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Notes

Determination of Two Photon Absorption Cross Section of Fluorescein Using a Mode Locked Titanium Sapphire Laser

Joon Myong SONG*, Takanori INOUE*, Hirofumi KAWAZUMI and Teiichiro OGAWA***

**Department of Molecular Science and Technology, Kyushu University, Kasuga-shi, Fukuoka 816–8580, Japan*
***Department of Industrial Chemistry, Kinki University in Kyushu, Iizuka 820–0011, Japan*

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Laser two-photon excited fluorescence has its own advantages in selection rule and backgrounds over one-photon excited (conventional) fluorescence.[1] Highly sensitive determinations based on two-photon excited fluorescence have been carried out using a high-intensity laser.[2,3] Characteristics of two-photon absorption should be clarified for better understanding of its mechanism and for developing wider analytical applications. There are several techniques to determine a two-photon absorption cross section.[4-6] However, its direct measurement is still difficult because of its small value.[7] Due to its low efficiency a sample in a high concentration had to be prepared for a determination of the two-photon absorption cross section, and the obtained value may be susceptible to self absorption.[6]

In this report, the two-photon absorption cross section of fluorescein at 800 nm has been determined by measuring the intensity ratio of one-photon excited fluorescence to two-photon excited fluorescence using a mode locked Ti-sapphire laser. Because of the use of the second harmonic of this laser as a light source of one-photon excitation, the present method is theoretically clearer and experimentally simpler than the other methods so far used. This report shows that the high peak power of the Ti:sapphire laser is more suitable for a determination of two-photon absorption cross section than a CW laser.

Experimental

The apparatus is essentially identical with that described in the previous paper.[3] In brief, a self mode-locked Ti-sappihire laser (Coherent Mira 900; pulsed width 180 fs, repetition rate 76 MHz) was tuned to 800 nm and focused on a BBO crystal for second harmonic generation (400 nm). The average power of the fundamental beam and the second harmonic beam was measured as 0.27 W and 0.10 W, respectively. Both of the beams were focused on a capillary cell (J&W Scientific, 100 μm i.d.) using a 10-X microscope objective. The polyimide coating of the capillary was removed at the irradiation position of the laser beam. The fluorescence was collected using another 10-X microscope objective which was set perpendicularly to the incident laser beam so that one-photon and two-photon excited fluorescence could be compared under identical experimental conditions. The fluorescence 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 was measured with a photomultiplier (Hamamatsu R5600U, quantum efficiency at 800 nm: $<10^{-5}$). The photocurrent was amplified and counted by a timer and counter (EG&G Ortec 871: 100 MHz).

The stock solution of fluorescein (1×10^{-4} M) was prepared in 20 mM phosphate buffer at pH 11. The sample solution with 1×10^{-6} M was prepared by a successive dilution of the stock solution and injected into the capillary cell by vacuum flushing.

Results and Discussion

Formulation and fluorescence intensity

The two-photon excited process is less efficient 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.

The observation of fluorescence consists of three processes: absorption of the incident radiation, emission from the excited state and the detection of photons.

J. M. S. present address: Department of Chemistry, Iowa State University, Ames, IA 50011, USA.
T. I. present address: Department of Applied Chemistry, Oita University, Tannohara, Oita 870–1124, Japan.
T. O. present address: Department of Molecular and Material Sciences, Kyushu University, Kasuga-shi, Fukuoka 816–8580, Japan.

The largest difference between one-photon excited and two-photon excited fluorescence lies in the first process, and thus a comparison between them should be carried out mainly for the first process.

The absorption rate (number of photons absorbed, N_a: photons s^{-1}) by one-photon (N_{a1}) and two-photon (N_{a2}) excitation by a pulsed laser can be expressed as follows:[8]

$$N_{a1}=\sigma P_{a1} C v/A_1 \quad (1)$$

$$N_{a2}=\delta P_{a2} P_{p2} C(l/A_2) \quad (2)$$

where σ is the absorption cross section (cm^2 $molecule^{-1}$), P_a is the average power of the laser (photons/s), C is the concentration (molecules cm^{-3}), v is the laser probe volume assuming the cylindrical geometry (cm^3), A is the cross-sectional area of the laser beam (cm^2), δ is the two-photon absorption cross section (cm^4 s $photon^{-1}$ $molecule^{-1}$), l is the cell length (cm) and P_p is the peak power of the laser (photons/s). The subscript 1 corresponds to the one-photon process and 2 to the two-photon process. P_p is related to P_a as $P_p=P_a/Rt$, where R is the repetition rate of the laser (pulses/s) and t is the laser pulse width (s/pulse).[9]

Although N_a is proportional to P_a at a low laser power for one-photon excitation, it may saturate at a high laser power. Meanwhile, the two-photon absorption rate depends on product of P_a and P_p, and increases up to much higher value of P_a than the one-photon process, because δ is very small. Then, the laser beam can be focused more tightly for two-photon excitation. Tighter focusing allows a better spatial filtering.

A typical lifetime of a fluorescent molecule is a few nanoseconds, and this is much larger than the laser pulse width (180 fs). Thus only one fluorescent photon can be observed from one molecule for one laser pulse. Therefore, the maximum value of N_a for a molecule should be 76×10^6 (photons $molecule^{-1}$ s^{-1}) in the present study. The laser power should be adjusted so that $\sigma P_a/A<76\times10^6$ for one-photon excitation and $\delta P_{a2} P_{p2}/A_2{}^2<76\times10^6$ for two-photon excitation.

