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Correlation Measurements by Advanced TCSPC Techniques

Introduction

If photons are emitted by thermal sources or by fluorescence of a large number of molecules the emission events are independent. The photons appear randomly, and the distribution of the time distance between subsequent photons drops exponentially with increasing time distance.

For light generated by nonlinear effects, by quantum dots [1, 2, 3, 4] or single molecules [5] this is not necessarily the case. When a single molecule has absorbed a photon it cannot absorb and emit a second photon until it has not returned to the ground state. Thus, the fluorescence photons are not longer independent. Instead, there is a correlation - or 'antibunching' - on the time scale of the fluorescence lifetime. An even more pronounced antibunching can be achieved in optically or electrically excited quantum dots. Investigation of these effects requires the detection and correlation of single photons on the ps and ns scale.

Fluorescence correlation spectroscopy (FCS) exploits the correlation between the fluorescence intensities in different time intervals or in different wavelength intervals [6-9]. A confocal or two-photon microscope setup is used to achieve a sample volume of the order 0.1 fl. At a concentration of 10^{-9} to 10^{-11} mol/l only a few molecules are in the focus. Diffusion processes cause a fluctuation of the number of molecules in the sample volume and therefore a fluctuation in the fluorescence intensity. In biological systems the autocorrelation function of the fluctuations gives information about the size of protein-dye clusters and about the mobility in membrane structures. If different protein structures are marked with different dyes a cross-correlation of the fluorescence signals (FCCS) shows whether the structures are linked or diffuse independently. Typical FCS and FCCS effects happen on a time scale from a few hundred nanoseconds to milliseconds.

Clusters of proteins and dyes can change there conformation which, in turn, induces changes in the fluorescence lifetime and the fluorescence anisotropy [10,11,12]. The lifetime changes can be due to changes in the quenching intensity, or, if two dyes with overlapping emission and absorption spectra are used, by fluorescence resonance energy transfer (FRET) [13,14]. Moreover, the fluorescence lifetime in conjunction with spectral information can be used to distinguish between different dyes and dye-protein clusters [15].

The borders between the correlation techniques are somewhat fuzzy. In almost any correlation experiment single molecules, a small number of molecules, or other single quantum structures are investigated. Structures such small almost inevitably show random changes, Brownian motion or other diffusion processes or light induced changes. Therefore, photon correlation effects are usually accompanied by intensity, lifetime and anisotropy fluctuations, and in typical FCS measurements photon correlation effects are present. Fortunately, advanced time-correlated single photon counting (TCSPC) techniques can be used to investigate all these effects, and, more important, to investigate them at the same time, in the same sample and in the same sample position. TCSPC is, in principle, able to record the detection times of the

individual photons of several detection channels with picosecond accuracy. With a suitable optical system TCSPC data contain information about FCS, FCCS, lifetime, antibunching, and anisotropy effects as well. The difference between the techniques is only how and on which time scale the photon data are correlated [11,12].

TCSPC Techniques

Conventional Time-Correlated Single Photon Counting - or TCSPC - is based on the detection of single photons of a periodical light signal, the measurement of the detection times of the individual photons and the reconstruction of the waveform from the individual time measurements [16,17].

The technique makes use of the fact that for low level, high repetition rate signals the light intensity is usually so low that the probability to detect one photon in one signal period is much less than one. Therefore, the detection of several photons can be neglected and the principle shown in fig. 1 be used.

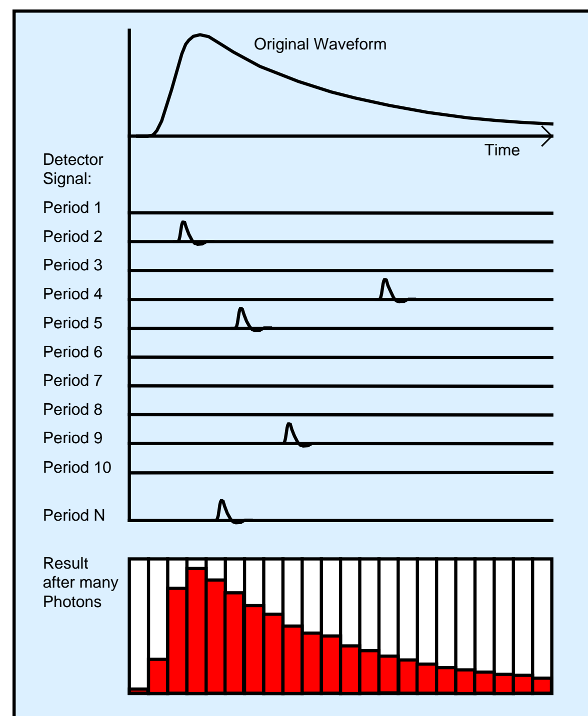


Fig. 1: Principle of the TCSPC technique

The detector signal consists of a train of randomly distributed pulses due to the detection of the individual photons. There are many signal periods without photons, other signal periods contain one photon pulse. Periods with more than one photons are very unlikely.

When a photon is detected, the time of the corresponding detector pulse is measured. The events are collected in a memory by adding a ‘1’ in a memory location with an address proportional to the detection time. After many photons, in the memory the histogram of the detection times, i.e. the waveform of the optical pulse builds up. Although this principle looks complicated at first glance, it has a number of striking benefits:

- The time resolution of TCSPC is limited by the transit time spread, not by the width of the output pulse of the detector. With fast MCP PMTs an instrument response width of less than 30 ps is achieved [16,17,18,24].
- TCSPC has a near-perfect counting efficiency and therefore achieves optimum signal-to-noise ratio for a given number of detected photons [19,20,21]
- TCSPC is able to record the signals from several detectors simultaneously [17,22-25]
- TCSPC can be combined with a fast scanning technique and therefore be used as a high resolution, high efficiency lifetime imaging (FLIM) technique in confocal and two-photon laser scanning microscopes [20,21,26,27]
- TCSPC is able to acquire fluorescence lifetime and fluorescence correlation data simultaneously [17]
- State-of-the-art TCSPC devices achieve count rates in the MHz range and acquisition times down to a few milliseconds [17,20,24]

Multi-Detector TCSPC

TCSPC multi-detector operation makes use of the fact that the simultaneous detection of photons in several detector channels is unlikely. Therefore, the single photon pulses from several detector channels - either individual detectors or the anodes of a multi-anode PMT - can be combined in a common timing pulse line. If a photon is detected in one of the channels the pulse is sent through the normal time-measurement circuitry of a single TCSPC channel. In the meantime an array of discriminators connected to the detector outputs generates a data word that indicates in which of the channels the photon was detected. This information is used to store the photons of the individual detector channels in separate blocks of the data memory [17, 22-23] (fig. 2).

