Sensors & Transducers, Vol. 271, Issue 4, December 2025, pp. 24-30

Sensors & Transducers

Published by IFSA Publishing, S. L., 2025
http://www.sensorsportal.com

Time-resolved Fluorescence by Point-by-point Acquisition with Python

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Received: 16 September 2025 / Revised: 11 Nov. 2025 / Accepted: 12 Dec. 2025 / Published: 30 Dec. 2025

Abstract: Currently, spectroscopic applications in the medical field for diagnosis disease requires specialized instrumentation. An example is time-resolved fluorescence, where fluorescence lifetime must be measured, demanding equipment capable of extracting times on the order of nanoseconds and picoseconds. In this work, we present the implementation of the sequential equivalent-time sampling technique to reconstruct and visualize the pulse width of a laser source. The system is based on a photo detector coupled to an oscilloscope that is fully controlled through a computer program developed in Python, allowing automated acquisition and data processing. This approach reduces the limitations imposed by real-time acquisition rates and enables the study of repetitive fast optical phenomena. The main objective is to establish a methodological and instrumental basis for future applications in fluorescence lifetime measurements of biological samples. To optimize resources, the system makes use of components from a transient absorption spectroscopy kit, thus taking advantage of the available instrumentation. The proposed implementation is versatile, cost-effective, and adaptable, making it possible to extend its functionality to biomedical fluorescence studies. In the long term, the system can be further improved and scaled, integrating more sensitive detectors, optimized electronics, and advanced data processing algorithms, with the aim of achieving reliable and accurate tools for medical diagnostics.

Keywords: Equivalent-time sampling, Oscilloscope control, Python instrumentation, Nanoseconds, Laser pulses, Fluorescence lifetime.

1. Introduction

One of the main challenges in treating diseases such as cancer is early diagnosis. For example, in the case of melanoma, early detection is extremely important to reduce mortality [1]. Time-resolved fluorescence is a technique that enables rapid and non-invasive diagnosis; however, its implementation requires specialized instrumentation, particularly for accurate fluorescence lifetime measurements. The challenge lies in the extremely short duration of this phenomenon, which occurs in the order of nanoseconds and picoseconds [2].

The measurement of such fluorescence lifetime currently requires expensive instrumentation with very high temporal resolution, making it less accessible. An alternative for measuring time-resolved fluorescence without such high-speed equipment is the sequential equivalent-time sampling technique, which allows capturing a sample at a different point in time

during each repetition of the phenomenon until the entire signal is reconstructed [3].

Therefore, in this work, the implementation of this technique is presented using a transient absorption kit, complemented by an oscilloscope and control via Python. This provides the groundwork for future projects focused on fluorescence lifetime measurements and demonstrates the feasibility of using this instrumentation to measure short time intervals. The main objective of the instrumentation is to enable the measurement of short fluorescence lifetimes, such as those of Rhodamine B, Rhodamine 6G, and fluorescein, which are all less than 5 ns [4] and comparable to the lifetime of flavin adenine dinucleotide (FAD), an endogenous molecule found in biological tissue. FAD, with a fluorescence lifetime of approximately 4 ns, is widely used in the diagnosis of various pathologies, including cancer [5].

Therefore, it is essential to properly configure and continually update the instrumentation to meet the temporal requirements of the technique and ensure accurate measurements for future biomedical applications.

2. Methods

2.1. Sequential Equivalent-time Sampling Technique

The acquisition of the signal using the sequential equivalent-time sampling technique requires multiple repetitions to reconstruct the waveform. The waveforms are obtained by taking one or more samples in each repetition, but at different points in time. In this case, the sampling is done sequentially, although it can also be performed randomly [6].

Fig. 1 illustrates the principle of sequential equivalent-time sampling, highlighting the time delay between each sample until the complete waveform of the signal is reconstructed.

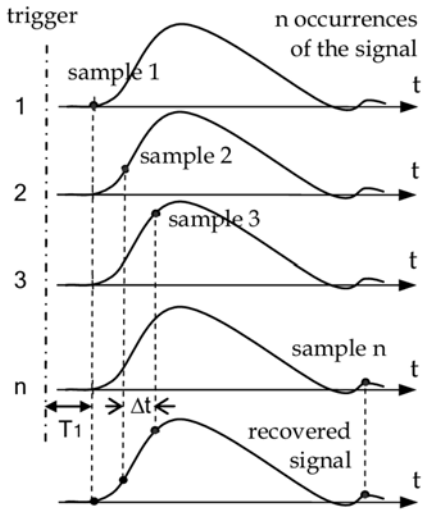

Fig. 1. Sequential equivalent-time sampling [7].

