Technical Note

PicoQuant

Time Tagged Time-Resolved Fluorescence Data Collection in Life Sciences

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Motivation

Fluorescence lifetime measurements in the time domain are commonly performed by means of Time-Correlated Single Photon Counting (TCSPC). Classical TCSPC is a histogramming technique based on precise timing and time binned counting of single photons emitted on pulsed laser excitation[1],[2]. However, in many fluorescence applications it is of great interest not only to obtain the fluorescence lifetime(s) of the fluorophore(s) but to record and use more information on the fluorescence dynamics. This is most often the case when very few or even single molecules are observed. For instance, in Fluorescence Correlation Spectroscopy (FCS), the information of interest is contained in the intensity fluctuations caused by the diffusion of fluorescent molecules through a small detection volume. These diffusion related fluctuations occur on a millisecond time scale and are typically analyzed by means of an autocorrelation of the fluctuation signal. This gives access to concentrations and diffusion constants and thereby to molecule mobility and/or size. Similarly, single molecules flushed through capillaries (e.g in DNA analysis applications) will emit short bursts of fluorescence, that are of interest for further analysis. The resulting fluorescence intensity dynamics on a time scale of milliseconds can be used to identify single molecule transits and to discriminate these events against background noise. Even with immobilized single molecules, characteristic blinking behavior of continuously excited molecules can be observed, again on a millisecond time scale. The analysis and interpretation of the underlying processes has become a research topic in its own. Since all these processes can be observed independently from the fast dynamics related to fluorescence lifetime, and even with CW illumination, the related techniques and instruments have until recently evolved rather independently. If both phenomena were of interest, researchers would usually conduct independent experiments. However, given the 'transient' nature of many single molecule experiments (consider, e.g., photobleaching), it turned out to be of great value to be able to link effects on both time scales in one experiment. For instance, in capillary flow experiments, the millisecond dynamics can help to identify a single molecule transit, while the picosecond to nanosecond dynamics (e.g., fluorescence lifetime) can be used to distinguish different species. This can be achieved by using a pulsed excitation source (e.g., picosecond pulsed laser), and a fluorescence detection set-up that allows for picosecond time resolution with respect to the excitation pulses.

In addition, the capability of intensity recording with sub-millisecond resolution must be ensured. The recording of the fast dynamics (fluorescence decay) is commonly implemented via TCSPC. The arrival times recorded in the TCSPC histogram are relative times between a laser excitation pulse and the corresponding fluorescence photon arrival times at the detector. These measurements are ideally resolved down to a few picoseconds. After collection of sufficient photon numbers, one or more fluorescence lifetimes can be calculated.

TCSPC can be implemented with a variety of instrumentation, but not many designs are suitable to record the slower intensity dynamics at the same time. This is because the intensity dynamics information is not available from conventional histograms

from TCSPC data, often collected over minutes. To solve the problem one can continuously collect histograms over very short intervals. This direct approach was successful in early capillary flow experiments, but is hampered by data acquisition bottle necks, since it generates large amounts of redundant data. This is because at the (necessary) short time slices per histogram, the histograms are mostly empty. Still they must be fully processed and stored. Even if the histogramming is performed in integrated hardware, e.g., on PC boards with dedicated memory, the redundant data processing is not very elegant and limits remain due to on-board memory constraints.

Furthermore, it is often desirable to have as much information as possible about all photon events for further analysis. Even histogramming with very fast time slices would reduce the original information content. It is therefore far more elegant to record each fluorescence photon as a separate event, and to consider fluorescence lifetime histogramming as one out of many analysis methods that can be applied to the photon stream, be it on-line or off-line.

Basic Concept

The desired capturing of the complete fluorescence dynamics can be achieved by recording the arrival times of all photons relative to the beginning of the experiment (time tag), in addition to the picosecond TCSPC timing relative to the excitation pulses. This is called Time-Tagged Time-Resolved (TTTR) mode[2]. Figure 1 shows the relationship of the time figures involved.

As in conventional TCSPC, a picosecond timing between laser pulse and fluorescence photon is obtained. In addition to that, in TTTR data collection a coarser timing is performed on each photon with respect to the start of the experiment. This is done with a digital counter running typically at 50 or 100 ns resolution. Most recent devices actually use the excitation pulses for this counter[6],[7],[8]. Since the classical TCSPC timing covers the picosecond time scale between laser pulses, the additional time tags allow covering the whole time range of seconds, minutes or even hours, thereby providing ultimate flexibility in further data analysis. The two timing figures (TCSPC time and time tag) are stored as one photon record. If the TCSPC device has more than one detector channel (either natively[7],[8] or by means of a router[6]) then the channel code is also stored in the photon record (Figure 2).

In order to work efficiently with current host computers, the photon record is typically chosen as a 32 bit structure. Current TTTR hardware designs are implemented in Field Programmable Gate Arrays (FPGA). A large First In First Out (FIFO) buffer is used to average out bursts and deliver a moderate constant data rate to the host interface. This way sufficient continuous sustained transfer rates are possible in real-time, even when photon rates are high. Most recent devices use PCI express or USB 3.0 where continuous transfer rates as high as 40 Mcps can be handled[7],[8].

TTTR with Multiple Detectors and Marker Signals

Fluorescence Lifetime Imaging (FLIM), which is a powerful extension to fluorescence imaging microscopy. In order to perform FLIM, the spatial origin of the photons must be recorded in addition to the TCSPC data. Conventional systems use a large arrays of on-board memory to accommodate the large amount of data generated due to the multidimensional matrix of pixel coordinates and lifetime histogram channels. Even with modern memory chips, this approach still used in some competing products is very limited in image size. Consequently, it is expensive, and implies loss of information. Furthermore, the time per pixel is usually limited. Still, in order to obtain lifetime information, a TCSPC histogram must be formed for each pixel. This makes it difficult to

