Becker & Hickl GmbH
Nahmitzer Damm 30
D-1277 Berlin
Tel. +49 30 787 56 32
Fax +49 30 787 57 34
email: info@becker-hickl.com
http://www.becker-hickl.com

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TCSPC Laser Scanning Microscopy

Upgrading laser scanning microscopes with the SPC-830 and SPC-730 TCSPC lifetime imaging modules

Since their broad introduction in the early 90s confocal and two-photon laser scanning microscopes have initiated a breakthrough in biomedical imaging [1,2,3,41]. The applicability of multi-photon excitation, the optical sectioning capability and the superior contrast of these instruments make them an ideal choice for fluorescence imaging of biological samples.

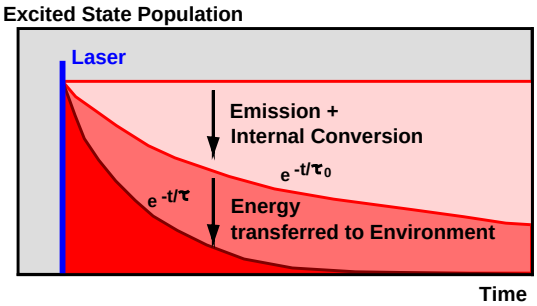
However, the fluorescence of organic molecules is not only characterised by the emission spectrum, it has also a characteristic lifetime. Any energy transfer between an excited molecule and its environment in a predictable way changes the fluorescence lifetime. Since the lifetime does not depend on the concentration of the chromophore fluorescence lifetime imaging is a direct approach to all effects that involve energy transfer. Typical examples are the mapping of cell parameters such as pH, ion concentrations or oxygen saturation by fluorescence quenching, or fluorescence resonance energy transfer (FRET) [4,5,6,7] between different chromophores in the cell. Furthermore, combined intensity / lifetime imaging is a powerful tool to distinguish between different fluorescence markers in multi-stained samples and between different natural fluorophores of the cells themselves. These components often have ill-defined fluorescence spectra but are clearly distinguished by their fluorescence lifetime.

Recording time-resolved fluorescence images can be achieved by combining a Laser Scanning Microscope with pulsed laser excitation and a new Time-Correlated Single Photon Counting (TCSPC) Imaging technique introduced by Becker & Hickl [8,9,10]. This note was written to assist upgrading of laser scanning microscopes for lifetime imaging.

Introduction

Biological Applications of Lifetime Techniques

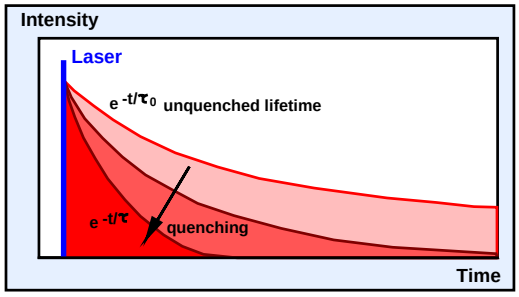
When a dye molecule absorbs a photon it goes into an excited state from which it can return by the emission of a fluorescence photon, by converting the absorbed energy internally, or by transferring the energy to the environment (figure right). The probability that one of these effects occurs is independent of the time after the excitation. If a large number of similar molecules with similar local environment is excited by a short laser pulse the fluorescence decay function is therefore single exponential. As long as no energy is transferred to the environment the lifetime is the 'natural fluorescence lifetime', τ_0 which is a constant for a given molecule and refraction index of the solvent. The fluorescence decay times of the fluorophores commonly used in microscopy are of the order of a few ns.



Return of molecules from the excited state

Fluorescence Quenching

If energy is transferred to the environment the actual fluorescence lifetime, τ, is less than the natural lifetime, τ_0. For almost all dyes the energy transfer rate depends more or less on the concentration of ions, on the oxygen concentration, on the pH value or on the binding to proteins in a cell [52]. Therefore, specifically designed dyes can be used to probe the concentration of biological relevant ions such as Na^+, Mg^{++}, or Ca^{++}, the oxygen concentration or the pH value inside a cell [54]. There is a direct relation between the lifetime and the quencher concentration (figure right).



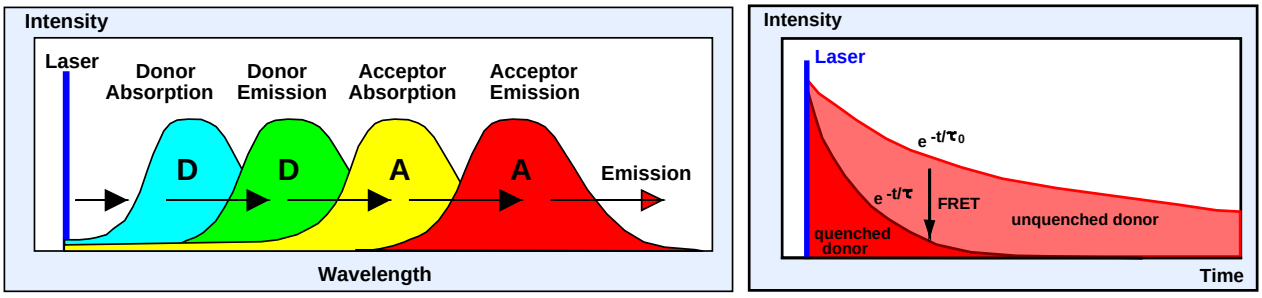
Fluorescence quenching

Fluorescence markers used to reveal particular protein structures in cells often bind to a variety of slightly different targets. Although this often does not cause significant changes in their spectral behaviour the lifetime can be clearly different due to different quenching efficiency [53,55]. Therefore the lifetime of markers can be used as an additional probe technique in cells. A wide variety of chromophores including CFP, GFP and YFP clearly show variations in their fluorescence lifetimes. Although most of these effects are not investigated in detail yet there is probably a large potential for 'intelligent' markers based on lifetime changes.

Fluorescence can be almost entirely quenched in aggregates of fluorophore molecules. Lifetimes as short as 20 ps have been found. The fast relaxation is often considered unfavourable for photodynamic therapy applications. However, if the aggregates monomerise inside a tumor cell the effect can be of particular interest [52].

Resonance Energy Transfer

A particularly efficient energy transfer process is fluorescence resonance energy transfer, or FRET [4,5,6,7]. FRET occurs if two different dyes are present with the emission band of the one dye overlapping the absorption band of the other. In this case the energy from the first dye, the donor, goes immediately into the second one, the acceptor. This results in a extremely efficient quenching of the donor fluorescence and, consequently, decrease of the donor lifetime, see figure below.



Fluorescence Resonance Energy Transfer (FRET)

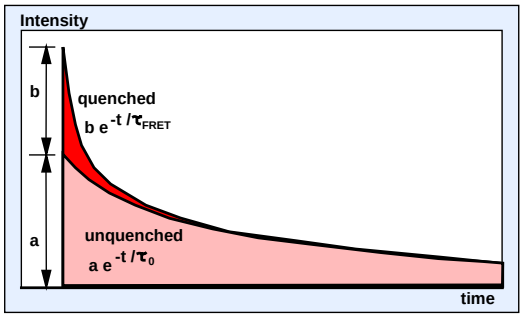
FRET works only over a distance shorter than a few nm. Therefore, it can be used to probe the distance between different subunits in the cell.

It is difficult to obtain quantitative FRET results from steady-state images. The fluorescence intensity does not only depend on the FRET efficiency but also on the unknown concentration of the dyes. Moreover, some of the acceptor molecules are excited directly, and the donor emission band extends into the acceptor emission. Up to eight measurements at different excitation wavelength and in different emission wavelength bands are required. FRET results can also be obtained by

measuring the donor fluorescence, then photobleaching the acceptor, and measuring the donor once more[6,7]. The FRET efficiency is given by the ratio of the two donor images. Of course, this procedure is barely acceptable for living cells.

In lifetime data, however, FRET shows up as a dramatical decrease of the donor lifetime [4, 11, 12, 13a, 13b].

The fluorescence decay functions contain the fluorescence of quenched and of unquenched donor molecules and are therefore double-exponential, see figure right. Qualitative FRET results can be obtained from the lifetime of a single exponential approximation of the decay curve. Quantitative measurements require double exponential decay analysis that delivers the lifetimes, τ_0 and $\tau_{FRET,}$ and the intensity factors, a and b, of the two decay components [12,13a,13b]. The relative numbers of quenched and unquenched molecules is given by the ratio of the two intensity components, b/a, while the average coupling efficiency of the FRET pairs is given by τ_0 / τ_{FRET}. In principle, both the ratio of quenched and unquenched molecules and the coupling efficiency can be derived from a single donor lifetime measurement.



Fluorescence decay components in FRET systems

Separation of Different Chromophores

Steady-state multi-wavelength techniques have been developed that efficiently separate different fluorescence markers by unmixing their fluorescence spectra [14]. However, not all marker combinations can efficiently be resolved. Even the well known GFP and YFP are difficult to unmix. Autofluorescence images of cells and tissue show a wide variety of fluorescence components with ill-defined, variable, and often unknown spectra. When spectral unmixing fails the components can usually be distinguished by their different lifetimes [15a,15b]. The relative concentration of two components can be determined by double-exponential decay analysis. Even if only a single exponential approximation, i.e. an average lifetime is measured, the contrast in the fluorescence images can be considerably improved. [15a,15b,16]. Moreover, changes in the relative concentration and the lifetime of autofluorescence components can possibly be used as diagnostic tools.

Significant progress can be expected from combining spectral unmixing and lifetime analysis. Recording of time- and wavelength resolved data is technically possible by TCSPC techniques [13a] (see also section ‘Multi Wavelength TCSPC Imaging’). Combining the two methods requires to develop suitable data analysis software.

Diffusion in Cells

Diffusion time constants in cells are usually in the ms range and below. They are usually determined by fluorescence correlation (FCS) techniques [17, 18]. The problem is that the correlation technique is a single point measurement and therefore cannot be combined with the fast scanning used in laser scanning microscopes. Moreover, the measurement is usually not done in the same setup as the cell imaging. This makes it difficult to identify the measured spot in a particular cell with sufficient accuracy. Basically the photon counting techniques used for lifetime measurement are able to run a combined FCS and Lifetime measurement at a single, well defined spot of the sample. This could not only help to solve the positioning problem but also to identify single marker molecules [19] and to reveal conformational changes of the diffusing marker/protein clusters [20]. Although FCS data recording is possible with the SPC-830 TCSPC imaging module [8] no combined FLIM / FCS setup has become known yet.

The Light Source

TCSPC fluorescence lifetime imaging requires a pulsed excitation source with a repetition rate in the 10 to 80 MHz range and a pulse width below 100 ps. The features of different light sources are discussed below.

Titanium-Sapphire Lasers

The ultimate solution is the femtosecond Ti:Sa laser. These lasers deliver pulses with 70 to 80 MHz repetition rate, 80 to 200 fs pulse width and up to several Watts average power. The wavelength is in the NIR from 780 nm to 950 nm. To excite the sample which usually absorbs below 500 nm, simultaneous two photon excitation is used. Due to the short pulse width and the high energy density in the focus of the microscope the two-photon process works very efficiently. Therefore the traditional frequency doubling of the Ti:Sa radiation is not often used for laser scanning microscopes.

Frequency Doubled Titanium-Sapphire Lasers

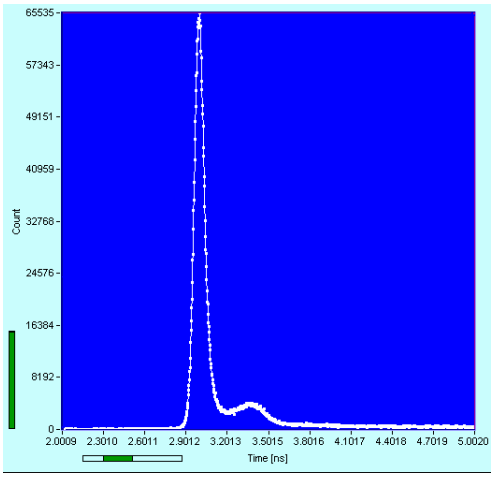
Frequency doubled titanium-sapphire lasers can be used to excite the sample via the traditional one-photon absorption. Frequency doubling is achieved by a nonlinear crystal. The output power is in the mW range. Less than 50µW are required to excite a typical sample so that the available power is by far sufficient. Whether one-photon or two-photon excitation gives less photobleaching is still under discussion [42,43,44,45].

Fibre Lasers

Another useful excitation source are fibre lasers. Fibre lasers are available for a wavelength of 780 nm and deliver pulses as short as 100 to 180 fs [21]. The average power is 10 to 20 mW. This is less than for the Ti:Sa laser but still sufficient for two-photon excitation. As a rule of thumb, the maximum useful power for biological samples and fs NIR excitation is 5 to 10 mW. A higher power kills the cells or cooks the sample. The benefit of the fibre laser is the small size, the high reliability and a lower price compared to the Ti:Sa laser. The drawback of the fibre laser is that is not tuneable.

