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Quantification of protein based on single-molecule counting by total internal reflection fluorescence microscopy with adsorption equilibrium

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Abstract

We developed a sensitive single-molecule imaging method for quantification of protein by total internal reflection fluorescence microscopy with adsorption equilibrium. In this method, the adsorption equilibrium of protein was achieved between solution and glass substrate. Then, fluorescence images of protein molecules in a evanescent wave field were taken by a highly sensitive electron multiplying charge coupled device. Finally, the number of fluorescent spots corresponding to the protein molecules in the images was counted. Alexa Fluor 488-labeled goat anti-rat IgG(H + L) was chosen as the model protein. The spot number showed an excellent linear relationship with protein concentration. The concentration linear range was 5.4×10^{-11} to 8.1×10^{-10} mol L^{-1}.

Keywords: Total internal reflection fluorescence microscopy; Single-molecule detection; Protein

1. Introduction

In the last decade, single-molecule detection (SMD) in aqueous solution has attracted a great deal of attention in the area of analytical chemistry due to ultra-low limit of detection (LOD) that depends on signal-to-noise (*S*/*N*) ratio. A variety of techniques such as dark-field microscopy [1], differential interference contrast microscopy [2], scanning electrochemical microscopy [3,4] and laser-induced fluorescence (LIF) detection have been used for SMD. Of these techniques, LIF detection is a powerful and useful technique suitable for SMD due to its broad applicability and high sensitivity. For SMD with LIF detection, signal can be amplified via enzyme-catalyzed reactions [5–7] or polymerase chain reaction (PCR) [8,9]. However, LOD for SMD in solution is mainly limited by high background from light scattering and fluorescent impurities, as ultrasensitive detectors such as avalanche photodiode and intensified charge coupled device (ICCD) are applied. It is possible to reduce Rayleigh scattering and Raman scattering using pulsed lasers and gated detectors [10–13]. Another way is to work with an ultrasmall probe volume to decrease the background. For this purpose, hydrodynamic focusing [10–13], levitated microdroplets [14–16], capillary or microchannel [5,7–9,17,18], confocal fluorescence microscope [18–23] and nanometer-scale pores [24] are commonly used with the probe volumes of 10^{-12} to 10^{-15} L^{-1}.

Total internal reflection fluorescence microscopy (TIRFM) is also an excellent technique for SMD in solution [25–32]. In this technique, an evanescent wave field is produced at an interface with a penetration depth of <350 nm depending on the light being used for excitation [33,34]. When fluorescent molecules enter the evanescent wave field, they are excited and imaged rapidly by ICCD. Therefore, the probe volume should be the product of the penetration depth and the fluorescent spot area occupied by a single molecule. For a penetration depth of 250 nm and one pixel of the ICCD, the probe volume is $\sim 3 \times 10^{-17}$ L^{-1} [27] much less than those in other techniques mentioned above, i.e. the *S*/*N* ratio is much better than those in other techniques. Thus, fluorescence or light scattering from solvent molecules becomes negligible. In 1976, Hirschfeid [35] first reported detection of a single polyethylenimine polymer containing 80–100 fluorescein isothiocyanate tags bound to γ-globulin supported on a microscope slide with TIRFM in a stationary, solvent-free system. Moerner and co-workers detected the diffusion of single dye molecules in and out of a polyacrylamide gel with TIRFM [25]. Yeung and co-workers directly measured single-molecule diffusion and

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photodecomposition in free solution, investigated the electrostatic trapping of protein molecules at a liquid–solid interface, provided a high-throughput single-molecule spectroscopy in free solution and observed anomalous single-molecule enzyme kinetics with prism-type TIRFM [26–30]. Singh-Zocchi et al. detected the nanometer-scale conformational changes of single DNA oligomers using prism-type TIRFM [31]. In all the above methods, TIRFM is used to investigate molecule characteristics, but not to quantify analyte concentrations. However, direct quantification for analytes of interest by counting molecules is possible. Fang and Tan reported a quantitative SMD method using TIRFM [32]. The fluorescent signals from fluorophores exited by the evanescent wave were collected by a microscope objective and detected by an ICCD. They applied this system to image rhodamine 6G (R6G) and R6G-labeled DNA molecules at the single-molecule level. Then, number of the bright spots in each image corresponding to the analyte of interest was counted. 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 this paper, we developed a new sensitive TIRFM for SMD, to reduce the low end of the linear relationship. Goat anti-rat IgG(H + L) labeled by Alexa Fluor 488 (IgG(H + L)-488) was chosen as the model protein. First, the solution of IgG(H + L)-488 was incubated with a glass microscope coverslip for a certain time. In this case, the adsorption equilibrium of IgG(H + L)-488 between the solution and the coverslip was achieved. Then, the image of the fluorescent spots in the evanescent wave field corresponding to the IgG(H + L)-488 molecules adsorbed on the coverslip and in the solution was taken by an objective-type total internal reflection fluorescence microscope equipped with an electron multiplying charge coupled device (EMCCD). The number of fluorescent spots in the image was a linear function of IgG(H + L)-488 concentration in the range of 5.4×10^{-11} mol L^{-1} to 8.1×10^{-10} mol L^{-1}. The low end of the linear relationship was 46-fold lower than that using TIRFM without adsorption reported in Ref [32].