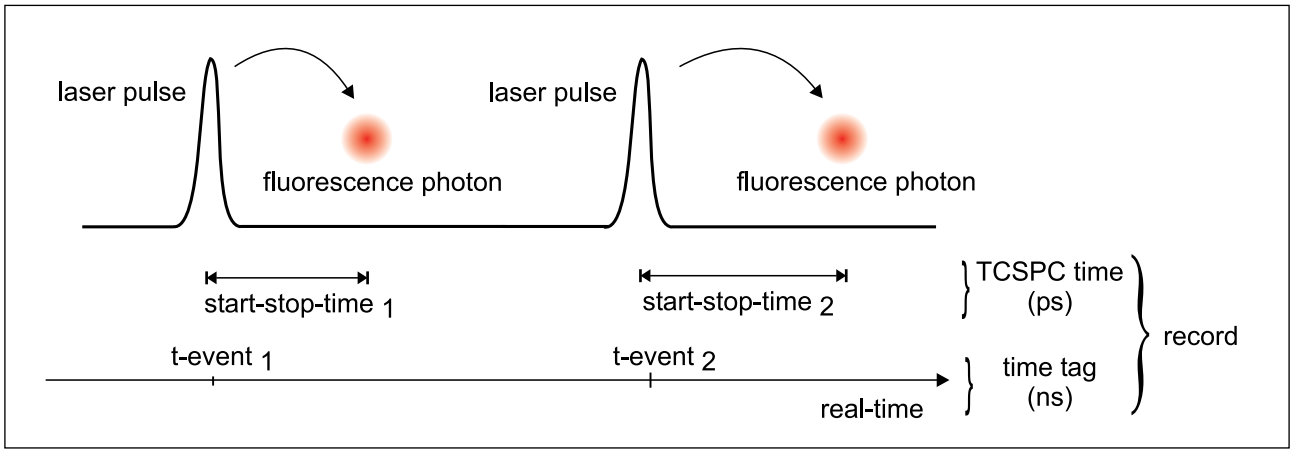

Figure 1: Timing figures in TTTR data acquisition.

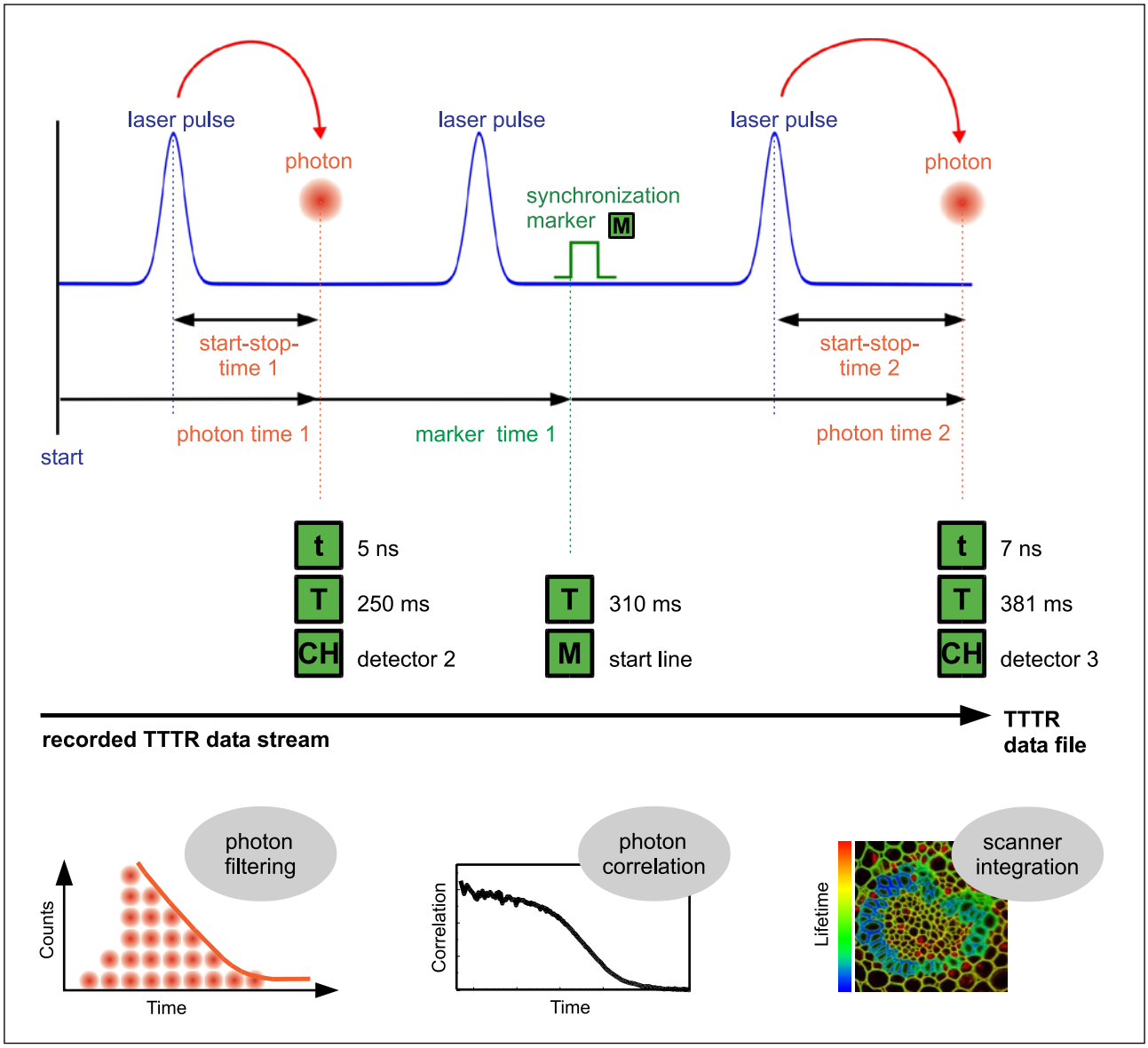

Figure 2: TTTR data acquisition scheme with routing and external markers.

construct suitable hardware at reasonable cost. To solve the problem much more elegantly, the TTTR data stream can be extended to contain markers for synchronization information derived from an imaging device, e.g., a piezo scanner. This makes possible to reconstruct the 3D image from the stream of TTTR records, since the relevant XYZ position of the scanner can be determined during the data analysis. The data generated is free of redundancy and can therefore be transferred in real-time, even if the scan speed is very fast, like, e.g., in Laser Scanning Microscopes (LSM). The image size is unlimited both in XYZ and in count depth. Since there are up to four such synchronization signals, all imaging applications can be implemented and even other experiment control signals can be recorded. This marker scheme is a very special feature of the PicoQuant TCSPC electronics[6],[7],[8]. It may be worth noting that this technology enabled PicoQuant to develop the leading edge MicroTime 200 Fluorescence Lifetime Microscope. Only much later the concept of TTTR markers was copied by competitors.

