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Three-Photon-Induced Fluorescence of Diphenylhexatriene in Solvents and Lipid Bilayers[3]

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We observed the emission of 1,6-diphenyl-1,3,5-hexatriene (DPH) when excited with the fundamental output of a fs Ti:sapphire laser at 860 nm. The emission spectra of DPH were identical to that observed for one-photon excitation at 287 nm. The dependence of the DPH emission intensity on laser power was cubic, indicating three-photon excitation of DPH at 860 nm. At a shorter wavelength of 810 nm, the dependence on laser power was quadratic, indicating a two-photon process. At an intermediate wavelength of 830 nm the mode of excitation was a mixture of two- and three-photon excitation. At 830 nm the anisotropy is no longer a molecular parameter, and the mode of excitation and anisotropy of DPH depends on laser power. Frequency-domain anisotropy decays of DPH in triacetin revealed the same rotational correlation times for two- and three-photon excitation. However, the time 0 anisotropy of DPH was larger for three-photon excitation than for two-photon excitation. Steady-state anisotropy data for DPH-labeled membranes revealed the same transition temperature for one- and three-photon excitation. These anisotropy data indicate that membrane heating was not significant with three-photon excitation and that three-photon excitation may thus be of practical usefulness in fluorescence spectroscopy and microscopy of membranes.

KEY WORDS: Fluorescence intensity decays; anisotropy decays; multiphoton excitation; three-photon excitation; two-photon excitation; diphenylhexatriene; membranes.

INTRODUCTION

Pulsed laser sources are routinely used as light sources in time-resolved fluorescence to study rotational motions of macromolecules. In most such applications the intense output of the dye or solid state lasers is frequency doubled or tripled to provide wavelengths suitable for excitation of the intrinsic or extrinsic fluorophores. In recent years there has been increased interest in the use of the more intense fundamental wavelength pulses for two-photon excitation. Two-photon induced fluorescence has been observed for a variety of biochemical fluorophores including the membrane probe DPH[3],[1,2] DNA-bound probes[3,4] and the tyrosine and tryptophan residues of proteins.[5,6] One reason for the interest in two-photon excitation is the prediction of different information in the resulting anisotropy decays[7–9] or information about the electronic properties of indoles.[10] A second reason for the interest in two-photon excitation is its use for intrinsic confocal excitation in fluorescence microscopy[11,12] and its use in localized photolysis of caged compounds.[13] These possibilities of

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[3] *Abbreviations used*: DPH, 1,6-diphenyl-1,3,5-hexatriene; chol, cholesterol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3[phospho-rac(1-glycerol); DMPG, 1,2-dimyristoyl-*sn*-glycero-3[phospho-rac(1-glycerol); FD, frequency domain.

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confocal excitation and localized photolysis are the result of the dependence of two-photon excitation on the square of the laser power, with the resulting excitation occurring only at the focal point of the laser beam. Two-photon excitation has also been observed with the CW infrared lasers used for optical manipulation of cells.[14,15] Hence, multiphoton excitation is of wide interest in both biophysics and cell biology.

In recent publications we showed that the use of long-wavelength femtosecond pulses from a Ti:sapphire laser can result in three-photon excitation. Three-photon excitation has been observed for 2,5-diphenyhexazole,[16] for the calcium probe Indo-1,[17,18] and for a tryptophan derivative.[19] We now show that the membrane probe DPH can be excited by a three-photon process upon illumination at 860 nm. This wavelength is readily available from a Ti:sapphire laser, suggesting the use of three-photon excitation in membrane biophysics or fluorescence microscopy.

THEORY

Fluorescence Anisotropy with Multiphoton Excitation

In studies of multiphoton excitation it is informative to examine the fluorescence anisotropy. A complete description of the anisotropy with two-photon excitation is complex[7–10] and requires consideration of the tensor properties of the transitions. However, we have found for some linear molecules, including DPH, that the two-photon transition appears to be colinear and have the same orientation as the one-photon transition. In this case the expected anisotropy can be easily predicted based on the usual orientation dependence of electronic transitions. For vertically polarized (z-axis) excitation with one or more photons the anisotropy is given by[20,21]

$$r_0(\theta,\beta) = \left(\frac{3}{2}\langle\cos^2\theta\rangle - \frac{1}{2}\right)\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \quad (1)$$