2. Experimental

2.1. Reagent and apparatus

Goat anti-rat IgG(H + L) labeled by Alexa Fluor 488 (2 mg mL^{-1}, MW = 148000, Molecular Probes, Eugene, OR, USA) was dissolved in phosphate-buffered saline (PBS) consisting of 150 mmol L^{-1} NaCl, 7.6 mmol L^{-1} Na_2HPO_4, and 2.4 mmol L^{-1} NaH_2PO_4 (pH = 7.4). Other chemicals (analytical grade) were purchased from standard reagent suppliers. PBS was prepared with doubly distilled water and filtered through a 0.22 μm filter twice. Before use, PBS was irradiated for 12 h by ultraviolet light.

TIRFM measurements were accomplished with an inverted microscope (Model IX81, Olympus, Tokyo, Japan) equipped with a high-numerical-aperture 100 × (1.65 NA) TIRFM oil-immersion objective (PlanApo TIRFM, Olympus, Tokyo, Japan), a fluorescence microscope control unit (IX2-UCB, Olympus, Tokyo, Japan), a multiline Ar ion laser with an output power of 10 mW for 488 nm (Melles Griot, Carlsbad, USA), a laser incidence angle adjustment knob, a mirror unit consisting of a 470 to 490 nm excitation filter (BP470-490), a 505 nm dichromatic mirror (DM 505), a 510 to 550 nm emission filter (BA510-550) and a 16-bit thermoelectrically cooled EMCCD (Cascade 512B, Tucson, Arizona, USA). In order to match the 100 × TIRFM oil-immersion objective in TIRFM experiments, special coverslips with a thickness of 0.13 mm (Olympus, Tokyo, Japan) and special immersion oil (Olympus, Tokyo, Japan) were used. IgG(H + L)-488 molecules were excited by the 488-nm line of the laser through the TIRFM oil-immersion objective. The fluorescence emitted by the IgG(H + L)-488 molecules was collected by the same objective and the fluorescent image was acquired by the EMCCD. Imaging acquisition was controlled by the MetaMorph software (Universal Imaging, Downingtown, PA, USA).

2.2. Single-molecule imaging using the objective-type TIRFM

To produce total internal reflection (TIR) of the laser beam at the interface between the solution and the coverslip, the incident angle of the laser beam was adjusted carefully by turning the laser incidence angle adjustment knob. First, the incident angle of the laser beam was adjusted roughly. A semitransparent paper was put between the mirror unit and the objective. The laser beam was adjusted away from the center axes. Two bright spots, corresponding to incident light and reflected light respectively, could be seen on the paper. The brightness of reflected light was weaker than that of incident light. When TIR was achieved, the brightness of both spots was nearly the same, because the laser was reflected totally. In this case, the distance between the two bright spots had a maximum. To check the TIR situaction, a coverslip was placed on the oil-immersion objective. After the coverslip was irradiated for 8 min by the laser with a 488 nm line, 3 μL L^{-1} of IgG(H + L)-488 solution was added onto the coverslip. The incident angle of the laser beam and the focus of the objective were adjusted finely until the clearest image with "twinkle" of light spots was observed, meaning that the fluorescently labeled molecules moved in or out the evanescent wave field. The image with the IgG(H + L)-488 molecules in the evanescent wave field was taken immediately by the EMCCD. The data on the image was analyzed by the MetaMorph software.

3. Results and discussion

3.1. Imaging of single IgG(H + L)-488 molecules

After PBS was irradiated for 12 h by ultraviolet light and the coverslip was irradiated for 8 min by laser light, the background noise was decreased greatly. The resolution of single IgG(H + L)-488 molecules in the fluorescence images depended on laser power and exposure time. When a laser power of 6 mW and an exposure time of 100 ms were used, the signal-to-noise ratio was 5.3. In this case, single IgG(H + L)-488 molecules could be identified easily. To acquire images of single