Pulsed Diode Lasers

A reasonable cost solution for one-photon excitation are pulsed diode lasers which are available for the blue and red spectral range [22, 23, 24, 25]. These lasers deliver pulses from 40 to 400 ps duration with up to 80 MHz repetition rate. The average power is up to a few mW. However, the pulse width increases with the power. For pulses shorter than 100 ps the average power is of the order of several 100 µW. The figure right shows the typical pulse shape of a 405 nm picosecond diode laser.



Pulse shape of a blue diode laser. Becker & Hickl BDL-405, recorded with R3809U MCP and SPC-830 TCSPC module. FWHM is 75 ps.

Pulsing diodes with less than 100ps width requires special driving techniques that are not commonly available. However, pulses as narrow as 300 ps from red diodes can easily be obtained by connecting a commercially available pulse generator to a bare laser diode.

Unfortunately the beam quality of diode lasers is not very good. Therefore it can be difficult to obtain a diffraction-limited resolution. However, if only the central part of the beam is used, the result can be quite acceptable. Discarding a large fraction of the

beam causes a considerable loss of power. This loss is, however, not substantial because 50 µW in the focal plane are sufficient to excite the sample.

Modulated CW Lasers

For signal processing techniques based on phase measurement of modulated signals [26,27,28] the commonly used Ar+ and He-Ne lasers in conjunction with an acousto-optical modulator can be used. Using modulated CW lasers in conjunction with photon counting techniques is not recommended. To fully exploit the performance of TCSPC techniques light pulses with ps duration are required rather than sinewave signals. Acousto-optical modulators are resonance systems unable to deliver sufficiently short pulses with high on/off ratio.

Mode-Locked CW Lasers

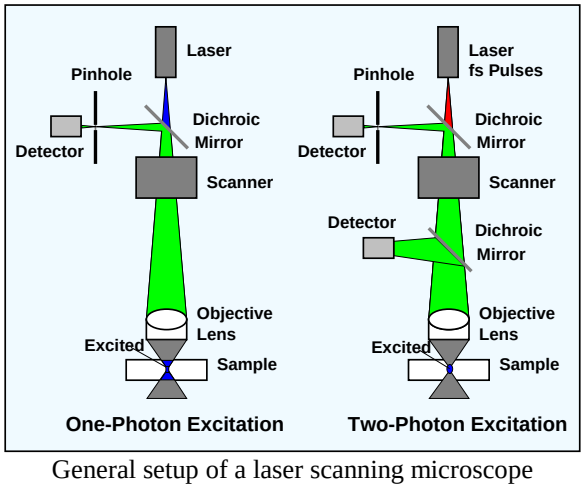
Ar+ lasers can be actively mode-locked. By introducing a modulator into the laser cavity pulses as short as 100 ps with 80 to 120 MHz repetition rate are obtained. The pulses can be used directly or to pump a jet-stream dye laser that delivers ps pulses at a wavelength tunable from 500 to 600 nm. Although the light from these lasers can be used for fluorescence excitation the systems are often unstable and require permanent maintenance, checking and re-adjustment. We strongly discourage to use these lasers as excitation sources for time-resolved microscopy.

Pulse Pickers and Cavity Dumpers

Pulse pickers and cavity dumpers are used to obtain a lower repetition rate from lasers running at a high, fixed repetition rate. For fluorescence measurements they are sometimes used to measure lifetimes longer than the original period of the laser. The problem of the devices is the limited on-off ratio and the electrical noise they often produce. It is by far better to take some incomplete decay into regard in the data analysis than to cope with electrical noise and satellite pulses. Furthermore, a low repetition rate reduces the useful count rate in photon counting setups and possibly increases photobleaching. Don’t use pulse pickers if they are not absolutely necessary.

The Microscope

The general optical principle of a laser scanning microscope is shown in the figure right.



General setup of a laser scanning microscope

The laser is fed into the optical path via a dichroic mirror and focused into the sample by the microscope objective lens. In the traditional confocal setup used for one-photon excitation (figure right, left part) the light from the sample goes back through the objective lens, through the scanner, is diverted by a dichroic mirror and goes through a pinhole in the upper image plane of the objective lens. Light from outside the focal plane is not focused into the pinhole and therefore substantially suppressed. X-Y imaging is achieved by optically scanning the laser spot over the sample, Z imaging (optical sectioning) by moving the sample or the microscope up and down. Depth resolution and high contrast due to suppression of light from outside the focal plane make the scanning microscope superior to the conventional wide-field microscope [1,2].

With a fs Ti:Sa laser the sample can be excited by two-photon absorption [3]. Excitation occurs only in the focus, so that no pinhole is required to reject light from outside the focal plane. Therefore, the fluorescence light need not be fed back through the scanner and through the pinhole.

Instead, it can be diverted by a dichroic mirror directly behind the microscope objective. This setup is called ‘non-descanned detection’ in contrast to the ‘descanned detection’ used for one-photon excitation.

Two-photon excitation in conjunction with non-descanned detection can be used to image tissue layers as deep as 500 µm. Since the scattering and the absorption at the wavelength of the two-photon excitation are small the laser beam penetrates through relatively thick tissue. Even if there is some loss on the way through the tissue it can easily compensated by increasing the laser power. The increased power does not cause much photodamage because the power density outside the focus is small. However, as long as there are enough ballistic (non-scattered) excitation photons in the focus the fluorescence is excited. Of course, the fluorescence photons are heavily scattered on their way out of the tissue and therefore emerge from a relatively large area of the sample surface which is out of the focus of the objective. However, for non-descanned detection there is no need to focus the fluorescence light anywhere. Therefore the fluorescence photons can be efficiently transferred to the detector.

It is sometimes believed that lifetime imaging is somehow connected to two-photon excitation. This is, of course, not correct. Depending on the signal processing technique used, lifetime imaging requires a pulsed or modulated laser. Although a Ti:Sa laser is the ideal source lifetime imaging is possible with the frequency doubled Ti:Sa laser [13a], with pulsed diode lasers [25a,25b], or other lasers delivering pulses with sub-ns duration and MHz repetition rate.

The Detector

Time-correlated single photon counting requires a detector capable to deliver an electrical pulse for a single detected photon. The detector must be fast enough to resolve the fluorescence decay time of typically 100 ps to 5 ns. Only photomultipliers (PMTs) [29] and single-photon avalanche photodiode modules [32] meet these requirements.

Built-in PMTs

Commercial scanning microscopes employ small side-window PMTs to detect the light from the sample. The PMTs are integrated in the scanning head. Usually there are several detection channels with separate PMTs. The PMTs are selected for low price and high sensitivity, not for time resolution in the single photon counting mode. Although the built-in detectors can be used for TCSPC imaging they do not deliver a good time resolution. Nevertheless, they can be used to distinguish between different dyes in multi-stained samples and to investigate other lifetime effects in the ns range. They are **not** useful for FRET measurements. The internal detectors should be operated at the maximum permissible supply voltage and with a HFAC26-10 preamplifier of Becker & Hickl.

PMH-100 PMT Module

The PMH-100 (see figure right) of Becker & Hickl is a rugged PMT module with an internal high-voltage generator, preamplifier, and overload warning circuit. It is connected directly to the SPC-730 or SPC-830 module. The PMH-100 yields a system response of 150 to 220 ps FWHM and can be used to measure lifetimes down to 100 to 200 ps. We recommend this detector as a startup solution. It is attached to the non-descanned port of the microscope or to a fibre output from the scanning head. The PMH-100 is available with bialkali and multialkali cathodes. The reduced spectral range of the bialkali cathode can be a benefit in two-photon systems when blocking of the laser line is a problem. Typical FRET effects are well

PMH-100 detector, about 1/2 natural size

detectable with the PMH-100. However, for quantitative FRET experiments we recommend the R3809U MCP-PMT (see below).

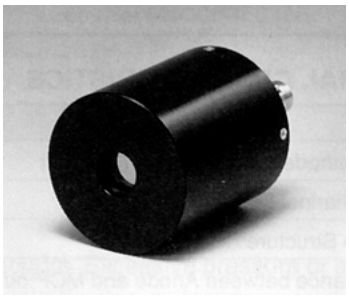
H5783 Photosensor Module

The Hamamatsu H5783P and H5773P Photosensor Modules incorporate a small size PMT and the HV power supply. They require a +12 V supply and some gain setting resistors only. The +12 V is available from the SPC-730 module or from the DCC-100 detector controller (see below). The time resolution is 150 to 220 ps FWHM. The H5783P and H5773P are available with bialkali and multialkali cathodes. For optimum results, use the '-P' type, which is specified for photon counting. Due to their small size, the H5783 and H7783 modules are a solution if a detector in the scanning head has to be replaced with a faster one. The modules should be operated with a HFAC-26-10 preamplifier of Becker & Hickl.

H5783 module, natural size

R3809U MCP PMT

The Hamamatsu R3809U MCP PMT [29, 30] achieves an FWHM below 30 ps. It is the ultimate solution for TCSPC scanning microscopes. It is clearly the best detector for FRET experiments. However, since MCPs are expensive and easily damaged it is neither a solution for beginners nor a low budget solution.



R3809U MCP, about 2/3 natural size

The R3809U is connected to the SPC-730 or SPC-830 via an HFAC-26-01 preamplifier. Furthermore, it requires a -3 kV high voltage power supply. Thus, the overall cost for one R3809U detection channel is of the order of $15,000 to $20,000, not including the SPC module. This is not very much compared to the price to the microscope and the laser, but a lot if the detector is damaged by maltreatment. Due to its relatively large size the R3809U should be attached to the non-descanned port of the microscope or to a fibre output from the scanning head.

The R3809U is available in different cathode versions. The most versatile one is the R3809U-50 with a multialkali cathode for the wavelength range from 180 to 820 nm. The R3809U-52 has a bialkali cathode and can be used up to 650m [30]. This limitation of the spectral range can be a benefit for two-photon systems when blocking of the laser line is a problem.

Gain control and overload shutdown of the R3809U can be achieved by the DCC-100 detector controller [31], see below.

A cooler is available for the R3809U. Cooling substantially reduces the dark count rate and therefore makes possible long acquisition times. However, before you install a cooler, make sure that your background signal really comes from the detector and not from poor blocking of excitation light or even from insufficient shielding of daylight.

SPCM-AQR Avalanche Photodiode Modules

The SPCM-AQR Avalanche Photodiodes Modules of EG&G / Perkin Elmer [32] have a high quantum efficiency in the NIR. This makes the modules exceptionally suitable for single molecule investigations. For precision decay time measurements they are less useful because their TCSPC system response is 500 ps wide and slightly dependent on the count rate. The SPCM-AQR is connected to the SPC-730/830 via an adapter available from Becker & Hickl.

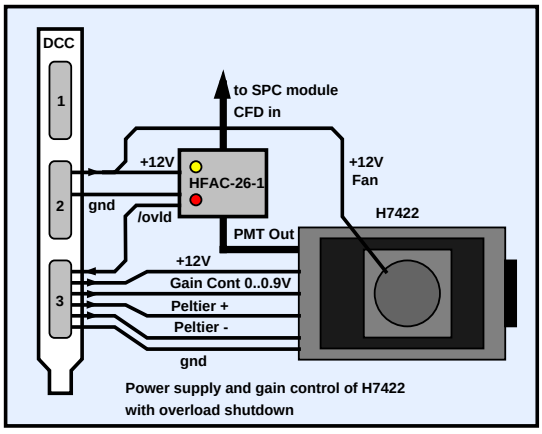
Hamamatsu H7422 Modules

The H7422 modules are high speed, high sensitivity PMT modules [33]. They contain a GaAs cathode photomultiplier along with a thermoelectric cooler and a high voltage generator. The H7422 modules feature excellent sensitivity in the visible and near-infrared region. The resolution in the TCSPC mode is typically 250 to 300 ps. The H7422 comes in different cathode versions for the wavelength range up to 1050 nm. For most microscope applications the H7422-40 is best. It has the highest quantum efficiency of all H7422 versions and is sensitive up to 750 nm. Above this wavelength the sensitivity drops rapidly. That means that the dyes typically used for cell staining can be measured, but there is a substantial suppression of the excitation line of a Ti:Sa laser used for two-photon excitation. If you need sensitivity up to 900 nm - which implies one photon excitation in the red or NIR range - you can use the H7422-50.

All H7422 modules should be handled with care because their cathodes can easily be damaged by overload. Exposure to daylight is not allowed even when the devices are switched off. Therefore, the H7422 should be used with an HFAC-26-1 preamplifier only. Power supply, gain control, overload shutdown and cooling is achieved by using the **bh** DCC-100 detector controller (see below).

The DCC-100 detector controller

The DCC-100 module [31] was designed to control detectors in conjunction with **bh** photon counters. It can be used to control the gain of the Hamamatsu H7422, H5783, H7422 or similar Hamamatsu photosensor modules by software. The gain of MCPs and PMTs can be controlled via an FuG HCN-14 high voltage power supply. In conjunction with **bh** preamplifiers overload shutdown of the detectors can be achieved. Furthermore, the DCC-100 delivers the current for thermoelectric coolers, e.g. for the Hamamatsu H7422. High current digital outputs are available for shutter or filter control. The DCC-100 is a PCI module for IBM compatible computers. It works under Windows 95, 98, 2000 and NT.



The figure right shows how a H7422 module is controlled via the DCC-100. For more information, please see DCC-100 data sheet and DCC-100 manual, www.becker-hickl.com.

