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Statistical Analysis of Fluorescence Correlation Spectroscopy of Ultra Low Concentration Molecules with a Confocal Microscope

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In this study, we simulated a statistical model of FCS (fluorescence correlation spectroscopy) based on a Poisson process to understand and explain observations of the experiment performed on molecules of ultra-low concentration by the home-built laser- scanning confocal microscope. The statistical model confirmed that the relative mean square amplitude of fluctuations is shown to be inversely proportional to the average number of molecules, even in the ultra-low concentration, if some conditions are satisfied. Signal-to-noise ratio and the variability of dwelling time under the confocal volume were found to be effective conditions for the experiment.

Keywords : fluorescent correlation spectroscopy, Poisson process, confocal microscope

OCIS codes : (110.0180) Microscopy ; (300.0300) Spectroscopy ; (300.6280) Spectroscopy, fluorescence and luminescence

It is important to understand the dynamics of molecules in biological systems such as biophysical processes of proteins and DNAs, which can be analyzed by measurement of diffusion coefficients, distance and reaction rates of molecules. FCS, one of the analyzing methods, exploits intensity fluctuations in the emission of a small number of fluorophore molecules. FCS is based on the statistical analysis of ACF (auto correlation function) of fluorescence fluctuations. ACF can give information about diffusion processes, conformational changes of protein complexes, and intramolecular dynamics [1-5].

In this study, in order to investigate the possibility that ACF can follow the trend that mean square amplitude of fluorescence signal is inversely proportional to the average number of molecules even in ultra-low concentration, we computed ACF of the fluorescence signals of nano using a home-built confocal microscope based on TCSPC (time correlated single photon counting) [6-7] and simulated a statistical model of ACF based on a Poisson process to explain the statistics of observations from the experiment. With the statistical model, we focused on two conditions such as signal-to-noise ratio and the dwelling time of molecules under the confocal volume.

If the number of molecules inside the confocal volume changes as a result of the diffusion of fluorophore molecules into and out of the confocal volume, the intensity fluctuation of the fluorescence signal occurrs. The normalized fluorescence correlation function of a fluctuating signal $F(t)$ is written as

$$G(\tau) = \frac{< \delta F(t)\delta F(t+\tau) >}{< F(t) >^2} \qquad (1)$$

where $\delta F(t)$ and $\delta F(t+\tau)$ are the amplitudes of fluctuations from the mean at time t and $t+\tau$ respectively,

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and $<F(t)>$ is the mean value of the signal. Thus, the autocorrelation is the normalized average product of the fluctuation of a signal from the mean at some time, t, with the fluctuation from the mean at some later time, $t+\tau$. The time, τ, is known as the delay time over which the fluctuations are compared [2]. The average number of molecules should be inversely proportional to the mean square amplitude of fluctuations which is equivalent to $G(0)$ as the following equation shows,

$$<N> = \frac{1}{[G(0)-1]} \tag{2}$$

where $<N>$ denotes the average number of molecules under the confocal volume, which is related to the sample concentration [1-4].

In our experiment, we computed ACF with measured fluorescence fluctuations of nano-spheres from 5 samples with different concentrations such as $1\times 10^3/\mu l$, $1\times 10^4/\mu l$, $1\times 10^5/\mu l$, $1\times 10^6/\mu l$, and $1\times 10^7/\mu l$. The ACF was calculated in every 1 second and averaged for 180 seconds, thus the total acquisition time is 180 seconds. Nano-spheres with the size of 0.02 μm, broad excitation bandwidth centered at 505 nm, and emission wavelength of 515 nm were obtained from Molecular Probes, Inc.. The five samples were prepared in phosphate buffered saline (pH 7.3) with detergent and pipetted 5 micro liters per each sample on the slide glass, and covered with a cover glass. As shown in Fig. 1, our confocal microscope system based on TCSPC consists of a 440 nm pulsed laser with average output power of 3.1 mW at a repetition rate of 40 MHz and the minimum pulse width of 59 pico seconds, 1 channel APD, a photon counter, a 40x (NA 0.6) PLAN objective, a bandpass filter with a passband of 515 ~ 569 nm, and a dichroic mirror with a reflectance bandwidth of 500 ~ 560 nm.