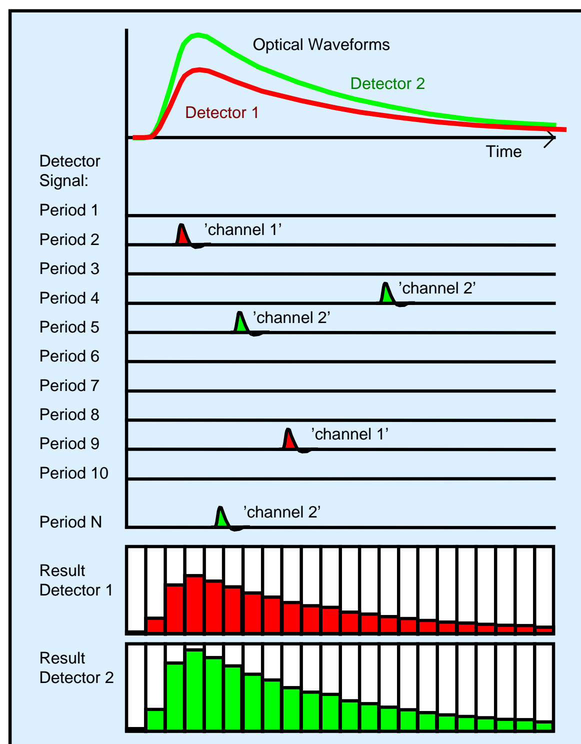


Fig. 2: Multi-detector TCSPC

Multi-detector TCSPC can be used to simultaneously obtain time- and wavelength resolution [20,21], to distinguish photons of different polarisation [10] or to record photons from different locations of a sample [24]. Because the photon pulses of all detectors are sent through the same TCSPC timing electronics [17] the photons of different detector channels cannot be directly cross-correlated at a time shorter than the dead time of the TCSPC device. The technique can, however, be used to obtain FCS data for the individual detector channels, fluorescence cross-correlation data between different channels at a time scale above a few 100 ns, and fluorescence lifetime data. In these cases it is of benefit that the time scales for all detector channels are exactly the same so that cross-correlation does not require any calibration.

It should be noted that multi-detector TCSPC does not involve any multiplexing or scanning process. Therefore the counting efficiency for each detector channel is still close to one, which means that the efficiency of a multi-detector TCSPC system can be considerably higher than for a single channel TCSPC device [19,20].

Fast Sequential Recording

Unlimited fast sequential recording of decay curves can be achieved by a dual memory structure. The principle is shown in fig. 3. Two identical memories are used. Both memories are large enough to accept a large number of subsequently recorded waveforms. The dual memory structure is used to alternately swap between the two memories and to read the data from one memory while the measurement writes new data into the other one [17]. Thus, a virtually unlimited number of decay curves can be recorded without any time gaps between subsequent curves.

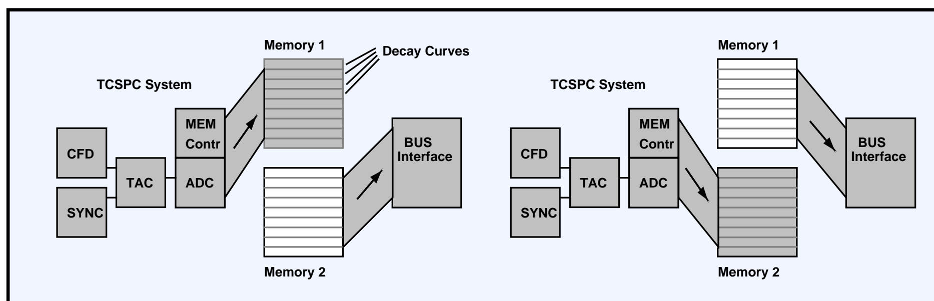


Fig. 3: Fast sequential recording

The 'Continuous Flow' mode for fast sequential recording is implemented in the bh SPC-430, SPC-630, and SPC-134 modules. The 'Continuous Flow' mode is strictly hardware controlled and thus provides an extremely accurate recording sequence. The collection time per curve can be less than 1 ms while maintaining unlimited continuous recording without gaps between subsequent decay curves. Fast sequential recording can be combined with multi-detector operation if several detectors and a router are used, see fig 4.

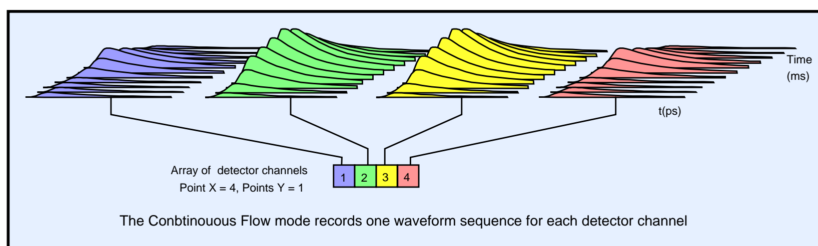


Fig. 4: Fast sequential recording with several detectors

Sequential recording works efficiently as long as a resolution of about 1 ms per step is sufficient. Therefore it can be used to investigate slow diffusion processes or to trace other changes in the fluorescence behaviour of molecules on this time scale. The SPC-430 module in the sequential recording (continuous flow) mode has been successfully used to identify molecules travelling through a capillary [28, 29]. The identification of DNA sequences by 'smart probes' is described in [30]. Probe molecules travelling through the laser focus cause photon bursts. Bust size, burst duration, and the fluorescence lifetime within the burst are used for discrimination. An algorithm to identify molecules by using sequential recording data is described in [31].

Sequential recording works well for collection times down to about 1 ms per curve. For times much shorter than the reciprocal photon count rate sequential recording becomes inefficient in terms of memory or hard disk space. Therefore sequential recording does not work well for correlation experiments on the nanosecond and microsecond scale.

TCSPC Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) exploits intensity fluctuations in the emission of a small number of chromophore molecules in a femtoliter sample volume [5-9]. The fluctuations are on a time scale from some 100 ns to several ms. To record intensity changes such fast, sequential recording of complete decay curves would be inefficient. Therefore, the bh SPC-630, SPC-134, and SPC-830 TCSPC modules have an FIFO or 'Time-Tag' mode that uses the device memory as a first-in-first-out (FIFO) buffer. Instead of building up the photon distribution over the detection time it records the full information about each individual photon [17]. The entry for each photon contains the time of the photon within the fluorescence decay curve (micro time), the time from the start of the experiment (macro time), and the number of the detector that detected the photon. The data structure is shown in fig. 5.

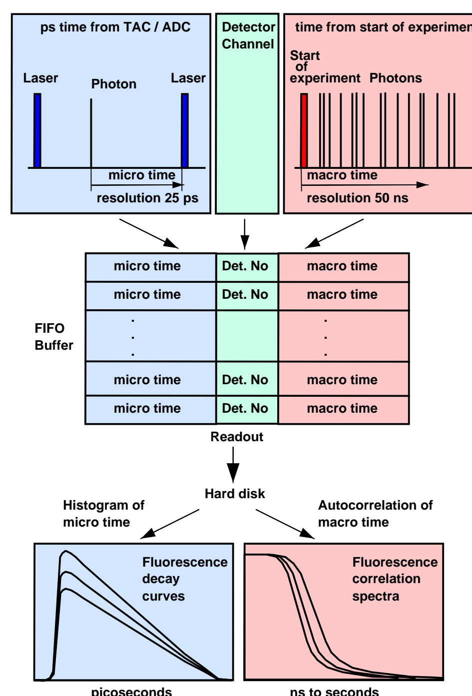


Fig. 5: Simultaneous FCS / lifetime data acquisition

The recorded data contain a wealth of information about diffusion, rotation, singlet and triplet effects in the investigated molecules. The simplest application is combined fluorescence lifetime and FCS recording. By correlating the macro times and building up a histogram of the micro times of the photons FCS curves and decay curves are obtained for each of the detection channels. Cross correlation of the macro times of different wavelength or polarisation channels delivers FCCS and anisotropy data. Higher order correlation of the micro times shows variations of the fluorescence lifetime between different molecules drifting through the focus or variations induced by conformational changes of dye-protein complexes [10-12].

