Expansion of intensity correlation spectroscopy for lifetime measurements— application to intracellular oxygen dynamics measurements

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Abstract. We report on a simple correlation method for lifetime measurements using a random modulated excitation light source. We use an intensity correlation function of emission for lifetime analyses. In this method, no reference timing of the excitation is required. We apply the correlation method to measure phosphorescence decays and successfully demonstrate in the analysis of the phosphorescence decay from Pd(II) porphine in HeLa cells under aerobic and anaerobic conditions to understand the oxygen dynamics in individual cells. The method is applicable to faster decay time measurements down to a nanosecond range when the detection system is improved. Current fluorescence correlation setups can easily be modified for lifetime measurements, expanding the applicability in biological problems. © 2007 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2717623]

Fluorescence correlation spectroscopy (FCS) is one of the most popular applications of the correlation methods in biological research. FCS usually analyzes fluorescence intensity correlation associated with the number fluctuation due to the random motion of fluorescent particles in a small region in a sample.[1] Since it is a noninvasive method to detect a very small region, FCS is widely applicable to the analysis of dynamic behaviors of fluorescence labeled molecules in living cells.[2,3] Efficient and sophisticate FCS setups with a laser scanning microscope (LSM) system are now commercially available and widely used in biological research. The combination with other measurements, such as fluorescence lifetime measurements, is also recognized to be very important to characterize much complex biological phenomena. In fact, a combination system with fluorescence lifetime measurements has been tested using a conventional technique, the time-correlated single photon counting method.[4] Such a multimodal FCS measurement system usually consists of components of different measurement systems. Therefore, it tends to become very complex and expensive, limiting biological applications, and in particular, some practical uses for medical applications.

We report on a new method to measure the decay function by a fluctuation analysis with a trigger-controlled pulsed source to obtain the decay kinetics, using a conventional FCS setup. The concept is simplification using one setup, which can measure both fluorescence correlation and decay functions. The intensity autocorrelation function of emission by random excitation pulses is calculated to extract decay kinetics. In this method, the reference timing, such as the timing of excitation pulses, is not required. We applied this method to phosphorescence decay measurements with Pd(II)-porphine [5,10,15,20-tetrakis(4-sulfonatophenyl)-porphine, Pd:TSPP] in solutions and in HeLa cells, and have demonstrated an analysis of the decay time in the cells.

The schematic diagram of the experimental setup is shown in Fig. 1. A frequency-doubled Q-switched Nd^{3+}:YAG laser light was injected from the Hg lamp port of an epifluorescence microscope (BX-50, Olympus, Tokyo, Japan) and focused into the sample by an objective lens (UPlanApo 60×, NA 1.2/water, or UPlanFl 10×, NA 0.3, Olympus). A standard green fluorescence filter set (U-MWG2, Olympus) was used for the phosphorescence detection. In the measurements *in vitro*, the lower magnification lens was used mainly because of the higher count rates. On the other hand, the higher one was used in the cell measurements and fluorescence correlation measurements. The laser pulse could be synchronized to a periodic trigger source or a random trigger source, generated by random dark counts from a photomultiplier tube. The periodic excitation causes a periodic feature of the correlation function, making analysis complex. Thus, we mainly used the random trigger source. An arbitrary excitation position in a cell was visually guided by eyes seeing both the excitation laser spot and the transilluminated image simultaneously. The organella, such as nucleus and cytoplasm, could be selected.

The emission collected by a 62.5-μm graded index fiber at the camera port of the microscope was detected by an avalanche single photon counting module (APD; SPCM-100, RCA). The arrival times of the photons were recorded by a hand-built photon arrival time recorder. A small Clark-type 200-μm ϕ oxygen electrode (POE20W/PO2-100, Inter Medical, Tokyo, Japan) was used in calibration measurements *in vitro*. The accuracy and linearity, according to the specifications, were ±3 Torr and ±2 Torr, respectively. All measurements were conducted at a room temperature of 24 to 27°C.

