THREE-PHOTON EXCITATION OF 2,5-BIS (4-BIPHENYL) OXAZOLE: STEADY STATE AND TIME RESOLVED INTENSITIES AND ANISOTROPIES

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ABSTRACT

Three-photon excitation of 2,5-bis(4-biphenyl) oxazole (BBO) was observed when it was excited with the fundamental output of a femtosecond Ti:sapphire laser above 820 nm. The emission spectrum of BBO was identical for one-, two-, and three-photon excitation at 320, 640, and 960 nm, respectively. In toluene and triacetin, the emission intensity of BBO depended on the square of the laser power for wavelengths below 820 nm and displayed a sharp transition to a cubic dependence at longer wavelengths. The spatial distribution of the emission of BBO with three-photon excitation was more strongly localized than for two-photon excitation of a coumarin fluorophore at the same wavelength. The same single exponential intensity decay was observed for one-, two-, and three-photon excitation. However, the frequency domain anisotropy decay with three-photon excitation at 960 nm revealed a larger time-zero anisotropy, larger differential polarized phase angle, and larger modulated anisotropy than is possible for two-photon excitation with colinear oscillators. In triacetin, the anisotropy is not constant for three-photon excitation at different wavelengths. Surprisingly, the fluorescence intensities for three-photon excitation were only about 100-fold less than for two-photon excitation. The increasing availability of Ti:sapphire lasers suggests that multiphoton excitation can become a common tool in fluorescence spectroscopy. © *1996 Society of Photo-Optical Instrumentation Engineers.*

Keywords fluorescence; three-photon excitation; two-photon excitation; time resolved; frequency domain; Ti:sapphire laser.

1 INTRODUCTION

Since the invention of the laser, two-photon spectroscopy has been used to study the excited state properties of organic and biochemical fluorophores.[1–3] By two-photon excitation we mean the simultaneous absorption of two red long-wavelength photons to result in excitation to the first singlet state (Scheme 1). In recent years the use of two-photon excitation ($2h\nu$) has been extended to studies of the time resolved fluorescence of biological macromolecules,[4–6] to fluorescence microscopy,[7–9] and to pattern photobleaching with evanescent illumination.[10] These applications are being made possible by the availability of mode-locked Ti:sapphire lasers, which provide femtosecond pulses over a range of wavelengths from 700 to 1000 nm.

We describe the observations of three-photon excitation of an organic fluorophore using the fundamental output of a femtosecond (fs) Ti:sapphi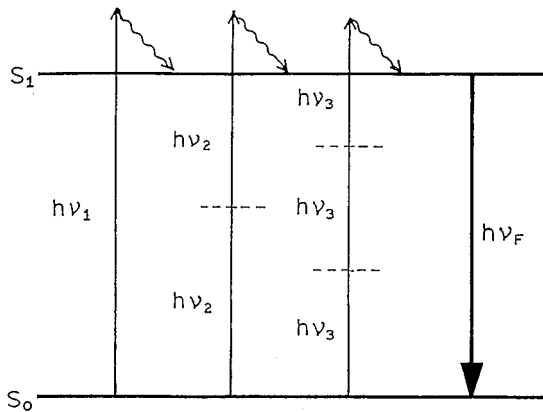re laser. In this case the fluorophore simultaneously absorbs three still longer NIR photons to reach the first excited state (Scheme 1). Three-photon excitation ($3h\nu$) has been observed previously for neat solutions of benzene[11–13] or for crystals of naphthalene.[14–15] It has recently been reported for an organic polymer[16] or a fluorophore solution[17] using the fundamental output of a Q-switched Nd:YAG laser at 1064 nm. We now show that three-photon-induced fluorescence can be observed in relatively dilute solutions of an organic fluorophore using femtosecond pulses from a mode-locked Ti:sapphire laser. This observation suggests that three-photon excitation can be used in biophysics and cellular imaging.

2 THEORY

In previous reports we demonstrated that emission anisotropy can be informative regarding the mode of excitation (i.e., one- or two-photon excitation). For colinear absorption and emission transition moments, the anisotropy is higher for two- than for

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Scheme 1 Jablonski diagram for one-, two-, and three-photon excitation.

