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Quantitative detection of antibody based on single-molecule counting by total internal reflection fluorescence microscopy with quantum dot labeling

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ABSTRACT

We presented a sensitive method to quantify antibody based on single-molecule counting by total internal reflection fluorescence microscopy with quantum dot labeling. In this method, the biotinylated monoclonal anti-human IgG molecules were immobilized on the silanized glass substrate surface. By the strong biotin–streptavidin affinity, streptavidin-coated quantum dots were labeled to the target molecules as fluorescent probe. Then, images of fluorescent spots in the evanescent wave field were obtained by a high-sensitivity electron multiplying charge-coupled device. Finally, the number of fluorescent spots corresponding to single molecules in the subframe images was counted, one by one. The linear range of 8.0×10^{-14} to 5.0×10^{-12} mol L^{-1} was obtained between the number of single molecules and the sample concentration.

1. Introduction

Single-molecule detection (SMD), based on the analysis of individual molecules, has attracted a great deal of attention in the fields of analytical chemistry and life science within the last decade [1,2]. A key factor for SMD in solution is to work with an ultrasmall probe volume to minimize the background effect [3,4]. To confine the probe volume, nanometer-scale pores [5], microchannels or capillary [6–8], confocal fluorescence microscopy [9–11], and total internal reflection microscopy (TIRFM) are valid techniques. Of these techniques, TIRFM is a powerful tool for SMD in solution. Total internal reflection (TIR) of the light generates an evanescent wave at an interface, which provides a penetration depth of a few hundred nanometers (<350 nm) [3,4,12]. Because of the small probe volume produced by the limited penetration depth [13], the background signal from the bulk is strongly reduced, and consequently the signal-to-noise (S/N) ratio is much better than those in other techniques. TIRFM has a good inherent axial resolution, and is also suitable for single fluorescence molecule detection at the interface [14].

All researches on quantitative analysis at the single-molecule level by TIRFM perform the counting of single fluorescent molecules [4,15–17]. Fang and Tan [15] developed a fluorescent method for single-molecule counting using an optical fiber probe and TIRFM. The fluorophores were excited by the evanescent wave field, and then the fluorescent signals were collected at a microscope and detected by an intensified charge-coupled device. Rhodamine 6G (R6G) and R6G-labeled DNA molecules were taken as the model molecules. The linear relationship between the average number of bright spots and the concentration was in the range of 2.5×10^{-9} to 1.7×10^{-8} mol L^{-1}. In our previous study [4], we developed a single-molecule imaging method for quantification of protein by TIRFM with adsorption equilibrium. The fluorescence images of single molecules were taken by an electron multiplying charge-coupled device (EMCCD). By counting the number of fluorescent spots corresponding to the protein molecules in the subframe images, a linear range of 5.4×10^{-11} to 8.1×10^{-10} mol L^{-1} was obtained between the spot number and protein concentration. Yeung and co-workers [16] developed a sensitive method to quantify human papilloma virus-16 DNA based on TIRFM that used a single-molecule imaging system. Both the single-probe scheme and the dual-probe method were employed to label target DNA. The limit of detection was 0.7 copy/cell with the single-probe method, and the linear dynamic range of 1.44–7000 copies/cell was obtained with the dual-probe model. Jin and co-workers [17] presented an ultrasensitive quantitative method for fluorescent molecules using a single-molecule counting approach. First, the fluorescent molecules were immobilized and accumulated on the coverslip using an electrochemical adsorption accumulation method, and then images of them were acquired by a TIRFM instrument in connection with an EMCCD. By

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counting the number of fluorescent spots corresponding to single molecules in 100 images, the linear range of 10^{-15} to 10^{-12} mol L^{-1} between the number of fluorescent molecules and the sample concentrations were obtained.

The fluorescent labels should be bright and stable enough in single-molecule fluorescence detection, which provides exquisite sensitivity (in terms of signal intensity and detection limit) [18], and guarantees the reliability of quantitative determination. Compared with organic dyes and fluorescent proteins, quantum dots (QDs) possess unique photophysics properties. These include broad and tunable absorption spectrum, narrow emission spectrum, high brilliance and photostability [19–21]. By taking advantage of these properties, several research groups have employed QDs for SMD studies [22–24] recently. For example, Nie and co-workers [22] reported the utility of QDs for real-time detection of single molecules in a microfluidic channel. They developed a nanoparticle "sandwich" assay using different QDs conjugated to antibodies that would recognize two binding sites on a single target molecule. A signal was detected when two different color QDs simultaneously recognized the target molecule. Quantitative studies indicated that the detection limit of the method was 10 target molecules (proteins, genes, or intact viruses).

Due to ultrasmall probe volume of TIFRM, the large diffusion rate and the short residence time of single molecules in the evanescent field [17], it is very difficult to capture single molecules in solution. To solve this problem, we immobilized the antibody molecules onto the silanized coverslip surface [25,26]. Then, the fluorescence label was performed via QDs labeled on antibody according to the strong biotin–streptavidin affinity. The images of fluorescent spots corresponding to the anti-human IgG molecules immobilized on the substrate surface were taken by an objective-type TIRF microscope equipped with an EMCCD. Based on counting of single fluorescence molecules, one by one, a linear relation between the total number of fluorescent spots and the anti-human IgG concentration was obtained in the range of 8.0×10^{-14} to 5.0×10^{-12} mol L^{-1}.

