Technical Note

Time-Correlated Single Photon Counting

Michael Wahl, PicoQuant GmbH

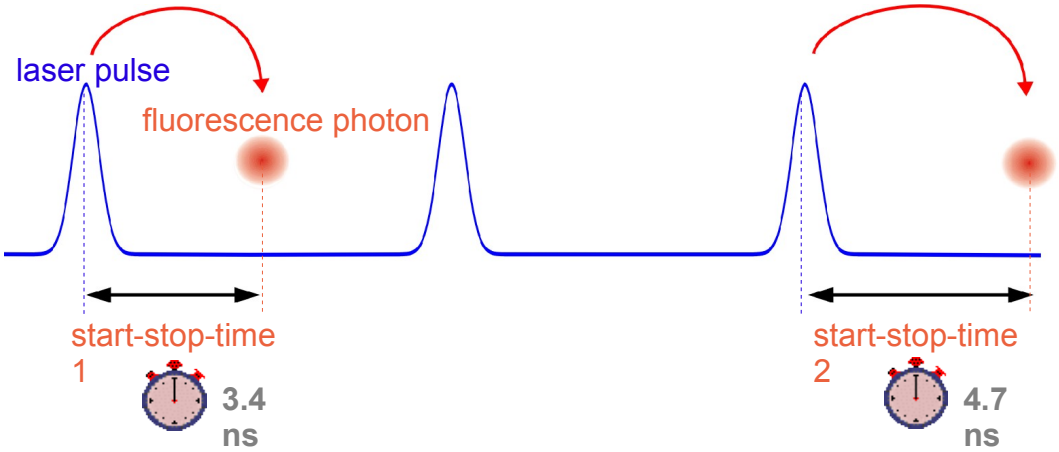
The Principle of Time-Correlated Single Photon Counting

Time-resolved fluorescence spectroscopy is a powerful analysis tool in fundamental physics as well as in the life sciences. Implementing it in the time domain requires recording the time dependent intensity profile of the emitted light upon excitation by a short flash of light, typically a laser pulse. While in principle, one could attempt to record the time decay profile of the signal from a single excitation-emission cycle, there are practical problems preventing such a simple solution in most cases. First of all, the decay to be recorded is very fast. Typical fluorescence from important organic fluorophores lasts only some hundred picoseconds to some tens of nanoseconds. In order to recover not only fluorescence lifetimes but also the decay shape, one must be able to resolve the recorded signal at least to such an extent, that the decay is represented by some tens of samples. For a decay of, e.g., 500 ps this means the transient recorder required would have to sample at e.g. 50 ps time steps.

This is hard to achieve with ordinary electronic transient recorders. Moreover, the light available may be simply too weak to sample an analog time decay. Indeed the signal may consist of just a few photons per excitation /emission cycle. Then the discrete nature of the signal itself prohibits analog sampling. Even if one has some reserve to increase the excitation power to obtain more fluorescence light, there will be limits, e.g. due to collection optic losses, spectral limits of detector sensitivity or photo-bleaching at higher excitation power. The solution for both problems is Time-Correlated Single Photon Counting (TCSPC). With periodic excitation (e.g. from a laser) it is possible to extend the data collection over multiple cycles and one can reconstruct the single cycle decay profile from single photon events collected over many cycles.

The method is based on the repetitive precisely timed registration of single photons of e.g. a fluorescence signal [1,2]. The reference for the timing is the corresponding excitation pulse. As a single photon sensitive detector a Photomultiplier Tube (PMT), Micro Channel Plate (MCP) or a Single Photon Avalanche Diode (SPAD) can be used. Provided that the probability of registering more than one photon per cycle is low, the histogram of photon arrivals per time bin represents the time decay one would have obtained from a “single shot” time-resolved analog recording. The precondition of single photon probability can (and must) be met by simply attenuating the light level at the sample if necessary.

The following diagrams illustrate how the histogram is formed over multiple cycles. In the example, fluorescence is excited by laser pulses. The time difference between excitation and emission is measured by electronics that act like a stopwatch. If the single photon probability condition is met, there will actually be no photons at all in many cycles. In the example this situation is shown after the second laser pulse. It should be noted that the occurrence of a photon or an empty cycle is entirely random and can only be described in terms of probabilities. Indeed, the same holds true for the individual stopwatch readings.



The histogram is collected in a block of memory, where one memory cell holds the photon counts for one corresponding time bin. These time bins are often referred to as time channels. The typical result is a histogram with an exponential drop of counts towards later times.

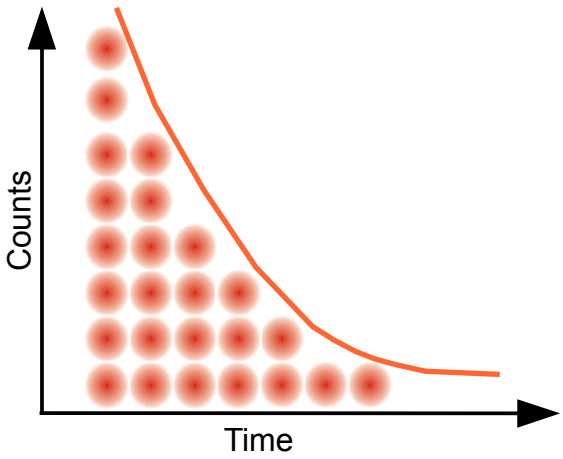


Fig. 2: Fluorescence lifetime histogram: exponential decay

In practice the registration of one photon involves the following steps: first the time difference between the photon event and the corresponding excitation pulse must be measured. For this purpose both events are converted to electrical signals. For the fluorescence photon this is done via the single photon detector mentioned before. For the excitation pulse it may be done via another detector if there is no electrical synchronisation signal (sync) supplied by the laser. Obviously, all conversion to electrical pulses must preserve the precise timing of the signals as accurately as possible.

The actual time difference measurement is done by means of fast electronics which provide a digital timing result. This digital timing result is then used to address the histogram memory so that each possible timing value corresponds to one memory cell or histogram bin. Finally the addressed histogram cell is incremented. All steps are carried out by fast electronics so that the processing time required for each photon event is as short as possible. When sufficient counts have been collected the histogram memory can be read out. The histogram data can then be used for display and e.g. fluorescence lifetime calculation. In the following sections we will expand on the various steps involved in the method and associated issues of importance.

