Real-time Fluorescence Lifetime Acquisition System (RfLAS)

Rok Dolenec,[1*] Andrej Seljak,[2]

[1]Institute Jozef Stefan, Jamova cesta 39, 1000 Ljubljana, Slovenia; [2]Fondazione Bruno Kessler, via Sommarive 18, 38123, Trento – Povo, Italy
*Corresponding author: rok.dolenec@ijs.si

ABSTRACT

Fluorescence lifetime (FL) measurements provide information on the molecular makeup of a sample. They are used in a wide range of applications in life sciences and industry. Typical techniques require expensive instrumentation and long acquisition times. The RfLAS project demonstrated a FL acquisition system that is extremely fast, compact and significantly less expensive than current approaches. Further integration of the system in a single chip will make FL measurements more attractive for industrial applications, such as pharmacy and food industry and make FL experiments more affordable for many fields of science and medicine.

Keywords: Fluorescence lifetime; silicon photomultiplier; waveform sampling

1. INTRODUCTION

Fluorescence is the emission of light by certain substances (fluorophores) after they are illuminated with light of specific excitation wavelengths. Measurements of the fluorescent light emitted by various samples are used in a very wide range of applications, such as imaging of cell structures, tracking of antibodies and DNA sequencing in biology, detection of cancer cells in medicine and quality control in pharmacy. Besides intensity, the fluorescence lifetime (FL) can also be measured, as pioneered in application of fluorescence lifetime imaging microscopy (FLIM). This has many advantages over the base method, such as independence from fluorophore concentration, reduced damage to the sample (photobleaching) and ability to measure properties of the microenvironment in which the fluorophore is located (pH, oxygenation…).

Currently, FL measurements require sophisticated and expensive instrumentation. Typically, the fluorescence lifetime is determined with time correlated single photon counting (TCSPC) method, which is intrinsically slow. Mature technological developments in the field of high energy physics (HEP) enable direct waveform sampling technology as important and a very cost effective tool for fast FL applications. By measuring the photodetector signal resulting from complete fluorescence response, FL can be estimated even from a single excitation pulse.

Real-time Fluorescence Lifetime Acquisition System (RfLAS) was assembled from low cost, commercially available components in order to demonstrate the feasibility of such approach. Calibrated FL standards with lifetimes in the range of 2 ns – 9 ns were used to test RfLAS accuracy and performance for different levels of available fluorescence light intensity and photodetector configurations. Using our prototype, we show that FL of all tested fluorescence standards could be measured with an accuracy better than 10% from only a single pulse of excitation light, which improves below 1% level by averaging over only a few tens of pulses. Therefore, RfLAS demonstrates that FL can be acquired practically in real-time for a much lower price point than current state of the art.

The three critical components – the photodetector, waveform sampler and data processing algorithms – lend themselves perfectly for implementation in a single chip. These are also areas of expertise of the authors, and the institutes they are affiliated with. The envisioned integrated detector would push the performance and robustness beyond the present state, and more importantly, using CMOS technology at scale, would collapse the price per unit, opening possibilities to use FL obtained information in much wider areas as currently available.

2. STATE OF THE ART

In TCSPC method, FL is determined from a histogram of measured time delays between excitation pulses and individual fluorescence photons, resulting from said excitations. If more than one photon is detected per pulse, the accuracy is degraded (pile-up effect), so the fluorescence signal has to be at a single photon level. The excitation pulse has to be repeated many times in order to obtain sufficient time delay histogram statistics,

leading to long acquisition times and possible photobleaching of the sample.

The acquisition times are even longer if imaging is required. In this case, laser excitation is scanned over the sample, and sufficient TCSPC statistics have to be accumulated for each scan position (image pixel). Alternative imaging approach is possible with single photon avalanche diode (SPAD) arrays, recently developed specifically for FL application with time-to-digital converters (TDC) implemented on a single chip. These devices have an intrinsic limiting factor, the sensitive area is somewhere between 1% and 20 % [1] as most of the space is used for electronics, and prototypes have a relatively small pixel count.

FL is also measured using frequency-domain technique, where it is derived from phase shift between modulated excitation illumination and resulting modulation in fluorescence signal, and gated detection, where FL is estimated from ratios of fluorescence signal at specific time gates.

