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A New Analytical Method to Determine the Purity of Synthetic Fluorophores using Single Molecule Detection Technique

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A new assay technique to distinguish between pure compounds and the isomeric mixtures has been suggested using single molecule (SM) fluorescence detection technique. Since the number of emission spots in a fluorophore-spread film prepared from a genuine dye solution was determined by experimental condition, the deviation of spot numbers from the expected values could be considered to be an indication of lower purity of the sample solution. The lower limit of sample concentration for this assay was determined to be 5×10^{-10} M to show uniform number of expected spots within 10% uncertainties in our experimental condition. An individual fluorescence intensity distribution for a mixture of isomers having doubly different emissivities was simulated by adding distributions obtained from Cy3 and nile red (NR) independently. The result indicated that the mixture could be identified from the pure compounds through the difference in the number of Gaussian functions to fit the distribution. This new assay technique can be applied to the purity test for synthetic biofluorophores which are usually prepared in small quantities not enough for classical ensemble assays.

Key words: Single molecule detection, Synthetic biofluorophores, Number of emission spots, Fluorescence quantum yield

INTRODUCTION

Since the first achievement of single molecule detection (SMD) at room temperature [1], this technique has been developed as a useful tool to attain individual molecular information [2]. SMD technique can be applied to elucidate the photophysical properties of molecular systems such as light emitting polymers [3,4], photonic wires [5], and biomolecules labeled with fluorophores [6,7]. Although the results from ensemble measurements are essential for investigating molecular properties, they often preclude some information by averaging and mixing all individual signals. SMD technique provides detailed information such as spectral shift [8], fluctuation of fluorescence intensity due to local environment [9], and temperature dependent line broadening [10] of single molecules that could not be seen in ensemble measurement.

One of the interesting applications of SMD technique is to observe the fluorescence property of individual molecule. By using an ensemble measurement, the fluorescence signals are obtained merely as average values. On the other hand, it is possible to observe emission intensities, spectra and their distribution of individual fluorophore using SMD technique. It has been demonstrated that subpopulation of a specific molecule in the mixture solution could be observed using SMD technique [11,12]. The intramolecular distance between donor and acceptor separated by DNA, as a rigid spacer, has been measured using single-pair fluorescence resonance energy transfer (spFRET) analysis, and the distance distribution could be separately obtained for two different DNA spacers having 7 and 14 bases [12]. Since there are free acceptors in solution, the distance between two fluorophores can be underestimated through the signal averaging over all fluorophores in the ensemble FRET measurement. On the other hand, subpopulations obtained using SMD technique provide information of individual distances as well as the mean value of total molecules. Free acceptors can be distinguished from single-pairs through different efficiencies in the spFRET intensity histogram.

Another example of SMD application is the quantification of product molecules generated by single enzymes or catalysts. It has been reported that the rates of fluorescent product generation by single enzymes can be monitored as the time-dependent increase of fluorescence intensity in the nanoscopic reactors [13]. The fluorescence intensities observed in the individual nanoreactor provided information about

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biological activities of single enzymes without the need of simulation on the experimental results.

Fluorescence techniques applicable to biological assays are becoming more important due to the development of small scale and high throughput assay techniques. Consequently, the demand for the synthetic biofluorophores as probes is increasing. Synthetic biofluorophore is usually prepared as an isomeric mixture of compounds having different geometry and emission intensities. However, some biofluorphores are not readily purified from the isomeric mixtures since it is not easy to find an adequate separation column for isomers having structural similarities. On the other hand, biological assay results performed using the synthetic fluorophores are usually interpreted assuming that the probes have the same structure and emission properties. Ignorance of the probe purity might mislead the interpretation of experimental results. Thus it is needed to check the purity of synthetic biofluorophores by using an adequate analysis technique.