where θ is the angle from the z axis, and β is the angle between the absorption and the emission transition moments. The subscript zero indicates the absence of rotational diffusion during the excited—state lifetime or the time 0 anisotropy. The average value of $\cos^2\theta$ depends upon the type of photoselection, and the value of $\langle\cos^2\theta\rangle$ is given by

$$\langle\cos^2\theta\rangle = \frac{\int_0^{\pi/2}\cos^2\theta\, f_i(\theta)\, d\theta}{\int_0^{\pi/2} f_i(\theta)\, d\theta} \quad (2)$$

where $f_i(\theta)$ is the directional distribution of the excited state.[21] For one-photon excitation this distribution is given by

$$f_1(\theta) = \cos^2\theta \sin\theta \quad (3)$$

For one-photon excitation Eq. (1) becomes

$$r_{01}(\beta) = \frac{2}{5}\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \quad (4)$$

The factor of 2/5 originates with $\cos^2\theta$ photoselection [Eq. (3)]. For collinear transitions ($\beta = 0$) the fundamental anisotropy (r_{01}) without rotational diffusion is 0.40. For nonzero values of β the one-photon anisotropy ranges from 0.40 to -0.20.

The anisotropy expected for two (r_{02})- or three (r_{03})-photon excitation can be calculated using

$$f_2(\theta) = \cos^4\theta \sin\theta \quad (5)$$

$$f_3(\theta) = \cos^6\theta \sin\theta \quad (6)$$

where the subscript (2 or 3) refers to two- or three-photon excitation. Substitution into Eq. (1) yields

$$r_{02}(\beta) = \frac{4}{7}\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \quad (7)$$

$$r_{03}(\beta) = \frac{2}{3}\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \quad (8)$$

For two- and three-photon excitation the maximal anisotropies for $\beta = 0$ are 0.571 and 0.667, respectively. Hence, for collinear transitions three-photon excitation is expected to result in a more highly oriented excited-state population. Observation of a larger anisotropy for three- versus two-photon excitation provides strong evidence for three-photon excitation. The anisotropy values for nonzero values of β are summarized elsewhere.[16]

In the presence of rotational diffusion the steady-state anisotropy (r_i) is lower than the time 0 anisotropy (r_{0i}), where i indicates the mode of excitation. The steady-state anisotropy is related to the value of r_{0i} and to the rotational correlation time (θ_r) by

$$\frac{r_{0i}}{r_i} = 1 + \frac{\tau}{\theta_r} \quad (9)$$

where τ is the mean decay time.

EXPERIMENTAL METHODS

DPH was obtained from Molecular Probes and used without further purification. The concentration of DPH

in triacetin was 1.5 m*M*. Lipid vesicles were prepared by the usual procedure of sonication with a probe-type sonicator near 40°C in 10 m*M* Tris buffer (pH 8.0), followed by centrifugation to remove large particles. The concentration of DPPG was 2.5 m*M*. The concentration of DMPG, with or without cholesterol (4:1 molar ratio), was 5 m*M*. DPH was added after membrane formation as a concentrated solution in tetrahydrofuran. The DPH-to-phospholipid molar ratio was near 1:50. Steady-state anisotropies with one-photon excitation at 350 nm were obtained using a SLM 8000 spectrofluorometer. Intensity and intensity-decay measurements were performed using magic-angle conditions. For emission spectra with one-photon excitation at 287 nm, we used a SLM 8000 spectrofluorometer with a 10-nm bandpass. For emission spectra with three-photon excitation at 860 nm, the signal was brought to the SLM Spectrofluorometer via a 600-μm single-mode optical fiber, 10 m long. Solutions were in equilibrium with air.

Frequency-domain intensity and anisotropy decays with three-photon excitation were obtained on instrumentation described previously.[22,23] Three-photon excitation was provided by a femtosecond mode-locked Tsunami Ti–sapphire laser from Spectra Physics. The repetition rate of 80 MHz was held fixed by the Lock-to-Clock accessory. The repetition rate was divided by 8 by the Lock-to-Clock electronics and used as the 10-MHz reference signal for the FD instrument. The pulse width was near 80 fs.

