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RESEARCH ARTICLE

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Fluorescence cross-correlation spectroscopy using single wavelength laser

Abstract In this paper, we first introduced the basic principle of fluorescence cross-correlation spectroscopy (FCCS) and then established an FCCS setup using a single wavelength laser. We systematically optimized the setup, and the detection volume reached about 0.7 fL. The home-built setup was successfully applied for the study of the binding reaction of human immunoglobulin G with goat antihuman immunoglobulin G. Using quantum dots (745 nm emission wavelength) and Rhodamine B (580 nm emission wavelength) as labeling probes and 532 nm laser beam as an excitation source, the cross-talk effect was almost completely suppressed. The molecule numbers in a highly focused volume, the concentration, and the diffusion time and hydrodynamic radii of the reaction products can be determined by FCCS system.

Keywords fluorescence cross-correlation spectroscopy, single-molecule detection, single laser excitation, quantum dot

With the rapid development of life sciences, people expect to study the conformational changes of biological macromolecules, the relationship between molecular structures and their functions at the single molecular level [1]. Furthermore, the molecular interaction is one of the research hotspots in this field [2, 3].

Currently, FCCS has become a powerful technique in single molecular detections due to its high sensitivity and short analysis times. This method has been used for measuring nucleic acid, protein conformational fluctuation [4–6], nucleic acid hybridization [7], protein interaction [8, 9], enzyme kinetics [10, 11], and the intracellular molecular interaction [12, 13]. Recently, some reviews have been published [14, 15], which introduced the theoretical background and improvements of FCCS.

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To date, most FCCS systems use either dual-color lasers or two-photon excitation systems that make the FCCS structure to be complicated and costly since an expensive two-photon laser is needed. In this article, we established a setup of FCCS using single wavelength laser on the basis of a confocal configuration. Using quantum dots and fluorescence dyes as the probes. we studied the immune reaction of human immunoglobulin G with goat antihuman immunoglobulin G as a reaction model by FCCS system.

1 Theoretical background of FCCS

FCCS was developed on the basis of fluorescence autocorrelation spectroscopy. This method not only possesses high sensitivity, but it also overcomes the limit in which their diffusion coefficients must differ by a factor of at least 1.6, in order to distinguish the two components in fluorescence correlation spectroscopy (FCS) analysis. We already introduced the methodologies [20] and applications [21–24] of FCS. Here, we simply described the theoretical background of FCCS [7, 11]. In the model of FCCS, there are two separation light channels (denoted by 1, 2). The fluorescence intensity fluctuations in two channels can be analyzed by cross-correlation function:

$$G_{12}(\tau) = \frac{\langle \delta F_1(t) \delta F_2(t+\tau) \rangle}{\langle F_1(t) \rangle \langle F_2(t) \rangle} \quad (1)$$

Whereas the fluorescence fluctuation $\delta F_1(t)$ is defined as the difference of the instantaneous fluorescence from the temporal mean in channel 1, $\delta F_2(t+\tau)$ is defined as the difference of the instantaneous fluoresce from the temporal mean in channel 2 after lag τ time. Taking into account the characteristics of the excitation and detection optics, a functional relation between the correlation function and the occupation times for translational diffusion for fluorescent species can be found.

$$G_{12}(\tau) = G_{12}(0)\left(1+\frac{\tau}{\tau_{D,12}}\right)^{-1}\left(1+\frac{\omega_0^2\tau}{Z_0^2\tau_{D,12}}\right)^{-\frac{1}{2}} \quad (2)$$

Whereas $\tau_{D,12}$ is average lateral transit time of the two-color binding molecules, which is defined as the τ value when $G_{12}(\tau)$ is equal to $G_{12}(0)/2$ as the same as in FCS model. The effective detection volume has an ellipsoidal shape. The ω_0 and Z_0 are the horizontal and vertical axes (are defined as where laser power value descended to $1/e^2$ from the center). Based on the above definition, the diffusion coefficient is then calculated on the basis of $D=\omega_0^2/(4\tau_{D,12})$. According to the Stokes-Einstein relation $R_h= kT/(6\pi\eta D)$, we can get the hydrodynamic radius R_h of the two-color binding molecule, where, D is diffusion coefficient, k is Boltzmann constant ($1.38\times10^{-23}\,\mathrm{J\cdot K^{-1}\cdot mol^{-1}}$), T is the absolute temperature, and η is the viscosity of the solvent.