Preamplifiers

Most MCPs and PMTs deliver pulses of 20 to 50 mV when operated at maximum gain. Although these pulses can easily be detected by the input discriminators of the SPC modules a preamplifier can improve the time resolution, the noise immunity, the threshold accuracy and the safety against damaging the SPC input. Furthermore, it can extend the detector lifetime because the detector can be operated at a lower gain and a lower average output current.

For all PMTs in TCSPC applications we recommend the **bh** HFAC-26 preamplifier. The HFAC-26 has 20 dB gain and 1.6 GHz bandwidth. The maximum linear output voltage is 1 V. Therefore, it amplifies the single photon pulses of a typical PMT or MCP without appreciable distortions. Furthermore, the HFAC-26 incorporates a detector overload detection circuit. This circuit measures

the average output current of the PMT and turns on a LED and activates a TTL signal when the maximum safe detector current is exceeded. In conjunction with a DCC-100 detector controller this signal can be used to shut down the high voltage of a PMT or MCP when an overload occurs.

Thus, even if the gain of the amplifier is not absolutely required the overload warning function helps you to make your measurement setup 'physicist proof'. If you use an MCP with your SPC module you should always connect it via an HFAC-26 preamplifier.

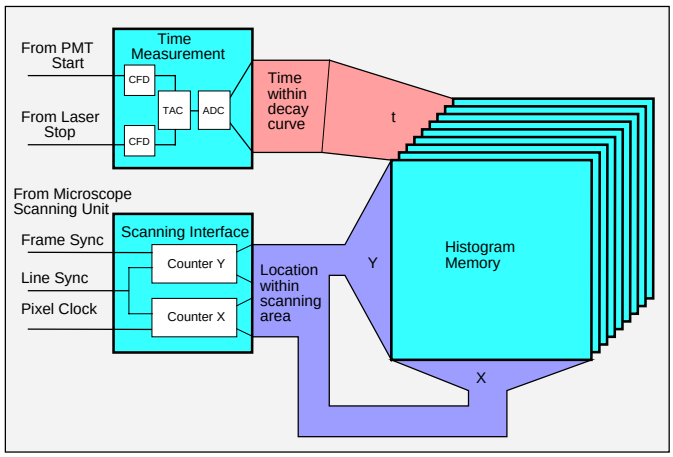
The HFAC-26 is available with different overload warning thresholds from 100 nA (for MCPs) to 100 uA (for large PMTs).

HFAC-26 Amplifier

The TCSPC Imaging Module

The structure of the SPC-730 and SPC-830 TCSPC modules [8] in the basic 'Scan Sync In' imaging mode is shown in the figure below.

The modules employ an advanced three-dimensional TCSPC technique featuring both high count rate and low differential nonlinearity [8, 9]. It contains the usual building blocks (CFDs, TAC, ADC) in the 'reversed start-stop' configuration together with a scanning interface and a large histogram memory integrated on one board.



Basic principle of the SPC-730 TCSPC Imaging module [8]

The Becker & Hickl SPC-730 TCSPC Imaging Module [8]

The TCSPC module receives the single photon pulses from the photomultiplier (PMT) of the microscope, the reference pulses from the laser and the Frame Sync, Line Sync and Pixel Clock signals from the scanning unit of the microscope. For each PMT pulse, i.e. for each photon, the TCSPC module determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address the histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over the scan coordinates, x, y, and the time, t, within the fluorescence decay function builds up. The result can be interpreted as a two-dimensional (x, y) array of fluorescence decay curves or as a sequence of fluorescence images for different times (t) after the excitation pulse.

Interestingly, there is practically no loss of photons in the TCSPC imaging process. As long as the photon detection rate is not too high all detected photons are processed and accumulated in the histogram, thus providing near-ideal signal-to-noise ratio and maximum sensitivity. This is a key advantage of TCSPC imaging compared to gated photon counting [49, 50], gated image intensifiers [25, 48] and modulation techniques [26, 27, 28]. A comparison of the efficiency of the different methods is given in [34].

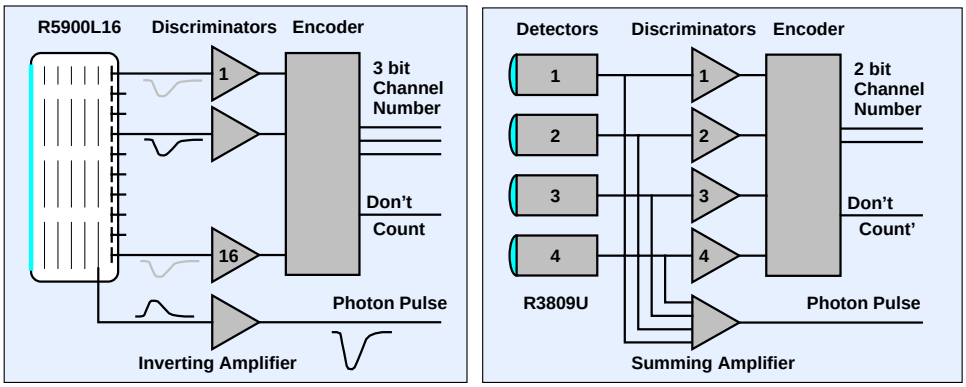
Another benefit of TCSPC imaging is the high time resolution. The time channel width can be as small as 820 fs. Decay times down to a few picoseconds can be determined with fast detectors. On the other hand, TCSPC imaging is flexible in that a large number of pixels can be measured with wide time channels or precision measurements can be done at a small number of pixels. Furthermore, the method can work with the fastest scanning speed of the microscope thus avoiding heat concentration in the excited spot of the sample.

Advocates of gated photon counting or phase flourometry sometimes claim that the count rate of the TCSPC method is too low for microscopy application. This ill reputation comes from older TCSPC devices built from nuclear instrumentation modules. The SPC-830 has a dead time of 125 ns yielding a maximum useful count rate of about 4 MHz. For comparison, living cells excited by two-photon excitation give a count rate of some 10,000 photons per second. Samples of non-living cells stained with highly fluorescent dyes can deliver up to 200,000 photons per second. A higher excitation intensity usually kills the cells or destroys the sample.

The SPC-730 and SPC-830 modules can be attached to almost any laser scanning microscope. The only requirement is that the scan control signals, Frame Sync, Line Sync and Pixel Clock, are available and a reasonably fast detector can be attached [11, 12, 13a, 13b, 16, 35, 36, 37].

Multi-Wavelength Imaging

TCSPC imaging can be combined with a multidetector technique [8, 10 ,38 ,39 ,40] and be used to record the photon density over time, wavelength and the coordinates of the scanning area [13a, 13b]. The principle of the multi-detector technique is shown in the figure below.

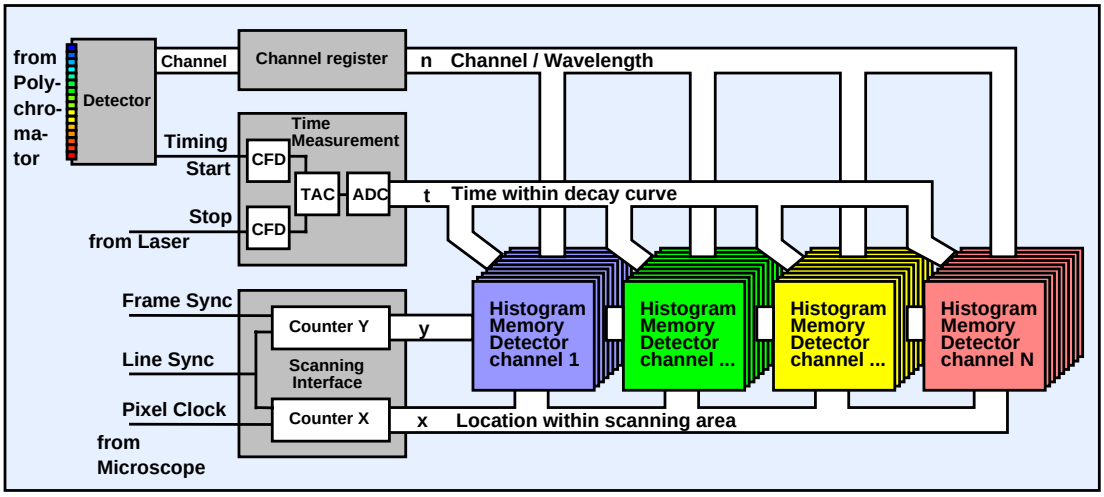


Multi-detector technique for TCSPC. Left: Multichannel PMT, Right: Individual PMTs

The technique works both with a multichannel PMT [13a, 39] and with several individual PMTs or MCPs [13b, 40]. It makes use of the fact that the detection of several photons in different detector channels in one laser period is unlikely. Therefore, the single photon pulses from all detector channels can be combined into a common photon pulse line and send through the normal time measurement procedure of the TCSPC module. The output of each PMT channel is connected to a discriminator. If the channel detects a photon the corresponding discriminator responds and sends a pulse to the subsequent encoding logic. The encoder delivers the number of the PMT channel that detected the photon. The channel number is used as an additional dimension in the multi-dimensional histogramming process of the TCSPC imaging technique.

Both the SPC-730 and the SPC-830 can be used for multi-wavelength imaging. The module structure in the multi-wavelength mode is shown in the figure below. The recording electronics

consists of a time measurement channel, a scanning interface, a detector channel register, and a histogram memory.



Multi-wavelength TCSPC lifetime imaging

Except for the additional wavelength coordinate the recording process is similar to the histogramming process for single-wavelength operation. For each photon, the time measurement channel determines the detection time (t) referred to the next laser pulse. The scanning interface determines the current location (x and y) of the laser spot in the scanning area. Synchronously with the detection of a photon, the detector channel number (n) for the current photon is read into the detector channel register. If a polychromator is used in front of the detector, n represents the wavelength of the detected photon.
The obtained values for t, x, y, and n are then used to address the histogram memory. Thus, in the memory the distribution of the photons over time, wavelength, and the image coordinates builds up. The result is a four-dimensional data array that can be interpreted as a set of image stacks for different wavelengths. Each stack contains a number of images for subsequent times after excitation.

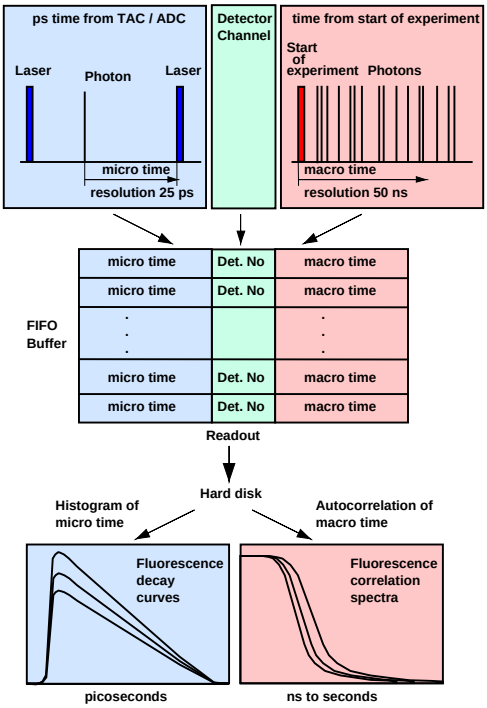
Multi-wavelength detection works with the SPC-730 and the SPC-830 as well. However, due to its much larger memory the SPC-830 is superior in terms of pixel numbers and time channels per pixel.

FCS data recording

Diffusion constants in cells can by determined by fluorescence correlation (FCS) techniques. Recording an FCS function requires to detect the fluorescence intensity in a fixed spot of the sample and to calculate the autocorrelation function of the intensity fluctuations or of the photon detection times [17,18]. Although FCS is not an imaging technique imaging is used in combination with FCS to locate the spot where an FCS measurement has to be run.

Generally, the TCSPC technique is not only able to record time-resolved images in laser scanning microscopes, but also to record FCS data. An FCS measurement can even be combined with a single-point lifetime measurement. The multi-detector technique described above for multi-wavelength TCSPC imaging is also available for FCS measurements. With the introduction of the SPC-830 it is not longer necessary to use independent modules for imaging and FCS. The SPC-830 works for imaging and FCS recording as well.

TCSPC FCS data recording does not build up a histogram as the TCSPC imaging techniques do. Instead, it records the full information about each photon. Each entry contains the time of the photon in the laser pulse sequence, the time from the start of the experiment, and the detector channel. The data structure is shown in the figure right. For each detector an individual correlation spectrum and a fluorescence decay curve can be calculated. An instrument like this was used to detect and identify single molecules on a substrate. By using four wavelength channels and a piezo scanning stage different molecules could be identified in a time of the order of 1 ms [19].



If several detectors are used to record the photons from different chromophores, the signals of these chromophores can be cross-correlated. The fluorescence cross-correlation spectrum shows whether the molecules of both chromophores and the associated protein structures are linked or diffuse independently.