The absorption property of fluorescent molecules is not altered in large extent after labeling on biomolecules. In case of Cy3 labeling, extinction coefficient of one emitter changes from 1.33×10^5 [14] to $1.31 \sim 1.44 \times 10^5$ $cm^{-1}M^{-1}$ (< 10 %) and the maximum absorption wavelength falls within 10 nm by attaching to DNA oligomers [15]. Thus it is difficult to distinguish a labeled oligomer from its isomeric compounds by absorption measurement. On the other hand, the fluorescence property is sensitive to the molecular structure of labeled oligomers. The fluorescence quantum yield of Cy3 changes from 0.04 [16] to 0.12 [15] (300% increase) when it is covalently attached to DNA oligomers. However, it is not also easy to distinguish a pure labeled oligomer from the isomeric mixtures using the ensemble fluorescence measure-ment since the experimental observable is an averaged signal. Without knowing the fluorescence quantum yields of diverse isomeric labeled oligomers, the emission study does not give any information about the purity of samples in ensemble measurement.

In this study, we have tried to develop a new assay technique to distinguish between a pure fluorescently labeled compound and isomeric mixtures using SM fluorescence detection technique. Since SMD provides detailed information about individual fluorophore, an analytical method for purity test using SMD could be suggested based on the observation of distributions. Although there has been studies on the ratiometric analysis using SMD [11,12], this is the first attempt to apply SMD technique for purity test of synthetic fluorophores. Since the application of SMD technique requires trace amount of samples in the order of $10^{-15} \sim 10^{-12}$ (femto- to pico-) moles, the technique would be applied for the quality analysis of synthetic biofluorophores which are usually prepared in a very small scale.

MATERIALS AND METHODS

Materials

Nile red (NR) (Molecular Probe) and Cy3 (Amersham Biosciences) were used without further purification. The NR solution was prepared in methanol, and the concentration was estimated from the absorption spectrum regarding the molar extinction coefficient [17]. The value of NR concentration for spin coating was in the range of $1.6\text{-}8.2 \times 10^{-10}$ M, and NR was finally diluted in poly(methylmethacrylate) (PMMA; Aldrich) in toluene solution (10 mg/ml). Solution of 150 μl NR in PMMA/toluene was spin coated at 2,000 rpm onto a rigorously cleaned coverglass (180 μm thick, Fisher Scientific). By spin coating the NR in PMMA/toluene solution, a polymer film was obtained in which individual NR molecules were embedded in PMMA matrix. In the concentration range for spin coating, NR molecules on the film were separated enough for SM fluorescence observation. For single Cy3 fluorescence detection, aqueous Cy3 solution of 2×10^{-8} M was spin coated on a PMMA film.

Experimental setup

An inverted microscope (Axiovert-25CFL, Carl Zeiss) was used for the main body of the SMD apparatus. The sample scanning was achieved by using an *xyz* scanner consists of a closed-loop, multi-axis Piezo-NanoPositioning system (Nanocube™ P-611.3S, Physik Instrumente). The excitation laser beam of 543.5 nm He-Ne laser (25LGR193-230, Melles Griot) or 514.5 nm Ar^+ laser (35LAP321-220, Melles Griot) was delivered to the input port of the microscope through a single-mode optical fiber (QSMJ-3S3S-320-2/125-10, Ocean Optics). The laser beam from the fiber was filtered by narrow-band interference filters (F10-546.1-4-1.00 or F03-514.5-4-1.00 CVI Laser Corp.), and then reflected up to the micro-scope objective lens (Plan-neofluar 100× NA=1.3, Carl Zeiss) by using a dichroic beam splitter (565DCXR or 530DCXR, Chroma). The laser beam was expanded to 6 mm in diameter before it impinged the excitation filter to get higher optical resolution [18]. Fluorescence signal from the sample was collected by the same microscope objective lens and focused through a 150 mm-focal length lens onto a single photon counting module (SPCM-AQR-13-FC, PerkinElmer Optoelectronics). Scattered light around the excitation wavelength was removed by inserting a holographic notch filter (543.5 or 514.5 nm SuperNotch-Plus™, Kaiser Optics) between the objective lens and the focusing lens. The electrical pulse from the photodetector (SPCM) was counted by using a computer plug-in counter board (PCI 6602, National Instruments). Sample stage scanning and data acquisition were controlled by using a visual basic program running on a Pentium PC.

