

Counting the Number of Fluorophores Labeled in Biomolecules by Observing the Fluorescence-Intensity Transient of a Single Molecule

Mira Park,[1,2] Hyong-Ha Kim,[3] Dongho Kim,*[,2] and Nam Woong Song*[,1]

[1]Optical Nano-Metrology Group, Division of Optical Metrology, Korea Research Institute of Standards and Science, Yuseong, P.O. Box 102, Daejeon 305-340, Korea

[2]National Creative Research Initiatives Center for Ultrafast Optical Characteristics Control and Department of Chemistry, Yonsei University, Seoul 120-749, Korea

[3]Organic and Bioanalysis Group, Division of Chemical Metrology and Materials Evaluation, Korea Research Institute of Standards and Science, Yuseong, P.O. Box 102, Daejeon 305-340, Korea

Received December 1, 2004; E-mail: nwsong@kriss.re.kr

By using the single molecule detection (SMD) technique based on laser scanning fluorescence microscopy, we have developed a method to count the number of labeled fluorophores in a single biomolecule. The number of labeled fluorophores in an individual molecule was measured by counting the number of discrete level jumps in the fluorescence-intensity transients, or Gaussian fitting of the fluorescence-intensity histogram. The instabilities of intensity transients due to humid environments of biomolecules were minimized by optimizing the matrix materials and sample-preparation condition and adding a strong triplet quencher, AET (2-aminoethanethiol). After validating the counting method and estimating the counting uncertainties through observations of a well-defined number of fluorophores, the distribution of the number of incorporated fluorophores in a DNA strand was observed. The advantages in biological analysis by using a single-molecule fluorescence technique have been considered.

Since the first optical detection of a single molecule labeled with multiple chromophores,[1] there has been a significant progress in the single-molecule detection (SMD) technique.[2–7] The results obtained from SMD provide detailed information, such as the individual molecular distribution of spectral shapes, and the quantum fluctuations in the fluorescence-intensity transient, which can not be observed by an ensemble measurement.[8–10] Thus, SMD is expected to provide us a technological breakthrough with numerous potential applications. The photophysical properties of a single molecule have been studied by optical microscopic methods, such as near-field scanning optical microscopy (NSOM) and confocal microscopy.[7] Although the NSOM technique offers optical resolution beyond the diffraction limit, it requires complicated control and delicate manipulation with a relatively low detection efficiency. Far-field optical microscopy techniques, such as laser-scanning confocal microscopy and the epifluorescence microscopy technique, provide simpler and more convenient methods for spatially resolved spectroscopy.[5,11–13] Therefore, most of the recent studies on single-molecule properties employ the laser-scanning confocal microscopy technique, and its technical importance is emphasized increasingly along with the application of SMD to biological molecules.

The optical properties of single fluorescent molecules, such as light-emitting polymers or chemical dyes, in various environments have been widely studied using the SMD technique.[14–19] One of the interesting applications in studies involving single molecule is to measure the number of fluorophores attached to a single biomolecule. By using an ensemble measurement, the number of labeled fluorophores in a single molecule can be obtained merely as an average value with a hidden distribution. On the contrary, the SMD technique enables one to directly measure the number of labeled fluorophores through individual observations, and provides information on the explicit distribution of the numbers. Detailed information about the number of labeled dyes suggests a better way to apply biofluorophores in biological analyses, especially in biochip assays.

In this work, we developed a method to count the number of fluorophores attached to individual biomolecules by observing the fluorescence-intensity transient of each molecule. The extent of uncertainty in counting fluorophore numbers attached to DNA strands was also estimated by observing DNA oligomers with a known number of attached dyes. Since the fluorescence-intensity transient of a single molecule fluctuates largely in certain environments, we have optimized the nanoenvironment surrounding the single molecules so as to minimize the intensity fluctuation, by changing the matrix materials, sample-preparation conditions, and adding a strong triplet-state quencher. There have been previous studies reported on the counting of labeled fluorophores by using mass spectrometry,[20] and fluorescence-intensity measurements of single molecules.[21] It has also been demonstrated that the number of fluorescent dyes in laser focal spots could be counted by observing the fluorescence-intensity transients using the SMD technique.[22,23] In this work, since the target molecule for counting was attached to a DNA strand, the result provides complementary information to previous works about the behavior of fluorescent molecules in a biomolecular environment.

Published on the web August 30, 2005; DOI 10.1246/bcsj.78.1612

Materials and Methods

Instrumentation. A laser scanning confocal microscope (LSCM) system based on an inverted microscope (Axiovert-25CFL, Carl Zeiss) was used for SMD application. Sample scanning was achieved by using an *x*–*y* scanning sample stage consisting of two electrostrictive actuators (AD-100, New Port) and a two-axis linear aluminum ball-bearing stage. The excitation laser beam of a 543.5 nm He–Ne laser (25 LGR 193-230, Melles Griot) was delivered to the input port of a confocal microscope through a single-mode optical fiber. The laser beam from the fiber was filtered by a narrow-band interference filter (F10-546.1-4-1.00, CVI Laser Corp.), and then reflected up to the microscope objective lens (Plan-neofluar 100× oil immersion, Carl Zeiss) by using a dichroic beam splitter (565DCXR, Chroma). The laser beam was expanded to 4 mm in diameter before it impinged the excitation filter to get higher optical resolution. When we recorded the fluorescence intensity of a single molecule at the focal point of the objective lens with linear scanning, the intensity vs distance curve exhibited a beam diameter of 230 nm in fwhm with a Gaussian fitting. Fluorescence from single molecules was collected by the same microscope objective lens and focused through a 150 mm focal length lens onto an APD detector in a single-photon counting module (SPCM-AQR-13-FC, Perkin-Elmer Optoelectronics). Scattered light around the excitation wavelength was removed by inserting a holographic notch filter (543.5 nm SuperNotch-Plus™, Kaiser Optics) between the objective lens and the focusing lens. The electrical pulses from the output of an APD detector was counted with a computer plug-in counter board (PCI 6602, National Instruments). Sample stage scanning and data acquisition were controlled by a personal computer based on a visual basic program.

