Note: Rapid measurement of fluorescence lifetimes using SiPM detection and waveform sampling

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In fluorescence spectroscopy and imaging, fluorescence lifetime measurement—assessing the average time fluorophores spend in their excited state before returning to their ground state—offers a number of advantages over quantifying fluorescence intensities that include resistance to photo-bleaching and independence from fluorophore concentration, excitation intensity, and measurement methodology. Despite growing interest, fluorescence lifetime techniques frequently mandate relatively complex instrumentation, slow data acquisition rates, and significant data analyses. In this work, we demonstrate the feasibility of measuring fluorescence lifetimes using off-the-shelf analog silicon photomultipliers and switched-capacitor array waveform sampling techniques, with precision matching that of much larger and more elaborate commercial instruments. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5003413>

To achieve greater sensitivity in fluorescence imaging, considerable effort has been made in developing techniques that do not rely upon spatially mapping a reporter's concentration via its fluorescence intensity; measurements that are inherently sensitive to excitation intensity, detection method, and photon scatter and absorption. Instead, many of these intensity-independent imaging techniques, like fluorescence lifetime imaging microscopy, focus on spatially mapping the characteristic time dependence of a reporter's fluorescence decay. Such approaches are not only independent of fluorophore concentration but are also considerably less prone to reporter photo-bleaching and can offer additional information regarding a reporter's local microenvironment (e.g., pH, oxygenation and ionic strength). Unfortunately, many implementations of fluorescence lifetime imaging—especially those operating in the time-domain—require complex equipment and suffer from relatively slow data acquisition rates that greatly restrict their wide-field real-time imaging utility. Recent trends, towards the use of multiple fluorescent probes to report multiple targets simultaneously, often face further constraints in measurement sensitivity that arise from the overlap of their various reporters' respective excitation and emission spectra—even when spectrally unmixed.¹

At present, one of the more versatile and accurate means of measuring fluorescence lifetimes is through the use of time-correlated single-photon counting (TCSPC).¹ In this technique, a pulsed laser is used to transiently excite the specimen and the ensuing fluorescence emission decay is recorded—one photon at a time. This process is repeated, with each photon's arrival time being histogrammed by a time-to-digital converter (TDC), to obtain the sample's fluorescence response curve. Subsequent curve fitting of the

decay portion of the sample's fluorescence response curve is then performed to derive the optical reporter's fluorescence lifetime. Critical to the validity of this approach are the inherent assumptions that, during each excitation cycle, the probability of having more than one fluorescence photon arrival (termed pile-up) is negligible and that the lifetime of the fluorophore is much shorter than the excitation cycle's duration. As such TCSPC approaches are inherently slow since, per excitation cycle, only one photon is detected—with many excitation cycles being required to build each fluorescence response curve. Lasers possessing higher repetition rates can be used to accelerate the rate of data acquisition. However, in doing so, one limits the length of fluorescence lifetimes that can be analyzed due to the next excitation pulse's arrival.

To date, the most commonly used detectors in TCSPC have been photomultiplier tubes (PMTs) and avalanche photodiodes (APDs) due to their high signal-to-noise ratios (SNRs). (The long duration “tail” of the APD's impulse response is of little detriment as only the initial fast rising portion of the fluorescence curve is used by the TDC.) Recently, however, silicon photomultipliers (SiPMs) have garnered attention for their potential use in fluorescence lifetime studies as they afford both the chief advantages of conventional silicon APDs (smaller size, lower voltage operation, and greater mechanical robustness) and the chief advantages of PMTs (greater SNR and stability). To this end, Tyndall *et al.*² developed a single CMOS chip that combined a digital SiPM with an array of TDCs to permit the detection of multiple fluorescence photons per excitation cycle and reduce, by over an order-of-magnitude, conventional TCSPC pile-up. The chip, not commercially available, also possessed embedded electronics for the on-board calculation of fluorescence lifetimes. Rocca *et al.*³ subsequently used this unique platform to achieve

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FIG. 1. (a) Board that contains two Hamamatsu S10931-050P SiPMs. (b) Configuration for fluorescence lifetime measurements. (c) A compact DRS4 evaluation board providing 4 waveform-sampling 5 GS/s channels.

real-time fluorescence lifetime detection for cell sorting during flow cytometry.