Parallel Operation of Several TCSPC modules

Although it seems unlikely that count rates much higher than 10^6 photons per second can be obtained from living cells for a longer period of time it cannot be excluded that the peak count rate in single spots with high chromphore saturation is higher. Count rates in excess of some 10^6 s^{-1} cause noticeable counting loss [8] and impose overload problems to most detectors. Although traditional PMTs still work at 10 MHz the timing performance may not longer meet the high standard of the TCSPC technique. MCP PMTs, i.e. the fastest detectors for TCSPC, are clearly overloaded above 1 MHz.

The solution to the count rate problem - if it exists - is to operate several TCSPC modules with individual detectors in parallel. Although no such application has become known yet the implementation is straightforward. All Becker & Hickl TCSPC modules are designed to work in packages of up to four devices [8, 40].

Recording efficiency

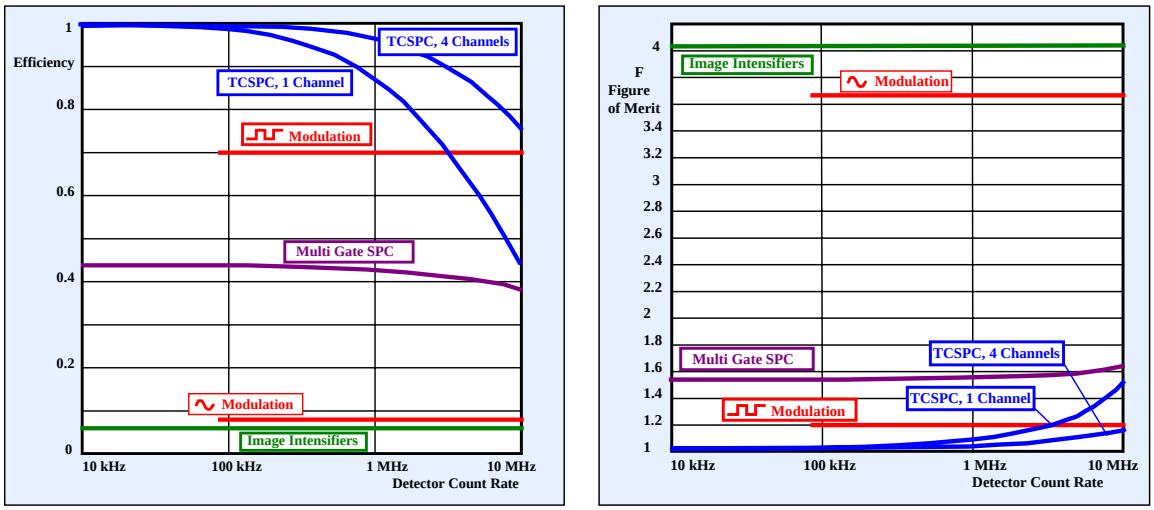
Different signal processing techniques differ considerably in terms of recording efficiency, i.e. in the exploitation of the detected photons. Taking into regard that the available number of photons is limited by photobleaching in the sample, recording efficiency is the most important parameter next to the time resolution. The quality of a signal recording technique can be described by a ‘figure of merit’ , F, [46, 47] that describes the ratio of the signal-to-noise ratio SNR of an ideal measurement to the signal-to-noise ration actually achieved, i.e.

$$F = \frac{SNR_{ideal}}{SNR_{act}}$$

Since the SNR is proportional to the square root of the number of detected photons, n, the efficiency of a technique in terms of photons required to obtain a given SNR is

$$E = 1 / F^2$$

A comparison of the figure of merit and the efficiency for single channel modulation techniques with sine wave and square wave modulation [26, 28], modulated and gated image intensifiers [27,48], multi-gate photon counting [49,50], for TCSPC with one channel and four parallel channels is given below. Figure of merit and efficiency for TCSPC are calculated from the known relation of counting loss, dead time and detector count rate [8]. The values for the other methods are from [47].



Counting efficiency and figure of merit F of TCSPC imaging compared to single channel modulation techniques, gated and modulated image intensifiers, and multi-gate photon counting.
F is the ratio of theoretical SNR to obtained SNR, F=1 means ideal SNR

TCSPC features a near-perfect figure of merit and counting efficiency up to a detector count rate of 1 MHz. Surprisingly, TCSPC imaging beats the other methods even for detector count rates of the order of 5 to 10 MHz.

Acquisition Time

The acquisition time for TCSPC lifetime measurements can vary in a wide range. In vivo lifetime measurements at the human ocular fundus in conjunction with an ophthalmologic scanner delivered single exponential lifetimes for an array of 128x128 pixels within a few seconds [15a,15b]. On the other hand, for the double exponential decay data of FRET measurements acquisition times were from 5 to 30 minutes were used [12].

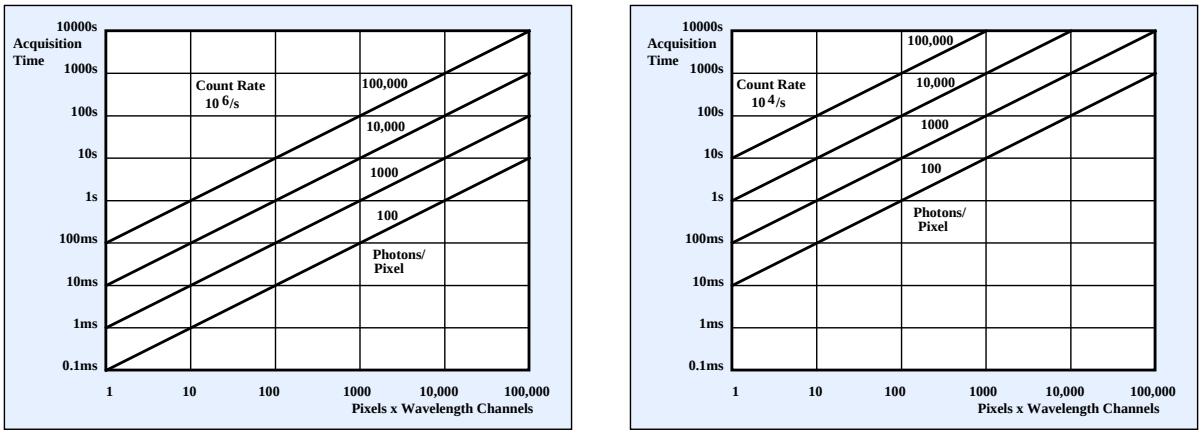
In practice the acquisition time depends on the required lifetime accuracy, on the number of lifetime components to be resolved, on the number of pixels and wavelength channels and on the count rate that can be obtained from the sample without photobleaching or photodamage.

The figure below shows the acquisition time as a function of the product of the number of pixels and the number of wavelength channels for different number of photons per pixel. The left diagram is for a high count rate of 10^6 /s. Count rates of this order can be obtained by one-photon excitation from living cells containing high chromophore concetrations. For two-photon excitation a count rate of 10^6 /s is unlikely and requires extremely stable samples stained with high efficiency chromphores. The right diagram is for a very low count rate of 10^4 /s. Count rates of this order are typical for autofluorescence of cells and tissue and for samples with low photostability.

The number of photons per pixel range from 100 for rough single exponential decay mapping to 10^5 for precision double exponential decay analysis [51].

The figure shows that relatively long acquisition times must be expected, particularly for large numbers of pixels and precision measurements of samples at a low count rate. It should, however, be pointed out that long acquisition times are not a special feature of TCSPC imaging - they simply

result from the fact that much more photons per pixel are required to determine the fluorescence lifetime than to record the fluorescence intensity.



Acquisition time for a count rate of 10^6 /s (left) and 10^4 /s (right) for different numbers of photons per pixel. Photons per pixel range from 100 for rough single exponential decay mapping to 10^5 for precision double exponential decay analysis.

Shorter acquisition times can be achieved if the scanning area is confined to a few pixels or only one pixel is measured. For single pixels the acquisition time can be in the ms or sub-ms range. That makes it possible to investigate diffusion processes by monitoring the lifetime change in a single pixel. Even fast FRET measurements appear feasible. FRET requires to resolve the components of a double exponential decay function. This requires 10,000 to 100,000 photons which can be obtained in 10 to 100 ms.

Image size

TCSPC imaging at the fast scan rate of a laser scanning microscope requires the decay curves of all pixels of the image to be held in the memory of the SPC module. This requires a huge amount of fast memory. Therefore, a tradeoff between image size, i.e. pixel numbers, the number of time channels and the number of detector channels has to be made. For the SPC-730 a maximum of 256x256 pixels can be recorded. Although this is usually enough for single-wavelength lifetime imaging simultaneous multi-wavelength imaging is possible only for a relatively small number of pixels [8].

With the introduction of the SPC-830 module memory space is not longer a severe constraint. Some combinations of image size, and number of time and wavelength channels for the SPC-830 are given in the table below.

Detector Channels	Resolution x,y	Time Channels	Min. Time Channel Width (ps)
1	2048 x 2048	4	50
1	1024 x 1024	16	12.5
1	512 x 512	64	3.125
1	256 x 256	256	0.8
1	128 x 128	1024	0.8
4	1024 x 1024	4	50
4	512 x 512	16	12.5
4	256 x 256	64	3.125
4	128 x 128	256	0.8
16	512 x 512	4	50
16	256 x 256	16	12.5
16	128 x 128	64	3.125
16	64 x 64	256	0.8

The SPC-830 can even be used as a steady state imaging photon counter, i.e. with only one time channel. The maximum resolution is then 4096 x 4096 pixels.

System Setup

Attaching the detector

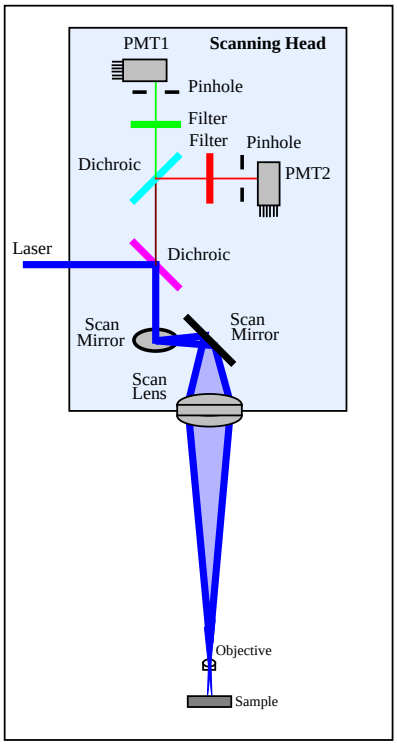
Generally there are four options for the detection channel. You can

- use one of the internal detectors of the microscope
- replace one of the internal detectors with a faster one
- use a fibre to feed the light from the scanning head to an external detector
- attach a detector to the non-descanned port of the microscope

Not all options are available in all microscopes. The following considerations can help to find the best solution for your microscope and your application.

Using the built-in detectors

The general principle of the scanning head of a laser scanning microscope is shown in the figure right.



The laser beam is coupled into the setup via a dichroic mirror. Than it is deflected by the scan mirrors. The scan lens focuses an image of the scan mirror axis into the microscope objective. Thus, the direction the laser takes through the objective changes as the mirrors tilt back and forth thus scanning the laser focus over the sample. The fluorescence light from the sample goes back through the microscope objective, the scan lens, and the scan mirrors. If the setup is correctly aligned the motion of the returned light beam is exactly compensated by the scan mirrors. The fluorescence light is separated into several detection channels by one or several additional dichroics. Filters are used to block the scattered laser light and to select the correct fluorescence wavelength. Pinholes in front of the detectors are used to suppress the light from outside the focal plane of the microscope objective.

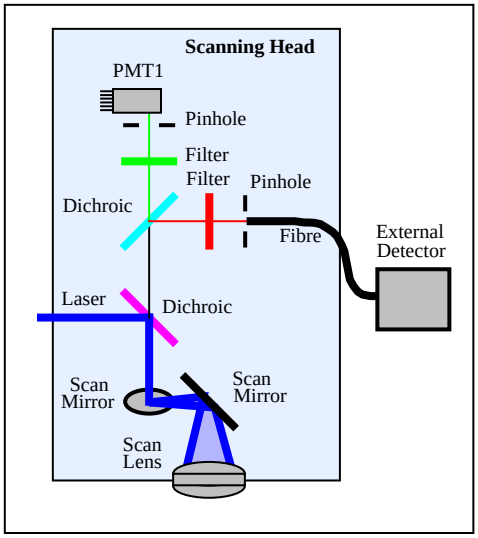
Various modifications of this setup are used in different microscopes. Transfer lenses or mirrors are used to image the rotation axis of the first scanning mirror into the axis of the second one, the laser beam diameter is changed to fit the diameter of the microscope objective, monochromators are used instead of the filters, and the size of the pinholes can be adjustable. Depending on the application and on the setup of the scanning head, detection via the scanning head PMTs has benefits and drawbacks.

- One-photon excitation requires the pinholes to suppress the fluorescence from outside the focal plane. Therefore, the detection path back through the scanning head is the only useful one for one-photon excitation.
- Two-photon excitation does not require the pinholes. The pinholes can even be troublesome because they suppress some light that leaves the sample slightly scattered. Therefore, the pinhole size is adjustable in good scanning heads. For single cell imaging there is no appreciable drawback of the detection path via the scanning head.
- For two-photon excitation, the complicated optical path trough the scanning head can introduce some loss of intensity due to the large number of lens and mirror surfaces. However, the scanning heads of state-of-the-art microscopes are optically near perfect so that there is no noticeable loss of photons. Furthermore, computer controlled selection of dichroics and filters or monochromators

available in the scanning heads helps to select the best wavelength range and to suppress scattered laser light. This often compensates for possible loss in the optical path.