RESULTS AND DISCUSSIONS

Conceptual background of the assay development

In the ensemble measurements, two samples having different compositions can give the same fluorescence

intensities. Figure 1 shows the schematic of model systems in which one is composed of two isomers (50:50) having different fluorescence quantum efficiencies (0 and 1) (sample A), and the other is composed of a single fluorophore having quantum efficiency of 0.5 (sample B). The ensemble fluorescence intensities of the two samples can be expressed as in Eqn. (1).

$$I_{F,A} = I_0 e\, \Phi_F c_A = I_0 e \times 1 \times 1/2 c_A + I_0 \varepsilon \times 0 \times 1/2\, c_A = 1/2\, I_0 \varepsilon\, c_A \quad (1a)$$

$$I_{F,B} = I_0 \varepsilon\, \Phi_F c_B = I_0 \varepsilon \times 0.5 \times c_B = 1/2\, I_0 \varepsilon\, c_B \quad (1b)$$

, here $I_{F,A}$ and $I_{F,B}$ are the fluorescence intensities of sample A and B, I_0 is the excitation intensity, ε is the molar extinction coefficient of fluorophores, Φ_F is the fluorescence quantum yield of fluorophores, and c_A and c_B are the concentrations of samples. Now we assume three factors as: (1) the same excitation intensity was used for fluorescence measurement; (2) two sample solutions have the same concentrations ($c_A = c_B$); and (3) the extinction coefficients are the same for three isomers; then the measured ensemble fluorescence intensities of two samples would be the same. However, if the fluorescence intensities of individual molecules were detected, the difference between two samples could be observed through the difference in the number of fluorescent spots. Spin coating of dye/polymer mixture solutions on a cover glass provides a film where dye molecules are embedded in polymer matrix [7,18]. Moreover, the number of fluorescent spots observed in a fixed area tends to be determined by the sample concentration for spin coating [18]. Thus, the number of fluorescent spots in the film of sample A would be half of that in the film of sample B, since 50% of molecules do not emit fluorescence in sample A. Furthermore, the center of fluorescence intensity distribution of sample A is expected to show twice as much as that of sample B. Such result can be used to distinguish a pure fluorophore and isomeric mixtures. If a sample consists of a single compound, the number of emission spots would follow the case in sample B. However, the isomeric mixture consisting of compounds having different fluorescence quantum yields would result in low number of emission spots than expected. If the number of emission spots can be expected at a defined experimental condition, it is possible to assess the sample purity through fluorescence quantum yield by observing the number of emission spots.

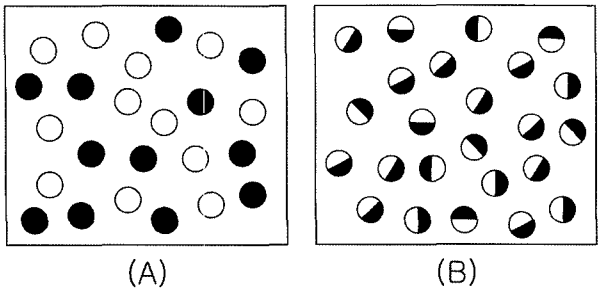

Figure 1. While the emission intensities of individual molecules exhibit three different levels (0, 0.5 and 1), two samples give the same fluorescence intensity in ensemble measurement. (A) 50:50 mixture of two isomers having same extinction coefficient but different fluorescence quantum yields; $\Phi_F = 0$ (●) and $\Phi_F = 1$ (○), (B) a pure sample having same extinction coefficient but different fluorescence quantum yield with the isomers in A; $\Phi_F = 0.5$ (◑).

