Two-photon fluorescence excitation cross sections of biomolecular probes from 690 to 960 nm

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We report on two-photon fluorescence excitation (TPE) action cross sections for five widely used molecular fluorophores. Measurements were performed by use of ultrashort (~100-fs) Ti:sapphire pulsed excitation over the range 690–960 nm. TPE spectra were obtained by comparison with a fluorescein calibration standard. Large cross sections were found for the cyanine reagent Cy 3 (~140 GM) and for Rhodamine 6G (~150 GM), both at 700 nm [1 GM = 10^{-50} (cm^4 s)/photon]. Several fluorophores show interesting and desirable blue shifts with respect to twice the one-photon absorption wavelength. Fluorophore fluorescence intensities showed no significant departure (±4%) from quadratic illumination power dependence, indicating genuine two-photon processes. Implications of these measurements for two-photon laser-scanning microscopy are discussed. © 1998 Optical Society of America

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1. Introduction

Two-photon laser scanning microscopy (TPLSM), first demonstrated by Denk *et al.*[1] at Cornell University, Ithaca, New York, is based on the simultaneous absorption by each fluorophore molecule of two photons in a single quantum event.[2] As commercial two-photon microscopes are currently available, TPLSM is finding increasing applications in biological research. One of the most important parameters needed for quantitative fluorescence microscopic studies is the excitation cross section of the fluorophore. Here we report on five dyes that compose a set of particular interest for biophysical applications and are already popular for conventional one-photon microscopy. For example, dextrans conjugated to various fluorophores are retained well within cells, which improves imaging, and are useful for determining membrane permeability and intercellular communication in neuronal tracing and for the monitoring of endocytosis. The carbocyanine dyes DiOC5 and DiOC6 have been used widely to stain mitochondrial membranes and to measure membrane potentials in fluorescence microscopy. The cyanine reagent Cy 3 is a highly fluorescent and highly water-soluble dye that is useful in a variety of biological applications; Rhodamine 6G, although widely used in confocal microscopy, has never, to our knowledge, been investigated for two-photon applications in the range of 690 to 960 nm.

Detailed knowledge of the two-photon excitation spectra of molecular fluorophores is required for determining which fluorophores are suitable for TPLSM. High values of the two-photon absorption (TPA) cross section render fluorophores efficient for nonlinear microscopic studies because the ratio of the energy absorbed to the input-energy flux through a sample is then high, minimizing possible photodamage of the sample under investigation. A large cross section is especially desirable when two-photon techniques are used to image molecular activities in living biological preparations.

In a continuation of our previous research,[3,4] we measured the two-photon fluorescence excitation (TPE) spectra of five prevalent molecular fluorophores with Ti:sapphire mode-locked laser excitation over the spectral range from 690 to 960 nm. The TPE cross section is proportional to the TPA cross section [see Eq. (1), below]. Our motivation was to provide a reliable database of two-photon cross sections for quantitative nonlinear microscopic studies. The absolute TPA cross sections and the TPE spectra

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of fluorescein were used as calibration standards throughout our investigation. Experimental results show insignificant departure from the quadratic dependence of the fluorescence intensity on the incident power, thus indicating that genuine two-photon processes occur.

A brief review of the theory is given in Section 2. The experimental procedure is described in Section 3. In Section 4 the main results, along with a discussion, can be found.

2. Two-Photon Excitation Theory

There are two ways of determining the two-photon cross section of a molecular fluorophore. One way is to measure the TPA cross section. This is done by the measurement of the amount of light being absorbed as it passes through the sample. Because high incident power is usually needed to generate a measurable signal, saturation, excited-state absorption, and photobleaching can complicate the interpretation of such measurements. Another procedure for determining two-photon cross sections is to measure the fluorescence signal generated by TPA. From the two-photon fluorescence signal a TPE action cross section σ_{TPE} can be determined. The TPE action cross section is linearly proportional to the TPA cross section σ_2, with the constant of proportionality being the fluorescence quantum efficiency of the fluorophore:

$$\sigma_{\mathrm{TPE}} = \eta_2 \sigma_2. \tag{1}$$

The number of photons absorbed per unit time by a two-photon process $[N(t)]$ in the focal volume of strongly focused illumination is related to the number of fluorescence photons $F(t)$ collected per unit time by the experimental apparatus:

$$F(t) = \frac{1}{2}\,\phi \eta_2 N(t), \tag{2}$$

where ϕ is the fluorescence collection efficiency of the experimental setup. The factor of $1/2$ accounts for the fact that two photons are absorbed from the optical field for each fluorophore excitation generated. The quantity measured experimentally is the time-averaged fluorescence emission $\langle F(t)\rangle$. Following the analysis of Xu *et al.*,[3] we find that, when the incident optical field is in the form of pulses, the time-averaged fluorescence detected can be expressed as

$$\langle F(t)\rangle \approx \frac{1}{2}\,\phi \eta_2 \sigma_2 C\,\frac{8_p}{f\tau}\,\frac{8\langle P(t)\rangle^2}{\pi\lambda/n}. \tag{3}$$

In expression (3), C is the fluorophore concentration (in cubic centimeters), n is the refractive index of the sample, $\langle P(t)\rangle$ is the time-averaged laser source power (in photons per second), and λ is the excitation wavelength (in centimeters). We have also written the degree of second-order temporal coherence of the light, $g^{(2)} \equiv \langle I_0{}^2(t)\rangle/\langle I_0(t)\rangle^2$ (where $\langle I_0\rangle$ is the average intensity at the focal point inside the sample), as $g_p/f\tau$, and where g_p is a dimensionless parameter of the order of unity that depends on the pulse shape, τ is the full temporal width at half-maximum (FWHM) of the excitation pulse, and f is the pulse-repetition rate of the laser.[3,4] The TPE action cross section, defined herein as $\eta_2\sigma_2$ [usually expressed in Goppert-Mayer units: 1 GM = 10^{-50} (cm^4 s)/photon], can thus be related to experimental variables by use of expression (3).

The fluorescence depends strongly on the temporal coherence of the optical field. Thus accurate determination of TPE action cross sections requires knowledge of $g^{(2)}$ in the focal region, which is a nontrivial task.[4] In these experiments the complications of the $g^{(2)}$ measurement inside the sample[5] were avoided by use of a standard calibration sample of known TPE action cross sections and spectra. Fluorescence signals from new molecular fluorophores were calibrated with respect to the fluorescence of the calibration sample, so the ratio of these fluorescence signals determines values of two-photon action cross sections for new fluorophores.

For a given excitation wavelength, $g^{(2)}$ is the same for both the calibration sample and the new fluorophore. The ratio of the experimentally measured fluorescence signals becomes

$$\frac{\langle F(t)\rangle_{\mathrm{cal}}}{\langle F(t)\rangle_{\mathrm{new}}} = \frac{\phi_{\mathrm{cal}}\eta_{2\mathrm{cal}}\sigma_{2\mathrm{cal}}C_{\mathrm{cal}}\langle P_{\mathrm{cal}}(t)\rangle^2 n_{\mathrm{cal}}}{\phi_{\mathrm{new}}\eta_{2\mathrm{new}}\sigma_{2\mathrm{new}}C_{\mathrm{new}}\langle P_{\mathrm{new}}(t)\rangle^2 n_{\mathrm{new}}}, \tag{4}$$

where $\langle P_{\mathrm{cal}}(t)\rangle$ and $\langle P_{\mathrm{new}}(t)\rangle$ are the incident powers for the calibration and the new fluorophores, respectively. The TPE action cross section of a new molecular fluorophore is then related to known experimental wavelength-dependent parameters, including the TPE action cross section of the calibration standard, as described by

