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Distribution of single molecules in polymer thin films

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Abstract

Distribution of fluorescent dye molecules in polymer thin (∼100 nm) films was investigated using far-field single-molecule video microscopy, by varying concentrations of dye molecules mixed in the polymer. Histograms of fluorescence photocounts of individual fluorescent spots showed wide distribution, varying in the number of fluorescent spots composed of one, two, three or group of molecules. The number of the molecules present in the fluorescent spots was also ascertained by fluorescence photobleaching experiments. Photocounts associated with maxima of the histograms were found to be independent of the concentrations; however, the number of occurrences associated with more than one molecule decreased with decreasing concentration. By reducing concentration as well as by mixing dye molecules into a polymer solution, fluorescent spots grouping more than one molecule were separated considerably into fluorescent spots including a single-molecule.

Keywords: Single molecules; Rhodamine B; Photobleaching

1. Introduction

Recent studies of single molecules using fluorescence microscopy [1–24] have revealed intrinsic properties of individual molecules averaged on and blurred under bulk measurements, thus, placing great importance on the study of single molecules in physical [4–10] and biological [5,11] science. One of the important advantages of single-molecule studies is probing directly heterogeneity of individual molecules. Also, fluorescence measurement provides a powerful tool of understanding ground and excited state properties of single molecules.

Study of single molecules in solution has limitations to follow up molecular processes due to solvent dynamic effects [12]. Immobilization of molecules on solid supports, such as glass or quarts surfaces [4–10,13–20], has been used for overcoming these effects. Because dye molecules directly attached on an air–solid interface are liable to fluorescence quenching and photobleaching [4,15,21–24], secondary supports such as polymers are used for avoiding fluorescence quenching and photobleaching [5,8,13,18–20]. A drop-and-drag method [9,10], spin coating a $\sim 10^{-9}$ M dye solution over a polymer film [13,17] as well as spin coating a polymer solution over the dye molecules on surfaces [10] were used for providing solid supports and also for minimizing photobleaching.

The use of a $\sim 10^{-9}$ M dye solution is a common practice of and a criterion for observing single molecules on a surface using light microscopy. The number of molecules in a fluorescent spot is estimated from the number of steps in photobleaching experiment [21–24]. However, ascertaining the number more than three was not achieved in these studies. To facilitate single-molecule studies, therefore, it is important to find experimental conditions that the occurrences of fluorescent spots including molecules more than three is small before photobleaching experiment. Also, digitized histograms of fluorescence photocounts of individual fluorescent spots provide evidence for detection of single molecules [8–10]. However, what change occurs on the distribution of single molecules when the dye concentration is changed, which is characterized by the digitized histograms, is unknown in the previous studies [10] because of the use of limited concentrations. Dependence of digitized histograms on the concentration of dye solutions was examined in [9]; however, the histograms in [9] were obtained from fluorescent spots on special sites sparsely dotted on a silicon surface. The single-molecule fluorescence on the special sites is free from quenching, whereas that on the site other than the special sites is strongly quenched [9]. Thus, dependence of the distribution of single molecules on the concentration of dye

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solutions should be examined using a quartz (or glass) surface, which is basically free from fluorescence quenching characteristics, in evaluating precisely the distribution of single molecules. Indeed, it is important to quantitatively evaluate the distribution of molecules on a surface for preparing single-molecule samples.

In the current work, concentration-dependent distribution of single molecules in a polymer film on a quartz surface was evaluated on the basis of the distribution of photocounts and the photobleaching of individual fluorescent spots. No previous work reported such quantitative evaluation of the grouping of molecules. Quantitative evaluation of the grouping of molecules, which is the key aspect of the current work, will provide us a solid foundation of preparing quantitatively molecular-dispersed samples before implementing single-molecule photochemical studies.

2. Experimental section

2.1. Materials

Quartz plates used in the current work were optically cleaned by sonication in a mild (∼1%) detergent solution for 30 min, followed by repeated washing with Milli-Q water, acetone (Wako, Tokyo), and ethanol (Wako) and then dried in a jet of nitrogen gas. Rhodamine B (rhB) was purchased from NACALAI (Kyoto) and polyvinyl alcohol (PVA, average molecular weight 500) from Wako. Solvents and reagents involved in this work were used as supplied.

Samples were prepared by spin coating (∼3700 rpm) of a 100 μl rhB solution (5–160 nM) in Milli-Q water, each containing 5 mM PVA, on optically cleaned quartz surfaces.

