

Title : Analog mean-delay method for the high-speed fluorescence lifetime measurements

Name : Youngjae Won

1. Introduction

The fluorescence lifetime imaging microscopy (FLIM) is widely used in many spectroscopic applications for biological studies [1~3]. In point scanning techniques for FLIM, the time-correlated single-photon counting (TCSPC) is a more common way to record a lifetime because it provides a high accuracy and a good photon economy even for measuring a short lifetime [1, 4, 5]. In this scheme, the accuracy is not limited by the finite temporal resolution of the photodetectors such as photo-multiplier tube (PMT) and a Geiger-mode avalanche photodiode (G-APD) because the arrival time of a single photon is determined individually in a single-photon counting manner, taking advantages of highly sensitive photodetectors. However, to minimize the pile-up effect for a better accuracy, the number of detected photons is limited to about 1 to 5 for every 100 excitation pulses. And the frequency of excitation pulse does not exceed 10 MHz in many spectroscopic applications to ensure that the fluorescence decay signal from one excitation pulse is not affected by that of neighboring excitation pulses. Because of this limitation of the photon detection rate the acquisition time for one image is needed so much. And it is hard to apply in study of dynamic phenomena within one second by SPC based method.

In this study, we present a new high-speed measurement method of fluorescence lifetime with a high level of accuracy and precision. In our method, signals are acquired by an analog acquisition manner and lifetimes are determined from the analog time-domain signals by taking the mean temporal delay of the fluorescence intensity signals so that this method is called *analog mean-delay (AMD) method*. Because the effect of IRF can be eliminated by mathematical process after detection of analog signal, detection of multiple photons in one excitation pulse can be allowed for enhancement of measurement speed. This enhanced measurement speed can be more than two-orders compared with a conventional TCSPC. The accuracy of our method is not hampered by the slow response of the photo-detector because of its deconvolution-like characteristic, and this makes our method applicable by using cost-effective measurements. In our method, high spatial resolution for imaging is also ensured because it is developed to be applied in a confocal or two-photon system. By the theoretical and experimental investigations, we have found our method can be well applied to the high-speed measurement of fluorescence lifetime with good accuracy, precision and measurement speed.

2. Method

In analog fluorescence signal detection, the detected fluorescence emission curve is determined by convolution of the exponentially fluorescence decaying function with the slow instrumental response function (IRF), i.e. the excitation pulse shape, the impulse response function of the photo-detector. The analog fluorescence signal and IRF signal can be expressed by

$$i_e(t) = \gamma \cdot \{I_{ex}(t) \otimes \Psi_\tau(t) \otimes I_{pd}(t)\} \quad (1)$$

$$i_{irf}(t) = \gamma \cdot \{I_{ex}(t) \otimes I_{pd}(t)\}, \quad (2)$$

where t is the time, $i_e(t)$ is the detected fluorescence signal, γ is the net conversion coefficient of an excitation photon to a detected photoelectron, $I_{ex}(t)$ is the excitation pulse shape, $\Psi_\tau(t)$ is the exponential fluorescence decay function, $I_{pd}(t)$ is the impulse response function of the photo-detector, and $i_{irf}(t)$ is the instrumental response function (IRF) of the system. The exponential fluorescence decay function $\Psi_\tau(t)$ is characterized by the mean lifetime τ for a single exponential decay so that it is represented by $\Psi_\tau(t) = \exp(-t/\tau) \cdot u(t)$, where $u(t)$ is unit step function. This can be regarded as probability distribution function (PDF) of random emitting time of a fluorescence photon. To extract the lifetime τ from $i_e(t)$, other convolution components $I_{ex}(t)$ and $I_{pd}(t)$ must be removed from right-hand side of Eq. (1). In statistical theory, convolution of the PDFs corresponds to a summation operation of the corresponding random variables. From this property, we can simply calculate the lifetime without concern of the effect of the IRF [6].