- The pinhole - even a very large one - helps to suppress straylight and optical reflections that often show up in time resolved data obtained by non-descanned detection.
- The most serious drawback of using the internal detectors is the poor time resolution. Unfortunately, replacing the detectors with faster ones is usually very difficult, if not impossible. The remedy is the fibre coupling option described in the next section.

Fibre Coupling



One of the internal detectors is removed and an optical fibre is attached instead. The light is fed to an external detector. Compared to the setup with all PMTs in the scanning head, this configuration has the benefit that any detector can be installed at the end of the fibre. There is no problem to use large detectors, coolers, or additional filters.

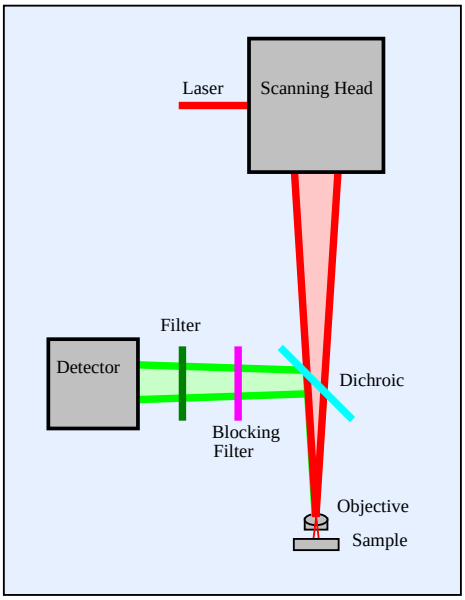
The drawback is that

- the coupling of the fluorescence light into the fibre may be inefficient, especially for a thin fibre in conjunction with two photon excitation and strongly scattering samples.
- the dispersion in the fibre can introduce some broadening of the system response. Due to the small aperture of the light beam behind the pinhole the effect is very small. We did not find a substantial loss of resolution of an R3809U MCP coupled trough a 1 m long fibre to a Zeiss LSM-510.

Some microscope manufacturers (e.g. Zeiss) offer a fibre coupling option for their scanning heads. In this case fibre coupling is an easy-to-use solution that works for one-photon and two-photon excitation as well. Detectors for the Zeiss LSM-510 fibre output are available from **bh**, see figure below.

PMH-100 H7422-40 R3809U
Detectors for the ‘Fibre Out’ version of the Zeiss LSM-510

Non-Descanned Detection



Microscope manufacturers claim that non-descanned detection (NDD) is the ultimate solution for two-photon excitation. This is certainly true for imaging of deep tissue layers. For single cell imaging the benefit of non-descanned detection is questionable - well designed scanning heads , e.g. that of the Zeiss LSM-510 - obtain almost the same efficiency as non-descanned detection with the same microscope. The optical path for non-descanned detection is shown in the figure right.

The fluorescence light is separated from the excitation by a dichroic mirror before it enters the scanning head. The light goes through a laser blocking filter and a wavelength selection filter directly to the detector. Although the principle is more or less the same in all microscopes, several modifications are possible. The setup of the dichroic can be reversed so that the scanned laser beam is reflected instead of the fluorescence signal. A demagnification lens can be used to shrink the illuminated area on the detector. Zeiss offers a 'Non Descanned Detection Module' with several detection channels and computer selectable dichroics and filters for the LSM-510.

The benefits and drawbacks of non-descanned detection are:

- Two-photon excitation in conjunction with NDD allows imaging as deep as 500 um into biologic tissue.
- For single cell layers there is no appreciable advantage in sensitivity compared to descanned detection. The scanning head optics in good microscopes works virtually without losses. If the pinhole diameter is adjustable (as it is in the Zeiss LSM-510) and single cells are investigated the advantage of NDD is questionable.
- NDD can be reasonably used only for two-photon excitation
- The selection of the dichroic and of the laser blocking filter is crucial. The scattered laser light is many orders of magnitude stronger than the fluorescence, and a suppression factor of 10^6 to 10^{10} is required. Therefore, make sure that the correct filters be inserted in your microscope and that your microscope supplier gives you appropriate support if you need changes.
- Since there is no pinhole the NDD setup is prone to optical reflections. Reflections between the filters or reflections from condenser lenses behind the sample are often found in the decay curves.
- Since the detection path is relatively open to straylight the detector can easily be overloaded. Daylight or light from monitors or control lamps must be carefully blocked from the microscope during the measurement. Furthermore, NDD setups are often not safe in terms of operator errors. Often a mercury or halogen lamps used to adjust the sample visually. If the lamp can be switched on when the detection path is open the detector is immediately destroyed. Therefore, special care has to be taken in order not to damage the detector.

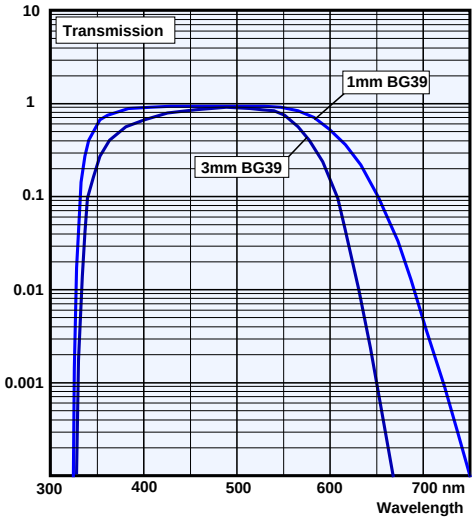
Two-Photon Systems

Optical System

Scattering of excitation light can be a serious problem in a two-photon microscope. Even if the optical system is perfect the excitation light scattered at the dichroic, at the microscope lens, and in

the sample is many orders of magnitude stronger than the fluorescence light. Therefore proper blocking of the excitation light is essential for two-photon fluorescence imaging.

The key to two-photon operation is the Schott BG39 filter. The characteristics of this filter is shown in the figure right. The filter efficiently blocks the excitation between 780 and 900 nm. A minimum of 1mm BG39 for bialkali detectors and a minimum of 3mm for multialkali detectors is required.



Transmission of BG39 Filter

Although dielectric filters have a sharper edge than the BG39 these filters usually fail to block the laser sufficiently. The reason may be that a dielectric filter reflects the light instead to absorb it. Therefore the laser light is scattered through the microscope and eventually arrives at the detector. Therefore, a dielectric filter should always be used in conjunction with a BG39.

Even with the BG39 filter scattered laser light often impairs the results of lifetime measurements. If the light comes from the sample it shows up as a sharp peak at the top of the fluorescence decay curves. If it comes from parts of the optical system the peak appears in a different position. If you see such effects although you have enough BG39 filters in the light path the reason may be:

Scattering at the microscope objective. To get diffraction limited resolution, the cross section of the laser beam is usually made larger than the microscope lens. A part of the laser beam hits the lens mount and is scattered into the detection path. Solution: Keep the beam diameter as small as possible - if you can. Or - if you are designing your own system - use a transfer lens and a diaphragm in front of the detector.

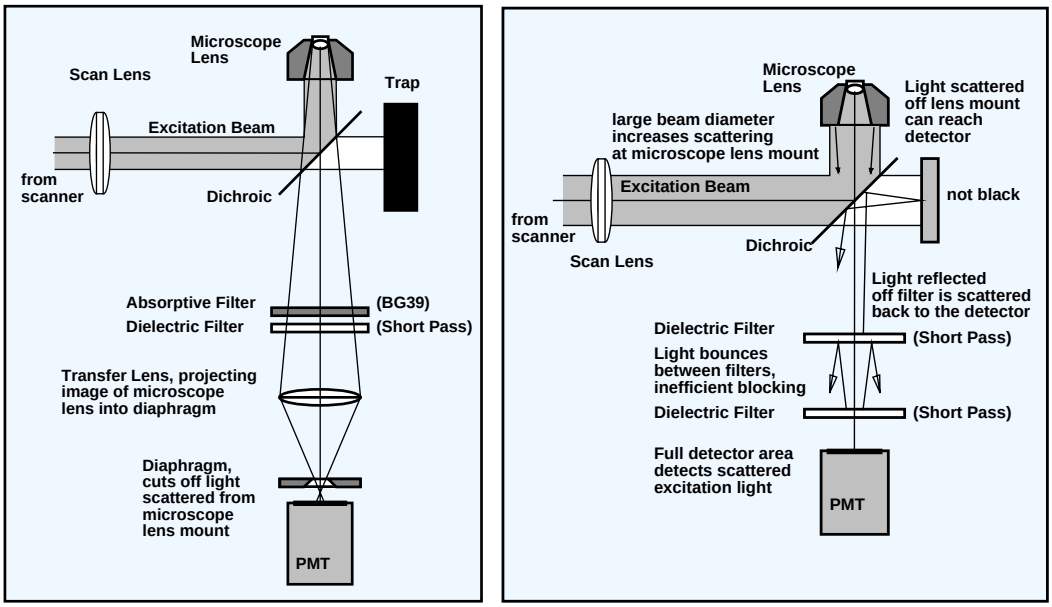
Reflection at a condenser lens. Usually the microscope has a condenser lens on the opposite side of the sample. Laser light that penetrates the sample can be reflected at this lens and directed back into the detection path. A simple solution is to shed the condenser during the measurement.

Dielectric blocking filters. In dielectric (dichroic) filters the blocked light is reflected. That means, it is scattered through the microscope. Therefore, the first filter should be an absorptive filter, e.g. a BG 39.

Stacks of dielectric filters. Do not stack dichroic filters to improve the blocking factor. The blocked light is reflected and bounces between the filters so that the blocking factor is less than the product of the blocking factors of the two filters. If dichroic filters have to be stacked, place absorptive filters (BG glass filters) between.

Insufficient Baffling: As far as possible, block straylight out of the detection path. The most critical places are the area around the microscope lens and the area behind the dichroic mirror. Excitation light from these areas can be diverted directly into the detection path. Make sure that the critical areas are black and insert baffles so that they are not directly seen by the detector.

A well-designed optical system for non-descanned detection and a design with features to be more or less avoided are shown in the figure below.



Well-designed NDD system (left) and features to be avoided (right)

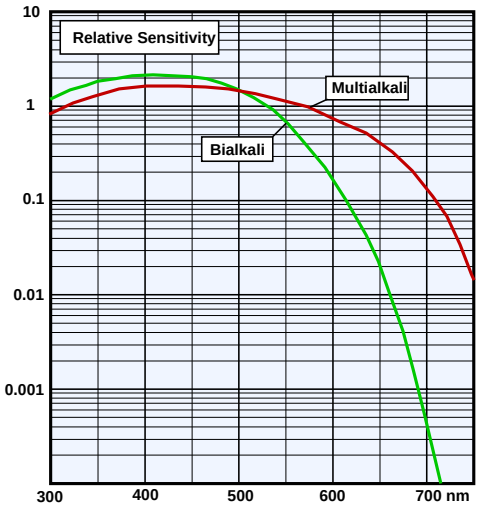
Selecting the Photocathode Version of the Detector

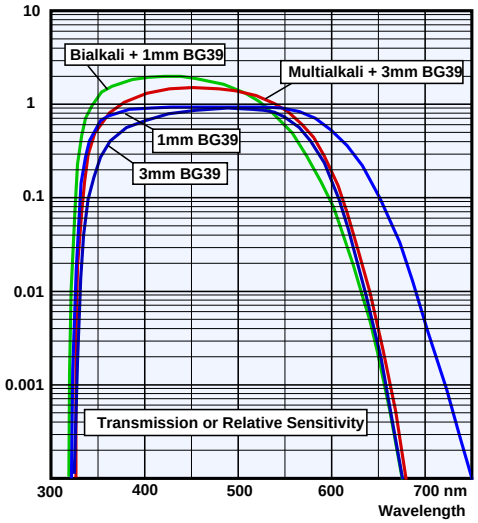
For most detectors two cathode versions exist - the bialkali and the multialkali cathode. The quantum efficiency and the spectral response function mainly depends on the cathode type. The sensitivity variation between different tubes of the same type is often of the same order as the differences between tubes of different types. The typical spectral response for the two cathode versions is shown right.

The dark count rate for the bialkali cathode is typically 20 to 80 counts per second. The multialkali cathode usually has 200 to 600 dark counts per second.

The sensitivity of the bialkali cathode drops sharply above 650nm. Therefore a 1 mm BG39 blocking filter is enough for this cathode. The multialkali tube has a sensitivity range up to 820nm and usually requires 3mm BG39. The figure right shows the spectral response of the bialkali cathode with 1mm BG39 and of the multialkali cathode with 3mm BG39.

