Single Molecule Detection by Laser Two-Photon Excited Fluorescence in a Capillary Flowing Cell

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The two-photon excited fluorescence of fluorescein and Rhodamine B in a flowing sample cell was observed using a self mode-locked Ti-sapphire laser (180 fs pulse). The detection limit of fluorescein was less than 2 molecules in the probe volume. The single molecule detection of Rhodamine was successfully performed in a flowing cell, and the number of photon bursts agreed approximately with that expected.

Keywords Laser, two-photon fluorescence, single molecule detection, flow cell

Laser-induced fluorescence has been a very sensitive analytical technique1, and one-molecule detection2–4 has been achieved. It has progressed so well that the greatest concern for a highly sensitive determination is not the increase in the signal but the decrease in laser light scattering. Two-photon excitation has two definite advantages for a highly sensitive detection. (1) The excitation wavelength and emission wavelength are far apart, and laser scattering at the cell wall and Raman scattering can be easily removed spectrally; this is especially the case for those molecules with a small Stokes shift. (2) The two-photon fluorescence occurs primarily at the focal point of a lens, and spatial isolation with microscope objectives can be used to maximize the signal-to-noise ratio. Two-photon excitation can provide information which is not obtainable by one-photon excitation. The two-photon spectrum may remove the Doppler broadening, thus allowing clear separation of the rotational lines of molecular fluorescence.5 Two-photon spectroscopy can reveal excited-state symmetry through polarization dependence for randomly oriented systems.6 Two-photon spectroscopy had limited use in analytical applications due to its low efficiency. Because two-photon excited fluorescence is proportional to the square of the laser intensity, recent improvements in the high-peak-intensity laser have, however, made the highly sensitive determinations using two-photon excitation a reality. Detection limits as low as 120 pM were obtained by two-photon excitation.7,9 A single-molecule detection by two-photon excited fluorescence was carried out on a glass plate using a Ti-sapphire laser (100 fs) and confocal geometry, where molecules diffusing into and out of the two-photon excitation volume produced fluorescence bursts.10 A detection limit of 3 pM has been reported using a microchannel plate, and only one molecule was expected to stay under observation at any given moment.11 Merits in a single molecule identification were compared for one-photon and two-photon excitation for molecules on a glass plate using a Ti-sapphire laser (300 fs). The detection efficiency of two-photon excitation was limited by continuum generation in the solvent.12 Fluorescence imaging of single molecules on a glass plate was carried out together with observation of single-molecule emission spectra.13 A single-molecule spectrum was also obtained in a solid matrix at cryogenic tempertures.14

In the previous paper, we have reported a two-photon fluorescence detector for capillary electrophoresis with a detection limit of 8.0 amol (1.4 fg) for coumarine 440 using an fs Ti-sapphire laser.15 In the present paper, we are reporting single molecule detection in a capillary flowing cell based on two-photon fluorescence. The signal intensity has been theoretically analyzed and compared with the experimental findings.

Experimental

Apparatus  
A schematic diagram of the experimental apparatus is shown in Fig. 1. A self mode-locked Ti-sapphire laser (Coherent Mira 900D; pulsed width 180 fs, repetition rate 76 MHz, wavelength 800 nm) pumped by an Ar-ion laser (Coherent Innova 310; 8 W) was tightly
focused on a capillary cell (J&W Scientific, 100 µm i.d.) using a 10-X microscopic objective. The fluorescence was observed through another 10-X microscopic objective perpendicular to the incident laser beam. The fluorescence emission was transmitted through two glass filters (Andover Y-44: short cut-off below 410 nm; Andover 040FG11: band path at 320 – 680 nm, and transmission at 800 nm is below 10⁻⁵) and measured with a photomultiplier (Hamamatsu R5600U, response time: 3 ns, quantum efficiency at 800 nm: <10⁻⁵). The photocurrent was amplified by a current amplifier (EG&G Ortec 9302: 100 MHz) and a constant fraction discriminator (EG&G Ortec 871: 100 MHz), and was counted by a timer and counter (EG&G Ortec 871: 100 MHz).

The amplified signal was also analyzed by a microcomputer (NEC PC-9801RX).

Sample preparations

Fluorescein, Rhodamine B, sodium dihydrogenphosphate, and sodium hydroxide were obtained from Kishida Chemicals and were used as received. The standard solution of fluorescein of 1×10⁻⁴ M was prepared in 20 mM phosphate buffer at pH 11. The stock solution of Rhodamine B of 1×10⁻⁴ M was prepared in 5 mM phosphate buffer solution at pH 9. The sample solutions were prepared by successive dilution of the standard solution. The polyimide coating of the capillary at the irradiation and observation region was removed; the distance from the positive electrode to the observation region was 18 cm. It was cleaned by vacuum-flushing with 0.1 M NaOH solution before each measurement. The sample solution of fluorescein was injected into the capillary by vacuum flushing. The sample solution of Rhodamine B was injected by electrophoresis at 1.5 kV/30 cm using a high voltage power supply (Matsusada Precision, HCZE-30PN 0.25).