Number of fluorescent spots vs. dye concentration

Single molecule fluorescence spot image of NR embedded in PMMA matrix was observed by excitation with 514.5 nm laser (Fig. 2A). The emission spots were considered to be originated from individual molecules based on the following evidences: (1) The number of fluorescent spots in a fixed area (10 × 10 μm) changed proportional to the solution concentrations (Fig. 2B); (2) The intensity transients of most emission spots (> 90%) exhibited a single discrete jump instead of gradual dimming (Fig. 2C); (3) Each spot showed a well-defined dipole orientation when observed with linearly polarized excitation. Many spots showed dramatic changes when the excitation polarization was switched perpendicularly.

The number of single fluorescent spots was counted in different NR-embedded PMMA films prepared with various sample concentrations for spin coating. The results are plotted in Fig. 2B, and summarized in Table 1. This experiment was

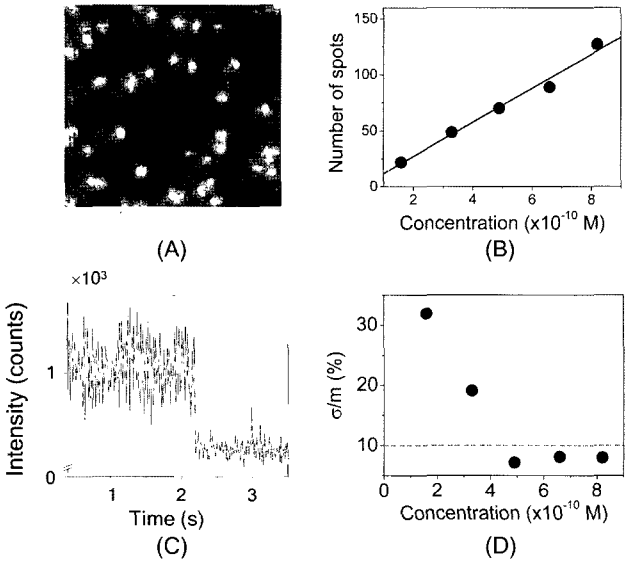

Figure 2. (A) Single molecule spot image of NR molecules embedded in a PMMA matrix. (The scale bar indicates 1 mm in length) (B) The plot of emission spot numbers *vs.* NR concentration for spin coating. (Spot numbers were counted in 10 × 10 μm area of the PMMA film). (C) Intensity transient of NR fluorescence exhibiting the discrete intensity jump which is a characteristic behavior of SM fluorescence. (D) Uncertainty (σ/m) for the number of fluorescent spots obtained from the spot numbers in 3 different regions.

Table 1. Number of emission spots [a] *vs.* NR solution concentration for spin coating

Concentration ($\times 10^{-10}$ M)	Number of spots (N)			[b] M_T	M_T - [c] M_P [d] (M_F)	[e] σ	σ/M_F (%)
	Area 1	Area 2	Area 3				
0	24	25	28	25.6	0	2.08	-[f] (8.1)
1.6	41	47	55	47.6	22	7.02	31.9
3.3	64	77	82	74.3	48.7	9.29	19.1
4.9	91	95	101	95.7	70.1	5.03	7.2
6.6	110	111	123	114.7	89.1	7.23	8.1
8.2	144	151	164	153	127.4	10.1	7.9

a) Spots in 10 × 10 μm area were counted.
b) Mean number of total fluorescent spots observed on NR/PMMA films.
[$M_T = (N_{area1} + N_{area2} + N_{area3})/3$]
c) Mean number of fluorescent spots on PMMA film.
d) Mean number of NR spots on NR/PMMA films.
e) Standard deviation of the spot numbers in three different regions.
f) σ/M(%) for PMMA spots obtained from the spot numbers of three different regions.

performed to test if the number of fluorescent spots in a fixed area can be determined by the spin coating conditions. The number of total emission spots was obtained by averaging the number of spots in three independent regions, and the average number of spots in a blank PMMA film was subtracted to obtain the NR spot numbers. As shown in Fig. 1B, the number of NR spots was linearly dependent on the concentration of NR solution for spin coating. This result means that the number of fluorophore spots can be predicted if the concentration of spin coating solution is known.