Currently, FL measurements require sophisticated and expensive setups, and certain time to reconstruct the FL. In case of imaging, a few frames per second can be achieved at best for sufficient image resolutions [2].

3. BREAKTHROUGH CHARACTER OF THE PROJECT

In our project we explore FL in combination with direct waveform sampling (DWS). After excitation light interacts with the sample, the output pulse of the photodetector is shaped as convolution of the instrument response function (IRF, itself a convolution of photodetector response and excitation pulse) and fluorescence decay. By comparing the measured pulse waveform with previously determined IRF, FL can be calculated. This approach requires a very fast photodetector and signal sampling electronics. Although micro channel plate photodetectors are suitable, these are not very robust, and are very expensive. The use of silicon photomultiplier (SiPM) photodetectors represent an unquestionable advantage. DWS on chips is a mature technology, which is in use at several HEP experiments in harsh environments. DWS has the potential to use the whole information carried by the fluorescence pulse. Thus, enough information to accurately measure the FL can be collected faster, with a smaller number of excitation pulses. When sufficient number of fluorescence photons can be detected, the FL can be reconstructed even after a single excitation pulse, enabling real-time acquisition.

The SiPMs have high photon detection efficiency, single photon sensitivity, they are robust, compact, and operate at safe voltage levels. The price for these devices on the commodity market ranges around 10€. DWS technology is nowadays available on commercially available chips offering multiple acquisition channels, which makes them very suitable for FL measuring applications. Waveform sampling of the SiPM output can be achieved with a sampling rate of 5 Gsamples/s using DRS4 [3] (domino ring sampler) chip, commercialized at a price around 100 € per channel. Besides the excitation light source, other required components do not present significant additional cost. Compared to the current commercially available systems, which cost on the order of 10.000 €, significant reduction in price is possible, with the benefit of having a simpler device that surpasses in acquisition times the state of the art. Availability of such cost effective real-time FL measurement would stimulate scientific research and increase efficiency of many industrial processes. Just some of the application benefiting are in medicine (differentiation of diseased from healthy tissues), pharmacy (monitoring of biopharmaceutical production), biology (in-vivo cell tracking) and cultural heritage (non-invasive studies of works of art).

4. PROJECT RESULTS

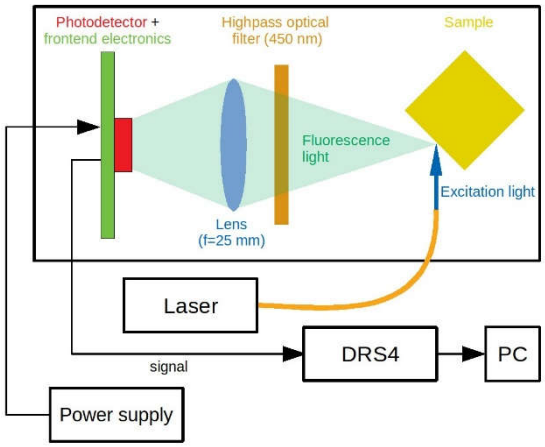
The RfLAS demonstrator was designed to measure FL of samples contained in standard spectroscopic cuvettes. The principles of operation are illustrated in Fig. 1. The laser excitation light is illuminating the sample. Fluorescence light emitted by the sample is collected with a simple bi-convex lens on the surface of the SiPM photodetector. A high pass optical filter is positioned in the light path, to block direct excitation light from contributing to fluorescence signal measurement, but is removed for IRF acquisition. The SiPM signal is digitized by the waveform sampler and processed on a computer.

Results obtained for three fluorescence standards are presented: fluorescein in a 1 mMol, pH = 1.0 solution and two commercial standards (SETA BioMedicals KS-370 and KS-405). For the commercial standards, the FL were provided by the producer to be (3.18 ± 0.04) ns and (9.07 ± 0.03) ns, for KS-370 and KS-405, respectively. FL of the fluorescein solution was measured to be (2.2 ± 0.1) ns by 2-photon laser excitation using a laser scanning confocal microscope via fast detection TCSPC system (Becker&Hickl).