Sample Preparation. Poly(methyl methacrylate) (PMMA) (MW 120000) and poly(vinyl alcohol) (PVA) (Avg. MW 30000–70000) were purchased from Sigma-Aldrich Co. HPLC-grade toluene (Merck) and deionized (DI) water were used as solvents for PMMA and PVA, respectively. Cy3-dUTP (Cy3-tagged deoxyuridine triphosphate) (Amersham Biosciences) was used as an example of a single dye-labeled biomolecule. A double labeled DNA oligomer, 5′-Cy3-oligonucleotide-Cy3 ph-3′ (oligonucleotide = GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG), was custom-order synthesized and purified by reverse-phase (RP18 column) HPLC (Integrated DNA Technology Inc.). An efficient triplet quencher, 2-aminoethanethiol (AET), was purchased from Sigma-Aldrich Co. The dye solution was prepared by dissolving dye powders in DI water. The optical density of the dye solution was measured to determine the concentration. The solution was then successively diluted down to 10^{-8}–10^{-10} M. Films for single-molecule fluorescence detection were prepared by spin coating (2000 rpm) the sample solution onto rigorously cleaned cover glasses or polymer films on cover glasses.

For Cy3 incorporation in a long DNA strand with multiple sites for possible dye labeling, genetically modified soybean (*Glycine max* RoundupReady®, Monsanto, USA) genomic DNA was amplified by PCR (polymerase chain reaction) in the presence of Cy3 labeled nucleotides. Two primers were designed to amplify a specific part of the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene. Cy3-dUTP and dTTP were added into the PCR reaction mixture at a ratio of 7:3 with the other dNTPs (deoxynucleotide triphosphate; dATP, dCTP, and dGTP) of adequate quantity. PCR was carried out in an automatic thermal cycler (DNA Engine Tetrad™, MJ Research) for 35 cycles. The initial cycle was 3 min at 94 °C, 20 s at 94 °C, 30 s at 53 °C, and 2 min at 72 °C, which was followed by 34 cycles of 20 s at 94 °C, 30 s at 53 °C, and 2 min at 72 °C. The 72 °C incubation in the last cycle was extended for an extra 10 min before the reaction samples were cooled to 4 °C. The final PCR product, *ds*DNA (double strand DNA) of 957 base pairs, was checked by electrophoresis on a 1% agarose gel to verify the correct size. The PCR product was purified with a spin column (Sephadex G-50, Amersham Pharmacia Biotech) to eliminate unincorporated Cy3-dUTP.

Results and Discussion

The fundamental difference of SMD from ensemble measurement lies in the fact that the result comes from only one molecule for each observation. The results from ensemble measurement consist of signals from multiple molecules (usually over millions of molecules). Thus, the fluorescence-intensity transients observed in SMD usually exhibit discrete levels, since the signal intensity is governed by the fluorescence quantum yield change of a single molecule.[10,12,14] Contrary to SMD, the results obtained via ensemble measurements usually show a continuous intensity decrease as the molecules become photobleached consecutively. In the case of light-emitting polymers, the intramolecular conformational change results in more than two discrete intensity levels for one molecule.[10] On the other hand, simple fluorophores usually show two intensity levels (bright and dark), resulting in one discrete jump, unless the molecules experience changes in the nanoenvironment during optical excitation. When a single molecule shows one discrete level jump, it is possible to count the number of attached fluorophores in a single molecule, since the number of down-steps in the intensity transient corresponds to the number of fluorophores.[12] However, in a humid environment, the fluorescence-intensity transient of a single molecule does not simply exhibit two levels due to the complex interaction between single molecules and the surrounding water molecules. Since most biomolecules are water-soluble and maintain their activities in aqueous solution, it is necessary to optimize the SMD condition in a humid environment for fluorescence stabilization. Thus, we tried to confine the number of level jumps in the fluorescence-intensity transient of a single biomolecule as one by changing the sample-preparation condition.

Figure 1a shows a scanned image of the fluorescence from a single Cy3-dUTP embedded in PVA film at room temperature. A Cy3-dUTP solution was prepared by dissolving the dye powder in DI water. The solution was successively diluted in DI water, and finally diluted in an aqueous PVA solution (1 mg/mL) to be a final concentration of 3×10^{-10} M. The film for SMD was prepared by spin coating of a Cy3-dUTP/PVA solution (150 μL) onto a cover glass. Since most biomolecules can be dissolved in water, the water-soluble polymer (PVA) was first tested as the matrix material for SMD.