2.2. Instrumentation

The photodiode used was the SIPM MicroFC-30020 from ONSEMI and a time rise of 0.6 ns with a measurement wavelength range of 400 to 1100 nm. Additionally, the data acquisition and control board, the National Instruments™ USB-6341 (10 MHz bandwidth, 500 kSa/s sampling rate), is used to control the repetition rate of the laser pulses [10].

Considering that the acquisition board does not have sufficient bandwidth and sampling rate, a KEYSIGHT oscilloscope, model DSOX1202G, with a bandwidth of 200 MHz and a sampling rate of 2 GS/s was also implemented [11].

Fig. 2 presents the configuration of the instrumentation employed in the experiments. An essential component is the oscilloscope, which is interfaced with the computer for control and data acquisition, and simultaneously connected to the laser module output to monitor each emitted pulse.

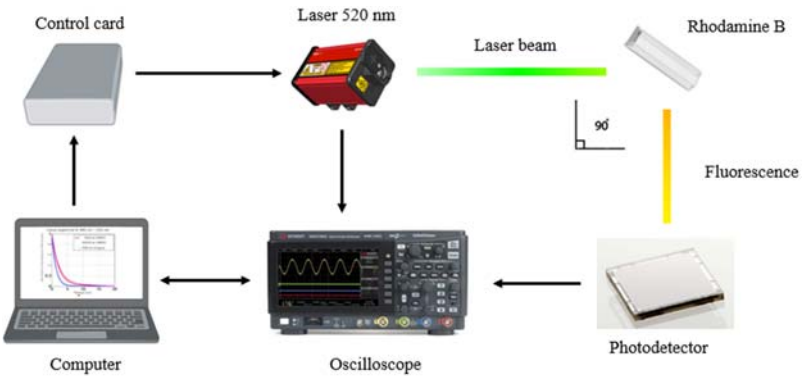

Fig. 2. Instrumentation diagram.

This configuration allows for synchronization between each pulse and signal capture at every instant. Furthermore, the fluorescence signal is being captured at a 90-degree angle in a quartz cell to avoid interference from both the laser transmission and the container material.

2.3. Python Code

The purpose of developing the control and acquisition code in Python was to create a flexible and open-source environment capable of integrating multiple instruments within the time-resolved fluorescence system. The Python program begins by establishing communication between the oscilloscope and the laser control board using the VISA protocol.

Subsequently, the signal scale is defined, followed by the data acquisition parameters are defined, which mainly include the number of samples per acquisition point, the total number of points to be taken, and the time interval between each point. All of these can be configured using the scale adjustment button. Once configured, the program developed in Python waits for the laser module to emit pulses to trigger the acquisition on channel 2 of oscilloscope and obtain the photodiode measurements point by point on channel 1.

To identify the process, Fig. 3 presents the Python code flowchart. Once the laser is turned on, data acquisition begins to determine the signal's scale. This ensures that the final signal, when reconstructed by the software, does not deviate from the original amplitude. Point-by-point acquisition can then begin. The total duration of the acquisition cycle depends on the number of points collected and the time interval between them; in this case, one point is acquired every 0.1 ns. After completing the signal sampling, the data is saved to a .csv file.

The signal reconstruction was performed by advancing the delay in 0.1 ns steps through a controlled shift of the oscilloscope time base. In each laser pulse repetition, a different point of the signal was recorded until the complete trace was obtained, ensuring the reproducibility of the sequential equivalent-time sampling technique.

The graphical user interface (GUI) was developed in Python using the Spyder (Anaconda) environment and the Tkinter library for window management and user interaction. Its design allows controlling the equivalent sequential acquisition process and visualizing the reconstructed signal in real time obtained from the oscilloscope.

The graphical user interface (GUI) allows the user to visualize the reconstructed signal at the end of the complete acquisition. In the GUI window, it is possible to modify the name and destination folder of the generated .csv file.