It should be pointed out that all this information is obtained from the same molecules of the same sample at the same time. This is a considerable benefit compared to FCS recording by a separate hardware correlator in combination with a conventional TCSPC lifetime experiments. Moreover, there are TCSPC modules which deliver fluorescence lifetime images with confocal and two-photon laser scanning microscopes and time-tag data as well. Therefore lifetime images of cells can be recorded, and correlation experiments be run in selected spots of the cell.

The TCSPC time tag mode is a powerful tool to investigate diffusion processes in cell membranes, reveal conformational changes of dye molecules attached to membranes or DNA, protein folding, or single molecule FRET effects, and to obtain structural information of subunits in cells by comparing their diffusion behaviour.

Classic Photon Correlation Experiments

With the TCSPC techniques described above a wide variety of correlation experiments are possible. A selection of the most frequently employed setups is described below.

Single-Detector Start-Stop Setup

A single detector correlation setup is shown in the fig. 6.

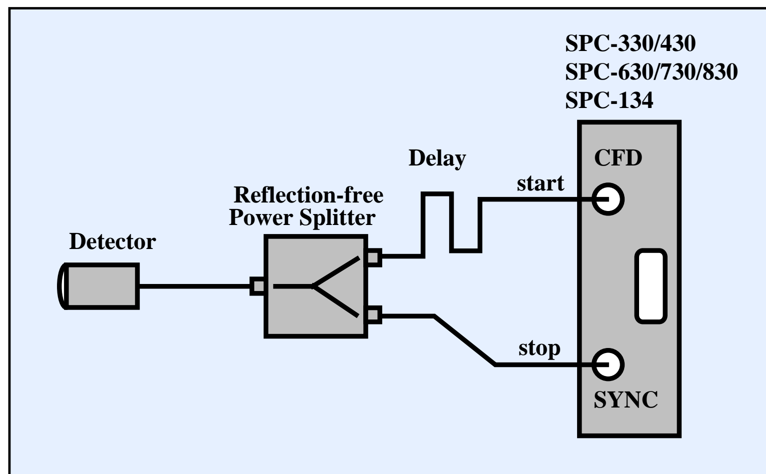


Fig. 6: Single detector photon correlation setup

The detector pulses are fed both to the start and the stop input of the SPC module. The 'start' pulse is delayed by a few ns (1 or 2 m 50 Ω cable). When the first photon is detected, it causes a stop pulse, and, a few ns later, a start pulse. The stop pulse is ignored by the SPC module because it was not started before. The start pulse starts the time measurement which runs until the next photon is detected. The stop pulse from this photon arrives first, therefore the second photon stops the time measurement. The start pulse from this photon arriving a few ns later is ignored because it is in the dead time of the SPC module. Therefore, the setup records a histogram of the time differences between pairs of photons.

The drawback of the single-detector setup is that the dead time of the detector makes it impossible to record photons appearing simultaneously or within a time sorter than a few ns. Moreover, the results are severely impaired by correlation effects in the detector itself. For several 100 ns after the detection of the first photon (the start photon) the detector is prone to produce afterpulses, and reflections and ripple in a PMT voltage divider cause ripple in the instrument response function. The single detector setup is therefore not used for classic photon correlation experiments. However, a single detector setup can be used for FCS experiments that correlate intensity fluctuations at a time scale longer than a few microseconds.

As for any TCSPC setup, the time resolution is limited by the transit-time spread (TTS) of the detector. Because start and stop are triggered by the same detector the resultant FWHM is about 1.4 the transit time spread of the detector.

Dual-Detector Start-Stop Setup

The problems of the single detector setup are avoided by the Hanbury-Brown Twiss setup [32]. The Hanbury-Brown Twiss setup is the basis of all TCSPC photon correlation experiments. The principle is shown in fig. 7.

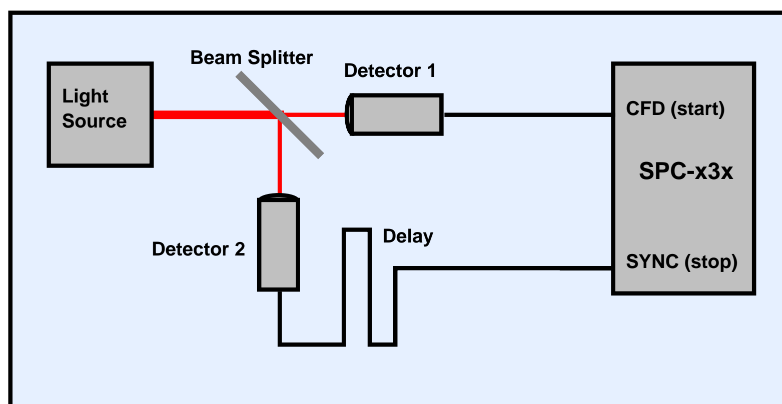


Fig. 7: Dual detector (Hanbury-Brown Twiss) photon correlation setup

The investigated light signal is split into two signals by a 1:1 beam splitter, and the two signals are fed to separate detectors. The detector signals are fed to the start and stop inputs of the TCSPC module. The stop pulses are delayed by a few ns to place the coincidence point in the centre of the recorded time interval. The setup delivers a histogram of the time differences between the photons at both detectors. Because separate detectors are used for start and stop there is no problem with the detector dead time and with the ripple due to start-stop crosstalk.

The efficiency of the setup is relatively low because only a fraction of the photons cause recordable events. There is only a 50% probability that two photons go to different detectors. Moreover, the probability that both photons are detected is the square of the effective quantum efficiency of the detectors and the optical system. Therefore it is important to use high quantum efficiency detectors for correlation experiments. Normally the SPCM-AQR avalanche photodiode detectors of Perkin Elmer are used [39]. However, these detectors have a count-rate- and wavelength-dependent response [18]. The changes can be of the order of 1 ns. It is therefore almost impossible to obtain a time resolution below 1 ns or to investigate changes in the correlation function versus intensity. Moreover, avalanche photodiode detectors emit a small amount of light when an avalanche is triggered. If light from the start detector reaches the stop detector it causes a false correlation peak.

Compared to the APD modules, conventional PMTs have a lower quantum efficiency but a shorter, stable, and almost wavelength-independent TCSPC response. However, PMTs with GaAsP photocathodes such as the Hamamatsu H7422-40 [41], have a quantum efficiency close to 40% between 400 and 500nm. We found that the H7422-40 had an efficiency comparable to or better than the SPCM-AQR in the wavelength range below 530 nm.

