To be published in Optics Letters:

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Authors: Serena Farina,Ivan Labanca,Giulia Acconcia,Alberto Ghezzi,Andrea Farina,Cosimo D'Andrea,Ivan Rech

Accepted: 15 November 21

Posted 15 November 21

DOI: https://doi.org/10.1364/OL.444815

Above pile-up fluorescence microscopy with a 32 Mcps single-channel time-resolved SPAD system

Serena Farina[1,*], **Ivan Labanca**[1], **Giulia Acconcia**[1], **Alberto Ghezzi**[2,3], **Andrea Farina**[3], **Cosimo D'Andrea**[2,4], **and Ivan Rech**[1]

[1] *Politecnico di Milano, Dipartimento di Elettronica, Informazione e Bioingegneria, via Golgi 40, 20133 Milan, Italy*
[2] *Politecnico di Milano, Dipartimento di Fisica, Piazza L. da Vinci 32, 20133 Milan, Italy*
[3] *Consiglio Nazionale delle Ricerche, Istituto di Fotonica e Nanotecnologie, Piazza L. da Vinci 32, 20133 Milan, Italy*
[4] *Center for Nano Science and Technology at PoliMi, Istituto Italiano di Tecnologia, via Pascoli 70/3, 20133 Milano, Italy*
[*] *Corresponding author: serena.farina@polimi.it*

Compiled November 15, 2021

One of the major drawbacks of Time-Correlated Single Photon Counting (TCSPC) is generally represented by pile-up distortion, that strongly bounds the maximum acquisition speed to few percents of the laser excitation rate. Based on a previous theoretical analysis, recently we presented the first low-distortion and high-speed TCSPC system capable of overcoming the pile-up limitation by perfectly matching the Single-Photon Avalanche Diode (SPAD) dead time to the laser period. In this work, we validate the proposed system in a standard fluorescence measurement by comparing experimental data with the reference theoretical framework. As a result, a count rate of 32 Mcps was achieved with a single-channel system still observing a negligible lifetime distortion.

http://dx.doi.org/10.1364/ao.XX.XXXXXX

Time-Correlated Single Photon Counting (TCSPC) is a time-resolved technique to perform optical pulse measurements in a wide variety of different applications, as Fluorescence Lifetime Imaging (FLIM) [1, 2], single-molecule analysis [3], underwater depth imaging [4] and many others. TCSPC experiments can indeed offer a much better sensitivity to analyze fast and faint light signals when compared to a classical analog acquisition technique [5].

Nevertheless, one of the major limitations of Time-Correlated Single Photon Counting is generally represented by pile-up distortion, that strongly bounds the maximum acquisition speed to few percents (1-5 %) of the laser excitation rate [5, 6]. For instance, a constrained maximum speed of 4 Mcps is adopted while employing a pulsed laser with a typical repetition rate of 80 MHz. Today, an overcoming of pile-up limitation is highly desirable as many advanced applications would greatly benefit from an increase in the measurement rate; among those it is worth mentioning single-pixel cameras [7–9] and fast FLIM techniques [10, 11].

Over the years, several approaches have been followed in literature to overcome the pile-up issue, ranging from the exploitation of multiple channels in parallel to detect more than one photon in one excitation period [6], to software corrections through post-processing algorithm [12]. A more radical and compact solution was presented by Cominelli *et al.* [13] in 2017, demonstrating that a perfect matching between the detector dead time and the laser period represents the key to avoid pile-up distortion even at high photon rates.

Based on this theoretical analysis, we recently developed a new TCSPC instrument that implements the proposed acquisition technique [14]. The overall system is constituted by two main modules: a Detection Head to host the Single-Photon Avalanche Diode (SPAD) and a timing module to acquire and digitally convert the photon timing information. In particular, the Detection Head features a fully-integrated Active Quenching Circuit (AQC) [15] capable of varying the applied dead time by acting on the quenching and reset control pins. Exploiting this feature, it is therefore possible to experimentally verify the relation between detector dead time and distortion.

More precisely, in this work we validate the novel low-distortion and high-speed TCSPC system [14], to finally demonstrate that the historical pile-up limitation can be overcome by matching the detector dead time to the laser period. To this aim, we performed a fluorescence measurement of fluorophore decay and we compared the obtained figures of merit (distortion and efficiency) with the numerically simulated graphs reported in the theoretical paper [13].