In our analysis, the diffusion of molecules is not taken into account. From FCS measurements, we confirmed that the diffusion time is sufficiently larger than the decay time. The correlation function of the emission intensity is given by $g(\tau)=\langle I(t)I(t+\tau)\rangle/\langle I(t)\rangle^2-1$. When the pulsed excitation is used and the timing of each pulse is randomized, the emission only correlates within the emission associated with each excitation pulse. When the emission decay is given by a multi-exponential decay function $F(t)=\Sigma_i a_i/\tau_i \exp(-t/\tau_i)$, where

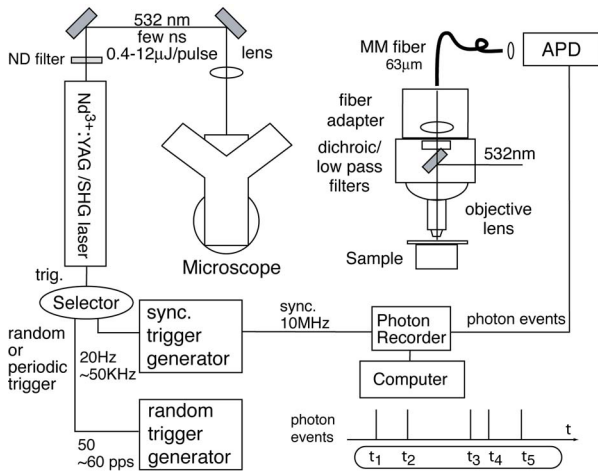*Tel: +81–11–706–3371; E-mail: gnishi@imd.es.hokudai.ac.jp

Fig. 1 Schematic diagram of the experimental setup for luminescence lifetime measurements. The system was based on a conventional FCS setup.

$\Sigma_i a_i = 1$ and τ_i is the lifetime, the correlation function can be calculated as

$$g(\tau) = \frac{1}{\nu}\sum_i \left(\sum_j \frac{a_j}{1 + \tau_j/\tau_i}\right)\frac{a_i}{\tau_i}\exp\left(-\frac{\tau}{\tau_i}\right), \qquad (1)$$

where ν is the average excitation rate. For a single exponential decay, the expression can be simplified as

$$g(\tau) = \frac{1}{2\nu\tau_0}\exp\left(-\frac{\tau}{\tau_0}\right). \qquad (2)$$

Thus, the correlation amplitude is inversely proportional to the average excitation rate and the decay time. When a very small region is observed, the diffusion of molecules also contributes both the decay and amplitude of the correlation function, and the theory should be extended. Note that the correlation function of the emission excited by periodic excitation pulses is not expressed by a simple multiexponential decay as Eq. (1), because of the periodicity.

Typical luminescence signals of 22 μM Pd:TSPP in 2.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) under decreasing oxygen concentration by a glucose-oxidase-catalase (GOx-Cat) system[5] are shown by photon burst traces in the inset of Fig. 2. With decreasing oxygen concentration, the burst frequency and the height of the burst were increased, as shown from the trace "a" to the trace "d" with average count rates from 0.33, 0.50, 0.89, and 1.29 kcps, respectively. The background count rate was 0.26 to 0.27 kcps. Since the count rate was small enough, the distortion due to the dead time[6,7] was not taken into account in the following analysis.