It includes four main buttons:

1. Turn On/Off Laser, which controls the laser activation;
2. Single Trigger for Scale, which performs an automatic capture to determine the proper oscilloscope scale;
3. Adjust Scale/Offset, allowing manual adjustment of these parameters if a different configuration is required;
4. Start Acquisition, which begins the full equivalent sequential acquisition process.

Additionally, the interface displays information about the oscilloscope auto scale, including the voltage per division (V/div), offset, and the measured signal limits (Y min and Y max).

In Fig. 4, the graphical interface can be observed. As described, it is simple in functionality but includes all the essential features required to acquire the signal necessary for measuring fluorescence lifetimes. Nevertheless, it is designed to be scalable, allowing the implementation of additional functions if desired by modifying the Python source code.

3. Experimentation

For the experimental stage, tests were carried out using a Rhodamine B solution as a reference fluorophore, excited by a 520 nm laser pulse with an approximate temporal width of 5 ns. A distilled water sample was also used as a control to compare the system's temporal response against a non-fluorescent medium.

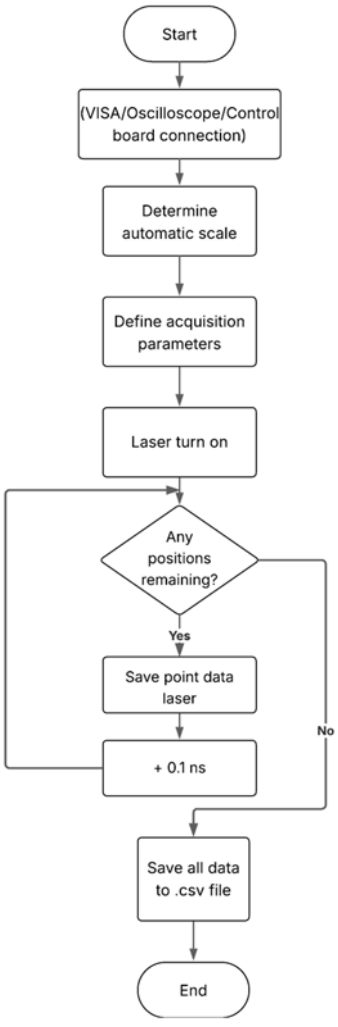

Fig. 3. Flowchart of Python code.

Measurements were performed using the equivalent sequential acquisition system, specifically designed to capture the temporal profile of the fluorescence pulse with high resolution. In this

method, the signal was recorded with a temporal step of 0.1 ns, operating at an acquisition frequency of 200 Hz. To enhance the signal-to-noise ratio and minimize fluctuations related to laser triggering and detection electronics, 16 samples were collected for each temporal delay, and these were subsequently averaged before reconstructing the complete signal. This approach enables accurate reconstruction of the fluorescence decay trace, effectively emulating equivalent-time sampling in the temporal domain.

Rhodamine B was selected because it is a well-characterized fluorophore known for its high quantum efficiency, strong visible emission, and typical lifetimes in the nanosecond range, making it an ideal standard for validating the time-resolved fluorescence instrumentation system.