As outlined briefly above, PicoQuant's TTTR hardware is designed to include channel information for multiple detectors. In case of the PicoHarp 300, four detector channels can be provided by means of a router[6]. This is a cost efficient solution but can have limitations in correlation experiments across channels. In case of the HydraHarp 400 and the TimeHarp 260 there are truly independent detector channels natively provided[7],[8]. From the perspective of TTTR mode it does not matter if the channel information comes from independent timing circuits. A numerical channel code is recorded in the data stream and can be recovered later. Having multiple detector channels available, one can record, e.g., different emission wavelengths or multiple polarization states in parallel. (Figure 3)

Typically, routing and synchronization information are fed to the TTTR hardware as TTL signals from the external devices. Figure 2 shows how such additional information is inserted in the TTTR data.

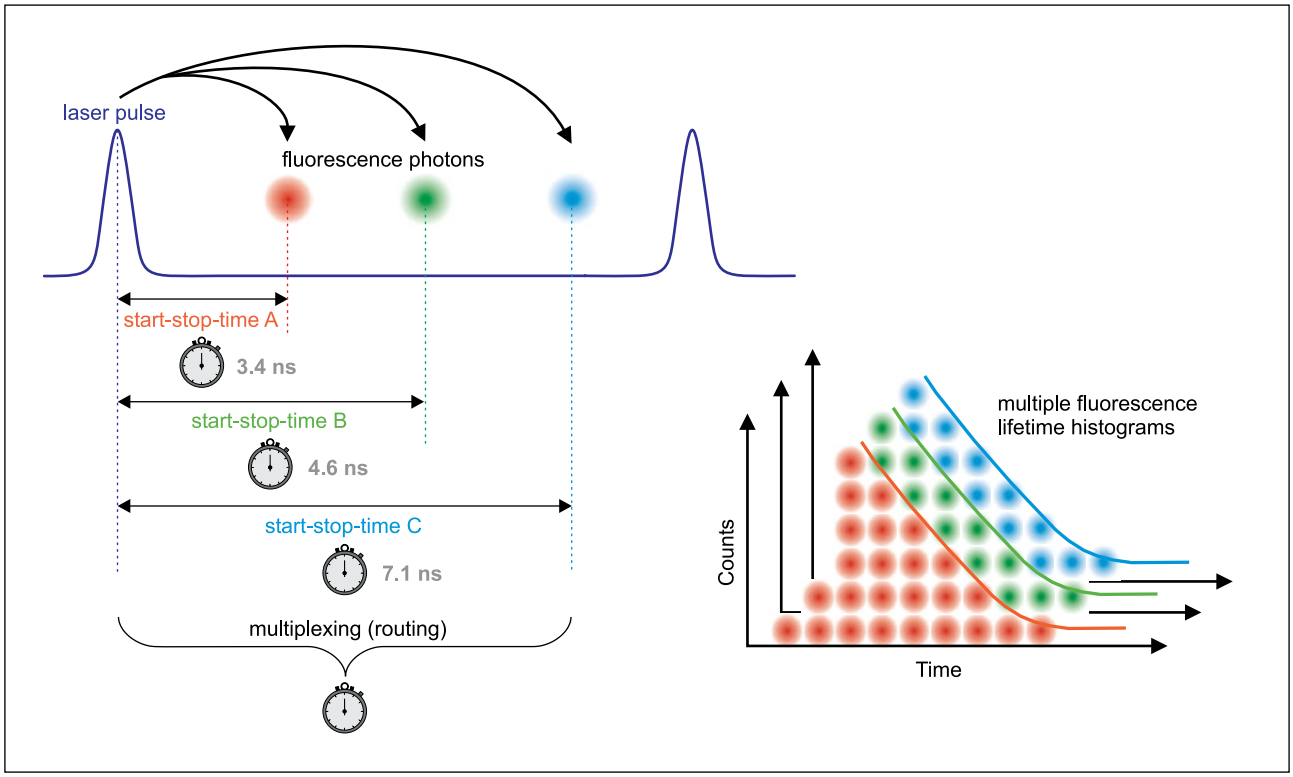

Figure 3: Multichannel TCSPC.

In an imaging application the external markers signals are typically connected such that they correspond to 'start of line' or 'start of pixel' in an image scan. Sequentially stepping through the data records it is then straight forward to form TCSPC histograms for all pixels and calculating their fluorescence lifetime(s). These can then be evaluated and color-coded according to their intensity as well as lifetime.

TTTR Data Analysis and Typical Applications

Time-Tagged Time-Resolved (TTTR) measurement mode allows to perform vastly different measurement tasks based on one single data format, yet without any sacrifice of information available from each single photon. This in turn allows to handle all measurement data in a standardized and yet flexible way. The concept is without redundancy in the data stream, but also without any loss of information, as opposed to, e.g., in onboard histogramming. Therefore, virtually all algorithms and methods for the analysis of fluorescence dynamics, such as intensity time trace analysis, burst analysis, lifetime histogramming, Fluorescence Correlation Spectroscopy (FCS), and Fluorescence Lifetime Imaging (FLIM) can be implemented. Figure 4 shows a summary of the methods that can be applied by using TTTR data.

Intensity traces over time, as traditionally obtained from Multi-Channel-Scalers (MCS), are obtained from TTTR data by evaluating only the time tags of the photon records. Sequentially stepping through the arrival times, all photons within the chosen time bins (typically milliseconds) are counted. This gives access to, e.g., single molecule bursts (in flow) or to blinking dynamics. The bursts can be further analyzed, e.g., by histogramming for burst height and frequency analysis.