$$\sigma_{2\mathrm{new}}(\lambda)\eta_{2\mathrm{new}} = \frac{\phi_{\mathrm{cal}}\eta_{2\mathrm{cal}}\sigma_{2\mathrm{cal}}(\lambda)C_{\mathrm{cal}}}{\phi_{\mathrm{new}}C_{\mathrm{new}}}\,\frac{\langle P_{\mathrm{cal}}(t)\rangle^2}{\langle P_{\mathrm{new}}(t)\rangle^2}\,\frac{\langle F(t)\rangle_{\mathrm{new}}}{\langle F(t)\rangle_{\mathrm{cal}}}\,\frac{n_{\mathrm{cal}}}{n_{\mathrm{new}}}. \tag{5}$$

3. Experimental Procedure

A. Apparatus

The experimental setup is similar to that used by Xu *et al.*[3,4] The excitation source consists of a mode-locked Ti:sapphire laser (the Tsunami, Spectra-Physics) pumped by an argon-ion laser (the BeamLok, Spectra-Physics). Two sets of intracavity mirrors are necessary to cover the spectral range from 690 to 960 nm. The laser beam is expanded to an approximately 1–1.5-cm diameter ($1/e^2$) and enters a light-protected rig that contains the optical components for the experiment. The light passes through a red excitation filter and overfills an achromatic microscope objective (Zeiss, Model Neofluoar 0.3 NA/10×) that focuses the excitation light into the sample and collects the fluorescence signal in an epi-illumination configuration. Pulse dispersion, chromatic aberration, and spherical aberration have been

Table 1. TPA Cross Sections of Fluorescein at Selected Wavelengths[a]

Wavelength (nm)	TPA Cross Section (GM)[b]
691	16
700	19
720	19
740	30
760	36
780	37
800	36
820	29
840	13
860	8
880	11
900	16
920	26
940	21
960	15

[a]Fluorescein in H_2O (pH $\cong$ 13). A TPE cross section is the product of the TPA cross section times 0.9.
[b]1 GM = 10^{-50} (cm^4 s)/photon.

found to have negligible effects on two-photon molecular excitation in the focal volume when these optics are used with 100-fs pulses.[6] The fluorescence signal is separated from the incident beam by a long-wave dichroic mirror (Chroma) with a reflectivity of >95% for $\lambda < 610$ nm. The fluorescence signal is filtered by the 2-cm path length of a 1-M $CuSO_4$ solution with an optical density of O.D. > 10 for $\lambda >$ 690 nm to prevent any residual excitation illumination from reaching the detector, which is a photomultiplier tube (Hamamatsu, Model R1924). The fluorescence emission is recorded and averaged for 10–20 s by a computer-controlled photon counter (Stanford Research Systems, Model SR400). Background photon levels of a few percent of signals are recorded and subtracted from the fluorescence signal. The power of the incident beam is monitored by means of a power meter, and we minimize any power fluctuations (<3%) by allowing the laser to warm up for several hours prior to all measurements. A computer-controlled spectrometer accurately selects the excitation wavelength and observes the pulse spectra with a FWHM of ~10 nm. Linearly polarized radiation was used throughout the experiments.

B. Calibration

We calibrated our TPE action cross-section measurements with respect to the absolute TPA cross sections of fluorescein as a function of wavelength.[4] The TPA cross-section data for fluorescein are listed in Table 1. Noting that the refractive indices are approximately equal and using fluorescein as our standard, we find that relation (5) becomes

$$\sigma_{2\mathrm{new}}(\lambda)\eta_{2\mathrm{new}} = \frac{\phi_{\mathrm{FL}}0.9\sigma_{2\mathrm{FL}}(\lambda)C_{\mathrm{FL}}\langle P_{\mathrm{FL}}(t)\rangle^2\langle F(t)\rangle_{\mathrm{new}}}{\phi_{\mathrm{new}}C_{\mathrm{new}}\langle P_{\mathrm{new}}(t)\rangle^2\langle F(t)\rangle_{\mathrm{FL}}}, \quad (6)$$

