TCSPC upgrade of a confocal FCS microscope

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We extended the measurement capabilities of the Carl Zeiss ConfoCor 1 FCS microscope by (a) using pulsed picosecond diode lasers instead of a continuous wave (CW) laser excitation, (b) introducing a fast single photon avalanche diode detector, and (c) exploiting the capabilities of the PicoQuant TimeHarp 200 board. When the time-tagged time-resolved (TTTR) mode of the TimeHarp is utilized, the complete fluorescence dynamics are recorded. That is, the time-evolution of the fluctuations and the fluorescence decay kinetics are captured simultaneously. Recording individual photon events (without on-the-fly data reduction like in hardware correlators) preserves the full information content of the measurement for virtually unlimited data analysis tasks and provides a much more detailed view of processes happening in the detection volume. For example, autocorrelation functions of dyes in a mixture can be separated and/or their cross-correlation can be investigated. These virtual two-channel measurements are performed utilizing a single detection channel setup. The time-resolved FCS is a powerful tool in biological studies and is demonstrated here on unilamellar vesicles giving clear evidence for Bodipy dye exchange between them. The described upgrade scenario is applicable to other confocal microscopes as well. In principle, any FCS system so far utilizing conventional CW lasers can benefit from pulsed excitation and the original functionality of the setup is fully preserved.
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I. INTRODUCTION

Fluorescence correlation spectroscopy (FCS) was first conceived in the early 1970s and has since become a very popular spectroscopic technique. FCS is based on statistical analysis of the temporal behavior of spontaneous fluorescence intensity. In practice, the method requires to detect the signal from a few molecules diffusing through (or undergoing other changes) in a very small detection volume. This leads to signal intensity fluctuations on a micro- to millisecond time scale, which are typically analyzed by means of an autocorrelation function. A reasonable realization of the standard confocal setup with favorable signal-to-noise ratio came in 1993.[1] It used stable lasers, an aberration-free epifluorescence confocal microscope with high numerical aperture objective, efficient interference filters, a sensitive single-photon detector, and a fast hardware correlator. This setup is still one of the most widely used and actually served as a basis for the first commercially available FCS instrument, the Carl Zeiss ConfoCor 1 FCS microscope.

Measurement and analysis of fluorescence decay kinetics by means of time-correlated single photon counting (TCSPC) is another well established technique to get insight into the molecular photophysics.[2,3] Time-correlated counting means repetitive measurements of the time between a laser excitation pulse and the corresponding fluorescence photon arrival, typically with subnanosecond resolution. By histogramming many of these relative times the fluorescence decay kinetics is reconstructed.

These techniques and instruments have evolved rather independently. Researchers interested in both phenomena (and time scales) usually conducted consecutive, independent experiments. However, given the transient nature and unrepeatability of e.g. a single molecule transit through the detection volume, it turned out to be of great value to combine both methods in one instrument. A simple and straightforward realization is presented here. It makes use of commercially available components: the sophisticated optics of the ConfoCor 1 confocal FCS microscope and the compact TCSPC electronics of the TimeHarp 200 TCSPC board. In the following sections we describe the hardware adaptation, the new data acquisition modes, software solutions and briefly demonstrate the capabilities of time-resolved FCS.

II. HARDWARE UPGRADE

A suitable pulsed diode laser head is coupled to a polarization maintaining single mode fiber, therefore easily interchangeable with the original fiber-coupled CW lasers usually used with ConfoCor. During the experiments described below we used a PicoQuant laser head emitting 639 nm light, but other options covering the wavelength range from 375 nm to 470 nm and from 630 nm to longer than 900 nm are available as well. These heads are interchangeable and

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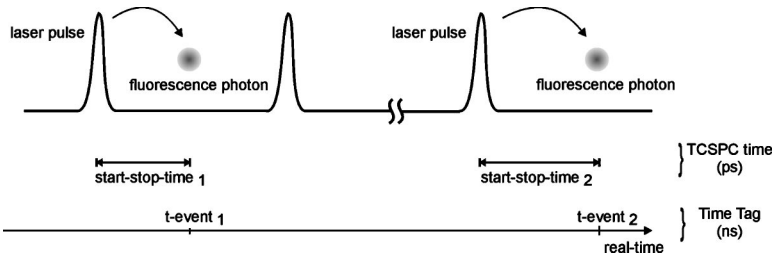

FIG. 1. The principle of the time-tagged time-resolved (TTTR) data acquisition mode.

can be driven from a single PDL800-B driver (one at a time). The driver also provides electrical pulses synchronous with the light pulses (SYNC signal) necessary to perform TCSPC data acquisition. The average excitation power that entered the objective was 970 μW at 40 MHz repetition rate. (The value is affected by the fiber coupling efficiency, as well as by the transmission of the excitation filter and optics. The corresponding optical power measured at the laser head output is 3.5 mW.)