Fluorescence lifetimes can be obtained by histogramming the TCSPC (start-stop) times and fitting of the resulting histogram, as in the conventional approach. In single molecule applications with very few counts per histogram, faster algorithms based on maximum likelihood criteria are used. When the objective is mere distinction of multiple species with a priori known fluorescence lifetimes, even simpler and more speed efficient algorithms can be employed.

The strength of the TTTR format is best exploited when both time figures are used together. For instance, one can first evaluate the MCS trace to identify single molecule bursts, and then use the TCSPC times within those bursts, to evaluate fluorescence lifetimes for individual bursts (Figure 5). If there are different molecular species with different fluorescence lifetimes, this can be used to distinguish them in real-time, e.g., in capillary flow approaches to DNA sequencing or substance screening. Vice versa, one can employ time gating on the TCSPC time before evaluating the intensity trace, i.e., one rejects all photons that do not fall in a time span that is likely to contain fluorescence photons. This time gating reduces noise from background and scattered excitation light.

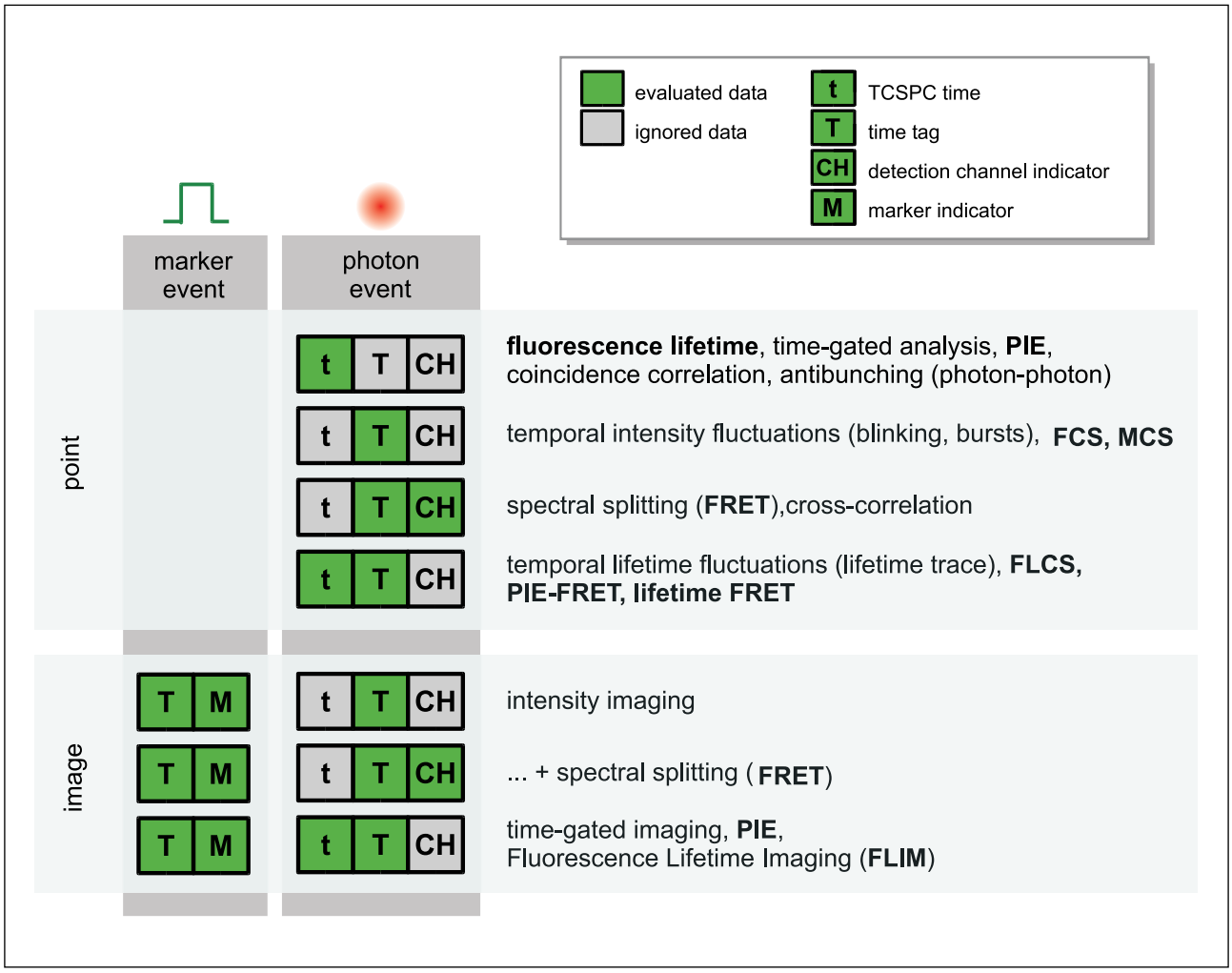

Figure 4: Data display and analysis methods applicable with TTTR data.

A powerful application of TTTR mode and time gating is FCS. Traditionally, FCS was performed by hardware correlators, because the computational demand of the correlation function is considerable, and results are often desired to be available in real-time. However, hardware correlators have some disadvantages. One is that they usually do not calculate the correlation function in the strict mathematical sense. This is because simplifications such as coarser binning towards longer lag times and data quantization (rounding) are employed in order to reduce the computation load and memory requirements. The other is that they perform an immediate (real-time) data reduction, that does not allow to recover the original data, and that prohibits to 'slice' the data if parts of it turn out to be unusable during the measurement. This is the case, e.g., in diffusion experiments, when large undesired particles enter the focal volume. The scatter or strong fluorescence from these particles will then immediately enter the previously collected correlation function and 'swamp' it irreversibly with artifacts. Having individual photon records available from TTTR mode, one can perform the correlation in software and select the 'good' data, or data of interest, as required. On modern computers and with