where 0.9 is the fluorescence quantum efficiency of fluorescein.[7] We assumed herein that the one-photon and the two-photon quantum efficiencies of fluorescein are equal, i.e., $\eta_1 = \eta_2$, and that the fluorescence quantum efficiency is constant over the spectral range of the experiment. The fluorescence detection efficiencies of our apparatus were calculated for each molecular fluorophore, taking into account the spectral responses of the filters, the dichroic beam splitter, the photomultiplier tube, and the fluorescence emission spectra of the fluorophores. The systematic uncertainty in the absolute cross-section values, which was estimated at ±30%, is mostly due to the uncertainty in the fluorescence collection and detection efficiencies. Relative values and shapes of excitation spectra are substantially more precise.

C. Sample Preparation

Throughout our experiments we used a 14.5-μM solution of fluorescein in H_2O (pH ~ 13) as our standard calibration sample. Four dyes—dextran–fluorescein (with a molecular weight of 10,000 for the Dextran), Rhodamine 6G, DiOC5 (3,3′-dipentyloxacarbocyanine iodite), and DiOC6 (3,3′-dihexyloxacarbocyanine iodite)—were purchased from Molecular Probes. The fifth dye, Cy 3, was purchased from Amersham Life Science. Stock solutions were first prepared and then diluted to 50–100 μM. The concentrations were calculated from the one-photon absorption (OPA) spectra by use of a spectrophotometer (Hewlett-Packard, Model HP8451A diode array spectrometer). The dye concentrations and the solvents used are listed in the captions of the figures, below. Deep-well slides (Fisher Scientific) and cover slips were used to assure that two-photon experiments were performed in the thick-sample limit.

4. Results and Discussion

The measured TPE action spectra are presented in Figs. 1 and 2. The TPA cross sections of fluorescein are presented in Table 1.[3] The dependence of the fluorescence signal on the incident power was investigated for all fluorophores at each excitation wavelength. Table 2 presents the measured exponent at several wavelengths for a typical dye, DiOC5. We note that the departure from perfect power-squared dependence was within ±4% for all fluorophores, indicating genuine two-photon events.

It is interesting to compare our TPE spectra with the two-photon spectra of other widely used fluorophores. The peaks in the TPE spectra of dextran–fluorescein and fluorescein are the same, as is shown by comparison with the plot of the TPE spectra of fluorescein also included in Fig. 1(a). Our results thus show that binding fluorescein onto dextrans does not affect the relative TPE spectra of the pair, although the magnitude of the action cross section is reduced, most likely as a result of self-quenching. Another interesting comparison is that between the cross sections of Cy 3 and those of the similar but symmetric molecule $DiIC_{18}$(3). We found the TPE spectrum of Cy 3 [Fig. 1(b)] to be very similar to that of $DiIC_{18}$(3). The action cross section of Rhodamine 6G [Fig. 1(c)] resembles that of Rh B, but its short-wavelength peak is even more accentuated. In Fig.

