Microscopic Chemical Imaging for Species-Selective Determination of Rhodamine Dyes Adsorbed on Microparticles.

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A fluorescence microscope equipped with an interferometer was used for spectrum imaging of microparticles of an ion-exchange resin adsorbing rhodamine 6G, rhodamine B, or rhodamine 101. Two-dimensional images with each pixel having a fluorescence emission spectrum were obtained for species-selective determination of the rhodamine dyes. These microparticles showed different peak positions in the emission spectra separated by 15nm, and two types of them had similar fluorescence intensity to each other. It is demonstrated that species of adsorbed dyes are clearly distinguishable as an image by using a spectrum-based image processing technique and that free molecules in solution can be distinguished from the adsorbed dyes. The spatial resolution and detection limit of the system were evaluated. This technique has a potential to selectively determine a small amount of target molecules in microscopic substances, in which a large amount of disturbing substance exist.

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There is an increasing interest in non-RI (radio isotope) diagnostics in a variety of fields especially in biology and biotechnology.1 The RI methods have been applied from basic researches to practical diagnostics and its importance does not decrease nowadays but requires of special facilities and specialists in treating RI promote developing methods with more convenience than the RI methods. Alternatives are required to have a competing sensitivity with the RI methods. A fluorescence method is one of the most promising alternatives.1

Fluorescence detection is one of the most sensitive methods for target molecules in a variety of materials such as liquid solution, gel, solid, and biological cells. Although the target molecules must have a relatively high fluorescent quantum yield, a lot of established methods are available to label a nonfluorescent target molecules with fluorescent chromophores by chemical and physical derivatization. However, when a sample contains a small amount of the target molecules and an extremely large amount of more or less luminescent substances, the fluorescence method suffers from background signals coming from the disturbing substances. This is often the case when we try to detect low-concentration target molecules in a living cell. The condition is much wrong for non-fluorescent target molecules in the sample cell. Non-specific bindings and excess reagents of labeling chromophores generate large fluorescence with almost the same spectrum characteristics.

A fluorescence spectrum of some fluorescent molecule is sensitive to the surrounding environment. Spectrum shift is observed depending on where the fluorescent chromophore is. Molecules with these chromophores have been used as a micro-environment probe.2 If such an environment-dependent character can be fully utilized to discriminate specifically bounded chromophores from non-specifically bounded or unbounded chromophores, a selectivity of target molecules will be enhanced greatly.

In this report we demonstrate the spectrum-based discrimination for microparticles of an ion exchange resin colored with three kinds of rhodamine dyes. A two-dimensional fluorescence digital image was measured with a microscope, of the mixed microparticles each of which contained one of the rhodamine dyes. Each pixel of the image has the information of an emission spectrum so that image processing was successfully made the discrimination possible. Difference of fluorescence spectrum between adsorbed and free dyes was examined. An in-plane resolution and detection limit of adsorbed rhodamine dyes were evaluated. Discussion is made on the performance of this method for species-selective determination of molecules adsorbed on micro-substances.

Experimental

Apparatus
A schematic illustration of the experimental setup is shown in Fig. 1. It consists of three parts: A light source for fluorescence excitation; inverted microscope (TE-200, Nikon) for microscopic observation; and an imaging spectrometer (SD-200, Shimadzu Co. and Applied Spectrum Imaging Co.). A 150-W halogen lamp was used as a light source with collimating lenses and a 10-cm monochromator. The monochromated light at 520 nm was guided by an optical fiber bundle to illuminate a sample on the microscope stage. To determine the detection limit a second harmonic light of a continuous-wave diode-pumped Nd ytrrium aluminum garnet laser (532 nm, 5 mW) was used. Fluorescence emitted from the sample was collected with an objective lens (Plan Fluoro,
was measured before the fluorescence measurements. From the difference of two absorbance spectra, the amount of dyes adsorbed to the resin was calculated. The efficiency of the ion exchange was from 76 to 95% for a dye solution of a concentration ranging from \(7 \times 10^{-6}\) to \(2 \times 10^{-5}\) M. Density of the resin was assumed to 1.1 g/cm\(^2\) and the number of the microparticles in the suspension was estimated, then the adsorbed amount was calculated.

**Reagents**

The ion-exchange resins are produced from Mitsubishi chemical Co. Resins with 11 \(\mu\)m in an averaged diameter (MIC-GEL CK10S) and those with 4 \(\mu\)m in an averaged diameter (MIC-GEL CK10M) are styrene-divinylbenzene based copolymer and have 10% of a cross-linking density. RhB and Rh101 (reagent grade, Nacalsi, Japan) are used as received. R6G is purified by recrystallization. Water is purified by Milli-Q system (Milli-Q Academic, Millipore) until the resistively reached higher than 18 M\(\Omega\)cm.

All experiments were performed at room temperature at atmospheric pressure.

**Results and Discussion**

**Discrimination of microparticles adsorbing different dyes**

Dry microparticles adsorbing R6G, RhB and Rh101 showed similar spectra to those in aqueous solutions but peak wavelengths were shifted to 585, 600, 620 nm, respectively. Such an environment-dependent peak shift of fluorescence emission spectra has been reported on rhodamine dyes in literature. Wet microparticles had the spectrum shifts of the same magnitudes as dry ones. It is expected that the spectrum similarity may make it difficult to identify what kind of rhodamine dyes are adsorbed on each microparticle. Figure 2a shows a fluorescence-image of mixed microparticles of 11 \(\mu\)m in diameter in water. An edge of water is clearly seen at the upper side of the Fig.2a. There are three types of microparticles but only two types (bright and dark particles) are distinguishable. It is because spectrum shift is too small to distinguish three from types of microparticles when they have the same intensity levels.