2.2. Methods

The distribution of rhB molecules in a PVA film was determined using far-field light microscopy [8]. The excitation source (10 mW) was the second harmonics (532 nm) of a cw Nd:YAG laser (Coherent DPSS 532) and was circularly polarized using a λ/4 plate for uniformly irradiating rhB molecules, irrespective of the transition-dipole direction of individual molecules. The colatitude of the laser beam was ∼65° with respect to the surface normal, which is parallel with the optical axis of a microscope (Nikon Optiphot XP). The fluorescence emission was determined using the microscope equipped with a 100× objective with NA 0.75 (Nikon), a long pass filter for red fluorescence, and a photon-counting video camera (Hamamatsu C2400-40).

Photobleaching experiments were done at high laser power excitation, by irradiating using a frequency-doubled (532 nm) diode pumped cw Nd:YVO_4 laser (Spectra Physics Millennia IIs), arranged with a second microscope.[1] The emitted fluorescence was determined using the microscope (Olympus IX70) equipped with a long pass filter for red fluorescence, a 100× objective with NA 0.95 (Olympus), an image intensifier (Video Scope VS4-1845), and a CCD camera (Hamamatsu C5985). Average thickness of the polymer film was found to be ∼100 nm using a surface profile measuring system (Veeco DEKTAK^{3}ST, CA, USA). All measurements were done at room temperature (296 K) in air.

3. Results and discussion

Generally, fluorescence image of dye molecules, deposited on a surface depends on the uniformity of the surface used as well as on the dye concentration selected. In the current study, uniformity of a quartz surface was achieved by coating polymer on the surface and a uniform distribution of dye molecules by mixing the rhB solution into a polymer solution. Figs. 1A and B show representative fluorescence images collected in 30 s accumulation from quartz surfaces, spin coated with 50 and 5 nM rhB solutions, respectively, each containing 5 mM PVA. Figs. 1A and B also show that many well-separated fluorescent spots were observed, mainly from single molecules and, the number of fluorescent spots decreased with decreasing dye concentration.

Figs. 2A–D show histograms of fluorescence photocounts from individual fluorescent spots of different concentrations of rhB in a PVA film. Note that the positions of photocount maxima remained almost constant, irrespective of the dye concentrations selected. Also, the photocounts of each maximum were approximately integral multiples of ∼200 counts, which is the lowest average photocount observed in these experiments. There are slight variations in the maxima in Figs. 2A–D more precisely, in Fig. 2A the maxima are at 240, 410, 670, and 850 photocounts while those in Figs. 2B–D are at 230, 400, 650, 950; 230, 390, 695, 950 and 185, 385, 580, 735 photocounts, respectively. It is safe to postulate that these variations in the photocount maxima originate from the difference in the fluorescence quantum yield from molecule to molecule,

[1] We used two microscopes for a practical reason, but not for a theoretical reason. To compare our previous work [8,9] with the current work and to evaluate quantitatively the number of fluorescence photocounts, we used a Nikon Optiphot XP microscope, equipped with a 30 mW and 532 nm cw Nd:YAG laser and a photon-counting video camera, for photon-counting and imaging experiments. On the other hand, we used a 2 W and 532 nm cw Nd:YVO_4 laser for photobleaching experiments, because higher excitation energy was needed to facilitate photobleaching. However, the use of photon-counting video camera is not good for photobleaching experiments that require high power excitation, because the photon-counting video camera we used is liable to saturation under high power excitation (see also [9]). Thus, we used a CCD camera coupled with an image intensifier for photobleaching experiments, because this combination is not liable to saturation. To implement separately photobleaching experiments, we used the other microscope, Olympus IX70. Theoretically, the use of the Nikon microscope is possible in photobleaching experiments however, we dislike repeated exchange of the two video cameras.