The fluorescent spots were considered to originate from individual molecules, rather than multiple molecules, based on several pieces of evidence. Firstly, the number of fluorescent spots changed linearly with the solution concentrations over the range of 1–20 $\times 10^{-10}$ M. Secondly, each spot showed a well-defined dipole orientation geometry when observed with linearly polarized excitation. Spots mostly showed dramatic changes in emission intensities when two intensities of the

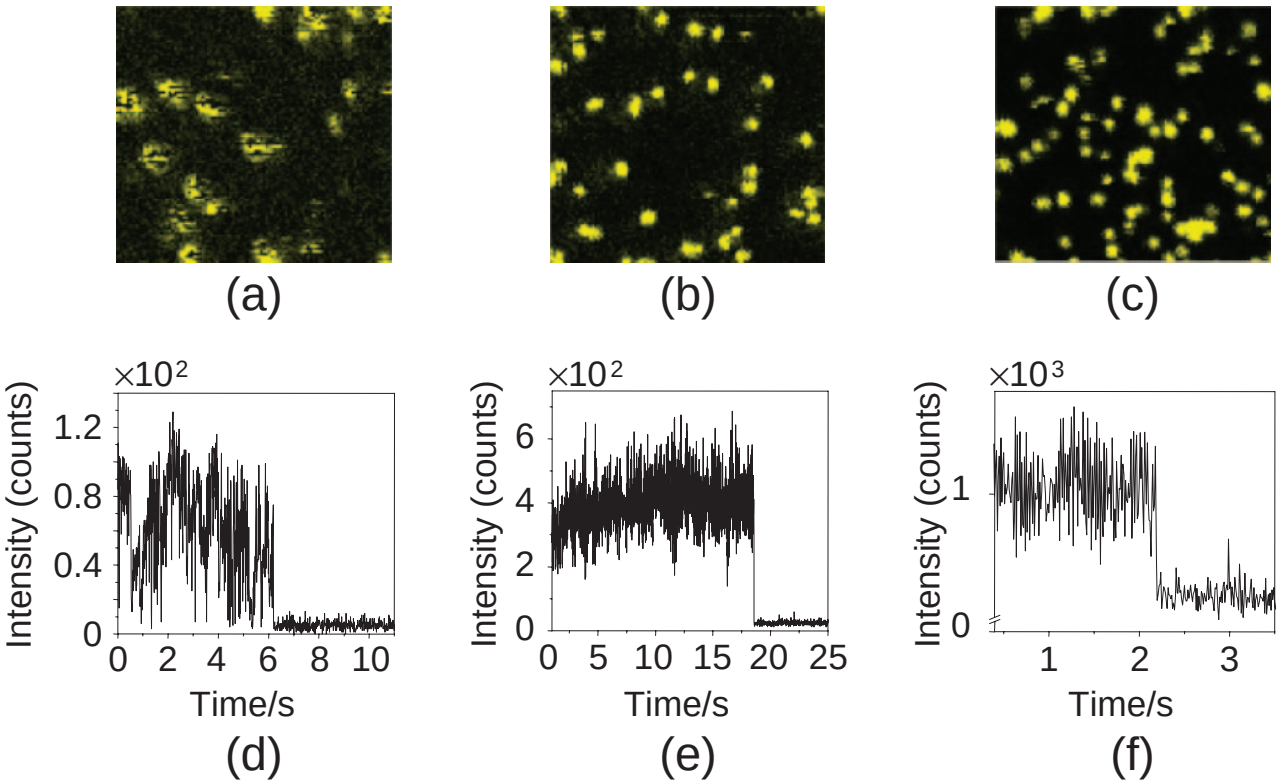

Fig. 1. Single molecule fluorescence images of Cy3-dUTP (a) embedded in PVA, and spin coated on PMMA (b) without and (c) with AET. Fluorescence-intensity transients of single Cy3-dUTP (d) in PVA and on PMMA (e) without and (f) with AET. Excitation was done at 543.5 nm at 1 μW for imaging and 15 μW (d, e) and 300 μW (f) for intensity transient measurement.

identical spot were compared for perpendicularly polarized excitation. Finally, bleaching of the spots did not occur as gradual dimming, but in a discrete way.

As shown in Fig. 1a, fluorescent spots embedded in a PVA matrix mostly showed dark stripes or bands due to the intensity fluctuations of a single Cy3 molecule during image scanning. Some of these spots disappeared after a second or third scan, possibly due to rapid photobleaching. When the fluorescence-intensity transient of each spot was observed, it exhibited a highly fluctuating intensity behavior (Fig. 1d). Intensity fluctuations, ranging from tens of milliseconds to several seconds in time scale, were observed. These fluctuations may have arisen from interactions between the molecule and the polymer matrix. It is thought that residual solvent molecules in the PVA film are responsible for this instability of the fluorescence-intensity transient.[24,25] Large fluctuations in the fluorescence-intensity transient made observing the discrete energy level jumps difficult.

Since water molecules are attributable to the unstable behavior of the fluorescence-intensity transient, we attempted to observe the single-molecule fluorescence of Cy3-dUTP in a hydrophobic environment using the PMMA matrix. Although most biomolecules are expected to lose their biological activities when dehydrated and surrounded by a hydrophobic environment, fluorescent dyes attached to the biomolecules retain their optical properties, enabling us to observe their fluorescence. Thus, it is worthwhile to observe the fluorescence from dry biomolecules in a hydrophobic environment as long as we are not interested in the intrinsic properties of the biomolecule, itself, but the properties of the attached fluorophores.