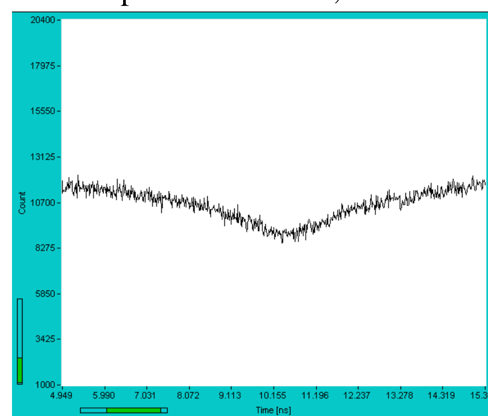


Fig. 8: Antibunching curve for Rhodamine 6G, CW excitation, one SPC-134 channel, H7422-40 detectors

The dual detector setup can be used with continuous excitation or with excitation by picosecond pulses. Continuous excitation delivers the usual antibunching curve. An example for organic molecules is shown in fig. 8. It was recorded with two H7422-40 modules connected to one channel of an BH SPC-134 TCSPC package.

Antibunching experiments at single quantum dots are described in [4]. The authors used SPCM-AQR modules as detectors and an older BH SPC-300 TCSPC module for data recording.

Antibunching experiments with picosecond lasers of high repetition rate deliver a number of correlation peaks which are spaced by the laser pulse interval. If the laser pulse width is much shorter than the fluorescence lifetime there is almost no chance that a single molecule is excited several times within one laser pulse. For a single molecule photon pairs with a spacing less than the laser pulse distance become extremely unlikely. Therefore, the ratio of the height of the central coincidence peak and the adjacent peaks is a indicator of the number of the molecules in the excited volume. Antibunching experiments with pulsed excitation are described in [4] and [33].

Multi-Detector Start-Stop Setup

The principle described above can be combined with the multi-detector - or 'routing' - capability of the SPC modules. Fig. 9 shows a setup that correlates the photons detected in several start detectors with the photons detected in one stop detector.

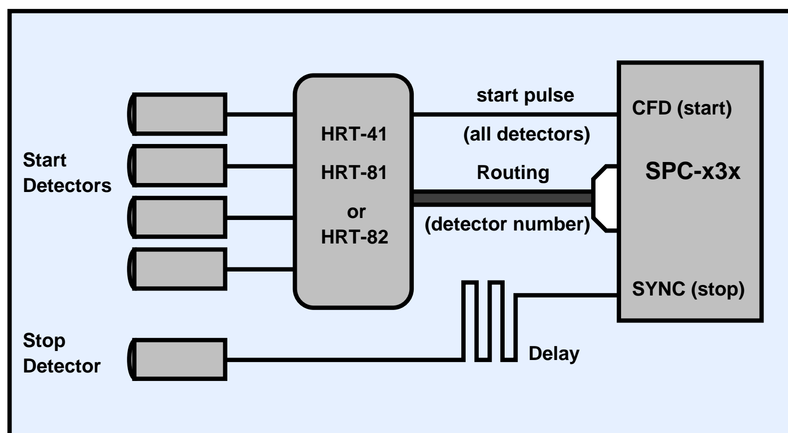


Fig. 9: Correlation setup with several start detectors

A HRT-41, HRT-81 or HRT-82 router [17,23,24] is used to connect several detectors to the start input of the SPC module. The setup can be used if the detection of several start photons for one stop photon is unlikely or can be excluded at all, i.e. if a single start photon can go different ways. If one of the detectors detects a photon the HRT module sends the resulting pulse into one common timing pulse line. The timing pulse is sent through the normal timing measurement procedure of the SPC module. In the meantime, the HRT module determines the detector which detected the photon and generates a digital 'routing' signal representing the number of the detector that detected the photon. This number is used to route the photons from different detectors into different histograms in the SPC memory, or, if the SPC works in the 'FIFO' mode, to mark the individually stored photons with the detector number.

The setup does not require that the stop photon actually appears after the start photon. It is only necessary that the stop pulse at the SYNC input of the SPC module arrives after the start pulse. This can easily be achieved by a delay cable of appropriate length in the SYNC channel.

The multi-detector technique can, with some restrictions, be used if both the start and the stop photon can by detected by several detectors. The setup is shown in fig. 10.

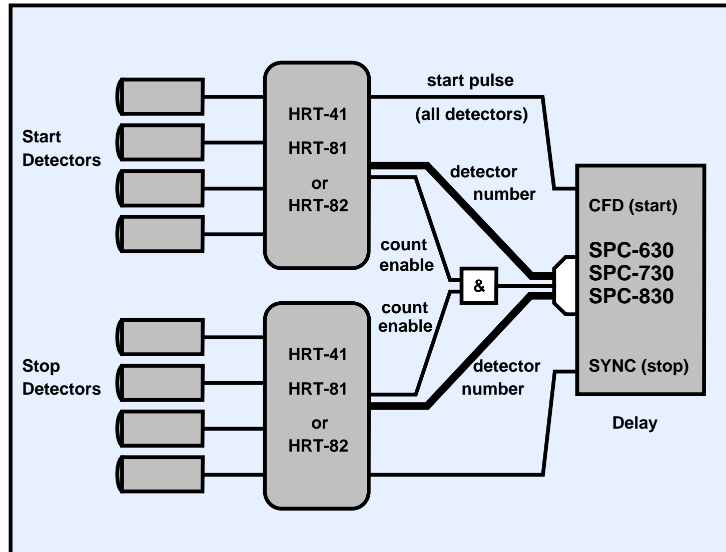


Fig. 10: Correlation setup with several start and stop detectors

The start detectors and the stop detectors are connected to the SPC module via HRT-41, HRT-81 or HRT-82 routers. The bits of the detector numbers delivered by both routers are connected to different routing bits of the SPC module. In the histogram mode of the SPC for each start and stop detector combination a separate histogram is built up. In the FIFO mode each start-stop event is marked with the start and stop detector number.

For each valid routing event the routers generate a 'count enable' signal. This signal is used to avoid misrouting photons if several detectors connected to one router trigger simultaneously. The count enable signals of both routers are combined via a logical AND, e.g. a 74HCT08 gate.

The dual-router setup requires that the routing bits and the count enable bits of both routers are valid at the same time. In standard HRT devices these signals are valid for approximately 40 ns. To assure a minimum overlap of 10 ns the start and stop events must be not more than 30 ns apart. Longer overlap times can be achieved by changes in the HTR device, but on the expense of reduced peak count rate.

Multi-Detector Start-Stop Experiments with the SPC-134

The SPC-134 module has four fully parallel TCSPC channels [17, 24]. Therefore it can be used to build up correlation experiments with four start detectors and one stop detector, with one start detector and four stop detectors, or with four independent dual-detector start-stop channels. Several configurations are shown in the fig. 11. Because the TCSPC channels are parallel it is not longer required that there is only one stop photon detected at a time.

