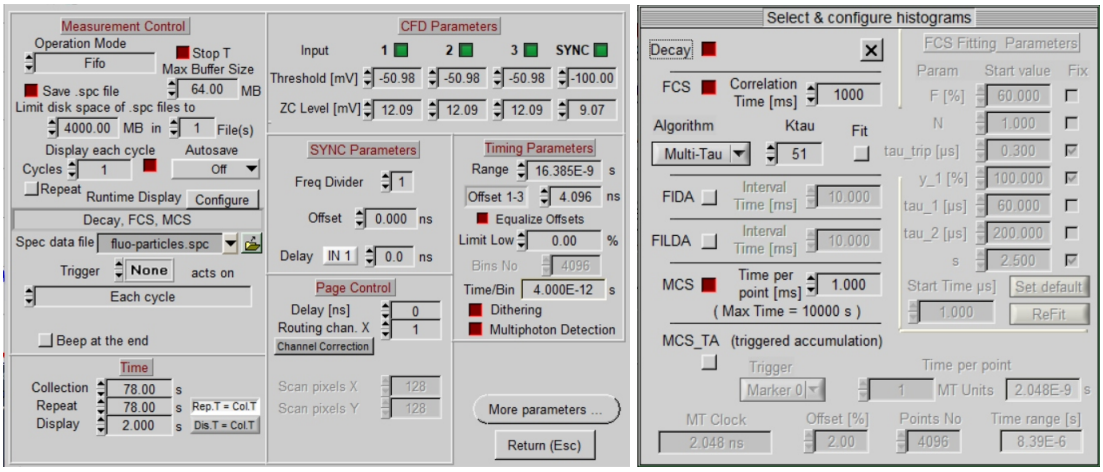


Fig. 57: FIFO Mode. Left: Typical system parameters. Right: Parameters for runtime calculations and runtime display.

More Parameters

The 'More Parameters' button opens a panel that contains parameters which are specific for the FIFO Mode. The panel is shown in Fig. 58.

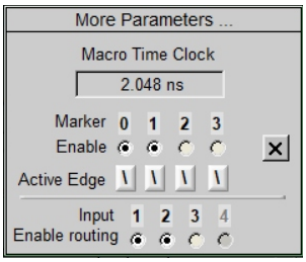


Fig. 58: 'More Parameters' in the FIFO mode

FIFO Absolute Time Mode

The absolute time mode is similar to the FIFO mode in that it creates a data stream of time- and parameter-tagged single photon data and sends them to SPCM. However, in the absolute time mode The SPC-QC hardware does determine relative times between the photons and the reference pulses at the SYNC input. Instead, it uses this input to record photons from a fourth detector. For each photon and each detector, the SPC-QC determines the time from the start of the recording, and the status of the routing bits. 'Markers' can be inserted in the data stream to tag the times of external events and associate them to the photons. SPCM stores these data for further analysis, and/or immediately calculates coincidence curves, auto- and cross-correlation functions, photon counting histograms, or time traces (or Multichannel Scaler, MCS curves) from them.

User Interface

A typical SPCM user interface configuration is shown in Fig. 59. The fluorescence of diffusing nanoparticles was detected in two spectral channels. The panel on the left shows the coincidences between the photons of the two channels on the nanosecond scale. The MCS trace, shown upper right, shows the burst of photon cause by particles travelling through the laser focus. An FCS curve is shown lower right. The drop of the curve (at about 10 ms correlation time) is an indicator of the dwell time of the particles in the focus.

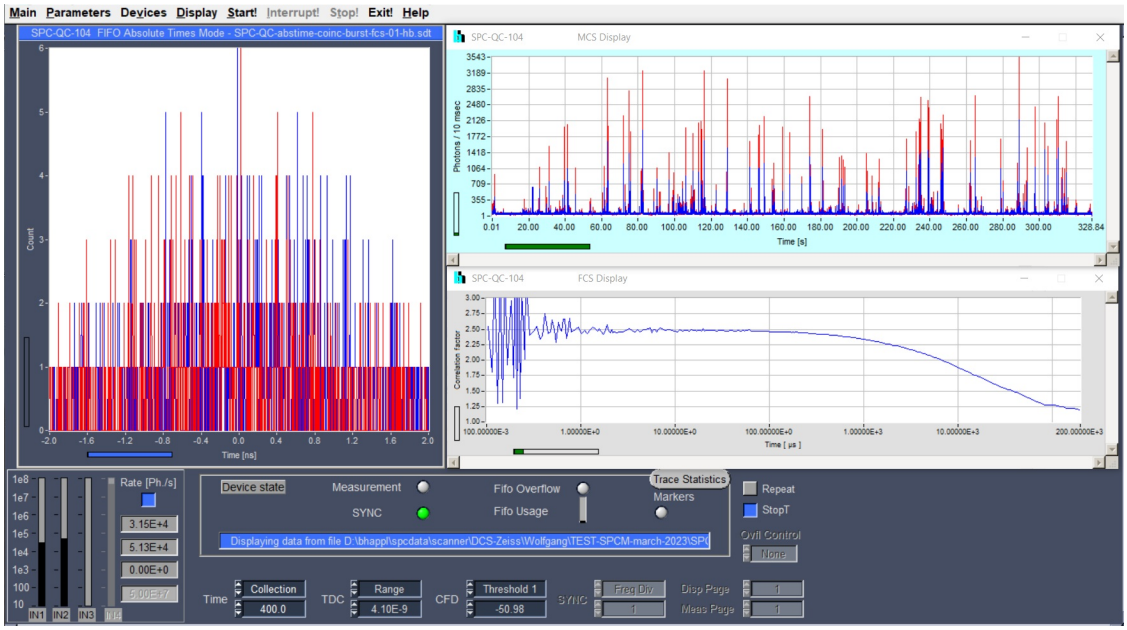


Fig. 59: Absolute-Time mode, SPCM user interface

System Parameters

Typical system parameters are shown in Fig. 60. Operation mode is 'FIFO Absolute Time'. The time-tagged data of the photons are store in a file 'coinc-burst.spc'. Three of the four input channels of the SPC-QC are enabled, the CFD parameters are set to record single-photon pulses from hybrid detectors. The timing (coincidence) range is 4.096 ns, the number of time bins is 1024. Time / Bin is thus 4 ps. The 'Configure' parameters are shown in Fig. 60, right. The parameters are set to build up and display coincidence curves, FCS curves, and MSC traces.

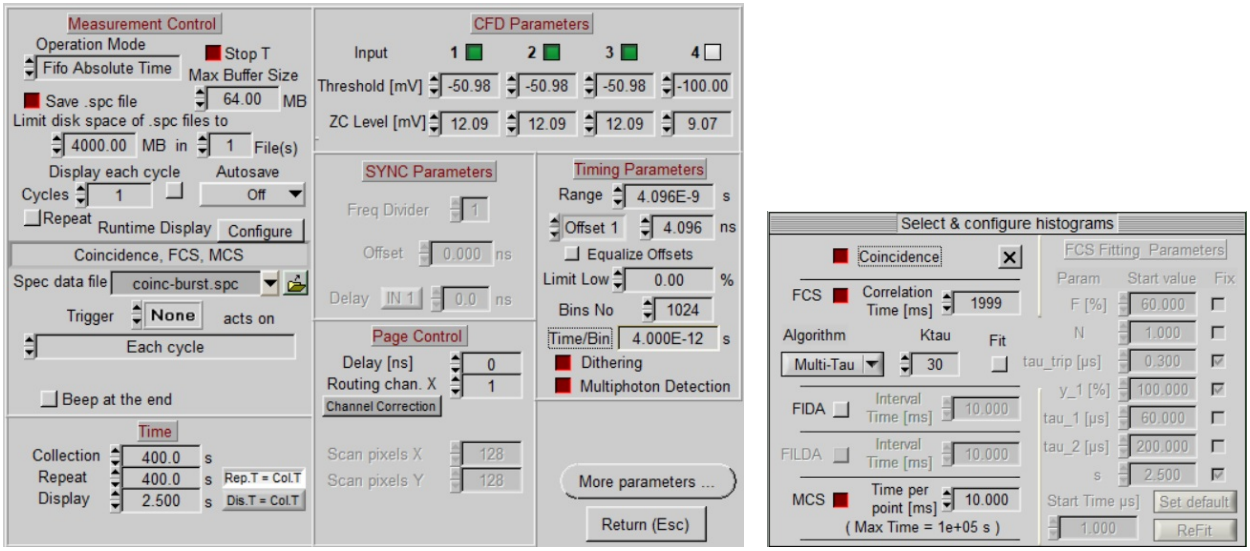


Fig. 60: Absolute-Time mode, system parameters.

More Parameters

The 'More Parameters' button opens a panel that contains parameters which are specific for the Absolute Time Mode. The panel is shown in Fig. 61.

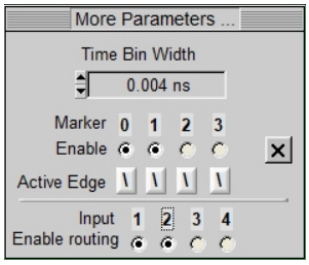


Fig. 61: 'More Parameters' in the Absolute Time mode

FIFO Imaging

The FIFO Imaging mode builds up FLIM data from a stream of time- and parameter-tagged single-photon and pixel, line, and frame marker data. Each pixel marker indicates the transition to the next pixel, each line marker the transition to the next line, and each frame marker the transition to the next frame. SPCM analyses these data, determines the times of the individual photons and the x-y position of the scanner in the moment of their detection, and builds up a photon distribution over these parameters. The recording process is illustrated in Fig. 25, page 23. The result is an array of pixels, each containing a fully resolved decay curve in a large number of time channels. Typical FLIM data formats are 512 x 512 to 2048 x 2048 pixels, with 256 to 1024 time channels per pixel. An example is shown in Fig. 62. It shows a typical user interface for FLIM measurement. Two FLIM images from two spectral channels are recorded in parallel, the image resolution is 1024 x 1024 pixels with 1024 time channels per pixel. A decay-curve window is displayed upper right, the control panel of a bh GVD-140 scan controller is open lower right.

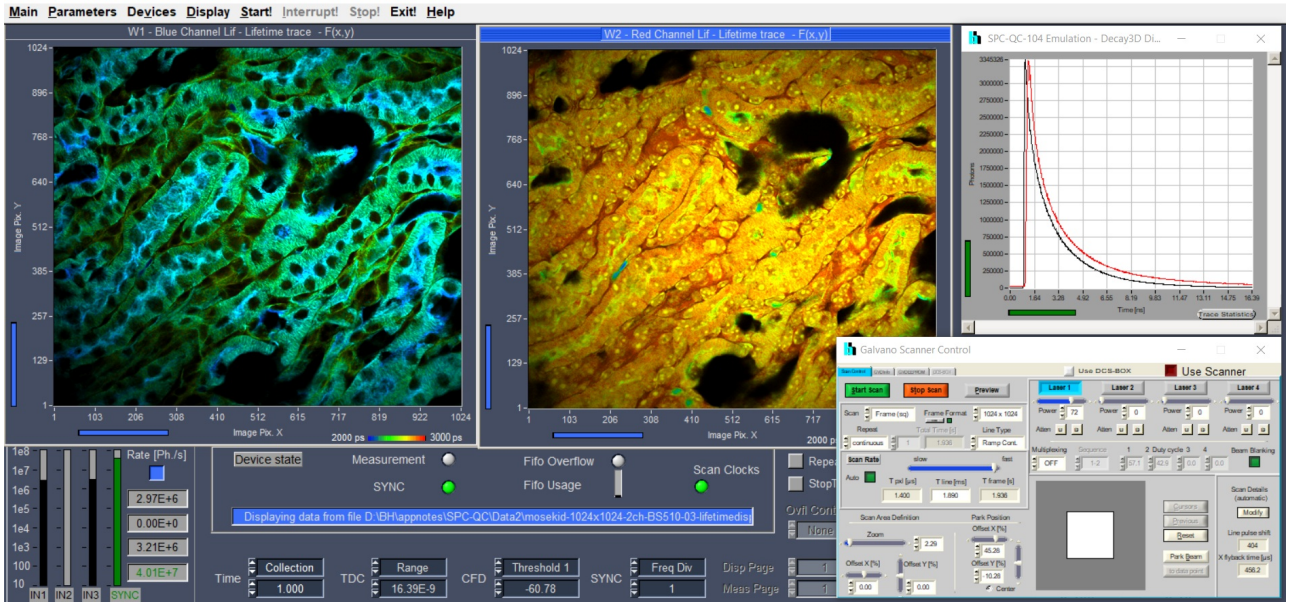


Fig. 62: FIFO Imaging Mode. User interface, two FLIM images with 1024 x 1024 pixels, decay curve window, and scanner control panel for DCS-120 system

System parameters for FLIM measurement are shown in Fig. 63. Operation Mode is 'FIFO Imaging'. Saving of single-photon data is 'off', autosave at the end of the measurement is 'off'. Sequential recording by the 'Cycle' function is 'off'. Collection time (lower left) is 20 seconds. The measurement can be stopped either after the defined collection time ('Stop T' 'on') or by a commend of the user ('Stop T' 'off'). In the setup shown the measurement is stopped by the user. The advantage is that the user can run the measurement until the number of photons in the image is high enough to provide the

desired lifetime accuracy. 'Stop T' should be used, however, if the intensities in several FLIM results must be comparable, or if a series of images are recorded.

The CFD parameters (centre top) are set for HPM-100 hybrid detectors, the Sync discriminator is set to detect the synchronisation pulses from the laser. Channel two is turned off because it is not used.

The timing parameters are set to cover a time range of 16 ns with 1024 time bins. The time bin width (Time/Bin) is then 16 ps. (To reduce the bin with to 4 ps either increase the 'Bins No.' from 1024 to 4096, or decrease the 'Range'.)

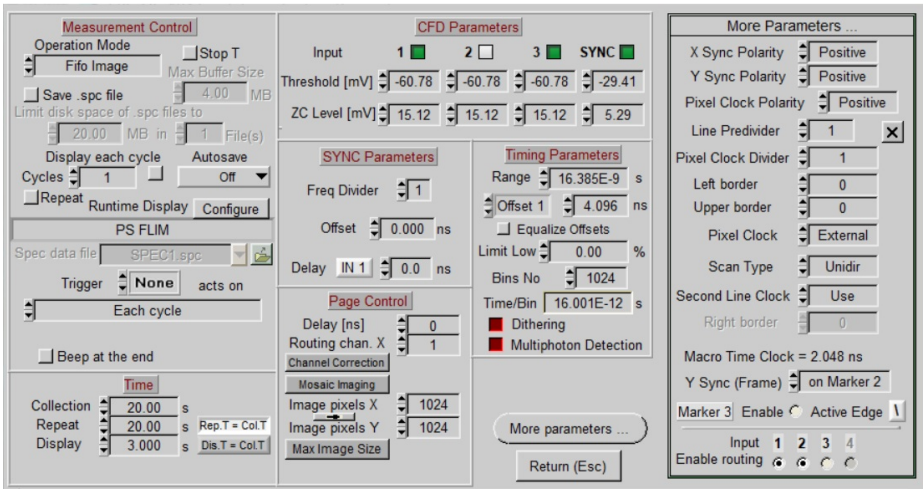


Fig. 63: System parameters for FLIM, DCS-120 scanner

Scan Parameters

A click on 'More Parameters' opens the panel shown in Fig. 63 on the right. The parameters define the interaction of the recording process with the scanning. They are shown for a DCS-120 confocal or multiphoton system. The polarity of the sync pulses is 'Positive', line and pixel clock dividers are '1'. That means that the FLIM image is recorded with the same number of pixels and lines the scanner is scanning. Pixel clock is 'External' - it comes from the scan controller. It can be set to 'internal' for scanning systems which have no pixel clock. (We discourage to use such systems for FLIM unless this is absolutely needed)

'Scan Type' is 'unidirectional'. Recording with bidirectional scanning is possible, but not recommended for various reasons [1]. Second Line Clock is on 'Use'. There are scanning systems which deliver a line clock also at the end of each line. In these cases it must be on 'Skip'. The recording procedure than skips every second line clock, avoiding that the line flyback is mistaken as a normal line of the scan. Marker 3 is disabled. It is used for 'FLITS' - a procedure to record fast dynamic lifetime effects by line scanning [1]. Routing inputs 1 and 2 are enabled for possible laser wavelength multiplexing.

System parameters for the bh FLIM systems for Zeiss laser scanning microscopes [7, 8] are shown in Fig. 64. The only difference to the DCS-120 setup is the pixel number of 512 x 512 and the 'Left Boarder' of 24 pixels. This adapts the recording to the default pixel number in the Zeiss Zen software. For LSM scans of 1024 x 1024 or 2048 x 2048 increase the pixel numbers in the system parameters. 'Left Boarder' = 24 accounts for the fact that the valid image area begins 24 pixels after the line clock. However, not all Zeiss LSMs need this 24-pixel delay, therefore you may have to use '0' for some systems.

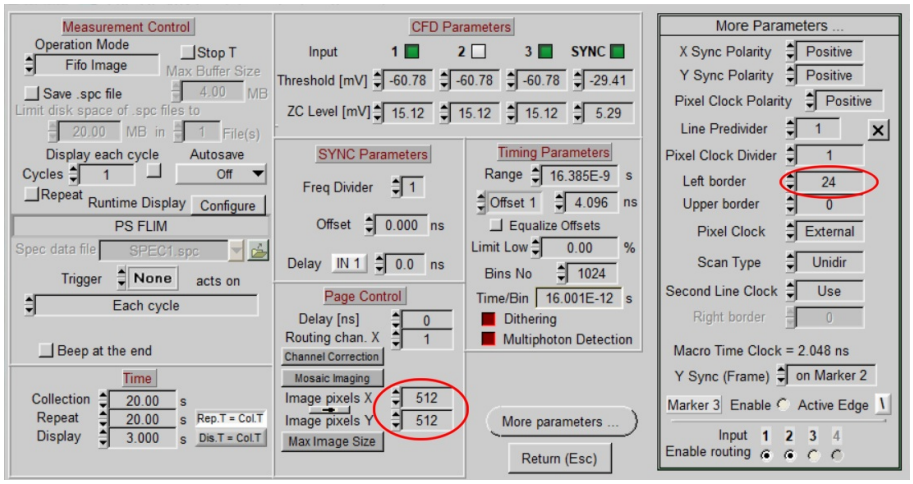


Fig. 64: System parameters for FLIM with the Zeiss LSM laser scanning microscopes

Zeiss FLIM users are often record FLIM with pixels numbers smaller than the scan in the microscope. You can do this by increasing 'Line Divider' and Pixel Clock Divider' in the 'More Parameters' panel. For example, 'Line divider' = 2 and 'Pixel clock divider' = 2 records a 256 x 256 pixel FLIM image with a 512 x 512 pixel LSM scan. The habit to reduce FLIM pixel numbers stems from the time of 32-bit windows when large FLIM images were difficult to handle. For modern 64-bit systems reducing the FLIM format is not recommended. If you have signal-to-noise problems better use binning in SPCImage NG data analysis [23].

With the SPC-QC module two or three FLIM images can be built up in parallel. Multi-wavelength detection with a MW-FLIM GaAsP detector is possible if the second and the third SPC-QC channels are not used.

Another extension of the FLIM process is 'Mosaic FLIM'. In combination with a motorised sample stage, SPCM records FLIM images of large sample areas without being limited by the limited field of view of the microscope lens. Moreover, Mosaic FLIM can be used to record extremely fast time series of FLIM images. By using periodic stimulation of a transient process in the sample and synchronisation of the mosaic recording with the stimulation, it is possible to accumulate such 'temporal mosaic' data over many stimulation periods. The speed of the mosaic series is then no longer limited by the decrease in the photon number. To use these functions, please refer to the 'bh TCSPC Handbook' [1], chapter 'SPCM Software'.

The FIFO Imaging mode is also able to build up lifetime images from MCS data. It can do so even simultaneously with the build-up of FLIM data. The result is simultaneous FLIM / PLIM, a method which has found wide application in live sciences since its introduction by bh in 2015. Please see bh TCSPC Handbook [1], chapter 'Simultaneous FLIM/PLIM'.

Measurement-Control Functions

Defining a Measurement Sequence

The measurement procedures described under 'Operation Modes' run a single measurement in the selected mode. It is in the responsibility of the user to stop the measurement when the desired number of photons have been recorded, to save the data, and possibly repeat the measurement under modified external experiment conditions. With the measurement-control functions measurements such actions

can be automised. The measurement can be stopped after a defined 'Collection Time' or when the histogram memory overflows, and the data can be saved automatically when the measurement is completed. A measurement can be started by a trigger pulse, measurements can be repeated, and the data of the individual measurement cycles can be saved into subsequent data files. A few of these functions are shown in Fig. 65 through Fig. 69.

Timing the Measurement

The timing of a measurement is controlled by the 'Time' parameters in combination with the 'Stop T' button, see Fig. 66, left. When 'Stop T' is set the measurement (or a measurement cycle) stops after the defined 'Collection Time'. 'Stop on Overflow' stops a 'Single' mode measurement when a data overflow in the maximum of the curve occurs. Please see Fig. 65, right. During the recording, intermediate results are displayed in intervals of 'Display Time'.

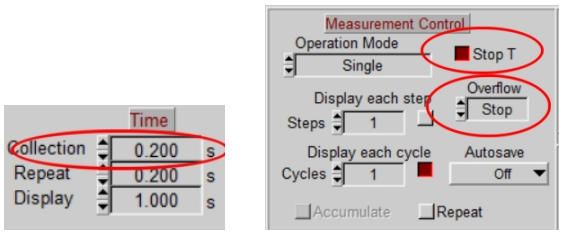


Fig. 65: Measurement timing.

Measurement Sequences

A sequential measurement can be defined by the 'Steps' and 'Cycles' functions, see Fig. 66, left and Fig. 67, left. 'Steps' defines a number of steps of a sequence of 'Single' mode recordings. Subsequent recordings are stored into subsequent memory pages (see 'Page Control' and 'Trace Parameters'). A new measurement step starts after a defined 'Repeat Time', see Fig. 66, second left. Data of a large number of steps are kept in the memory and saved when the defined number of steps has been completed. Saving can be performed by an 'Autosave' function, see Fig. 66, second right. The file name is defined under 'Spec Data File', see Fig. 66, right. The data are saved automatically at the end of the stepping sequence, see Fig. 66, second right. The file name is defined under 'Spec data file', see Fig. 66, right.

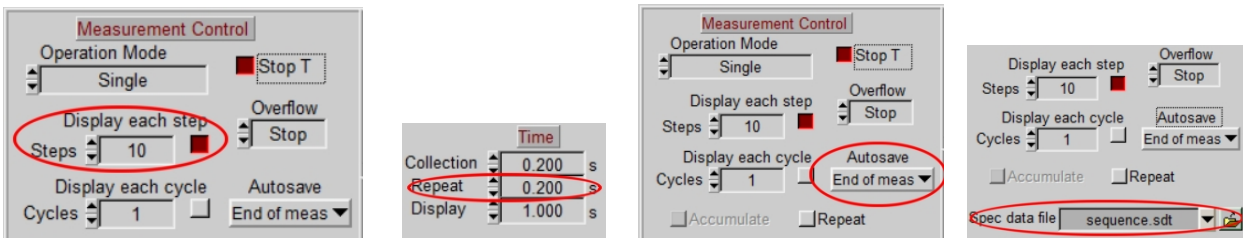


Fig. 66: Steps of a measurement sequence, Time control parameters, Autosave function, and file definition for Autosave

The page stepping sequence can be repeated by a defined number of 'cycles' see Fig. 67, left, and the cycles saved into subsequent data files, see Fig. 67, second right. The file name is defined under 'Spec Data File', see Fig. 67, right. The file name gets extended by a number, and automatically counts up for every new cycle.