Figure 1b shows a scanned image of fluorescence from single Cy3-dUTPs on PMMA film. The Cy3-dUTP solution of 2×10^{-8} M (150 μL) was spun onto a thin PMMA film obtained by prior spin coating of PMMA/toluene (1 mg/mL; 150 μL) solution onto a cover glass. The film was allowed to dry for at least 10 min in the ambient atmosphere to remove the residual solvent molecules. The resulting single-molecule film showed a lower (two orders of magnitude) density of fluorescent spots than that obtained by spin coating the fluorophore/polymer mixture solution onto a cover glass. This might be related to the physical properties, such as viscosity, molecular weight, and solvent volatility, which govern the interaction between the solution and the coated surface.

The fluorescence intensity of single Cy3-dUTPs on PMMA film was significantly stronger than that of single Cy3-dUTP molecules embedded in the PVA matrix (Figs. 1d and 1e). Fluorescence spots showed a bright, circular shape without any obvious dark stripes or bands. Within a relatively short time scale, the fluorescence-intensity transient became more stable, showing less fluctuations than that of the embedded molecules in PVA. As a result of less fluctuation in the fluorescence intensity, Cy3-dUTP molecules showed a discrete intensity drop to the background level. However, the intensity-level variation on a relatively long time scale (i.e., several seconds) still existed. Changes in the fluorescence-intensity transient might be attributed to several factors, such as changes in the nanoenvironment by the molecular conformational change, triplet-excited state lifetime, oxygen concentration, and charge

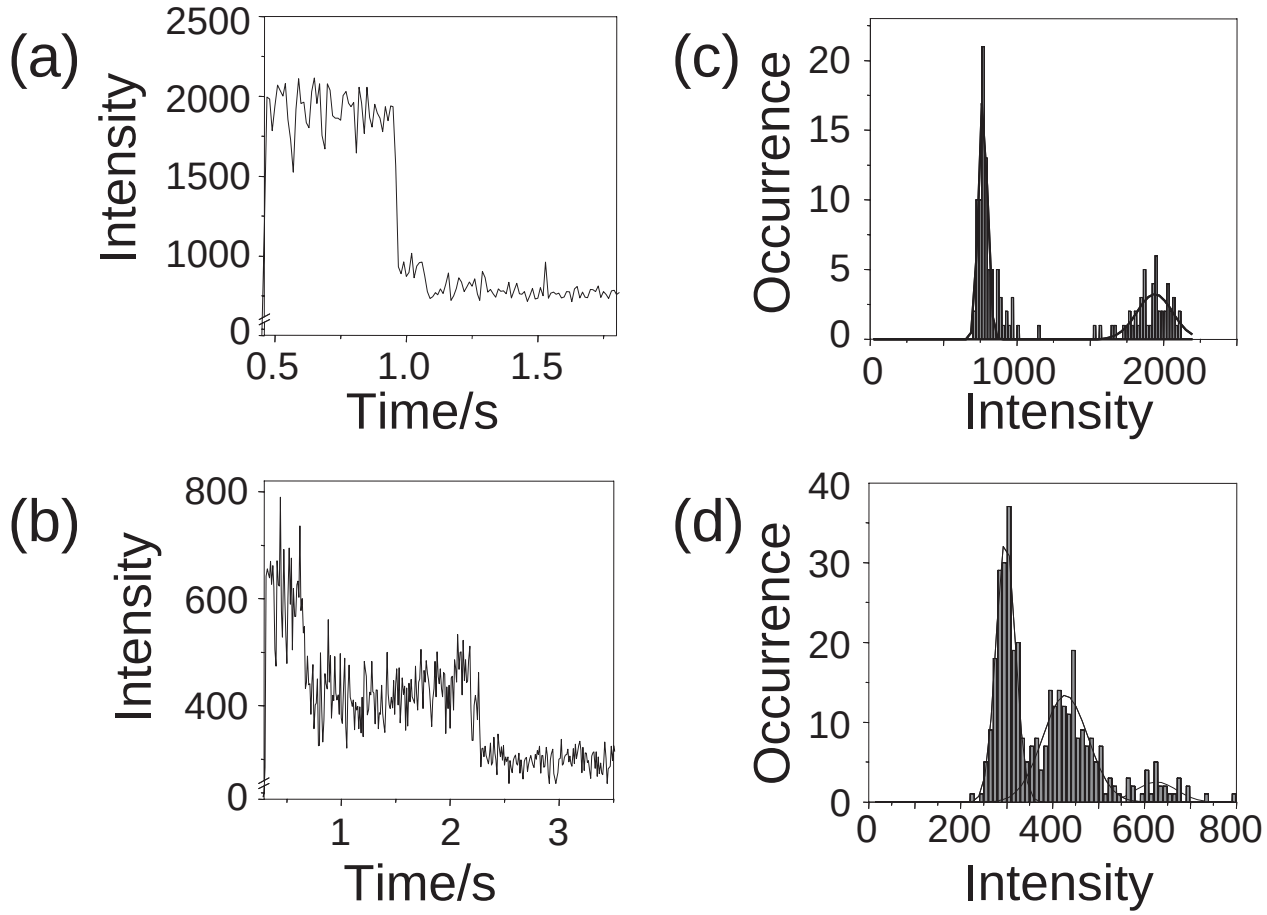

Fig. 2. The fluorescence-intensity transients of (a) a single labeled molecule (Cy3-dUTP) by 300 μW and (b) double labeled molecule (5′-Cy3-oligonucleotides-Cy3 ph-3′) by 180 μW excitation at 543.5 nm. The Gaussian fit results on the intensity histograms of (c) a single labeled molecule (Cy3-dUTP) and (d) double labeled molecule (5′-Cy3-oligonucleotides-Cy3 ph-3′).