For three-photon excitation the fundamental output of the Ti:sapphire (810–860 nm) was brought directly to the sample compartment and focused with a laser-quality lens (2-cm focal length). The emission was isolated with a Corning 4-96 filter which transmits from 380 to 550 nm. The signals from the solvents or lipids alone were less than 0.5% of that observed in the presence of DPH. We expect that the probe concentration could be decreased with improved focusing and/or higher laser power. The 1-to-50 ratio was chosen to keep the background less than 0.5%. For measurements of the dependence of the emission on laser intensity the peak power was attenuated with neutral density filters. To avoid any effects of widening the laser pulses by the neutral density filters, a single filter of the same design and thickness, but varying optical density, was used for the intensity measurements at various peak powers.

The intensity decays of DPH in triacetin were single exponentials. For anisotropy decays of DPH in triacetin, we used the single-correlation time anisotropy decay model

$$r(t) = r_{0i}\, e^{-t/\theta_r} \qquad (10)$$

where θ_r is the rotational correlation time, r_{0i} is the time 0 anisotropy, and the subscript i indicates the number of simultaneously absorbed photons. The values of r_0 and θ_r were recovered from least-squares analysis of the differential polarized phase angles and modulated anisotropies.[24]

RESULTS

In previous experiments we found that several fluorophores yielded the usual ultraviolet or visible emission upon illumination with the fundamental output of a fs Ti:sapphire laser operating from 760 to 900 nm.[16–18] For these probes the wavelengths were too long for two-photon excitation, and the emission was shown to result from three-photon excitation. To date, three-photon induced fluorescence has not been observed for a membrane-bound fluorophore. Hence we decided to examine the most widely used membrane probe DPH for possible excitation by simultaneous absorption of three long wavelength photons. We first examined a solution of DPH in triacetin. In this solvent DPH displays a high quantum yield and a single-exponential intensity decay.[1]

Upon illumination of the DPH solution at 860 nm one can visually observe a blue emission at the focal point of the excitation. This emission is highly localized in the center of the cuvette, with no apparent emission from the regions before and after the focal point. This localization of the emission suggest a multiphoton process since the emission does not occur from regions of the laser beam where the light is not focused and the local intensity is lower.

We examined the emission spectrum resulting from focused illumination at 860 nm. This emission spectrum (Fig. 1) was essentially identical to that observed for DPH with one-photon excitation at 287 nm. This excitation wavelength was chosen to be one-third of 860 nm. DPH displays the same emission spectrum when excited at 287 nm and at the more usual wavelength near 360 nm. The signal was obviously due to DPH because the signal observed from triacetin alone was less than 0.5% of that observed for the DPH solution.

DPH does not display one-photon absorption above 420 nm. Hence it seemed unlikely that DPH would display two-photon excitation at wavelengths above 840 nm. To determine the mode of excitation we examined the dependence of the DPH emission intensity on the laser power. At 860 nm the emission intensity was dependent on the cube of the laser power (Fig. 2), which indicated that DPH displayed three-photon excitation. At

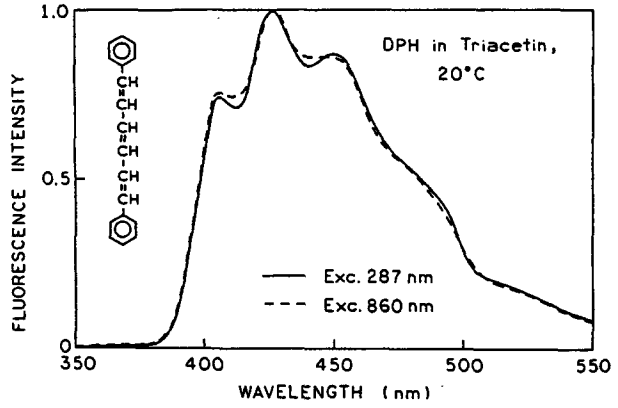

Fig. 1. Emission spectra of DPH with one-photon (287-nm) and three-photon (860-nm) excitation.