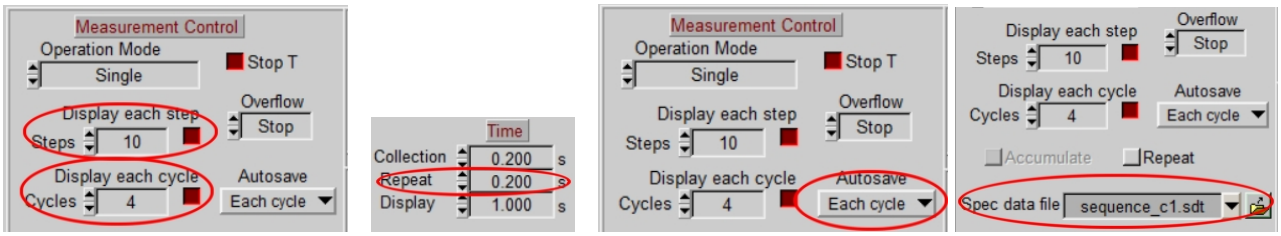


Fig. 67: Steps and cycles of a measurement sequence, Time control parameters, Autosave function, and file definition for Autosave

In the FIFO and FIFO Imaging Modes, and 'Absolute Time' modes measurement 'pages' and, consequently, the page stepping function do not exist - the data are too complex to be stored in simple 'memory pages'. However, the measurement can be repeated for a defined number of measurement 'Cycles', see Fig. 68, left. With the autosave function the results of the measurement cycles can be saved in subsequent data files, see Fig. 68, middle. The file names are defined under 'Spec data file', Fig. 68, right. As for the cycles in the 'Single' mode, the file name gets extended by a number, and automatically counts up for every new cycle.

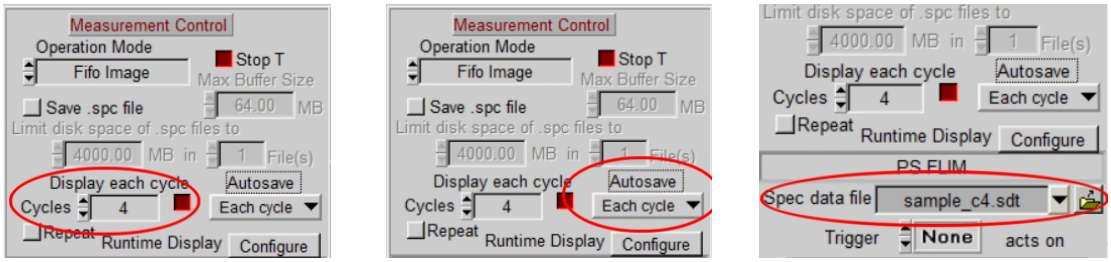


Fig. 68: Cycle function in the FIFO, FIFO Imaging, and Absolute Time mode

Saving Time-Tagged Photon Data

In the FIFO, FIFO Imaging, and 'Absolute Time' modes time- and parameter tagged data of the individual photons can be saved. Saving of the data is enabled as shown in Fig. 69, left. The name of the single-photon file is specified as shown in Fig. 69, right. The file extension is .spc. SPC files can become very large, therefore the file size can be limited to a reasonable number of megabytes. When this size is exceeded the measurement stops.

Fig. 69: Saving time-tagged single-photon data

Page Control

Page Control contains parameters related to the data structure of the results and the associated system configuration. 'Delay' is the time after the detection of a photon when the routing signals are read. 'Routing chan. X' and 'Routing chan. Y' are the numbers of routing channels in X and Y direction. Image pixels X and Y is the pixel format for lifetime images. Channel Correction opens a table by which the delay non-uniformity of the channels of a multi-wavelength detector can be corrected.

Mosaic Imaging opens a menu that defines the format of image mosaics, see [1]. 'Max Image Size' opens a menu to define the memory space claimed by SPCM. The 'Page Control' section is different for different operation modes. Examples are shown in Fig. 70.

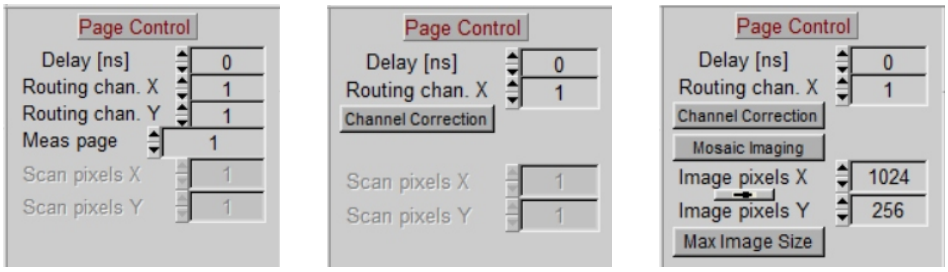


Fig. 70: Page Control section of SPCM system parameters. Left to right: Single Mode, FIFO Mode, FIFO Imaging Mode.

The Page Control parameters are the same as for other bh SPC modules. Please refer to [1] for details.

CFD Parameters of Detector and Sync Inputs

The CFD-parameter panel shown in Fig. 71. It contains the threshold and zero-cross levels of the input discriminators. In the Single, Oscilloscope, FIFO and FIFO Imaging mode it contains the discriminator settings for the three detector inputs and for the Sync input, in the Absolute Time mode it contains the settings for the four detector inputs. All inputs can be enabled or disabled by the buttons above the discriminator parameters. It is recommended to turn off inputs which have high-rate signals connected and are not used.

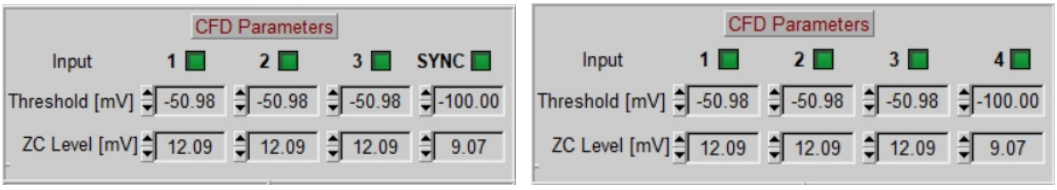


Fig. 71: CFD Parameters. Left: Single, Oscilloscope, FIFO and FIFO Imaging mode. Right: Absolute-Time mode

SYNC Timing Parameters

The 'SYNC Parameter' section of the System Parameters is shown in Fig. 72. It contains the Sync 'Frequency Divider' ratio, an 'Offset' to shift the effective timing reference in time, and the setting for a USB-controlled delay unit (see page 86) which may be connected in the SYNC signal path. With frequency-divider ratios larger than one several signal periods of the optical signals are recorded.

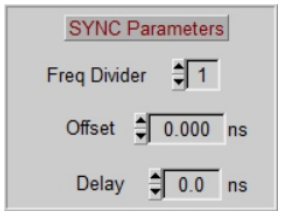


Fig. 72: SYNC Parameters

TDC Parameters

The TDC-parameter section controls the parameters for time measurement in the TDCs. 'Range' is the time-conversion range, 'Offset' the position of a recorded waveform inside the observation time interval, and 'Range' is the length of the observation time interval. Examples are shown in Fig. 48 and Fig. 49, page 35. 'Bins. No'. is the number of time channels, and 'Time/Bin' the time-channel width resulting from the selected combination of 'Range' and 'Bins No'. Please note that the minimum time-channel width is 4 ps. Therefore not all combinations of parameters are possible. SPCM therefore corrects the user inputs and suggests parameter combinations which are within the capabilities of the hardware. Examples are shown in Fig. 73 and Fig. 74.

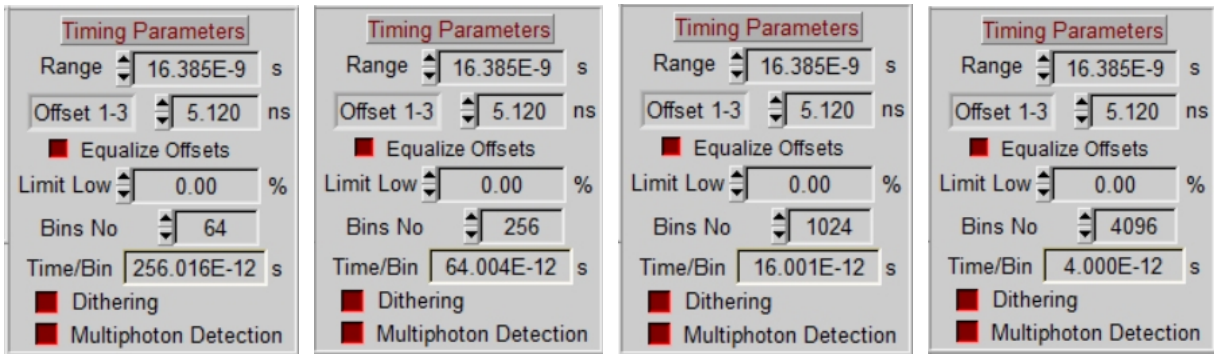


Fig. 73: TDC settings, different Channel Number with same Range. The combinations yield different Time/Channel.

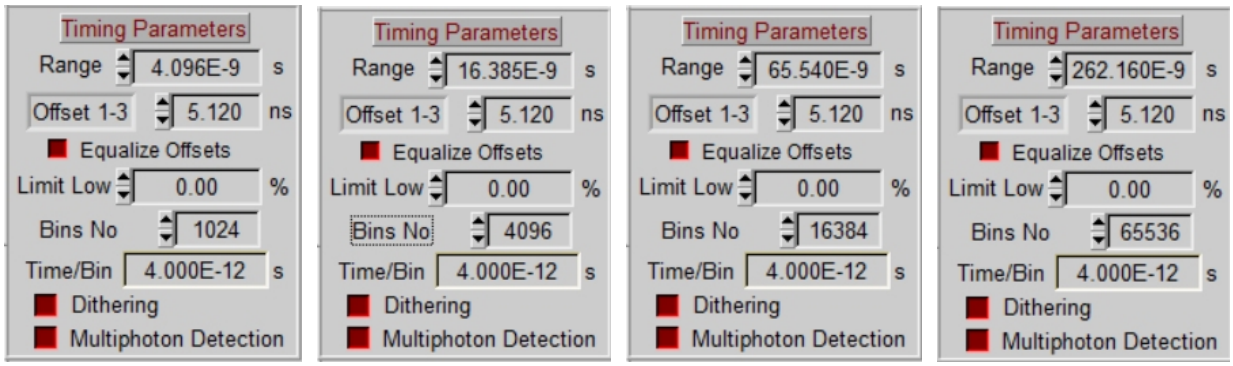


Fig. 74: TDC settings, different Range with same Time/Channel, different Channel Number.

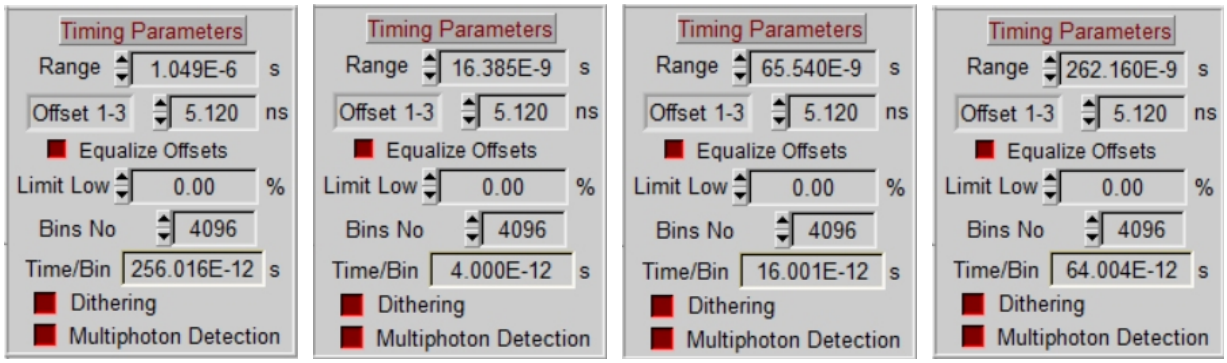


Fig. 75: TDC settings, different Range with same Channel Number, different Time/Channel

Not all combinations are available for all operation modes. For examples, the maximum channel number for FIFO Imaging is 4096, and for the FIFO mode channel number is always 4096.

More Parameters

'More Parameters' opens a panel showing parameters which are specific to the selected operation mode. For example, in the FIFO Imaging mode the 'More Parameters' define the synchronisation of the recording with the frame, line, and pixel clock pulses of the scanner, the pixel clock source and the type of the scan (unidirectional or bidirectional). In the FIFO mode and in the Absolute Time mode the More Parameters panel selects marker inputs, active edges, and routing bits. The parameters in this panel are explained with the individual operation modes.

Display Functions

The bh TCSPC modules record multi-dimensional data. In the simplest case, the result of a measurement may just be a single fluorescence decay curve or a single waveform of another optical signal. However, a measurement can also produce several curves simultaneously. In case of the SPC-QC, the curves can represent data recorded by different channels of the module, data recorded by several detectors connected to a single channel via a router, data recorded by several channels of a bh multi-wavelength detector, data recorded with several multiplexed lasers, or data from subsequent steps of a time-series recording procedure. Several such data sets can be recorded in different 'memory pages'. Moreover, the curves may not only represent waveforms of light signals at all but FCS curves, photon counting histograms, or time-resolved spectra. A few examples of the display of TCSPC curves are shown in Fig. 76.

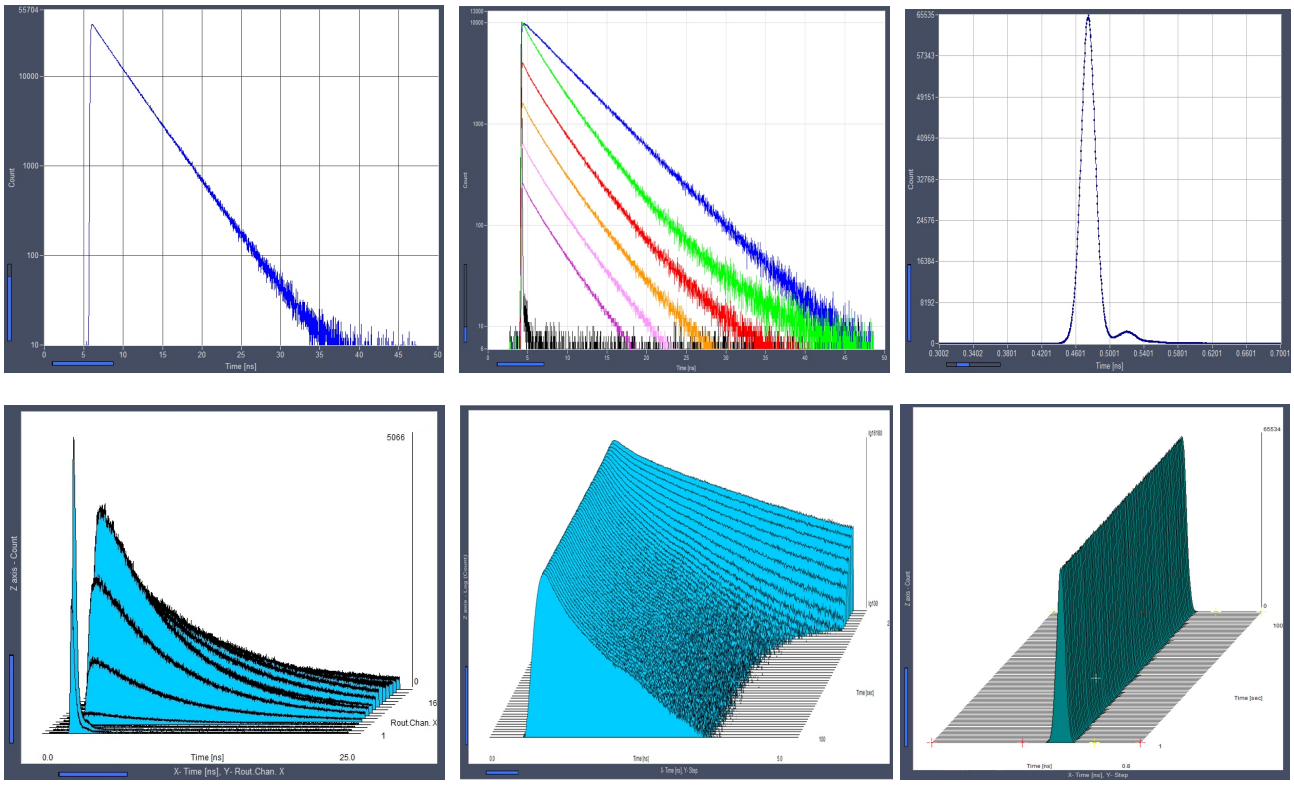


Fig. 76: Curve display in SPCM. Upper row: 2D curve display. Lower row: 3D curve display.

The structure of TCSPC data becomes even be more complex when an SPC module is used in the imaging mode. A single TCSPC image is an array of pixels, with each pixel containing a decay curve in form of photon numbers in consecutive time channels. Several such data arrays may exist if a multi-wavelength detector is used, or if the measurement system is using routing or laser multiplexing. Even more, mosaic imaging delivers a two-dimensional array of TCSPC images. Data with more than two dimensions cannot easily be displayed by a two-dimensional display device or printer. The data must be projected in a single plane, and the way this projection is performed must be suitably selected. Examples are shown in Fig. 77.

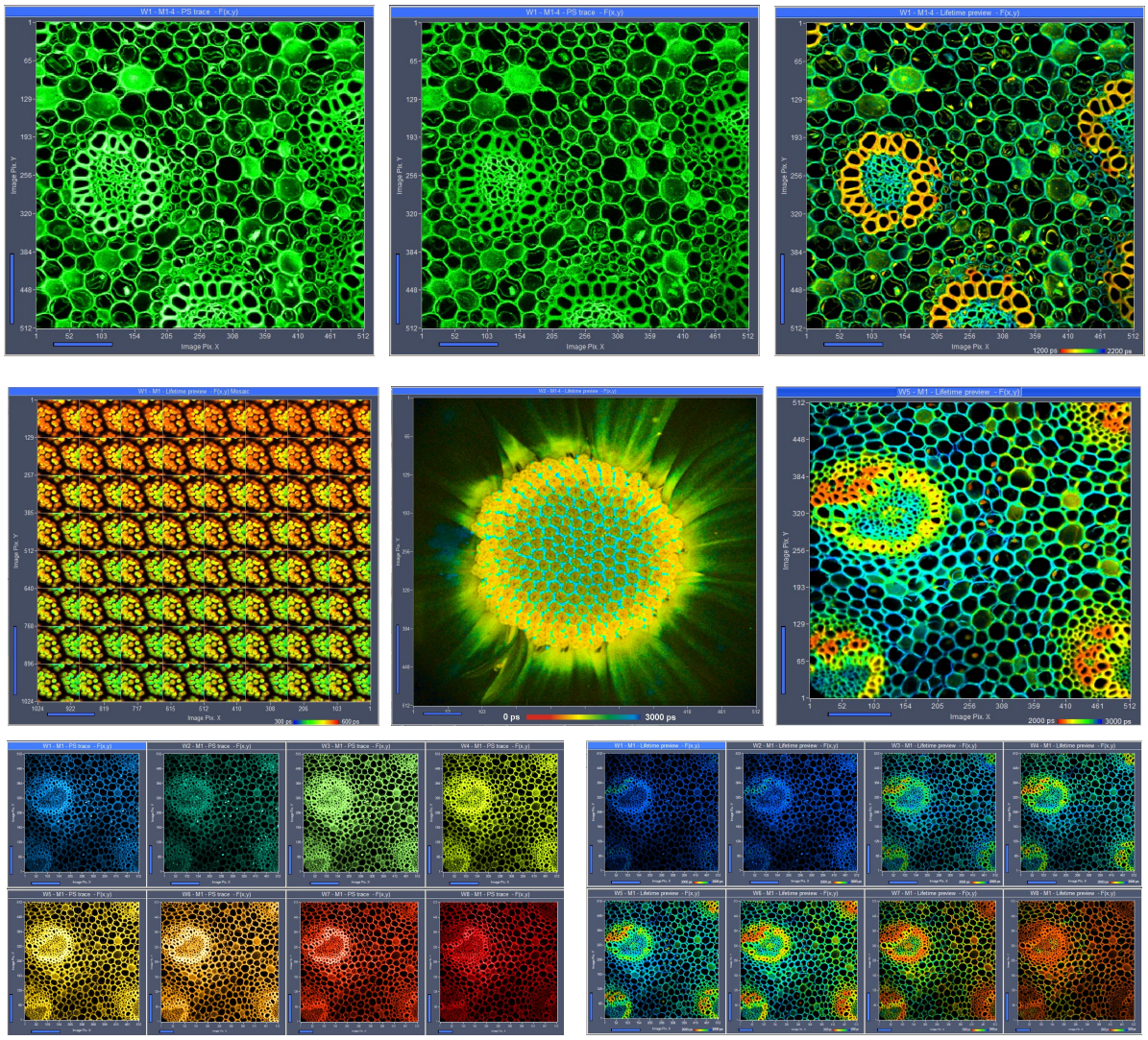


Fig. 77: Examples of SPCM image display. Upper row: Intensity image, gated intensity image (of late photons), lifetime image. Middle row: Mosaic FLIM image, image from several combined channels of a fast FLIM system, single image from multi-wavelength FLIM data. Lower row: Multi-wavelength intensity image in 8 routing windows selecting 2 adjacent channels each, colours adjusted to real emission wavelengths, multi-wavelength image, colour-coded lifetime.

Display of 2D Data

Decay curves recorded in a single channel or in several channels of an SPC-QC module, data recorded by several detectors, by several channels of a bh multi-wavelength detector, by several multiplexed lasers, or data from the steps of a time-series recording procedure can be displayed by the 2D display functions of the SPCM software. The 2D function puts the curves in a single waveform diagram, as shown in Fig. 76. The display of 2D data is controlled by the 2D Trace Parameters in combination with the Display Parameters. The 2D Trace Parameters determine which data are displayed, the Display Parameters determine how they are displayed. The parameters act both on the display of data in the SPCM main window and on the '2D Curve' routine under 'Display' in the top task bar of SPCM.

Selection of the Data to be Displayed

The data to be displayed are selected in the 'Trace Parameter' panel. Up to 16 Traces (curves) can be defined. The traces can be curves from different memory pages into which the data have been recorded (Fig. 78, left), curves from different routing channels of an SPC or SPC-QC module (Fig. 78, middle), or curves from different channel of an SPC-QC module (Fig. 78, right).

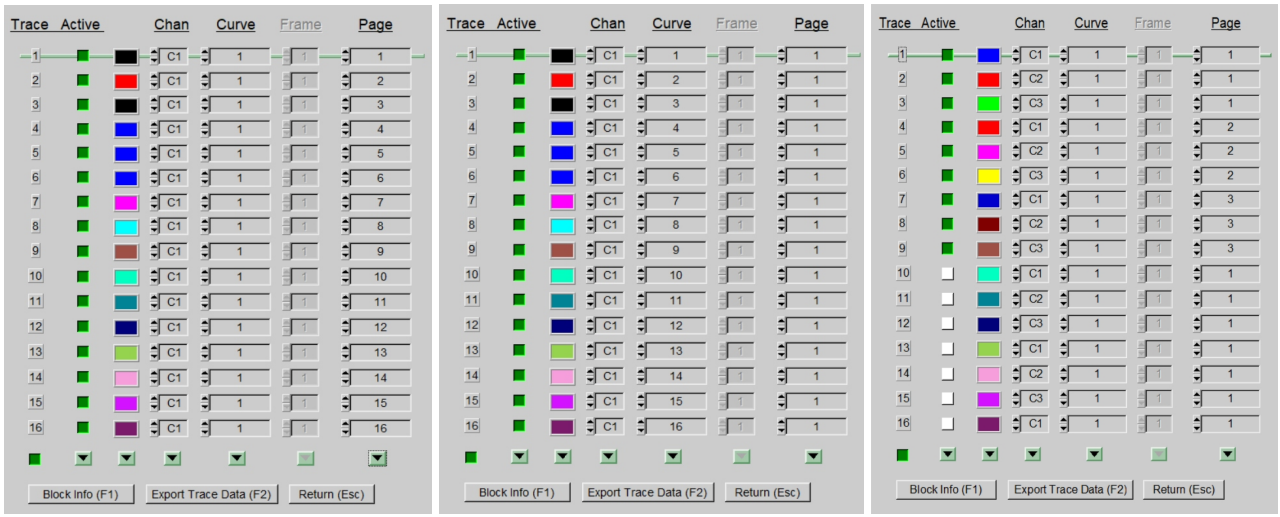


Fig. 78: Examples of trace parameter definitions. Subsequent measurement pages (left), curves from subsequent routing channels (middle), curves from different channels of an SPC-QC module in three different measurement pages (right).