distribution around the molecule. Since the triplet state is related to the quantum-yield change by photo-oxidation,[26,27] blinking by a nonemissive excited state,[9,18,28,29] and photobleaching by successive photoexcitation, a triplet state quencher, such as AET, may decrease the fluorescence-intensity fluctuation by reducing the triplet-state lifetime.[5]

Figures 1c and 1f show the stabilized effect of single-molecule fluorescence resulting from the addition of a strong triplet quencher, AET, around Cy3-dUTP on PMMA film. AET (10 mg/mL) was dissolved in an aqueous solution of Cy3-dUTP prior to spin-coating on PMMA film. The addition of AET resulted in an increase of the average number of total photons detected from individual molecules from 3×10^5 to 5×10^5. This result indicates that the addition of a triplet quencher depletes the triplet-state population, which possibly leads to reduced photobleaching.[5] As depicted in Figs. 1b and 1c, comparison of the contrast ratios between fluorescence spots and the background can lead to a possible deduction that the fluorescence intensity of a single Cy3-dUTP increased as AET was added to the samples. Cy3 molecules in the AET treated film appeared brighter with a significantly darker background. Stabilization of the intensity transient was also achieved by AET addition, as shown in Fig. 1f. The intensity transient was stable and exhibited neither a short nor long-time scale fluctuation, displaying a distinct discrete jump corresponding to the photobleaching of each molecule. We adopted this condition to measure the number of photobleaching steps in a single fluorescent biomolecule. In this case, we could easily observe the number of photobleaching events for a biomolecule labeled with two independent fluorophores.

To examine the applicability of single molecule fluorescence transient measurement in counting the number of labeled fluorophores, we observed the number of discrete jumps in the fluorescence-intensity transient of single and double-labeled biomolecules. Scanned fluorescence images and their intensity transients were acquired from Cy3-dUTP (single labeled) and 30-mer oligonucleotide with two Cy3 dyes attached at each end (double labeled).

In the case of single-labeled molecules, 88% of the observed samples showed a single-step intensity jump in the fluorescence-intensity transients. Figure 2a shows a representative fluorescence-intensity transient from a single Cy3-dUTP. A single and instantaneous intensity drop after 1 s is considered as photobleaching of a single Cy3-dUTP. The intensity histogram of the transient gives information of single-molecule fluorescence, as shown in Fig. 2c.[16] When the intensity histogram was fitted with Gaussian functions, two distinct intensity levels could be observed in most of the examined molecules

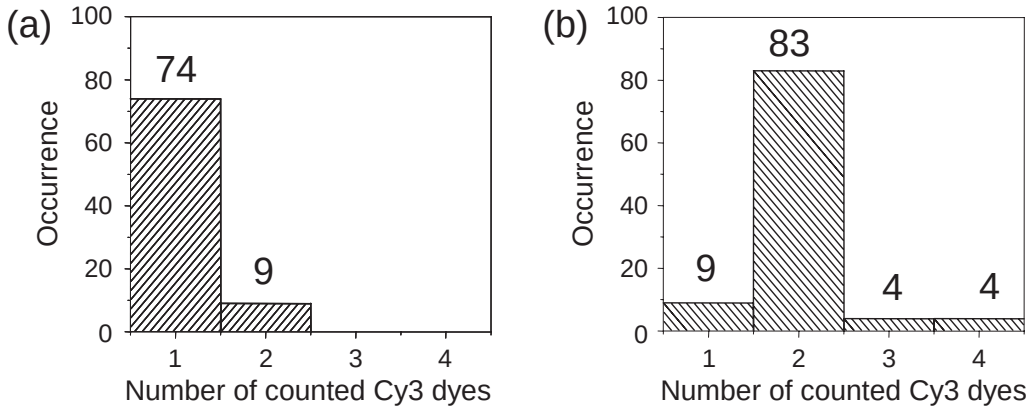

Fig. 3. Histogram of the number of fluorophores in a single molecule counted by using fluorescence-intensity transient measurement. The total number of observed molecules are (a) 83 for a single labeled molecule (Cy3-dUTP) and (b) 100 for double labeled molecule (5′-Cy3-oligonucleotides-Cy3 ph-3′).

(88%) corresponding to the emissive and background intensity levels, respectively.

Two-step intensity jumps, as shown in Fig. 2b, were observed from 83% of the double-labeled oligonucleotides. The intensity transients exhibited two well-resolved steps due to the sequential photobleaching of two independent fluorophores. In this case, the intensity histogram was fitted by three Gaussian functions corresponding to the sum of two fluorophores, a single fluorophore and the background intensity level, respectively (Fig. 2d).