1. EXPERIMENTAL SETUP

The experimental setup is shown in Fig. 1. The illumination source is a supercontinuum laser (NKT Photonics, SuperK EXTREME EXW-12) with a repetition rate equal to 80 MHz. The employed laser features a plug-in acousto-optical tunable filter (AOTF, NKT Photonics SuperK SELECT multi-line tunable filter, 10 nm FWHM bandwidth), that allows to select an emitting wavelength within the absorption bandwidth of the desired fluorophore. The filtered laser beam is coupled from the AOTF

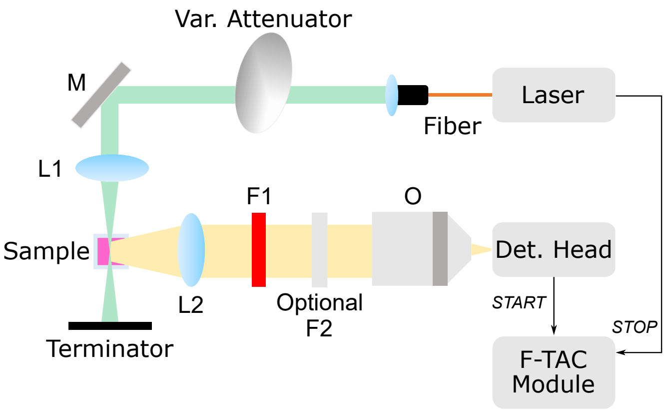


Fig. 1. Fluorescence experimental setup for the validation of the ultra-fast and low-distortion single-channel TCSPC system. L, lens; M, mirror; F, filter; O, objective.

graded-index fiber (NKT SuperK FD10) to a step-index fiber (300 μm diameter, NA 0.4); it is then collimated and finally passed through a circular neutral density variable attenuator. The selected attenuator is driven by a stepper rotating motor, that regulates the light transmitted to the fluorescent sample. With high attenuation, the average number of photons impinging on the detector is kept well below the pile-up limit, thus constituting a benchmark for fluorescence lifetime extraction. Conversely, to demonstrate the novel measurement technique, experiments were carried out by progressively increasing the incoming photon rates above pile-up. A spherical lens (L1, f=60 mm) focuses the transmitted light on the chosen fluorescent sample and the collected fluorescence, selected by a long-pass filter F1 (Thorlabs FELH0550), is then focused by a 10x microscope objective O (Melles Griot 160/0.17 10x/0.25) on a 50-μm custom-technology SPAD detector, placed in the system Detection Head. The Detection Head directly delivers the START signal to the TCSPC timing module, that receives the STOP signal from the laser itself. Furthermore, an optional neutral density filter F2 can be included in the setup when performing efficiency measurements, as will be clearly explained later.

Finally, it is worth noting that two major aspects have been taken into account while mounting and optimizing the described setup. First of all, it is of utmost importance to focus the final light spot exclusively on the active SPAD, as to avoid the arise of photocurrent in the dummy detector [14]. Otherwise, the undesired current of the dummy cell would have induced a variation of the timing electronic front-end threshold at different photon rates. Secondly, the setup is intended to deliver all the light power to the detector, in order to allow the highest acquisition rates predicted by theory (30-40 Mcps) [13].

The test samples consisted of two cuvettes, one filled with a solution of ethyl alcohol and Rhodamine B and the other one with ethyl alcohol and 4–dicyanomethylene–2–methyl–6–p–dimethylaminostyryl–4H–pyran (DCM). The chosen fluorophores feature a lifetime of respectively about 2.7 ns and 1.7 ns [16] and are excited at 520 nm.

2. EXPERIMENTAL MEASUREMENTS

As a first experiment, we evaluated the exponential lifetime distortion with respect to the detector dead time (Fig.2) at high photon rates (32 Mcps), as numerically simulated in the theoretical article. The distortion error was calculated as follows:

$$E[\%] = \frac{\tau_{meas} - \tau_{ref}}{\tau_{ref}} \cdot 100 \quad \textbf{(1)}$$

and it represents the percentage deviation of a measured decay, compared to the actual one. Considering that the fluorophore lifetime can slightly vary depending on the environmental and temperature conditions, we decided to take as the reference lifetime (τ_{ref}) the one measured with the exact same setup and instrument, yet with a count rate below pile-up limit (i.e. 2.8 Mcps), thus corresponding to a theoretical lifetime distortion of 0.8 %.