Display Range and Curve Style

The display range, linear or logarithmic scale, and the style of the curves is defined in the 'Display Parameters'. Examples are shown in the figures below. The display parameters also allow the user to define a grid for the display. The autoscale button automatically adjusts the display range to the maximum of the largest curve in the display.

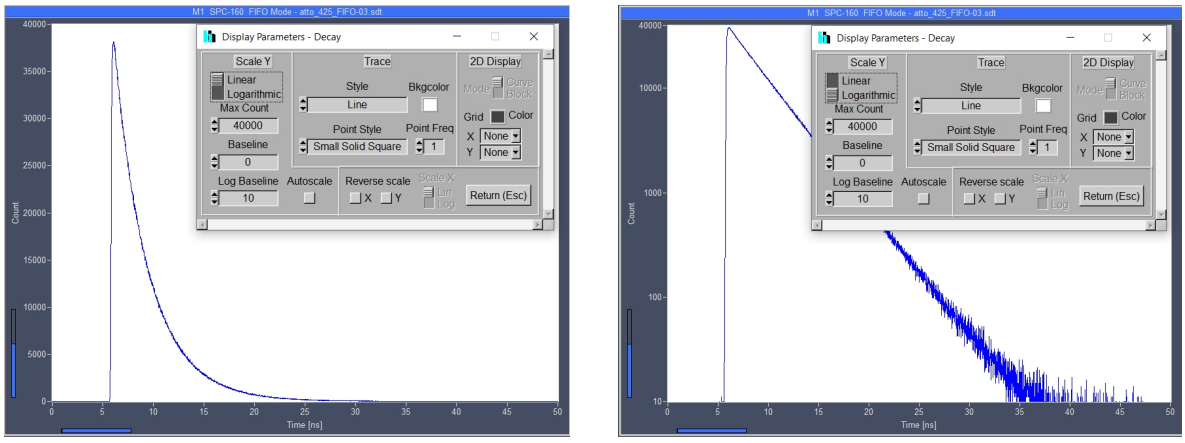


Fig. 79: Curve display for various display-parameter settings. Linear and logarithmic scale.

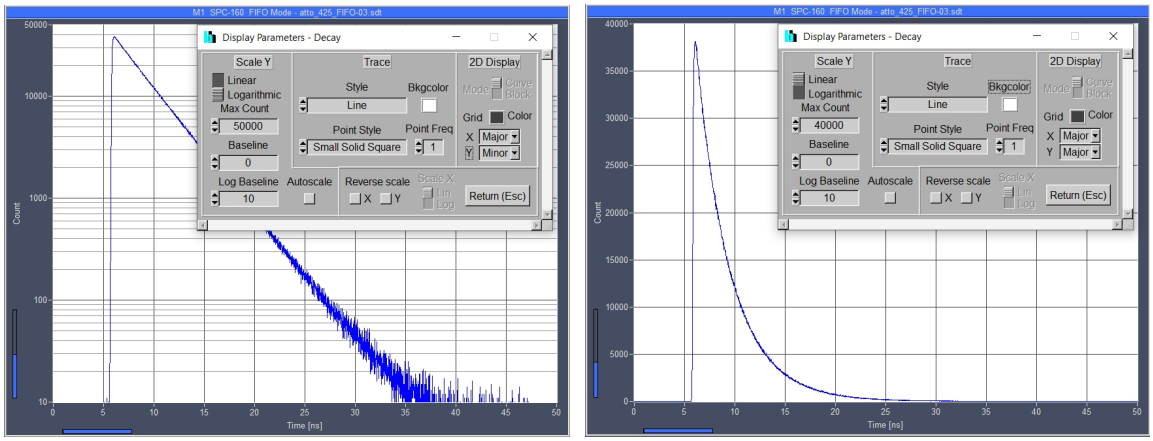


Fig. 80: Curve display for various display-parameter settings. Linear and logarithmic grid.

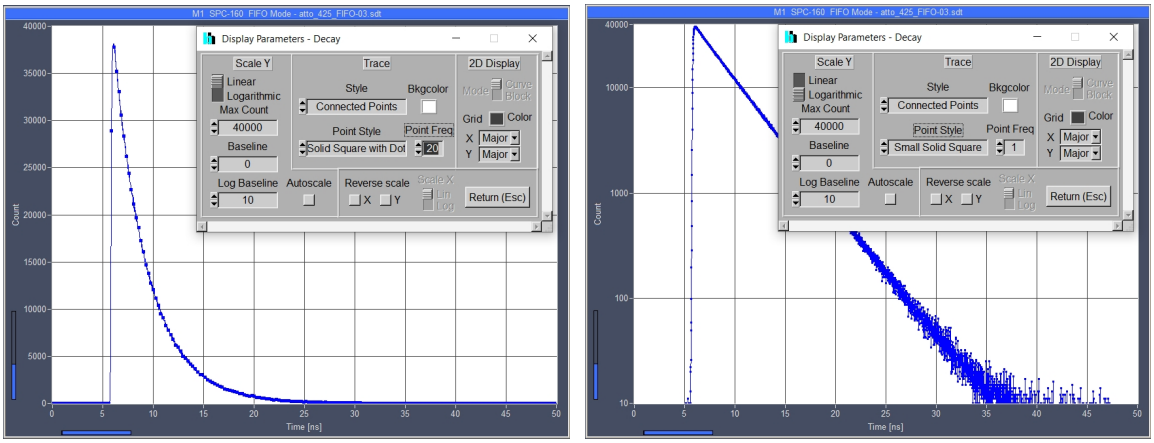


Fig. 81: Curve display for various display-parameter settings. Curve style 'Connected Points', different point frequency.

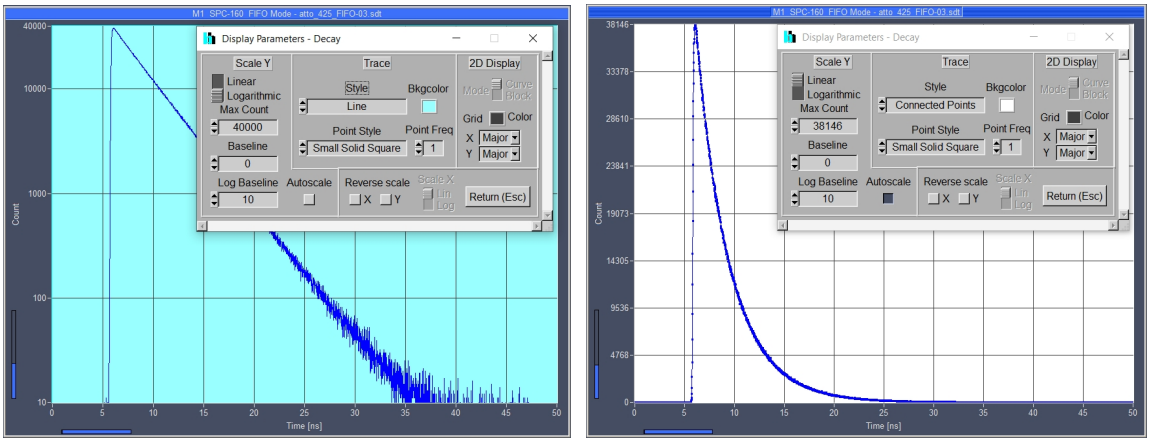


Fig. 82: 2D display for different display-parameters. Different background colours.

Display of 3D Data

The 3D display function is used to display data from sequential measurements, multi-detector measurements, multi-wavelength measurements, or from combinations of these. The corresponding photon distributions may be built up versus several variables, such as an externally varied parameter, the time in a fluorescence decay, the time in a sequence of decay curves, or the wavelength.

Display Modes

Examples of the display of 3D data are shown in Fig. 83 and Fig. 85. Fig. 83 shows a sequence of decay curves recorded by scanning the wavelength of a monochromator, Fig. 85 shows 3D data recorded with a 16-channel PML-GaAsP multi-wavelength detector. There are two different modes for 3D display. The data can be displayed as an array of curves (curve mode, shown left) and as a pseudo image in which the brightness and the colour represent the photon numbers (colour-intensity mode, shown right).

Display Parameters

The display mode and display style are controlled by the 'Display Parameters'. Parameters corresponding to the display configurations of Fig. 83 and Fig. 85 are shown in Fig. 84 and Fig. 86. Display mode is 3D Curves (left) and Colour Intensity (right). Offset X and Offset Y are the offsets of the subsequent curves in the 3D Curve mode. The colour bar associates the colours of the colour-intensity mode to the count numbers in the time channels. The number of colours can be changed by 'No. of Colours', the colours can be selected from a colour palette. Click into the colour bar to do this. An additional 'Mode' parameter defines the third coordinate of the display. It is 'f(x,y,param)' for the monochromator scan, and f(t,x) for the multi-wavelength recording. Please see [1] for further details.

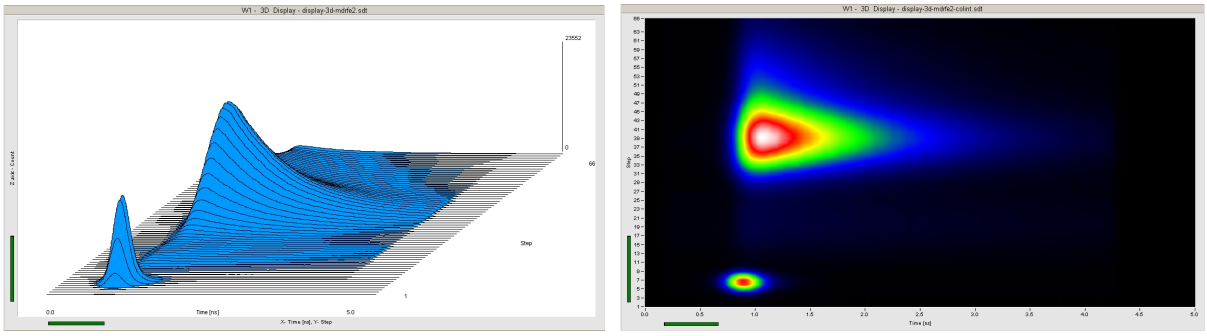


Fig. 83: Result of f(t,ext) measurement, wavelength scan by monochromator. Left curve mode, right colour intensity mode

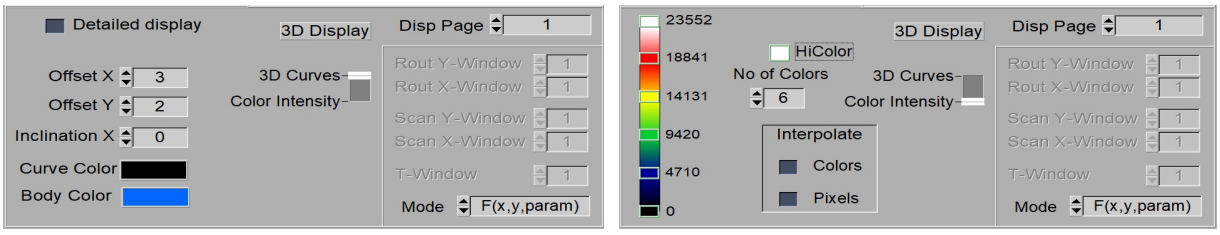


Fig. 84: Display parameters for Fig. 83. Left: Curve mode. Right: Colour-intensity mode.

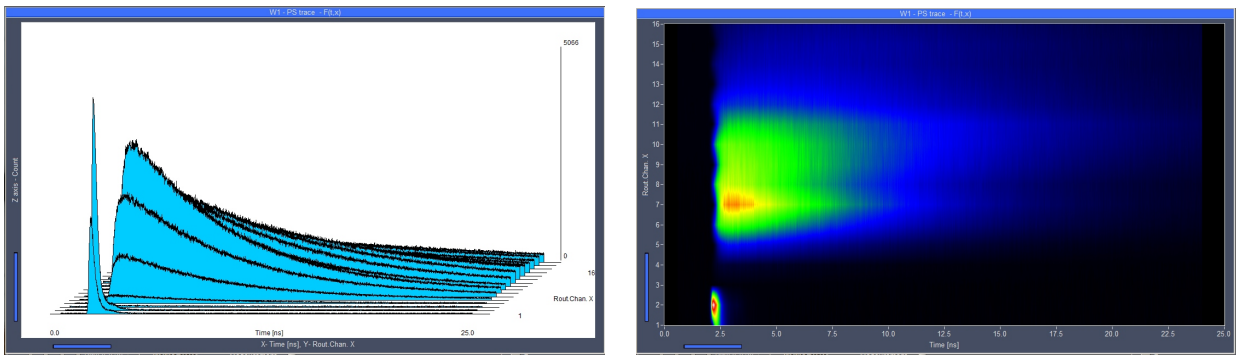


Fig. 85: Result of f(t,x) multi-wavelength measurement with the PML-16 GaAsP detector. Left Curve Mode, right colour intensity mode

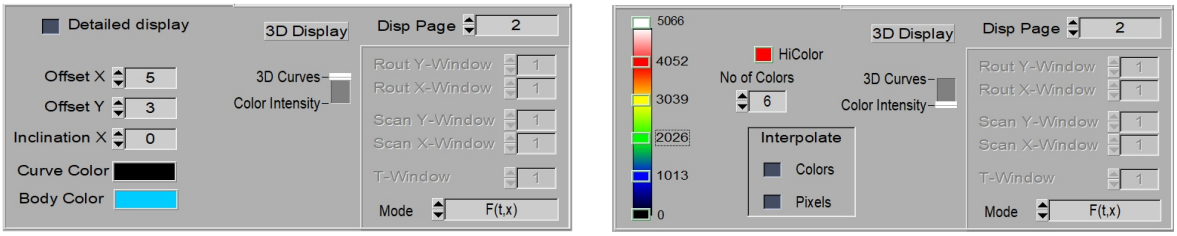


Fig. 86: Display parameters for the data in Fig. 85. Left: Curve mode. Right: Colour-intensity mode.

Display of FLIM Data

The results of a FLIM measurement are multi-dimensional data arrays. In the simplest case, the dimensions are the two coordinates of the scanning area, X and Y, and the time in a fluorescence or phosphorescence decay, t. However, FLIM data can be more complex. There may be additional coordinates, such as the detector-channel number in multi-wavelength FLIM data, the time step within a series of recordings, or other parameters which were assigned to the photons in the recording process. It may be necessary to display the data in different parameter spaces, e.g. as pure intensity images, as time-gated images, or as colour-coded fluorescence lifetime images. It may even be desired to display the data not as images but as sequences of decay curves integrated over selected spatial windows within the images. The SPCM software allows the user to define up to eight display windows for data of different source, different data type or different projection within the data parameter space. The display of the data is controlled by the '3D Trace Parameters', the 'Window Parameters' and the 'Display Parameters'. This section explains the parameters setup for the display of FLIM data for typical FIFO Imaging measurements and demonstrates it at typical examples.

Intensity Images from FLIM Data

One Image from One Channel of an SPC-QC Module

Fig. 87 shows the parameters for displaying an intensity image from the combined photon numbers in all time channels of a single FLIM image. In the 3D Trace parameters, only one display window (W1) is enabled. The data type to be displayed is ps FLIM, the data come from TCSPC module M1, and the display mode is F(x,y). The Window parameters (bottom left) define a single Time Window, from time channel 1 to 256, and two spatial windows, both from pixel 1 to pixel 1024. In other words, the windows incorporate the entire FLIM data array.

The Display Parameters, Fig. 88, define a colour-intensity image with linear intensity scale. The intensity is coded by colour, the colour scale goes from black over red to white. The image is displayed for Time Window 1 - this is the only time window defined (see Window Intervals, Fig. 87). It contains the photons of all time channels (from 1 to 256). The SPCM Main panel with an image defined by these parameters is shown in Fig. 89.

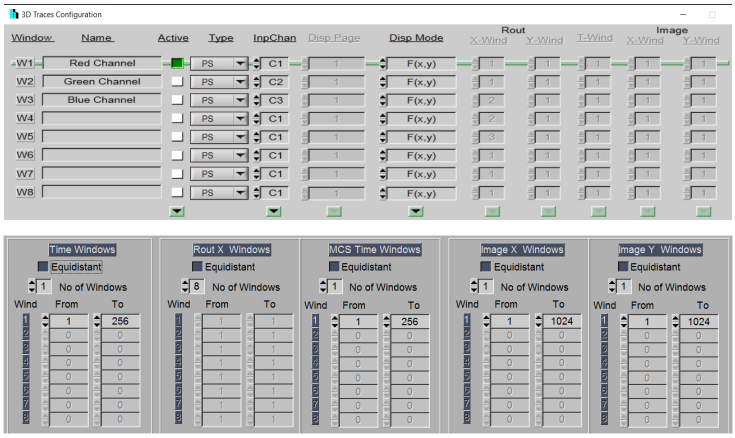


Fig. 87: 3D Trace parameters and Window Parameters for the display of a single image

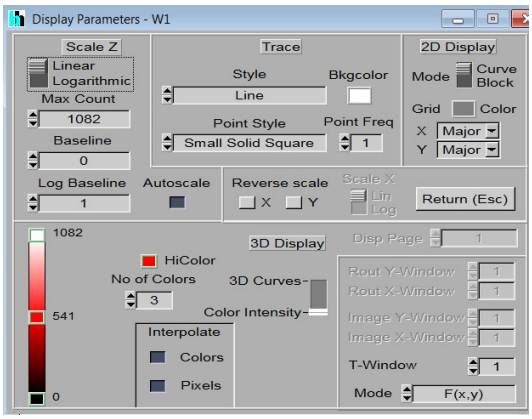


Fig. 88: Display parameters

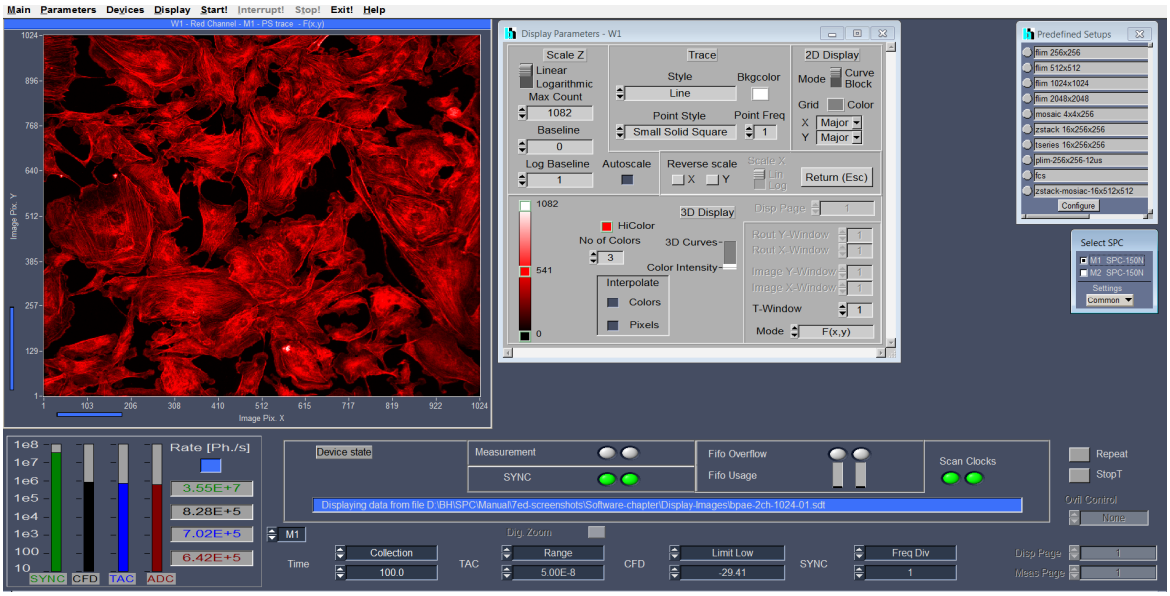


Fig. 89: SPCM main panel with the image defined by the parameters shown above

Images from Two Channels of an SPC-QC Module

The parameter setup for the display of two images from different channels is shown in Fig. 90. The Trace Parameters define two display windows. They contain data from two SPC modules, M1, and

M2. The window parameters are the same as for a single image - there is only one time window, containing the photons of all TCSPC time channels from 1 to 256.

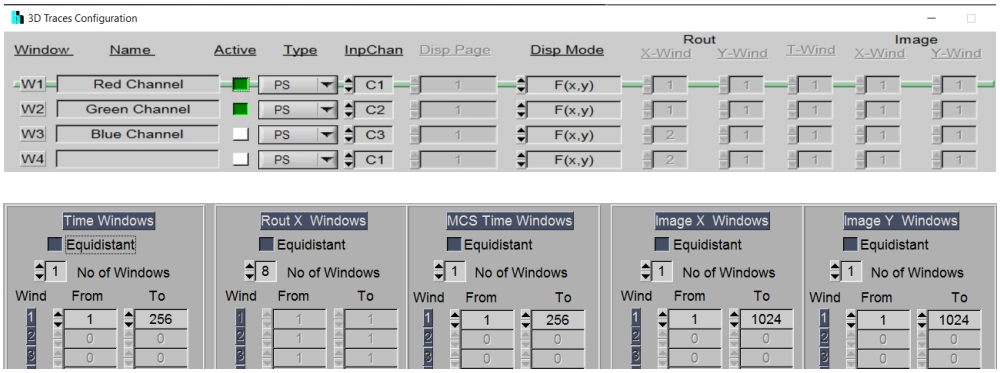


Fig. 90: 3D Trace parameters and Window parameters for the display of the images from two SPC modules, M1 and M2

The two images are displayed with separate display parameters, see Fig. 91. The SPCM main panel with the two images is shown in Fig. 92.