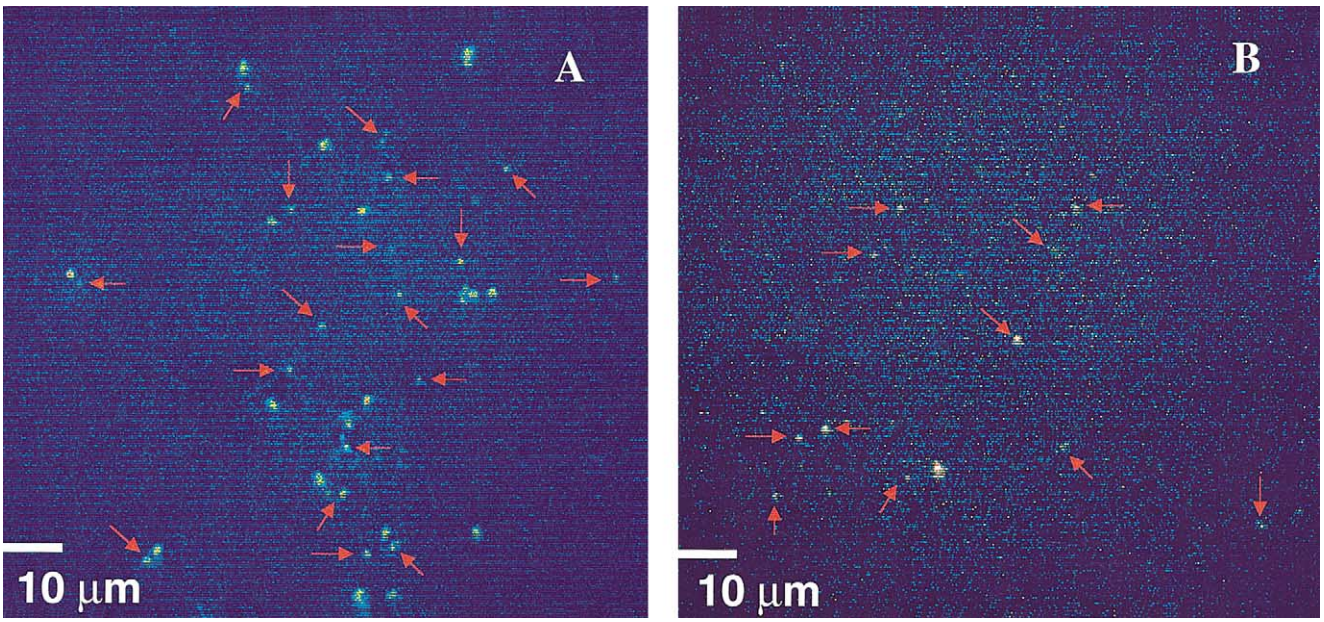

Fig. 1. Representative fluorescence images of single rhB molecules in a polymer film, prepared by spin coating (A) 50 nM; (B) 5 nM aqueous rhB solutions containing 5 mM PVA. Arrows represent spots corresponding to ~200 photocounts/30 s.