In the experiment, fluorescence fluctuations of the two channels were simultaneously analyzed and cross-correlated. We first obtained the molecular number of channel 1 and 2 (N_{11} and N_{22}) and then calculated the molecular number of two-color bounding complex (N_{12}) based on following function:

$$N_{12} = 1/[G_{12}(0)\cdot N_{11}\cdot N_{22}]$$

Finally, we obtained the concentration of two-color binding complex according to N_{12} and the detection volume (decided by ω_0 and Z_0).

2 Experiments

2.1 Instrumentation and materials

The home-built FCCS system is shown in Fig. 1. In brief, the 532 nm YVO4 laser (Ion Laser Technology Co., Ltd. Shanghai) was attenuated by a circular neutral density filter (HB Optical Technology Co. Ltd. Shenyang). After it was expanded, the laser beam was reflected by a dichroic mirror (550DRLP, Omega Optical, USA) in an inverted fluorescence microscope (IX71, Olympus, Japan), afterwhich, it was focused into a sample solution by a water immersion objective (UplanApo, 60×NA1.2, Olympus, Japan). About 30 μL sample was placed on a cover slip (0.13 – 0.17 mm thickness, Sigma-Aldrich, USA). The excited fluorescence signal collected by the same objective passed through the dichroic mirror and then was split by another dichroic mirror (610DRLP, Omega Optical, USA). The short wavelength fluorescence was reflected by the mirror, filtered by a band-pass filter (565WB20, Omega Optical, USA), and finally collected after passing the 30 μm pinhole by avalanche photodiodes (SPCM-AQR14, Perkin-Elmer EG&G, Canada). The long wavelength florescence passed through the mirror, filtered by a long-pass filter (700 ALP, Omega Optical, USA), and finally collected after passing the 70 μm pinhole by another avalanche photodiodes. The yielded signals in the two channels were tracked and correlated by a real time correlator (ALV-5000/EPP, ALV-GmbH, Germany). The recording time per sample was 30 s. One mg/mL Rhodamine 6G and isothiocyanate Rhodamine B (Invitrogen Co.) solution were prepared and diluted according to the requirements of experiments. One mg/mL human immunoglobulin G and goat antihuman immunoglobulin G (Dingguo Biology Co. Ltd.) were prepared and kept at −20°C. Water-soluble quantum dots (QD745, emission wavelength 745 nm) were prepared as described in the reference [25]. All solutions were prepared with ultrapure water (18.2 MΩ) purified on Millipore Simplicity (Millipore).

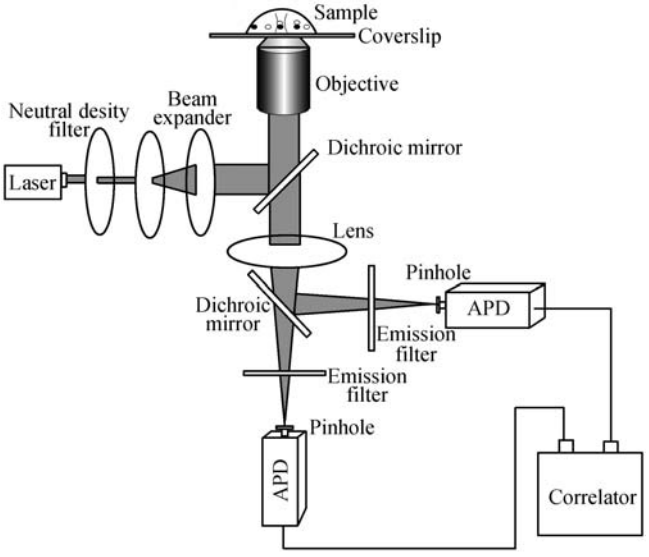

Fig. 1 FCCS setup with single wavelength laser

2.2 Methods

IgG was labeled with isothiocyanate Rhodamine B (RBITC) in carbonate buffer (0.2 mmol/L, pH 9.16). IgG reacted with RBITC at room temperature for 3–5 h. The mixture was purified using ultrafiltration membrane (NMWL 50 000, Millipore) by centrifugation three times at 8000 rpm. In this process, the free dyes could be removed, which was characterized by capillary electrophoresis. The goat antihuman IgG was conjugated at room temperature with QD745 for 3–5 h based on the adsorption of QDs to antibody. The free QD745 could be removed using an ultrafiltration membrane (NMWL 100000, Millipore) by centrifugation three times at 8000 rpm. When the antibodies were mixed at the different ratios, the immunocomplexes was formed. Then, 30 μL mixed solution was placed on a coverslip, and FCCS measurements were preformed on a home-build FCCS system.

