Single Molecule Detection of Cyanine Dye at the Dodecane–Water Interface

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A single molecule of 1,1'-dioctadecyl-3,3',3''-tetramethylindocarbocyanine (DiI) was detected at the dodecane–water interface by means of a laser-induced fluorimetry. Total internal reflection microscopy was used for the detection of the fluorescence from a single DiI molecule adsorbed at the dodecane–water interface. A bundle of photon signals observed during 16 ms with the integration time of 2 µs indicated the crossing of a single DiI molecule in an observation area in the microscopic detection. The duration of the photon bundle suggested the possibility to calculate the lateral diffusion coefficient of the single molecule at the liquid–liquid interface.

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The detection of single molecules is the only ultimate method that enables us to observe the behavior of individual molecules. Various techniques for the detection of single molecules have been developed, most of them utilizing laser-induced fluorescence spectroscopy. The detection of single molecules in solutions1-3 and at solid–liquid interfaces4 has been already reported. On the other hand, there have been no studies on the single molecule detection at liquid–liquid interfaces so far. The liquid–liquid interfaces have been recognized as the specific reaction fields in the solvent extraction5,6, liquid membrane sensor and phase transfer catalysis7. However, detailed structural properties of the interface and the mechanism of interfacial reactions have not been elucidated at a single molecular level yet. In the present study, the detection of a single dye molecule adsorbed at the dodecane–water interface was examined by means of laser-induced fluorescence microscopy.

In the laser-induced fluorescence method, an intrinsic fluorescence signal has to be discriminated from background signals including a scattered light. Since the fluorescence emitted by a single molecule is extremely weak, the reduction of the background signals is essential for the detection of a single molecule. In order to lower the background, the total internal reflection technique2 was adopted in the present study. The dodecane–water interface adsorbed by target fluorescent molecules was prepared in a thin and flat glass cell. When a laser beam is irradiated from the organic phase side to the interface with an incident angle of larger than the critical angle (69°), the light is totally reflected at the interface and only the evanescent region at the interface can be observed. Using this method, we could detect quite weak fluorescence with an extremely low background.

The purpose of the present study is to develop the method for the detection of a single molecule adsorbed at the liquid–liquid interface for the first time.

**Experimental**

**Chemicals and sample preparation**

The chemicals used in this study were obtained from commercial sources: 1,1'-dioctadecyl-3,3',3''-tetramethylindocarbocyanine (DiI) perchlorate from Molecular Probes, dodecane (GR) and glycerin (GR) from Nacalai Tesque (Japan). Water was distilled and purified with a Milli-Q purification system (Milli-Q SP. TOC., Millipore). Solid DiI was dissolved in dodecane and the solution was diluted at the concentration of $7.9 \times 10^{-10} - 1.3 \times 10^{-11}$ mol dm$^{-3}$.

![Fig. 1 A two-phase microcell made from a bored slideglass, a bored coverslip and coverslips. A right angle prism was attached on the cell for the total internal reflection excitation.](image)

**Microcell**

A flat microcell was made by sticking a bored (10 mm in diameter) slideglass and a bored (5 mm in diameter) coverslip on another coverslip (see Fig. 1). Water (2.7 mm$^3$) was filled in the lower depression in the cell and then the DiI dodecane solution (63 mm$^3$) was filled in the upper depression in the cell to make a thin aqueous phase of 0.14 mm in thickness. Finally, a new coverslip was put on the dodecane phase to close the cell and a right angle quartz prism (2 cm × 2 cm × 2 cm) was attached on it.
The gap between the prism and the coverslip was filled with glycerin. By this way, the dodecane–water interface was made in the microcell and it could be adjusted to the focal plane of an inverted microscope.

Fig. 2  Schematic diagram of the total internal reflection fluorescence microscopy for the single molecule detection. APD is an avalanche photodiode detector.

**Total internal reflection fluorescence microscopy**

The total internal reflection fluorescence microscope system was set up as shown in Fig. 2. The apparatus consisted of an inverted microscope (TE300, Nikon), a cw-Nd:YAG laser (532 nm, TEM₀₀, 50 mW, 0.6 mm in beam diameter, model 4301-050, Uniphase) and an avalanche photodiode detector (SPCM-AQR-16, EG&G Canada), which provided quantum efficiencies of about 65 % around 570 nm with a dead time of 36 ns. The laser beam was passed through a λ/2 plate and was focused by a lens (focal length, 4 cm) to the interface through the prism. An s-polarized incident light was irradiated at the interface with the λ/2 plate. The radius of the laser beam was reduced to about 15 μm at the dodecane–water interface. Fluorescence emitted by the DiI molecules adsorbed at the interface was collected by an oil immersion objective (CFI Plan Apo 60×H, NA = 1.4, Nikon) and was focused on the detector after passing through a bandpass filter (570DF30; path range, 555 – 585 nm; Omega Optical). Time-dependent photon counting was carried out with a multichannel scaler (MCS-plus, EG&G Ortec). The minimum integration time per one channel was 2 μs, and the maximum number of channels was 8192. In almost all measurements, the integration time for one channel was set to 2 μs and thus the detection time for each measurement was about 16 ms.