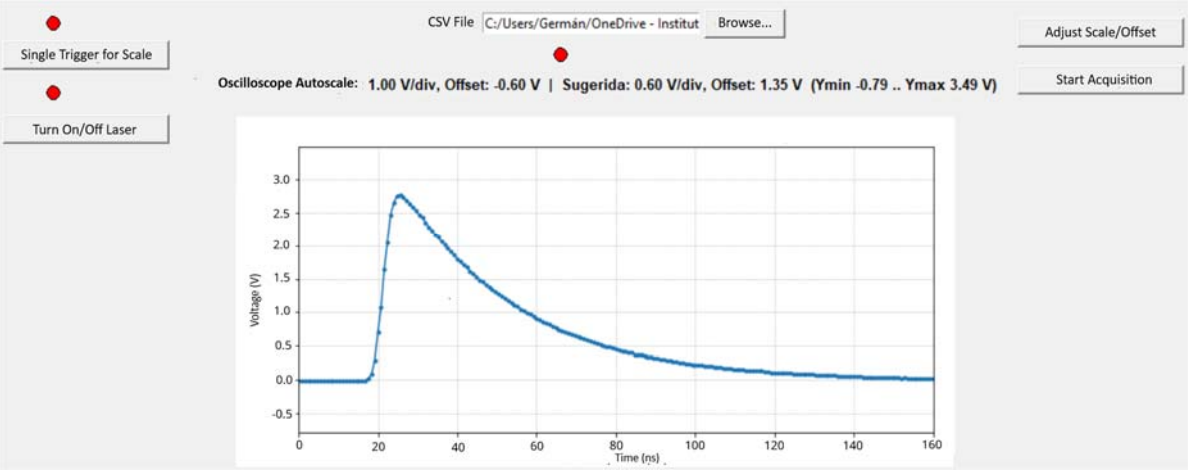

Fig. 4. Graphical Interface.

In contrast, the distilled water sample serves as a baseline with no fluorescence signal, ensuring that the detected emission originates exclusively from the excited sample and not from reflections or instrumental artifacts.

Fig. 5 shows the experimental setup assembled on the optical table, where the fluorescence pulse acquisition was performed. The system consists of a 520 nm laser module emitting pulses of approximately 5 ns duration, directed toward a quartz cuvette containing the Rhodamine B solution.

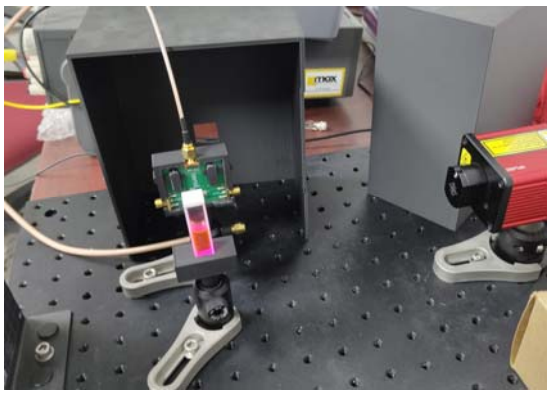

Fig. 5. Tests were performed on Rhodamine B with a 520 nm laser module.

The generated fluorescence emission was detected by an ONSEMI silicon photomultiplier (SiPM), coupled through SMA connectors with a characteristic impedance of 50 Ω to ensure proper signal matching. The detector was mounted on an adjustable holder to achieve optimal optical alignment and maximize fluorescence signal collection.

The setup was mounted on a vibration-isolated optical table to maintain stability during data acquisition. Additionally, black shielding panels were used along the optical path to minimize ambient light interference and unwanted reflections. This experimental configuration enabled the collection of signals with a high signal-to-noise ratio, suitable for temporal characterization and fluorescence lifetime analysis.

To remove undesired high-frequency components and detect noise, the acquired signals were processed using a band-pass filter ranging from 5 to 250 MHz. This filtering step preserves only the frequency components associated with the actual fluorescence response, effectively reducing electrical noise and spurious oscillations introduced by the oscilloscope or the detection circuit.

Subsequently, Fig. 6 shows the filtered and temporally aligned signals corresponding to the measurements of distilled water and Rhodamine B. As observed, both traces exhibit good overlap in the initial rising region, indicating proper synchronization and signal processing.

After filtering and alignment, the acquired signals were processed through deconvolution using the Laguerre method, with the objective of removing the contribution of the Instrument Response Function (IRF) and obtaining the actual fluorescence response of the fluorophore. This method relies on expanding

the measured signal into a series of orthogonal Laguerre functions, which allows a stable and efficient representation of the temporal decay even in the presence of noise. The choice of this approach lies in its capability to accurately reconstruct fluorescence dynamics without requiring prior knowledge of the decay model.

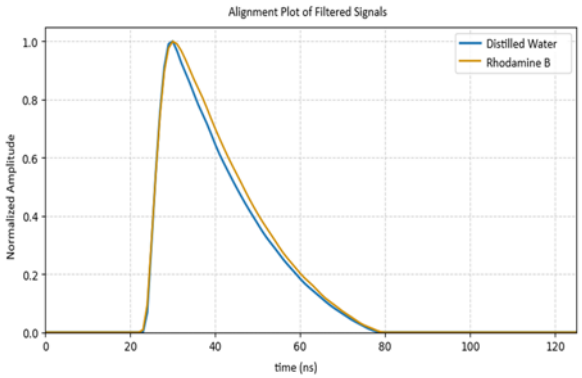

Fig. 6. Alignment Plot of Filtered Signals.