Count rates and single photon statistics

It was mentioned that it is necessary to maintain a low probability of registering more than one photon per cycle. This is to guarantee that the histogram of photon arrivals represents the time decay one would have obtained from a single shot time-resolved analog recording. The reason for this is briefly the following: Detector and electronics have a “dead” time for at least some nanoseconds after a photon event. Because of these dead times TCSPC systems are usually designed to register only one photon per excitation cycle. If now the number of photons occurring in one excitation cycle were typically >1, the system would very often register the first photon but miss the following ones. This would lead to an over-representation of early photons in the histogram, an effect called ‘pile-up’. It is therefore crucial to keep the probability of cycles with more than one photon low.

To quantify this demand one has to set acceptable error limits for the lifetime measurement and apply some statistics. For practical purposes one may use the following rule of thumb: In order to maintain single photon statistics, on average only one in 20..100 excitation pulses should generate a count at the detector. In other words: the average count rate at the detector should be at most 1..5% of the excitation rate. E.g. with the diode laser PDL 800-B, pulsed at 80 MHz repetition rate, the average detector count rate should not exceed 4 MHz. This leads to another issue: the count rate the system (of both detector and electronics) can handle. Indeed 4 MHz are stretching the limits of many detectors and certainly are way beyond the capabilities of most conventional NIM based TCSPC systems. On the other hand, one wants high count rates, in order to acquire fluorescence decay histograms quickly. This may be of particular importance where dynamic lifetime changes or fast molecule transitions are to be studied or where large numbers of lifetime samples must be collected (e.g. in 2D scanning configurations). PMTs (dependent on the design) can handle count rates of up to 1..10 Millions of counts per second (cps), standard (passively quenched) SPADs saturate at a few hundred kcps. Oldfashioned NIM based TCSPC electronics can handle a maximum of 50,000 to 500,000 cps. With modern integrated TCSPC designs count rates over 10 Mcps can be achieved. It is also worth noting that the actual count arrival times of course are random so that there can be bursts of high count rate and periods of low count rates. Bursts of photons may still exceed the average rate. This should be kept in mind when an experiment is planned. Even if an instrument can accommodate the average rate, it may drop photons in bursts. This is why the length of the dead-time is of interest too. This quantity describes the time the system cannot register photons while it is processing a previous photon event. The term is applicable to both detectors and electronics. Dead-time or insufficient throughput of the electronics are usually not of detrimental effect on the decay histogram or, more precisely, the lifetime to be extracted from the latter. However, the photon losses prolong the acquisition time or deteriorate the SNR if the acquisition time remains fixed. In

applications where the photon burst density must be evaluated (e.g. for molecule transition detection or imaging) long dead-times can be a problem.

Timing resolution

The characteristic of a complete TCSPC system that summarizes its overall timing precision is its Instrument Response Function (IRF). The basic idea is that if the system is ideal, i.e. has an infinitely sharp excitation pulse and infinitely accurate detectors and electronics, it should have an infinitely narrow IRF. Any deviation from this ideal results in a broadening of the IRF. Before looking into how the individual error contributions add up, the most critical sources shall be introduced here.

The weakest component in terms of timing resolution in TCSPC measurements will usually be the detector. However, as opposed to analog transient recording, the time resolution of TCSPC is not limited by the pulse response of the detector. Only the timing accuracy of registering a photon determines the TCSPC resolution. The timing accuracy is limited by the timing uncertainty the detector introduces in the conversion from a photon to an electrical pulse. This timing error (or uncertainty) can be as much as 10 times smaller than the detectors pulse response. The timing uncertainties are usually quantified by specifying the r.m.s. error or the Full Width Half Maximum (FWHM) of the timing error distribution. Note that these two notations are related but not identical. In the case of a Gaussian error distribution the FWHM value is twice as large as the corresponding r.m.s. value. Good but also expensive detectors, notably MCPs, can achieve timing uncertainties as small as 25 ps FWHM. Cheaper PMTs or SPADs may introduce uncertainties of 200 to 400 ps FWHM. More recent SPADs can show timing uncertainties as small as 30 ps FWHM.

The second most critical source of IRF broadening usually is the excitation source. While most laser sources can provide sufficiently short pulses, it is also necessary to obtain an electrical timing reference signal (sync) to compare the fluorescence photon signal with. Where this signal is derived from depends on the excitation source. With gain switched diode lasers (e.g. PDL 800-B) a low jitter electrical sync signal is readily available. The signal type used here is commonly a narrow negative pulse of -800 mV into 50 Ohms (NIM standard). The very sharp falling edge is synchronous with the laser pulse (<3 ps r.m.s. jitter for the PDL 800-B). With other lasers (e.g. many Ti:Sa lasers) a second detector must be used to derive a sync signal from the optical pulse train. This is commonly done with a fast photo diode (APD or PIN diode, e.g. the TDA 200). The light for this reference detector must be coupled out from the excitation laser beam e.g. by means of some semi-transparent mirror. The reference detector must be chosen and set up carefully as it also contributes to the overall timing error.

Another source of timing error is the timing jitter of the electronic components used for TCSPC. This is caused by the finite rise/fall-time of the electric signals used for the time measurement. At the trigger point of e.g. compactors, logic gates etc. the amplitude noise (thermal noise, interference etc.) always present on these signals is transformed to a corresponding timing error (phase noise). However the contribution of the electronics to the total timing error usually is relatively small. Modern TCSPC electronics cause an r.m.s. jitter of <10 ps. Nevertheless it is always a good idea to keep the RF noise low. This is why signal leads should be properly shielded coax cables and strong sources of RF interference should be kept away from the TCSPC detector and electronics.

The contribution of the time spread introduced by the individual components of a TCSPC system to the total IRF width strongly depends on their relative magnitude. Strictly, the total IRF is the convolution of all component IRFs. An estimate of the overall IRF width can be obtained from the geometric sum of the individual components e.g. as r.m.s. error or FWHM (Full Width Half Maximum) values according to statistical error propagation laws:

$$e_{IRF\,system} \approx \sqrt{\sum e^2_{component}} \qquad (1)$$

Obviously, due to the squares in the sum, the total will be more than proportionally dominated by the largest component. It is therefore of little value to improve a component that is already relatively good. If e.g. the detector has an IRF width of 200 ps, shortening of the laser pulse from 50 ps to 40 ps is practically of no effect.