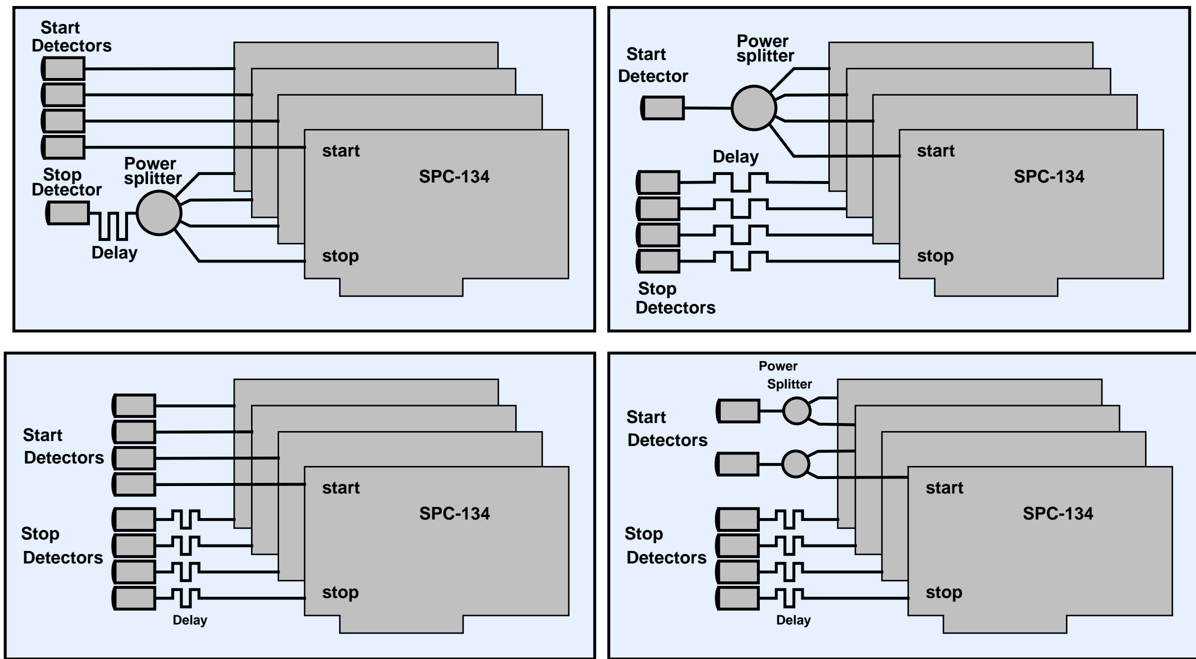


Fig. 11: Correlation experiments with the SPC-134

Fluorescence Correlation Spectroscopy (FCS)

Single-Detector FCS / Lifetime Experiments

A typical FCS setup is shown in Fig. 12. A laser beam is focused into the sample through a microscope objective. The fluorescence light from the sample is collected by the same microscope lens, separated from the laser by a dichroic mirror, and fed through a pinhole in the upper image plane of the microscope lens. Only light from the focal plan fits through the pinhole, therefore the sample volume can be confined to less than 1 femtoliter. The fluorescence light from this sample volume is fed to a detector.

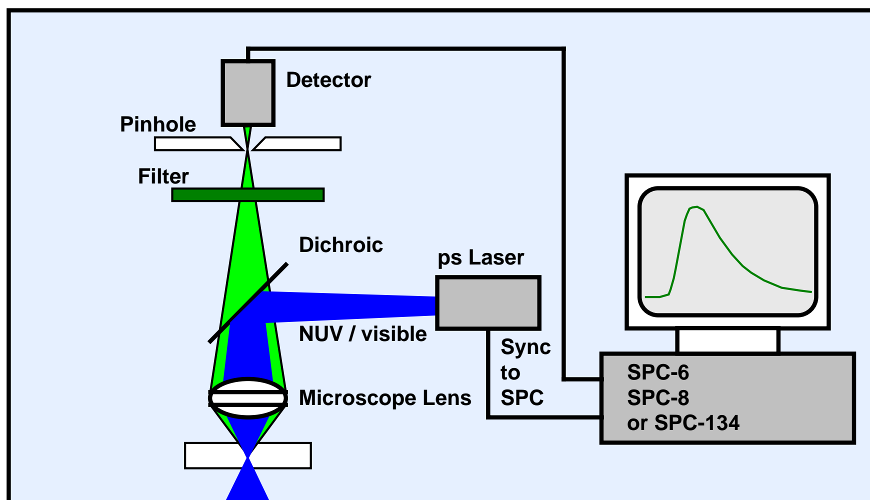


Fig. 12: Single-detector FCS / lifetime experiment

The single photon pulses from the detector are fed to an SPC-630, SPC-830, or SPC-134 TCSPC module. The module is operated in the 'FIFO' mode, i.e. for each photon the time referred to a reference pulse from the laser and the time from the start of the experiment is recorded.

If a high repetition rate pulsed laser is used, from the recorded data the fluorescence decay curve and the FCS in any time interval within the measurement time can be obtained. The decay curve is obtained by calculating a histogram of the photon density over the times of the photons within the laser pulse sequence (the 'micro-times'), the FCS by calculating the autocorrelation of the times of the photons from the start of the experiment (the 'macro-times'). The benefit of the setup compared to a conventional FCS hardware correlator is that the fluorescence lifetime data are available from the same data set. Moreover, the lifetime data originate from exactly the same molecules as the FCS data and can therefore be used to distinguish between different molecules and different binding and conformation states of dye-protein complexes. Another benefit of the technique is that the correlation spectrum can be calculated over any time interval within the measurement. It is therefore easier to trace temporal changes in the FCS data.

A single detector FCS / lifetime setup has been used to identify different species of DsRed on the single molecule level [34] . Decay times as short as 5 ps could be determined.

The TCSPC-FCS technique can also be used in conjunction with a continuous laser. Of course, in this case a meaningful micro-time does not exist, and no lifetime data are obtained. The setup is shown in fig. 13.

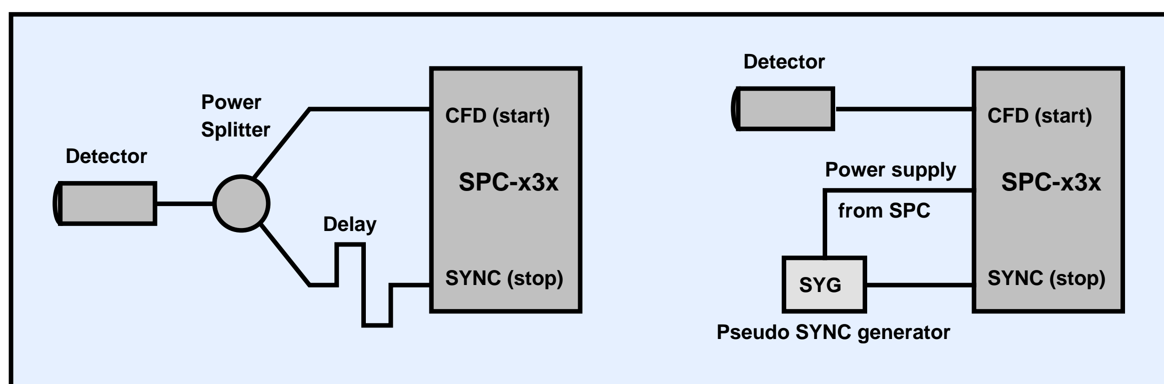


Fig. 13: SYNC (stop pulse) generation for FCS with continuous laser

Because the TCSPC module needs a synchronisation pulse to finish the time measurement for a recorded photon an artificial stop pulse must be provided. This can be the delayed detector pulse itself, or a pulse from a pseudo-sync generator. A suitable generator is available for the bh TCSPC modules.