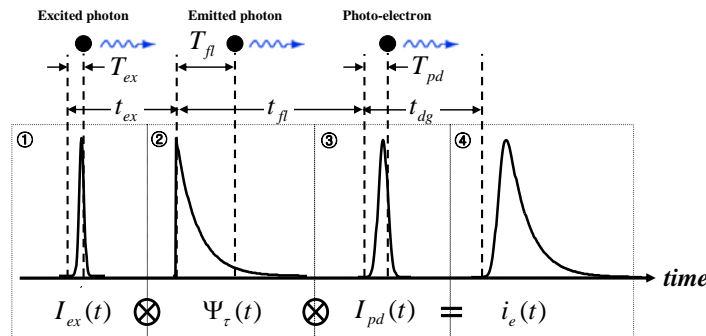


Fig. 1 Schematic for timing about the processes of ①excitation, ②fluorescence emission, ③photo-detection, and ④photoelectron acquisition. Each process occurs serially in time. For a detected photoelectron, the final temporal delay is a summation of various random and deterministic variables of time.

Fig. 1 shows the schematic for timing about the processes of excitation, fluorescence emission, photo-detection, and photoelectron acquisition. Each process occurs serially in time. The final temporal information of a photoelectron is determined by various random and deterministic variables of time. Random variables of time are represented by a capital letter and these have their own characteristic PDFs as a function of time such as $\Psi_e(t)$, and deterministic variables of time are represented by a small letter. First, an excitation photon is delivered to a sample with a delay of t_{ex} , and this photon is absorbed by a fluorescence molecule with a delay of random time T_{ex} . After very short time of photon-electron relaxation within energy states of the fluorescence molecule, the electron stays in the lowest excitation state before emitting to the photon with a delay of random time T_{fl} . And then, the emitted photon is delivered to a photodetector with a delay of time t_{fl} . At photodetector, the detected photon is converted to the much of electrons, and these electrons are spread with a delay of time T_{pd} . Each of the photoelectrons is delivered to the digitizer with a delay of t_{dg} and the photoelectron is acquired by the digitizer. The arrival time of the photoelectron can be represented by

$$T_e = (t_{ex} + T_{ex}) + (T_{fl} + t_{fl}) + (T_{pd}) + (t_{dg}) \quad (3)$$

Each part of right-hand side of Eq. (3) corresponds to the each steps ①, ②, ③, and ④ in Fig. 1. The distribution of random variables of time T_e , T_{ex} , T_{fl} , and T_{pd} can be followed by PDFs which correspond to the finally acquired electric pulse $i_e(t)$, excitation pulse $I_{ex}(t)$, fluorescence decay function $\Psi_e(t)$ and the impulse response function of the photo-detector $I_{pd}(t)$, respectively. With the same process, the arrival time of a final photoelectron (T_e^0) for IRF is also represented by

$$T_e^0 = (t_{ex} + T_{ex}) + (t_{fl}) + (T_{pd}) + (t_{dg}) \quad (4)$$

In this case, the T_e^0 is correspond to the $i_{irf}(t)$. For a random variable of temporal delay of T , the expected or average value denoted by $\langle T \rangle$ is called the mean delay of T i.e. the time average of the corresponding PDF. From Eq. (3) and Eq. (4), we can derive following relation by the linearity of the expectation operator,

$$\langle T_e \rangle - \langle T_e^0 \rangle = \langle T_{fl} \rangle = \tau \quad (5)$$

By the definition of the average value of random variables with corresponding PDF, Eq. (5) can be expressed as

$$\tau = \langle T_e \rangle - \langle T_e^0 \rangle = \left(\frac{\int t \cdot i_e(t) dt}{\int i_e(t) dt} \right) - \left(\frac{\int t \cdot i_{irf}(t) dt}{\int i_{irf}(t) dt} \right) \quad (6)$$

Eq. (6) denote that the effect of IRF can be eliminated from two signals, $i_e(t)$ and $i_{irf}(t)$, and this fact shows that the measurement of fluorescence lifetime in our method is independent of slow instrumental response of the system. Therefore, inexpensive photo-detectors with slow response can be used and Gaussian low-pass filters with narrow-bandwidth can be also added in measurement setup to reduce sampling rate for data acquisition. Low sampling rate provide a chance to use an inexpensive digitizer and reduce a power of data which can help to decrease data processing time.