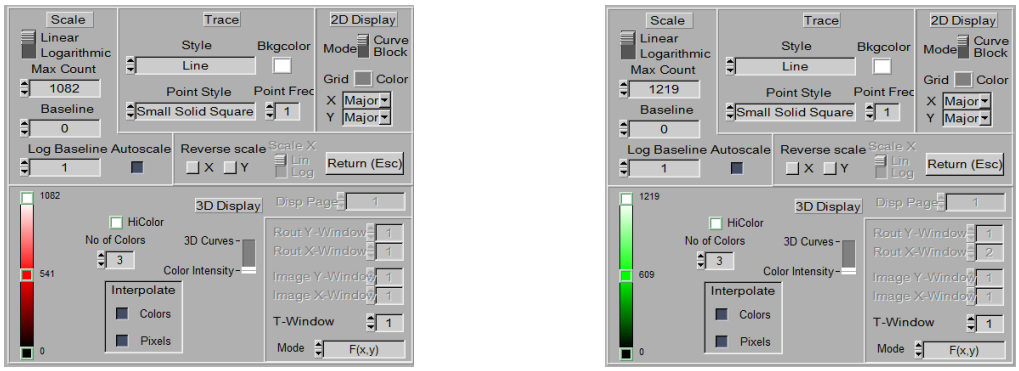


Fig. 91: Display parameters for the two images defined by the parameters in Fig. 90

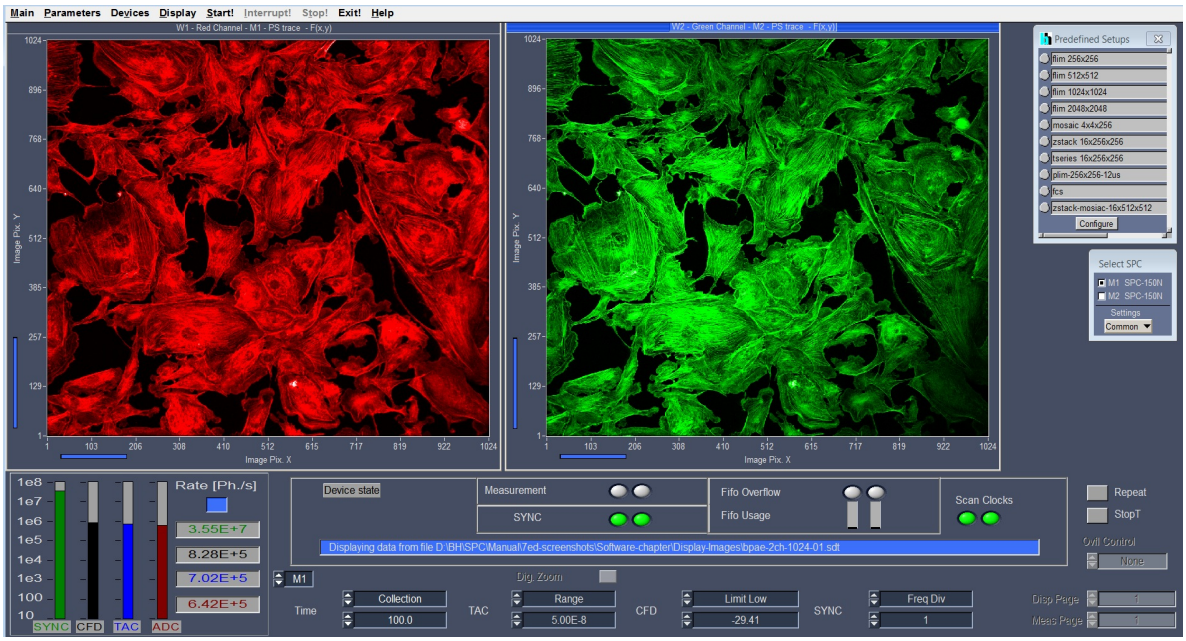


Fig. 92 SPCM main panel. Two images for separate SPC-QC channels.

Intensity Images from Routing or Laser-Multiplexing Channels

Images from several routing channels are displayed as shown in Fig. 93. The 3D Trace Parameters define several display windows for different routing channels. The data in these channels come from two detectors connected to channel 1 and channel 2 of the SPC-QC (Em 560 and Em 405), the routing bits come from the multiplexing of two lasers (Ex 488 and Ex 405). The 3D trace parameters define the number of display windows used, and the information to be displayed in the windows. The Window parameters define which routing channels or groups of routing channels are displayed in the windows. The 3D Trace parameters and Window parameters and the images created by this setup are shown in Fig. 93.

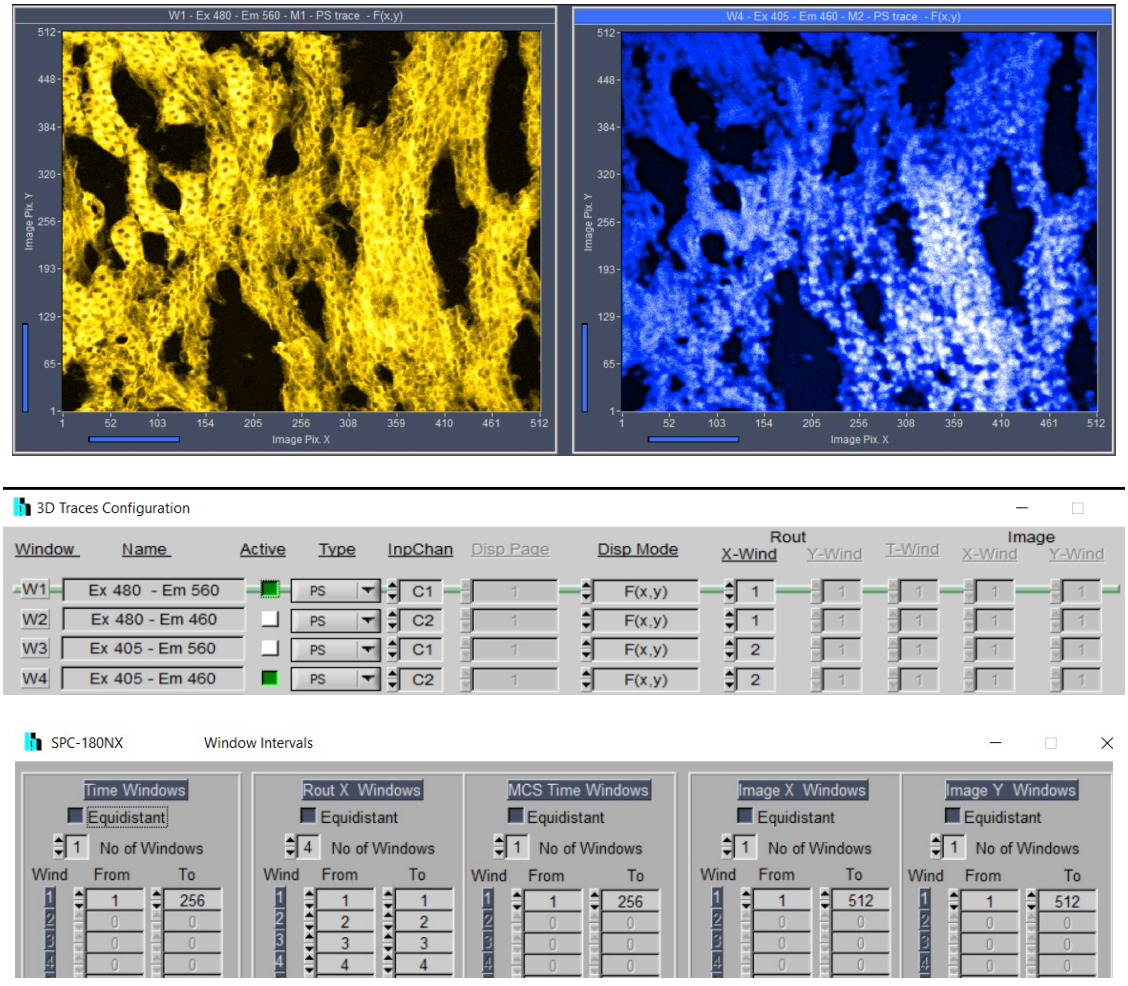


Fig. 93: Two images from a dual-channel wavelength-multiplexing system, intensity display. 3D trace parameters and Window parameters shown at the bottom. Four combinations of excitation and emission wavelength exist, two of them are switched active in the 3D Trace parameters and displayed in two image windows of SPCM.

Multi-Wavelength Intensity Images

The display of multi-wavelength FLIM data from a 16-channel detector is shown in Fig. 94 trough Fig. 96. The trace parameters define eight display windows. Data type is ps FLIM. The individual images in the display windows are derived from subsequent ‘Routing Windows’. Each routing window contains the data of two subsequent routing channels, see 'Window Parameters'. This way, data of every two wavelength channels are combined in one image.

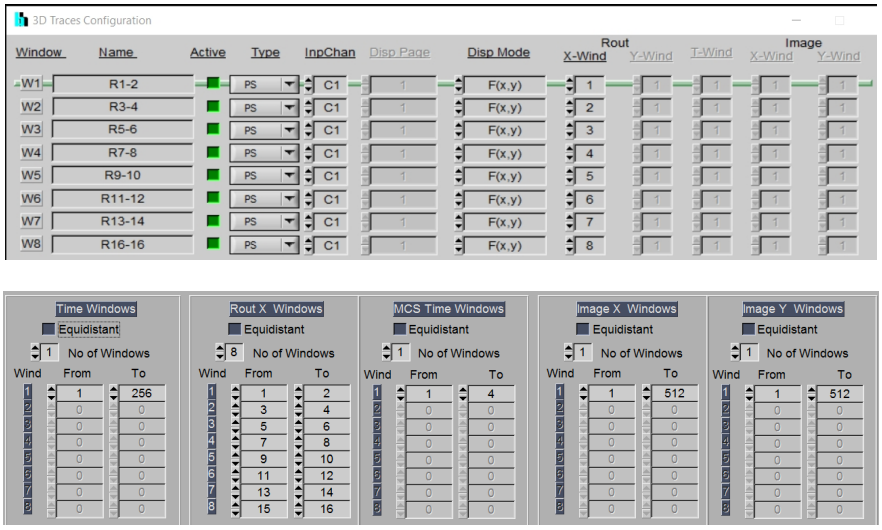


Fig. 94: 3D Trace Parameters and Window Parameters for multi-wavelength FLIM

The individual images have separate display parameters. The display parameters for the first three images are shown in Fig. 95. They contain the colour definition for the individual images, and the routing window. Since the intensities in different wavelength channels can vary over a wide range. Autoscale is turned on for all images. The main panel of SPCM with the eight images is shown in Fig. 96.

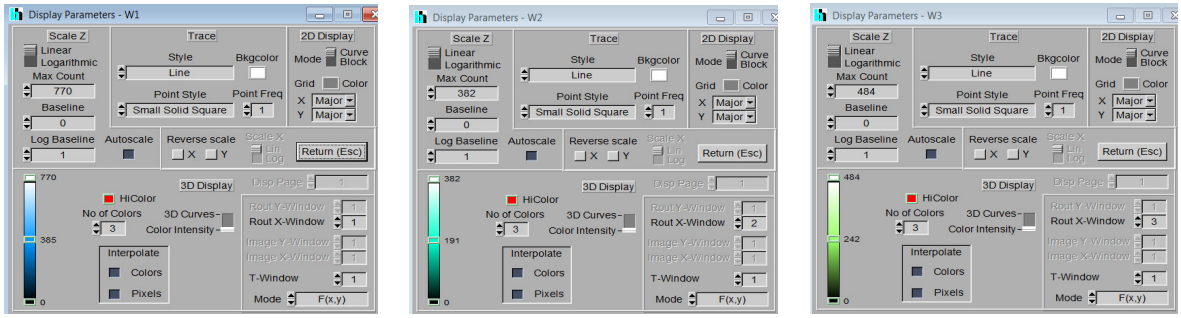


Fig. 95: Display parameters in the first three display channels of a multi-wavelength measurement

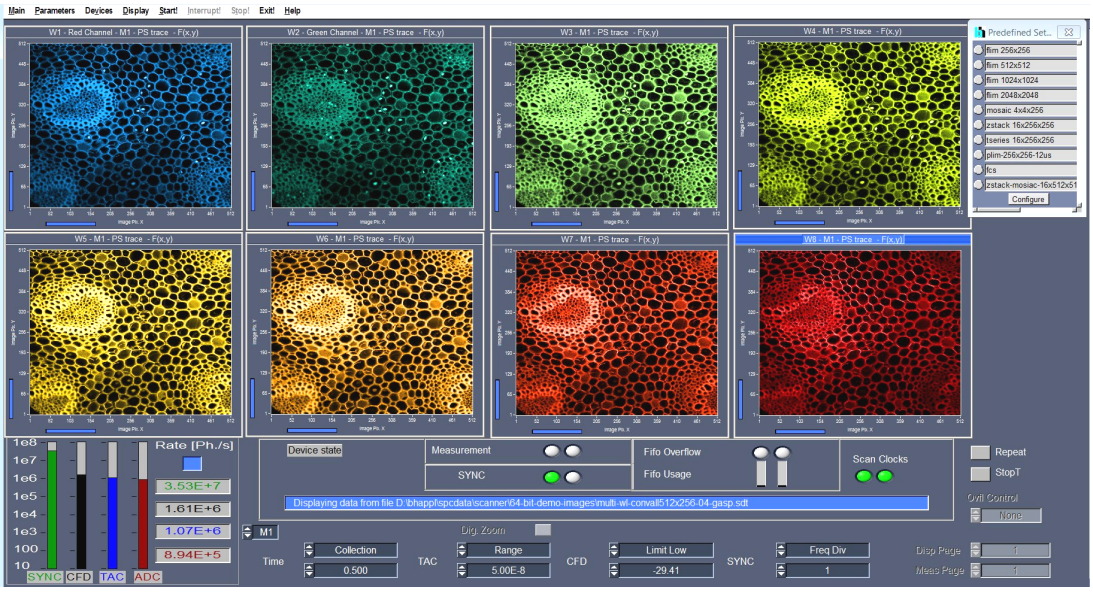


Fig. 96: SPCM main panel for multi-wavelength FLIM

Colour-Coded Lifetime Images

Fig. 97 shows the definition of the parameters for run-time lifetime display for a two-channel SPC-QC system. For online-lifetime display of colour-coded lifetime images, select 'Type' = 'LIFET' in the 3D trace parameters.

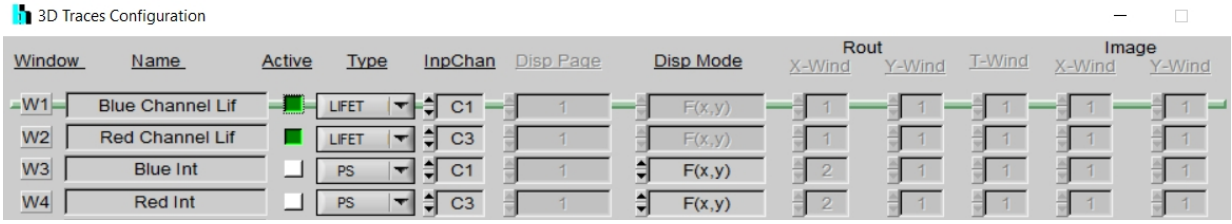


Fig. 97: 3D trace parameters for run-time display of lifetime images. Two SPC-QC channels.

The 'Type' definition need not be identical in all channels. For example, you can display a lifetime in one channel together with an intensity image in the same channel or in another channel, and switch the images 'Active' or inactive on demand.

The display of lifetime-image display requires more parameters than the display of intensity images. The online-lifetime function calculates the first moment of the decay data in the pixels, and subtracts the first moment (the centroid) of the IRF from it [1, 24]. Therefore the temporal location of the IRF must be known. Moreover, the display range for the lifetime, the direction of the colour scale must be known. These parameters are defined in the 'Display Parameters', see Fig. 98. As usual, there is a separate set of display parameters for each image. In the lower part of the panels the lifetime range, the direction of the colour bar, the brightness and the contrast, and the reference moment for the IRF is defined. The reference moment can be determined with SPCImage or calculated from a reference FLIM file of a sample with known fluorescence lifetime. For principle of run-time lifetime calculation please see [24] or section 'Fast Online FLIM' in the bh TCSPC Handbook. The main panel of the SPCM software with the lifetime images of two SPC channels is shown in Fig. 99.

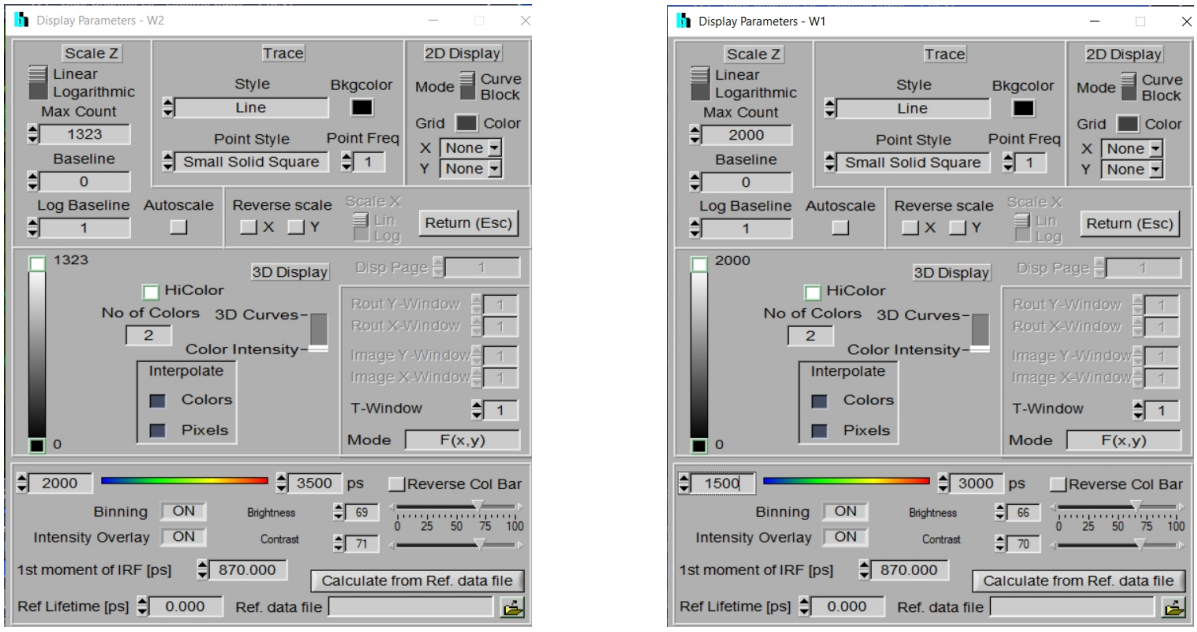


Fig. 98: Display parameters for run-time lifetime display. Two channels with different lifetime range.

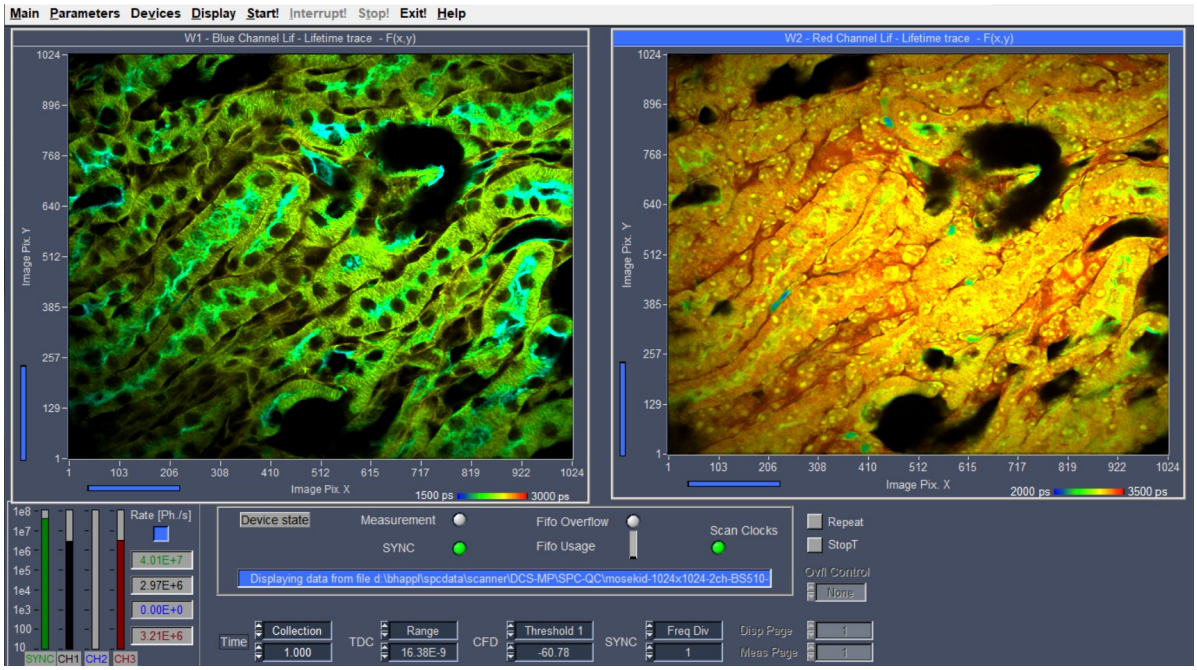


Fig. 99: SPCM main panel with run-time lifetime calculation. SPC-QC, channels 1 and 3.

Display of Decay Data in Point or Region of Interest

A right-mouse click into one of the image in the SPCM main panel opens a small menu from where you can access the image cursors, the display parameters, trace parameters, window parameters, etc. A click into 'Show Decays' opens a decay-curve window as shown in Fig. 100, right.

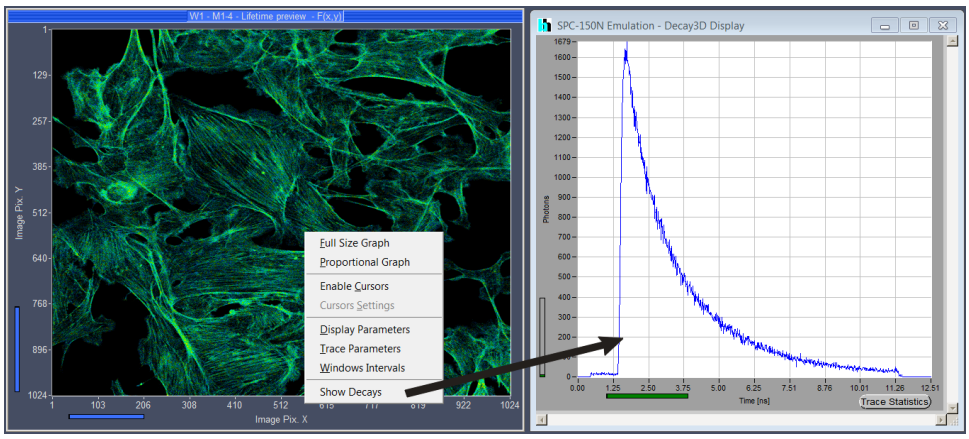


Fig. 100: A right mouse click into an image and a click on 'Show Decays' opens the decay curve panel shown on the right.

The SPCM main panel with the decay curve window is shown in Fig. 101. The curves can be displayed for all active image windows, and for individual regions of interest (ROIs), points of interest (POIs), or for the entire image (ALL). The ROIs are defined by the image cursors, the POI by the 'Data Point'. A right mouse click into the decay window opens a panel with the trace parameters for the individual curves. The panel can be seen in Fig. 101, lower right. You can add or remove curves, activate or de-activate curves, define colours for the curves, define whether a curve is from an ROI or a POI, and from which image display window the curve comes. The coordinates of the POI and of the ROI cursors are displayed on the right. As an example, Fig. 101 displays decay curve for two recording channels within two regions of interest.