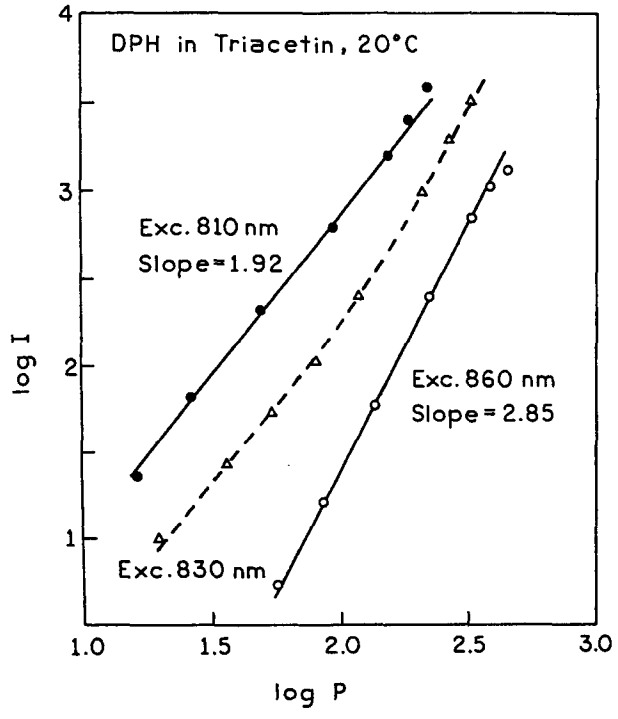

Fig. 2. Dependence of the emission intensity of DPH on laser power for excitation at 810, 830, and 860 nm.

a somewhat shorter wavelength of 810 nm the DPH intensity depended on the square of the laser power, indicative of two-photon excitation. Based on our experience with several fluorophores we found two-photon excitation whenever the first excited state could be reached with two photons, and three-photon excitation only at longer wavelengths. Three-photon excitation has been observed only at wavelengths longer than twice the single-photon absorption. At an intermediate wavelength of 830 nm the mode of excitation was between two- and three-photon excitation. This suggests that under our experimental conditions, some of the DPH molecules display simultaneous absorption of two photons, and other DPH molecules display three-photon excitation.

Our observations of two-photon excitation of DPH at 810 nm and three-photon excitation at 860 nm seems to be in agreement with the pioneering studies of Fang *et al.*[25] These workers found that DPH displayed two-photon absorption, with a maximum near 400 nm and a two-photon absorption tail to 420 nm.

At this time we are not able to estimate the cross sections for three-photon excitation of DPH. However, it appears that DPH displays a low three-photon cross section. The fluorescence signal for DPH with 860-nm excitation is about 100-fold smaller than that shown by indo-1 upon three-photon excitation at 850 and 885 nm.[17] Additional experimentation is required to understand further which fluorophores display high or low three-photon cross sections.

In previous studies of three-photon excitation we noticed that it was necessary to stir the sample to obtain a stable signal.[16–18] For DPH in triacetin the signal was surprisingly stable without stirring. Upon illumination of 860 nm the signal from DPH showed an initial decrease in intensity of less than 2%, after which the signal remained constant with continued illumination. Upon stirring, the signal increased to the initial level. These results suggest that DPH is reasonably stable under the conditions needed for three-photon excitation and that triacetin has negligible single- or multiphoton absorption at 860 nm.

It is important to note that the intensity of DPH with three-photon excitation is about 100-fold less than that found for two-photon excitation (Fig. 2). These comparable intensities are surprising and suggest that three-photon excitation can be useful in fluorescence spectroscopy and microscopy. However, we do not know whether the wavelengths of 810 and 860 nm are optimal or poor wavelengths for multiphoton excitation of DPH. We were not able to compare the DPH intensity with two-photon excitation at shorter wavelengths because of the limited tuning range of our Ti:sapphire laser. Shorter wavelengths were available from a picosecond dye laser, but the wider pulse width of the picosecond dye laser precludes comparison with the fs Ti:sapphire laser. Further studies are needed to better understand the relative signal levels that can be expected for two- and three-photon excitation of biochemical fluorophores.