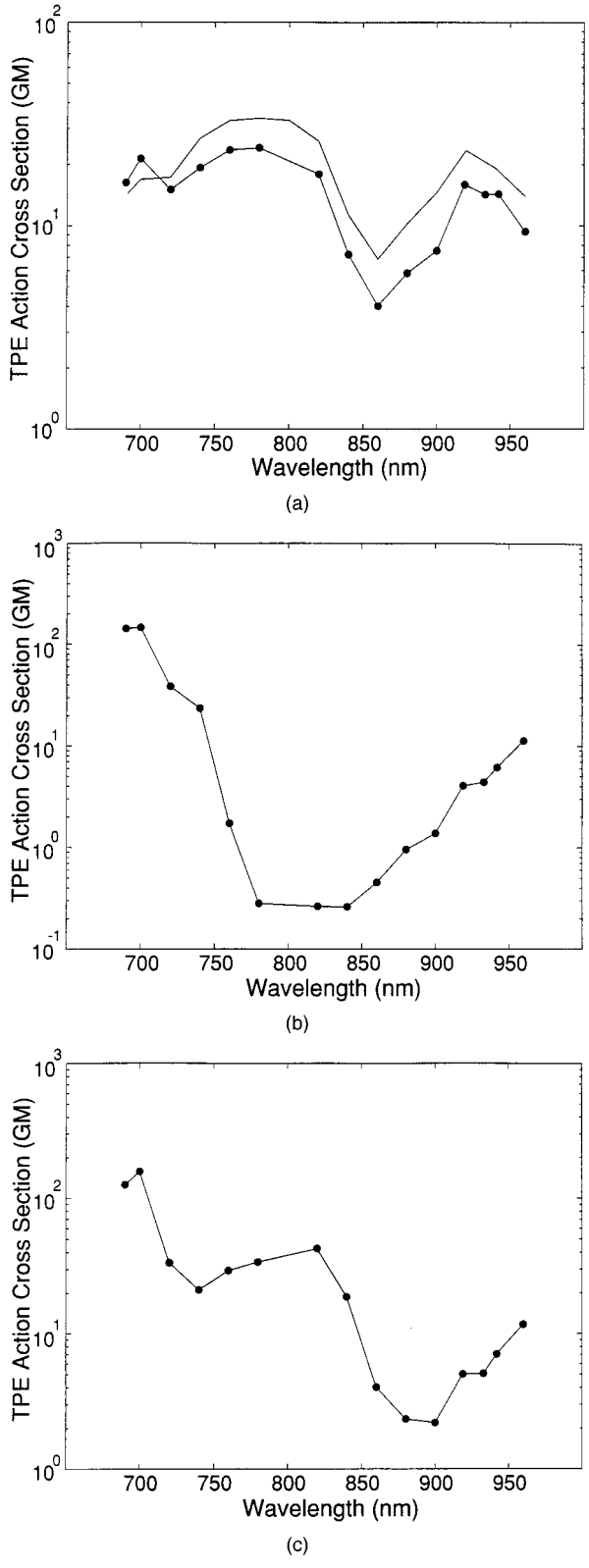

Fig. 1. Plots of the TPE action cross sections (filled circles) for (a) dextran–fluorescein (dextran MW of 10,000) at a concentration of 123 μM and dissolved in H_2O at pH ~ 11 (compare the curve with the TPE cross sections for fluorescein listed in Table 1), (b) Cy 3 on a logarithmic scale at a concentration of 65 μM dissolved in Na_2CO_3 buffer at pH ~ 9.3, and (c) Rhodamine 6G on a logarithmic scale at a concentration of 110 μM dissolved in MeOH. The TPE cross section is defined as $\eta_2\sigma_2$ (see text). 1 GM = 10^{-50} (cm^4 s)/photon.