The measurement was carried out by progressively increasing the detector dead time, by acquiring the emitted decay curves and estimating their corresponding lifetimes. Since the Detection Head features a high-performance Active Quenching Circuit (AQC) [15], it is possible to fine-tune the applied dead time by acting on voltage signals that directly control the duration of the sensor quenching and reset time. This operation is easily performed through an external Universal Asynchronous Receiver-Transmitter (UART) connection with the power board of the Detection Head module. The sensor reset time was set to a fixed value, in order to achieve the shortest possible reset interval and to consequently maximize the system efficiency[13]. Instead in both the distortion and the subsequent efficiency experiments, we swept the detector dead time from almost 12 ns up to over 30 ns, corresponding to a quenching voltage control between 0.12 V and 0.87 V. In this way, it was possible to cover more than an entire laser period, including two main points of interest, corresponding to a dead time of 12.5 ns and 25 ns. Indeed, according to the theory, dead times that are integer multiples of the laser period correspond to distortionless measurements, for the reason that a single exponential repetition is entirely masked and the periodicity of the experiment is thus preserved. It is worth mentioning that the relation between the quenching control voltage and the resulting dead time is not linear; for this reason, we built a lookup table that allowed us to remap the measured lifetime distortion as a function of the dead time.

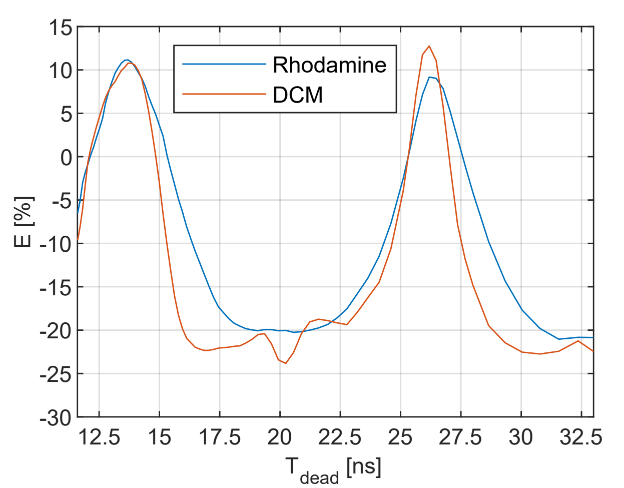


Fig. 2. Lifetime distortion error of Rhodamine B and DCM is reported with respect to detector dead time with a photon rate of 32 Mcps. By choosing an integer multiple of the laser period as dead time (12.5 ns, 25 ns, etc.), almost zero distortion can be achieved even far above pile-up.

The obtained experimental curves (Fig.2) faithfully follow the periodical behaviour of the simulated results already reported in Cominelli *et al.* [13]. In particular, for both the Rhodamine B and DCM fluorophores, the graph features two fixed points characterized by almost zero distortion in correspondence of the integer multiples of the laser period, i.e. 12.5 ns and 25 ns. Two other zero distortion intersections are present, still they can not be used in practical experiments as they directly depend on experimental parameters, such as the measured lifetime. The obtained results clearly validate the previous theory and demonstrates that pile-up limitation is definitely overcome in our single-channel TCSPC system, provided that a matched dead time is employed for the SPAD detector. The system is thus paving the way to those advanced applications where high-speed represents an enabling factor.

To better illustrate how pile-up distortion rises with variable dead time, in Fig. 3 we reported some significant exponential curves acquired from Rhodamine B. The reference curve (black) was measured at low count rates below pile-up (2.8 Mcps) with a non-matched dead time. Similar curves (overlapping) have been found at high rates (32 Mcps) but with a dead time equal to 12.5 ns. At this point, we increased the dead time still keeping the high rate, thus obtaining the distorted curves reported in the figure. In this scenario, two different cases can be distinguished: when the dead time exceeds the laser period by a quantity that is comparable to the time span of fluorescence, a remarkable change of slope is observed in the central part; on the contrary, with longer dead times the distorted curves are described by the classic pile-up model [5]. It is worth noting that all the obtained curves are in good accordance with previous theoretical studies presented in [6, 13], where the detector dead time was considered as the unique source of pile-up. In our system indeed the timing dead time can be considered negligible thanks to the adopted timing architecture [17].

Besides demonstrating the effectiveness of dead time matching on lifetime distortion, it is of utmost importance to quantify the counting efficiency of our system, as to show that high rates can be actually achieved with the proposed technique. To this aim, two different measurements have been carried out on Rhodamine B and the obtained results are reported in Fig. 4.