Table 1 Fundamental anisotropies and differential phase angles for colinear transitions with one-, two-, and three-photon excitation.

Observable	One-photon	Two-photon	Three-photon
r_{0i} or $r_{\omega i}$	0.40	0.57	0.67[a]
r_{0i}/r_{01}	1.00	1.43	1.67
Δ_ω (degrees)	30	41.8	48.6

[a] An incorrect value was reported in Ref. 18. The correct value of r_{0i} is given above and in Ref. 27. The angle β is assumed equal to zero.

one-photon excitation.[18] For vertically polarized excitation, the fundamental or time-zero anisotropy is given by

$$r_{0i}(\beta)=(\tfrac{3}{2}\langle\cos^2\theta\rangle_i-\tfrac{1}{2})(\tfrac{3}{2}\cos^2\beta-\tfrac{1}{2}), \qquad (1)$$

where θ is the angle from the z axis, β is the angle between the absorption and emission transition moments, the subscript zero indicates the absence of rotational diffusion during the excited state lifetime or the time-zero anisotropy, and the subscript i indicates the number of absorbed photons. The average value of $\cos^2\theta$ depends upon the type of photoselection. The potentially larger anisotropy for two-photon excitation originates with $\cos^4\theta$ photoselection, compared with $\cos^2\theta$ for one-photon excitation.

For any number (i) of simultaneous absorption events, the value of $\langle\cos^2\theta\rangle_i$ is given by

$$\langle\cos^2\theta\rangle_i=\frac{2i+1}{2i+3}. \qquad (2)$$

For colinear transitions where $\beta=0$, the fundamental anisotropy is given by

$$r_{0i}=\frac{2i}{2i+3}. \qquad (3)$$

For two- and three-photon excitation, the maximal anisotropies for $\beta=0$ are 0.57 and 0.67, respectively (Table 1). Hence, for colinear 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. However, we note that a larger number of absorbed photons does not necessarily imply a higher anisotropy. Low anisotropies have been observed for two-photon excitation of indole, tryptophan,[19] and tyrosine,[20] and have been explained in terms of the tensor properties of the transitions.[21]

The above description of the time-zero anisotropy resulting from two- and three-photon excitation [Eqs. (1)–(3)] is rather simplified and ignores the tensor nature of the transitions.[21–24] For instance, under selected circumstances the anisotropy with two-photon excitation can be as high as 0.61,[21–22] compared with the value of 0.57 for colinear transitions (Table 1). However, the simple description given above is adequate for these initial studies of two- and three-photon excitation of BBO.

The effects of a higher time-zero anisotropy can also be seen in the frequency domain anisotropy decays. In these cases one measures the phase angle difference (Δ_ω) between the polarized components of the emission when the sample is excited with polarized light modulated at a circular frequency ω. One also measures the ratio of the polarized components of the emission (Λ_ω), where Λ_ω is the ratio of the amplitudes of the modulated emission parallel and perpendicular to the excitation polarization. This ratio can be presented as the modulated anisotropy.[25]

$$r_\omega=\frac{\Lambda_\omega-1}{\Lambda_\omega+1}. \qquad (4)$$

The value of Δ_ω and r_ω can be used to demonstrate the mode of excitation. In particular, the highest possible value of Δ_ω for ω approaching infinity is given by[26]

$$\tan\Delta_{0i}=\left\{\frac{3r_{0i}}{2[(1+2r_{0i})(1-r_{0i})]^{1/2}}\right\}, \qquad (5)$$