In the present work, we demonstrate, for the first time, the feasibility of making fluorescence lifetime measurements using off-the-shelf analog silicon photomultipliers (SiPMs)⁴ and switched-capacitor array based waveform sampling techniques.^{5,6} Comparison of the fluorescence lifetimes derived from this simple cost-effective system to those made on a much larger and more elaborate commercial instrument revealed a surprisingly high degree of correlation and reproducibility.

The optical apparatus used in this work is shown in Fig. 1. A 405 nm pulsed laser diode (PiL040X, A.L.S. GmbH) was operated at a repetition rate of 1 MHz, generating 45 ps duration pulses that averaged less than 2 mW total optical output power. A collimation lens was then used to inject the diode's output into an optical fiber at the proximal end, while another collimation lens was used to expand the distal end's emerging light into a 2 mm diameter beam of low divergence—for sample illumination within a semi-micro cuvette. A discrete SiPM (S10931-050P Hamamatsu) was positioned such that it accepted fluorescently emitted light from the specimen, orthogonal to the incident excitation beam.

The SiPM's output was fed directly to a 4-channel DRS4 (Domino Ring Sampler ver. 4, Paul Scherrer Institute—PSI) evaluation board using a method previously reported by us for precise timing correction, with up to 30 ps timing resolution.^{5,6} The DRS4 chip has 8 sampling channels, each 1024 cells deep, that can be daisy-chained to attain virtually unlimited sampling depth at frequencies ranging from 0.7 GS/s (giga-samples per second) to 5 GS/s. On-chip phase locked loop (PLL) is used to stabilize the sampling frequency with a jitter of less than 30 ps. The stored voltage samples of the capacitor cells were read by a 14-bit ADC running at 33 MHz to yield sample readout times of 30 ns. A 31 μ s latency time was observed in reading out all the 1024 samples used in this study; a period easily reduced by reading fewer samples.

Transmission measurements of identical path length were made of a cuvette that contained our fluorescent specimens' solvent (ethanol *sans* fluorophore) to derive our system's instrument response function (IRF), in analogy to those employed by others,⁷

$$f_I(t) = \begin{cases} (A_1 + A_2)(t - t_0/t_r), & t_0 \leq t \leq t_0 + t_r, \\ A_1 e^{-t-(t_r-t_0)/\tau_l^{(1)}} + A_2 e^{-t-(t_r-t_0)/\tau_l^{(2)}}, & t_0 + t_r \leq t, \end{cases}$$

where t_0 is the time at which the optical pulse commences and t_r is the rise-time to the maximal detected intensity. Nonlinear curve fitting of the above equations to the measured data

FIG. 2. Normalized/digitized fluorescence response curves for various dyes studied: Coumarin 6 (blue), Coumarin 314 (green), and Coumarin 343 (black), as well as system's IRF (red).

yielded $A_1 = 2.87 \times 10^{-2}$, $\tau_l^{(1)} = 30.91$ ns, $A_2 = -4.02 \times 10^{-4}$, $\tau_l^{(2)} = 261.8$ ns, and $t_r = 2.36$ ns.

As shown in Fig. 2, fluorescence response curves were recorded and fitted for each fluorescent dye dissolved in ethanol—Coumarin 6 (blue), Coumarin 314 (green), and Coumarin 343 (black)—and the following model was used to derive each reporter's fluorescence lifetime τ_{dye} :

$$f_{dye}(t) = A f_I(t) \otimes \left[e^{-t/\tau_{dye}} \cdot U(t) \right], \quad U(t) = \begin{cases} 1, & t \geq 0, \\ 0, & t < 0. \end{cases}$$

Thus, in contrast to conventional TCSPC techniques, our approach acquires a complete fluorescence response curve for each laser excitation, thereby accelerating data acquisition. Although the SiPM's response recovery is relatively slow, and the fluorescence response curve is a convolution of the specimen's fluorescence emission curve with the IRF, precise fluorescence lifetimes can readily and rapidly be obtained, as we demonstrate below. Muretta *et al.*⁸ reported a somewhat similar approach albeit with a more sophisticated, crate-based high-speed digitizer for waveform sampling of a fast PMT's output.

