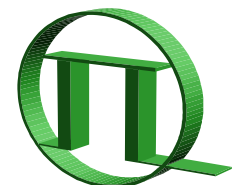


Technical Note

Time Tagged Time-Resolved fluorescence data collection

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Motivation

Fluorescence lifetime measurements in the time domain are commonly performed by means of Time-Correlated Single Photon Counting (TCSPC). This is a histogramming technique based on precise timing and time binned counting of single photons emitted on pulsed laser excitation [1]. 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 fluorophore 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 independent 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 laser), and a fluorescence detection setup 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.

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.

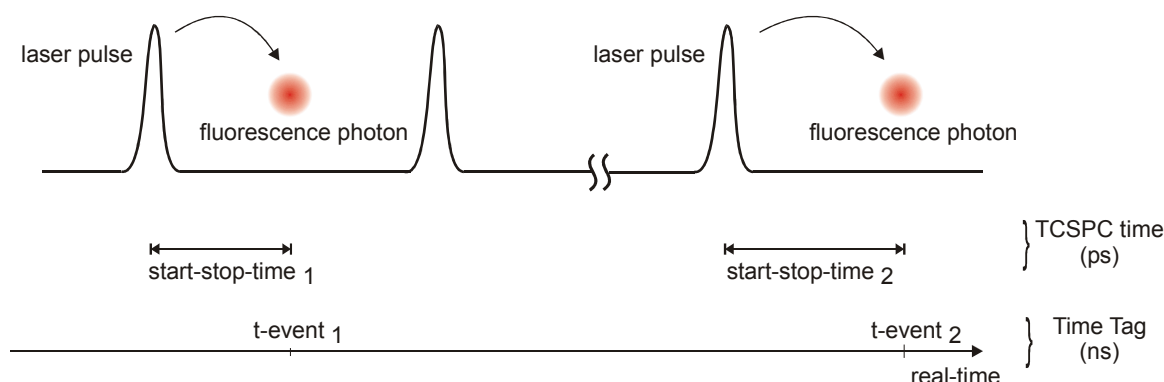


Fig. 1 timing figures in TTTR data acquisition

As in conventional TCSPC, a picosecond timing between laser pulse and fluorescence photon is obtained. In addition to that, a coarser timing is performed on each photon with respect to the start of the experiment. This is done with a digital counter running at typically 50 or 100 ns resolution. Even though this is much higher than what most applications mentioned above would require, modern hardware provides this resolution at no extra cost. Since the TCSPC timing typically covers the time scale just below 100 ns, it is indeed sensible to choose a time tag resolution just above that range, thereby covering the whole time range for ultimate flexibility in further data analysis. The two timing figures (TCSPC time and Time Tag) are stored as one photon record. 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 hardware First In First Out (FIFO) buffer for 128 k events 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.

TTTR Data Analysis

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, like 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 2 shows a summary of the methods that can be used with TTTR data.

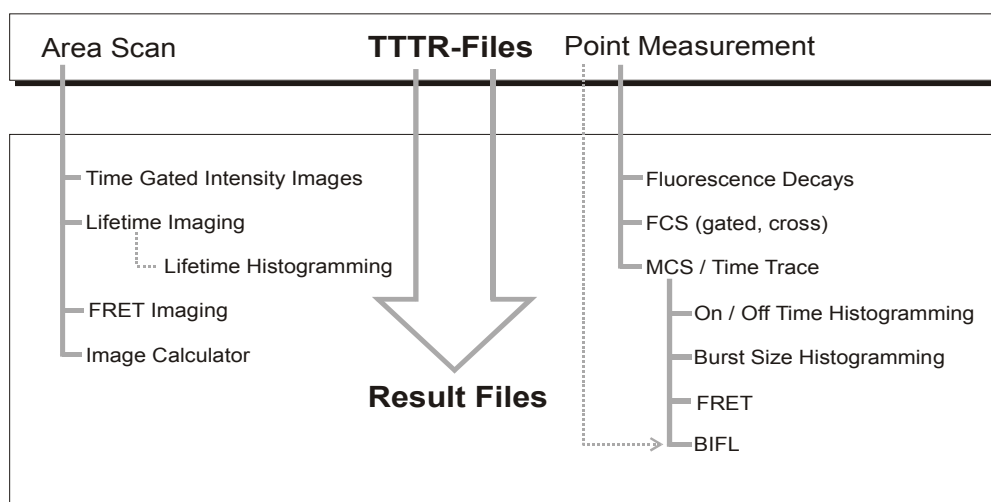


Fig. 2 Analysis methods applicable with TTTR data

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 may be used. When the objective is mere distinction of multiple species with a priori known fluorescence lifetimes, even more efficient pattern matching algorithms can be employed.

The strength of the TTTR format is used 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. If there are different molecular species with different fluorescence lifetime, 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 probable to contain fluorescence photons. This reduces noise from background and scattered excitation light.

Another powerful application of TTTR mode 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 recently developed fast algorithms it is possible to perform the correlation even in (or near) 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 again employ time gating on the TCSPC time to reject scatter and background noise. More complex algorithms are the subject of ongoing research. It has recently been shown, that TTTR based FCS 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.

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. Conventional systems use a large arrays of on-board memory to accommodate the large amount of data generated due to the 3-dimensional matrix of pixel co-ordinates and lifetime histogram channels. Even with modern memory chips, this approach 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 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 2D image from the stream of TTTR records, since the relevant XY 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

XY and in count depth. Since there can be multiple such synchronization signals, all imaging applications can be implemented and even other experiment control signals can be recorded. Furthermore, TTTR hardware can be designed to include routing information for multiple detectors. This means the origin of the photon event (i.e. which detector it came from) is also recorded in the data stream and can be recovered later. Having multiple detector channels available, one can record e.g. different emission wavelengths or polarization states in parallel. Typically, routing and synchronization information are fed to the TTTR hardware as TTL signals from the external devices. Figure 3 shows how such additional information is inserted in the TTTR data.

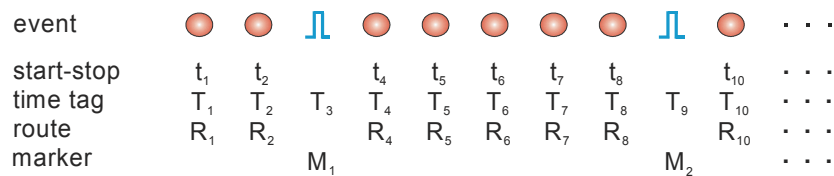


Fig. 3 Principle of TTTR acquisition with routing and external markers

The red bullets denote photon events, while blue pulses denote external markers signals, such as '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 evaluating their fluorescence lifetime(s).

There are many further applications of the TTTR approach, and the techniques based on it. Important examples include Fluorescence Recovery after Photobleaching (FRAP) and Förster Resonance Energy Transfer (FRET). Due to the virtually unlimited choices in data analysis, TTTR mode is a very powerful key to molecular imaging. Because allows to combine fluorescence imaging with thorough analysis of fluorescence dynamics, the method is used in the most advanced time resolved fluorescence microscopes available today [5].

Further reading

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