Fig. 2 shows the ACFs of 5 samples implemented with our confocal microscope system. The $G(0)$ values in the experiment were 19.2, 17.9, 13.7, 13.2, and 12.5, respectively from low to high concentration, and corresponding $<N>$ values are 5.49×10^{-2}, 5.92×10^{-2}, 7.87×10^{-2}, 8.20×10^{-2}, and 8.70×10^{-2}. By the way, the computed $<N>$ values of nano-spheres from effective confocal volume are 1.6×10^{-6}, 1.6×10^{-5}, 1.6×10^{-4}, 1.6×10^{-3} and 1.6×10^{-2}. As a result, it has the tendency that $G(0)$ is inversely proportional to $<N>$, but is not exactly fit to the Eq. (2).

To determine the meaning of this experiment, we simulated the observed ACF pattern computed from fluorescence fluctuations of nano-spheres with different concentrations. A Poisson distribution is generally accepted as the standard process to describe random discrete events of the burst of fluorescence signal because a randomly moving molecule is independent in the focal volume and is written as follows [1-4].

$$f(k,\lambda) = \frac{\lambda^k e^{-\lambda}}{k!} \tag{3}$$

where $f(k,\lambda)$ is a Poisson distribution of k fluorophores being present in the focal volume, k is the number of molecules coming in and out of the confocal volume, and λ is the expected number of k during the observation time.

By assuming that each event can be referred to as a Bernoulli experiment, we simulated FCS experiments with the restriction that the total number of sample is 1000. We take a Gaussian form in order to simulate a fluorescence burst profile with various widths.

At first, we adopted a homogeneous Poisson process to examine the relationship between $G(0)$ and $<N>$ as in Eq. (2) for the various signal-to-noise ratios as shown in Fig. 3. The signal-to-noise ratio of the experiment was acquired from the intensity ratio of the small-

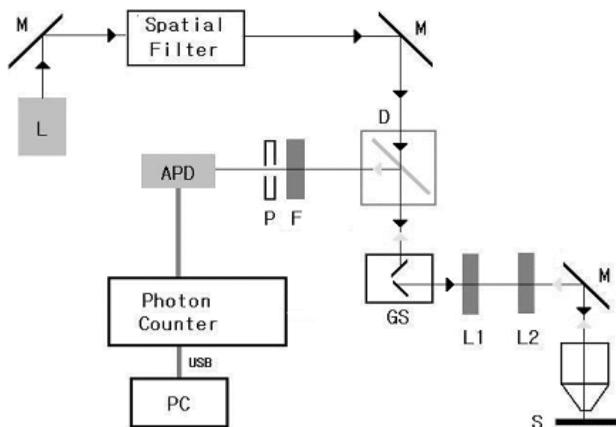

FIG. 1. Schematic diagram of the scanning confocal microscope system (L: laser, M: mirror, GS: galvano scanning mirrors, D: dichroic miror, L1: scan lens, L2: tube lens, F: filter, P: pinhole, S: Sample)

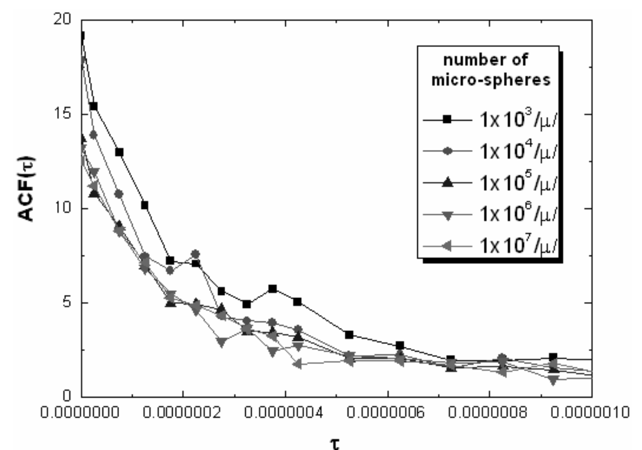

FIG. 2. Experimental result of ACF(τ) vs. correlation time lag τ according to the various number of nano-spheres.

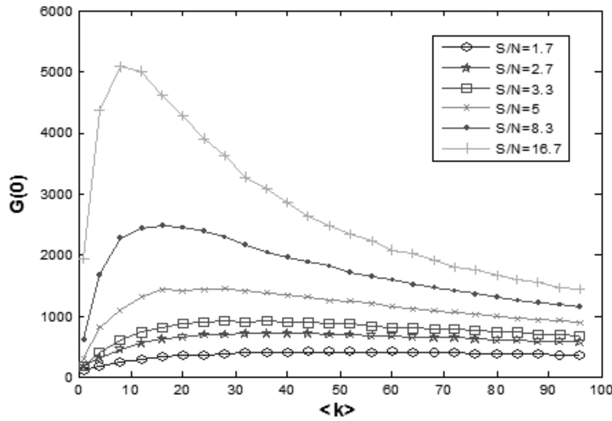

FIG. 3. The plot of simulation of $G(0)$ vs. $<k>$ with Poisson process for different signal-to-noise ratios. The burst width is set to 4.