where r_{0i} are the maximal values for one-, two- or three-photon excitation. Hence, for colinear transitions, the maximal differential phase angles are 30, 41.8, and 48.6 deg, respectively. Observation of a differential polarized phase angle larger than 41.8 deg can be regarded as proof of excitation by more than two photons (Table 1). The values of r_ω for low frequencies can be interpreted as a steady-state anisotropy, and the limiting value for ω approaching infinity is comparable to the time-zero anisotropy. Values of r_ω greater than 0.57 demonstrate excitation with more than two photons, subject to the caveat mentioned above for two-photon tensors.[21–22]
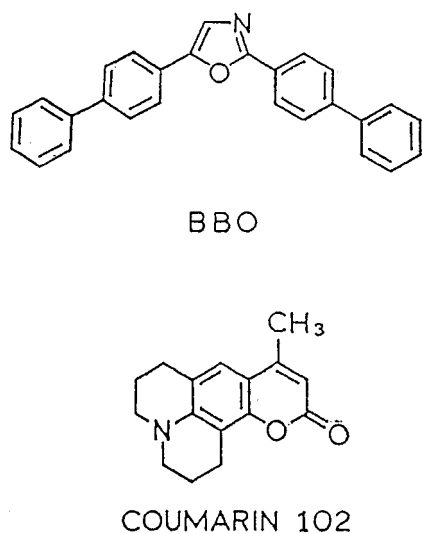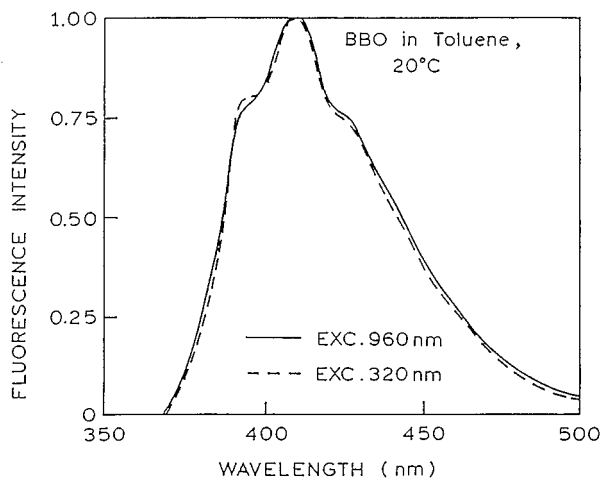
Scheme 2 Structures of 2,5-dibiphenylyloxazole (BBO) and coumarin 102 (C102).

Fig. 1 Emission spectra of BBO in toluene with excitation at 320 and 960 nm.

3 MATERIALS AND METHODS

BBO was obtained from Aldrich, 99%, scintillation grade, and was used without further purification. Coumarin 102, 99%, laser grade, was obtained from Eastman-Kodak. The chemical structures are shown in Scheme 2. For one-photon excitation of BBO we used either the frequency-doubled output of a pyridine 2 dye laser near 350 nm or the arc lamp on the steady-state fluorometer. Excitation from 580 to 740 nm was provided by the fundamental output of rhodamine 6G or pyridine 2 dye lasers, which were synchronously pumped by a mode-locked argon ion laser. The repetition rate was 3.976 MHz with a pulse width near 6 ps. Excitation from 750 to 1000 nm was provided by an fs mode-locked Ti:sapphire laser from Spectra Physics. The repetition rate of 80 MHz was held fixed by the Loc-to-Clock accessory. The repetition rate was divided by 8 by the Loc-to-Clock electronics, and used as the 10-MHz reference signal for the frequency domain (FD) instrument. The pulse width was near 80 fs.

The fundamental outputs of the Ti-sapphire or the dye lasers were brought directly to the sample compartment and focused with a laser-quality lens (2-cm focal length) to a spot with an approximate 20 μ diameter. The maximum illumination intensity was 800 mW, resulting in a maximum incident power of 200 kW/cm^2. Given the 80 MHz repetition rate of the Ti:sapphire laser, and 80 fs pulse width, the peak intensity was calculated to be 3×10^{10} W/cm^2 or 1.2×10^{27} photons/cm^{2}s. For intensity and anisotropy decay measurements, the emission was isolated with a stack of two Corning 4-96 filters which transmitted from 380 to 560 nm, combined with a short-wave pass filter SWP620 from Spindler Hoyer. The samples were stirred during the measurements, which was necessary to obtain a stable signal. The concentration of BBO in toluene or tri-

acetin for the intensity and anisotropy decay measurements was near $2 \times 10^{-5} M$, as determined from the optical density near 1.0 at 340 nm and the extinction coefficient near 50,000 M^{-1} cm^{-1}.[28] Slightly higher concentrations of BBO near $5 \times 10^{-5} M$ were used to measure the emission spectra.