Multi-Detector FCS / Lifetime Experiments

The multi-detector technique described above under 'Multi-Detector TCSPC' can be used to obtain FCS / lifetime data in different wavelength intervals, under different polarisation angles, and from different chromophores. Two possible setups are shown in fig. 14.

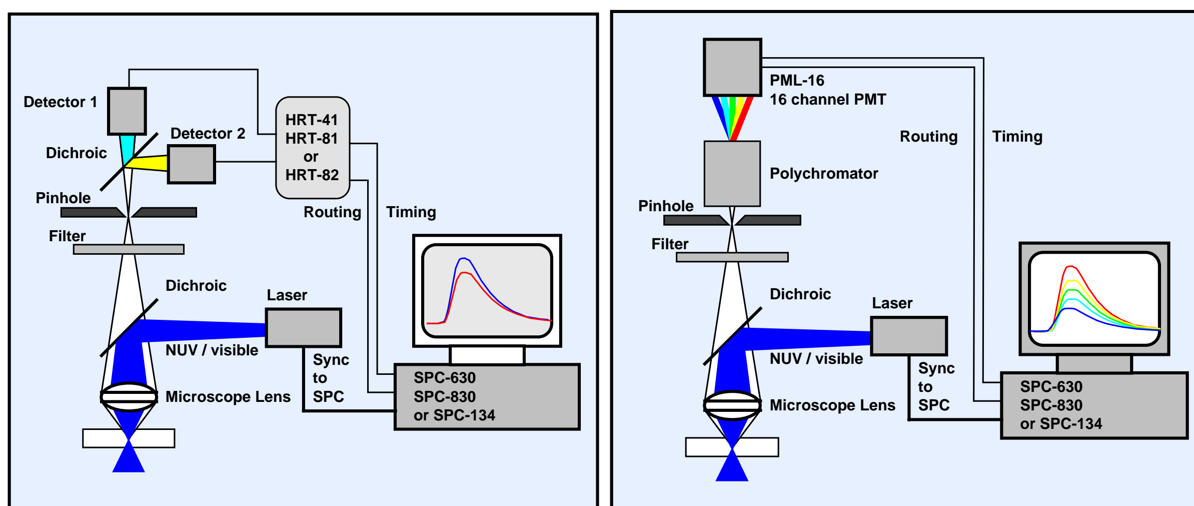


Fig. 14 Multi-colour FCS / lifetime experiments

If only two detectors are used the wavelength intervals can be selected by a dichroic beamsplitter and appropriate filters (fig. 14, left). If the setup is used for FRET experiments or for two chromophores with overlapping absorption spectra, one laser is sufficient to excite the fluorescence in both emission bands. If two dyes are used that cannot be excited by the same laser wavelength, two lasers have to be used. For continuous lasers this is mainly a problem of alignment. Pulsed lasers have to be synchronised, which is complicated if solid state or dye lasers are used. However, synchronisation can easily be achieved by using laser diodes which are driven by the same pulse generator.

Multi-detector FCS / lifetime experiments are used to obtain information about conformational changes of dye-protein structures, for single molecule FRET experiments, protein folding, to distinguish between different chromphores, and to reveal links between different protein structures in cells [11].

A multi-detector setup in combination with an older SPC-402 FIFO mode card has been used to monitor fluorescence quenching due to conformational changes of the citrate carrier CitS labelled by Alexa 546/568 [35]. Identification of single molecules by detecting the fluorescence in four wavelength intervals simultaneously was shown in [15]. Using time and wavelength information of the detected photons improved the identification efficiency dramatically.

The same principle shown in fig. 14, left, can be used for anisotropy measurements. A polarising beamsplitter is then used instead of the dichroic.

A complete fluorescence spectrum can be resolved by a PML-16 16-channel detector head [22] attached to the output of a polychromator (fig. 14, right).

Two-Photon FCS

The small sample volume required for typical FCS experiments can also achieved by two-photon excitation [7]. A femtosecond Ti:Sa laser is used to excite the sample. Because there is no appreciable excitation outside the focal plane of the microscope lens a small sample volume is achieved without a pinhole. This makes the optical setup very simple. Due to the high power density in the focus of a high numerical aperture microscope lens and the short pulse width of the Ti:Sa laser two-photon excitation works with remarkable efficiency. However, blocking the extremely intense excitation light from the detector requires good

filters in front of the detector [21]. Moreover, higher-order effects like three photon absorption and excited state absorption can be a source for increased photobleaching in the sample.

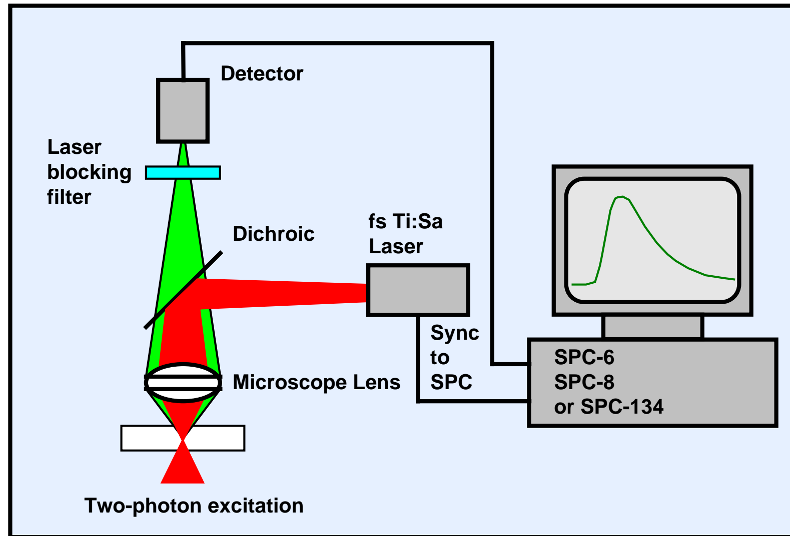


Fig. 14: Two-photon FCS / lifetime experiment

Of course, two photon excitation can used also in conjunction with TCSPC multi-detector operation. Multi-colour FCS / lifetime experiments and polarisation measurements can be built up in the same way as described for one-photon excitation.

Combination of Correlation Techniques

The correlation techniques described above use different approaches for antibunching and FCS experiments. FCS recording is based on 'time-tagging' the recorded photons, anti-bunching is based on recording the times between photon pairs. Although the same TCSPC modules can be used for these experiments, antibunching and FCS data are not obtained simultaneously. Several ways to combine the techniques are shown below.

Start-Stop Experiment in Time-Tag Mode

A combination of the start-stop experiment with time-tag recording is shown in fig. 15.