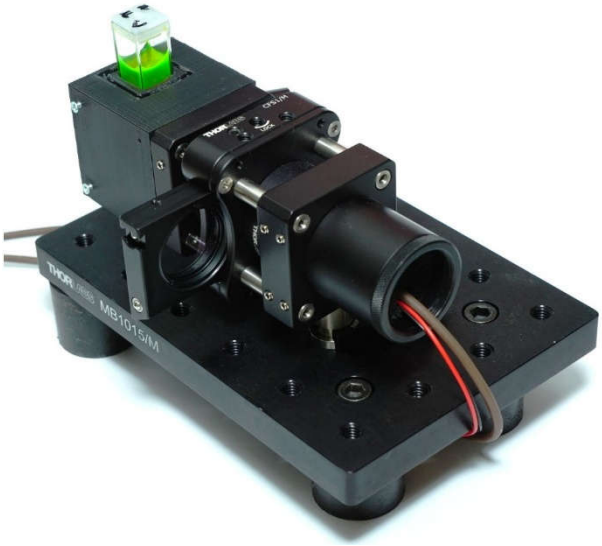
Two SiPMs produced by Fondazione Bruno Kessler (FBK) were tested: NUV-HD low field device, with 15 µm micro cells and six 1 mm^2 SiPMs connected in parallel, and NUV-HD high field device, with 40 µm micro cells and 4x4 mm^2 active area. While the 15 µm device has better dynamic range and timing response, the SiPM with 40 µm micro cells has better photon detection efficiency and could be used when ultimate efficiency is needed. The SiPMs were operated at bias voltages of 40.0 V (8.0 V overvoltage) and 35.2 V (6.5 V overvoltage), for 15 µm and 40 µm device, respectively.

As a source of excitation light 403 nm laser (PiLas diode laser system EIG1000D, pulse width

35 ps FWHM) was used. The laser intensity was controlled with neutral density filters and the light was guided to the sample using multi-mode optical fibre forming a spot of about 1 mm radius at sample surface.



(a)



(b)

Fig. 1. Schematic (a) and photo (b) of the RfLAS demonstrator.

Waveform sampling was achieved using a DRS4 evaluation board. One channel running at 5 G samples/s was used, with the acquisition triggered by the SiPM signal itself.

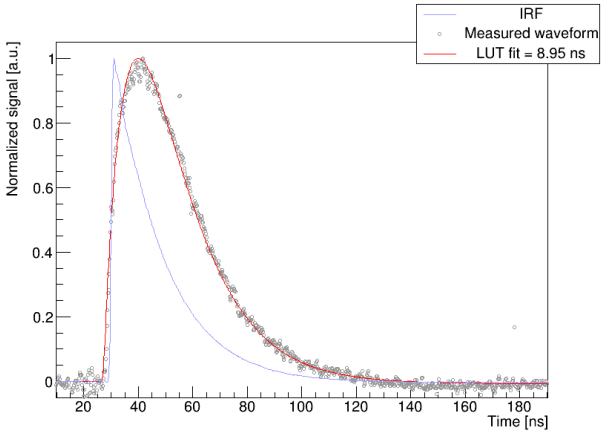
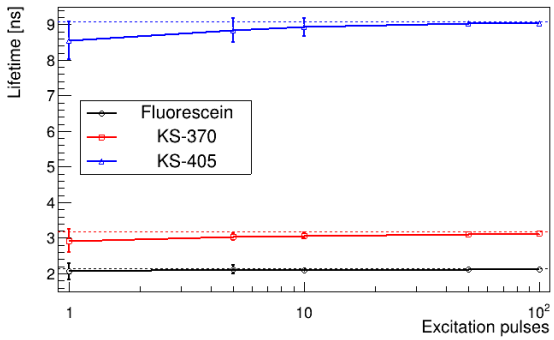


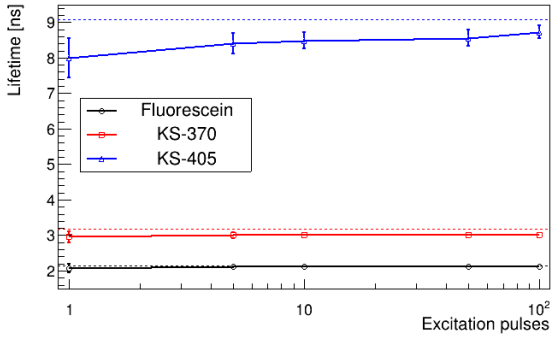
Fig. 2. Example of data analysis: the instrument response function (blue line), fluorescence response waveform acquired from single excitation pulse (grey dots) and resulting lifetime fit (red line). Here, KS-405 standard and SiPM with 15 µm micro cells were used.