Other pulsed lasers can be used as well, however, they must meet the following criteria: stable repetition rate on the order of MHz, sub-ns pulse duration, stable average power, availability of low-jitter electrical pulses synchronous with the optical pulses. The new light source must be guided to the entry port precisely aligned to the optical axis.

It is not necessary to change the optical hardware whatsoever, but suitable filter sets are needed for each excitation wavelength. With the 639 nm laser diode we use a z640/20x excitation filter, a z640rdc dichroic mirror and a HQ685/50m emission filter (Chroma Technology, Rockingham, USA).

The original photon detector (EG&G, Salem, MA, provided by Carl Zeiss) of the ConfoCor 1 was replaced by a Perkin–Elmer SPCM-AQR-13-FC single photon avalanche diode (SPAD). This was necessary to achieve the fast electrical response to photon impact required by TCSPC. In our case, the FWHM of the final instrument response function (IRF) is 450 ps, including the optical duration of the laser pulse and the electronic timing jitter; it is determined mainly by the SPAD's timing uncertainty. Alternatively, other photon counting detectors (e.g., compact photomultiplier tubes) can be used, however, the mentioned SPAD module has the advantages of high quantum yield and being compatible mechanically and electronically.

The PicoQuant TimeHarp 200 TCSPC board is a plug-and-play computer board with dedicated software.[4,9] It occupies one PCI slot. One SMA connector receives the SYNC signal from the laser diode driver. In our case, the photon signal (i.e., the SPAD's output) is fed directly to the TimeHarp's START input through a 20 dB inline rf attenuator. Having only one detector, the multipurpose sub-D connector (control port) remains unused. When utilizing two or more detectors (e.g., dual color or polarization resolved detection), this port is used to connect a router (PRT400 from PicoQuant GmbH).

We have found it convenient to split the TTL output signal of the SPAD by a power splitter (i.e., reflection-free T-pad) and to use the system's original multiple-tau hardware autocorrelator (ALV Laser, Langen, Germany) and the TimeHarp simultaneously. Because data acquisition at high signal count rate demands significant throughput of the PC's data bus, a modern host PC is recommended. In principle, both PC boards can be operated independently in the same PC, but because the original hardware autocorrelator has an old-fashioned ISA interface, we use two computers.

III. DATA ACQUISITION MODES AND ANALYSIS

The data acquisition mode of pivotal importance is the so-called time-tagged time-resolved (TTTR) mode.[4,5] In principle, this is an advanced version of TCSPC based on recording individual photon events without on-line data reduction. In addition to the time between the excitation pulse and fluorescence photon detection (TCSPC time with down to 35 ps resolution), a coarser timing is performed relative to the start of the experiment (time-tag with 100 ns resolution) and, simultaneously, routing bits are generated that identify the actual detector channel. These three pieces of information are gathered for all detected photons and stored as one photon record (refer to Fig. 1). In addition, markers signaling external events (e.g., scanner movement) can be inserted into the data stream. Recording of individual events is of crucial importance, because it makes possible to apply later virtually any algorithm or data analysis method of photon dynamics.[5–8]

The TTTR data format is well documented[9] and writing custom analysis routines is facilitated by various demo source codes for reading TTTR files.

The usefulness of this concept becomes clear when compared to the conventional way of obtaining the autocorrelation curve. The standard ConfoCor system is equipped by a hardware correlator, because the computational demand of the correlation function is considerable, and results are often desired to be available in real-time. A drawback of this solution is that it performs an immediate (real-time) data reduction, that does not allow to recover the original intensity 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. Furthermore, off-line analysis can be repeated infinitely with variations in the analysis approach, if in-depth investigations in basic research are desired. However, TTTR data recording does not automatically imply off-line analysis. On modern computers and with recently developed fast al-

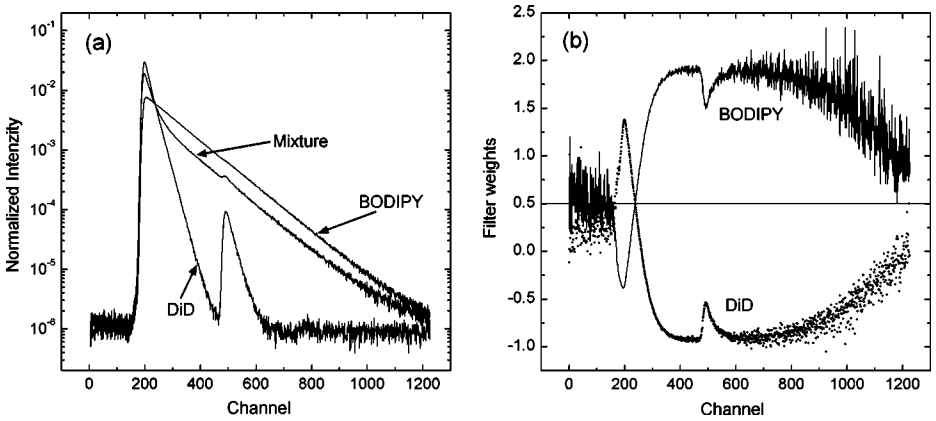

FIG. 2. (a) Area normalized TCSPC histograms of pure BODIPY and DiD in methanol. The histogram of one of their mixtures is also shown. (b) Statistical filters derived from these histograms. The width of one channel is 39 ps. See the text for the explanation of features around the 500th channel.