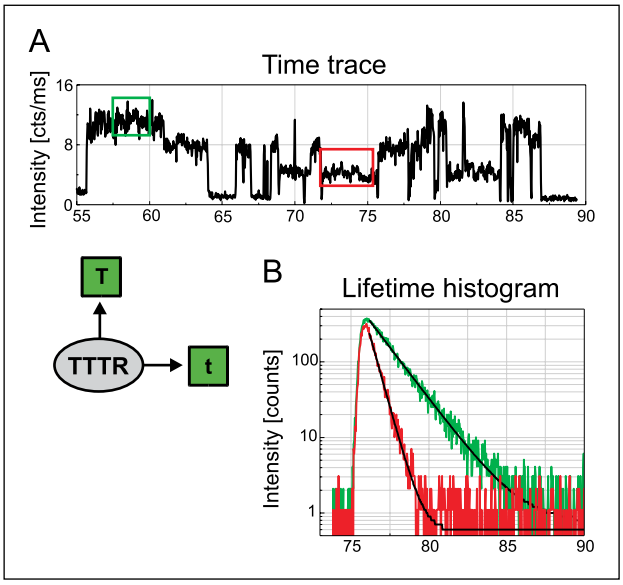

Figure 5: Single molecule lifetime variations over time evaluated by using TTTR. (A) Time trace and (B) fluorescence lifetime histogram of single Cy5 molecules, acquired with a MicroTime 200 using pulsed 635 nm excitation. The lifetime of the two selected molecules (green and red region in MCS trace and decay) and was fitted to 1.6 ns and 0.65 ns, respectively.

recently developed fast algorithms it is possible to perform the correlation even in real-time[3]. Furthermore, off-line analysis can be repeated infinitely with variations in the analysis approach, if in-depth investigations in basic research are desired. Finally, the ultimate strength in TTTR based FCS analysis is again the combination of the two time figures. As a first useful approach, one can employ time gating on the TCSPC time to reject scatter and background noise. More complex algorithms are subject of ongoing research. One very powerful algorithm of this kind is called Fluorescence Lifetime Correlation Spectroscopy. It uses TTTR data to implement a lifetime-weighted or 'filtered' variant of FCS that allows to separate different molecular species in a mixture, in one single FCS measurement[4]. By filtering the photon events according to their TCSPC time before they enter the correlation, one can obtain the separated FCS curves of the species (Figure 6). However, the mathematical idea behind FLCS is much more general. Indeed the filter concept can also be used to suppress detector artifacts (afterpulsing) and background noise.

Yet another elegant application of TTTR mode is in Fluorescence Lifetime Imaging (FLIM), which is a powerful extension to fluorescence imaging microscopy. In order to perform FLIM, the spatial origin of the photons must be recorded in addition to the TCSPC data. As outlined above, this is done by feeding position marker signals from a scan stage into the TTTR data stream. This permits the reconstruction of 2D or 3D images from the collected photon records. Once such imaging capability is set up, there are many further methods that can be taken over to imaging with the TTTR approach. Important examples include Förster Resonance Energy Transfer (FRET) and fluorescence anisotropy methods (Figure 7).

Indeed, due to the virtually unlimited choices in data analysis, TTTR mode is a very powerful key to molecular imaging. Because it allows to combine fluorescence imaging with, e.g., Multiparameter Fluorescence Detection[5] for thorough analysis of fluorescence dynamics, the method is used in the most advanced time resolved fluorescence microscopes available today[6]. (Figure 8)

Distinction of Specialized TTTR Data Acquisition Modes

The basic concept of TTTR data collection we introduced in the previous sections is what historically evolved first. In modern instruments today this scheme is referred to as T3 mode. There is actually another mode we call T2 mode. In the following we describe some more details of these two modes of time tagged data collection. Essentially the distinction of the two modes has to do with the role of the SYNC input of the TCSPC device, i.e., the timing input where typically the laser synchronization pulses are fed in.

Imagine, we had the option of creating a 'perfect' instrument that would be able to capture ALL timing events regardless of what they are, i.e., laser excitation events or photon events from different detectors. If we can capture all these events with the