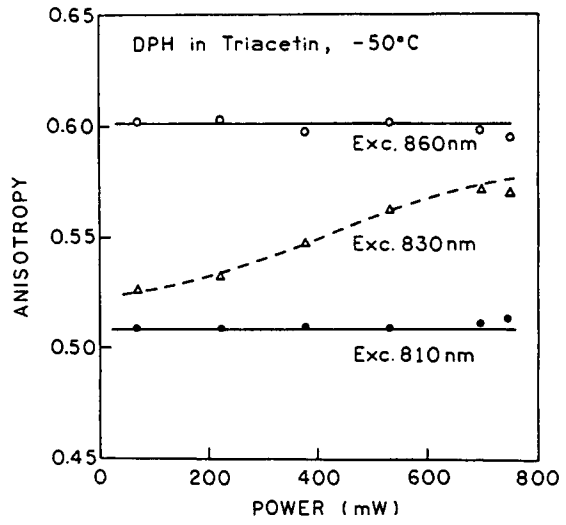

Fig. 3. Dependence of the anisotropy of DPH in triacetin, −50°C, on laser power at 810, 830, and 860 nm.

We next examined the anisotropy of DPH in frozen solution where rotational diffusion is not significant during the excited-state lifetime (Fig. 3). For three-photon excitation at 860 nm the anisotropy is 0.609, independent of laser power. This value is higher than the anisotropy observed for two-photon excitation at 810 nm (Fig. 3) or at 716 nm (Table I). The higher anisotropy for 860-nm excitation supports our claim of three-photon excitation of DPH. One could claim that the third harmonic is being generated in the solution by the intense illumination, which in turn excites the DPH. However, excitation by the third harmonic could not yield a anisotropy higher than the one-photon value of 0.37. Also, the observed three-photon value of 0.609 is higher than that possible for two-photon excitation with colinear transitions, which is 0.57.

For colinear transitions, or equivalently a single dominant term in the transition tensor, the anisotropies for one-, two-, and three-photon excitation are expected to show relative values of 1.0, 1.429, and 1.667, respectively (Table II). The relative values observed for DPH are 1.0, 1.39, and 1.62, which are close to the predicted values. Hence the anisotropy data demonstrate three-photon excitation of DPH. The stability of the DPH signal in this vitrified solvent, where diffusive replacement of the DPH in the focal point does not occur, shows the stability of DPH with three-photon excitation.

In contrast to the power-independent anisotropies of DPH with 810- or 860-nm excitation, the anisotropy of DPH depends on laser power with 830-nm illumination (Fig. 3). This dependence on power is due to the mode of excitation changing from mostly a two-photon process at low laser power to a three-photon process at higher laser power. This result indicates that the anisotropy of DPH is not a fundamental parameter at 830 nm but, instead, depends on the precise excitation conditions. The dependence on power can complicate the interpretation of anisotropies or anisotropy decays when the mode of excitation is not clearly a two- or three-photon process. Alternatively, the dependence on laser power could be a valuable tool for quantifying the local intensity of the focused laser excitation, particularly in fluorescence microscopy where numerous optical elements can attenuate and/or broaden the laser pulses.

We questioned whether the focused excitation at 860 nm resulted in local heating of the sample. The intensity decay of DPH in triacetin was a single exponential with a decay time of 7.2 ns for one-, two-, and three-photon excitation (Table I). Local heating and/or photochemical effects are likely to result in a more complex intensity decay than in the absence of such effects. Frequency-domain anisotropy decays of DPH are shown in Fig. 4 for two-photon (810-nm) and three-photon (860-nm) excitation. The recovered time 0 anisotropies are in agreement with those measured in frozen solution. The same rotational correlation times were observed for both wavelengths (Table I) and agree with those reported previously for one-photon excitation under the same conditions.[1] Local heating of the sample would be expected to decrease the correlation time, so that these results suggest the absence of heating with three-photon excitation.

The differential polarized phase angles shown in Fig. 4 provide additional evidence for three-photon excitation. As shown elsewhere[2] the maximum possible differential phase angle (Δ) for any light modulation frequency is given by

$$\tan \Delta_i = \frac{3r_{0i}}{2[(1 + 2r_{0i})(1 - r_{0i})]^{1/2}} \qquad (11)$$

where i indicates the number of simultaneously absorbed photons. These values are summarized in Table II for completely colinear transitions ($r_{01} = 0.4$) and for the r_{0i} values observed for DPH (Fig. 3). We note that the r_{0i} values for DPH in frozen solution (Table II) may not precisely match those recovered from the frequency-domain anisotropy decays (Table I) due to the limits of experimental accuracy. The maximum value for two-photon excitation with $r_{02} = 0.571$ is 41.8°. For the measured r_{02} values of 0.528 the maximum two-photon value