3. Experiment and results

In our AMD method, a lifetime can be extracted by two signals, fluorescence signal $i_e(t)$ and instrumental response signal $i_{irf}(t)$. We acquired these two signals, $i_e(t)$ and $i_{irf}(t)$, in the same time by separation with an optical delay line. Fig. 2 shows a schematic diagram for lifetime measurement in our method. Used excitation laser was inexpensive semiconductor gain-switching pulse laser with 635 nm of operation wavelength and ~800 ps of pulse width. To cut unwanted light, two of laser line filters (LLF) and a long pass filter (LPF) were used at the source part, IRF part, and fluorescence output part, respectively. Injected excitation pulses were divided into two paths at the beam splitter. One part was going to sample for fluorescence signal corresponding to Eq. 3 and another was going through direct path for IRF signal corresponding to Eq. 4. Optical delay line by long optical fiber made two signals to be temporally separated. From this, deterministic time delay t_d (199.3 ns) was added in Eq. 4. Used photodetector in our experiment was low-cost photo-multiplier tube (PMT, R7400U-20, Hamamatsu) with 11.7 % of quantum efficiency, and its time response was 5.4 ns. Because the fluorescence lifetime extraction is independent to the slow response function of the system addition to the electric gaussian low-pass filter (GLPF) after PMT is allowed to reduce the sampling rate of the digitizer. We designed the GLPF to make the signals passing through GLPF have no more than 50 MHz of frequency components so that 100 MS/s of the sampling rate can be allowed by nyquist-shannon sampling theorem [7]. In our study, the used digitizer was PCI-5114 (National Instruments) with 100 MS/s of the sampling rate and 8 bit of the amplitude resolution.

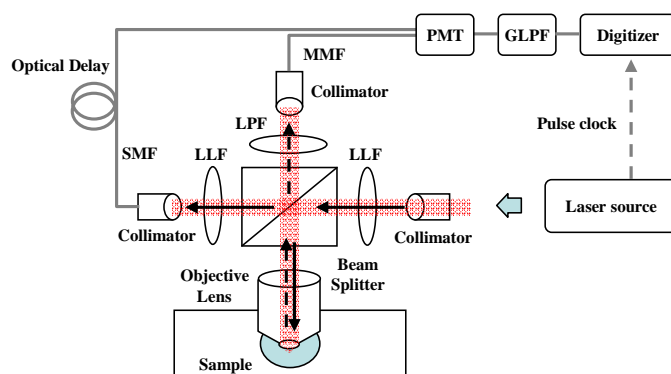


Fig. 2. Schematic diagram for lifetime measurement

The accuracy of our method was evaluated by two kinds of fluorophores, Alexa Fluor 633TM (Invitrogen) and CY5 (Amersham Biosciences). It is known that Alexa Fluor 633 has a relatively long lifetime of 3.2 ns in water and CY5 has a short lifetime of 1.0 ns in phosphate buffered saline (PBS) [8]. In our experiment, those fluorophores were diluted by PBS and were placed on glass plates as sample specimens. In order to demonstrate the usefulness of our method as a tool for measurement of fluorescence lifetime, TCSPC method was compared because it provided high accuracy for lifetime measurement.

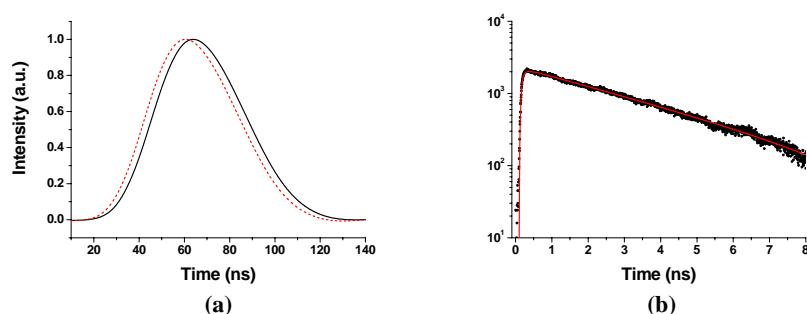


Fig. 3. (a) Signals for our mean-delay method (b) Signals for TCSPC; Used fluorophore : Alexa Fluor 633TM