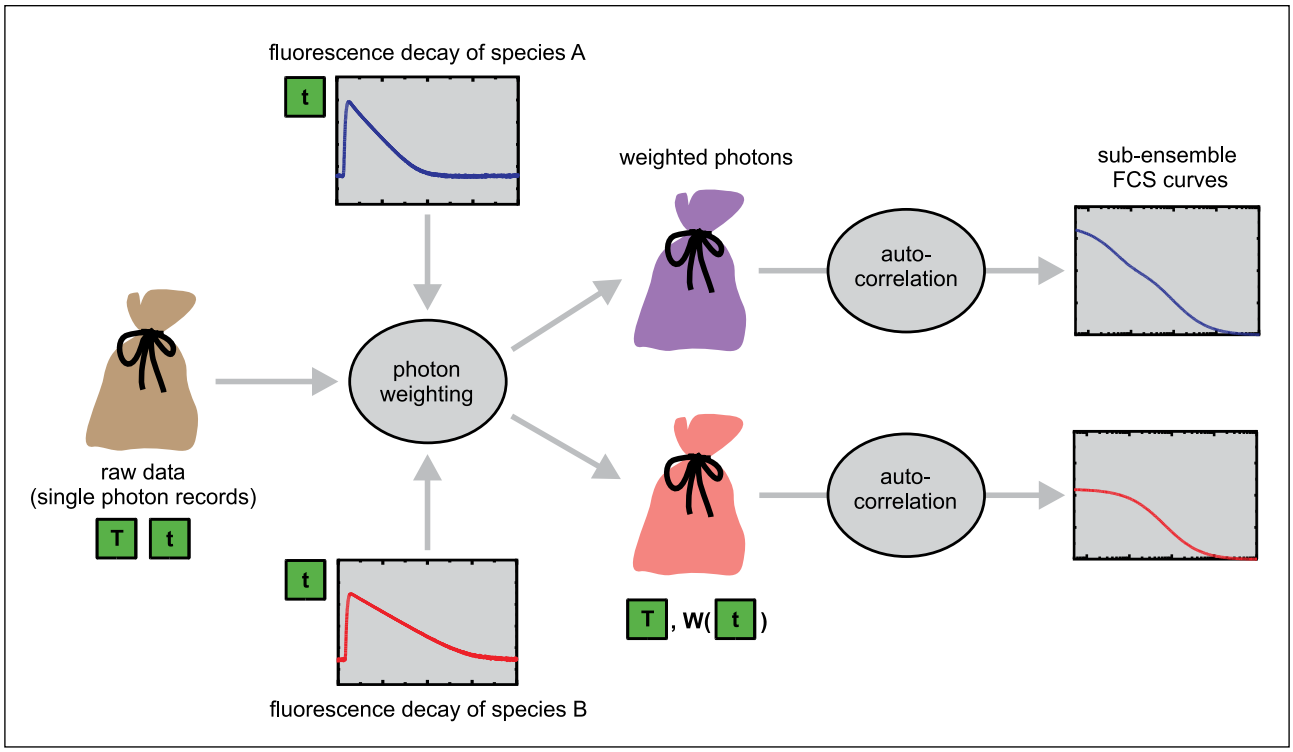

Figure 6: Simplified principle of FLCS (Fluorescence Lifetime Correlation Spectroscopy).

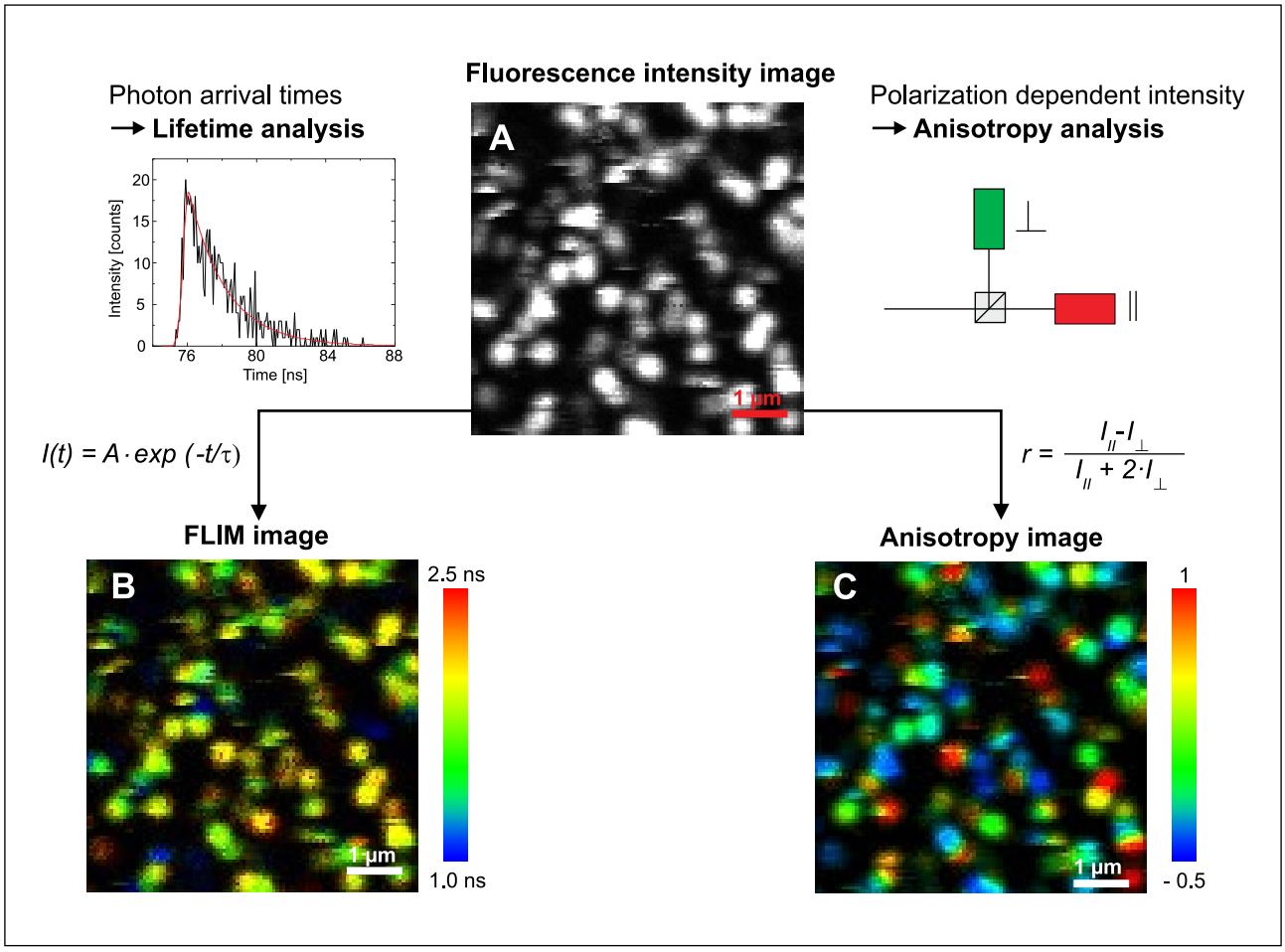

Figure 7: Examples for image analysis possibilities based on TTTR data acquisition. (A) Fluorescence intensity image, (B) FLIM image and (C) Anisotropy image of single Cy5 fluorescent molecules, acquired with a MicroTime 200 using pulsed 635 nm excitation and two detection channels for parallel and perpendicular polarization.