Table I. Intensity and Anisotropy Decays for DPH in Triacetin at 40°C

λ_{exc} (nm)	Mode of excitation[a]	τ (ns)	θ_r (ns)	r_{0i}[b]	r_{0i}[c]
358[d]	1	7.27	0.67 (0.64 – 0.69)[e]	0.371 (0.368 – 0.374)	0.376
810	2	7.22	0.58 (0.56 – 0.61)	0.532 (0.530 – 0.534)	0.523
716[d]	2	7.21	0.61 (0.59 – 0.63)	0.523 (0.518 – 0.526)	0.518
860	3	7.20	0.56 (0.53 – 0.60)	0.628 (0.621 – 0.634)	0.609

[a]Number of simultaneously absorbed photons.
[b]Time 0, anisotropy from analysis of the frequency-domain data.
[c]The fundamental anisotropy measured at −50°C in triacetin.
[d]From Ref. 1.
[e]Confidence intervals obtained from the least-squares analysis [26].

Table II. Comparison of Measured and Calculated Anisotropies for One-, Two-, and Three-Photon Excitation

Excitation (nm)	Model of excitation[a]	Measured[b]			Calculated[c]		
		r_{0i}	r_{0i}/itr_{01}	Δ_i	r_{0i}	r_{0i}/r_{01}	Δ_i
360	1	0.376[d]	1.00	28.3°	0.4	1.00	30°
716	2	0.518[d]	1.38	38.1°	0.57	1.43	41.8°
810	2	0.523	1.39	38.5°	0.57	1.43	41.8°
860	3	0.609	1.62	44.7°	0.67	1.67	48.6°

[a]Number of simultaneous absorbed photon.
[b]Experimental values in frozen solution, triacetin at −50°C. We estimate these values to be accurate to ± 0.005.
[c]Calculated values for colinear transitions [Eqs. (4), (7), and (8)].
[d]From Refs. 1 and 2.

is 38.8°. The highest observed value of DPH is 42.5°, indicating that excitation at 860 nm is due to more than two photons.

We next examined the steady-state anisotropies of DPH-labeled membranes, to determine whether three-photon excitation could still be observed. We reasoned that adverse effects of the focused illumination on the bilayers would be revealed by a change in the temperature-dependent anisotropies and/or transitions temperatures. Anisotropies of DPH are shown in Fig. 5 for DPPG bilayers and in Fig. 6 for bilayers of DMPG or DMPG/chol. Also shown are the anisotropies observed with one-photon excitation. As expected, the anisotropies are higher for three-photon as compared with one-photon excitation. However, the temperature-dependent profiles and transition temperatures are the same for one- and three-photon excitation. This result indicates that the

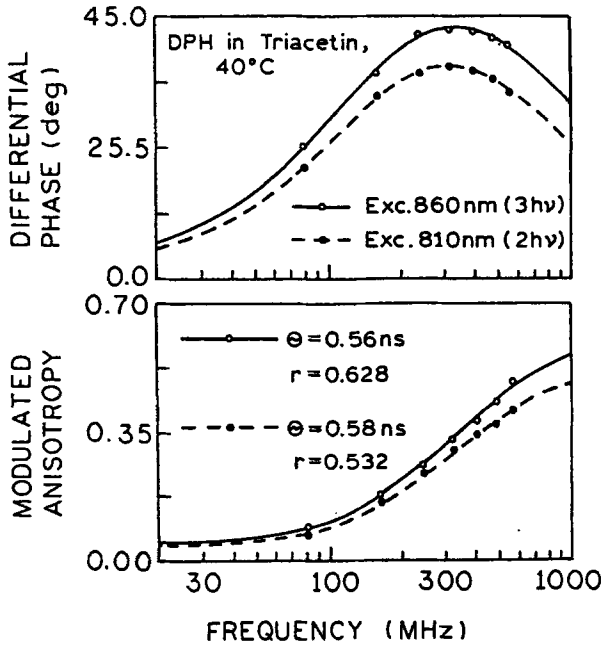

Fig. 4. Frequency-domain anisotropy decay of DPH in triacetin at 40°C for two-photon (810-nm) and three-photon (860-nm) excitation.

bilayers are not significantly heated with three-photon excitation. Hence it appears that three-photon excitation could be observed in DPH-labeled cells in a fluorescence microscope without significant damage. However, in order to keep the signal stable, we had to stir the sample with a magnetic stirrer. At this time we do not know if photostability will be a problem in microscopy, where the excited volume is considerably smaller than in our experiments.