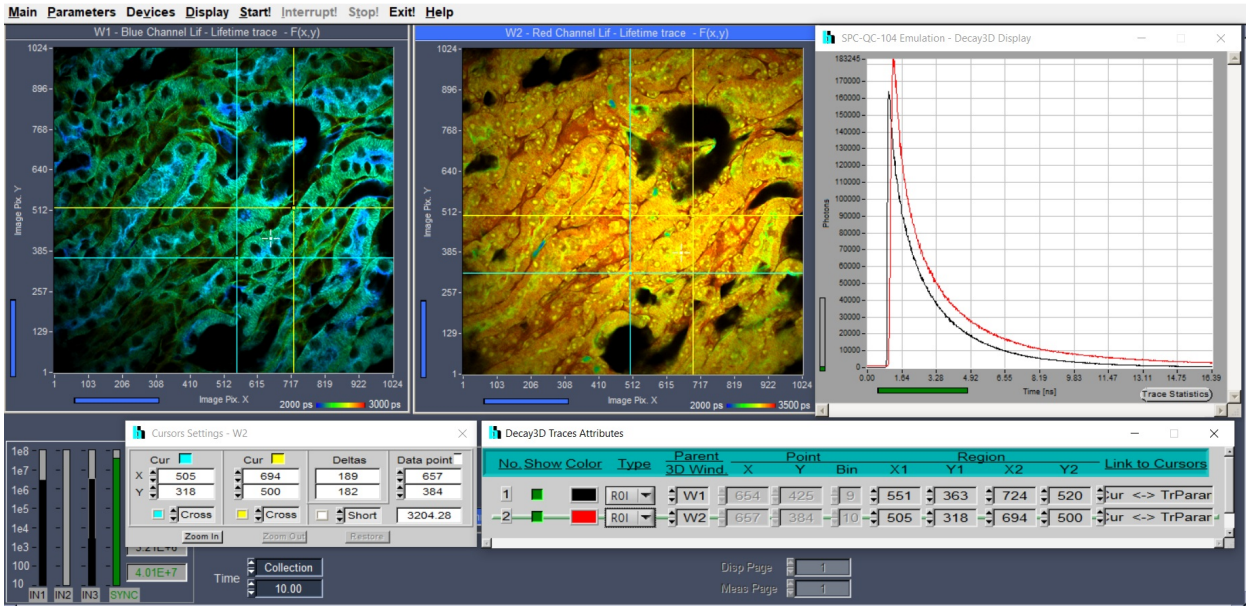


Fig. 101: SPCM Main Panel with lifetime images and decay curve window

The decay-curve display works for all images that contain decay data in their pixels. These can be intensity images, time-gated intensity images, lifetime images, combined images from several SPC modules or SPC-QC channels, or images from different routing channels.

The display of the decay curves itself is controlled by the 'Display Parameters' the same way as for decay curves recorded in the Single, Oscilloscope, or FIFO mode. The display scale can be linear or logarithmic, an autoscale function is available, and the curves can be displayed as individual data points, lines, or data points connected by lines. Please see 'Display of 2D Data'.

Save and Load Functions

Save and Load functions are the same as for other modules of the bh SPC series. You can save and load measurement data together with the setup parameters or only setup parameters. In the first case, SPCM creates .sdt files, in the second case .set files. The save and load panels are shown in Fig. 102. Please see bh TCSPC Handbook for details.

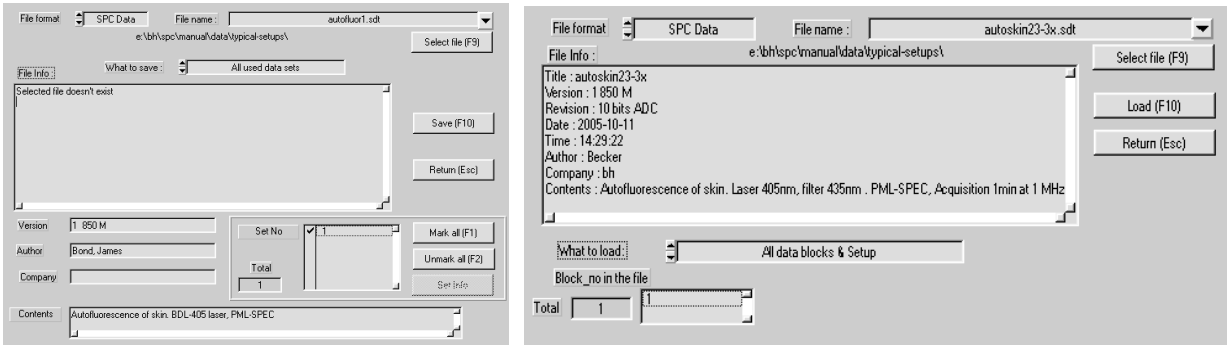


Fig. 102: SPCM functions for loading and saving data and setup parameters

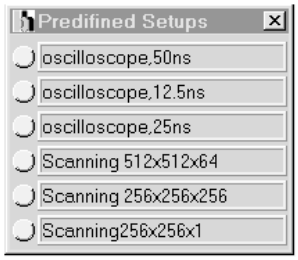
Autosave at SPCM Exit

When you are exiting SPCM the setup data are automatically saved into an 'auto.set' file. When you later open SPCM the setup is automatically loaded so that the system starts with the parameters of the previous SPCM session.

Predefined Setups

Setups of frequently used system configurations can be added to a list of ‘Predefined Setups’. Changing between these setups then requires only a mouse click.

To use the predefined setup function, click on ‘Main’, ‘Load Predefined Setups’. This opens the panel shown below. A setup is loaded by clicking on the button left of the name of the setup.



To add or delete setups to or from the list, or to change the names of the setups, click into one of the name fields with the right mouse key. This opens the panel shown in Fig. 103.

To add a setup, click on the disk symbol right of the ‘File Name’ field. Select the files you want to put into the list of predefined setups, and click on the ‘Add’ button. Every setup has a user-defined ‘nickname’. The default nickname is the file name of the .set file. To change the nickname, click into the nickname filed and edit the name. Then click on ‘Replace’.

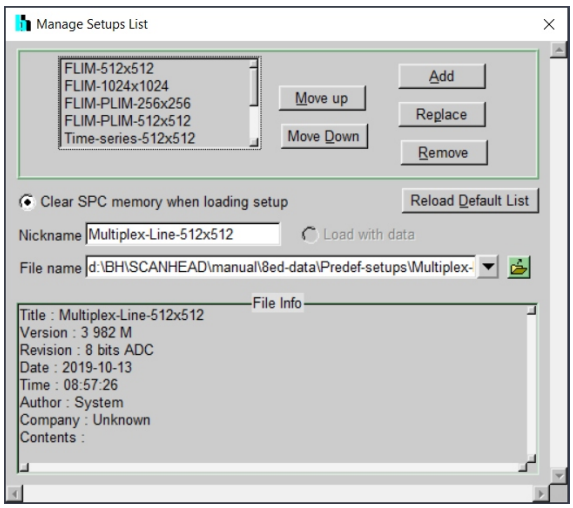


Fig. 103: Editing the list of predefined setups

Predefined setups are extremely useful to switch between different operation modes and system configurations without the need of setting individual system or user-interface parameters.

The Predefined Setup can also be used to make TCSPC / FLIM experiments extremely simple. An example is shown in Fig. 104. The instrument is a FLIM system that has only four setups: One for recording 'Small Images', one for 'Medium Images', one for 'Large Images', and one for recording a 'Single Curve'. Even pixel numbers and time-channel numbers are hidden. By clicking on the desired setup all system parameters are set. All that remains for the user to do is to start the measurement.

Such extremely simplified predefined setups are a perfect way to configure the system to the needs to users who consider themselves great scientists and therefore refuse to spend any thoughts on the technical backgrounds of their experiments.

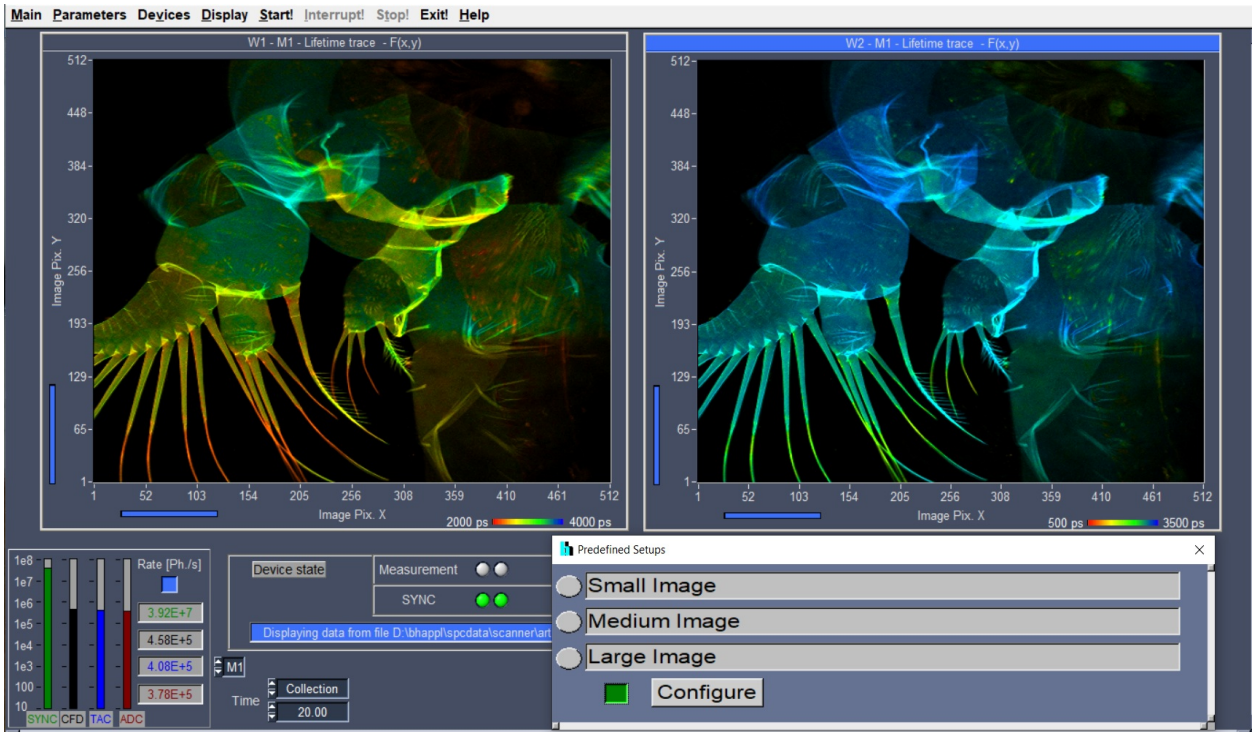


Fig. 104: FLIM system simplified down to just three different image sizes. All other system and display parameters are pre-set.

SPCImage NG Data Analysis Software

General Functions

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. In addition to FLIM data, SPCImage NG processes single-curve decay data, multi-wavelength data, excitation-multiplexed data, PLIM data, mosaic FLIM data, and other multi-dimensional TCSPC data sets. SPCImage NG provides decay models with one, two, or three exponential components, incomplete-decay models, and a shifted-component model. Another important feature is advanced IRF modelling, making it unnecessary to record IRFs for the individual FLIM data sets. 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 or the 2D parameter histograms. Pixels with similar phasor or 2D parameter signature can be combined for high-accuracy time-domain analysis, resulting in photon numbers known only from cuvette-based lifetime experiments. The following section gives a brief overview on SPCImage NG. For a comprehensive description please see [4]. Please see also [5] and [23].

The main panel of SPCImage in its basic configuration is shown in Fig. 105.

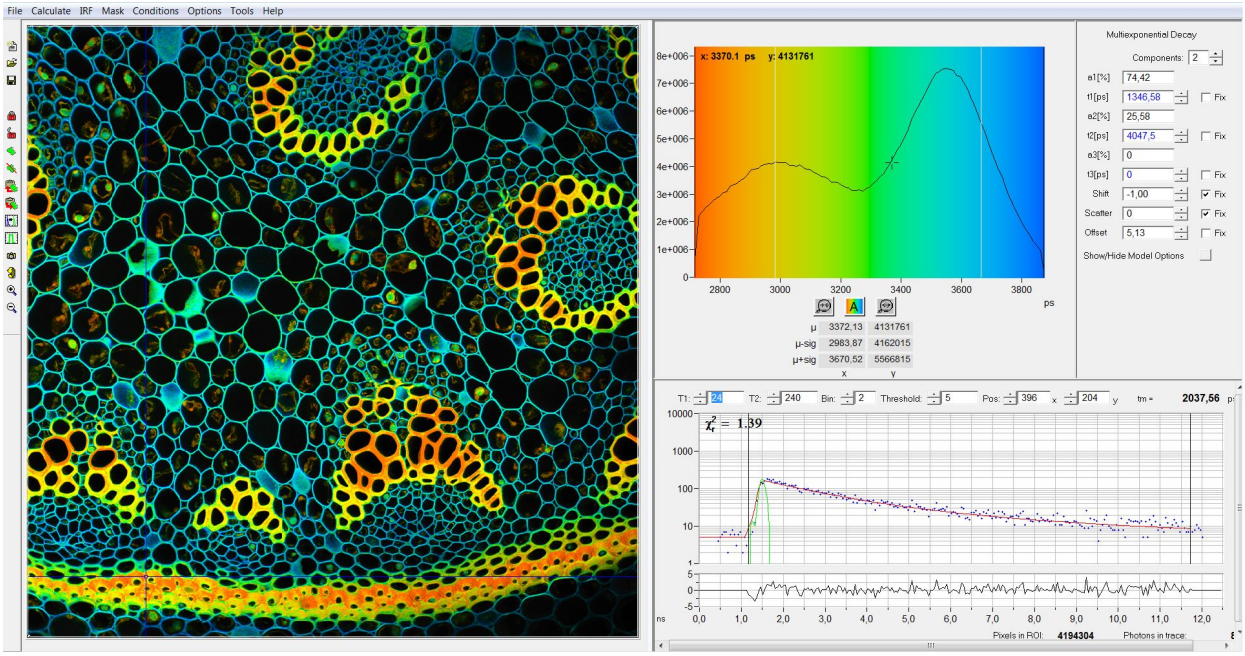


Fig. 105: Main panel of SPCImage NG

Combination with Phasor Plot

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. 106.

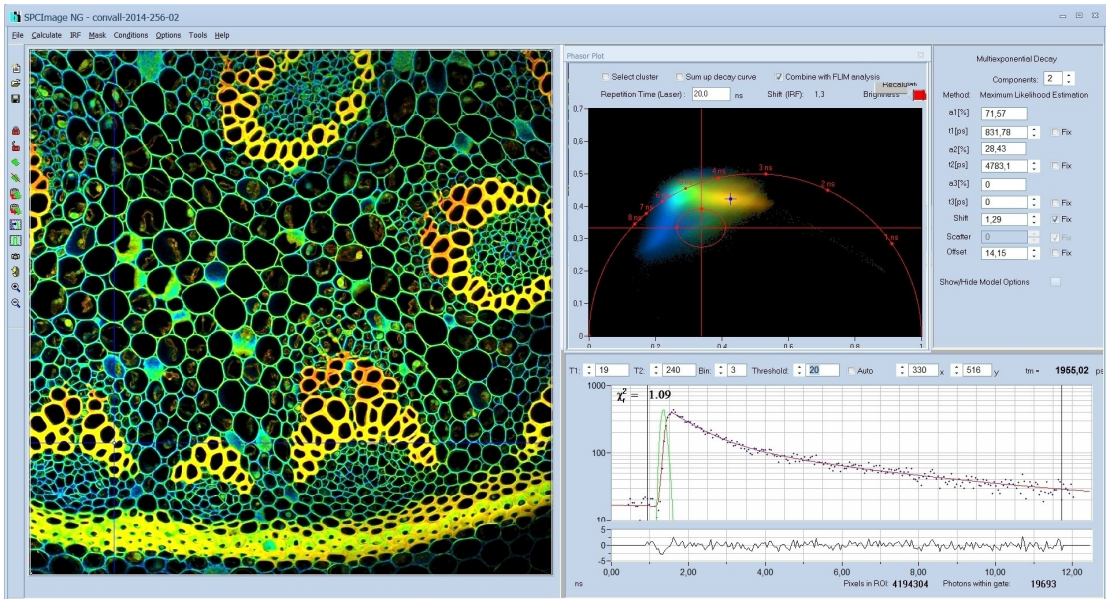


Fig. 106: Combination of time-domain analysis (left and lower right) and phasor plot (upper right)

Images of Decay Parameters

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. 107 through Fig. 110.

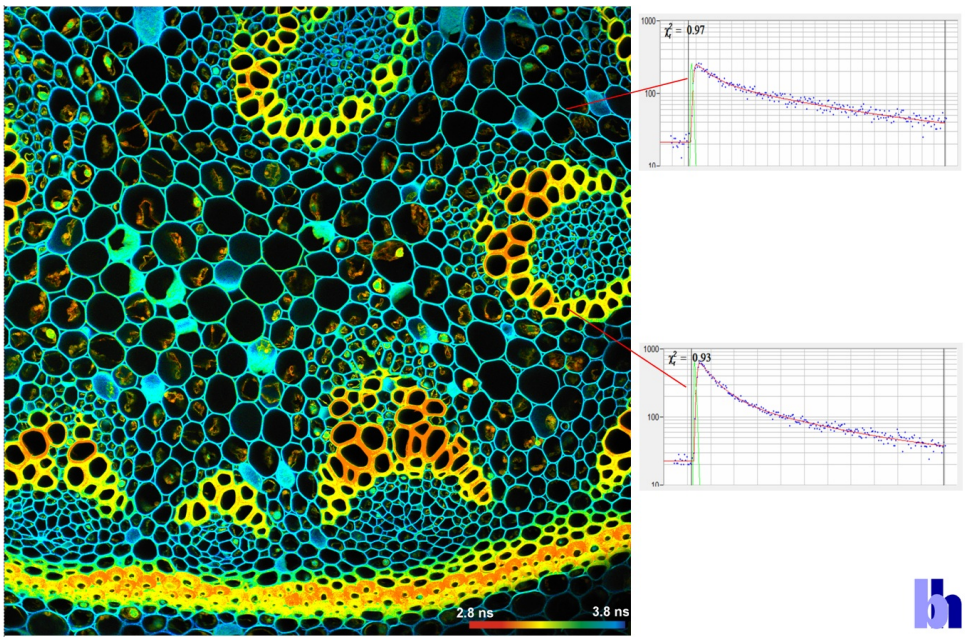


Fig. 107: Image of the amplitude-weighted lifetime, t_m, of a double-exponential decay. Right: Fluorescence decay curves in selected pixels.

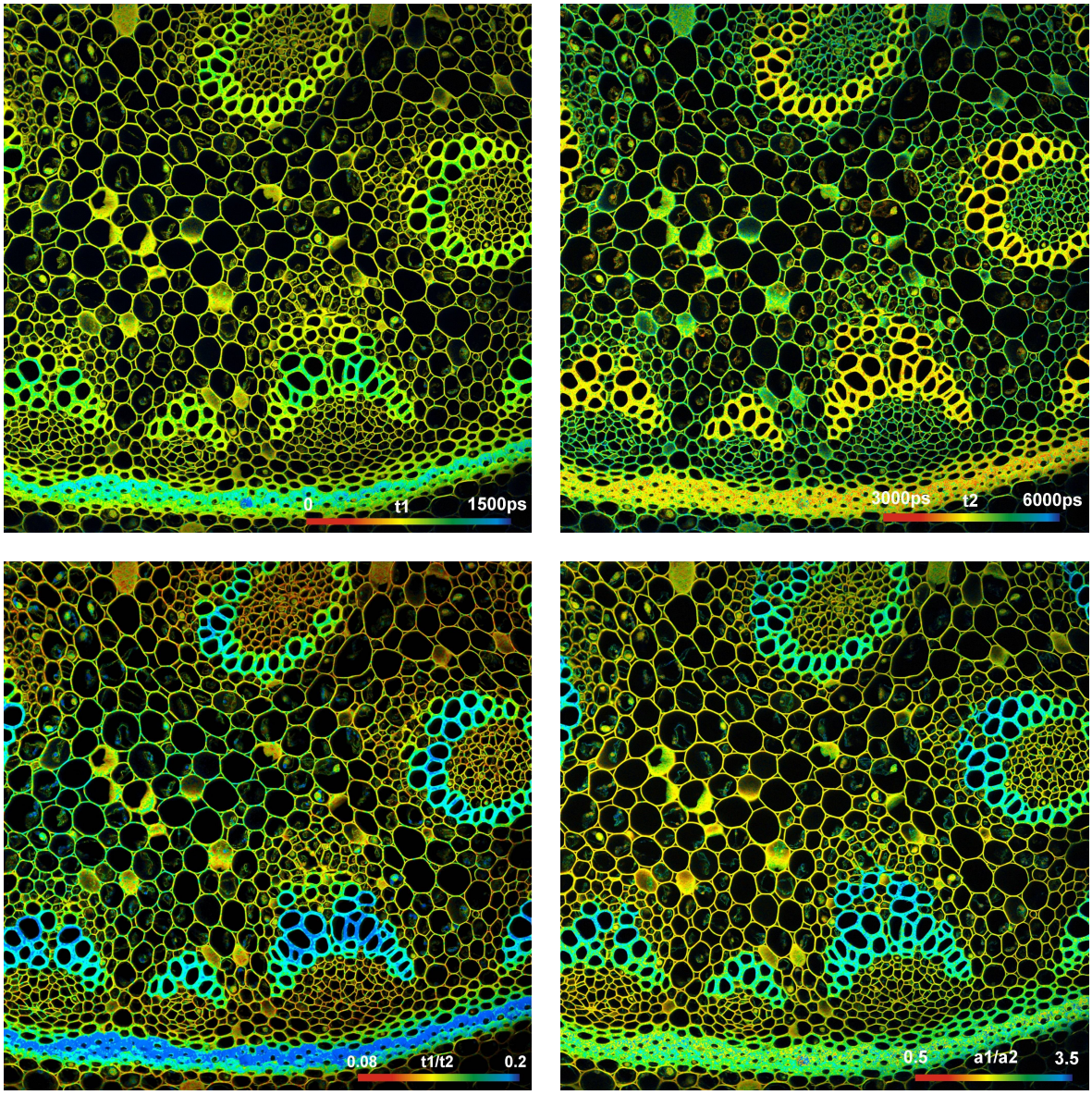


Fig. 108: Upper row: Images of the lifetimes of the fast component, t_1, and the slow component, t_2, of a double-exponential decay. Lower row: Images of the amplitude ratio, a_1/a_2, and the lifetime ratio, t_1/t_2, of the fast and the slow decay component.

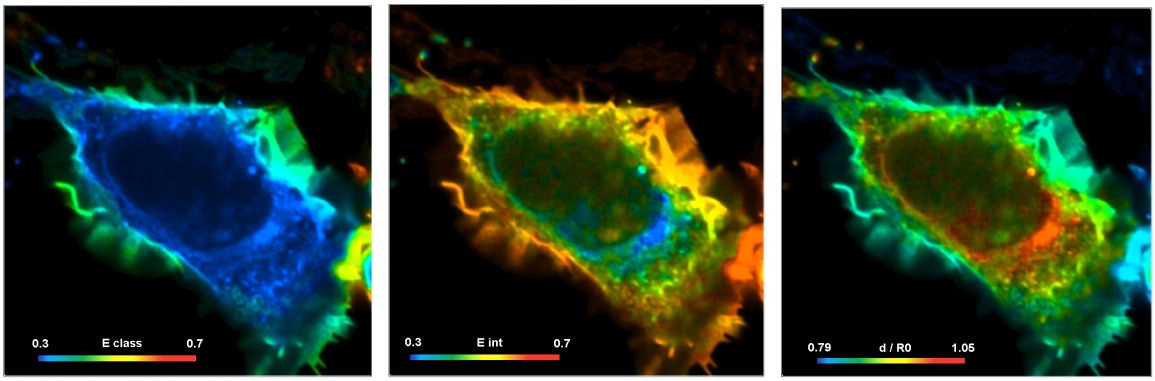


Fig. 109: Cell with interacting proteins, labelled with a FRET donor and a FRET acceptor. Left to right: Classic FRET efficiency, FRET efficiency of interacting donor fraction, FRET distance

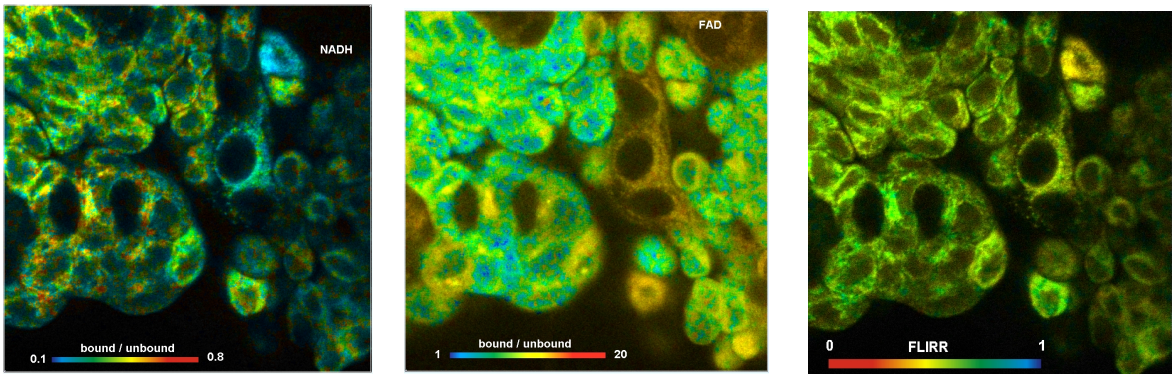


Fig. 110: Metabolic FLIM. Bound/unbound ratio of NADH, Bound/unbound ratio of FAD, Fluorescence-Lifetime Redox Ratio, FLIRR.