Apart from predicting the approximate IRF width according to Eqn. 1 one can of course measure it. The typical approach is to place a scattering medium in the sample compartment so that there is no fluorescence but only some scattered excitation light reaching the detector. The IRF measurement is not only a means of optimizing and testing the instrument. It also serves as an input to data analysis with „deconvolution“ and is therefore a frequent measurement task. It was mentioned earlier that the total IRF is the convolution of all component IRFs. Similarly, the measured fluorescence decay is the convolution of the „true“ physical process of exponential decay with the IRF. With this theoretical model it is possible to extract the parameters of the „true“ decay process [3]. This is often referred to as „deconvolution“ although it should be noted that the term is not mathematically precise in this context. The procedure that most data anaysis programs actually perform is an iterative reconvolution.

Having established the role of the IRF and possibly having determined it for a given instrument leads to the question what the actual lifetime measurement resolution of the instrument will be. Unfortunately it is difficult to specify a general lower limit on the fluorescence lifetime that can be measured by a given TCSPC instrument. Apart from the instrument response function and noise, factors such as quantum yield, fluorophore concentration, and decay kinetics will affect the measurement. However, as a rule of thumb one can assume that under favourable conditions, most importatntly sufficient counts in the histogram, lifetimes down to 1/10 of the IRF width (FWHM) can still be recovered via iterative reconvolution.

A final time-resolution related issue worth noting here is the bin width of the TCSPC histogram. As outlined above, the analog electronic processing of the timing signals (detector, amplifiers, etc.) creates a continuous (e.g. Gaussian) distribution around the true value. In order to form a histogram, at some point the timing results must be quantised into discrete time bins. This quantisation introduces another random error that can be detrimental if chosen too coarse. The time quantisation step width (i.e. the bin width) must therefore be small compared to the width of the analog error distribution. As a minimum from the information theoretical point of view one would assume the Nyquist frequency. I.e. an analog signal should be sampled at least at twice the highest frequency contained in it. The high frequency content depends on the shape of the distribution. In a basic approximation this can reasonably be assumed to be Gaussian and therefore having very little high frequency content. For practical purposes there is usually no point in collecting the histogram at time resolutions much higher than 1/10 of the width of the analog error distribution. Nevertheless, a good histogram resolution is helpful in data analysis with iterative reconvolution.

Photon counting detectors

Photomultiplier Tube (PMT)

A PMT consists of a light-sensitive photocathode that generates electrons when exposed to light. These electrons are directed onto a charged electrode called a dynode. The collision of the electrons with the dynode produces additional electrons. Since each electron that strikes the dynode causes several electrons to be emitted, there is a multiplication effect. After further amplification by multiple dynodes, the electrons are collected at the anode of the PMT and output as a current. The current is directly proportional to the intensity of light striking the photocathode. Because of the multiplicative effect of the dynode chain, the PMT is a photoelectron amplifier of high sensitivity and remarkably low noise. PMTs have a wide dynamic range, i.e. they can also measure relatively high levels of light. They furthermore are very fast, so rapid successive events can be reliably monitored. PMTs are also quite robust. The high voltage driving the tube may be varied to change the sensitivity of the PMT.

When the light levels are as low as in TCSPC the PMT „sees“ only individual photons. One photon on the photocathode produce a short output pulse containing millions of photoelectrons. PMTs can therefore be used as single photon detectors. In photon counting mode, individual photons that strike the photocathode of the PMT are registered. Each photon event gives rise to an electrical pulse at the output. The number of pulses, or counts per second, is proportional to the light impinging upon the PMT. As the number of photon events increase at higher light levels, it will become difficult to differentiate between individual pulses and the photon counting detector will become non-linear. Dependent on the PMT design this usually occurs at 1-10 millions of counts per second.

The timing uncertainty between photon arrival and electrical output is small enough to permit time-resolved photon counting at a sub-nanosecond scale. In single photon counting mode the tube is typically operated at a constant high voltage where the PMT is most sensitive.

PMTs usually operate between the blue and red regions of the visible spectrum, with greater quantum efficiency in the blue-green region, depending upon photo-cathode materials. Typical peak quantum efficiencies are about 25%. For spectroscopy experiments in the ultraviolet and visible region of the spectrum, a photomultiplier tube is very well suited. In the near infrared the sensitivity drops off rapidly. Optimized cathode materials can be used to push this limit, which may on the other hand lead to increased noise. The latter can to some extent be reduced by cooling.

Because of noise from various sources in the tube, the output of the PMT may contain pulses that are not related to the light input. These are referred to as dark counts. The detection system can to some extent reject these spurious pulses by means of electronic discriminator circuitry. This discrimination is based on the probability that some of the noise generated pulses (those from the dynodes) exhibit lower signal levels than pulses from a photon event.

Microchannel Plate PMT (MCP)

A microchannel plate PMT is also a sensitive photon detector. It consists of an array of glass capillaries (10-25 µm inner diameter) that are coated on the inside with a electron-emissive material. The capillaries are biased at a high voltage applied across their length. Like in the PMT, an electron that strikes the inside wall of one of the capillaries creates an avalanche of secondary electrons. This cascading effect creates a gain of 103 to 106 and produces a current pulse

at the output. Due to the confined paths the timing jitter of MCPs is sufficiently small to perform time-resolved photon counting on a sub-nanosecond-scale, usually outperforming PMTs. Good but also expensive MCPs can achieve timing uncertainties as low as 25 ps. Microchannel plates are also used as an intensifier for low-intensity light detection with array detectors.

Avalanche Photo Diode (APD)

APDs are the semiconductor equivalent of PMTs. Generally, APDs may be used for ultra-low light detection (optical powers <1 pW), and can be used in either "linear" mode (bias voltage slightly less than the breakdown voltage) at gains up to about 500, or as photon-counters in the so-called "Geiger" mode (biased slightly above the breakdown voltage). In the case of the latter, the term gain is meaningless. A single photon may trigger an avalanche of about 108 carriers but one is not interested in the output current or voltage because it carries no information other than „there was a photon“. Instead, in this mode the device can be used as a detector for photon counting with very accurate timing of the photon arrival. In this context APDs are referred to as Single Photon Avalanche Diodes (SPAD). Widespread commercial products attain timing accuracies on the order of 400 ps FWHM. Single-photon detection probabilities of up to approximately 50% are possible. Maximum quantum efficiencies reported are about 80%. More recent SPAD designs focus on timing resolution and can achieve timing accuracies down to 30 ps but are less sensitive at the red end of the spectrum. The dark count rate (noise) of SPADs strongly depends on the active area. In SPADs it is much smaller than in PMTs, which can make optical interfacing difficult.