A pinhole of 50 μm in diameter was placed in front of the detector to restrict the observation area. The diameter of the observation area, dₐₜ, at the interface was confined to 830 nm that was calculated simply by dividing the diameter of the pinhole by the magnification of the objective. The value of 830 nm was larger than the diffraction limit of light. All of the molecules in the observation area could be irradiated with the laser light, because the observation area was much narrower than the area irradiated by the laser beam. The laser energy density at the observation area was calculated as about 2.6 × 10⁵ W cm⁻² by using the maximum power of the Gaussian laser beam profile, the reflection efficiency of the two mirrors, the transparency of the lens and the prism, and the incident angle to the interface (72°). All the experimental procedures were carried out in a thermostated room at 25±1°C.

**Results and Discussion**

**Adsorption of DiI at the dodecane–water interface**

DiI is a monovalent cation with two long alkyl-chains and is expected to be adsorbed substantially at the dodecane–water interface. An interface (1.0 cm² in area) was formed between 2.9 × 10⁻⁹ mol dm⁻³ DiI dodecane solution (1.00 cm³) and water (1.17 cm³) in a standard 1 cm × 1 cm quartz cell. The fluorescence spectra from this interface was measured by a conventional fluorescence spectrophotometer (Hitachi 650-40) under the total internal reflection (TIR) condition described previously with the excitation wavelength of 532 nm. The TIR fluorescence intensity at the maximum wavelength of 573 nm became constant after 150 min from the formation of the interface. No fluorescence was detected from the dodecane phase. Thus, it was confirmed that all of the DiI molecules were adsorbed at the interface and the interfacial concentration of DiI was determined as 2.9 × 10⁻¹² mol cm⁻². For the measurement of a single DiI molecule, the initial concentration of DiI was lower than that for the TIR fluorescence measurement. The measurement was started after leaving the two phases for 4 h to attain the interfacial adsorption. Orange fluorescence of DiI was observed by eyes from the laser trajectory in the dodecane phase just after the formation of the two–phase, but fluorescence of DiI was observed only from the interface after 4 h. The number of observable DiI molecules was calculated from the interfacial concentration and the observation area, assuming that all of the molecules were adsorbed at the interface.

**S/N ratio and efficiencies**

A serious problem concerning the detection of single molecule fluorescence is a high background due to laser scattering. We repeated the measurement of 16 ms for 100 times to evaluate the signal to background noise ratio. The average count rate of the background was 9 counts/16 ms for the dodecane–water interface without any solutes, whereas 254 counts/16 ms was observed in the case that the observation area contained 1.2 DiI molecules on average. This result means that the observed photon count corresponds to about 30 photons emitted by molecules on average. With the cycle time and the detection efficiency, the observable photon counts from one molecule was calculated as 3 % by multiplying the quantum efficiency of the avalanche photodiode detectors by the fluorescence collection efficiency, which was calculated13 by the NA value, the transparency of the objective lens, the transparency of the filter for the DiI emission spectrum, and the efficiency of all the other optics between the objective and the detector. Hence, it can be estimated that one observed photon count corresponds to about 30 photons emitted by molecules on average. With the cycle time and the detection efficiency, the observable photon counts from one molecule were estimated to be about 700 counts/16 ms and the magnitude of this value was as high as that of the observed one (200 counts/16 ms).
In conclusion, we detected the fluorescence emitted by a single fluorescent DiI molecule at the liquid–liquid interface for the first time. The kinematic natures and nano-properties of various interfaces will be revealed by extending the single-molecule-probing approach proposed in the present study.

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References and Footnotes
14. The radius of DiI molecule was calculated as $7.0 \times 10^{-8}$ cm by assuming as a sphere with the molecular volume of DiI, which was calculated by using the additivity for volume of components consisting of the molecule. A. F. M. Barton, “CRC Handbook of Solubility Parameters and Other Cohesion Parameters”, 1983, CRC Press, Boca Raton, 64. The D value of DiI in dodecane was estimated using the Stokes-Einstein equation and the viscosity of dodecane ($1.38 \times 10^{-3}$ Pa s).