gorithms it is possible to perform the autocorrelation in real-time,[10,11] thus completely substituting the functionality of the hardware correlator.

A conventional FCS measurement based on TTTR mode as described above utilizes the information contained in the time-tag only, and as a matter of course can be performed equally well with CW excitation. Picosecond pulsed excitation, however, makes possible to exploit the TCSPC time as well. The standard TimeHarp application software (bundled with the board) supports various time-resolved data acquisition modes. Among other things, the actual fluorescence decay kinetics can be displayed on-line (and saved afterwards) by means of repetitive on-board TCSPC histogramming. This so-called Oscilloscope Mode is very useful when setting up the measurement, for example during targeting the detection volume when the sample is not a simple liquid. Combining the information available from the two timescales offers interesting possibilities. For example, intensity trace over time (as traditionally obtained from multichannel scalers) can be 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 single molecule bursts. These can be further analyzed e.g. by histogramming for burst height and frequency analysis. Fluorescence lifetimes can be obtained for each burst by histogramming the relevant TCSPC (start-stop) times and fitting the resulting histogram, as in the conventional TCSPC approach. All these algorithms are available as a stand alone software package (SW-MT 1/2 from PicoQuant GmbH).

The ultimate strength in the TTTR based FCS analysis is the combination of the two time figures, but now in a different context. As a first useful approach, one can employ time-gating on the TCSPC time, for example to reject scattered light or background noise.[12] Going far beyond simple gating, it has recently been shown that the TCSPC timing information in TTTR data allows statistical separation of autocorrelation functions of different molecular species in a mixture, if their fluorescence decays are different.[13] This is possible in a single channel FCS measurement. The separation is done using a special weighting of every photon event according to its TCSPC time. This way, the separated autocorrelation curve of each species or their cross-correlation curve can be calculated. We implemented this method, called time-resolved FCS (TR-FCS hereafter). A windows dynamic link library containing auto- and cross-correlation algorithms for TR-FCS was written in C language and a graphical interface to it was developed using the capabilities of OriginPro 7.0 (OriginLab Corporation). The time consumption of the autocorrelation algorithm scales linearly with the runtime of the experiment and with the square of the intensity. For example, to autocorrelate a 40 s measurement with an average count rate of 130 kHz (i.e. 5.2 million events) takes approximately 7 s on a moderately fast computer.

IV. EXPERIMENTS

The first experiment demonstrates the ability of TR-FCS to separate the autocorrelation curves of dyes with different lifetimes and thus simultaneously track their concentration in a mixture. As a model system we have chosen a mixture of two noninteracting dyes in methanol: BODIPY® 630/650-X, STP ester, sodium salt (BODIPY hereafter) with a lifetime of 4.4 ns, and $DiIC_{18}(5)$ oil (1,1′-dioctadecyl-3,3,3′,3′ tetramethyl-indodicarbocyanine perchlorate, DiD hereafter) with a lifetime of 0.9 ns. First, the solutions of pure dyes were measured, then in five steps a small amount of concentrated BODIPY solution was added to the DiD stock solution.

The separation of autocorrelation curves begins with TCSPC histogramming of the TTTR data. The histograms of pure dyes (available from independent measurements) and that of the analyzed mixture are normalized to a unit area [Fig. 2(a)]. From these "decay curves," statistical filters are derived[13] for each dye in the given mixture [Fig. 2(b)]. It is interesting to note that the parasitic signal peaking at around the 500th channel (an artifact due to the detector in our early setup) has no influence on the results. This is because the TCSPC filtering of photon events is based on the difference of the decay curves without any assumption on their functional forms.