Other and novel detectors

The field of photon detectors is still evolving. Recent developments that are beginning to emerge as usable products include so called silicon PMTs, Hybrid PMTs, superconducting nanowire detectors and APDs with sufficient gain for single photon detection in analog mode. Each of these detectors have their specific benefits and shortcomings. Only a very brief overview can be given here. Silicon PMTs are essentially arrays of SPADs, all coupled to a common output. This has the benefit of creating a large area detector that can even resolve photon numbers. The drawback is increased dark count rate and relatively poor timing accuracy. Hybrid PMTs make use of a combination of a PMT front end followed by an APD structure. The benefits are good timing and virtually zero afterpulsing while the need for very high voltage is a disadvantage. Superconducting nanowires (typically made from NbN) can be used to create photon detectors with excellent timing performance and high sensitivity reaching into the infrared. The shortcomings for practical purposes are the extreme cooling requirements and the low fill factor of the wire structures, making it difficult to achieve good collection efficiencies. Another class of potentially interesting detectors are recently emerging APDs with very high gain. In combination with a matched electronic amplifier they have been shown to detect single photons. As opposed to Geiger mode, this avoids afterpulsing and allows very fast counting rates. The disadvantage is a high dark count rate, currently way too high for any practical TCSPC application.

Basic principles behind the TCSPC electronics

Conventional TCSPC systems consist of the following building blocks (Fig. 3):

The CFD is used to extract precise timing information from the electrical detector pulses that may vary in amplitude. This way the overall system IRF may be tuned to become narrower. The same could not be achieved with a simple threshold detector (comparator). Particularly with PMTs, constant fraction discrimination is very important as their pulse amplitudes vary significantly. The figures 4 and 5 show a comparison between level trigger and CFD operation.

The most common way of implementing a CFD is the comparison of the original detector signal with an amplified and delayed version of itself. The signal derived from this comparison changes its polarity exactly when a constant fraction of the detector pulse height is reached. The zero crossing point of this signal is therefore suitable to derive a timing signal independent from the amplitude of the input pulse. This is done by a subsequent comparison of this signal with a settable zero level, the so called zero cross trigger. Making this level settable allows to adapt to the noise

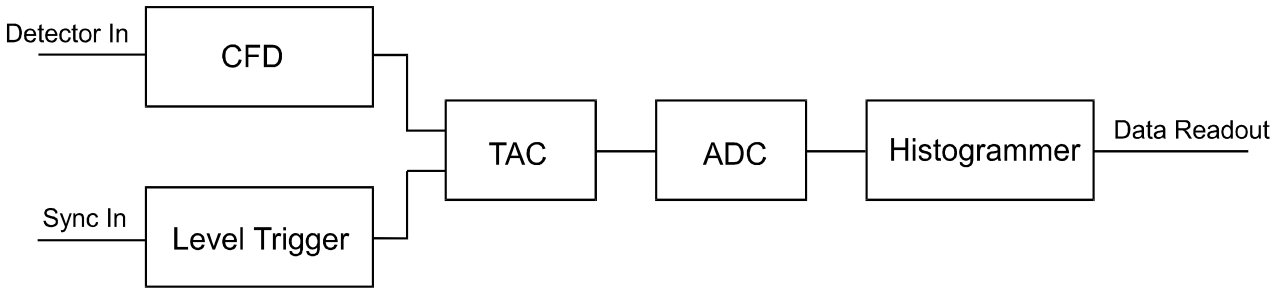


Fig. 3

levels in the given signal, since in principle an infinitely small signal could trigger the zero cross comparator. Typical CFDs furthermore permit the setting of a so called discriminator level, determining the lower limit the detector pulse amplitude must pass. Random background noise pulses can thereby be suppressed. Particularly pulses originating from random electrons generated at the dynodes of the PMT can be suppressed as they had less time to amplify, so that their output pulses are small.

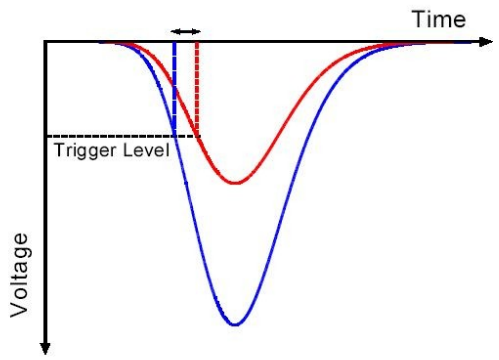


Fig. 4: Constant level trigger

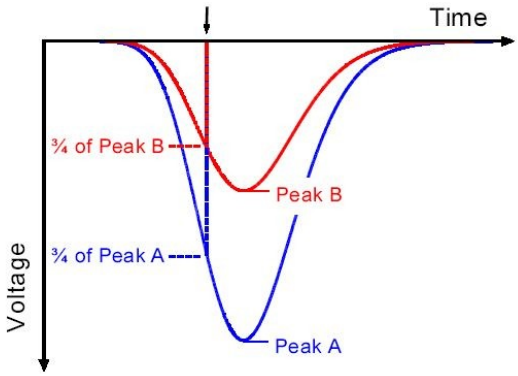


Fig. 5: Constant fraction trigger

Similar as for the detector signal, the sync signal must be made available to the timing circuitry. Since the sync pulses are usually of well-defined amplitude and shape, a simple settable comparator (level trigger) is sufficient to adapt to different sync sources.

In the classical design the signals from the CFD and SYNC trigger are fed to a Time to Amplitude Converter (TAC). This circuit is essentially a highly linear ramp generator that is started by one signal and stopped by the other. The result is a voltage proportional to the time difference between the two signals.

The voltage obtained from the TAC is then fed to an Analog to Digital Converter (ADC) which provides the digital timing value used to address the histogrammer. The ADC must be very fast in order to keep the dead time of the system short. Furthermore it must guarantee a very good linearity, over the full range as well as differentially. These are criteria difficult to meet simultaneously, particularly with ADCs of high resolution (e.g. 12 bits) as is desirable for TCSPC over many histogram channels. Furthermore, the TAC range is very limited.