The first experiment was performed concurrently with lifetime distortion estimation and consisted in the measurement of the average number of recorded photons per period (P_{rec}) when varying dead time. The obtained experimental graph (Fig. 4a) features a step behaviour that is consistent with both theory and intuitive explanation: a rapid decrease in efficiency is indeed present around integer multiples of the laser period and corresponds to a progressive masking of the fluorescence pulse by dead time. Once the whole light signal is masked, the achieved efficiency reaches an almost constant plateau until the subsequent integer multiple of the laser period is reached. Some small differences can be noticed with respect to the theoretical graph, in particular concerning the slight slope of the first plateau and the expected values for P_{rec}. Nevertheless, these discrepancies can be attributed to the fact that the theoretical graph is still not taking into account the photons impinging during the detector reset phase, while our practical measurement also involves this non-ideal effect.

To better visualize the efficiency reduction introduced by photons in the reset phase, we performed one last measurement and we plotted the average number P_{rec} of recorded photons in a period as a function of the average number P of impinging photons in a period (Fig. 4b) with a dead time of 12.5 ns. Due to setup limitations, it is not feasible to directly measure the

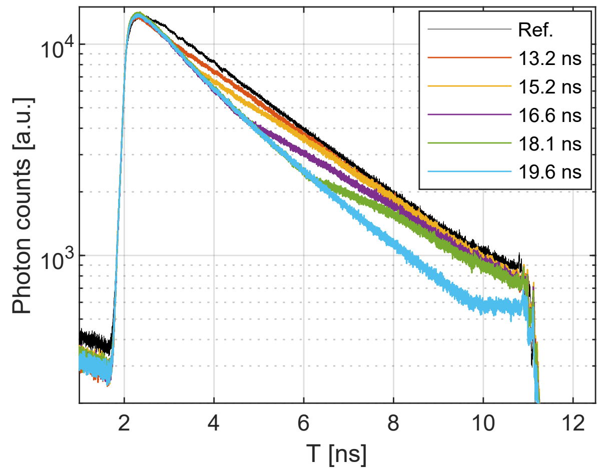


Fig. 3. Exponential waveforms of Rhodamine B showing how pile-up distortion affects lifetime estimation at different dead times. The reference curve (mono-exponential fitting with $\tau = 2.78ns$ and $\chi^2_R = 0.992$) is acquired with a count rate well below pile-up, while the other curves are acquired at 32 Mcps.

value of the actual photons impinging on the 50-μm detector active area, i.e. the value P. For this reason, we resorted to an indirect estimation of the P quantity by inserting an optional neutral density filter, namely F2, in the experimental setup. On the other hand, the value of the recorded photons P_{rec} could be easily retrieved from the TCSPC system itself.

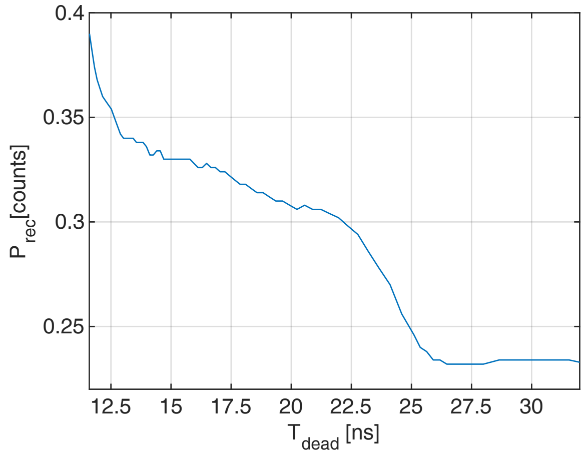
As a first step, we characterized the F2 filter attenuation parameter A at very low rates (50 kcps), by measuring the ratio between the incoming photon rates with the filter and without it. If both the acquired photon rates are sufficiently low, the system is not experimenting any counting loss related to the finite dead time, and the calculated attenuation faithfully replicates the real value. Afterwards, we progressively increased the optical power and once again we measured the achieved photon rates both with and without the filter. In this case, the value of P_{rec} simply corresponds to the photon count rate without filter normalized by the laser excitation rate. Conversely, the value of P can be inferred by applying to the photons counts with filter both the effect of the filter itself and the frequency normalization, as in the subsequent equation:

$$P[counts] = \frac{C_{filter}[Mcps]}{A \cdot f_{laser}[MHz]} \qquad (2)$$

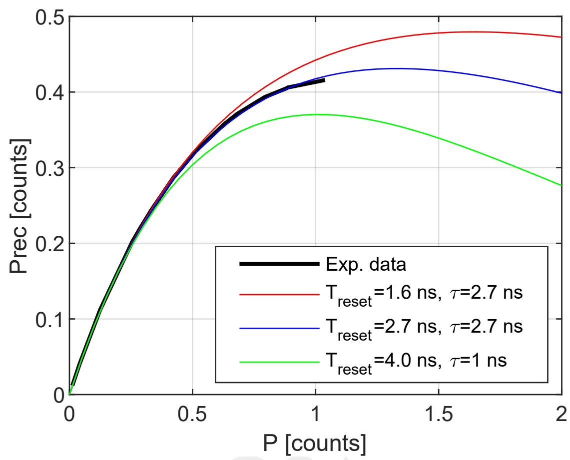
where C_{filter} represents the count rate with filter, A is the filter attenuation and f_{laser} is the laser frequency. In order to guarantee a correct estimation of the P value, the photon rate achieved with filter should be sufficiently low, as to avoid counting loss phenomena. For this reason, it is important to select a filter that is capable to attenuate also the highest photon rates corresponding for instance to a P value of 1 (i.e. 80 Mcps) or higher.

The described procedure was repeated with different optical powers and the obtained experimental curve (Fig. 4b in black) was compared toward the theoretical equation derived in [13], that describes the relationship between P and P_{rec}:

$$P_{rec} = \frac{P}{exp\left[P \cdot \left(1 - e^{-\frac{T_{reset}}{\tau}}\right)\right] + \frac{exp\left[P \cdot \left(1 - e^{-\frac{T_{reset}}{\tau}}\right) - 1\right]}{e^{\frac{T_{reset}}{\tau}} - 1}} \qquad (3)$$



(a) Average number of photons recorded in a period with respect to detector dead time. In correspondence of the integer multiples of the laser period a steep decrease in P_{rec} is observed.



(b) Average number of photons recorded (P_{rec}) in a period with respect to the average number of photons impinging (P) in a period with a dead time of 12.5 ns. With P=1 a count rate of 32 Mcps is reached.

Fig. 4. Efficiency measurements for the novel TCSCP system

where T_{reset} represents the finite reset interval of the SPAD detector and τ is the fluorophore lifetime. The green and lower curve in Fig. 4b is the estimation presented in the theoretical article, considering T_{reset}= 4 ns and τ= 1 ns. This represents a worst case scenario, due to the high value of T_{reset} and the small value of τ. The red and higher curve employs our experimental and system parameters, i.e. T_{reset}= 1.6 ns from previous measurements [14] and τ= 2.7 ns of Rhodamine B. Finally, the blue curve represents the best interpolation for the experimental data, obtained from Eq. 3 by varying the T_{reset} value with the fixed lifetime of Rhodamine B. The small difference between the previously measured reset value (1.6 ns) and the inferred one (2.7 ns) could be attributed to second-order effects or non-idealities, like photon masking during reset, that are not taken into account in the mathematical derivation. Despite this, the obtained results show a good accordance with the theoretical predictions also for the efficiency performance. A significant increase is indeed achieved in the count rate, going from the 5% acquisition speed limitation imposed by pile-up to about 40% speed with the novel low-distortion technique.

In conclusion, in this paper, we presented the experimental validation of a novel high-speed and low distortion timing system, that allows us to definitely overcome pile-up limitation in TCSPC experiments. By precisely selecting the dead time, the acquisition rate of a single TCSPC channel was pushed up to 32 Mcps in a standard fluorescence measurement. Even though the instrument is specifically designed to target FLIM experiments (e.g. Förster Resonance Energy Transfer), we believe that the proposed approach is highly promising towards the speed-up of many other advanced applications, such as single-pixel cameras. Future work will then be devoted to the extension of the technique to a multichannel system to bring the advantages of this single-channel approach into a typical parallel channel configuration.

Funding. Consiglio Nazionale delle Ricerche (3710, Bi-lateral project CNR-RS); Regione Lombardia (NEWMED, POR FESR 2014-2020); Horizon 2020 Framework Programme (777222).

Disclosures. The authors declare that there are no conflicts of interest related to this article.

Data availability. Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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