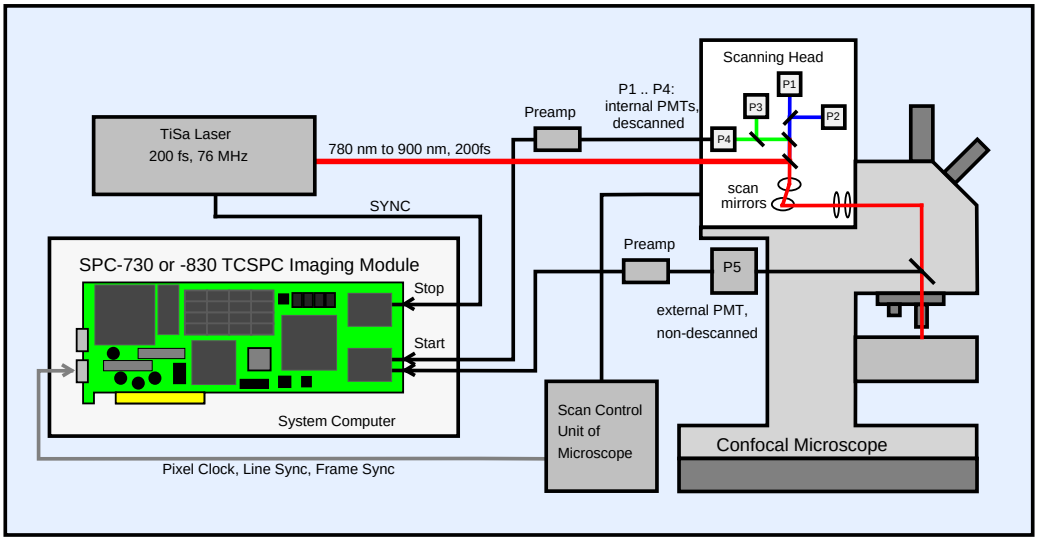
Interestingly, there is almost no difference in the wavelength range for the two cathode/filter combinations. It is probably better to sacrifice a few nanometers in the red and take advantage of the higher blue sensitivity and the lower dark count rate of the bialkali cathode.





System Connections

The typical setup of the TCSPC microscope is shown in the figure below. A Ti:Sa laser delivers femtosecond pulses in the wavelength range from 780 nm to 950 nm.



General setup of the TCSPC Laser Scanning Microscope

The microscope scans the sample in the x-y plane providing an image in the focal plane of the objective lens. 3 D imaging is achieved by changing the depth of the focus in the sample.

Data acquisition is accomplished by the Becker & Hickl SPC-830 or SPC-730 TCSPC imaging module [8]. The CFD input receives the single photon pulses of the PMT. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from the reference diode of the Ti:Sa laser.

The SPC module gets the scan control signals, Pixel Clock, Line Sync, and Frame Sync, from the scan controller of the microscope. Connecting the TCSPC module does not require any modification in the microscope electronics and software. The scan parameters are controlled in the usual way via the microscope software. The normal scan speed, the zoom function, region of interest setting, etc. can be used also when recording lifetime images. Furthermore, the SPC-730 and SPC-830 can be run in parallel with the standard image recording electronics of the microscope. Basically, steady state images and lifetime images in different spectral ranges can be obtained at the same time.

Due to the simple interfacing the SPC-730 and SPC-830 TCSPC imaging modules can be adapted to almost any laser scanning microscope [11, 12, 13a, 13b, 16, 35, 36, 37]. The only requirement is that Frame Sync and Line Sync signals with TTL or CMOS levels can be made available. The Pixel Clock signal is not absolutely required. If a pixel clock is not available it can be generated in the SPC module.

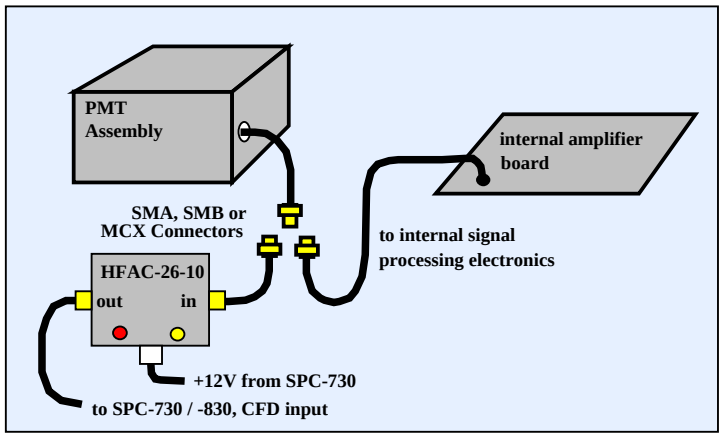
Detector Signals

Detectors in the Scanning Head

Generally, using the internal detectors of the microscope scanning head for FLIM is not recommended. The detectors are usually selected for high efficiency and low cost, not for time resolution. Moreover, opening the scanning head poses the risk of deadjusting the optics. If you have to use the detectors for whatever reason, please follow the instructions below.

In most microscopes the internal PMTs are connected to preamplifiers designed for video signal bandwidth. The output signals of these amplifiers are too slow for photon counting. Therefore, the PMT output must be made directly available. The PMT output is connected to a 50 Ω coaxial cable.

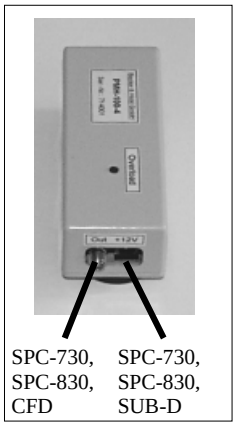
Best case, there is a small coaxial connector (SMB, SMA, MCX, Lemo, etc.) that you can use to disconnect the PMT from the internal amplifier and to connect it via a HFAC-26-10 preamplifier to the SPC-730 or SPC-830 module. Worst case, you have to desolder the cable either from the PMT assembly or from the internal amplifier board. We recommend to insert a connector into the cable so that you can easily connect the detector either to the scanning head electronics or to the SPC system.



Using the internal detectors for FLIM

Warning: Do not connect or disconnect the PMT signal line when the PMT operating voltage is switched on. There is not only danger of electrical shock, you can also damage the preamplifiers.

PMH-100 detector head



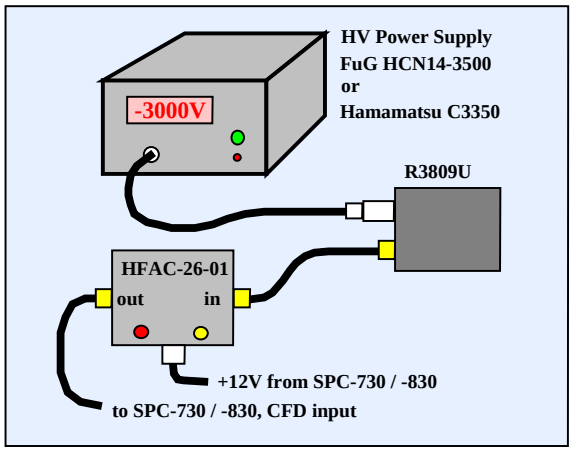
Connecting the PMH-100 is very simple. Connect its +12V power supply to pin 10 of the lower sub-D connector of the SPC-730 or -830 module. Connect a 50 Ω SMA cable from the detector output to the CFD input of the SPC-730 or -830. You need not make the cables yourself, they are delivered with the PMH-100 if it is ordered together with an SPC-730 or SPC-830 module.

The PMH-100 gives an optical and acoustical overload warning if the maximum output current of the PMT is exceeded. Please shut down the light or disconnect the +12 V immediately if you see the LED turning on or hear the overload beep.

The PMH-100 can also be connected via the DCC-100 detector controller card. However, since there is no overload signal available from the PMH-100 it cannot be automatically shut down on overload. Up to three detectors can, however, switched on and off via the DCC-100.

R3809U MCP

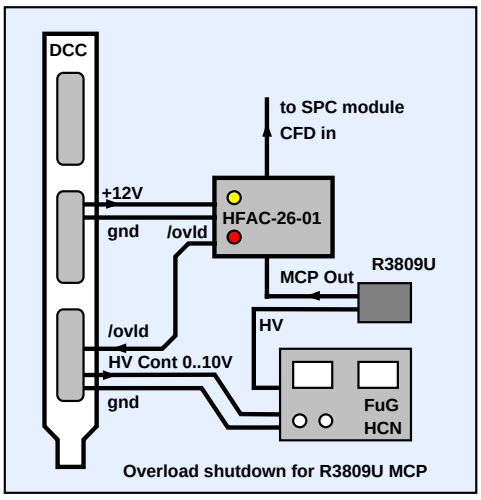
The Hamamatsu R3809U MCP requires a high voltage power supply and an HFA-26-01 preamplifier. The connections for a simple setup are shown in the figure right.



Simple connection of R3809U MCP. The HFAC-16 preamp indicates overload conditions

Although the R3809U can be operated at up to 3400 V a supply voltage of 3000 V is sufficient to get a system response below 30 ps and excellent counting efficiency. Therefore, an operating voltage of 3000V should not be exceeded in order to achieve a maximum lifetime of the detector. Suitable power supplies are the Hamamatsu C3350 (available for 220 V and 127 V) and the FuG HCN14-3500. If you connect another power supply, please make sure that the output voltage is negative.

MCP PMTs are very sensitive to overload. The maximum permitted average output current is only 100 nA. Exceeding this value does not damage the MCP immediately but reduces the lifetime of the device if the overload persists for a longer time. The Becker & Hickl HFAC-26-01 preamplifier measures the output current of the MCP and turns on an overload warning LED if a current of 100 nA is exceeded. The count rate at which the MCP output current reached 100 nA depends on the gain of the MCP and is between 200,000 and 800,000 counts per second. In other words, if the overload lamp turns on at a relatively low count rate, you have got a good MCP with high gain. If you cannot reach a sufficiently high count rate without getting overload you can reduce the high voltage and the CFD threshold.



Controlling the R3809U MCP via the DCC-100 detector controller. The R3809U is shut down if overload occurs.

Maximum safety against MCP damage is achieved by controlling the MCP via the DCC-100 detector controller. The connections are shown in the figure right. An FuG HCN-14-3500 is used as an high voltage power supply for the R3809U. The HCN-14 is available with a 0 to 10 V control input. The high voltage is proportional to the control voltage. Thus, the detector operating voltage can be controlled by the DCC-100 and be shut down on overload. For overload detection, the HFAC-26-01 preamplifier with an overload threshold of 100 nA is used.

Warning: Do not connect or disconnect the output signal line when the MCP operating voltage is switched on. There is not only danger of electrical shock, you can also damage the preamplifier. Please make sure that the connection between the MCP and the amplifier is reliable. Moreover, make sure that the HV cable and the HV connectors be in a good condition. Any interruption in the ground return path of the HV supply can put the detector case, the signal cables, the preamplifier, etc. on high voltage. Therefore be careful please - touching 3000 V can ruin you the whole day.

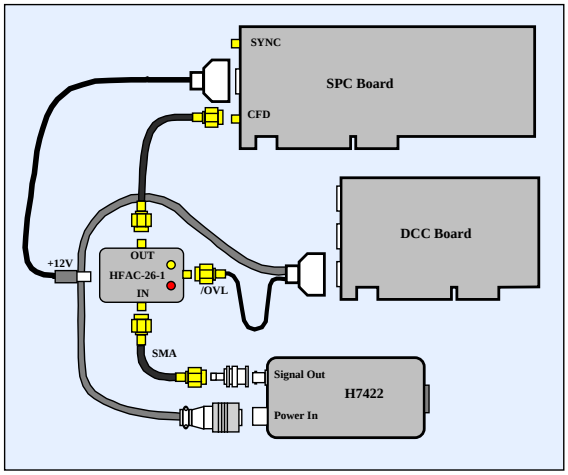
H7422

The H7422 needs a +12V power supply, a gain control voltage, and a high power current source to drive the internal peltier cooler. Therefore, the most practicable solution is to operate it in conjunction with the DCC-100 detector controller. The connections to the DCC-100 are shown in

the figure below. The detector is connected to ‘Connector 3’ of the DCC-100. The DCC-100 delivers the +12 V operating voltage, the 0 to 0.9 V gain control voltage, and the current for the thermoelectric cooler.

The HFAC-26-1 amplifier monitors the output current of the H7422. If an average current of 1 uA is exceeded the /ovld signal of the HFAC-26 goes to ‘low’. This sets the overload flip-flop in the DCC, and the gain control voltage and the +12 V at connector 3 are shut down.

Notice: The H7422 has its own overload shutdown. If the H7422 shuts down internally for whatever reason, it refuses to work unless it is reactivated by cycling the +12 V operating voltage. Cycling power can be awkward with a standard +12V power supply but is easily possible with the DCC-100.



Connecting the H7422 detector

Don’t connect or disconnect the signal cable from the detector to the preamplifier when the detector is switched on. This can destroy the amplifier. Moreover, make sure that the +12 V of the HFAC-26 is connected and switched on. Otherwise the HFAC may not work, and the overload protection is inactive.

Scan Control Signals

The scan control signals synchronise the data acquisition in the SPC-730 module with the scanning action of the microscope. Three signals are required:

- Frame Clock indicates the start of the next frame
- Line Clock indicates the start of the next line
- Pixel Clock indicates the start of the next pixel

The SPC-730 and SPC-830 need TTL or CMOS compatible pulses. The duration and the polarity of the pulses are not essential. The signals are connected to the upper sub-D connector of the SPC module. The pin assignment of this connector is shown below.