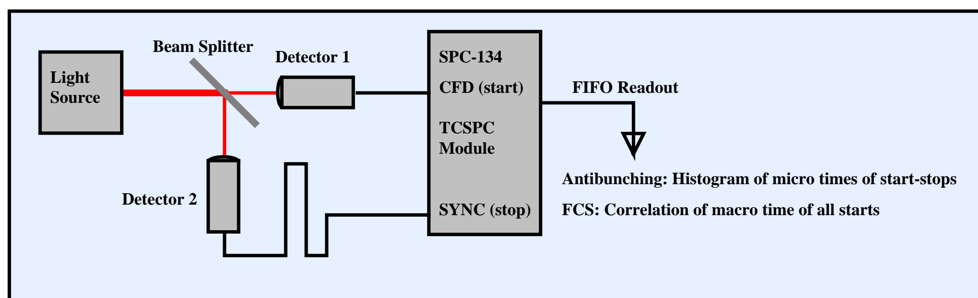


Fig. 15 Start-stop experiment in FIFO (time-tag) mode

As in the classic antibunching experiment, the light is by split in two almost equal parts which are detected in separate detectors. One detector is used for starting, the other for stopping the time measurement in the TCSPC module. The start-stop events are recorded in the FIFO or time-tag mode, i.e. the start-stop time (micro time) and the time from the start of the experiment (macro time) are recorded for each photon pair individually. The anti-bunching

curve is obtained by calculating a histogram of the micro times, the FCS curve by correlating the macro times.

Unfortunately this approach has a flaw: Most of the photons emitted by the sample cause either a start without a stop, or a stop without a start. Only a small fraction of the photons cause complete start-stop events and are recorded. The low efficiency makes the FCS recording practically useless.

However, if not only complete start-stop events are recorded but also start-only events, the setup works with reasonable efficiency. Exactly this happens in the TCSPC channels of the SPC-134 package. Different from the other SPC modules, which read the macro timer with the stop pulse, the SPC-134 reads the macro timer with the start. Therefore a start-only event initiates a normal timing and recording sequence, which, because there is no stop, does not deliver a reasonable micro time. The entry in the FIFO contains a correct macro time but no micro time and is marked by an 'invalid' bit. By using only 'valid' entries for the antibunching histogram and all entries for the FCS calculation correct results are obtained.

Synchronisation of TCSPC modules

A dual TCSPC setup with two synchronised, fully parallel TCSPC modules is shown in fig. 16.

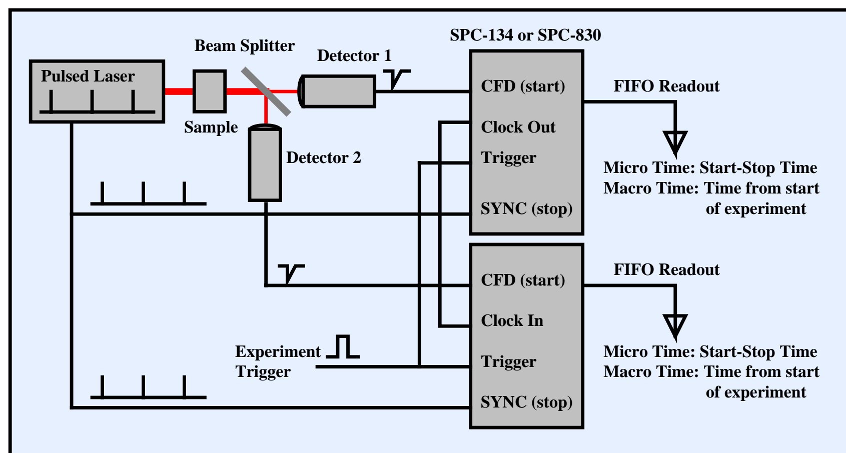


Fig. 16 Correlation setup with synchronised TCSPC modules

The events detected in the two detectors are processed in separate TCSPC modules. The start pulses of the TCSPC modules come from the detectors, the stop pulses from the pulsed excitation source, or, if a CW laser is used, from an external clock signal source.

The modules work in the FIFO (time tag) mode and deliver the micro times, the macro times, and, if several detectors are connected to each module, the detector channel number for each photon. In principle, the micro and macro times of the photons detected in the detectors of different modules can be correlated by analysing the FIFO data. To make the detection times comparable in all modules, the internal macro time clocks of the modules must be synchronised, and the recording must be started simultaneously in all modules. In the SPC-134 or in a multi-SPC-830 system this can, in principle, be achieved by a clock interconnection between the modules and applying a start pulse to the experiment trigger input.

The problem of the inter-module correlation is that the macro time clocks in different modules are phase-shifted so that photons detected simultaneously can be recorded one macro time period earlier or later. Moreover, the micro time scales of the modules may be slightly

different, and the micro times may be shifted due to different transit times in the detectors, cables and TCSPC modules. Correcting all these effects is extremely difficult.

Routing of Delayed Detector Signals

Normally the TCSPC multi-detector or 'routing' technique relies on the fact that the detection of several photons in different detectors in a short time interval is unlikely so that such events can be ignored. The ignoring of multi-photon events is no problem for normal lifetime measurements and in fact helps to reduce pile-up effects at low pulse repetition rates [17]. However, signals from detectors connected to one TCSPC module via the same router cannot be correlated at a time scale shorter than the dead time of the TCSPC module. A way to avoid this problem is shown in [33]. The principle is shown in fig. 17.