To calculate the FL, a fast look-up-table (LUT) approach was used. First, the measured IRF was convoluted with single exponential decay functions, for a predetermined set of lifetimes, and the so obtained LUT of possible waveforms was saved to memory. Then, the acquired fluorescence signal waveform was compared to the LUT set, and the closest matching lifetime was obtained using simple root-mean-square fitting. This algorithm is fast and hardware inexpensive, but proved to be reliable and robust. Examples of the measured waveforms and the resulting fit are shown in Fig. 2.

The performance of FL measurements using RfLAS is summarized in Fig. 3. The FLs were calculated for different number of excitation pulses, used to form an average fluorescence response waveform fed into the fitting algorithm. With 15 µm micro cell SiPM (Fig. 3a), excellent consistency with the reference values obtained using state of the art techniques was achieved. With all three standards, lifetimes in the range of 2 ns – 9 ns were acquired with an accuracy better than 10 % when single excitation pulse was used. By averaging over a larger number of excitation pulses, accuracy better than 1 % was possible. With the 40 µm micro cell SiPM (Fig. 3b), larger discrepancies were observed, due to lower dynamic range of the SiPM and relatively large light pulses used. With a larger fraction of SiPM micro cells triggered by the initial phase of the light pulse, and still in recovery during later phases, the shape of the waveform is biased towards shorter lifetimes. Another artefact was observed in the systematically lower FL measured with smaller number of excitation pulses. This was also observed in [4] and needs further study.



(a) 15 µm micro cells



(b) 40 µm micro cells

Fig. 3. Fluorescence lifetimes acquired using RfLAS, for waveforms averaged over different numbers of excitation pulses in case of SiPM with 15 µm (a) and 40 µm micro cells (b). The reference values for the three standards are shown as dashed lines.

The system was also tested using more cost effective laser LED excitation, and other SiPMs and fluorophores (results not shown here). The accuracy of the RfLAS was comparable to results shown here even for slower excitation and for a wide range of samples tested, demonstrating potential for a wide applicability of this method.

5. FUTURE PROJECT VISION

RfLAS project uses mature technology developed for HEP, and aims it primarily at medical, biomedical, biotechnology and pharmaceutical fields, all of which experience significant market growth. We expect this technology to enter the biotech market, which alone is expected to hit 727b$ in 2025 [5], providing tangible benefits for society. Many emerging applications require sensors with a wide field of view, good spatial resolution and very fast acquisition times - a parameter envelope not yet reached by present research. Our goals are to develop a device that uses a wide field illuminator (diffused laser) and a wide field detector, using a single laser pulse, capable of continuous sub millisecond frame rates.

5.1. Technology Scaling

In our development plan, firstly, from the selected off the shelf components, a highly integrated multi-channel version of the Phase I device will be built. It will be fully decoupled from laboratory equipment; therefore, it can be lent or sold to early adopters. These are crucial for us, we need early feedback, dissemination, and to validate and demonstrate the device in a real operational environment. An extremely important aspect is also presence on the market. Having a community of users, and a device that can be demonstrated in real operating environments will create the foundation for the next step.

Second, having built up the necessary experience, and deep understanding of the system, we will make an integrated scalable sensor, the real breakthrough in FL high speed imaging. The sensor will integrate efficiency optimized SiPMs, bump bonded to the electronics wafer, which could be produced in different technologies, with different performances, for different applications.

Taking in consideration mass production, these sensors can be made at a very competitive price. CMOS technology is also very affordable at scale, has a known roadmap and is very well supported. These factors provide a secure path to aggregate scalable solutions.

5.2. Project Synergies and Outreach

During Phase I, we discussed cooperation with potential users and partners, including another ATTRACT project. We will be able to quickly form a consortium capable of advancing RfLAS. Laying the foundation in the dissemination program, we should build quickly a community of users to provide application test cases and feedback, and most importantly increment to TRL 5-7.