1	+5V (max. 100mA)	**9**	**Line Clock**
2	Routing Signal, /R 7 or ARMED[2]	10	+12V (max. 60mA)
3	Routing Signal, /R 8 or TRGD[2]	11	-12V (max. 60mA)
4	Routing Signal, /R 9 or MEASURE[2]	**12**	**Pixel Clock**
5	**Ground**	13	TRIGGER[3]
6	-5V (max. 100mA)	14	CNTE2 (CNTE=CNTE1&CNTE2)
7	Routing Signal, /R 10	**15**	**Ground**
8	**Frame Clock**		

Frame Clock, Line Clock and Pixel Clock pulses are used in all scanning microscopes. The question is only whether they are available externally at an unused connector.

Cables for the Zeiss LSM-510 Axiovert and LSM-510 Axioplan, the Leica SP2, and the Biorad Radiance 2100 are available from Becker & Hickl. For other microscopes please contact Becker & Hickl under info@becker-hickl.com or phone +49 / 30 787 56 32.

Some microscopes have only the Frame Clock and the Line Clock available. In this case you can work with a pixel clock signal generated in the SPC module. This works since both the scanning speed of the microscope and the frequency of the synthetic pixel clock are constant. However, some

microscopes use a non-uniform pixel clock to compensate for nonlinearity in the line scan. In this case you may find some distortion in the SPC image if you use a synthetic pixel clock.

If you make your own cable, please make sure that you don't accidentally connect the +12V, -5V or -12V of the SPC-730/830 to a scan control output of your microscope. This would almost surely damage the microscope electronics.

Synchronisation with the Laser

The synchronisation signal from the laser is required to provide a stop signal for the time measurement of the individual photon detection events (please see also SPC manual, [1]).

Most Ti:Sa lasers deliver a monitor signal that can be used for synchronisation. The SPC-730 and -830 modules need negative pulses of 100 to 500 mV amplitude and < 2ns risetime. The signal should have a stable amplitude and be free of AC components from the laser power supply and transients from the scanning head. If the pulses from your laser are positive, please use an inverting transformer available from Becker & Hickl.

If there is no suitable signal from the laser please use the PHD-400-N photodiode module of Becker & Hickl. A reflection of the Ti:Sa laser beam at a glass surface focused to the diode chip is sufficient to generate pulses of -100 mV (please see also SPC manual, [8]).

First Light

Detecting the first Photons

If you have a minimum of experience with optical detectors it should be no problem for you to put the SPC system into operation. In this case proceed as described below. Otherwise please suppress your aversion against manuals and read the section ‘Getting Started’ in the SPC manual [1].

Adjusting the Sync Signal

Start the SPC software. Set ‘Sync Threshold’ = -50mV, ‘Sync Zero Cross’ = -20mV, ‘Sync Frequency Divider’ = 4

If you use a photodiode module for synchronisation: Adjust the photodiode until 'SYNC OK' is displayed and the Sync rate corresponds to the repetition rate of your laser. If necessary, change ‘Sync Threshold’ and ‘Sync Zero Cross’. The current indicator at the photodiode module should go to about 10% of full scale for a 70 to 80 MHz laser. Caution: the current indicator of the PHD-100 is active only when the output is connected to the SPC module.

If the Sync signal comes from the laser: If necessary, change ‘Sync Threshold’ and ‘Sync Zero Cross’ until the displayed Sync rate corresponds to the repetition rate of your laser. Make sure that the pulses from the laser are negative.

Adjusting the detector

Do not give any light to your sample. Darken the room in order not to overload the detector by daylight leaking into the optical path.

Set ‘CFD limit low’ = 50mV, ‘CFD Zero Cross’ = 0

PMH-100 detector: Connect the +12V from the SPC card to the detector. Make sure that there is no overload warning (LED turned on or overload alert beep). You should see a CFD count rate of 100 to 500 counts per second. If you have a much higher count rate you probably have room light leaking into the detector.

R3809U MCP: Carefully increase the operating voltage. Watch the overload LEDs at the HFAC-26-01 amplifier. (Please make sure that you have the right amplifier. It must be the HFAC-26-**01** with 100nA overload current.) Stop the procedure if the LED turns on and find the way the daylight leaks into the detector. If everything is correct you should have a CFD rate of 100 to 500 counts per second at -3000 V.

Internal PMT: Proceed as described for the R3809U. The maximum supply voltage for the internal detectors is usually -900 to -1000V. The internal PMTs of some microscopes are prone to noise pickup from the scanning system. The effect shows up as a high count rate present only when the scanning is active. The count rate does not depend on the detector operating voltage. If you have effects like this, all you can do is to increase the CFD threshold until the false counts disappear and to operate the detector at a gain as high as possible.

When the detector voltage has been set up, start an SPC measurement in the Oscilloscope Mode. Use the parameters shown below:

System Parameters:	System Parameters:	Display Parameters:	Trace Parameters:
Operation Mode: Oscilloscope	TAC Range: 50ns	Scale Y: Linear	Trace 1: Active, Curve 1, Page 1
Overflow: Stop	TAC Gain: 1	Max Count: 65535	
Trigger: None	TAC Offset: 6%	Baseline: 0	
Coll Time: 0.5s	TAC limit Low: 8%	Point Freq: 1	
Display Time: 100s	TAC Limit High: 92%	Style: Line	
CFD Limit L: 50mV	ADC Resolution: 1024 or 4096	2D Display Mode: Curve	
CFD ZC Level: 0	Count Increment: 100		
SYNC: ZC Level -20mV	Memory Offset: 0		
SYNC Threshold: -50mV	Dith Rng: 1/16		
SYNC Freq Divider: 4	Routing Channels X,Y: 1		
Scan Pixels X,Y: 1	Page: 1		

As long as there is no excitation at the sample you should see virtually nothing except perhaps for some single photons scattered over the time axis. If you have more that a few thousand photons per second you are detecting daylight.

Give light to the sample. Be careful with the intensity. Even if you use one of the internal detectors of the microscope you may be surprised of the sensitivity of the PMT in the photon counting mode. On the screen you should see three or four subsequent signal periods of your fluorescence signal. Adjust the light intensity to a count rate of 100,000 counts per second or less.

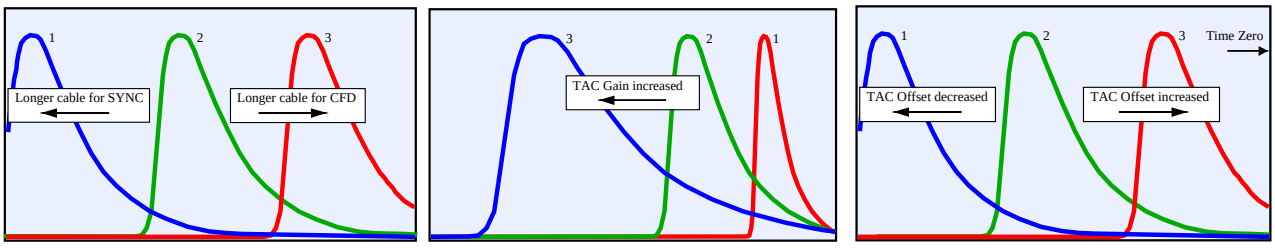
Change CFD Limit Low. The count rate decreases with increasing threshold, but the shape of the system response improves. Set Limit Low that you get about 80% of the maximum count rate.

Adjusting time scale and delay

Set ‘Sync Frequency Divider’ = 1. It can happen that you see only a part of the decay curve now. Change the length of the SYNC cable until you see the decay curve well inside the last (right) 10 ns of the display window. 1 ns corresponds to a cable length of 20 cm. Alternatively you can change the length of the cable from the preamplifier or the position of the photodiode module in the optical path. Caution: Don’t connect or disconnect the PMT when the high voltage is switched on.

Increase ‘TAC Gain’ to stretch the decay curve over the full display window. The most appropriate gain is 5. This gives a display window of 10 ns and covers nearly one laser period. You can use a higher TAC gain to get finer time bins. Use ‘TAC Offset’ to centre the decay curve in the display window.

For high TAC gain it can be nessecary to shift the curve let or right by changing ‘TAC Offset’. Please see SPC manual for fine adjustment of TAC parameters.

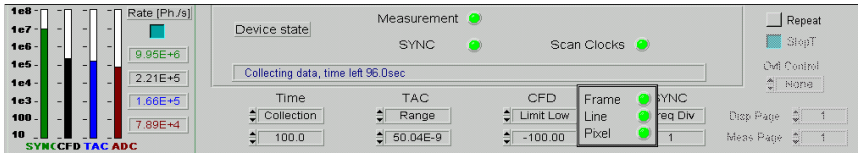


Effect of CFD and SYNC cable length, TAC Offset, and TAC Gain

Don’t forget to ave the result. Use ‘Main’, ‘Save’, options ‘SPC Data’, ‘All used data sets’. Advice: Please don’t name the file ‘test1.sdt’. Nine of ten files we receive for inspection are named ‘test1.sdt’ - you might get a strange answer.

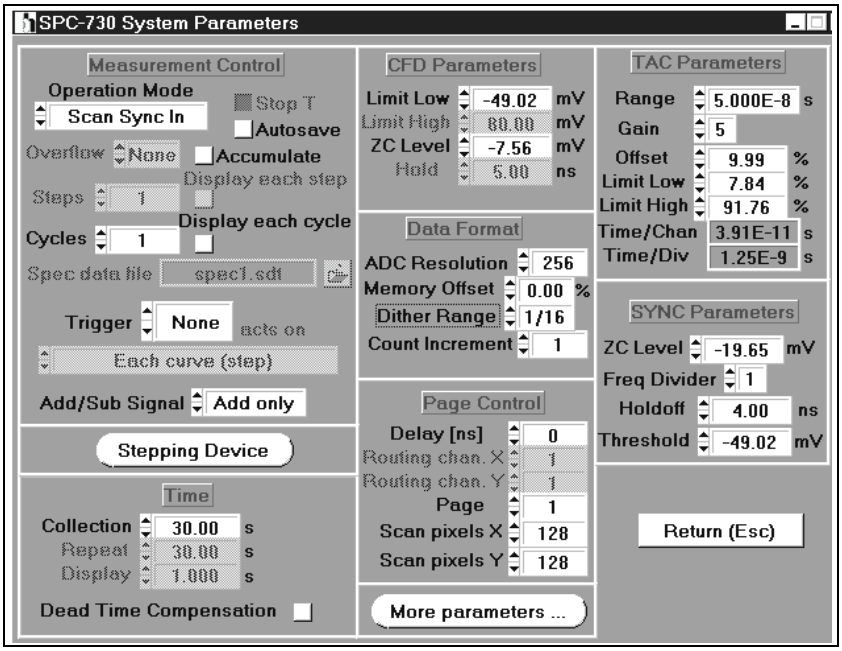
Recording the first image

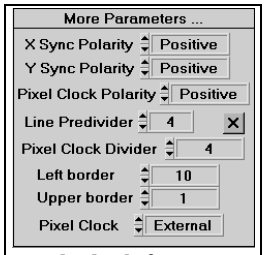
If the scan control signals are not connected yet, connect them now. If you have an SPC-830 or a newer SPC-730 in the ‘Scan Sync In’ mode the state of the scan control signals - frame clock, line clock and pixel clock - is displayed, see figure below.



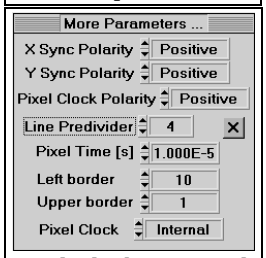
The ‘Scan Clocks’ lamp turns on when all used scan control pulses - i.e. Frame and Line Clock for internal pixel clock and Frame, Line and Pixel Clock for external pixel clock - are present. To display the state of the individual clocks, move the mouse cursor on the ‘Scan Clocks’ lamp. This opens a window with lamps for the individual clocks.

Except for the SYNC, CFD and TAC settings adjusted as shown in the last section, set the SPC-730/830 parameters as shown below:

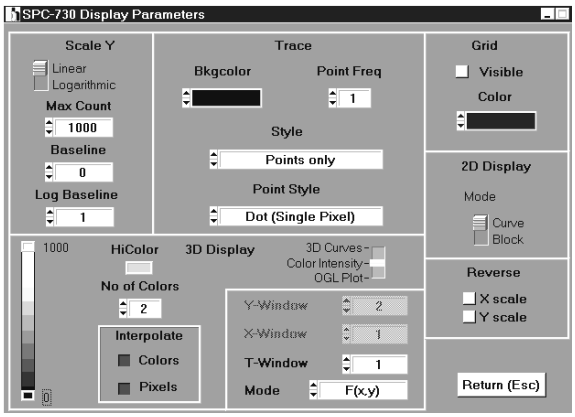


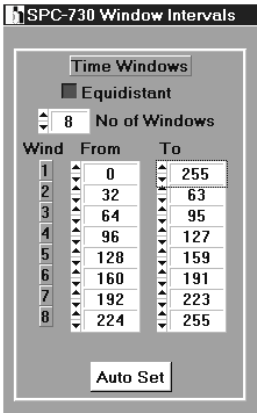


Pixel Clock from Microscope



Pixel Clock generated in the SPC-730





These settings give a full size TCSPC image of 128×128 pixels for a 512×512 resolution of the microscope. However, if you do not have a pixel clock from the microscope and thus have to generate the pixel clock in the SPC module, the pixel time of 10 µs is only a rough estimate. It can

happen that the real pixel dwell time is longer or shorter resulting in an image that is horizontally stretched or shrunk.