The histogrammer has to increment each histogram memory cell whose digital address in the histogram memory it receives from the ADC. This is commonly done by fast digital logic e.g. in the form of Field Programmable Gate Arrays (FPGA) or a microprocessor. Since the histogram memory at some point also must be available for data readout, the histogrammer must stop processing incoming data. This prevents continuous data collection. Sophisticated TCSPC systems solve this problem by switching between two or more memory blocks, so that one is always available for incoming data.

While this section so far outlined the typical structure of conventional TCSPC systems, it is worth noting that the tasks performed by TAC and ADC can be carried out by a single fully digital circuit, a so called Time to Digital Converter (TDC). These circuits can measure time differences based on the delay times of signals in semiconductor logic gates or the conductor strips between them. The relative delay times in different gate chains can be used to determine time differences well below the actual gate delay. Other TDC designs use interpolation techniques between the pulses of a coarser clock. This permits exceptionally small, compact and affordable TCSPC solutions, as the circuits can be implemented as Application Specific Integrated Circuits (ASICS) at low cost and high reliability. All PicoQuant TCSPC systems make use of such a design. The historical starting point of the whole family of TDC based TCSPC systems was the TimeHarp 100 permitting a digital resolution of <40 ps (<150 ps analog FWHM). This resolution was well matched to the excitation pulse widths possible with diode lasers and the resolution permitted by affordable compact PMTs (e.g. IRF 200 ps). Later we introduced the TimeHarp 200 with PCI interface and slightly improved resolution. Figure 6 shows a basic block diagram of the TimeHarp 100/200. The TAC and ADC have been replaced by a TDC.

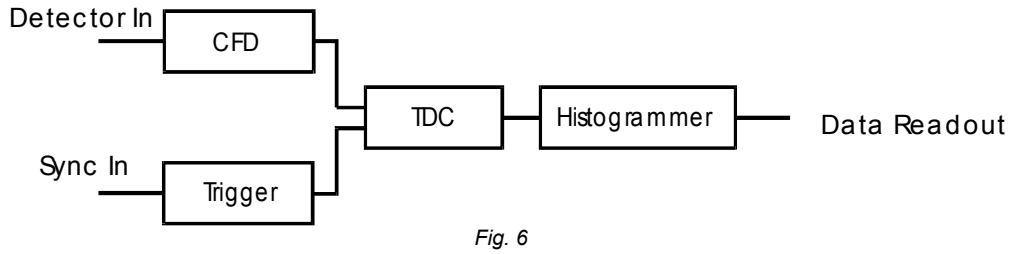


Fig. 6

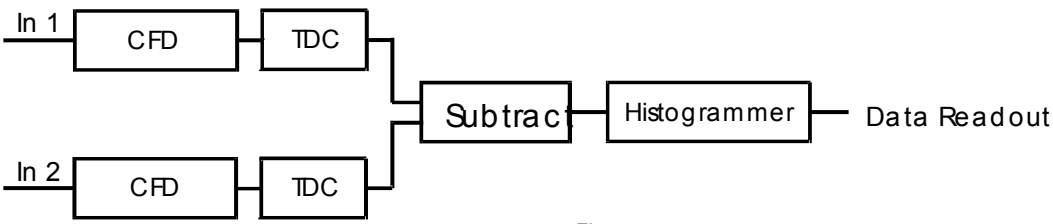


Fig. 7

The next significant steps were the PicoHarp 300 with 4 ps digital resolution and host interfacing via USB and finally the HydraHarp 400 with 1 ps digital resolution and multiple truly parallel channels. These new devices are fundamentally different in design. Instead of operating like a stopwatch, they have independent TDCs for each input channel. If a timing difference is needed (like in classical histogramming) it can be obtained by simple arithmetics in hardware. Figure 7 shows this for the PicoHarp 300 in a block diagram.

Observe the symmetry of the input channels, now both having a CFD. The symmetry as well as the separation of the input channels allows many advanced TCSPC concepts that will be discussed in a separate section further below. For the time being we will first take a look at a TCSPC setup that is fairly independent from the design of the TCSPC electronics used.

Experimental setup for fluorescence decay measurements with TCSPC

Figure 8 shows a typical setup for fluorescence lifetime measurements with TCSPC. The picosecond diode laser (here PDL 800-B) is running on its internal clock (settable at 2.5, 5, 10, 20 or 40 MHz). The driver box is physically separate from the actual laser head, which is attached via a flexible lead. This permits to conveniently place the small laser head anywhere in the optical setup.

The light pulses of typically 50 ps FWHM, are directed at the sample cuvette, possibly via some appropriate optics. A neutral density filter is used to attenuate the light levels to maintain single photon statistics at the detector. Upon excitation, the fluorescent sample will emit light at a longer wavelength than that of the excitation light. The fluorescence light is filtered out against scattered excitation light by means of an optical cut-off filter. Then it is directed to the photon detector, again possibly via some appropriate collection optics, e.g. a microscope objective or just a lens. For timing accuracies of 200 ps FWHM (permitting lifetime measurements even shorter than this via reconvolution) a cheap PMT is sufficient. The electrical signal obtained from the detector (e.g. a small negative pulse of -20 mV) is fed to a pre-amplifier and then to the TCSPC electronics via a standard 50 Ohms coax cable. In this example the complete TCSPC electronics are contained on a single PC-board (TimeHarp 200). Other models are designed as separate boxes connected via USB.

The laser driver also provides the electric sync signal needed for the photon arrival time measurement. This signal (NIM standard, a narrow pulse of -800 mV) is fed to the TCSPC electronics via a standard 50 Ohms coax cable.

Figure 9a shows two fluorescence decay curves obtained with such a simple setup.