The laser probe volume was assumed to be cylindrically symmetric.[5] In a confocal limit where the incident laser was focused to the diffraction-limited radius, A can be expressed as

$$A=\pi\omega_0{}^2[1+(\lambda z/\pi\omega_0{}^2)] \quad (3)$$

where z is the distance on the beam propagation axis (cm) from the focus, and ω_0 is the diffraction limited radius at z=0.[10] Thus, $(l/A_2)=[\tan^{-1}(d/b)-\tan^{-1}(-d/b)]/2\lambda$, where d is the length of the cell, and b is $2\pi\omega_0{}^2/\lambda$ (the confocal distance:13.2 μm). If $b<<d$, $(l/A)=\pi/2\lambda$. For one-photon excitation, v was set as the laser probe volume in the capillary.

The fluorescence signal (N_f) can be expressed as follows:

$$N_{f1}=KN_{a1}\phi_1 \quad (4)$$

$$N_{f2}=1/2KN_{a2}\phi_2 \quad (5)$$

where K is the collection efficiency of fluorescence photons, and ϕ is the fluorescence quantum yield: K should be independent of the excitation process, because the same apparatus is used. The fluorescence quantum yield of fluorescein would be identical for one-photon and two-photon excitation, because the final excited state is the same since the molecule has no center of symmetry: thus, $\phi_1=\phi_2=\phi$. Because two photons are necessary for exciting two-photon fluorescence, 1/2 is introduced in Eq. (5). Then,

$$(N_{f1}/N_{f2})=(N_{a1}/(1/2)N_{a2})$$
$$=(\sigma P_{a1} C v/A_1)/(1/2)\delta P_{a2} P_{p2} C(l/A_2) \quad (6)$$

Any difference in fluorescence intensity should be due to the difference in the absorption rate.

In a usual case N_{f1} is larger than N_{f2}. In order to obtain a comparable value of N_{a2} with N_{a1}, P_{p2} should be as large as possible. P_{p2} of the fs-pulsed laser used in the present study is 7.3×10^4 W, which is about 5 orders of magnitude larger than that of a CW laser of identical P_a (1 W). Thus a mode-locked fs laser would be able to overcome the small value of the two-photon absorption cross section (δ) and would be very useful as a light source of two-photon excitation.[11]

Determination of the two-photon absorption cross section

The two-photon absorption cross section has been determined for fluorescein and several other fluorophores.[12] Although this is an important parameter for the two-photon excitation process, substantial disagreement among published values often exists.[12] We have determined the two-photon absorption cross section of fluorescein on the basis of the Eq. (6).

The fluorescence intensity of fluorescein was measured and compared for one-photon excitation to that of two-photon excitation, as summarized in Table 1. The concentration of fluorescein was 1×10^{-6} M. The cross-sectional area of the laser beam was set as the diffraction limited radius: $\omega_0=1.2\times10^{-4}$ cm for 400 nm, 1.3×10^{-4} cm for 800 nm. The one-photon absorption cross section was determined to be 5.3×10^{-18} cm^2/molecule by measuring the molar absorption coefficient using a conventional absorption spectrophotometer. $P_{a2}=2.0\times10^{17}$ photons/s for 0.1 W at 400 nm. $P_p=8.1\times10^{22}$ photons/s at 800 nm. The observed value of (N_{f1}/N_{f2}) was 2.4. The two-photon absorption cross section (δ) can be obtained by comparing the fluorescence intensity based on Eq. (6) as 5.4×10^{-49} cm^4 s $photon^{-1}$ $molecule^{-1}$.

Comparison

The recent development of a mode-locked Ti-sapphire fs laser has increased efficiency of two-photon excitation enough to make it possible to measure two-

Table 1 Spectroscopic properties of fluorescein in an aqueous solution at pH=11

Absorption maximum	490 nm
Fluorescein maximum	516 nm
Fluorescein quantum yield	0.9
Fluorescence lifetime[13]	4.2 ns
Fluorescence intensity (one-photon excitation)	3.3×10^{6} cps
Fluorescence intensity (two-photon excitation)	1.4×10^{6} cps
One-photon absorption cross section at 400 nm	5.3×10^{-18} cm^2/molecule
Two-photon absorption cross section at 800 nm	5.4×10^{-49} cm^4/photon molecule

photon absorption cross section directly despite its very low value. Compared to CW laser, the Ti-sipphire fs laser has much higher second order temporal coherence, which provides two-photon excitation at low average laser power.[12] This can reduce the photodecomposition of fluorophores remarkably, which increases the accuracy of the measurement of the two-photon absorption cross section.

Xu *et el.*[12] measured two-photon absorption cross sections of fluorescein as 3.8×10^{-49} cm^4 s $photon^{-1}$ $molecule^{-1}$, which is approximately identical with the present value. The largest problem in their method lies in the accuracy in obtaining the collection efficiency of the two-photon excited fluorescence. The difficulty in obtaining the collection efficiency can be solved if the two-photon absorption cross section is determined based on the ratio of one-photon excited fluorescence to two-photon excited fluorescence using the same optical system. The second important problem in an accurate measurement is the two quantum efficiencies, ϕ_1 and ϕ_2. They are identical only when the excited state is the same for one-photon and two-photon excitation.

Two-photon ionization cross section of fluoranthene was reported[14] to be 2.5×10^{-54}, which is considerably smaller than the two-photon absorption cross section.

The method used in the present study is simple and seems to be ideal in the determination of the two-photon absorption cross section.

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