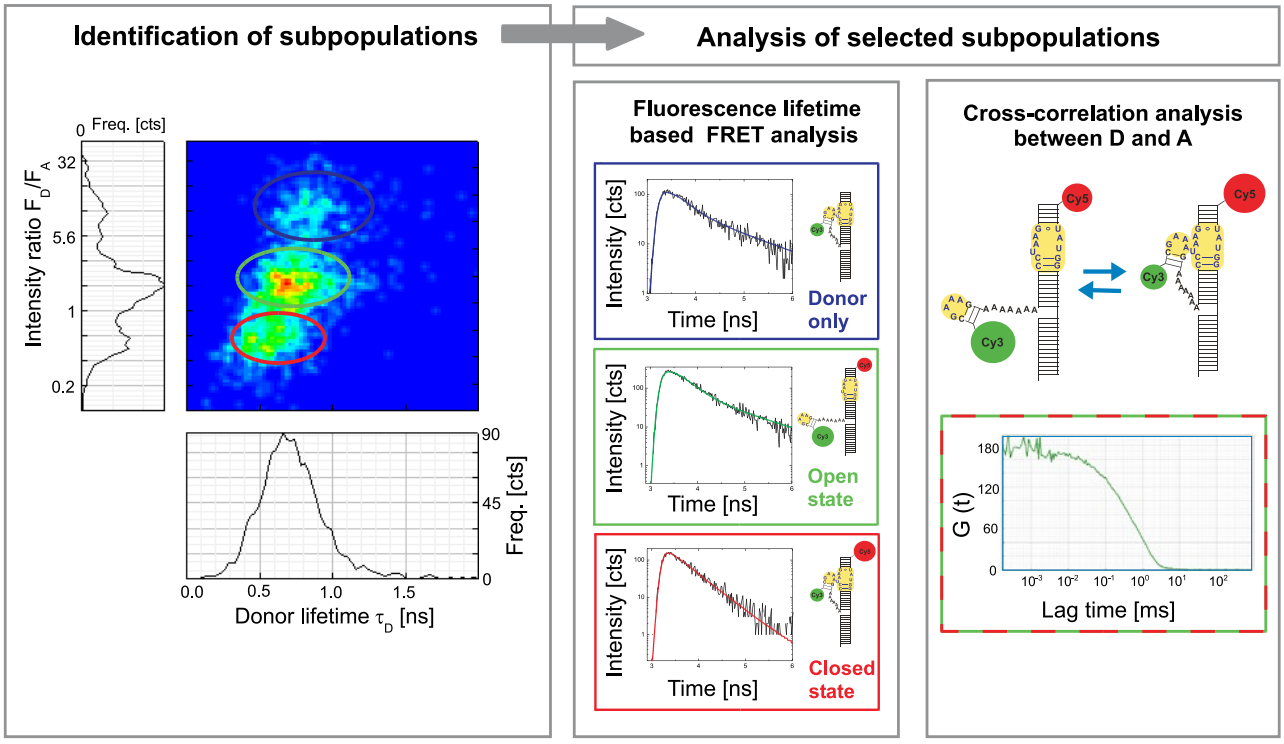

Figure 8: Multiparameter Fluorescence Detection (MPD) followed by combined FRET and cross-correlation analysis for investigation of molecular folding dynamics. In this example, the flexible tetraloop and tetraloop receptor were labeled with donor Cy3 and acceptor Cy5, respectively, to monitor the Mg 2+-driven RNA folding.

best possible resolution of our TDCs (Time-to-Digital-Converters) over an unlimited time span then it no longer necessary to distinguish a fine and coarse timing in the data records. Everything could then be calculated from the raw event times. This is what T2 mode is about. We will take a closer look at this mode now, but we will see that T3 mode is still needed in practice.

T2 Mode

In T2 mode all timing inputs of the TCSPC device are functionally identical. There is no dedication of the SYNC input channel to a sync signal from a laser (Figure 9A). Instead, it can be used for an additional detector signal. The events from all channels are recorded independently and treated equally. In each case an event record is generated that contains information about the channel it came from and the arrival time of the event with respect to the overall measurement start. The time tags are recorded with the highest resolution the hardware supports (currently 1, 4, or 25 ps for the different PicoQuant TCSPC devices)[6],[7],[8].

Each T2 mode event record consists of 32 bits. Going by the example of the data format of the TimeHarp 260 and HydraHarp 400, there are 6 bits for the channel number and 25 bits for the time tag. If the time tag overflows, a special overflow record is inserted in the data stream, so that upon processing of the data stream a theoretically infinite time span can be recovered at full resolution. Dead times exist only within each channel but not across the channels. Therefore, cross correlations can be calculated down to zero lag time. This allows powerful applications such as coincidence correlation and FCS with lag times from picoseconds to hours. Autocorrelations can also be calculated at the full resolution but of course only starting from lag times larger than the dead time.

The 32 bit event records are queued in a FIFO (First In First Out) buffer capable of holding several millions of event records. The FIFO input is fast enough to accept records at the full speed of the TDCs. This means, even during a fast burst no events will likely be dropped except those lost in the dead time. The FIFO output is continuously read by the host PC, thereby making room for fresh incoming events. Even if the average read rate of the host PC is limited, bursts with much higher rate can be recorded for some time. Only if the average count rate over a long period of time exceeds the readout speed of the PC, a FIFO overrun could occur. In case of a FIFO overrun the measurement must be aborted because data integrity cannot be maintained. However, on a modern and well configured PC a sustained average count rates of 40 Mcps are possible with the TimeHarp 260 and HydraHarp 400. This total transfer rate must be shared by the inputs used. For all practically relevant photon detection applications the effective rate per channel is more than sufficient.