For additional dissemination, we intend to leverage one of the strong points of our technology, its simplicity. We will take an abundant amount of knowledge gained during Phase I, and develop an open source, open hardware, single channel FL acquisition toolkit, composed of hardware solutions based on off the shelf components, data acquisition software and library of end-user experience. The feedback and exposure will directly benefit the project, and increase the speed of development.

5.3. Technology application and demonstration cases

Measurement of FL is a still growing field of research with many applications not realized. A technique, improved in acquisition speed, and even more importantly, lower entry cost, has the potential to advance many fields of science and open new industrial applications. We have discussed concrete applications

with potential users, including a pharmaceutical production company, high tech company developing monitoring and metrology technology for food industry and national health institute. With just this batch of early adopters, RfLAS would improve development and monitoring of biopharmaceuticals production; increase the quality of food available to consumer and reduce wasted food by measuring the ripeness of fruits and detecting presence of bacteria on food products; advance the accuracy and speed of diagnostics of histological samples; and contribute to a wide range of material science research.

5.4. Technology commercialization

We are in the process of obtaining IP protection for the core aspects of our development, with patent applications currently filed in UK and European offices. We are in talks with two companies interested in technology, with one we are in the process of signing NDA. Other private entities expressed interest for the development of front ends and data display software. The multichannel instrument will support our commitment to advance as quickly as possible to the integration step of our development program, to enable the community and users to have on disposal a price competitive and robust instrument for their application.

5.5. Envisioned risks

Our main target is the development program of highly integrated sensors, potentially having some degree of data processing on chip. Modelling, design, production, assembly and testing of such devices are, in a vast majority, also areas of expertise of the authors [6, 7], and the institutes they are affiliated with. We intend to prepare a simulation of such a device, to predict its performance and share the performance envelope with early adopters to shape its final form. The physical aspect requires multiple R&D cycles which is slow and costly. To mitigate the failure in this task, we will start by assembling some of the ideas we already have on low cost CMOS fabs and unveil potential issues toward high integration. At each iteration, interested users shall be able to test our devices in their respective environments.

5.6. Liaison with Student Teams and Socio-Economic Study

Our groups are open for collaborations, and look forward to establish reliable partnership with users, partners and stakeholders. Our plan envisages their presence from the very beginning and will provide support in their future endeavours, by providing them with better and more advanced instruments. Of special interest are Master students, the next generation of STEM engineers, which will, one hopes, adopt our technology. It is very rewarding having the possibility to empower the younger generation, and give them tools to cover the fear of missing out new opportunities in such an early stage, searching for other possible applications of the developed chip, that may include PET, encrypted LIDAR, and other machine vision applications.

6. ACKNOWLEDGEMENT

Authors thank Rok Podlipec for reference measurements of fluorescein standards.

This project has received funding from the ATTRACT project funded by the EC under Grant Agreement 777222

7. REFERENCES

[1] Bruschini, C. et al., 2019, Single-photon avalanche diode imagers in biophotonics: review and outlook, Light: Science & Applications, Nature.

[2] Liu, X. et al., 2019. Fast fluorescence lifetime imaging techniques: A review on challenge and development, Journal of Innovative Optical Health Sciences, 12(5): pp. 1930003.

[3] Ritt, S. et al., 2010, Application of the DRS chip for fast waveform digitizing, Nuclear instrument and methods in physics research A, 623, pp. 486 – 488.

[4] Tsai, H. M. et al., 2017. Note: Rapid measurement of fluorescence lifetimes using SiPM detection and waveform sampling, Review of scientific instruments, 88: pp. 096107.

[5] Biotechnology market analysis accessed 11/8/2020 https://www.grandviewresearch.com/press-release/global-biotechnology-market

[6] Seljak, A., et. al., 2018, Prototype readout system for a multi Mpixels UV single-photon imaging detector capable of space flight operation, JINST 13(02): pp. T02003-T02003.

[7] Dolenec, R., et al., 2017, Ultrafast detection in particle physics and positron emission tomography using SiPMs, Nuclear instrument and methods in physics research A, 876, pp. 257 – 259.