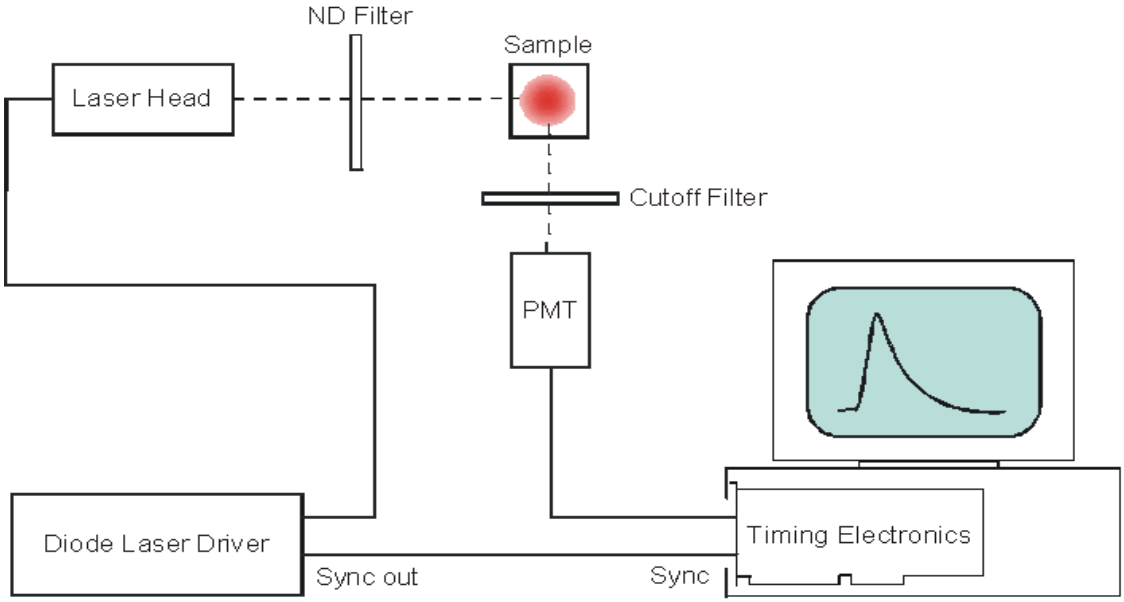


Fig. 8

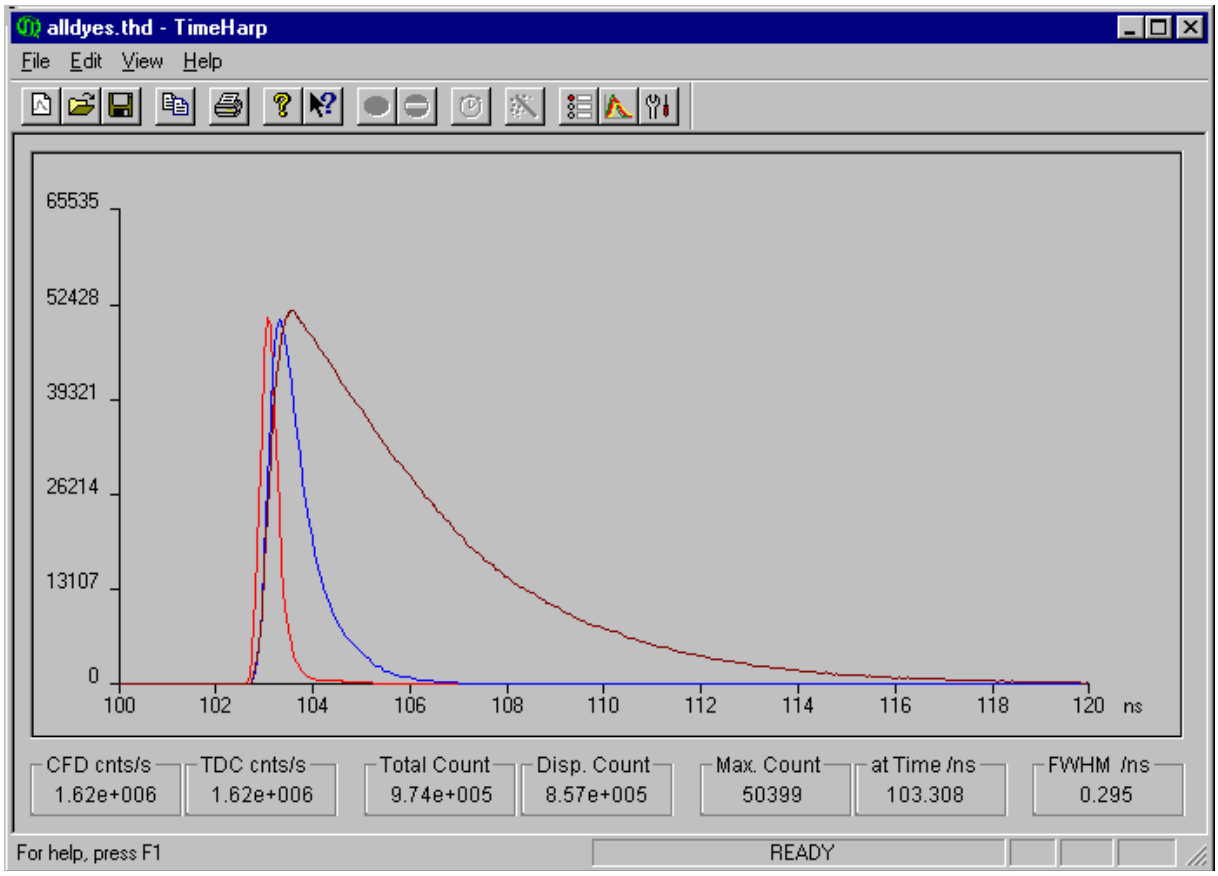

alldyes.thd - TimeHarp
File Edit View Help
65535
52428
39321
26214
13107
0
100 102 104 106 108 110 112 114 116 118 120 ns
CFD cnts/s 1.62e+006
TDC cnts/s 1.62e+006
Total Count 9.74e+005
Disp. Count 8.57e+005
Max. Count 50399
at Time /ns 103.308
FWHM /ns 0.295
For help, press F1
READY