Figure 3 shows a histogram of the number of intensity jumps in the fluorescence-intensity transient of the single and double-labeled molecules. For molecules labeled with one fluorophore, 88% of the measured transients showed one-step photobleaching, and 12% showed two-step photobleaching. Two-step bleaching from Cy3-dUTP might be attributable to two reasons. One is the spatial coexistence of two Cy3-dUTP's in the laser spot, which is larger than Cy3-UTP molecules by more than thousand times. Although we confirmed the single-molecule detection condition based on several pieces of evidence, mentioned above, a small fraction of the molecules could possibly be located close to the others in the laser spot leading to two-step bleaching. The other reason can be spectral diffusion of single molecules that are not tightly bound to the surface, and can easily change their position within 30 nm by optical excitation.[4,30] Spectral diffusion might result in different fluorescence-intensity levels in a single molecule for a fixed wavelength excitation, since the absorption coefficient and emission quantum yield vary within the nanoenvironment.[24,25]

In the case of double-labeled molecules, ca. 83% of the measured transients showed two-step photobleaching, and 17% of the transients showed either one- or multi-step photobleaching (Fig. 3b). For one-step photobleaching species, one can assume that one of the two fluorophores was inactivated or photobleached fairly quickly without exhibiting a discrete jump. In fact, several single fluorophore spots did not seem to fluoresce due to fast photobleaching. It is expected that a major portion of the single-molecule population is subjected to early photobleaching by laser irradiation, since the photobleaching process occurs with the binary distribution probability. A minor fraction (4%) showing four-step photobleaching might be attributed to two partially hybridized oligonucleotides that contain four independent fluorophores. Three-step photobleaching may originate from two partially hybridized oligonucleotides with one of the four fluorophores inactivated.

As described above, it is possible to directly count the number of fluorophores labeled in a single biomolecule with ca. 20% uncertainty by observing the fluorescence-intensity transients of individual molecules.

We examined this method to measure the distribution of the number of fluorophores in biomolecules with multiple labels. We measured the number of Cy3 dyes incorporated in a DNA strand after 35 cycles of PCR amplification. PCR was performed to amplify a certain region of the EPSPS gene using GM soybean genomic DNA as a template. In the PCR mixture, Cy3-dUTP was added for incorporation into the newly synthesized DNA strands. Target DNA synthesis was confirmed by electrophoresis with ethidium bromide staining. Since the PCR reaction resulted in a relatively poor product yield and inefficient incorporation, the quantity of incorporated Cy3 dyes could not be analyzed by measuring the optical absorption. When the fluorescence spectrum was observed at 543.5 nm excitation, both the PCR product solution and the aqueous Cy3-dUTP solution appeared to have the same spectra.

The low efficiency of PCR and Cy3 incorporation may be attributed to two factors. First, a low efficiency of DNA polymerization is expected in the presence of dUTP instead of dTTP. Second, the interference of labeled dyes may decrease the efficiency of PCR amplification. The substitution of dTTP to dUTP in the PCR mixture led to a product yield that was too low, and thus it was not visible as an obvious fluorescent band on the electrophoretic gel. On the other hand, when PCR amplification was performed in the presence of dTTP, the target DNA was observable as a bright fluorescent band on the electrophoretic gel. This result shows that dUTP was not the best choice for in vitro DNA amplification. It would be necessary to optimize other PCR conditions, such as the annealing temperature, choice of polymerase, and buffer composition, in order to increase the PCR product yield when using dUTP as one of dNTPs. Due to the modified three-dimensional structure, the polymerase may not recognize Cy3-dUTP in the same