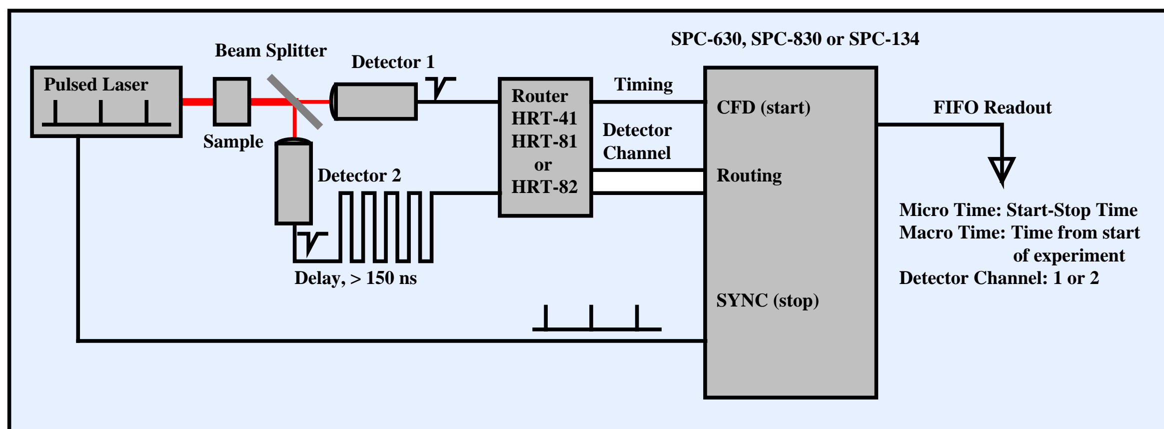


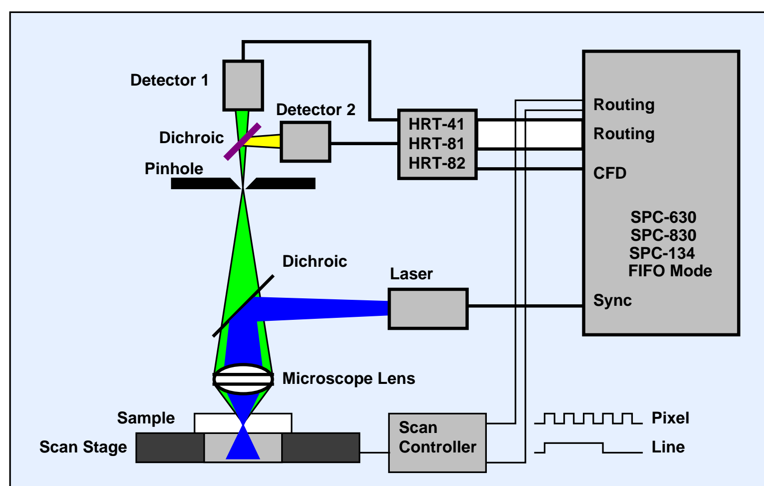
Fig. 17: Routing of delayed detector signals

Several detectors are connected via a router to the same TCSPC module. The photon pulses from the second detector are delayed by more than the dead time of the TCSPC module. More than two detectors can be used if their delay lines are different by more than the module dead time. The stop pulses for the SPC module come from the pulsed laser, or, if a CW laser is used, from an external clock generator. Due to the different delay of the detector signals photons detected simultaneously do not arrive simultaneously at the router inputs. Therefore, photons detected in the same laser pulse period are recorded and stored in the FIFO data file. The differences in the macro times caused by the delay lines in front of the router are known and can easily be corrected when the photons are correlated.

The setup was successfully used to track the fluorescence lifetime, the number of molecules in the laser focus, and the intensity fluctuations simultaneously [33].

Scanning FCS

A frequently asked question is how FCS data recording can be combined with scanning a sample. It should be noted here that it is generally impossible to scan a sample at a pixel rate of the order of the photon times to be correlated. FCS is therefore incompatible with the pixel dwell times used in laser scanning microscopes. However, if a sample is scanned at sufficiently low speed, FCS images - or at least line scans - can be obtained. A solution is shown in the figure below.



Combination of FCS with slow scanning

A scan stage is used to scan the sample in one or two directions. The scan controller delivers two control signals - a 'pixel' signal that changes its state at the transition from one pixel to the next, and a 'line' signal that changes its state at the transition from one line to the next. These signals are fed into two of the unused routing input bits of the SPC module. The scan rate is made slow enough to get a full FCS recording in each pixel. This requires a time from 100 ms to several seconds. In this time a large number of photons are recorded so that all transitions of the 'pixel' and 'line' signals appear in the data stream. Therefore, the scanning action can be tracked by analysing the state of the pixel and line signals in the data file, and separate FCS functions for the individual pixels can be calculated.

Of course, the described procedure can also be used in a commercial scanning microscope if a sufficiently slow scan speed can be selected. The scan control pulses (line clock and pixel clock) must be divided by two, which can be easily accomplished by a single SN 74 HCT74 device.

Detectors for Correlation Experiments

Detectors for photon correlation experiments and FCS are usually selected for maximum detection efficiency. In the visible and NIR range the highest efficiency is achieved by single-photon avalanche photodiodes (SPADs). These diodes are operated above the breakdown voltage [36-38]. If a photon is detected, it releases an avalanche breakdown which can easily be detected by a fast discriminator. Although the principle looks simple the practical realisation as anything but simple. The diode temperature and diode voltage must be held stable with high accuracy, and the normal operation after an avalanche breakdown must be restored by an active quenching circuit. At present the only commercially available SPAD detector is the SPCM-AQR module of Perkin Elmer [39]. Unfortunately this module is not designed for time-correlated single photon counting. We found that the delay from the photon detection to the output pulse depends on the count rate and can vary by almost 1 ns, particularly for newer devices [18]. The TCSPC instrument response function (IRF) has a width of 0.5 to more than 1 ns. Moreover, the width of the response depends on the wavelength. Although the SPCM-AQR is clearly the best detector for FCS it cannot be recommended for photon correlation experiments with ns and ps resolution or for combined FCS / lifetime experiments. Lifetime and correlation time results obtained with these modules

must be suspected to be impaired by detector effects, particularly if an dependence of the lifetime or correlation time on the intensity or burst size is found.

Compared to the APD modules, PMTs have a lower quantum efficiency but a shorter, stable, and almost wavelength-independent TCSPC response [18]. Unfortunately most PMTs are plagued by afterpulsing, i.e. after the detection of a photon the dark count probability is increased for a few 100 ns to some µs [18]. The reason is believed to be ion feedback in the tube. Afterpulsing is particularly unpleasant in FCS experiments because it happens in the same time range as the shortest FCS phenomena. Interestingly, the Hamamatsu R3809U MCP [40] and one Hamamatsu R5900-L16 multi-anode PMT were found to be free of afterpulsing [18]. With its 25 to 30 ps TCSPC instrument response width the R3809U is the fastest detector currently available. The R5900-L16 is used in the bh PML-16 multichannel PMT head [22] and yields between 150 and 220 ps instrument response width. At least these two detectors can be expected to work for FCS, photon correlation, and fluorescence lifetime experiments as well.

Both the R3809U and the R5900-L16 have conventional bialkali and multialkali cathodes with a peak quantum efficiency around 20 %. However, Hamamatsu is gradually introducing the new GaAsP and GaAs cathodes. The new cathode materials have a quantum efficiency up to 40 %.

A ready-to-use solution is the H7422 photosensor module [33] that is already available with the new cathodes. The efficiency of the H7422-40 was found comparable to the SPCM-AQR at 530 nm. Between 400 and 500 nm it is clearly higher than for the SPCM-AQR. Although the H7422 shows some afterpulsing, it appears applicable for FCS if it is operated at reduced gain [18]. The width of the TCSPC instrument response function is 300 to 350 ps fwhm and remains stable for count rates exceeding 10^6 /s. The H7422-40 is currently the best detector for antibunching experiments and combined FCS / lifetime experiments below 530 nm.

Summary

Advanced TCSPC techniques are available for conventional dual-detector photon correlation experiments, for multi-detector correlation experiments and for fluorescence correlation spectroscopy. Moreover, the picosecond time resolution and the multi-detector capability of TCSPC can be used for combined FCS / lifetime experiments and for multi-colour FCS combined with fluorescence lifetime and fluorescence anisotropy. Main applications are light emission by quantum dots, single molecule detection and identification, diffusion processes in cells, localisation of cell subunits marked with different dyes, conformational changes of protein / dye complexes, protein folding, and single molecule FRET. The bottleneck of the new techniques is the lack of detectors featuring high efficiency in the red and NIR region, stable and short transit time spread, and low afterpulse probability.

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