Fig. 9a

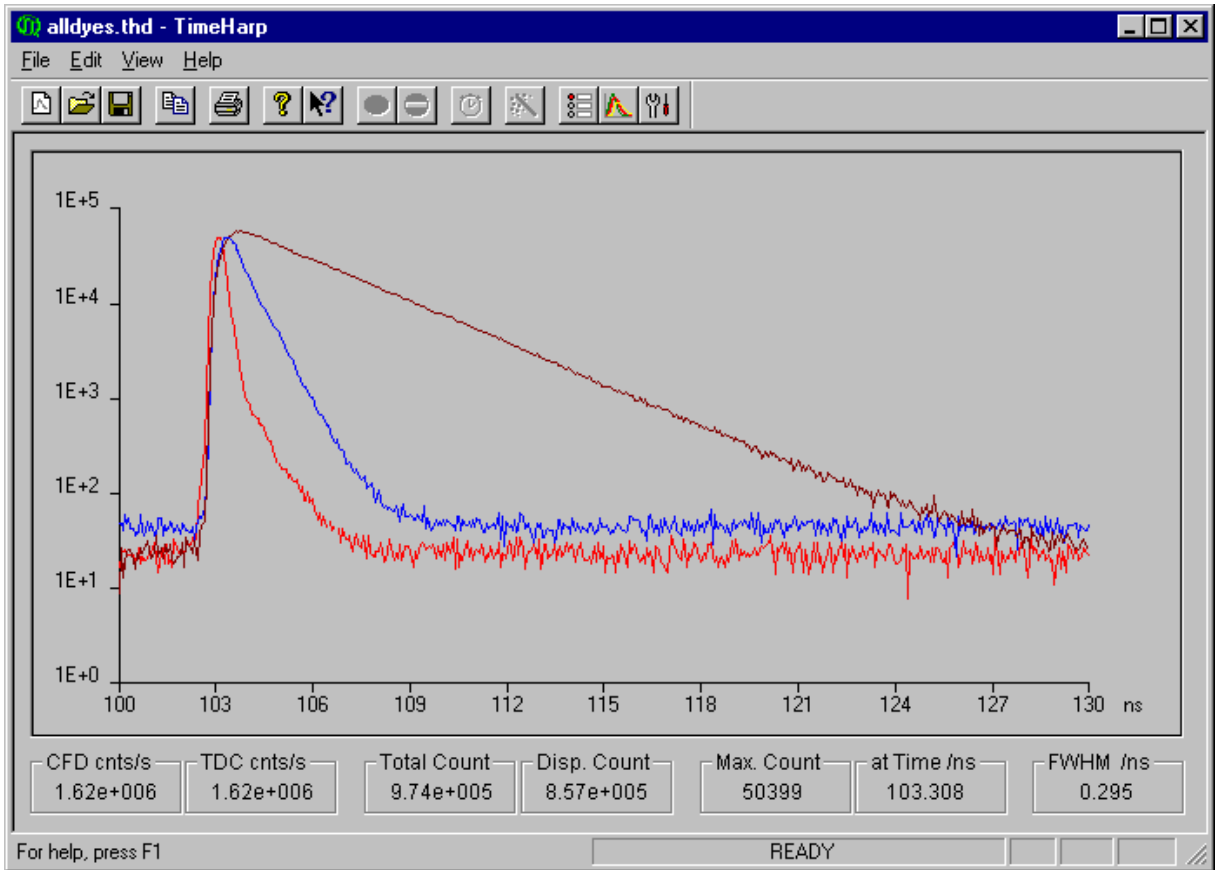

alldyes.thd - TimeHarp
File Edit View Help
1E+5
1E+4
1E+3
1E+2
1E+1
1E+0
100 103 106 109 112 115 118 121 124 127 130 ns
CFD cnts/s 1.62e+006
TDC cnts/s 1.62e+006
Total Count 9.74e+005
Disp. Count 8.57e+005
Max. Count 50399
at Time /ns 103.308
FWHM /ns 0.295
For help, press F1
READY

Fig. 9b

The narrowest curve (red) represents the system IRF, dominated by the detector. The second widest curve (blue) is a fluorescence decay from a solution of Toluidine Blue, a fluorescent dye with relatively short fluorescence lifetime. The widest curve (brown) is from Oxazin 4, another typical fluorescent dye. The excitation source was a PDL 800 at 80 MHz repetition rate.

A second plot in logarithmic scale reveals the nearly perfect exponential nature of the decay curves, as one would expect them (Fig. 9b) Note that this is even without a deconvolution of the relatively broad IRF (300 ps).

The approximate mono-exponential fluorescence lifetime can be obtained from a simple comparison of two points in the exponential display with count rates in the ratio of e:1 (e.g. 27,180:10,000). In this particular experiment this results in a lifetime estimate of 500 ps for Toluidine Blue and 3.1 ns for Oxazin.

For a precise measurement one would perform an iterative reconvolution fit taking into account the IRF. This would result in slightly smaller lifetimes since in this experiment the IRF is nearly as broad as the lifetime to be measured (at least for Toluidine). Nevertheless one can measure lifetimes significantly smaller than the IRF with this method. Additionally the r.m.s. residue from the fit can be used to assess the quality of the fit and thereby the reliability of the lifetime measurement. The FluoFit decay analysis software package from PicoQuant provides this functionality. Of course it is easy to measure long lifetimes with or without reconvolution, since the IRF is of less influence.

Reverse start-stop mode

So far we have always assumed that the time delay measurement should be nicely causal, i.e. the laser pulse causes a photon event and therefore we measure the time delay between laser pulse and subsequent photon event. However, there are practical reasons to give up this convenient concept. The reasons are in the high repetition rates of the typical excitation lasers: Since the time measurement circuit cannot know in advance, whether there will be a fluorescence photon, it would have to start a time measurement upon each laser pulse. Typical conversion times of conventional TCSPC electronics were in the region of 0.5 to 2 µs, and despite significant improvement, they are still 350 ns for the TimeHarp 100/200. Therefore any excitation rate in excess of ~3 MHz would overrun the time measurement circuits. In fact they would most of the time be busy with conversions that never complete, because there is no photon event at all in most cycles. The solution is in the precondition of the single photon counting statistics that must be maintained anyhow. By simply reversing the start and stop signals in the time measurement, the conversion rates are only as high as the actual photon rates generated by the fluorescent sample. These are (and must be) only about 1..5% of the excitation rates and can therefore be handled easily. The consequence of this approach, however, is that the times measured are not those between laser pulse and corresponding photon event but those between photon event and the next laser pulse. This is not too much of a problem, since the laser excitation is periodical and the measured times are directly related to the ones actually needed. As simple as this may sound, there may be yet more problems if the excitation period is very long. This may indeed be of practical relevance, e.g. with flash lamps (<100 kHz). The reverse Start-Stop Principle as explained so far would lead to time delays as long as e.g. 10 ms for 100 kHz excitation rate. These delays are much too long to be measured by conventional TCSPC electronics based on TAC/ADC while the region of interest in this time span (i.e. the actual fluorescence decay) is as short as a few hundred nanoseconds. TDCs generally can measure much longer time spans at high resolution but since there is usually a limited number of histogram bins the system may run into the same limitation. Again, there is a solution: One just has to delay the sync signal corresponding to the true excitation pulse relative to the photon detector signal. This can be done just by a few metres of cable or some sufficient optical path. The detector pulse can thereby 'overtake' the sync pulse and reach the timing electronics first. There it can start the time measurement and the sync pulse arriving later will stop the measurement. This works fine because the cable delays etc. remain constant.