Once the deconvolved fluorescence signal was obtained, a nonlinear exponential fitting of the decay was performed using the Levenberg–Marquardt algorithm, to estimate the corresponding fluorescence lifetimes (τ) in Fig. 7. This algorithm combines the advantages of the least-squares and gradient-descent methods, providing robust convergence even for experimental data with moderate noise levels.

To ensure the reproducibility and consistency of the results, five independent experimental measurements were conducted under identical excitation and detection conditions. From the set of obtained lifetime values, both the standard deviation and the average fluorescence lifetime were calculated, allowing the evaluation of experimental dispersion and the verification of system stability. This statistical analysis is essential to confirm the accuracy and repeatability of the fluorescence lifetime measurements.

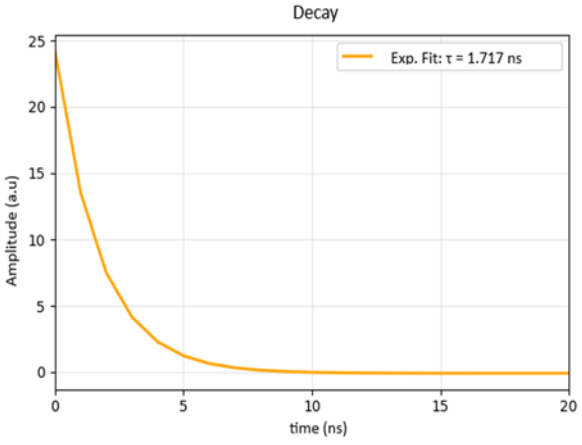

Fig. 7. Rhodamine Decay after Exponential Fit.

4. Results and Discussions

Table 1 presents the obtained fluorescence lifetime values of Rhodamine B, determined using the point-by-point sequential acquisition technique implemented with the developed system. For each measurement, the signal was acquired with a 0.1 ns temporal step, filtered within 5–250 MHz, deconvolved using the Laguerre basis, and finally fitted to a monoexponentially decay model through the Levenberg–Marquardt algorithm.

The results show good consistency among measurements, with estimated lifetimes ranging from 1.63 ns to 1.76 ns, and an average lifetime of 1.69 ns with a standard deviation of ± 0.05 ns. These values fall within the range reported in the literature for Rhodamine B (1.6 – 1.8 ns), confirming the accuracy and reliability of the developed instrumentation system [4]. The low standard deviation indicates high temporal stability and repeatability of the equivalent sequential acquisition method, as well as proper synchronization between the laser pulse and the detection system.

Table 1. Point-by-point results table.

Reference Lifetime (ns)	Estimated Lifetime (ns)	NMSE (%)	Standard Deviation (ns)	Average Lifetime (ns)
1.6 – 1.8	1.71	0.86	± 0.05	1.69
	1.63	0.53		
	1.76	0.84		
	1.65	0.27		
	1.74	0.59		

Notably, the use of the point-by-point sampling technique enabled the reconstruction of the temporal trace with a resolution superior to that of direct sampling methods, by exploiting the principle of Equivalent-Time Sampling (ETS). This approach, combined with multiple sample averaging for each temporal delay, significantly reduced noise and improved the quality of the measured decay, thereby enhancing the accuracy of the exponential fitting.

Complementarily, Table 2 shows the results obtained from the signals directly acquired from the oscilloscope, recorded during the system's auto scale procedure. In this case, the fluorescence lifetime of Rhodamine B was determined by applying the same exponential fitting (Levenberg–Marquardt) to the waveform captured in a single-shot acquisition, without using the point-by-point sequential technique.

The estimated values exhibit greater dispersion, with lifetimes ranging from 1.41 ns to 2.62 ns, and a standard deviation of ± 0.47 ns, resulting in an average lifetime of 1.90 ns. This value exceeds the upper bound of the literature range (1.6 – 1.8 ns), which, together with the increased variability, reflects the limitations of direct sampling and the sensitivity to instantaneous fluctuations in the laser or detection system. This upward deviation is consistent with single-shot captures that are more affected by

background noise, uncorrected reflections, or variations in oscilloscope scaling and offset during measurement.