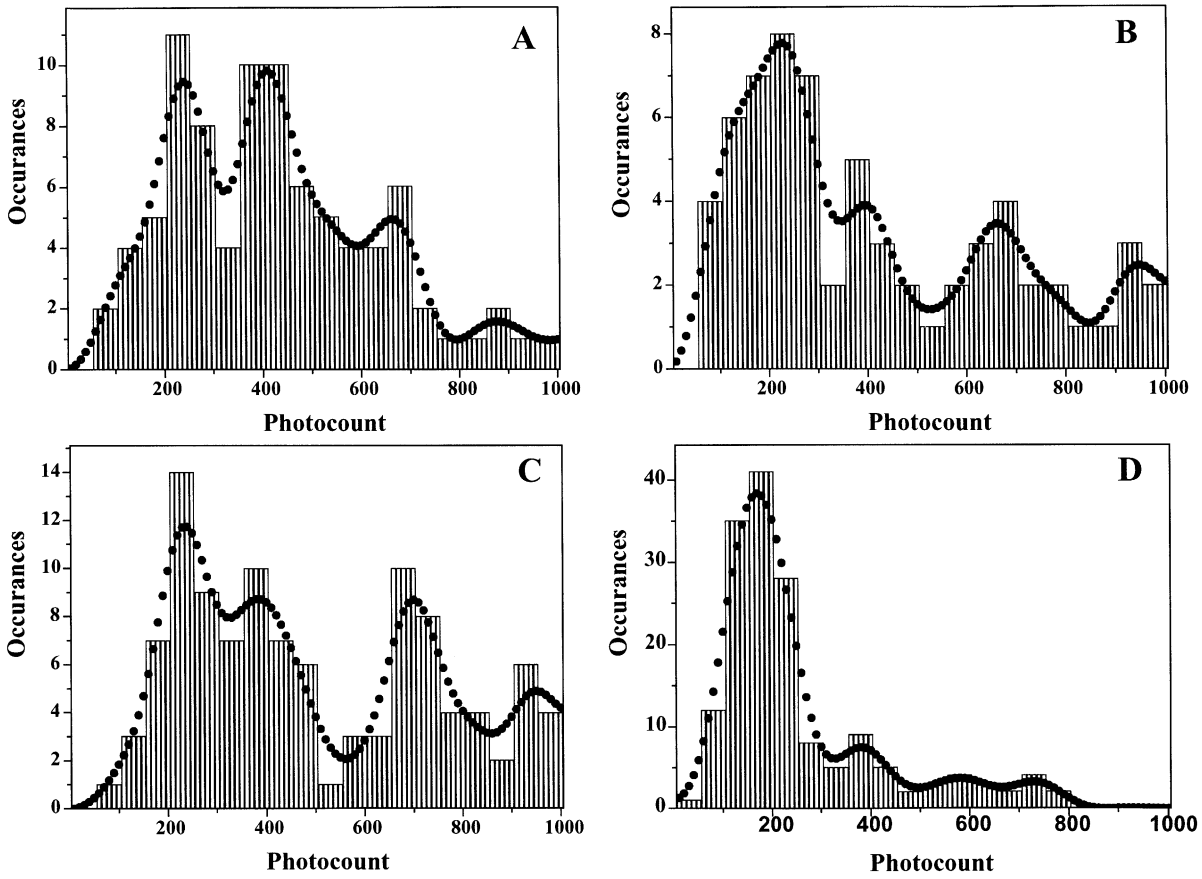

Fig. 2. Histograms of fluorescence photocounts from individual fluorescent spots in a PVA film containing different concentrations of rhB: (A) 160 nM; (B) 100 nM; (C) 50 nM; (D) 5 nM. The closed circles represent averaged curves for photocounts maxima.

depending on their nano-environment. These results show that the occurrences corresponding to ∼200 photocounts/30 s come from single rhB molecules and the others from groups composed of two, three, and four molecules.

The assignment of ∼200 counts to a single rhB molecule is also evident from the effect of concentration from 160 to 5 nM on the number of occurrences. Indeed, the number of occurrences corresponding to groups of molecules decreased with increasing 200-count occurrences (Figs. 2A–D) with decreasing concentration. In contrast to the wide distribution of fluorescence photocounts in histograms obtained in earlier report [8], a periodic and narrower distribution of photocounts was observed in the current work. Also, the distribution of photocounts, for example in [8], is attributed to the difference in site-dependent interaction between the dye molecules and the polymer host. This difference was characterized from the fluorescence lifetime measurements at different sites. Although, the current observation of occurrence of maxima at regular photocount intervals is similar to those reported in [9,10], in the current work, we confirmed the origin of the periodicity in the photocount distribution as from the presence of one, two, three, or large grouping of molecules by studying the photochemical kinetics of different spots. Compared to the earlier drop-and-drag method, the change in the distribution of photocounts in the histograms clearly reflected the change in the rhB concentration used. This parallelism is probably due to the quantitative control of the concentration in the current work. In the drop-and-drag method, what amount of dye molecules is actually prepared on the surface is not rigorously determined.

To identify the number of rhB molecules in each fluorescent spot, the samples were irradiated using a 400 mW laser. RhB molecules underwent photobleaching in 10–50 s (Figs. 3A–D), depending on the difference in their local environment. The difference in the photochemical lifetimes observed for single molecules indicate that the photochemical properties of individual molecules vary from each other depending on their nano-environment. The decrease in intensity involved in the photobleaching varied slightly from spot to spot (Figs. 3A–D), which is in consistent with the variations in the photocount maxima in the histograms (Figs. 2A–D). This difference in photocounts may be attributed to the variation in fluorescence quantum yield either from molecule to molecule or from site to site. The number of molecules in a fluorescent spot is also estimated from the number of steps in photobleaching experiment, as shown in Fig. 3. However, the observation in Fig. 3D shows that identifying the number more than three was impractical.