We also estimated the standard deviation of spot number counting in a film by comparing the number of spots in three different regions. As shown in Fig. 2D and Table 1, uncertainties of counting estimated from σ/m became lower than 10% when the sample concentration for spin coating was higher than 4.9×10^{-10} M, or the number of spots exceeded 70 in the defined area. Here, σ and *m* are the standard deviation and mean value of counted spot numbers in three different regions of a film, respectively. This result is related with the statistical aspect in dealing with the SMD results that *ca.* 80 molecules are needed to get uniform observables within 10% error range [18]. Although the number of required molecules was deduced for the uniformity of average intensities in ref. 18, a similar condition is expected in determining minimum requirement of uniform spot numbers in a fixed area.

Number of fluorescent spots at a fixed dye concentration

From the experiment of spot number *vs.* concentration, it was found that the solution concentration should be higher than~5×10^{-10} M to show uniform spot numbers within 10% error in a film. Furthermore, it has been reported that the thickness of spin coated polymer film depends on the polymer concentration, solvent volatility and spin speed [19,20]. With a uniform thickness of polymer film, the number density of dyes embedded in polymer film becomes a function of dye concentration. Since the number of dyes in a fixed area corresponds to the product of number density of dyes and the volume of polymer films, the number of emission spots would be a constant if all the parameters for spin coating such as the concentration of dye and polymer, solvent used and the spin speed are fixed. The uniformity of detected spot numbers was tested for different films prepared by spin coating of dye solution with same concentration solutions. The number of spots found in six different films prepared with 4.9×10^{-10} M NR/PMMA/toluene solution is summarized in Table 2 and drawn in Fig. 3. As shown in Fig. 3, the number of spots showed uniform values within 10% of the average over all films. In other words, 18 regions selected from 6 different films exhibited uniform number of emission spots within 10 % error range. Based on the above results, the number of emitting dyes observed in the polymer films can be predicted if the concentration of dye solution is known from the absorption measurement, and the sample consists of single component.

Now we can distinguish two samples in Fig. 1 by counting

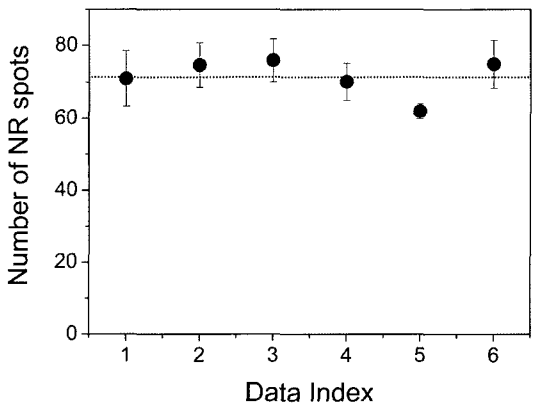

Figure 3. Average number of NR spots observed in 10 × 10 μm of the film. Six different NR-embedded PMMA films have been observed. The error bar indicates the standard deviation of counting spots among three different areas in a film.

Table 2. Number of emission spots[a] observed in NR/PMMA film spin coated[b] on glass.

Trial number	Number of spots (N)			[c] M_T	M_T-[d] M_P [e] (M_F)	[f] σ	σ/M_F (%)
	Area 1	Area 2	Area 3				
1	95	104	89	96	70.9	7.55	10.7
2	93	101	105	99.7	74.6	6.11	8.2
3	103	94	105	101	75.9	5.86	7.7
4	91	95	101	95.7	70.1	5.03	7.2
5	87	90	86	87.7	62	2.08	3.4
6	107	99	94	100	74.9	6.56	8.8

a) Spots in 10 × 10 μm area were counted.
b) 4.9×10^{-10} M NR in PMMA/toluene (10 mg/ml) was spin coated.
c) Mean number of total fluorescent spots observed on NR/PMMA films.
[$M_T = (N_{area1} + N_{area2} + N_{area3})/3$]
d) Mean number of fluorescent spots on PMMA film.
e) Mean number of NR spots on NR/PMMA films.
f) Standard deviation of the spot numbers in three different regions.