3 Results and discussion

3.1 FCCS system

In the FCCS assays, cross-talk effect, which is the fluorescence from one channel that can be detected in the other channel, significantly influences the reliability of

FCCS results. In order to avoid this effect, the dye-pair emission wavelengths must be markedly different. Unfortunately, these dye-pair are very rare. Therefore, two lasers are usually necessitated in the construction of FCCS system. However, in two lasers FCCS system, we meet the problem on how to adjust two laser focuses to overlap.

In this article, the 532 nm laser beam was used as the excitation source. Quantum dots (745 nm) and Rhodamine B (580 nm) were used as fluorescence probes. Single wavelength laser FCCS was successfully realized due to the use of unique optical property of quantum dots. Quantum dots can be excited at a wide wavelength range since they have broad absorption spectra. In this study, QD745 can be excited by 532 nm laser, and the maximum emission wavelength is at 745 nm. The photoluminescence spectra of Rhodamine B and QD745 as well as the transmission spectral curves of the filter and mirror are shown in Fig. 2. The 610-nm dichroic mirror was used to split the fluorescence. The light in channels 1 and 2 were filtered by a 565 WB 20 band-pass filter and 700 nm long-pass filter. In this case, the cross-talk effect was near-completely suppressed. The results indicated that the fluorescence intensity ratio of Rhodamine B in channel 2 to channel 1 was 1/12 and the fluorescence intensity ratio of QD745 in the channel 1 to the channel 2 was 1/80.

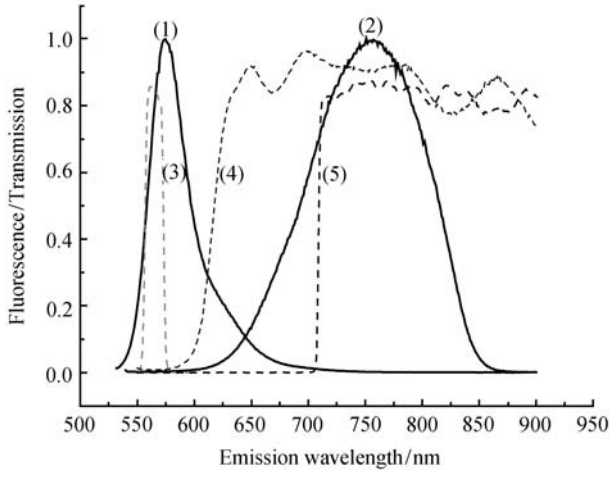

Fig. 2 Emission spectra of Rhodamine B and QD745
(1) Emission spectra of Rhodamine B, (2) Emission spectra of QD745, (3) Emission filter for channel 1,(4) Dichroic mirror, (5) Emission filter for channel 2

Figure 3 shows the correlation curves of immune complex including three curves. Curve 1 is the fluorescence autocorrelation curve of Rhodamine B, curve 2 is the fluorescence autocorrelation curve of QD745 and curve 3 is the FCCS curve of immune complex.

3.2 Detection Volume

The accurate measurement of the detection volume is the precondition to calculate the molecule concentration. The

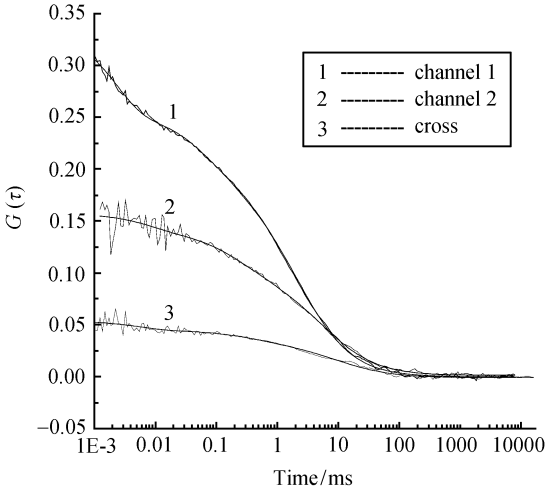

Fig. 3 FCS curves in channel 1, 2 and FCCS curve

structure parameter z_0/ω_0 were determined using 1.0×10^{-8} mol/L Rhodamine 6G, assuming its diffusion constant of $D=2.8\times10^{-6}$ cm^2/s in water. The recording time was 30 s repeated 11 times. The data were plugged into formula 2. The obtained ω_0 and z_0 were 0.26 μm and 1.89 μm, and their RSDs were 0.8 % and 5.6 % ($n=11$) respectively. The detection volume in channel 1 was 0.7 fL according to the formula $V_{eff}=\pi^{3/2}\omega_0^2 z_0$ [7].