When compared to the results obtained through the point-by-point acquisition technique, a notable improvement in stability and accuracy is observed. The sequential method achieves a much smaller standard deviation (± 0.05 ns) and an average lifetime closer to the reference value (1.69 ns), demonstrating higher reproducibility and robustness against electronic noise.

Table 2. Oscilloscope results table.

Reference Lifetime (ns)	Estimated Lifetime (ns)	NMSE (%)	Standard Deviation (ns)	Average Lifetime (ns)
1.6 – 1.8	1.84	2.56	± 0.47	1.90
	1.41	1.53		
	2.62	1.82		
	2.07	1.97		
	1.59	1.93		

This improvement arises because the point-by-point approach incorporates multiple averaging at each temporal delay, significantly enhancing the signal-to-noise ratio and minimizing the impact of small variations in laser pulse intensity or detector response. In contrast, direct oscilloscope acquisition depends on a single instantaneous capture, where random noise or scale mismatches can introduce considerable fitting errors, affecting the calculated lifetime. Overall, the comparison between both methods demonstrates that the developed instrumentation based on equivalent sequential sampling provides a substantial improvement in the accuracy and repeatability of fluorescence measurements, establishing it as a reliable tool for the temporal characterization of fluorophores and biological tissues.

As shown in Fig. 6, which displays the filtered and temporally aligned signals of Rhodamine B and distilled water, both traces exhibit a very small temporal offset. This behavior occurs because the overall instrumental response, including the detection electronics and the photodiode temporal response, has a duration comparable to the fluorophore fluorescence lifetime. As a result, the fluorescence emission is partially masked by the system's own response, making the characteristic decay difficult to distinguish visually.

Nevertheless, by applying the Laguerre deconvolution method followed by a nonlinear exponential fit (Levenberg–Marquardt algorithm), it is possible to mathematically isolate the fluorescence contribution and accurately determine the fluorescence lifetime. This demonstrates the system's capability to resolve fast decay dynamics, even when the measured pulse is dominated by the detector's response. The near overlap of both curves in the initial region confirms the excellent synchronization and temporal stability of the setup, while the subtle differences in the decay tail contain the necessary information to extract the true lifetime of the fluorophore.

8. Conclusions

The results obtained in this experimental stage demonstrate the feasibility and accuracy of the developed instrumentation system for fluorescence lifetime measurements using the point-by-point sequential acquisition technique. The implemented methodology enabled high-fidelity reconstruction of the temporal signal, effectively compensating for the limitations imposed by instrumental response and detection noise.

The comparative analysis between measurements directly obtained from the oscilloscope and those acquired using the point-by-point method revealed a significant improvement in stability and repeatability. While direct recordings exhibited higher dispersion and an average lifetime above the reference range (1.90 ns), the sequential system achieved an average value of 1.69 ± 0.05 ns, fully consistent with the literature values for Rhodamine B (1.6 – 1.8 ns). This confirms the effectiveness of averaging, filtering, and Laguerre deconvolution processes, which accurately isolate the fluorescence component even when it is partially overlapped by the detector response.

Furthermore, the overlaid signals shown in Fig. 6 validate the proper synchronization and temporal stability of the laser–detector system. Although the instrumental response partially masks the fluorescence emission, the application of mathematical deconvolution and exponential fitting methods allowed reliable determination of the lifetime, demonstrating the system's capability to resolve fast decays in the nanosecond range.

Overall, the results confirm that the proposed setup constitutes a robust and precise platform for time-resolved fluorescence studies, with performance comparable to that of commercial high-end instruments while maintaining a more accessible and flexible architecture.

As future work, it is proposed to apply this methodology to ex vivo biological tissue samples preserved in paraffin, to evaluate the fluorescence response of real tissues and validate the system's efficiency under conditions closer to biomedical environments. Extending the study to endogenous fluorophores naturally present in tissue will strengthen the applicability of the system in optical diagnostics and bio photonic characterization, establishing it as a cost-effective, high-temporal-resolution experimental tool for advanced biomedical research.

Acknowledgements

This work was supported by a grant from the National Polytechnic Institute. Germán Gerardo Sánchez Hernández has a scholarship from SIP-IPN

through the Master's Program in Engineering and Design of Sustainable Systems.

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