the number of emitting dyes in a fixed area of a fluorophore-spread film. When we observe the film prepared from an isomeric mixture in which either one is non-fluorescing, the number of counted fluorophores would be less than expected values. Consequently, the number of spots in the film from sample A would be half of that from sample B. This assay technique can be applied for the quality analysis of synthetic biofluorophores. Synthetic biofluorophores, such as fluorescently labeled DNA oligomers or proteins, are usually synthesized in small scales (< a few μmol), and it is not easy to find an adequate column for efficient isomer separation. Sample purity can be assessed by mass spectrometry and absorption measurements, however, such analysis techniques may not be sufficient to identify the purity of samples. SMD of individual fluorophores provides a simple way to distinguish isomers having different fluorescence quantum yields. Deviation from the expected spot numbers in a fluorophore-spread film can be considered to be an indication of lower purity of the synthesized biofluorophores.

Fluorescence quantum yield vs. SM fluorescence intensity

Two isomers having the same extinction coefficient but different fluorescence quantum yield exhibit different emission intensities in ensemble measurement. Similarly, it is expected that the intensity distribution is different between those isomers in SM fluorescence measurements. Since the SMD result is usually obtained as a distribution of which the average value corresponds to the ensemble measurement, different emission intensity in ensemble measurement would be pronounced as a different distribution in SMD. We observed individual fluorescence intensities of Cy3 and NR molecules to compare the intensity distributions of two fluorophores having different emissivities. Molar extinction coefficients of Cy3 and NR are 133,000 $cm^{-1}M^{-1}$ at 544.3 nm [14] and 38,000 $cm^{-1}M^{-1}$ at 519.4 nm [17] and fluorescence quantum yields are 0.04 [16] and 0.7 [21], respectively. From the extinction coefficients and fluorescence quantum yields, the relative ratio of Cy3 emissivities compared with NR is 0.2 when the molecules are excited at the absorption maxima. If we adopt excitation laser light of 543.5 nm He-Ne output for Cy3 and 514.5 nm Ar^+ output for NR, the excitation lines correspond to the absorption maxima of each molecule, and the absorbance can be approximated as the maximum extinction coefficients. The emissivity of a single molecule can be expressed as follows,

$$E_F = 1{,}000 \times I_o \Phi_F \varepsilon / hn N_A \quad (2)$$

where E_F is emissivity of the molecule, I_o is the excitation laser power in $W \cdot cm^{-2}$, Φ_F is the fluorescence quantum yield of the molecule, ε is the molar extinction coefficient of the molecule, h is the Planck's constant, ν is the excitation photon frequency and N_A is the Avogadro's number. Then, the emissivity becomes the number of photons emitted by a single molecule. If we use 3 μW for excitation, and assume that the laser beam diameter at focus is 230 nm [18], the E_F values of Cy3 and NR correspond to 1.75×10^5 and 3.57×10^5 photons·s^{-1}, respectively. Since the polarity of PMMA matrix is similar to that of acetonitrile, the fluorescence quantum yield of NR in PMMA is assumed to be 0.3 which was obtained in the acetonitrile solution [22, 23]. Considering the detection efficiency of our instrument (0.1), the expected values of detected photons in 10 ms gate time are 175 and 357 for Cy3 and NR, respectively. Thus, it is expected that the ratio of 0.5 would be obtained between Cy3 and NR emissivities by using SM fluorescence detection. This result led us to conclude that the difference in emissivities of both fluorophores could be expressed in the intensity distributions obtained by SMD.