When all parameters are set, start the measurement and wait. The measurement should stop after the specified ‘Collection Time’, i.e. after 30 seconds. In practice it can take a few seconds longer because after the end of the collection time the acquisition is continued until the current frame is completed. If the measurement stops correctly, the frame clock, line clock and pixel clock signals arrive at the SCP-730/830 module. If the measurement does not stop one or several clock signals are missing.

If you do not see an image after the measurement has stopped, please reduce the ‘Max Count’ setting in the display parameters. Some image should become visible.

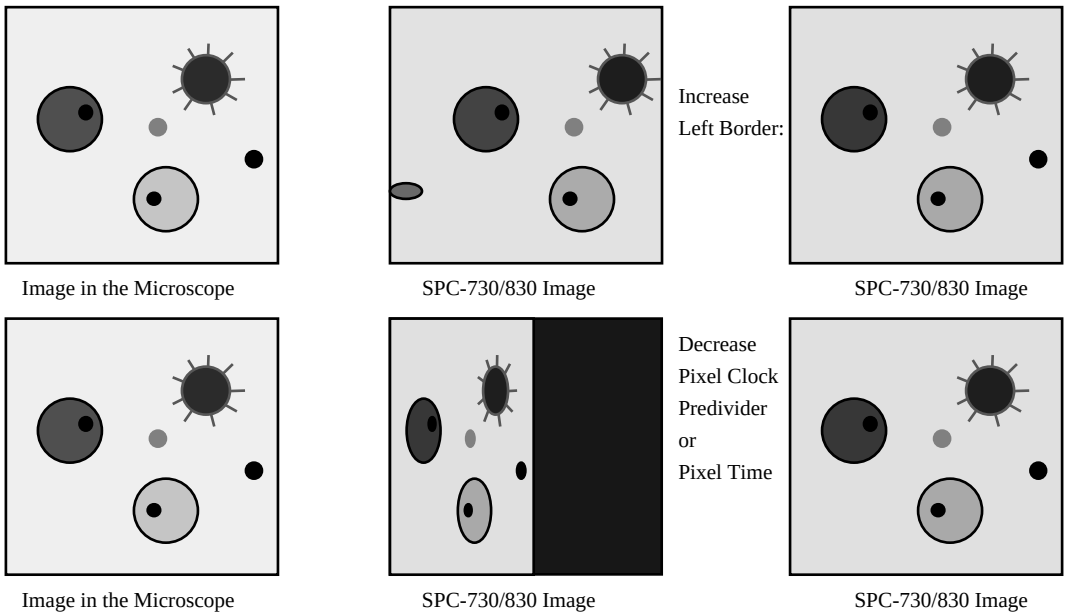
With the display parameter and window parameter configuration shown above, you get an image in the T Window 1 (specified in the ‘Display Parameters’). This window covers all time channels of the decay curves stored in the individual pixels. Another seven T windows are defined in the Window Parameters. With the setting above, the T Windows 2 through 8 contain subsequent time intervals of the time axis. As you step through these T Windows by changing the T Window number in the display parameters you see the image appearing with the laser pulse and fading as the fluorescence decays.

Do not forget to save the result. This saves also the setup parameters so that you can restore the system setup of the first successful measurement at any time.

If you do not get a reasonable image, please make sure that the parameters are set as shown above and check the scan control signals with an oscilloscope.

Adjusting image size and image location

The first images recorded with the settings shown under ‘Recording the first images’ can still have a wrong size, show a wrong part of the scan or be reversed in X or Y. Some typical effects and the way of correction are shown below.



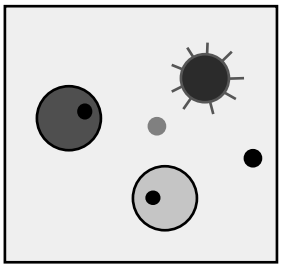
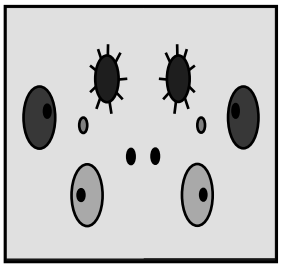
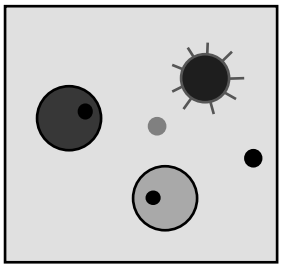


Image in the Microscope



SPC-730/830 Image

Decrease Pixel Clock Predivider or Pixel Time



SPC-730/830 Image

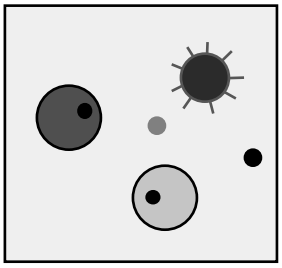
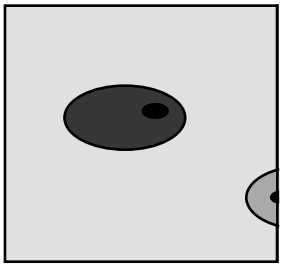
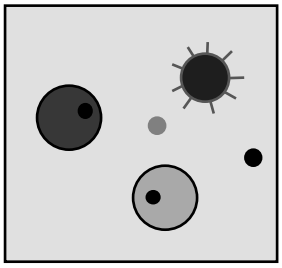


Image in the Microscope



SPC-730/830 Image

Increase Pixel Clock Predivider or Pixel Time



SPC-730/830 Image

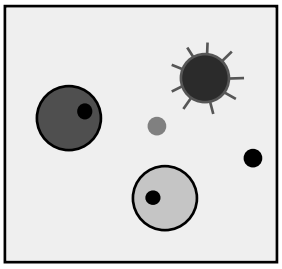
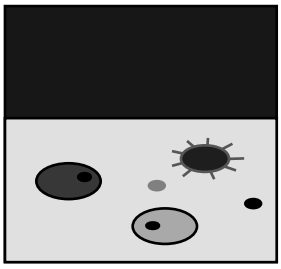
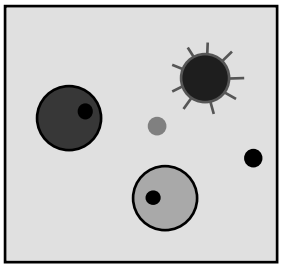


Image in the Microscope



SPC-730 Image

Decrease Line Clock Divider



SPC-730 Image

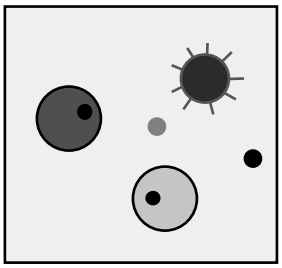
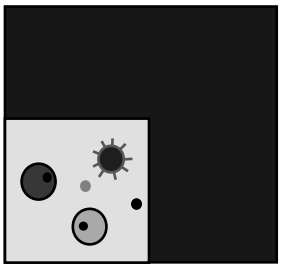
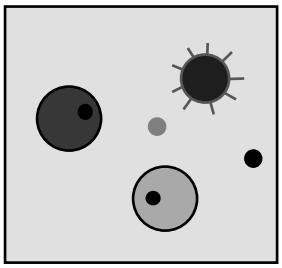


Image in the Microscope



SPC-730 Image

Decrease Line Clock Divider and Pixel Clock Predivider or Pixel Time



SPC-730 Image

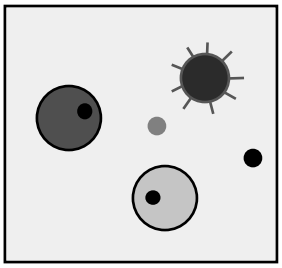
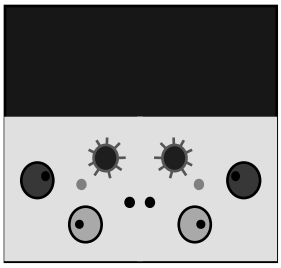
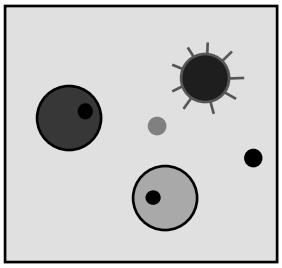


Image in the Microscope



SPC-730 Image

Decrease Line Clock Divider and Pixel Clock Predivider or Pixel Time



SPC-730 Image

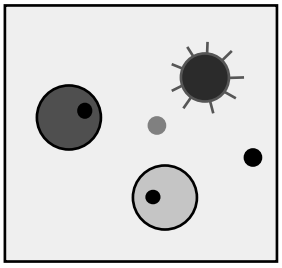
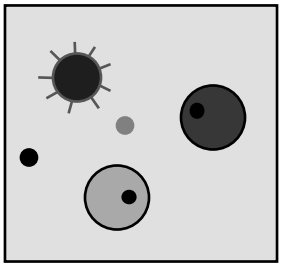
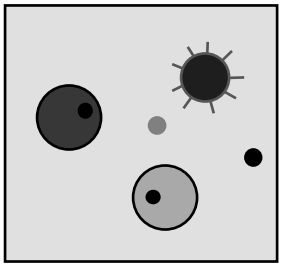


Image in the Microscope



SPC-730 Image

Set Reverse X in Display Parameters



SPC-730 Image

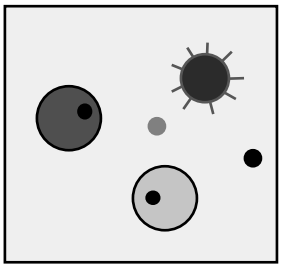
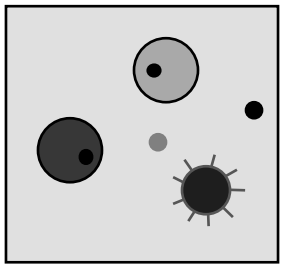
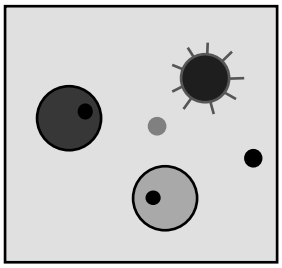


Image in the Microscope



SPC-730 Image

Set
Reverse Y
in Display
Parameters



SPC-730 Image

Some microscopes, e.g. the Leica SP1, deliver pixel clock pulses both at the start and at the end of each line. The result is an image as shown in the figure below. If you have effects like this please contact BH. We have adapters to transform odd scan control signals into useful frame and line pulses.

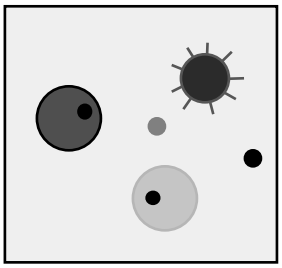
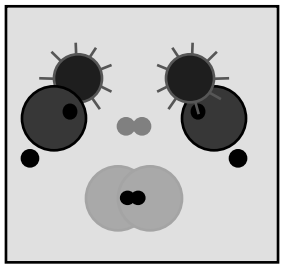
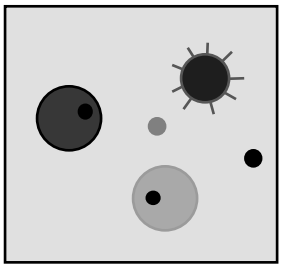


Image in the Microscope



SPC-730/830 Image

Contact
BH for
Adapter



SPC-730/830 Image

Assistance through bh

Software updates, new manual versions and application notes about new applications are available from our web site www.becker-hickl.de. Furthermore, we are pleased to support you in all problems concerning the measurement of fast electrical or optical signals. This includes discussions of new applications, the installation of the SPC modules, their application to your measurement problem, the technical environment and physical problems related to short time measurement techniques. Simply call us or send us an email.

Should there be a problem with your SPC module, please contact us. To fix the problem we ask you to send us a data file (.sdt) of the questionable measurement or (if a measurement is not possible) a setup file (.set) with your system settings. Furthermore, please add as much as possible of the following information:

Description of the Problem
SPC Module Type and Serial Number
Software Version
Type of the Microscope
Detector type, Operating voltage of the detector, PMT Cathode type
Preamplifier type, Gain, Bandwidth etc.
Laser System: Type, Repetition Rate, Wavelength, Power
SYNC Signal Generation: Photodiode, Amplitude, Rise Time
System Connections: Cable Lengths, Ground Connections. Add a drawing if necessary.
Environment: Possible Noise Sources
Your personal data: E-mail, Telephone Number, Postal Address

The fastest way is to send us an email with the data file(s) attached. We will check your system settings and – if necessary – reproduce your problem in our lab. We will send you an answer within one or two days.

Becker & Hickl GmbH
Nahmitzer Damm 30
12277 Berlin
Tel. +49 / 30 / 787 56 32
FAX +49 / 30 / 787 57 34
http://www.becker-hickl.com
email: info@becker-hickl.com
or becker@becker-hickl.com

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