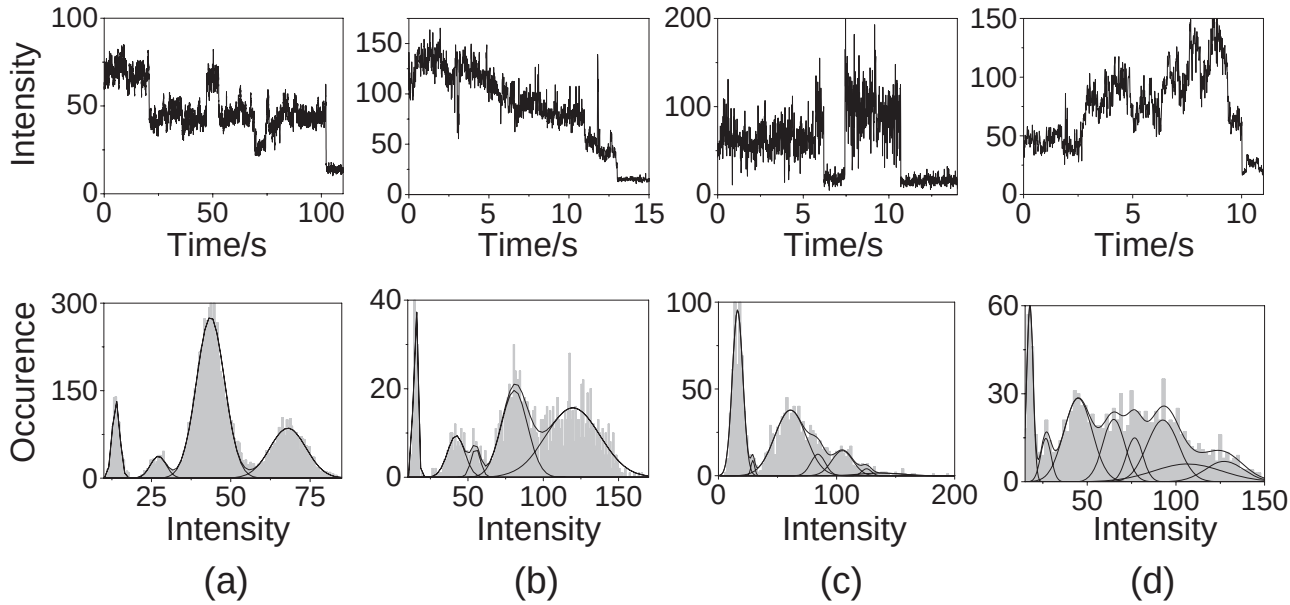

Fig. 4. Representative fluorescence-intensity transients and intensity histograms of DNA strands obtained by PCR amplification for Cy3 incorporation observed by 1 μW excitation at 543.5 nm. The numbers of incorporated dyes were obtained from the Gaussian fit for the intensity histograms. Obtained Cy3 incorporation numbers correspond to 3 (a), 4 (b), 5 (b), and 7 (d), respectively.

way as dUTP, and therefore Cy3-dUTP could possibly be an inhibiting component.

Individual DNA strands were observable as bright spots by detecting the fluorescence image. Since the concentration of DNA was lower than the optically measurable level, the sample solution was prepared arbitrarily by successive dilution until the spin-coated sample exhibited isolated single spots. The density of DNA spots within a fixed area decreased linearly as the samples were diluted with a factor of 1/50–1/1000, starting from the original solution. An AET solution was used as the solvent for dilution. Several representative diagrams of the fluorescence-intensity transients, their histograms, and the Gaussian fit results are shown in Fig. 4. As the number of incorporated Cy3 dyes increased, it became more difficult to distinguish the intensity levels expressed by the contribution of active fluorophores. Thus, we counted the number of incorporated Cy3 dyes by using the Gaussian fitting for the intensity histogram, as shown in Fig. 4.

Figure 5 shows the result of counting the number of incorporated dyes in a DNA strand by using a Gaussian fit. By using the SMD technique, the distribution of the number of incorporation could be measured. The incorporation number distribution ranged from one to eleven, and a DNA strand including four Cy3 dyes appeared to be the most probable PCR product. Although there are 362 potential sites for incorporation (adenine and thymine bases) over ca. 1000 base pairs, the maximum number of incorporated dyes was measured to be eleven. This value corresponds to a maximum of ~3% incorporation among the total available sites. This low incorporation efficiency is consistent with the poor Cy3-dUTP utilization compared with dTTP during PCR. As we simultaneously added Cy3-dUTP and dTTP in the PCR mixture, competition between two dNTPs may have resulted in a major incorporation of dTTP and a trace incorporation of Cy3-dUTP.

Information, such as the individual number distribution of

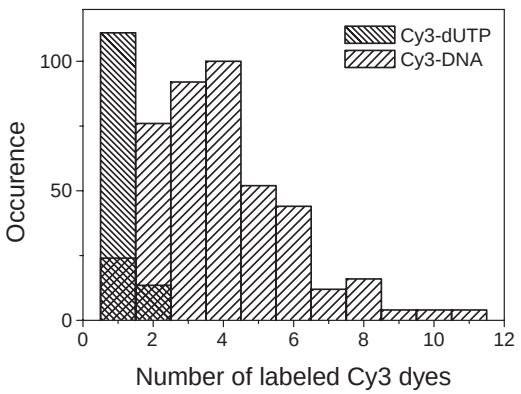

Fig. 5. Histogram of the number of fluorophores incorporated in DNA strands. Results from Cy3-dUTP are plotted for comparison. The total number of observed molecules was 214.

incorporated dyes, is impossible to be attained by an ensemble measurement where only one value corresponding to the average number of incorporated dyes in DNA strands could be obtained. An identical average value can be observed from both a wide distribution with a large width and a well-defined narrow distribution in a histogram. The detailed information of the distribution is buried under the representative values, such as the average or the measured quantity in the ensemble measurement. Once we obtain detailed information about the distribution by using SMD, it is possible to modify the experimental condition, and then observe the effect of the modification. This procedure can suggest a method to increase the PCR efficiency, or drive the reaction towards a specific result, such as narrowing the distribution in the number of dye incorporations.

These results are related to DNA quantification in two as-

pects. One is lowering the concentration of the detection limit; the other is accurate quantification. Efficient dye incorporation during PCR will produce a bright *c*DNA for biochip applications. This might contribute to trace analysis of target DNAs in biological and medical applications, since brighter fluorescence of a single DNA strand can lower the concentration of the detection limit for target DNAs. A narrow distribution of incorporation will promote the quantification of DNA in trace amounts. If dye-incorporated DNAs can be synthesized to have a uniform number of labels per molecule, the detected fluorescence signal on a biochip would increase linearly as the concentration of DNA increases. On the other hand, the difference in the number of dye-incorporation would result in uncertainties in DNA quantification when the target DNA concentration is relatively low. In a simplest picture, one hundred DNA strands incorporated with ten dye molecules will exhibit the same fluorescence intensity as one thousand DNA strands, each labeled by a single dye molecule. Thus, two *c*DNAs with different incorporation may result in a quantification error of as much as 1000% in DNA assessment. Such an error would be small, or negligible, when the target DNA concentration is relatively high, because the distribution of dye-incorporation would remain uniform once the concentration exceeds a certain level. In this case, the detected fluorescence signal on a biochip would also increase linearly with the increase of DNA concentration, despite different incorporation numbers. Since the importance of quantitative analysis on DNA with low abundance becomes more emphasized, it is necessary to develop assay techniques that are able to perform the quantitative analysis of a trace amount. In this aspect, it will be useful to develop a method to synthesize bright DNA with a narrow distribution of dye incorporation in the near future. Since information concerning the dye incorporation number distribution can be uniquely obtained by utilizing the SMD technique, it is also necessary to develop and expand applications of SMD in biological analysis.