For maximum throughput, T2 mode data streams

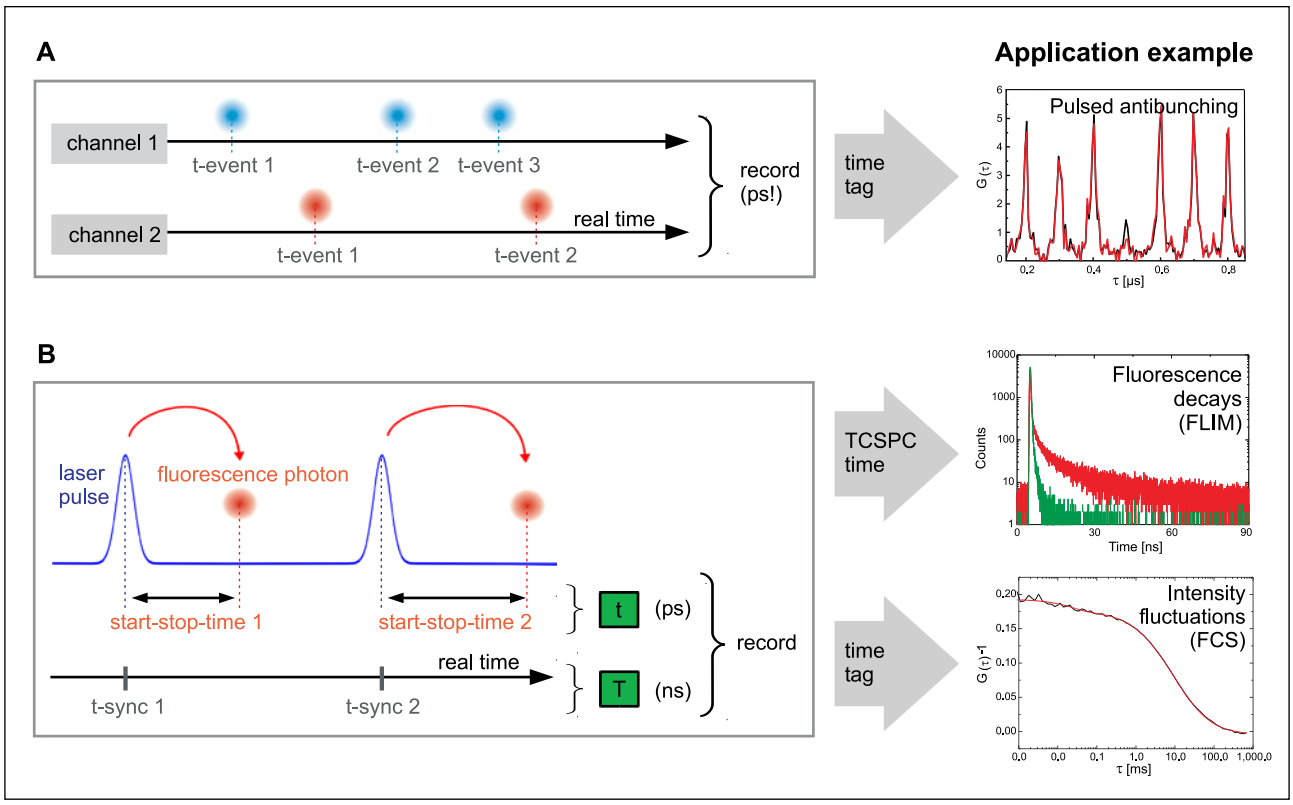

Figure 9: TTTR data acquisition modes. (A) T2 mode. (B) T3 mode.

are normally written directly to disk, without preview other than count rate and progress display. However, it is also possible to analyze incoming data 'on the fly'. The generic data acquisition software provided at no extra cost includes a basic real-time correlator for preview during a T2 mode measurement. The high-end software SymPhoTime 64 offers a similar real-time correlator and many more advanced off-line analysis methods.

T3 Mode

In T3 mode the SYNC input is dedicated to a periodic sync signal, typically from a laser (Figure 9B). As far as the experimental set-up is concerned, this is similar to classical TCSPC histogramming. The main objective is to allow high sync rates which could not be handled in T2 mode due to TDC dead time and bus throughput limits. Accommodating the high sync rates in T3 mode is achieved as follows: First, a sync divider is employed as in histogramming mode. This reduces the sync rate so that the channel dead time is no longer a problem. The remaining problem is now that even with the divider, the sync event rate may still be too high for collecting all individual sync events like ordinary T2 mode events. Considering that sync events are not of primary interest, the solution is to record them only if they arrive in the context of a photon event on any of the input channels. The event record is then composed of two timing figures: 1) the start-stop timing difference between the photon event and the last sync event, and 2) the arrival time of the event pair on the overall experiment time scale (the time tag). The latter is obtained by simply counting sync pulses. From the T3 mode event records it is therefore possible to precisely determine which sync period a photon event belongs to. Since the sync period is also known precisely, this furthermore allows to reconstruct the arrival time of the photon with respect to the overall experiment time.

Each T3 mode event record consists of 32 bits. Going by the example of the data format of the TimeHarp 260 and HydraHarp 400, there are 6 bits for the channel number, 15 bits for the start-stop time and 10 bits for the sync counter. If the counter overflows, a special overflow record is inserted in the data stream, so that upon processing of the data stream a theoretically infinite time span can be recovered. The 15 bits for the start-stop time difference cover a time span of 32,768×R where R is the chosen resolution. For example, at the highest possible resolution of the HydraHarp 400 (1 ps) this results in a span of 32 ns. If the time difference between photon and the last sync event is larger, the photon event cannot be recorded. This is the same as in histogramming mode, where the number of bins is larger but also finite. However, by choosing a suitable sync rate and a compatible resolution R, it is possible to reasonably accommodate all relevant experiment scenarios. R can be chosen in doubling steps of the card's base resolution.

Dead time in T3 mode is the same as in the other modes (hardware model dependent). Within each photon channel, autocorrelations can be calculated meaningfully only starting from lag times larger than the dead time. Across channels dead time does not affect the correlation so that meaningful results can be obtained at the chosen resolution, all the way down to zero lag time.

The 32 bit event records are queued in a FIFO (First In First Out) buffer just like in T2 mode. The FIFO input is fast enough to accept records at the full speed of the time-to-digital converters. Therefore, even during a fast burst no events will likely be dropped except those lost in the dead time. The FIFO output is continuously read by the host PC, thereby making room for new incoming events. Even if the average read rate of the host PC is limited, bursts with much higher rate can be recorded for some time. Only if the average count rate over a long period of time exceeds the readout speed of the PC, a FIFO overrun could occur. In case of a FIFO overrun the measurement must be aborted because data integrity cannot be maintained. However, on a modern and well configured PC a sustained average count rates of 40 Mcps are possible (TimeHarp 260 and HydraHarp 400). This total transfer rate must be shared if the device has two detector input channels. For all practically relevant photon detection applications it is more than sufficient.

For maximum throughput, T3 mode data streams are normally written directly to disk. However, it is also possible to analyze incoming data 'on the fly'. One such analysis method (typically used for FCS) is the on-line correlation implemented in the device's native software. Other specialized analysis methods are provided by the SymPhoTime 64 software offered by PicoQuant.

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