In addition to the functions described above SPCImage NG analyses single fluorescence decay curves, data from excitation-multiplexed FLIM, multi-wavelength FLIM, spatial and temporal mosaic FLIM, and simultaneously recorded FLIM/PLIM. 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 [Fig. 2]. 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 .tif images.

Maximum-Likelihood Algorithm

Different than previous versions, SPCImage NG uses a maximum-likelihood estimation (MLE) process to determine the decay parameters in the pixels. In contrast to the frequently-used weighted least-square (WLS) fit, MLE is based on calculating the probability that the values of the model function correctly represent the data points of the decay function. 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 unavoidable for the least-square fit. Please see [1], chapter 'SPCImage NG Data Analysis Software'.

Modelling of the Instrument-Response Function

Recording the 'Instrument Response Function' (IRF) is a permanent problem of time-resolved fluorescence spectroscopy. Recording the IRF in a FLIM system is difficult, and often impossible. As a result, there is rarely an IRF that was recorded in a FLIM system and represents the temporal behaviour of the system correctly. Therefore, SPCImage NG does away with IRF recording altogether. Instead, the IRF is extracted from the FLIM data themselves. Earlier SPCImage versions had an 'Auto IRF', which was derived from the rising edge of the fluorescence decay function. The 'Auto IRF' has been used successfully for more than 20 years. It works well for decay functions which are not too far from a single-exponential function but has deficiencies if very fast decay components are present. A new approach introduced by SPCImage NG is the 'Synthetic IRF'. It is created by modelling the IRF with a generic function. The exact parameters of this function are determined by fitting it to the FLIM data together with the selected decay model. The results of this procedure are so good that an accurate IRF is obtained even for decay functions containing ultra-fast components.

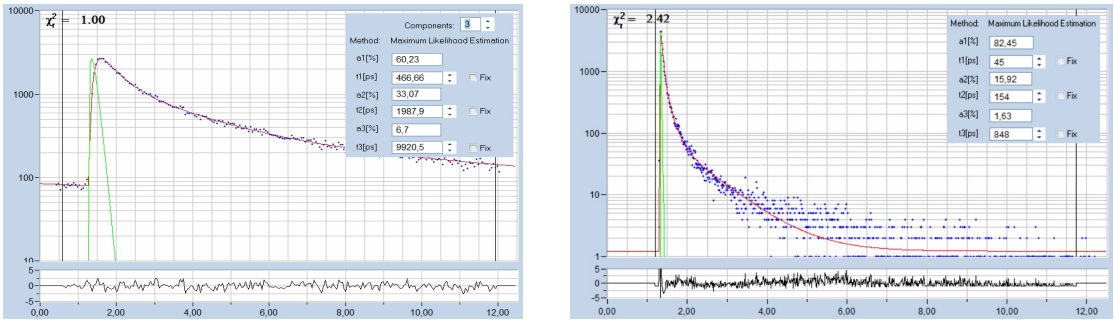


Fig. 111: Analysis with synthetic IRF. Left: Fluorescence excited by diode laser. Right: Ti:Sa laser, sample with extremely fast decay component. Green curve IRF, blue dots data points, red curve fit with triple-exponential decay model.

Decay Models

SPCImage NG provides single-, double-, and triple-exponential decay models. An ‘Incomplete Decay’ option is available to determine long fluorescence lifetimes within the short pulse period of the Ti:Sa laser of a multiphoton system. SPCImage NG provides also a 'Shifted-Component' model. In this model, the decay components of a multi-exponential model functions can be shifted in time by predefined values. The model is used for ophthalmic FLIM, where different decay components come from different depth within the eye. For details please see [1].

GPU Processing

Data recorded with bh FLIM systems 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 [1]. An example is shown in Fig. 112.

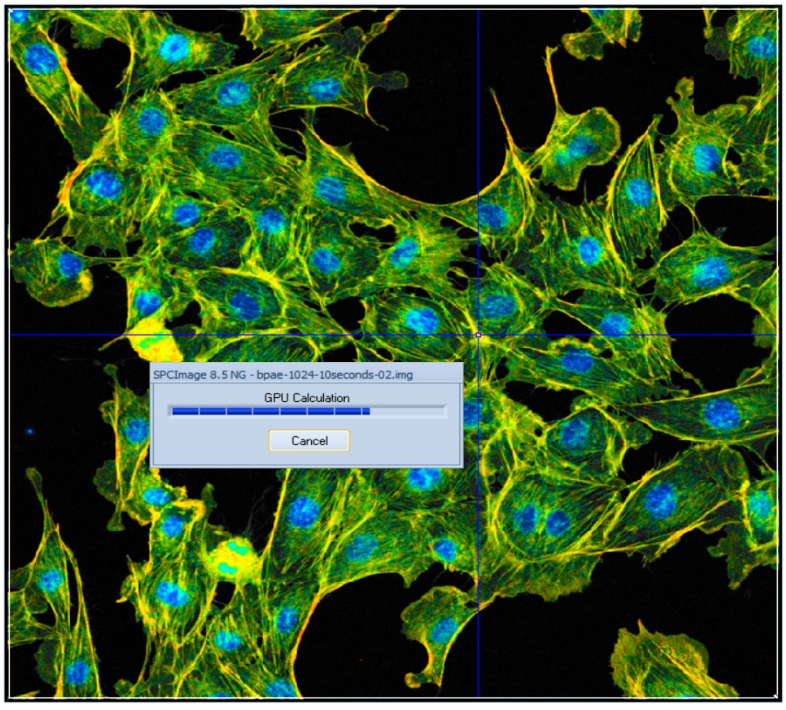


Fig. 112: A lifetime image with 1024 x 1024 pixels and 1024 time channel per pixel. The image was calculated on an NVIDIA GPU in 5 seconds.

Processing such amounts of data by the CPU of even a fast computer takes tens of minutes. SPCImage NG therefore runs the data analysis on a GPU (Graphics Processor Unit). 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. The image shown in Fig. 112 was calculated on a medium-speed NVIDIA GPU within five seconds. Conventional data analysis of this image takes about 10 minutes.

Loading Data into SPCImage NG

Loading SPCImage Data

Data which have already been analysed and saved by SPCImage are loaded via the normal load / open function of Windows. The extension of these files is '.img'. Click into 'File', 'Open', and select the desired file from the file selection panel shown in Fig. 113.

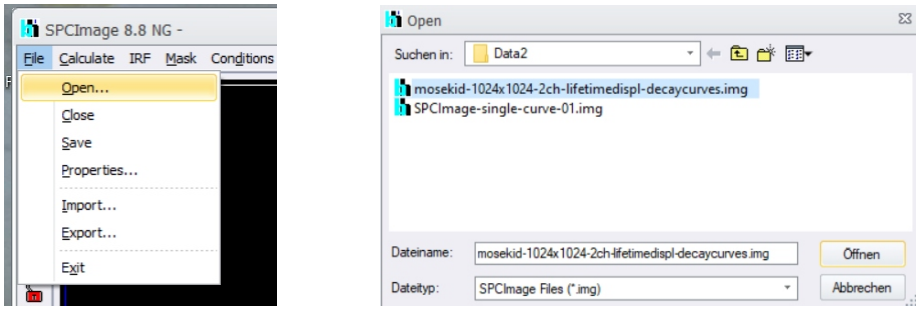


Fig. 113: Loading data which have been analysed and saved by SPCImage.

Importing SPCM Data

Importing .sdt files of FLIM data

FLIM Files from SPCM (.sdt format) are loaded into SPCImage via the 'Import' function. A click into ‘Main’, ‘Import’, (see Fig. 114, left) opens a file selection panel, see Fig. 114, middle. Select the desired file from this panel and click on 'Open'. This opens the 'Import Options' panel shown in Fig. 114, right. For standard import operations, SPCImage detects the number of SPC modules and the number of routing channels and suggests import options which load all combinations of modules and routing channels. Unless you want to load subsets of the data or change the assignment of routing channels, you can just click 'OK' and get the data imported.

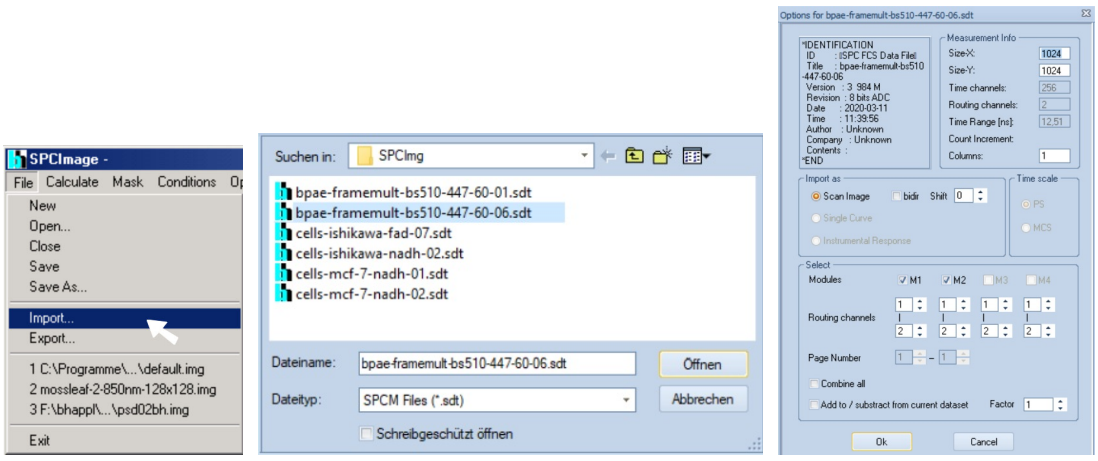


Fig. 114: Importing .sdt FLIM data. Left: Import of an .sdt file via ‘File’, ‘Import’. Middle: File selection panel. Right: Import options panel.

It can happen that the .sdt data contain combinations of modules, routing channels and page numbers which you do not want to import. In that case, you can disable or enable modules, and select ranges of routing channels which you want to import. There is also an option to combine the data of all modules and channels, or to add or subtract data from the current data set. These options should be handled with care. They require that all data are recorded with the same IRF position and IRF shape. They are mainly used in emergency situations, and to rescue data which were recorded with wrong SPCM system parameters.

Importing FLIM Data From SPCM via 'Send Data' Function

FLIM data can be send directly from SPCM to SPCImage. The ‘Send Data to SPCImage’ function of the SPCM software is illustrated in Fig. 115. The function automatically opens SPCImage and transfers the data. In the ‘Application Options’ of the SPCM software you can select whether you want to transfer the data of all active display windows or only the data of the selected one. To select a display window, first click into the image that you want to analyse, see Fig. 115, left. Then transfer the data by clicking into ‘Main’, ‘Send data to SPCImage’, see Fig. 115, right.

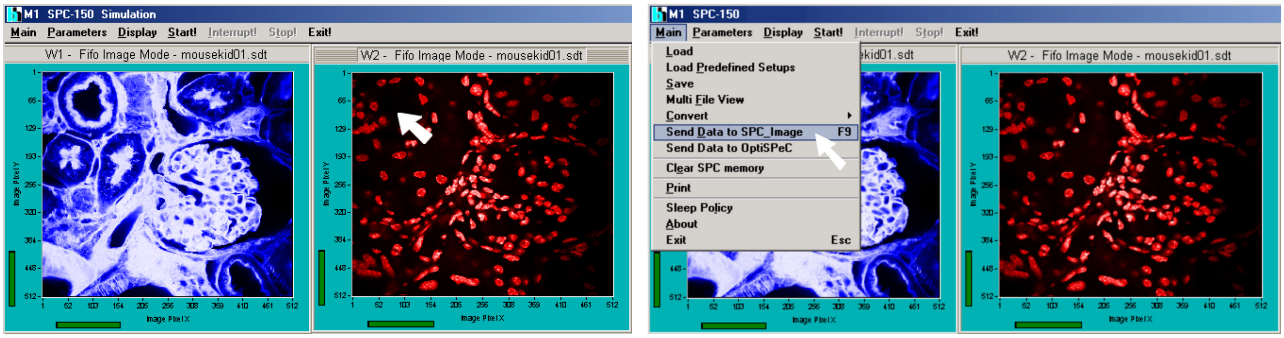


Fig. 115: Sending data from SPCImage into SPCImage. Left: Selection of the data to be analysed. Right: Sending the selected part of the data to SPCImage

SPCM Panel after Import of FLIM Data

After importing .sdt data into SPCImage the data are displayed as shown in Fig. 116. An intensity image is shown left, a decay curve at the cursor position bottom right. The image does not display colour-coded lifetimes because these have not been calculated yet. A decay-parameter histogram is shown upper right. It is empty because no decay analysis has been performed yet. In the upper right, the decay model can be specified. After loading new data the model function is single exponential ('Components' = 1). This is the default. For double- or triple-exponential decay models change 'Components' to 2 or 3. For other model options, such as 'Incomplete Decay' or 'Shifted-Components' please click into 'Options', 'Model' and select the desired features.

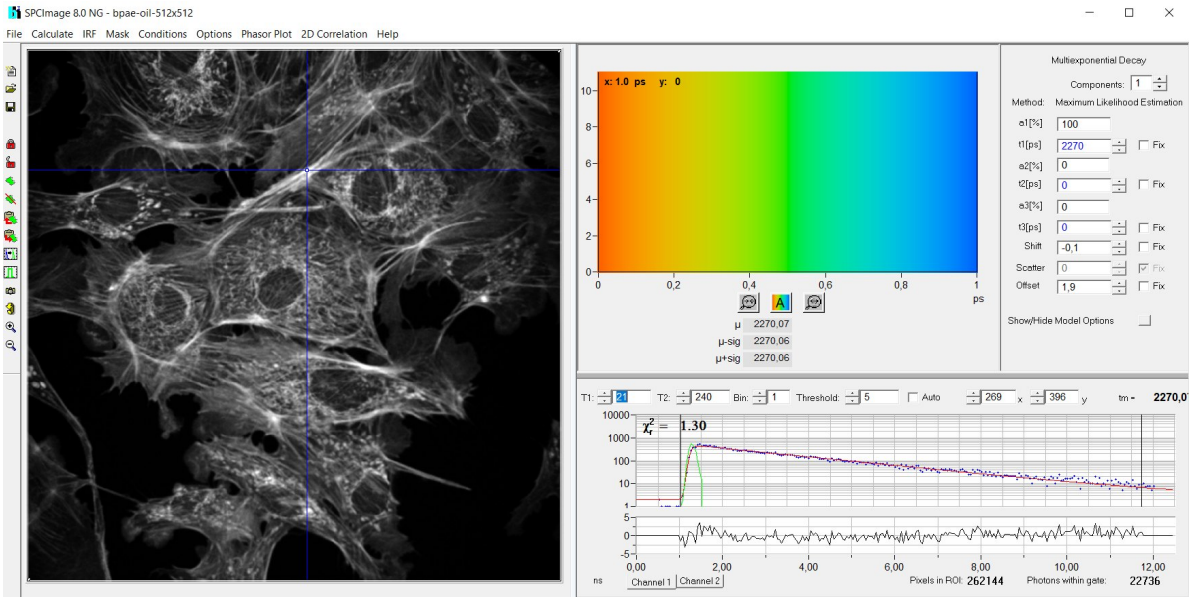


Fig. 116: SPCImage panel after importing raw data

Importing .sdt Files of Single Curves

Single-Curve decay data recorded in traditional cuvette-based setups can be imported into SPCImage and analysed with the normal set of models and model options. There are two ways to import the data into SPCImage. Import via the normal import function of SPCImage is shown in Fig. 117.

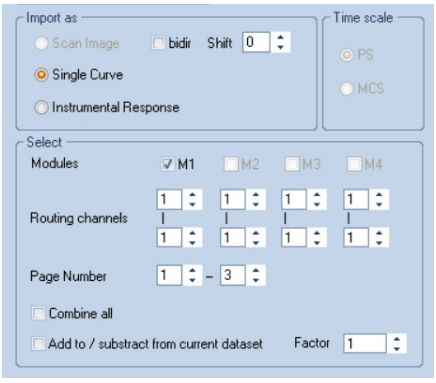


Fig. 117: Import of single-curve data via the import function of SPCImage

Select 'Single Curve', select the SPC module from which you want to import data, select a page or a range of 'Pages' and click OK to import the decay curves.

Importing Single-Curve Data From SPCM via 'Send Data' Function

Data can also be transferred to SPCImage by the 'Send Data' of SPCM. Click into ‘Main’, ‘Send Data to SPCImage’. This opens a select panel in the SPCM curve window, see Fig. 118. It contains the numbers of pages (traces) which are active in the '2D Trace Parameters'. Select the curve(s) you want to send, and click on ‘OK’. After that, SPCImage will open and display the decay curve(s) that have been sent to it, see Fig. 119.

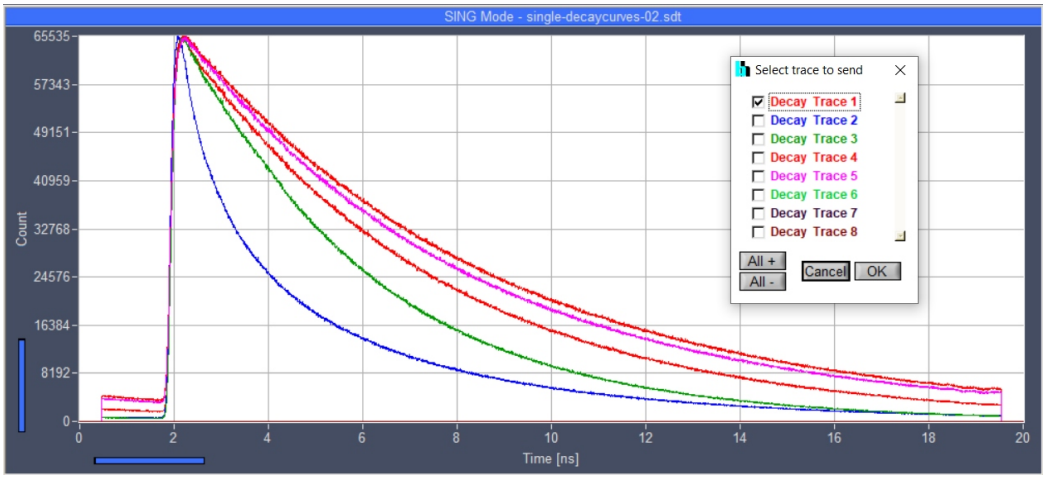


Fig. 118: Sending single curves from SPCM to SPCImage

SPC Image Panel after Import of Curve Data

After importing the data, SPCImage will display the decay curve(s) that have been imported or sent to, see Fig. 119. Of course, the image display panel and the parameter-histogram panel will remain empty. There is nothing that could reasonably be displayed in these windows. We suggest to reduce the size of these windows to provide maximum space for the display of the curve. SPCImage immediately runs a fit of the curve with the default single-exponential model. The blue dots are the data points, the red curve is the fit result, and the green curve is the IRF. The decay parameters are displayed in the upper right of the SPCImage panel. For selection of double- or triple-exponential decay models change the number of 'Components' to two or three. For other model options, such as 'incomplete decay' or 'shifted-components' please click into 'Options', 'Model' and select the desired features [4].

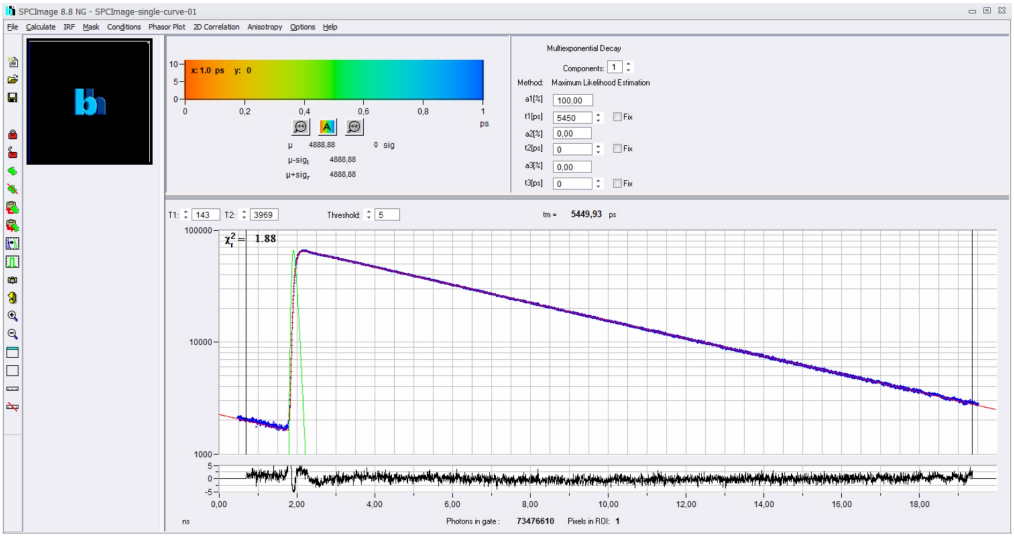


Fig. 119: SPCImage panel after importing a single decay curve. Single-exponential fit with synthetic IRF.

Single-Curve Data from Scanning Experiments

Single-curve data can also be obtained by scanning a sample in a FLIM system. A dish with a solution of the dye to be investigated is placed on the microscope stage, and FLIM data of the solution are recorded. To analyse such data, import them into SPCImage by the normal import procedure for FLIM data. Click the (lock) button to combine the decay data of the entire image area (or of an area selected by the image cursors) into a single decay curve. Select the desired decay model type, model

parameters and fit parameters, and choose or create an IRF [4]. The fit process starts instantly (no need to start 'Calculate'), and the decay parameters are shown in the upper right of the SPCImage panel. An example is shown in Fig. 120.