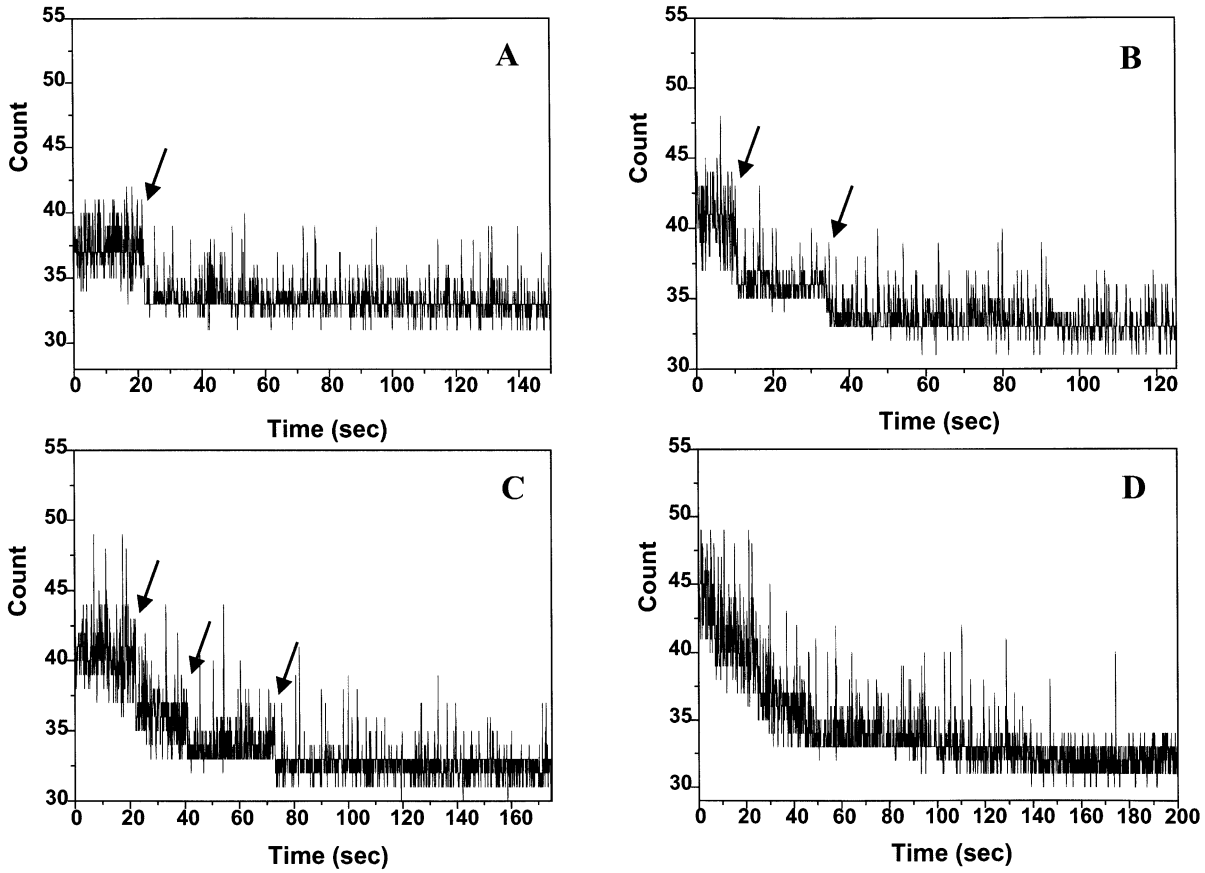

Fig. 3. Change in fluorescence photocount (count per 200 ms) with time obtained from rhB (5 nM) in a PVA film, showing photobleaching of one, two, three, and group of molecules.

To facilitate single-molecule studies, it is important to find experimental conditions that the number of occurrences larger than three is small, as shown in Fig. 2D. It is clear from Fig. 3 that different spots showed different kinetics of photobleaching. The one-, two-, three- or multi-step photobleaching observed indicate that the polymer film includes one, two, three, or larger grouping of rhB molecules. These observations are in consistent with the observed histograms in Fig. 2, showing distribution of one, two, three, and four rhB molecules.

4. Conclusion

The digitized histograms reported in the current work is different from those obtained in the previous work [9,10], in which grouping of adsorbed molecules was considerably kept even when reducing the concentration by a factor of 100. In contrast, in the current work grouping of several molecules was considerably separated into single molecules by reducing the concentration as well as by mixing a dye solution into a polymer solution. Thus, the validity of spin coating a $\sim 10^{-9}$ M droplet of a dye solution for preparing single-molecule samples is quantitatively demonstrated.

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