The correlation functions calculated from the photon counts were shown in Fig. 2(a). The correlation amplitude was corrected by the factor $[I/(I-I_{back})]^2$, where I and I_{back} are the observed and background count rates, respectively. The correlation functions below 1 μs were significantly distorted due to the dead time of the instrument, about 200 ns, and was not used in the fitting analyses. The functions shifted to a slower time and the amplitude became small with the
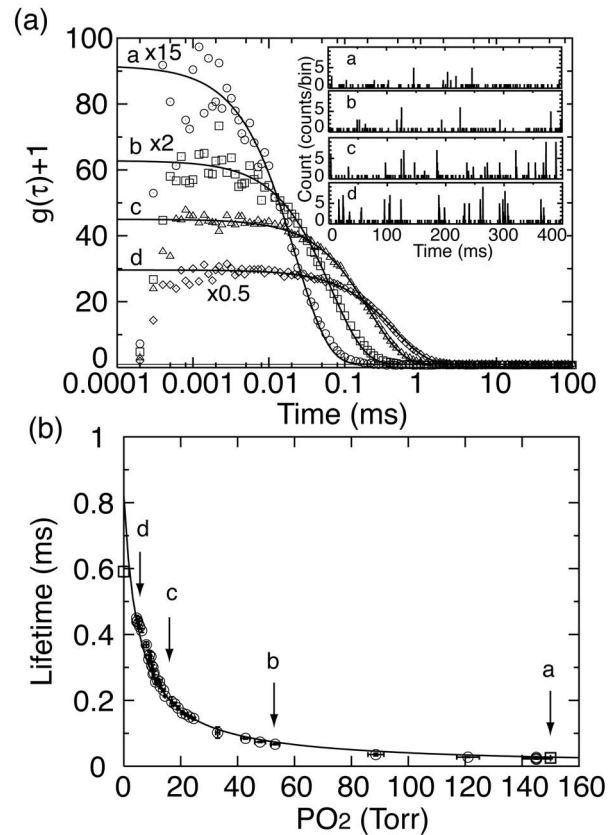

Fig. 2 Intensity correlation measurements *in vitro*. (a) Correlation functions and fitting results by single exponential functions. Oxygen concentrations were decreased in the order of circles, boxes, triangles, and diamonds corresponding to the photon counting traces in the inset from "a" to "d," respectively. The bin size was 100 μs. The correlation functions were calculated by the photon counting traces. The lifetimes are indicated by arrows in the bottom figure. (b) Phosphorescence lifetime as a function of oxygen partial pressure PO_2 measured by an oxygen electrode (circles). The measurements under aerobic and anaerobic conditions were using a sample chamber sealed by a cover glass without the oxygen electrode without and with the GOx-Cat system, respectively (boxes). The error bars of PO_2 and the lifetime indicate the PO_2 change during the phosphorescence measurement and the standard deviation estimated by the error matrix of the fitting results, respectively.

decrease in oxygen concentration. The correlation functions were well fitted by single exponential functions in the region over 0.9 to 1 μs to 100 ms, as shown by lines in Fig. 2(a), and eventually the phosphorescence decay was a single exponential. From the correlation amplitude and the lifetime, the average excitation rate of 55 sec^{-1} could be estimated from $g(0) = (2\nu\tau_0)^{-1}$. This value is in good agreement with the excitation pulse rate of 52 sec^{-1}.

The lifetimes determined under aerobic and anaerobic conditions are 25.2 ± 2.7 μs and 591 ± 16 μs, as shown by boxes in Fig. 2(b), respectively. The uncertainty of the lifetime under aerobic condition might be determined by the data statistics and the dead time distortion of the correlation function. On the other hand, the uncertainty under anaerobic condition might be determined by the data statistics and the uncontrolled leaking of oxygen from outside the chamber. From the uncertainties, we can consider that our method determines the
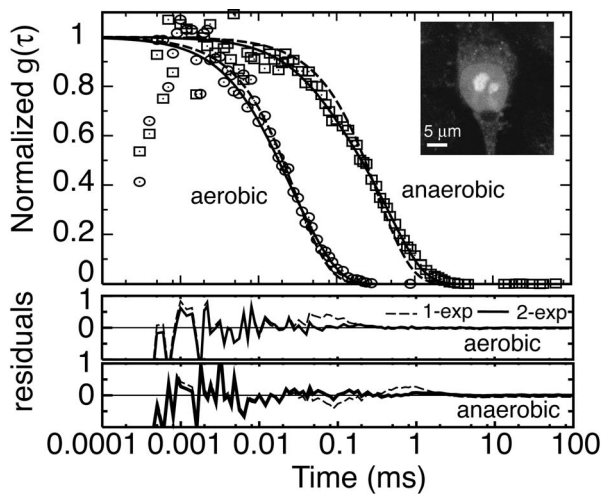
Fig. 3 Measurements with HeLa cells stained by Pd:TSPP. Typical decay curves of the phosphorescence correlation function, the residuals of the fittings, and a LSM image of phosphorescence (inset). The circles and boxes show the decay curves under aerobic and anaerobic conditions, respectively. The broken and solid lines denote the fitting results by single and double exponential functions, respectively. The LSM image was obtained under aerobic condition.