Since it is very difficult to find a conventional organic dye whose excitation wavelength is 532 nm and emission wavelength is more than 700 nm, the above method cannot be used to measure the detection volume in channel 2. Instead, we used an indirect method to measure the detection volume in channel 2. In detail, Rhodamine B was first used to determine the detection volume on a 590-nm long-pass filter in the same method as the method described above. Afterwhich, the Rhodamine B sample was replaced by QD745; the number of particles was recorded in the same condition. Last, 590 nm long-pass filter was replaced by 700 nm long-pass filter; the number of QD745 particles was recorded again. Based on the ratio of the particle numbers in twice measurements, the volume in channel 2 could be obtained indirectly to be 1.5 fL.

Since it was dependent on the intersection of two channels, the detection volume in FCCS system about was 0.7 fL, which was decided by the smaller volume in two channels.

3.3 Reproducibility

The labeled human immunoglobulin G was mixed with goat antihuman immunoglobulin G at the different ratios, and the mixture was determined after dilution with a buffer. To study the reproducibility of our system, the samples were determined seven times. The concentrations of the complex were calculated on the basis of the formula

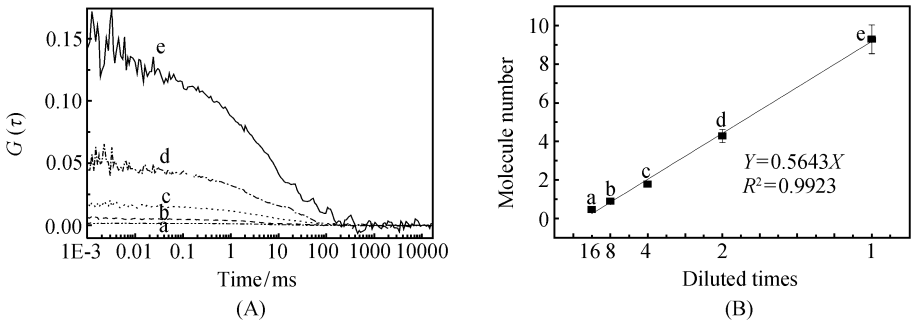

Fig. 4 FCCS curves of reaction products (A) and relation ship between molecular number and diluting times of the solution after the binding reaction (B). Concentration of reaction product/ (mol·L^{-1}) : (a) 1.14×10^{-9} ; (b) 2.07×10^{-9} ; (c) 4.27×10^{-9} ; (d) 1.02×10^{-8} ; (e) 2.21×10^{-8}

$C_{12} = \dfrac{N_{12}}{V * N_A}$, where V is cross volume, and N_A is Avogadro's constant. The results showed that this method has good reproducibility, and the RSD of the concentrations was less than 8.2%.

3.4 Reliability

The immune complexes were analyzed by FCCS after they were diluted to various concentrations. The results are shown in Fig. 4(A). The amplitude $G_{12}(0)$ increased with the increase of immune complex concentration. A good linear relation (R^2= 0.992) was obtained by plotting the molecular number to dilution times [Fig. 4(B)]. The molecular number in the detection volume is 0.4–10. This data illustrated that our FCCS system was an effective one.

The Normalized FCCS curves are shown in Fig. 5, which were obtained by normalized treatment from Fig. 4. We observed that different concentration complex had the same diffusion coefficient ($\tau_{D,12}$ (4.138±0.019) ms). This result illustrated that the conformation of the complex had no change. The diffusion coefficient was calculated to be 4.08×10^{-12} m^2/s, and the hydrodynamic radius of the reaction product was about 54 nm.

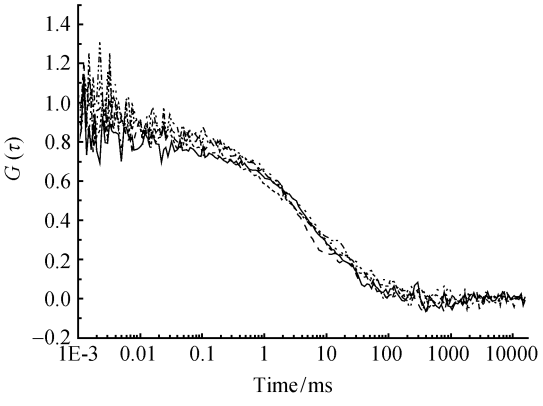

Fig. 5 Normalized FCCS curves of the immune complex

4 Conclusion

In this article, we established a setup of single wavelength FCCS and systematically investigated some parameters of the FCCS system. Using this system we successfully obtained the molecule number concentration, diffusion coefficient, and hydrodynamic radius of the immune complex. Our results demonstrated that single wavelength FCCS was easily realized and the cross-talk effect was almost completely suppressed by using quantum dots as labeling probes.

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