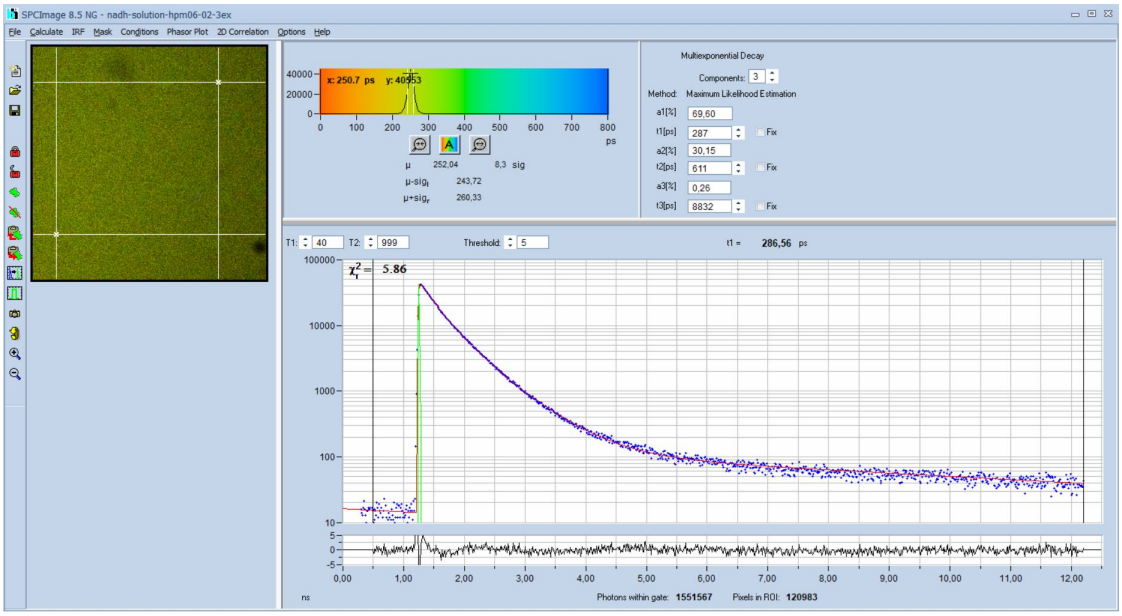


Fig. 120: Combination of decay data from an image area into a single curve, and data analysis with triple-exponential model. NADH solution, DCS-120 MP system with HPM-100-06 detector.

Calculating a Lifetime Image

Starting the Calculation

In principle, you can start a lifetime analysis immediately from the state shown in Fig. 116. (There is even an option to do this automatically, see 'Preferences' panel [4]) To start the analysis, click into ‘Calculate’, ‘Decay Matrix’, 'selected channel' or, if you have loaded data with several channels 'all channels'. This starts the fit process. A progress bar shows the advance of the calculation as the procedure runs through the pixels. If there is a GPU in the computer (we strongly recommend this) the calculation will complete within a few seconds, if there is no GPU it can take several minutes. What you get is a colour-coded lifetime image calculated with default model parameters of SPCImage. The decay model will be single-exponential, the IRF will be 'Auto', and the lifetime and intensity ranges will be set automatically.

Fig. 121: Starting the fit procedure for all pixels of the image

Single-Exponential Analysis

The number of decay components used in the analysis is defined in the upper right of the SPCImage panel. FLIM analysis with a single-exponential model (or with default parameters) is shown in Fig. 122. Number of 'Components' is 1. The procedure delivers a single lifetime, t_1. This lifetime is used for colour-coding the image. The lifetimes in the pixels will be correct and reasonably accurate. However, the fluorescence decay in biological objects is rarely single-exponential. Normally there are several decay components in each pixel, either from different fluorophores, or from one fluorophore in different molecular environment. Often the information is in the composition of the decay rather than in an average ('apparent') lifetime. Single-exponential analysis is therefore unlikely to deliver the maximum of information you can obtain from your raw data.

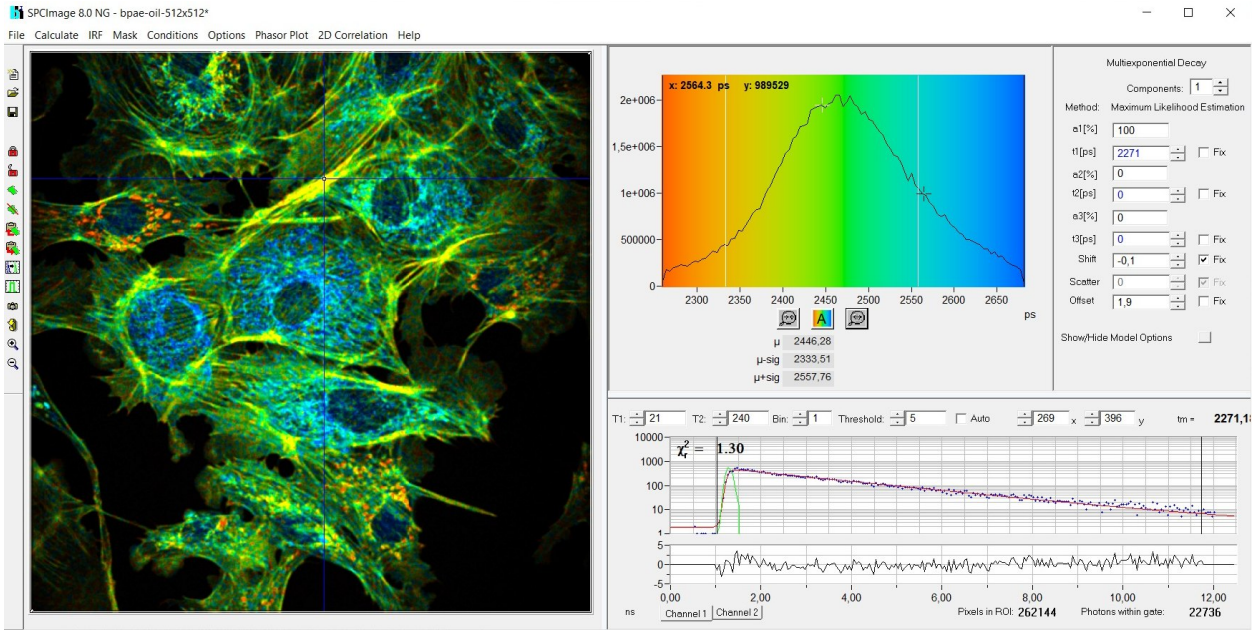


Fig. 122: SPCImage panel after calculating the lifetime image with the default settings

Multi-Exponential Analysis

Multi-exponential decay analysis is shown in Fig. 123. In the upper right, analysis with three exponential components has been selected. For every pixel, the analysis procedure delivers three lifetimes, t_1, t_2, t_3, and three amplitudes, a_1, a_2, a_3, for the three decay components. The display routine of SPCImage can display each of these parameters, ratios of the parameters, and intensity- or amplitude-weighted averages of the component lifetimes. The default is the amplitude-weighted mean lifetime, t_m. The display routine has functions to further refine the images, for example by manually adjusting the colour scale and the intensity scale, or by creating time-gated images. Please see [4].

Also the model function can be further refined, such as by the 'Incomplete Decay' or the 'Shifted-Components' option. It is also possible to fix one or two of the decay components to values which are a priori known. Please see [4] for details.

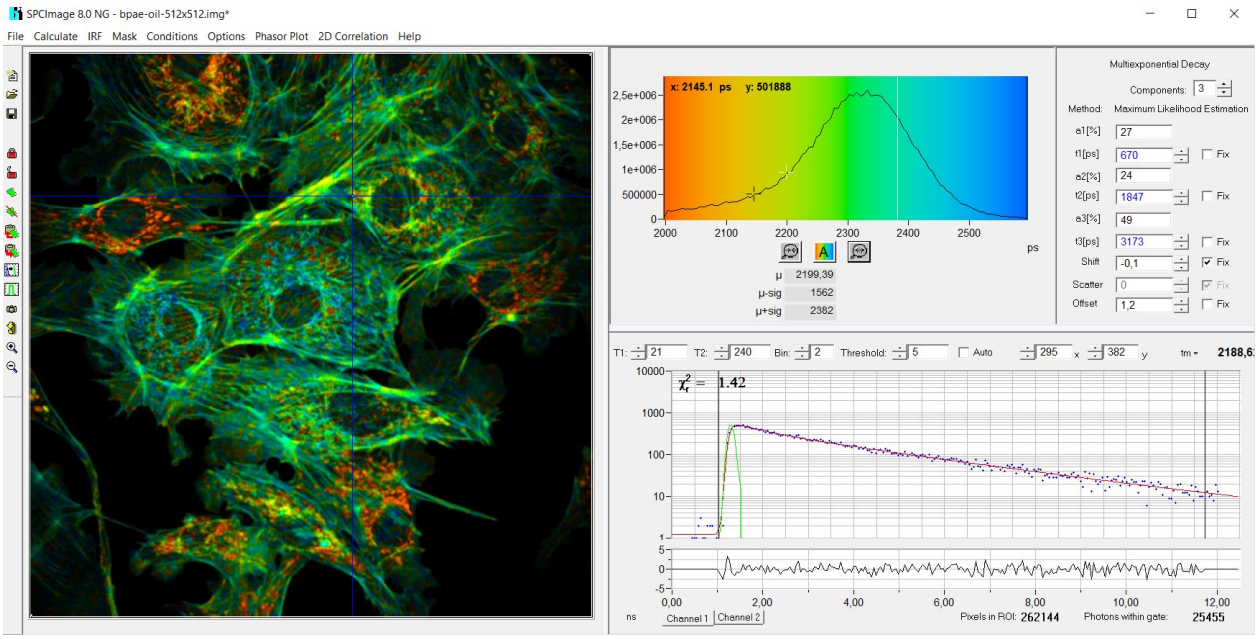


Fig. 123: Triple-exponential decay analysis. Model selection and decay parameters at cursor position shown in the upper right. Analysis with three exponential components has been selected, the amplitude-weighted lifetime, t_m, is shown.

Parameter Histogram

A histogram of the selected decay parameter over the pixels of the image is shown above the decay-curve window. It shows how frequent a given value of the parameter appears in the image. The parameter is the same that was selected for colour-coding the image. It can be the amplitude-weighted lifetime (as in Fig. 123), the intensity-weighted lifetime, a component lifetime, a component amplitude, or a ratio of two of these parameters. The parameter histogram can be displayed for selected regions of interest. The parameter histogram has two cursors, which interact with the display function. In combination with the , , and buttons a desired parameter range can be selected, and the colour scale adjusted accordingly.

Other SPCImage NG Functions

SPCImage NG has many more functions than can be covered in this abbreviated description. There are different Model Options, IRF Options, Display Modes, Parameter and Intensity Histograms, and 2-Dimensional Histograms of combinations of two selected decay parameters. There is a Phasor Plot, Image Segmentation by the Phasor Plot and by 2D Histograms, ROI Selection, Combination of Decay Data in ROIs and Segmented Image Areas, Binning of Decay Data, Analysis of Special Data Types, and Batch Processing of Series of FLIM recordings. To use these functions, please see chapter 'SPCImage NG Data Analysis Software' in the bh TCSPC Handbook [4].

Peripheral Components

Every TCSPC system needs peripheral components, such as lasers, detectors, and experiment control devices. A variety of such devices is available from bh. All are compatible with the SPC and SPC-QC modules. Frequently used peripheral devices are described below. For details please see TCSPC Handbook and individual data sheets and manuals.

BDS-SM Series Lasers

The BDS-SM series picosecond diode lasers [25] are OEM-size modules with dimensions of only 40 mm x 40 mm x 110 mm. For stand-alone operation a heat sink is added, the dimensions including the heat sink are 40 mm x 40 mm x 110 mm, see Fig. 125. The lasers contain the entire driver electronics. They are operated from a simple +12 V power supply and can be controlled via a bh DCC-100 card, a GVD-120 or GVD-140 card, or via an independent manual-control box. The lasers are available both with free-beam and single-mode fibre output. The pulse width is on the order of 40 to 90 ps, FWHM, the pulse repetition rate can be switched between 80 MHz, 50 MHz, 20 MHz, and CW. All the typical diode laser wavelengths from 375 nm to 785 nm are available. The output power is stabilised by an internal regulation loop, and fast on-off switching is implemented. The lasers have a synchronisation output to the bh TCSPC modules and a trigger input for synchronisation with external clock sources.

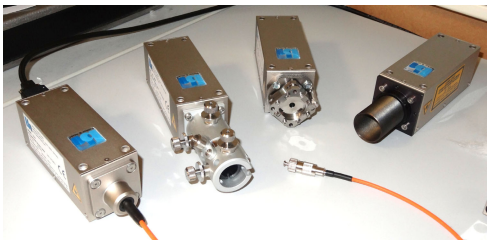


Fig. 124: BDS-SM series laser with pig-tail single-mode fibre, Qioptiq Kineflex adapter, Lasos Precision Connector, and free-beam output through a C-Mount adapter

BDU-SM USB-Controlled Picosecond/CW Diode Lasers

The BDU-SM family picosecond / CW diode lasers are fully controlled and powered via a USB interface. Available wavelengths range from 375 nm to 785 nm. The lasers are available with elliptical or circular free-beam output, and with single-mode fibre output. As the BDS lasers, the BDU lasers feature extraordinarily high timing stability and intensity stability. Repetition rates are 20, 50, 80 MHz, or CW. Optical power at 80 MHz is typically 3 to 5 mW. Pulse width is 40 ps to 300 ps, depending on the wavelength version and selected power. The laser, a typical pulse shape, and the control panel are shown in Fig. 125

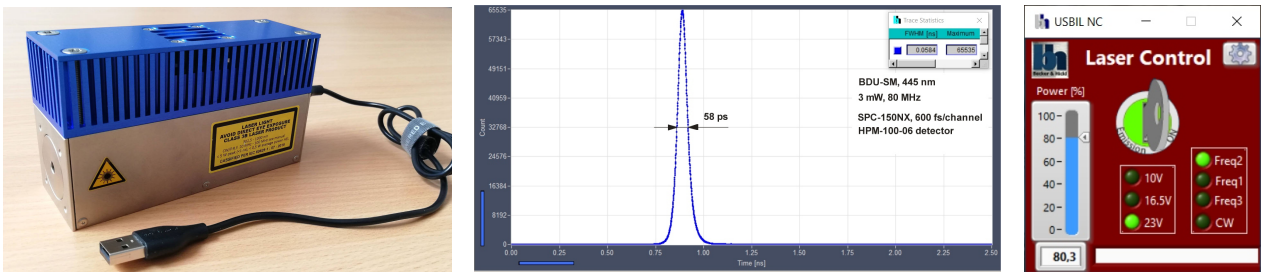


Fig. 125: BDU-SM series picosecond diode laser

Detectors

bh TCSPC devices work with almost any single-photon detector. For some detectors an electrical attenuator or a pulse inverter may be required. An overview on detectors, their working principles and their performance with TCSPC can be found in the bh TCSPC Handbook [1], chapter 'Detectors for

TCSPC'. IRF widths and IRF shapes given there are recorded with SPC-150 through SPC-180 series TCSPC modules. These modules have electrical IRF widths on the order of 6 ps to 2.8 ps, FWHM. Compared with the transit-time-spread of the detectors this is negligible. Recorded with short laser pulses, the IRFs are therefore close to the true IRF of the detector itself.

For the SPC-QC modules this is not necessarily the case. The electrical IRF width is about 45 ps, FWHM, and thus not negligible compared with the IRF width of fast detectors. With the detector IRF width, W_{irfd}, and the electrical IRF width of the SPC-QC, W_{irfel}, the system IRF width, W_{irf} can be estimated by

$$W_{irf} \approx \mathrm{SQRT}\,(W_{irfd}^2 + W_{irfel}^2)$$

That means the electrical IRF has a noticiable influence on the system IRF only for detectors faster than about 100 ps. These are the HPM-100-06 and -07 hybrid detectors, MCP PMTs [26], some fast single-photon avalanche diodes (SPADs), and superconducting single-photon detectors (SSPDs).

Hybrid Detectors

The bh HPM-series hybrid detectors feature high sensitivity, high time resolution, low dark count rate, and almost complete absence of afterpulsing [28]. With their introduction in 2009 they quickly replaced PMT detectors in almost all high-end TCSPC applications. More than 900 pieces were manufactured since 2009. The most frequently used one is the HPM-100-40. With its GaAsP cathode, almost 50% quantum efficiency, and 90 to 110 ps IRF width it is an almost ideal detector for time-resolved laser scanning microscopy [27, 28]. With the introduction of GaAs cathode versions the application was extended into NIRS spectroscopy [29]. Finally, with the ultra-fast bialkali and multialkali versions, the HPM-100 modules joined the exclusive club of sub-20-ps detectors, which previously was entirely governed by MCPs and SSPDs [26, 32, 33]. Photos of the HPM-detector are shown in Fig. 126.

Fig. 126: Left: bh HPM-100 hybrid PMT module. Left to right: Versions with C-Mount adapter, version with Zeiss BIG adapter, fibre-version, version with thermoelectric cooler

The HPM-100 modules come in different cathode versions. Detectors with different cathodes have different IRF width. Typical values (FWHM) are listed in the table below.

	Cathode Type	Wavelength Range	Peak Quantum Efficiency	IRF Width, SPC-150NX	IRF Width, SPC-QC
HPM-100-06	Bialkali	300 - 600 nm	20 %	18 ps	46 ps
HPM-100-07	Multialkali	250 - 800 nm	20 %	18 ps	46 ps
HPM-100-40	GaAsP	300 - 720 nm	48 %	90 - 130 ps	100 - 140 ps
HPM-100-41	GaAsP	300 - 800 nm	30 %	110 - 150 ps	120 - 150 ps
HPM-100-50	GaAs	400 - 900 nm	20 %	130 - 200 ps	140 - 200 ps

IRFs for an HPM-100-06 detector and an HPM-100-40 detector recorded with an SPC-QC are shown in Fig. 127. The test light source was a Toptica Femto-Fibre Pro femtosecond fibre laser. SHG was used to frequency-double the test light from 785 nm to 379 nm.

Fig. 127: IRF of HPM-100-06 (left) and HPM-100-40 (right) with SPC-QC-104. 100 ps/div, recorded at 379 nm.

PMC-150 and PMCS-150 Cooled PMT Modules

The PMC-150 and the PMCS-150 are cooled PMT modules. They are based on Hamamatsu photosensor modules. These modules contain a miniature PMT together with a high-voltage generator. The modules come in different cathode versions, please see data sheets on www.becker-hickl.com. The detectors are shown in Fig. 128. The IRF width depends on the illuminated area. When the entire active are is illuminated the IRF is about 130 ps (FWHM) wide, see Fig. 129.

Fig. 128: Left: PMC 150 detector. Right: PMCS-150 detector.

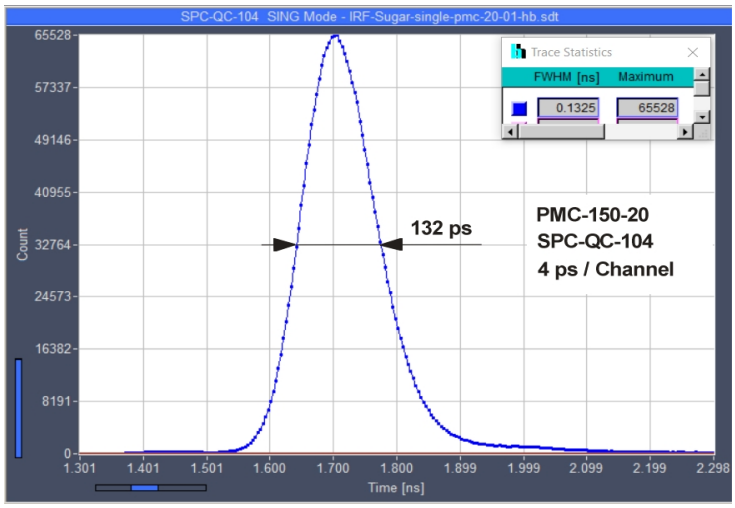


Fig. 129: IRF of a PMC-150-20, 100 ps/div., recorded with SPC-QC. Full cathode illuminated.

Zeiss BIG-2 Detector

The Zeiss BIG-2 detector is part of the Zeiss LSM series laser scanning microscopes. The detector contains two PMTs with GaAsP cathodes with a dichroic beamsplitter between them. The BiG-2 has an electrical output for the detector signal. There are contradicting reports about the applicability to TCSPC FLIM. Our own results have been positive [30]. The output amplitude for a single photon is on the order of +300 mV. With an A-PPI-D pulse inverter it is perfectly compatible with the bh TCSPC FLIM systems and, thus, with the SPC-QC-104. The IRFs shown in Fig. 130 were recorded with SPC-150 modules. The width is about 250 ps, FWHM. The same IRF width can be excepted with the SPC-QC-104 module.

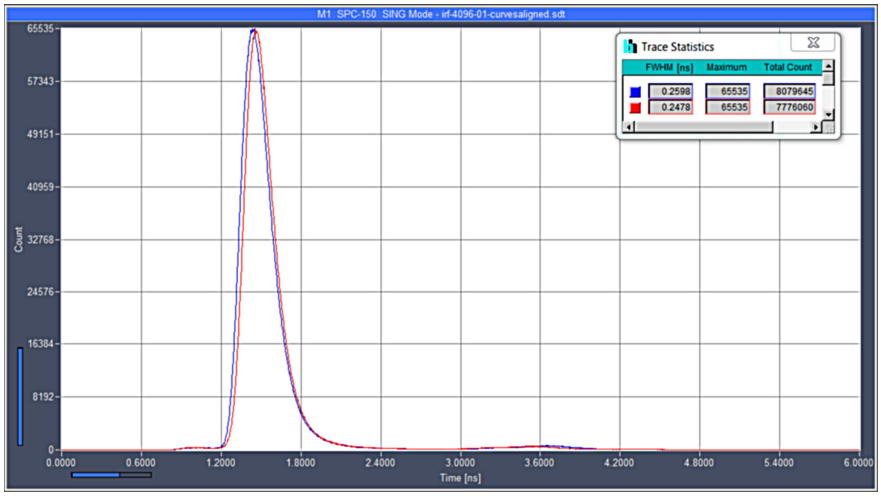


Fig. 130: IRF of the two channels of the Zeiss BiG-2 detector

Multi-Wavelength Detectors

The bh PML-16C and PML-16 GaAsP detectors provide 16 simultaneously recording channels for TCSPC. The detectors are shown in Fig. 131, left and second left. Multi-wavelength detection assemblies with the PML-16 detectors are shown in Fig. 131, second right and right.

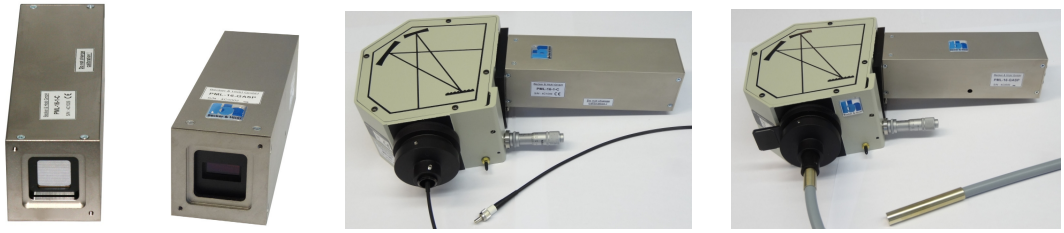


Fig. 131: Left to right: PML-16C, PML-16 GaAsP, PML-SPEC assembly with fibre input, MW-FLIM assembly with fibre-bundle input.

The detectors are operated via a DCC-100 detector controller card. They are directly compatible with the bh SPC and SPC-QC modules. IRFs for the PML-16-C and the PML-16 GaAsP are shown in Fig. 132. The IRF width is 150 ps FWHM for the PML-16-C, and 203 ps for the PML-16 GaAsP. Because the IRFs are relatively wide there is no perceptible difference between the SPC series modules and the SPC-QC-104. For further details please see bh TCSPC Handbook.