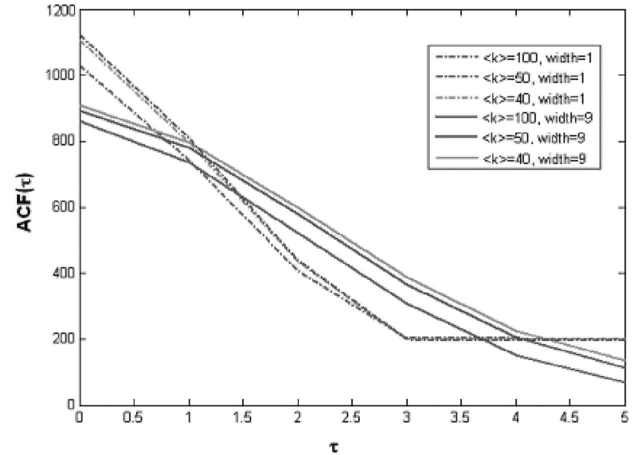

FIG. 4. The plot of simulation of ACF(τ) vs. correlation time lag τ by varying the burst width for 3 different $<k>$.

est number of nano-spheres($1\times 10^{3}\mu l$) over the phosphate buffered saline. So, the lowest signal-to-noise ratio of the experiment is 2.7. Poisson process with various signal- to-noise ratios was simulated by varying the signal strength. Herein the signal strength is given as the strength relative to the background noise. "Homogeneous" means that the number of occurrences, which is the number of molecules, in the confocal volume is uniform. With this simulation, we can find the minimum number of molecules coming in and out of the confocal volume given the signal-to-noise ratio from the experiment. By simply varying $<k>$, the mean value of the number of molecules, we can simulate the different concentrations of molecules diffused in and out of the confocal volume, which is related to $<N>$. With the simulated fluorescence signals for the various signal-to- noise ratio, we computed $G(0)$ values and plotted them as a function of $<k>$ in Fig. 3. In order that Eq. (2) is valid, the $G(0)$ should be decreased as $<k>$ increases. Fig. 3 shows that Eq. (2) can be valid only when the $<k>$ is above a certain value. It means that there is the minimum concentration for the given signal-to-noise ratio for Eq. (2) to be valid. According to the result of simulation, for higher signal-to-noise ratio, the minimum concentration is higher than that of lower signal-to-noise ratio. In our experiment of the signal-to-noise ratio of 2.7, Fig. 3 shows that Eq. (2) is valid from the $<k>$ value of around between 40 and 50, which is the average number of molecules coming in and out of the confocal volume during the acquisition time. The acquisition time of our experiment is 180 seconds, and the average number of molecules per one second under the confocal volume is in between 0.22 and 0.28. From this result we presumed that in our experiment, the number of molecules coming in and out of the confocal volume during one second should be above 0.22. As a result, we can find that if the signal-to-noise ratio is above a certain value, Eq. (2) can be valid, even in low concentration.

In addition, we varied the width of a burst profile model of the fluorescence signal to investigate the effect of dwelling time of nano-spheres under the confocal volume. The width of a burst means the width of the fluctuation produced by diffusion into and out of the confocal volume. The fluorescence signal model was modified by varying the width of the Poisson distribution. This modification can represent the variety of dynamics of diffusing molecules under the confocal volume. The longer width of a burst profile means the longer dwelling time. The dwelling time is important because we detect accumulated signals during the acquisition time. Moreover, there is a possibility that individual molecules can stay under the confocal volume during a certain period, even if the probability of existence of molecules under the confocal volume is extremely low. Fig. 4 shows the ACF graph with lag times according to various $<k>$ and width of the same signal-to-noise ratio signals as the experiment. With the width of one, the lowest width of the simulated signal, ACF is not consistent with the inverse of concentration, which means that $G(0)$, that is, ACF of zero lag time is not inversely related to the concentration. But, in case of width of 9, $G(0)$ values increase as the concentration decreases. This characteristic seems to support the idea that if the dwelling time of molecules under the confocal volume is long enough, the relationship of Eq. (2) holds good for the low concentration.

In conclusion, we found that if the signal-to-noise ratio and the dwelling time under the confocal volume are well selected, the ACFs of ultra low concentration molecules can follow the trend that the $G(0)$ is inversely proportional to the average number of molecules in the confocal volume.

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