Fig. 3 shows the signals for lifetime measurement of our mean-delay method (a) and TCSPC (b). In Fig. 3 (a), Dot graph is IRF signal shifted as temporal delay and solid graph is fluorescence signal, and each signal is normalized by peak. In this experiment, GLPF was used for 100 MS/s of sampling rate, and this sampling rate was increased by spline interpolation in data processing. Due to narrow bandwidth of GLPF, IRF and fluorescence pulse were broadened. In TCSPC, used excitation source was MIRA 900 (Ti:sapphire, Coherent) with ~ 120 fs of pulse duration and the fluorescence decaying curve was measured by R3809(MCP-PMT, Hamamatsu) and PicoHarp 300 (TCSPC module, PicoQuant). The number of detected photons was more than 10^6 in both cases. Adding to Alexa Fluor 633, lifetime of CY5 was also measured by both methods. Experimental results are listed in table 1. As we can see, the calculated lifetimes by both methods are almost same each other.

		Alexa Fluor 633	CY5
Measured fluorescence lifetime, τ	TCSPC	3.28 ns	1.08 ns
	Analog mean-delay (100MS/s)	3.27 ns	1.05 ns
Number of detected photons, N	Analog mean-delay (100MS/s)	1.1×10^3	1.1×10^3
Stand. Dev., $\Delta\tau$		126 ps	81 ps
Figure of merit, F		1.28	2.55

Table 1. Fluorescence lifetime obtained by analog mean-delay method and TCSPC

Table 1 also shows the precision performance of our analog mean-delay method. The precision performance can be evaluated by Figure of merit (F), defined as

$$F = \frac{\Delta\tau}{\tau} \cdot \sqrt{N}, \quad (7)$$

where τ , $\Delta\tau$, and N are the lifetime, standard deviation of the measured lifetimes and the number of detected photons involved with a lifetime determination, respectively. Due to the fundamental shot noise, F is larger than unity. Higher F -values than unity represents worse performance because it means there are other noise effects except for shot noise [9]. In this experiment, each fluorescence pulse for single lifetime determination was acquired by averaging of 48 pulses, and the number of detected photons was 1.1×10^3 . For the mean delay of the IRF signal, the signal was acquired by averaging of all pulses for accurate information. The high intensity of detected IRF signal can induce more noise effects such as a dark count in the PMT. To reduce the dark count in PMT, approximately 2 of the number of photons in every pulse period were used to acquire the IRF signal. The IRF signal can be acquired accurately by a number of averaging although the photo-detection rate is low. The acquisition time was taken by 17.6 μ s for the single lifetime determination, and this can be reduced to a few μ s with the higher fluorescence signal and decreasing the number of averaging. The standard deviation for lifetime was examined by 113 of frequencies. From information of τ , $\Delta\tau$, and N , F was calculated in both cases, Alexa Fluor 633 and CY5. The figure of merit was very good ($F=1.28$) for a long fluorescence lifetime of 3.27 ns, almost reaching the theoretical limit of $F=1$. Even though it is degraded for a short lifetime of 1.05 ns ($F=2.55$) this value is acceptable in most lifetime measurement applications. Because the excessive noise can be involved with the mean-delay calculations by long integration window of 45 ns, F is worse in the case of short lifetime.

The speed of signal acquisition is very fast in our analog mean-delay method. Collecting a set of data for determining a 3.2 ns fluorescence lifetime with a $\pm 3\%$ random error was completed in 17.6 μ s or 48 pulse periods, in which $\sim 1,100$ -photon signal is acquired. Here, the photon detection rate was about 68 MHz. Thus it takes 1.8 seconds to acquire an image composed of 100,000 pixels (316×316 pixels). The acquisition speed can be further improved by irradiating more excitation intensity and increasing the fluorescence photon rate with ease. For a single-channel TCSPC instrument, it takes longer than 100,000 pulse periods for a lifetime determination to obtain an equivalent result. Even for 10 times higher pulse rate of 27 MHz that is around the typical pulse rate for measuring a relatively long lifetime, 3.7 ms needs to be spent to acquire a set of photon counts for a single lifetime determination. The measurement speed of our analog mean-delay method is more than 2 or 3 orders of magnitude faster than the conventional TCSPC.

4. References

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