To minimize the deleterious effects that small hardware instabilities had on curve fitting—occasionally manifested in long decay-time data—we constrained the temporal window of fluorescence decay curve fitting to 268 ns based upon empirical findings and fitting convergence rates. Fluorescence response curve fits made with this temporal sampling constraint, averaged over 10 000 excitations for 20 nM concentrations of all 3 Coumarin dyes investigated, are presented in Fig. 3. Comparison of these data to those obtained, on the same samples, from a ChronosFD fluorescence lifetime spectrometer (ISS, Inc.) via single-exponential decay fitting revealed excellent agreement, with our system-derived/ChronosFD fluorescence lifetimes being 2.69 ns/2.64 ns for Coumarin 6, 3.41 ns/3.38 ns for Coumarin 314, and 3.97 ns/4.00 ns for Coumarin 343.

Figure 3 shows the typical fitting performance of our technique over ~ 10 -15 lifetime periods. Statistical analyses of fluorescence decay fittings revealed non-randomly distributed residuals for all but the lowest concentration of fluorophores studied, when employing datasets comprised of 10 or more excitation pulses. The non-random nature of these residuals,

FIG. 3. Lifetime fitting analyses of 3 Coumarin fluorophores at 20 nM using 10^4 excitations/fitting: Coumarin 6 (blue), Coumarin 314 (green), and Coumarin 343 (black). Corresponding residuals for each fluorophore appear in the lower panel.

currently under investigation, suggests a bias in IRF fitting that might be obviated by direct inclusion of the raw IRF data in fitting of the fluorescence lifetime τ_{dye} .

To verify that our derived fluorescence lifetimes possessed no fluorophore concentration dependence, we evaluated 4 different concentrations of each fluorophore, averaged over 10 000 excitation events. As shown in Fig. 4, no significant concentration dependence was observed in derived fluorescence lifetimes. To assess our algorithm's precision and stability in deriving fluorescence lifetimes at low fluorophore concentrations, we then conducted a series of titration studies, also summarized in Fig. 4. In these studies, we found that our algorithm accurately determined fluorescence lifetimes down to ~ 2 nM, but demonstrated a modest (within measurement error) dependence on the number of events sampled when fluorophore concentrations were reduced to 0.5 nM levels—posited to reflect, in part, the consequences of the small specimen volume excited and sampled by our SiPM's optical fiber and its impact on sampling statistics.

In summary, we have demonstrated, for the first time, the feasibility of making rapid fluorescence lifetime measurements using analog silicon photomultipliers and waveform sampling techniques. Single exponential fitting of the convolution of the system's response function with each dye's intrinsic fluorescence intensity decay function yielded fluorescence lifetimes in close agreement with those derived from measurements made on a bulky commercial TCSPC-based system. Moreover, to achieve 10% fluorescence lifetime

FIG. 4. Derived fluorescence lifetime as a function of the number of laser excitation pulses (events) sampled. Coumarin 6 (blue), Coumarin 314 (green), and Coumarin 343 (black).

accuracy in single-exponential decay analyses, each lifetime measurement via TCSPC takes ~ 250 μ s (~ 200 photons)— $8\times$ longer than the DRS4's latency period—while comparable multi-exponential decay analyses via TCSPC mandate 1-2 orders-of-magnitude more photons and time.⁹ Current studies are aimed at improving fluorescence lifetime precision, characterizing noise, expediting signal processing/fitting, proofing against dyes with multi-exponential decays, and extension of the technique to endoscopy and laparoscopy.

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