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Parallelized fluorescence lifetime imaging microscopy (FLIM) based on photon reassignment

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ABSTRACT

We propose a novel parallelized fluorescence lifetime imaging microscopy (FLIM) method based on photon reassignment with a detector array and time-correlated single photon counting (TCSPC) array. The detector array composed of seven avalanche photon diodes (APDs) substitutes for original pinhole and detector to constitute an imaging scanning microscopy (ISM) system. The introduction of parallel detection can solve the pile-up effect caused by the dead-time in TCSPC measurement, which can enhance the effective photon detection efficiency and notably increase the fluorescence lifetime imaging speed. More importantly, the resolution and signal–noise ratio (SNR) can be enhanced based on photon reassignment. The simulation results embody the superiority of our FLIM system, and the experiments with different samples validate the improvement of the resolution and speed of fluorescence lifetime imaging. The experiment results show that the resolution of our method is enhanced by a factor of 1.5 and the imaging speed can be at least increased by a factor of 2.5 compared with conventional confocal TCSPC FLIM system. We envision that the proposed FLIM method based on parallel detection will allow for super-resolution fluorescence lifetime real-time imaging of live cell.

1. Introduction

Fluorescence microscopy has become an indispensable tool in modern biology and medicine due to the fact that the optical imaging is non-ionizing, non-destructive and minimally invasive [1,2]. Fluorescence lifetime imaging microscopy (FLIM) is a key fluorescence microscopy technique to map the environment and interaction of fluorescent probes, which can provide information about the localization of specific fluorophores and variations of fluorophore microenvironment surrounding living cell such as ion concentration, pH, oxygen concentration, refractive index, viscosity, and temperature. [3,4] FLIM techniques can not only sense the fluorescence intensity of fluorescent molecules, but also measure the decay rate (or lifetime) of the fluorescence. The measurement techniques of FLIM are generally subdivided into time-domain (TD) methods by time-correlated single photon counting (TCSPC) [5–7] and frequency-domain (FD) methods using lock-in detection [8–10]. FD FLIM is attractive for its rapid acquisition, easy implementation and reduced bandwidth requirement [8,11], but its imaging resolution is limited. Nevertheless, for TD FLIM, the combination of TCSPC and confocal laser scanning meets almost all the imaging requirements ideally, such as high lifetime accuracy, resolution of multi-exponential decay profiles, simultaneous recording in several wavelength intervals and optical sectioning ability [4]. However, the counting rate of a TCSPC is limited by pile-up effects and signal processing time of the time-measurement circuity [12–14], resulting in relatively low effective photon detection efficiency and long acquisition time.

In 1988, Shepard described a new method called imaging scanning microscopy (ISM) which simply used a detector array to substitute for pinhole and single detector in confocal system leading to the resolution and signal–noise ratio (SNR) improvement [15]. Based on photon reassignment, the signals from all detectors in the detector array are measured and reassigned to its particular image point instead of integrated directly as in confocal microscopy [16]. By reassigning each images optically [17,18] or through postprocessing back to the excitation axis and summing them up, an image with enhanced resolution and SNR can be obtained. Considering using the detector array and TCSPC array in FLIM, the pile-up effect caused by dead time of the detectors and timing electronics can be sufficiently suppressed. Therefore, the imaging speed of FLIM with parallelized detector and TCSPC should be improved significantly [19,20]. And combined with the principle of photon reassignment, the imaging resolution and SNR is enhanced simultaneously.

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In this paper, we propose a novel parallelized FLIM method based on photon reassignment. The introduction of the detector array and TCSPC array can solve the pile-up effect caused by the dead time of the electronics and improve the imaging resolution based on photon reassignment, which is analyzed and proved theoretically and experimentally. The parallel detection in our system is composed of seven avalanche photon diodes (APDs) and seven TCSPCs, and the APD array in our system is used to substitute the pinhole and detector in conventional confocal system, forming an imaging scanning microscopy (ISM) system. Each TCSPC could record the photons from the corresponding APD to acquire the fluorescence decay. Therefore, the proposed FLIM system could realize the improvement of resolution and signal-to-noise ratio (SNR), and the imaging speed could be boosted significantly for its enhancement of the effective photon detection efficiency.

2. Methods

Quantitatively, the fluorescence lifetime could be defined as the average dwell time of a fluorescent molecule in its excited state after absorbing a photon. In the experiment, the fluorescence lifetime is expressed as the time delay between absorbing the exciting pulse and emitting a photon, which follows an exponential decay distribution that can be derived ideally as

$$I(t) = I_0 e^{-t/\tau}, \tag{1}$$

where I_0 is the intensity at time $t = 0$ and τ is the fluorescence lifetime. Theoretically, the main sources of pile-up in modern TCSPC system are the dead-time of detector and timer [14]. In our system, however, the dead-time of detectors is the main factor owing to the fact that the dead-time of detectors (APD, 40 ns) is much longer than timer's dead-time (10 ns). After the first photon in any given excitation being detected and timed by TCSPC system, the next photons that arrive late in the decay would have a higher probability of being lost due to the pile-up effect. The pile-up effect should be described as

$$I(t,\mu) = I_0 e^{-t/\tau} e^{-\mu(1-e^{-t/\tau})}, \tag{2}$$

where μ is the average photon-rate and the excess part $e^{-\mu(1-e^{-t/\tau})}$ resulting from pile-up effect would distort the decay histogram towards a shorter lifetime mathematically [12]. In the process of experiment, the average intensity obtained is the function of the excitation period, T. Considering that T is assumed to be much longer than τ, the ideal function can be derived as [21]

$$\bar{I} = 1/T \times \int_0^T I(t)dt = \tau I_0(1 - e^{-T/\tau})/T \approx \tau I_0/T. \tag{3}$$