Using the filter functions (i.e., weights), the autocorrelation curve for each dye component is calculated from the TTTR data file of the mixture (Fig. 3). By fitting the obtained autocorrelation curves with a model of free diffusion (taking into account the internal dynamics of DiD),[14] particle numbers were obtained and plotted against the real concentrations (see the inlets in Fig. 3). It is important to mention, that the diffusion times of the dyes are 72 μs for BODIPY and

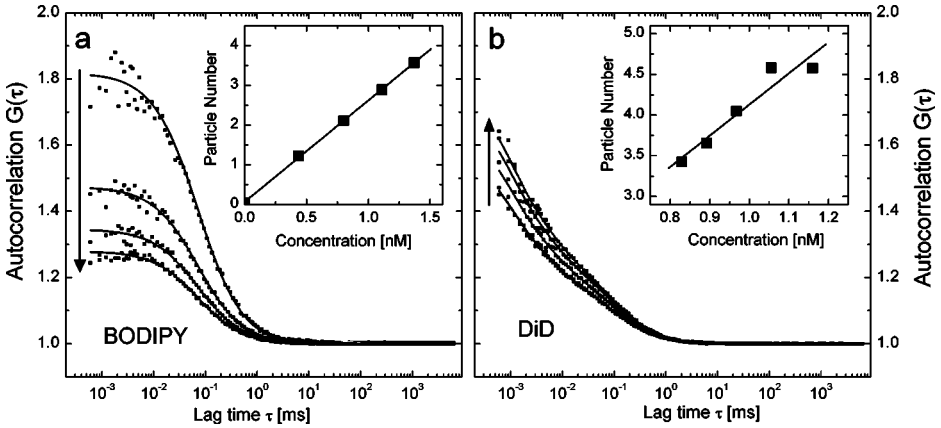

FIG. 3. To the methanol solution of DiD, small amounts of concentrated methanol solution of BODIPY were gradually added. (a) The amplitude of separated autocorrelation curve of BODIPY decreases, which means an increase of the concentration (see the inset graph). (b) The separated autocorrelation curve of DiD show gradually increasing amplitude (i.e., decrease of concentration) caused by dilution of the DiD stock solution by BODIPY addition. DiD in methanol show *cis–trans* isomerization, hence the fast initial decay of these FCS curves.

84 μs for DiD, which means they are irresolvable by fitting the model of two freely diffusing particles to the autocorrelation curve of the mixture.

In a second experiment we demonstrate the utility of TR-FCS approach to obtain the cross-correlation function in a single-channel measurement. The model system consists of two differently sized unilamellar phospholipid vesicles, each labeled with a different fluorescent compound. The observed process is the transfer of one of the labels to the other vesicle type.

Large unilamellar vesicles (LUVs) were labeled with water-insoluble DiD and small unilamellar vesicles (SUVs) were labeled with water-soluble BODIPY. Water solutions of these vesicles were measured separately to obtain the TCSPC histograms for the statistical filters (data not shown). When these solutions are mixed, a new equilibrium is established. The water soluble BODIPY contaminates the DiD labeled LUVs, whereas the water insoluble DiD molecules are expected to remain in LUVs. The mixture was measured after 20 min incubation time. Figure 4 shows the separated autocorrelation curves of the components, as well as their calculated cross-correlation.

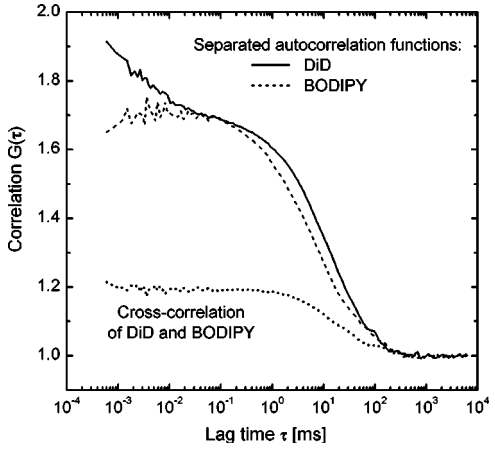

FIG. 4. Auto- and cross-correlation function of components of a vesicle mixture obtained by a single-color, single detection channel measurement. LUVs were labeled with water-insoluble DiD, whereas SUVs were labeled with water-soluble BODIPY. The significant amplitude of the cross-correlation function is caused by the interchange of water-soluble BODIPY molecules between the two kinds of vesicles.

The autocorrelation function for DiD reveals a single diffusing species with a fitted diffusion time identical to the one obtained from conventional FCS measurement of the pure LUV solution. (Again, the internal dynamics of DiD should be taken into account.[14]) On the other hand, analysis of the autocorrelation function for BODIPY shows the presence of three different dye populations. While the short diffusion time agrees with the diffusion time of free BODIPY dissolved in water, the two longer diffusion times indicate that both LUVs and SUVs are labeled with BODIPY. The suggested transfer of BODIPY from SUVs to LUVs finds its evidence in the cross-correlation function. Its diffusional part clearly shows the exclusive presence of double labeled LUVs, i.e. there is indeed no transfer of DiD from LUVs to SUVs. It is evident that such a quantitative characterization of supramolecular communication is very useful in studies of physiologically relevant processes.

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