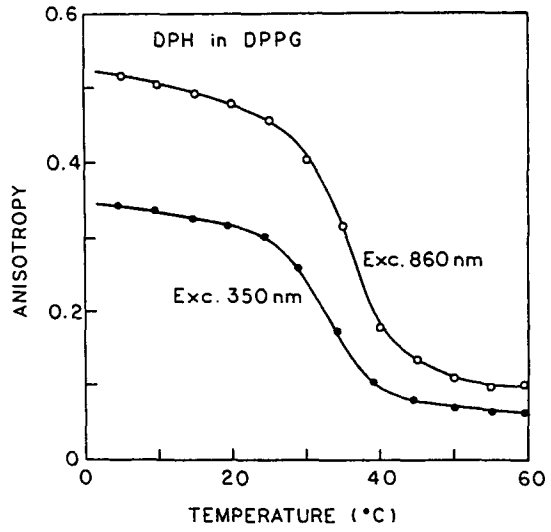

Fig. 5. Temperature-dependent anisotropies of DPH in DPPG for three-photon excitation at 860 nm and one-photon excitation at 350 nm.

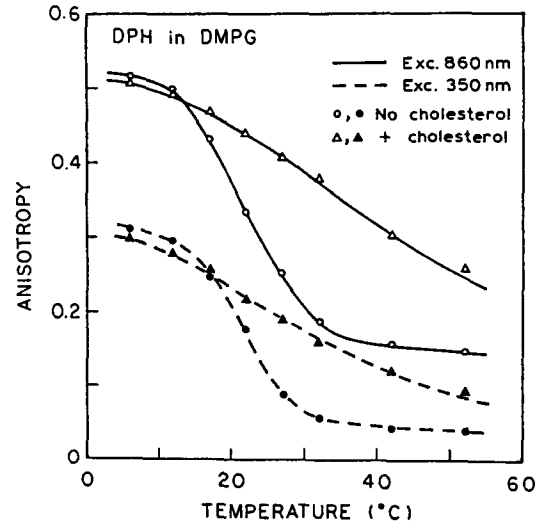

Fig. 6. Temperature-dependent anisotropies of DPH in DMPG and DMPG/chol bilayers for three-photon excitation at 860 nm and one-photon excitation at 350 nm.

DISCUSSION

What are the potential advantages of three-photon excitation? One possible advantage is higher spatial resolution in microscopy resulting from the cubic dependence on laser power.[17] Other potential advantages include selective excitation of fluorophores which display high three-photon cross sections or additional information in the anisotropy decays. While some theory has appeared for anisotropy decays with two-photon excitation,[7–9] to the best of our knowledge the theory of anisotropy decays with three-photon excitation has not been explored.

Three-photon excitation may have a technical advantage because it can be accomplished with the fundamental output of a Ti:sapphire laser near the peak of the tuning curves. These mode-locked lasers readily give femtosecond pulses. There are presently efforts to push the tuning curves of the Ti:sapphire laser to shorter wavelengths below 700 nm to reach suitable two-photon cross sections. Comparable signal levels may be achieved by relying on three-photon excitation with longer wavelengths.

And finally, we note that anisotropy measurements may be useful for determining the mode of excitation in multiphoton microscopy. Two-photon excitation has been reported with CW lasers used for optical manipulation of cells,[14,15] and informal reports have appeared on three-photon excitation of DNA-bound probes under similar conditions. At present it is unclear whether these conditions yielded true multiphoton excitation, or whether the observed signals are due to second or third harmonic generation by the optical systems. Three-multiphoton excitation of DPH, Indo-1[18] and other fluorophores[16] results in higher anisotropies, which can be used to determine better the mode of excitation.

In summary, we suggest that three-photon excitation be considered in fluorescence spectroscopy and confocal microscopy with multiphoton excitation.

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