Similarly, with pile-up effect, the integral part is substituted by Eq. (2) and the average intensity is expressed approximately as

$$\begin{aligned} \bar{I}'(\mu) &= 1/T \times \int_0^T I(t,\mu)dt = \tau I_0(1 - e^{-\mu(1-e^{-T/\tau})})/\mu T \\ &\approx \tau I_0(1 - e^{-\mu})/\mu T. \end{aligned} \tag{4}$$

Therefore, if we simply define the effective photon detection efficiency (α) as the ratio between these two average intensity, it can be obtained in traditional confocal FLIM system as

$$\alpha = \bar{I}'(\mu)/\bar{I} = 1 - e^{-\mu}/\mu. \tag{5}$$

Fig. 1(b) shows the influence of pile-up effect on effective photon detection efficiency. Increasing μ from 0.1 to 1.0 and 5.0, the detection efficiency of single detector represented by black solid line drops from 95% to 64% and 20%, respectively. Therefore, in conventional confocal FLIM system, to avoid the distortion of the final fluorescence decay curves caused by the high detected photon counting rates, low counting rates is often adapted, resulting in the long acquisition time in scanning microscopy [18]. By means of parallel detection in FLIM system, pile-up effect is suppressed efficiently in that the photon lost by one detector can be possibly detected and timed by another detector and TCSPC in parallel detection. Therefore, the effective detection efficiency could be related to the spatial photon distribution in the detection plane. The quantum nature of light determines a Poisson distribution of the photons [14], as a results, the spatial photon probability distribution is expressed as

$$p(x,y) \sim Poisson(f(x,y)), \tag{6}$$

where $f(x,y)$ represents the system point spread function (PSF). Therefore, the probability distribution $p(x,y)$ could satisfy the Bessel function due to the vectorial character with high NA, and the average intensity obtained by the ith detector is derived as

$$\bar{I}_i'(\mu) = \tau I_0(1 - e^{-p_i\mu})/p_i\mu T, \tag{7}$$

where p_i is the probability that the ith detector in the detector array detect a photon, which is the sum of $p(x,y)$ in Eq. (6) of the positions in the zone of ith detector. And finally the effective photon detection efficiency can be rewritten as

$$\alpha' = \sum_i p_i \bar{I}_i'(\mu)/\bar{I} = \sum_i [p_i \times (1 - e^{-\mu p_i}/\mu p_i)]. \tag{8}$$

The simulation results are shown in Fig. 1 with 80 MHz repetition rate and high photon count rates at 50 Mpc/s. The fluorescence decay simulated is composed by convolving the lifetime decay of 3 ns and instrumental respond function (IRF) with the full width at half-maximum (FWHM) of 1 ns. Based on Eqs. (5) and (8), the relationship between effective photon detection efficiency (α) and photon rate (μ) is shown in Fig. 1(b), which highlights the parallel detection's superiority in suppressing the pile-up effect prominently. The curves of the detector array with different detector numbers shown in Fig. 1(a) indicate that more detectors obtain higher effective photon detection efficiency. Fig. 1(c) and (d) show that more detectors can detect and time more photons obviously with high photon rate ($\mu = 5$) and parallel detection effectively solves the trouble of lifetime decay distortion caused by pile-up effect with single detector. When the photon rate is low ($\mu = 1$), parallel detection with 7 detectors only need shorter dwell time than single detector to obtain the ideal lifetime decay marked as "No pile-up" in Fig. 2 with enough photons, which is shown in Fig. 1(e) and (f). The simulation results reflect that parallel detection with 7 detectors achieves around 2.7 times improvement in imaging speed compared with conventional confocal TCSPC system.

Considering imaging scanning microscopy, each detector on the detector array owns independent effective PSF that is expressed as the product of the excitation PSF (PSF_{exc}) and detection PSF (PSF_{em}) of the imaging system,

$$f_i(x,y) = PSF_{exc} \cdot (PSF_{em} \otimes PH_i(x,y)) \tag{9}$$

where $PH_i(x,y)$ stand for the equivalent size and location of each detector. For the detectors that are displaced from optical axis, their effective PSF would narrower than that in the optical axis, which reflects the gain in confocal resolution that is obtained as the contribution of higher frequencies is slightly stronger in the displaced elements. However, the amplitude of the displaced PSF would reduce meaning that the image intensities will be weaker than the center detector. As 7 detectors are used in our parallelized FLIM system shown in the inset of Fig. 3, here the process of photon reassignment is simulated and the effective PSF profiles of the center and displaced detector are shown in Fig. 2(a). By photon reassignment, the measured PSFs are reassigned to the correct position by an appreciate shift that is derived as

$$d_{eff} = \alpha \cdot d, \tag{10}$$

where d is the spatial displacement between the displaced detector and center detector, d_{eff} is the distance between the effective PSFs of displaced and center detector, and α is the scale factor of the

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