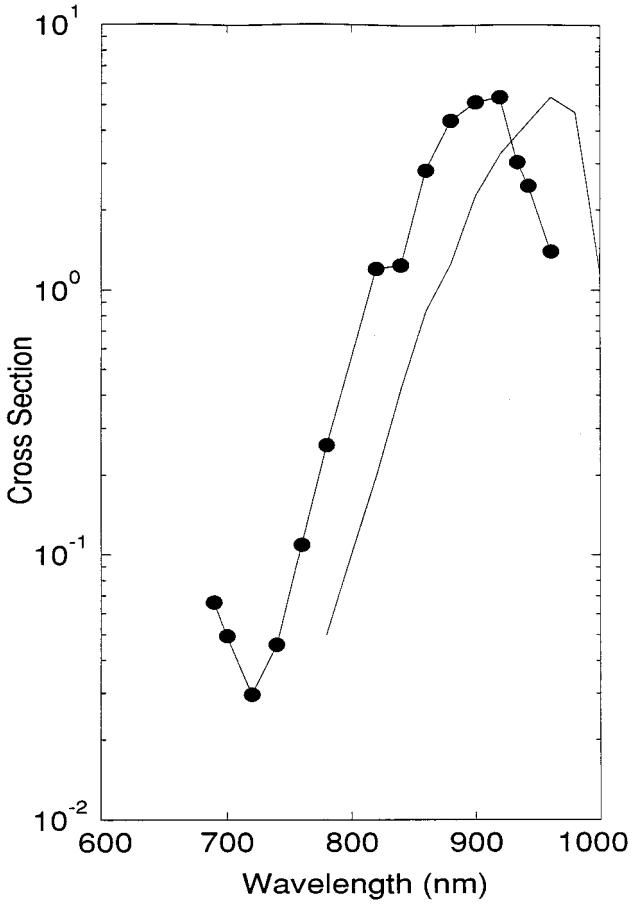

Fig. 2. TPE action spectra (filled circles) compared with the OPA spectra (dashed curve) of DiOC5 at a concentration of 81 μM dissolved in MeOH. Vertical-axis units: arbitrary for the OPA spectra and GM for the TPE spectra. Horizontal-axis units: wavelengths as shown for the TPE spectra; the OPA spectra wavelengths have been doubled as plotted. The TPE spectrum of DiOC6 is identical to the DiOC5 TPE spectrum within the limits of the experimental error (see text).

2 we show only the spectra of DiOC5 because its TPE cross sections are identical, well within experimental error, to those of DiOC6.

An interesting feature of the results is that the TPE peaks are blue shifted with respect to the OPA peak at twice the wavelength. The widely used car-

Table 2. Slope in the Logarithmic Plot of Fluorescence Versus the Incident Intensity at Several Excitation Wavelengths for DiOC5[a]

	Wavelength λ (nm)							
	690	720	740	820	860	880	900	960
Slope	1.98	1.96	1.98	1.98	1.96	2.04	1.98	1.96

[a]The deviation of the fluorescence signal from the power square dependence is within ±4% for all fluorophores investigated. The slope is given by

$$\log\left[\frac{F_1(I_1)}{F_2(I_2)}\right] = m \log\frac{I_1}{I_2},$$

where F_1 and F_2 are the fluorescence signals at intensities I_1 and I_2, respectively.

bocyanine dyes, DiOC5 and DiOC6, display this behavior. Figure 2 shows the blue shift for DiOC5, and a similar blue shift was found for DiOC6. The explanation for these blue shifts for two-photon-induced transitions is that the parity selection rules favor excitation to higher energy levels than do the respective one-photon-induced transitions. The strong peaks of the apparently blue-shifted TPE spectra tend to appear at wavelengths corresponding to twice the wavelengths of weak peaks in the one-photon excitation spectra. The 700-nm peak of Rhodamine 6G is even more enhanced relative to the longer-wavelength peaks around 800 and 1000 nm than had been found in Rh B. Vibrational coupling between the S_1 and the S_2 excited states, however, favors internal conversion to S_1 before fluorescence emission, so the emission spectra excited by OPA and TPA coincide. The blue shifts are a desirable practical consequence of parity selection rules. This permits a higher spatial resolution for TPLSM than would be predicted by the assumption that TPE occurs at twice the wavelength of OPA. These blue shifts allow the fluorophores to be excited efficiently with widely available and benign near-infrared wavelengths that are short enough to avoid absorption by water and long enough to reduce the risk of photodamage in biological samples.

We have determined the TPE action cross sections of five frequently used molecular fluorophores over the excitation range from 690 to 960 nm by using mode-locked Ti:sapphire excitation. Large TPE action cross sections were found for Rhodamine 6G (~150 GM) and Cy 3 (~140 GM), both at 700 nm. We confirmed the quadratic dependence of the two-photon excited fluorescence on the incident power at all wavelengths within $\pm 4\%$. On the basis of molecular structure, theoretical prediction of fluorophores with high multiphoton cross sections remains a challenge.

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