oxygen concentration with about 10% accuracy and a few percent accuracy under aerobic and anaerobic conditions, respectively. Thus the Clark-type electrode is better than our method under aerobic condition. In contrast, our method is better than the electrode under anaerobic condition.

The phosphorescence lifetimes obtained by the fitting were shown in Fig. 2(b). The curve shows a fitting result with the Stern-Volmer relationship $\tau^{-1} - \tau_0^{-1} = k_q[O_2]$, where τ_0 and k_q are the lifetime in the absence of oxygen and the quenching constant, respectively. The fitting was mostly in agreement with the data and yielded $\tau_0 = 0.826 \pm 0.034$ ms and $k_q = 0.233 \pm 0.006$ ms^{-1} Torr^{-1}. The quenching constant is in very good agreement with the previous report.[8] On the other hand, τ_0 was larger than our measurement value 0.59 ms under anaerobic condition and other reported values. This might be due to the error or the offset of the oxygen electrode.

Before the correlation measurements of HeLa cells, we confirmed that the cells could be stained. A phosphorescence image of HeLa cells, incubated with a 1-mM Pd:TSPP PBS solution for about 1 h, is shown in the inset of Fig. 3. Interestingly, the nucleus, in particular the nucleolus, were stained strongly. Some of the cells were not or very weakly stained. The variety of staining level of the cells might be related to the relatively high hydrophobic property of Pd:TSPP and an unknown transport mechanism.

The correlation functions of the phosphorescence at a point in cytoplasm in a cell under aerobic and anaerobic conditions are shown in Fig. 3. The count rates of the aerobic and anaerobic measurements were 0.28 and 0.44 kcps, respectively. The background without Pd:TSPP was about 0.27 kcps. The correlation amplitude was normalized to 1 and the correlation offset was subtracted.

The correlation curve under aerobic condition could be fitted by a single exponential function, and the difference be-

tween the single and double exponential fittings was very small, as shown by the residual plots. In contrast, it is clearly shown that the correlation curves under anaerobic condition involve two decay components. From the statistical viewpoint, the correlation curve under aerobic condition cannot be attributed to double exponential decay. However, the correlation curve under anaerobic condition suggests an existence of two different components. The large noise and the distortion of correlation function by dead time might obscure the details of the correlation curve under aerobic condition.

Commercially available FCS systems can be easily modified for our proposed method. The continuous-wave excitation laser of the system only needs to be replaced by a pulsed excitation source, which can be randomly triggered. Modifications of the detection system, however, are not required. This is very cost effective. Optimizations of our system may also allow measurements of small change of the oxygen concentration in a small volume in a single cell. The shorter time range of our system is limited by the detector and the recorder. However, the time range can be extended when a cross-correlation method[9] and a faster recorder are used. Furthermore, the applicability of our method is not restricted to microscope systems. For instance, a fiber-based lifetime measurement system can be replaced by our method. One interesting possibility is a combination with a fiber-based FCS,[10] which will extend the applicability to *in-vivo* systems. In conclusion, our method is not only for an extension to a fluorescence correlation system, but also for general lifetime measurement systems in biological research from microscopic to macroscopic scales.

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