Advanced TCSPC

Historically, the primary goal of TCSPC was the determination of fluorescence lifetimes upon optical excitation by a short light pulse [1,2]. This goal is still important today and therefore has a strong influence on instrument design. However, modifications and extensions of the early designs allow for the recovery of much more information from the detected photons and enable entirely new applications.

Classical TCSPC for fluorescence lifetime measurements only uses the short term difference between excitation and emission. It was soon realized that other aspects of the photon arrival times were of equally great value in the context of single molecule fluorescence detection and spectroscopy. For instance, in single molecule experiments in flow capillaries; an important option is to identify the molecules passing through the detection volume based on their fluorescence lifetime. Each molecule transit is detected as a burst of fluorescence photons. Each time such a transit is detected its fluorescence decay time has to be determined. Also in the area of single molecule detection and spectroscopy, photon coincidence

correlation techniques were adopted to observe antibunching effects that can be used to determine the number of observed emitters as well as the fluorescence lifetime.

Another important method that makes use of temporal photon density fluctuations over a wider time range is Fluorescence Correlation Spectroscopy (FCS). From the fluorescence intensity fluctuations of molecules diffusing through a confocal volume, one can obtain information about the diffusion constant and the number of molecules in the observed volume. This allows sensitive fluorescence assays based on molecule mobility and co-localization. Due to the small numbers of molecules, the photon count rates in FCS are fairly small. Therefore, the only practical way of collecting the data is by means of single photon counting. In order to obtain the time resolution of interest for the diffusion processes, counting with microsecond resolution is required. Hardware correlators for FCS can be implemented very efficiently and recent designs are widely used. However, these instruments are dedicated to correlation with nanosecond resolution at best, and cannot perform picosecond TCSPC.

The requirements of all these analytical techniques based on single photon timing data have much in common. Indeed, all of them can be implemented with the same experimental setup and are based on photon arrival times. A first step towards unified instrumentation for this purpose was a modification of classical TCSPC electronics. The start-stop timing circuitry is used as previously, providing the required picosecond resolution for TCSPC. In order to maintain the information embedded in the temporal patterns of photon arrivals the events are stored as separate records. In addition to that, a coarser timing (time tagging) is performed on each photon event with respect to the start of the experiment. This is called Time-Tagged Time-Resolved (TTTR) data collection [4].

In classical TTTR the different time scales are processed and used rather independently. However, it is of great interest to obtain high resolution timing on the overall scale, i.e. combining coarse and fine timing into one global arrival time figure per event, with picosecond resolution. In a most generic approach, without implicit assumptions on start and stop events, one would ideally just collect precise time stamps of all events of interest (excitation, emission or others) with the highest possible throughput and temporal resolution, and then perform the desired analysis on the original event times. Ideally this would be done on independent channels, so that between channels even dead time effects can be eliminated. These requirements are met by the PicoHarp and HydraHarp TCSPC systems from PicoQuant. Their radically new design enables temporal analysis from picosecond to second time scale, thereby covering almost all dynamic effects of the photophysics of fluorescing molecules. This is achieved by means of independent TDC timing channels allowing picosecond cross-correlations and very high throughput [5,6]. In addition to this enhanced functionality, the new designs eliminate the need for operating in reverse start-stop mode.

PicoQuant TCSPC electronics and system integration

Besides the fast timing electronics for acquisition of e.g. time resolved fluorescence decay profiles, PicoQuant provides pulsed diode lasers and other light sources, bringing the technology to a degree of compactness and ease of use unseen before. This permits the transfer of revolutionary methods from the lab to real life industry applications e.g. in quality control or high throughput screening.

The PicoQuant TCSPC systems contain all components previously accommodated in bulky NIM racks. Nevertheless they outperform conventional systems in many parameters. Due to a versatile design they support many useful measurement modes such as oscilloscope mode for on-the-fly optical alignment or continuous and time tagging modes. Hardware synchronisation pins permit real-time scanning setups with sub-millisecond stepping. DLL libraries as well as demo code are available for custom programming or system integration. A powerful data analysis software for time tagged data is also available.

Apart from supplying stand-alone components, PicoQuant develops complete instruments and supports system integration for specific research applications as well as for OEM needs. Of course we also do not leave the individual user alone with the sometimes tricky task of combining the components for a TCSPC system. We provide help, suggestions and professional consultancy to the individual researcher in the chemistry or biophysics lab as well as to the developer of an industry application. Just call or contact us by email.

References and further reading

[1] Lakowicz, J. R. , Principles of Fluorescence Spectroscopy, 3rd Edition
Springer Science+Business Media, New York, 2006

[2] O'Connor, D.V.O., Phillips, D., Time-correlated Single Photon Counting
Academic Press, London, 1984

[3] O'Connor, D.V.O., Ware, W.R., Andre, J.C.,
"Deconvolution of fluorescence decay curves. A critical comparison of techniques",
J. *Phys. Chem.* **83**, 1333-1343, 1979

[4] Wahl, M., Technical Note on TTTR, PicoQuant, 2004

[5] Wahl M., Rahn H.-J., Gregor I., Erdmann R., Enderlein J.,
"Dead-time optimized time-correlated photon counting instrument with synchronized, independent timing channels", *Review of Scientific Instruments*, **78**, 033106, 2007

[6] Wahl M., Rahn H.-J., Röhlicke T., Kell G., Nettels D., Hillger F., Schuler B., Erdmann R.,
"Scalable time-correlated photon counting system with multiple independent input channels", Review of Scientific Instruments, **79**, 123113, 2008

Copyright of this document belongs to PicoQuant GmbH. No parts of it may be reproduced, translated or transferred to third parties without written permission of PicoQuant GmbH. All Information given here is reliable to our best knowledge. However, no responsibility is assumed for possible inaccuracies or ommisions. Specifications and external appearences are subject to change without notice.

PicoQuant

PicoQuant GmbH
Rudower Chaussee 29 (IGZ)
12489 Berlin
Germany

Phone +49-(0)30-6392-6560
Fax +49-(0)30-6392-6561
Email info@picoquant.com
WWW http://www.picoquant.com