2. Experimental

2.1. Reagents and apparatus

Monoclonal anti-human IgG–biotin conjugate (Clone HP-6017) and 3-glycidyloxypropyltrimethoxysilane were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). QD605–streptavidin conjugates were obtained from Invitrogen Co. (Eugene, OK, USA). Water-free toluene was the product of distilled toluene. Other reagents and chemicals (analytical grade) were acquired from standard reagent suppliers. All buffer solutions were prepared with pure water and filtered through a 0.22-μm filter twice. Microscope cover glasses (22 mm × 22 mm) were purchased from Cole-Parmer (Illinois, USA).

The TIRFM imaging system was performed with an inverted microscope (Model IX81, Olympus, Tokyo, Japan) equipped with a high-numerical-aperture 60× (1.45 NA) oil-immersion objective lens (PlanApo TIRFM, Olympus, Tokyo, Japan), a fluorescence microscope control unit (IX2-UCB, Olympus, Tokyo, Japan), a solid laser with λ = 488 nm (30 mW, Melles Griot, Carlsbad, CA), a laser incidence angle adjustment knob, a mirror unit consisting of a 470–490-nm excitation filter (BP470-490), a 505-nm dichromatic mirror (DM 505), a >580-nm emission filter (IF580) and a 16-bit thermoelectrically cooled EMCCD (Cascade 512B, Tucson, AZ, USA). Fluorescent QDs were excited by the 488-nm line of the laser for TIRFM. The EMCCD was used for collecting the fluorescent images. Imaging acquisition and data analysis were performed by the MetaMorph software (Universal Imaging, Downingtown, PA, USA).

2.2. Substrate preparation

Microscope cover glasses were soaked in chromic acid for 24 h, extensively rinsed with water. Then, the glasses were cleaned by sonication in a freshly prepared mixture of abstergent and water (1:10, v/v) for 1 h, soaked in for 12 h, and thoroughly cleaned with water. Next steps were cleaned by sonication each in acetone, ethanol, and pure water for 20 min, activated in 1:1:1 hydrochloric acid, hydrogenperoxide, and pure water in an ultrasonic bath for 30 min, and then cleaned with pure water for 20 min. Finally, the activated glasses were dried at 120 °C for 20 min in the baking oven.

The pretreated glasses were silanized in a 6% (v/v) solution of GOPS in water-free toluene at 95 °C for 10 h, cooled at room temperature for 12 h. The silanized glasses were then treated by sonication in toluene, ethanol, and pure water. After dried with nitrogen stream and stored in a dryer for 15 min at 75 °C, the glasses were stored in a desiccator at room temperature.

2.3. Anti-human IgG immobilization

Each concentration (5.0×10^{-12}, 2.0×10^{-12}, 1.0×10^{-12}, 5.0×10^{-13}, 8.0×10^{-14} mol L^{-1}) of anti-human IgG was added onto the freshly prepared substrate surface in 25 μL phosphate buffered saline (PBS, pH 7.4) solution. The substrate was immediately placed in a sealed Petri dish at room temperature for 24 h. Finally, it was rinsed with pure water for 30 s, and dried under a nitrogen stream.

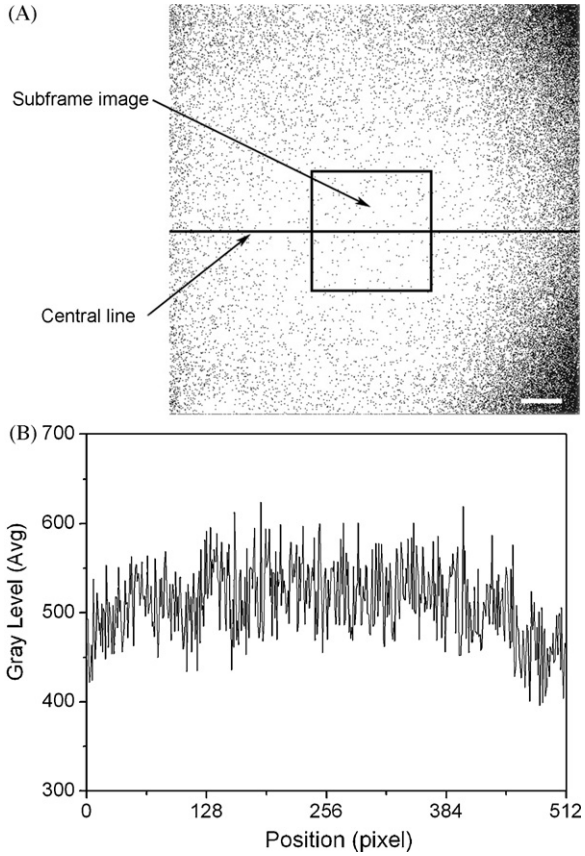

Fig. 1. (A) Fluorescence image of silanized substrates with borate buffer solution and (B) distribution of fluorescence intensity in the *x* direction along the central line of the bright spot indicated in (A). Scale bar, 50 pixel. Conditions: laser power, 30 mW; excitation wavelength, 488 nm; emission wavelength, >580 nm. Image acquirement: readout speed, 5 MHz; exposure time, 120 ms.