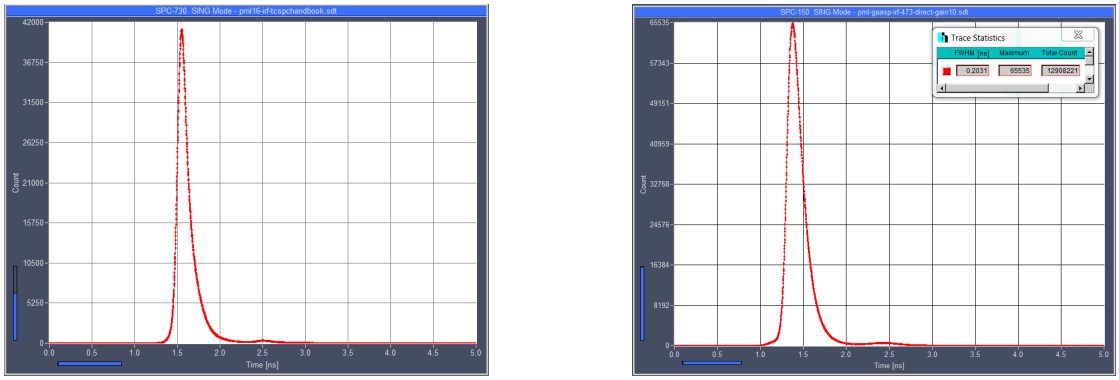


Fig. 132: IRF of PML detectors, one channel of 16. Left: PML-16C, FWHM = 150 ps. Right: PML-16 GaAsP, FWHM = 203 ps

SPADs

SPADs (Single-Photon Avalanche Photodiodes) connect to the SPC and SPC-QC cards without problems. For most of them a pulse inverter and an attenuator is required. These parts are available from bh. Real IRF widths of SPADs range from below 10 ps [31] for very small devices to more than 500 ps for large-area devices. 'Low Noise' (low dark count) versions tend to have wider IRFs than standard versions. An IRF obtained from an id Quantique id100-20 ultra-low noise detector is shown in Fig. 133, left, an IRF from a Perkin-Elmer (Excelitas) SPCM-AQR low-noise detector in Fig. 133, right.

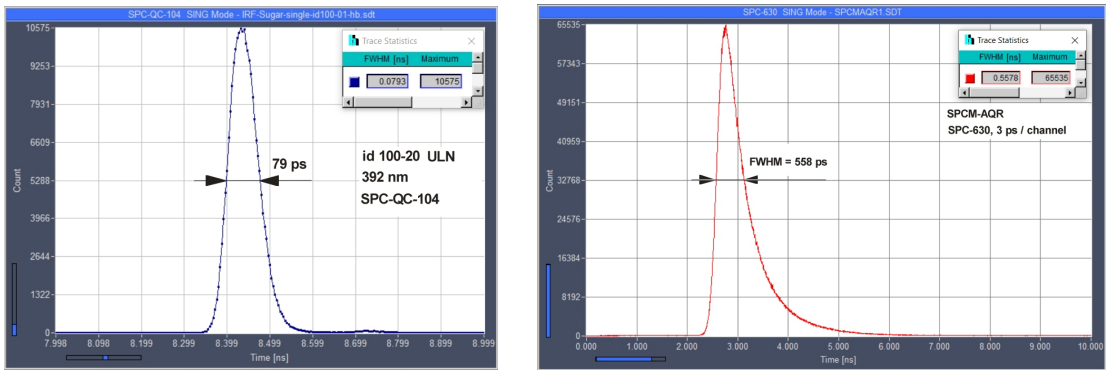


Fig. 133: Left: IRF of an id Quantique id100-20 low noise detector. Right: IRF of an Excelitas / Perkin Elmer SPCM-AQR detector, low-noise version. Note different time scale.

More examples can be found in the bh TCSPC Handbook [1]. As for the HPM detectors, there is no significant difference between the SPC and the SPC-QC modules for detectors with real IRF widths above about 100 ps.

SSPDs

SSPDs (Superconducting Single Photon Detectors) have extremely fast IRFs. Two examples are shown in Fig. 134. One is from a SCONTEL TCORPS-UF-10 detector [32], the other from a single-nanowire SSPD of JPL, Pasadena [33].

Fig. 134: IRF of a SCONTEL TCORPS-UF-10 SSPD (left) and IRF of a single-nanowire SSPD of Jet Propulsion Laboratory (JPL). Both recorded with SPC-150NXX module. Note different time scale.

It is obvious that the time resolutions of detectors this fast cannot be exploited by an SPC-QC or any other TDC-based timing instrument. Only the SPC-150NXX and the SPC-180NXX come close to the timing resolution required for SSPDs. Unless other detector parameters than the time resolution are important SPC-series TCSPC modules should be used instead of the SPC-QC-104.

DCC-100 and DCC-100-PCIe Detector / Laser Controllers

The DCC-100 card is used to control detectors and/or lasers of bh TCSPC systems. It is able to control the BDL-SMC, BDL-SMN, and BDS-SM ps diode lasers, and to control electro-mechanical shutters. Please see bh TCSPC Handbook for technical details. The software panel of the DCC-100 is shown in Fig. 135.

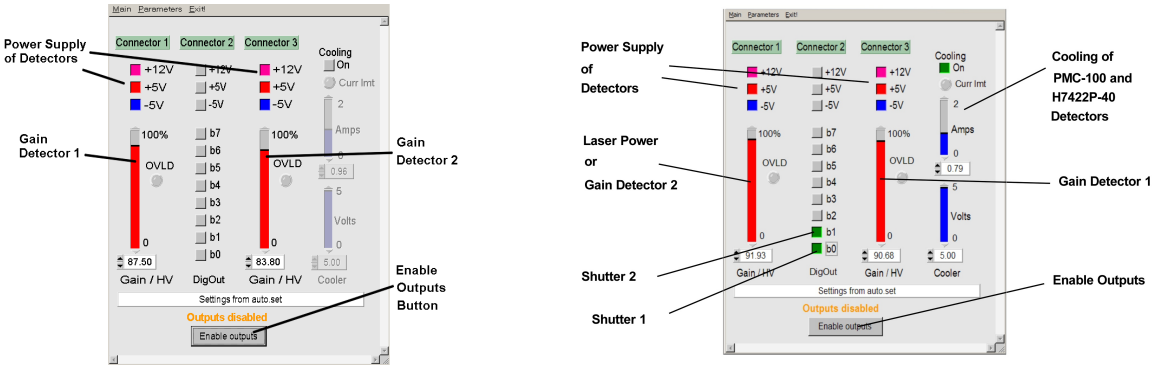


Fig. 135: Detector control panel. Left: Control of two detectors. Right: Control of one laser and one detector.

DCU-400 and DCU-800 USB Detector / Laser Controllers

The DCU devices are Detector / Laser Controllers with up to four (DCU-400) and up to eight (DCU-800) outputs. All outputs can control bh detectors or bh lasers. Except for a larger number of channels, the user interface is the same as for the DCC-100. Photos are shown in Fig. 136.

Fig. 136: DCU-400 and DCU-800 USB-controlled Detector / Laser controllers, front view and back view

GVD-120 and GVD-140 Scan Controllers

The GVD-120 and GVD-140 san controller modules are used to control fast galvanometer scanners or piezo scan stages with analog input. The devices also control the scanner of the bh DCS-120 Confocal and Multiphoton Scanning FLIM System [6]. In addition to driving the scanners the GVD devices control bh BDS and BDL picosecond diode lasers. The GVD-120 controls two, the GVD-140 four lasers. Laser control includes beam blanking during the flyback of the scanner, intensity control, laser on-off switching, and laser multiplexing. With the GVD cards, the operation of the scanner and the lasers is fully integrated in the SPCM software. The user interfaces are shown in Fig. 137. For technical details please see [1] and [6].

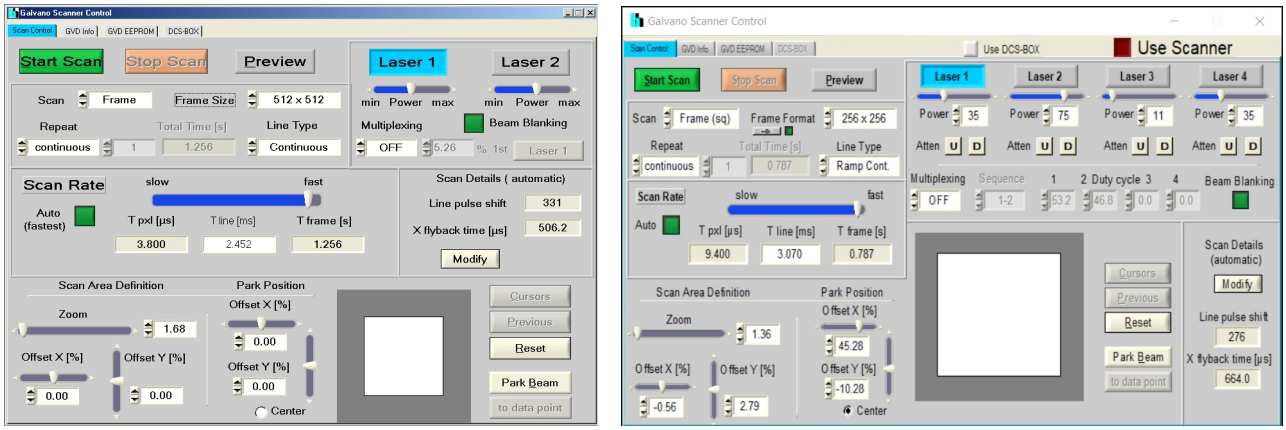


Fig. 137: GVD control panel. Left: GVD-120. Right: GVD-140

DCS-120 Scan Head

The DCS-120 scan head is used to scan objects with focused laser beams and return fluorescence signals from the object to two detectors. The scan head has two optical inputs for bh BDL-SMN or BDS-SM lasers, two confocal detection paths, internal beamsplitters and filters, individually selectable pinholes, and two optical outputs to detectors. The detectors are attached to the back of the scan head. Scanning is performed by fast galvanometer mirrors. The scan head is controlled via a GVD-120 or GVD-140 scan controller card. The user interface is fully integrated in SPCM, see above, Fig. 137. A photo of the scan head is shown in Fig. 138.

Fig. 138: DCS-120 scan head

Normally, the scanner is part of the bh DCS-120 Confocal and Multiphoton FLIM System, see [6]. However, it can be used also independently of this system. Its use is not only attractive to solve user-specific FLIM problems but also to upgrade conventional microscopes with FLIM. The DCS-120 scan head can also be a real alternative to - often desperate - attempts to attach FLIM systems to scanning microscopes which are not designed for this kind of operation. In these cases, a DCS-based system may be not only easier to set up but also perform better. Moreover, the DCS design results in a fully integrated system, operated from one piece of software, and from one computer. For details please see bh TCSPC Handbook [1] and handbook of the DCS-120 system [6].

DB-32 SYNC Delay Box

The DB-32 is a USB-controlled passive delay box. It is used to adapt the signal transit time in the SYNC path or in a detector path of a TCSPC system to different optical configurations. Moreover, it can switch between to different SYNC sources or two different detectors. The SYNC Delay Box is shown in Fig. 139.

Fig. 139: Sync Delay Box

The delay and the signal source are selected via a ‘Delay’ parameter in the SYNC parameter part of the System Parameters, or directly via the SYNC field in the main panel, see Fig. 140.

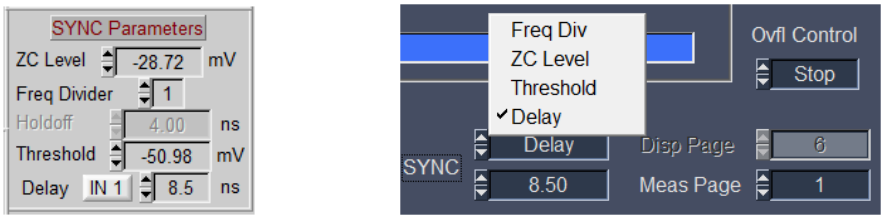


Fig. 140: Delay setting in the system parameters (left) and in the SYNC field of the main panel (right)

SIS 2x4 USB-Controlled Signal Switch

The SIS 2x4 module contains two 4:1 or 1:4 USB-controlled signal switches. The device is used to select between different detectors and different SYNC sources. Different hardware configurations of a TCSPC system can thus be realised and selected by loading the corresponding setup data via the 'Load' function or the Predefined Setup panel. The SIS 2x4 is shown in Fig. 141, left, the control panel in Fig. 141, right.

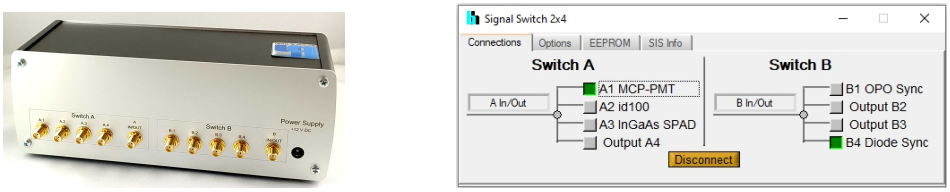


Fig. 141: SIS 2x4 USB-controlled signal switch

DDG-210 Pulse Generator Card

The DDG-210 pulse generator card is used in systems which do not contain a GVD card to on-off modulate lasers for phosphorescence decay measurements and phosphorescence lifetime imaging. The DDG-210 panel is shown Fig. 142. The card generates pulses to the laser or to an AOM of a laser, and a routing signal to the SPC module. The pulses can be generated in a free-running mode, or be triggered by the pixel clock of a scanner. The DDG-210 is part of the bh FLIM systems for the Zeiss LSM 710 to 980 family laser scanning microscopes [7]. Please see [7] and bh TCSPC Handbook [1] for further details.

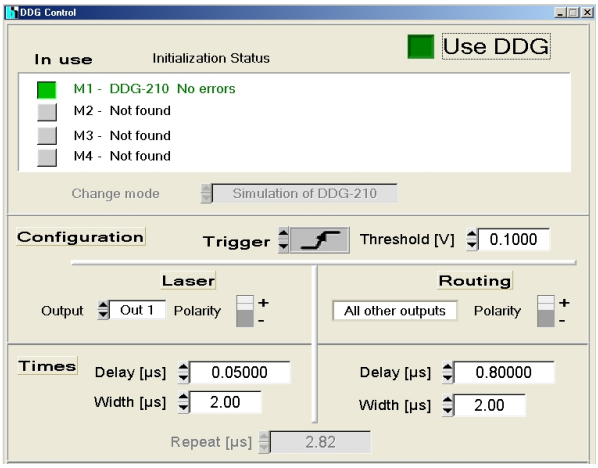


Fig. 142: DDG-210 control panel

Ti:Sa Laser and AOM Control

The SPCM software is able to control a Ti:Sa laser and an AOM. The control panel opens by a click into ‘Devices’, 'Ti:Sa laser & AOM Control’. The control panel is shown in Fig. 143

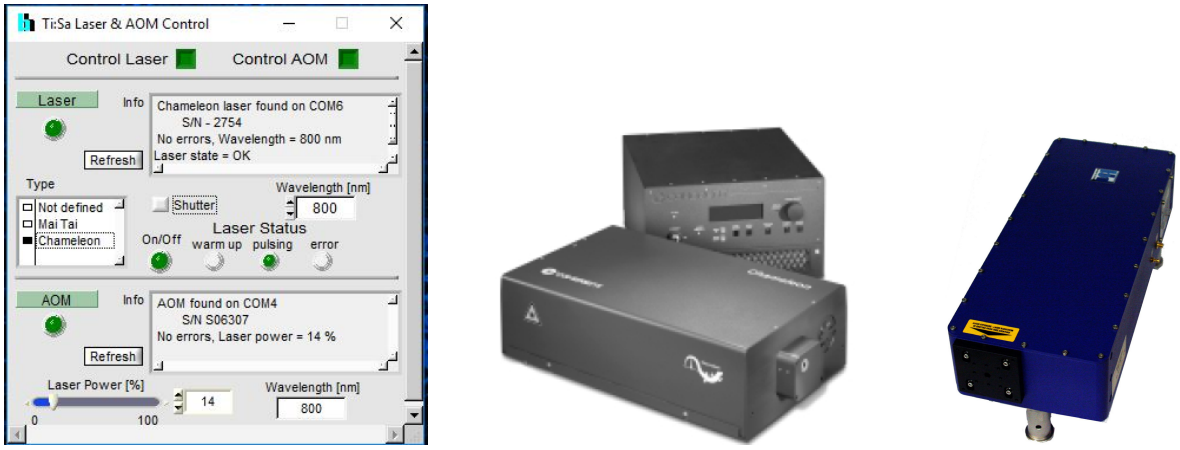


Fig. 143: Left: Ti:Sa laser and AOM control panel. Right: Coherent Chameleon laser and bh AOM

The laser is controlled in the upper part of the panel. The type of Ti:Sa laser must be selected under ‘Laser’, ‘Type’. The system computer connects to the laser via a USB interface. The laser panel controls the shutter of the laser and the wavelength, and displays the status of the laser.

The lower part of the panel controls the bh AOM. Since an AOM is wavelength-dependent the AOM controller has to know the wavelength selected for the laser. If both the laser and the AOM are controlled from SPCM the wavelengths for the laser and the AOM are automatically coupled.

Motor Stage

SPCM is able to control a motorised sample stage. The stage can be used to control the position of a sample manually or to record mosaics of images in cooperation with the GVD-120 or GVD-140 scan controller of the bh DCS-120 system.

The motor stage control panel is shown in Fig. 144. The panel shown left lets the user manually control the sample position. When a DCS scan is started the system records a normal FLIM image in the selected area of the sample. The panel on the right has 'Tile Imaging' enabled. The motor stage interacts with the scanner, and, in cooperation with the DCS scanner, records a spatial mosaic of images. Please see [1] for details.

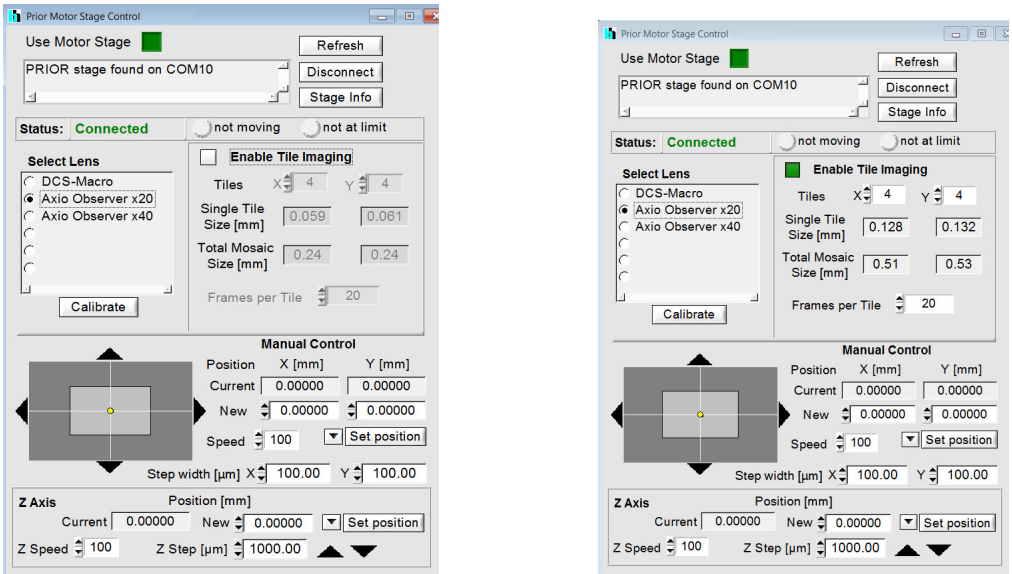


Fig. 144: Control panel for motor stage. Left: Normal scanning, manual control of motor stage. Tile imaging disabled. Right: Tile imaging enabled. Spatial mosaic scanning and manual control of stage.

Fig. 145 shows how the optical scanner interacts with the motor stage. When Tile Imaging is enabled the step width of the motor stage automatically adjusts to the scan area (Zoom factor) selected in the DCS scanner panel. When the measurement is started the SPC system records a mosaic of FLIM images the elements of which have the same x and y size as the step width of the motor stage. The result is a large image consisting of individual scans offset by the motor-stage step width. The data of the individual elements of the mosaic (or tiles) can be accumulated over a selectable number of frames (20 in Fig. 145). The number of frames per tile can be selected both in the motor stage panel and in the scanner panel. It is automatically adjusted to the same value.

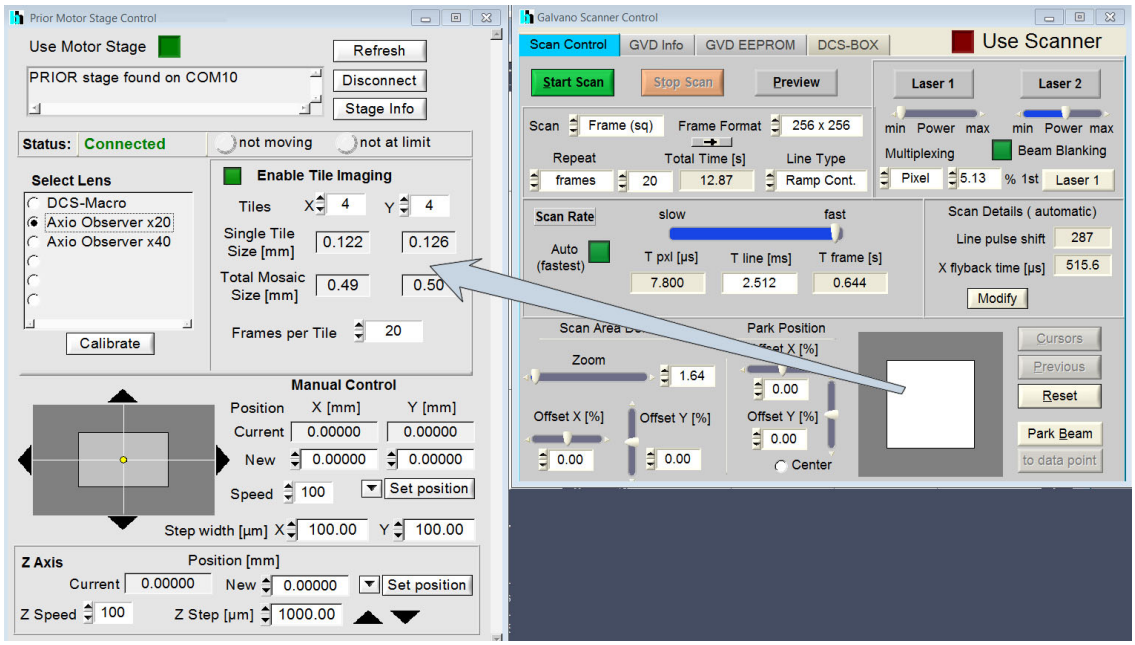


Fig. 145: Interaction of the motor stage with the DCS-120 scanner

Support

We are happy to give you any possible support to the use of your TCSPC devices. Support starts with help for finding the most promising experimental strategy to solve a given scientific problem, choosing the right system components to implement this strategy, and choosing data recording and data analysis procedures that yield a maximum of information from the experiment. Best case, support should start even before you buy the system components. Once you have the wrong laser and the wrong detectors the best technical support cannot correct the mistakes. Therefore contact us before it is too late.

An important component of support is the bh TCSPC Handbook. It presents solid information on any level: Detection of fast low-level light signals, TCSPC principles, noise sources and their minimisation, detectors and detector principles, setup of experiments, multi-dimensional features of advanced TCSPC and TCSPC FLIM, applications, parameter setup, and data analysis. Moreover, the book contains more than 1200 references related to the bh TCSPC and FLIM devices and their application. Please take a look into this book - if you want a printed copy please request one from bh.

If you have problems with your TCSPC module or with the experiment in which it is used, please contact us under info@becker-hickl.com. Please do not waste your and our time preparing screen shots or lengthy power-point documents to describe the problem. What we need is a data file. Only from the data file we can conclude what is wrong with your system. We can even reproduce a possible problem here.

If you can't save the data (.sdt) send us a setup file (.set). Screen shots are only appropriate if nothing else is working.

In most cases, however, the source of a problem is not directly in the TCSPC module but in the experimental setup. Therefore add a short description of the problem, the setup, and the application for which it is used. If necessary, we can remotely troubleshoot your system via 'Team Viewer' software. Please contact us - we will come back to you with the details.

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