Conclusion

The photobleaching of single fluorescent molecules was investigated under various polymer matrix conditions. After optimizing the composition of the polymer matrix, the condition of the sample preparation and by the addition of a triplet quencher, counting the number of fluorophores in a single biomolecule was possible with ca. 20% uncertainty. The application of this method was exemplified in obtaining detailed information on the distribution of the number of incorporated dyes in DNA strands. This result would help to perform efficient dye incorporation into DNA strands with uniform fluorescence intensities.

This work has been financially supported by the Basic Research Project funded by the Government Contribution (NWS), and the National Creative Research Initiatives Program of the Korea Science and Engineering Foundation of Korea (DK).

Supporting Information

Fluorescence image of single DNA strands which were incorporated with Cy3 dyes through PCR reaction. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

References

1 T. Hirschfeld, *Appl. Opt.*, **15**, 2965 (1976).
2 D. C. Nguyen, R. A. Keller, J. H. Jett, and J. C. Martin, *Anal. Chem.*, **59**, 2158 (1987).
3 W. E. Moerner and L. Kador, *Phys. Rev. Lett.*, **62**, 2535 (1989).
4 X. S. Xie and R. C. Dunn, *Science*, **265**, 361 (1994).
5 S. Nie, D. T. Chiu, and R. N. Zare, *Science*, **266**, 1018 (1994).
6 X. S. Xie, *Acc. Chem. Res.*, **29**, 598 (1996).
7 S. Weiss, *Science*, **283**, 1676 (1999).
8 L. Ying and X. S. Xie, *J. Phys. Chem. B*, **102**, 10399 (1998).
9 W.-T. Yip, D. Hu, J. Y. David, A. V. Bout, and P. F. Barbara, *J. Phys. Chem. A*, **102**, 7564 (1998).
10 D. Hu, J. Yu, and P. F. Barbara, *J. Am. Chem. Soc.*, **121**, 6936 (1999).
11 J. K. Trautman and J. J. Macklin, *Chem. Phys.*, **205**, 221 (1996).
12 T. Funatsu, Y. Hanada, M. Tokunaga, K. Saito, and T. Yanagida, *Nature*, **374**, 555 (1995).
13 J. J. Macklin, J. K. Trautman, T. D. Harris, and L. E. Brus, *Science*, **272**, 255 (1996).
14 A. Van Orden, N. P. Machara, P. M. Goodwin, and R. A. Keller, *Anal. Chem.*, **70**, 1444 (1998).
15 M. Köllner and J. J. Wolfrum, *Chem. Phys. Lett.*, **200**, 199 (1992).
16 D. A. Bout, W.-T. Yip, D.-K. Hu, T. M. Swager, and P. F. Barbara, *Science*, **277**, 1074 (1997).
17 M. Wu, P. M. Goodwin, W. P. Ambrose, and R. A. Keller, *J. Phys. Chem.*, **100**, 17406 (1996).
18 K. D. Weston, M. Dyck, P. Tinnefeld, C. Muller, D. P. Herten, and M. Sauer, *Anal. Chem.*, **74**, 5342 (2002).
19 B. Lounis and W. E. Moerner, *Nature*, **407**, 491 (2000).
20 D. Pinto, E. A. Arriaga, R. M. Schoenherr, S. S. Chou, and N. J. Dovichi, *J. Chromatogr., B*, **793**, 107 (2003).
21 T. Schmidt, G. J. Schütz, H. J. Gruber, and H. Schindler, *Anal. Chem.*, **68**, 4397 (1996).
22 M. Ishikawa, O. Yogi, J. Y. Ye, T. Yasuda, and Y. Maruyama, *Anal. Chem.*, **70**, 5198 (1998).
23 V. Biju, M. Takeuchi, K. Umemura, M. Gad, and M. Ishikawa, *Jpn. J. Appl. Phys.*, **41**, 1579 (2002).
24 F. Köhn, J. Hofkens, U.-M. Wiesler, M. Cotlet, M. Van der Auweraer, K. Müllen, and F. C. De Schryver, *Chem.—Eur. J.*, **7**, 4126 (2001).
25 Y. Hou, A. M. Bardo, C. Martinez, and D. A. Higgins, *J. Phys. Chem. B*, **104**, 212 (2000).
26 J. Yu, D. Hu, and P. F. Barbara, *Science*, **289**, 1327 (2000).
27 T. Ha, T. Enderle, D. S. Chemla, P. R. Selvin, and S. Weiss, *Chem. Phys. Lett.*, **271**, 1 (1997).
28 K. D. Weston, P. J. Carson, H. Metiu, and S. K. Buratto, *J. Chem. Phys.*, **109**, 7474 (1998).
29 D. S. English, A. Furube, and P. F. Barara, *Chem. Phys. Lett.*, **324**, 15 (2000).
30 V. P. Biju, J. Y. Ye, and M. Ishikawa, *J. Phys. Chem. B*, **107**, 10729 (2003).