Figure 4 shows the distributions of *ca.* 600 individual fluorescence intensities of each Cy3 and NR molecules in PMMA matrices. The average (and maximum) intensities obtained from the distributions correspond to 129 (350) and 324 (735) for Cy3 and NR, respectively. Higher emission

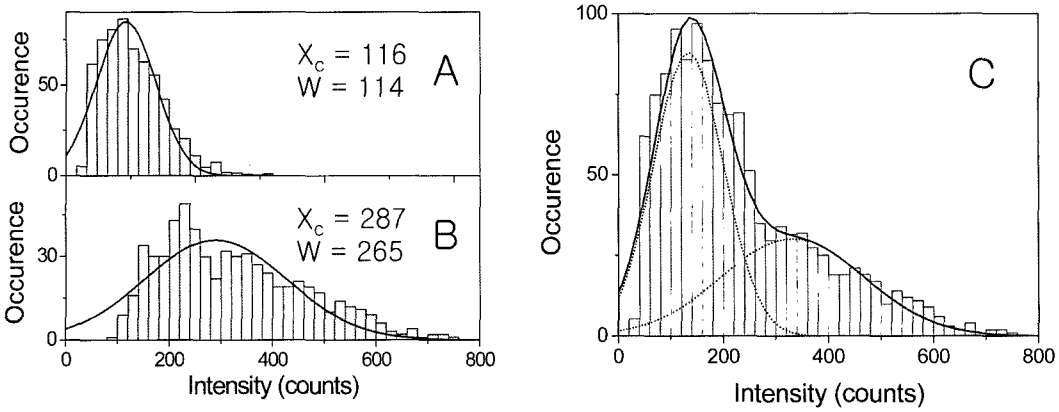

Figure 4. (A) Fluorescence intensity histogram of single Cy3 molecules on PMMA film excited by 3 μW of 543.5 nm light. (B) Fluorescence intensity histogram of single NR molecules embedded in PMMA film excited by 3 μW of 514.5 nm. (C) Distribution obtained by adding Cy3 and NR distributions; The lines represent the results of Gaussian fitting. Both distributions in A and B could be fitted with individual single Gaussian functions, and the distribution in C needed two Gaussian functions for fitting.

intensity values than expected in the above consideration might be attributable to the quantum yield change of fluorophores due to the polarity and rigidity variation in nanoscale environments of PMMA matrix. The fluorescence quantum yield of Cy3 varied from 0.04 to 0.12 depending on the substituent molecules [15], and that of NR varied from 0.08 to 0.7 depending on the polarity of environments [21-23]. The ratio of mean emissivities obtained from the distribution was 0.4. As shown in Fig. 4A and B, two fluorophores exhibited different intensity distributions considering the mean value and width. Each distribution was fitted with single Gaussian functions exhibiting different means and widths. NR molecules showed broader distribution with higher intensity mean value (Fig. 4B). When we added two distributions, two Gaussian functions were needed to fit for the resultant distribution (Fig. 4C). This result means that the fluorescence intensity distribution of single molecules prepared from a single compound can be fitted with a single Gaussian curve, while that from a mixture consisting of different isomers would be fitted with more than two Gaussian functions. Thus, it is also possible to distinguish a pure compound and the isomeric mixture by observing the SM fluorescence intensity distribution.

CONCLUSION

In this study, we have demonstrated that SMD can provide a new assay technique for distinguishing pure compounds and the isomeric mixtures. In case, the spectroscopic analysis using absorption and column separation fail to identify the isomeric mixture, SM fluorescence observation can give information about the existence of isomers having different emissivities. Since the absorption properties of fluorophores such as extinction coefficient is not much altered (< 10%) by substitution or environmental changes, single molecules in the analyzing samples are expected to absorb same quantity of light, however, the molecules are expected to emit different intensities of light - ranging up to 300% - depending on the molecular structures of substituent. Analysis on the isomeric mixtures having large difference in fluorescence quantum yield would result in deviation of emitting spot numbers in a fixed area from the expected values. On the other hand, the mixture of isomers having relatively small emissivity difference can be identified from the SM fluorescence intensity distribution which would not be fitted with single Gaussian function.

This new assay technique can be effectively applied for the purity analysis of synthetic biofluorescent probes prepared in small scale (< a few μmols) which is not enough for the usual ensemble assay techniques.

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