Results and Discussion

Probe volume

The two-photon excited fluorescence is less efficient than the one-photon excited fluorescence and has not been used much for highly sensitive determinations. Because the efficiency of a two-photon process is proportional to the square of the peak intensity of the laser, two-photon excitation has become more attractive using a recently-developed high-intensity laser. In a confocal limit where the incident laser was focused to the diffraction-limited radius \((\omega_0; 1.36 \mu m \text{ for } 800 \text{ nm})\), 73% of the total two-photon fluorescence is emitted within the path length of \(4\pi \omega_0^2/\lambda\) (29 µm, \(\lambda\): the laser wavelength). In this limit the probe volume can be set as a circular column of the radium of 1.36 µm and the length of 29 µm and was calculated to be 0.17 pl.

Highly sensitive detection of fluorescein

Fluorescein in the phosphate buffer was excited at 800 nm, and the two-photon fluorescence was observed at 320 – 680 nm. The analytical curve of fluorescein was linear over 4 orders of magnitude above the detection limit as is shown in Fig. 2. Because the background noise was about 100 count/s and its fluctuation was about 10 count/s, we could extend the analytical curve where the fluorescence photon count was 35 photon/s \((S/N=3)\) and determined the detection limit to be 1.5×10⁻¹¹ M. This value indicates that there are less than 2 molecules in the probe volume in the confocal limit. This value is better than the previous results obtained in solution samples.⁷,⁸

Single molecule detection of Rhodamine B

A flowing stream is a useful device to support analyte molecules for a single molecule detection. It has been maintained mainly by mechanical syringe pumps⁹,¹⁰, but the electrophoresis was used in the present work. The basic concept by Soper et al.¹⁶ and Lee et al.¹⁷ was applied to a single molecule detection of Rhodamine B.
in the flowing stream except that the present study was based on two-photon excitation. The molecular average transit time \( t_0 \) through the laser probe volume can be expressed with the average linear flow velocity \( v_a \) as follows:

\[
t_0 = \frac{\pi \alpha}{4v_a}
\]

Based on the elution time of a neutral molecule, 2-amino anthracene, \( v_a \) was found to be about 0.010 cm/s. Then, \( t_0 \) was about 10 ms. The concentration of Rhodamine B was \( 1 \times 10^{-12} \) M, and the average number of molecules in the probe volume, \( N_p \), was 0.102.

The probability, \( P(n) \), of \( n \) mol occupying the probe volume is given by the following equation based on the Poisson distribution:

\[
P(n) = \left( \frac{k\lambda}{n!} \right)^n e^{-k\lambda/n!}
\]

where \( k \) is the number of time segment per transit time, and \( \lambda \) is the average number of molecules in probe volume per time segment. The integration time of the counter (time segment) was 2.5 ms and thus \( k = 4 \); this indicated that the emitted photons should be distributed over four consecutive time segments when a single molecule flowed through the probe volume. Then \( \lambda = 0.0255 \). Then Eq.(2) indicates \( P(0) = 0.903 \), \( P(1) = 0.092 \) and \( P(2) = 0.005 \). The probability that more than 2 molecules stayed in the probe volume was negligibly small. Accordingly, the concentration of Rhodamine B was low enough to assure one-molecule detection. When the molecules pass through the probe volume, the average number of events \( (N_{ev} : \text{the number of time segments with any signal counts from probe molecules}) \) can be calculated as follows:

\[
N_{ev} = N_p T k / t_0
\]

where \( T \) is the measurement time. In the present case, \( N_p = 0.102 \), \( T = 2500 \) ms, \( k = 4 \) and \( t_0 = 10 \) ms, and then \( N_{ev} = 102 \). As expected in the probe volume calculation, this value corresponds to 73% of the total events, and we would expect about 140 events for the measurement time of 2500 ms. It is efficient to use the weighted quadratic summing (WQS) filter for the enhancement of the visibility of photon bursts from single molecules. The WQS filter algorithm can be expressed as follows:

\[
S(t) = \sum_{t=0}^{T} \omega(t) \cdot d(t+\tau)^2
\]

where \( d(t+\tau) \) is the raw data at time \( t+\tau \) and \( \omega(\tau) \) is a weighing factor, which is typically given by \( (\tau+1)/k \) for \( \tau = 0 \) to \( \tau = k-1 \) and by 0 otherwise.

Figure 3 shows the photon bursts for 1 pM Rhodamine B solution (b) and the solution blank (a) using \( s(t) \). The experimental result indicated 156 events during 2500 ms, which was approximately equal to the expected value (140). A slight increase in the observed value may indicate that the laser was not focused as tightly as expected and the probe volume would be slightly larger than expected.

The present paper indicates that a single molecule counting is possible based on the two-photon excited fluorescence and a flowing sample. Thus the two-photon process could be as sensitive as one-photon fluorescence using a fs laser and tight focusing.

References

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