Emission spectra in Fig. 2b of the points A, D and E show that these points have different spectra from each other. Spectrum difference between the points A and D is less than 20 nm so that it is hard to recognize difference between particles A and D only from the as-observed fluorescence image. The points A and B, C and D, and E and F showed the same peak positions of fluorescence spectra at around 585, 600, and 620 nm, respectively, although the maximum intensity of each spectrum was different. A, B, C, and D showed the similar intensity levels than E and F. It can be concluded that dyes adsorbed on the microparticles A and B, C and D, and E and F are RhB, R6G, and Rh101, respectively.

Because difference exists in their spectra, it is possible to enhance it by an image processing technique. A result of an image processing is shown in Fig. 2c, in which brightness \(B(x,y)\) of a pixel at the coordinates \((x,y)\) is given as

\[
B(x,y) = \frac{40 \times \sigma[S_{\text{norm}}(x,y,\lambda)] S_{\text{stand}}(\lambda) + S_{\text{back}}(x,y)}{S_{\text{norm}}(x,y,\lambda)}
\]

(1)

where \(S_{\text{norm}}(x,y,\lambda)\) is the maximum intensity of the image, \(\sigma[S_{\text{norm}}(x,y,\lambda)]\) represents the standard deviation between the normalized spectrum at \((x,y)\), \(S_{\text{stand}}(\lambda, x,y)\), and the normalized standard spectrum, \(S_{\text{stand}}(\lambda, x,y)\). The image processing software used is SpectraCube provided by Applied
Spectral Imaging Co. The image-processing function of Eq. 1 calculates similarity of the spectrum at \((x,y)\) to the normalized standard spectrum, \(S_{standard}(x,y)\). The spectrum at the point \(A\) was selected as the standard. It is clearly demonstrated that there are three types of microparticles in Fig. 1c: The microparticles A and B, C and D, and E and F can be classified into the same types, respectively.

An adsorbed amount of dyes was calculated to 1.5 fmol per particle. Even with the small amount and with the small differences in the emission spectra, labeling of the microparticles are found possible.

Observation of dye-adsorbed microparticles in the dye solution

It is often the case in detecting a small amount of dye-derivatized target molecules in a biological cell that the target molecules are surrounded by a large amount of the other molecules having a spectral similarity or of free dye molecules, while selective determination of the target molecules is urgently required. Figure 3a shows a fluorescence image of Rh101 adsorbing microparticles in an aqueous solution of Rh101 with a concentration as high as the solution shows the same level of fluorescence intensity as the microparticles. Fluorescence spectra obtained along the line in Fig. 3a are shown in Fig. 3b as an image in which vertical and lateral positions correspond to the spatial position and wavelength, respectively. A spectrum shift between the Rh101 dyes in solution and adsorbed dyes on the microparticles is clearly observed. The result suggests that spectrum-based discrimination of bounded dyes from free dyes can be performed for a fluorescent dye such as the rhodamine dye, with the spectrum imaging method.

Spatial resolution and the detection limit.

Spatial resolution and the detection limit will limit the performance when the method is applied to the detection of some target substances with a size of a sub-nanometer: i.e., virus in a living cell.

Spatial resolution of the present system was roughly evaluated. An edge of a fluorescence image of an 11 μm particle razed from
10 to 90 % of the maximum intensity within 2.5 μm in length when the intensity was measured along a line crossing the center of the particle. With the objective lens having a magnification of 40, the size of each pixel corresponded to a square with the one-side length of 0.23 μm. Because the pixel size is small in comparison with 2.5 μm, the intensity profile along the line crossing the center of the particle directly related to the spatial resolution but was influenced by the inhomogeneous fluorescence intensity of the sample itself because the sample was spherical. We conclude the spatial resolution of the system was better than 2.5 μm, and it can be improved if we use a high-magnification objective lens.

Because the microscope we used was not based on a confocal optical system, a good spatial resolution in depth direction was not expected. However, it was observed for each microparticle with a diameter of 4 μm that a positional change of the sample 4 μm up or down from the focal plane made the edge of a microparticle out of focus. The long optical path through the interferometer may cause the relatively good resolution in depth direction.

The detection limit was evaluated with R6G adsorbed microparticles with a diameter of 4 μm by using a 532 nm laser light source. Fluorescence image was measured and intensity at the center of each microparticle and at the peak wavelength was averaged over more than 30 microparticles for the samples prepared from a dye solution with a certain concentration. This is because fluorescence intensity of each microparticle varied 30% of the averaged value almost independent of the magnitude of the averaged value. It seems that each microparticle had different characteristics in terms of the dye cation exchange although we could not specify whether the inhomogeneity of the microparticles among the samples is induced during sample preparation or it is intrinsic to the microparticles themselves. The calibration curve showed a good linearity up to 65 amol per particle of adsorbed dye amount, and the detection limit determined as the three times of standard variation was 5.0 amol per particle.

Summary
A microscopic fluorescence spectrum imaging was performed of microparticles adsorbing three types of rhodamine dyes for the species-selective determination of a small amount of target molecules in a microscopic substance. These microparticles showed different peak positions in the emission spectra separated by 15nm, and two of them had similar fluorescence intensity to each other. Even under wrong conditions, it is demonstrated that species of adsorbed dyes are clearly distinguishable as an image by using a spectrum-based image processing technique and that free molecules in solution can be distinguished from the adsorbed dyes. The spatial resolution and detection limit of the system were evaluated as well. This technique has a good potential to selectively determine a small amount of target molecules in microscopic substances containing a large amount of disturbing substance. This method would be successfully applied to count a certain type of virus in a biological cell.

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References