Frequency domain intensity and anisotropy decays were obtained on the instrumentation described previously[29,30] using magic angle polarization conditions. The frequency domain intensity and anisotropy data were analyzed by nonlinear least squares.[25,31] The intensity and anisotropy decays were analyzed in terms of single exponential models.

$$I(t) = I_0 \exp(-t/\tau_i) \qquad (6)$$

$$r(t) = r_{0i} \exp(-t/\phi_i), \qquad (7)$$

where τ_i is the decay time and ϕ_i is the rotational correlation time. The subscript i refers to the number of photons in the excitation process.

For emission spectra we used an SLM AB-2 spectrofluorometer with a 4-nm bandpass. Solutions were in equilibrium with air. The signals from the solvents alone were less than 0.1% of that observed in the presence of BBO. 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. All measurements were performed at room temperature near 20 °C.
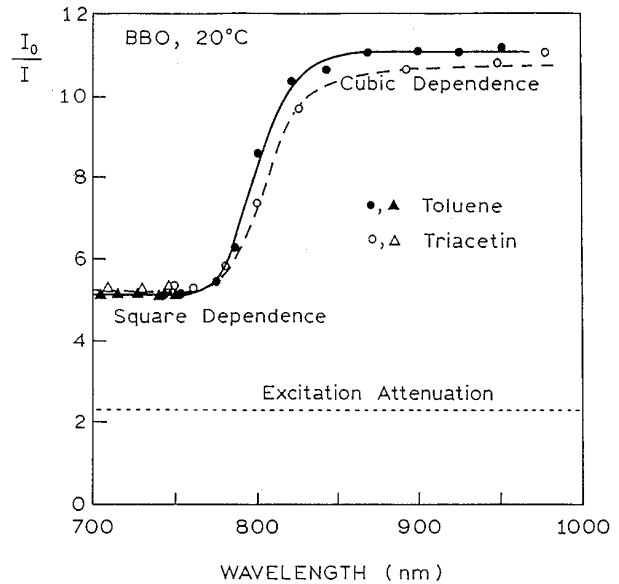
4 RESULTS

Emission spectra of BBO in toluene are shown in Figure 1 for one- and three-photon excitation at 320
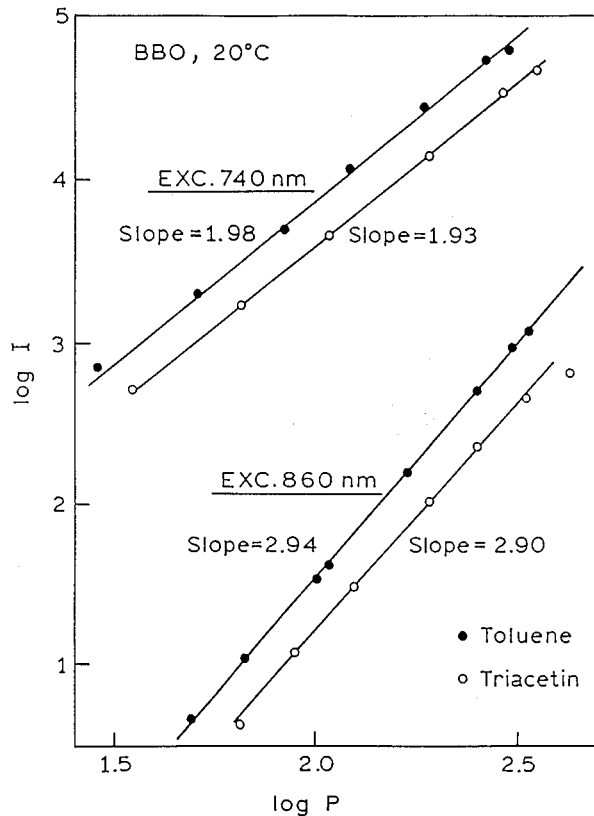
Fig. 2 Dependence of the emission intensity of BBO in toluene (●) and triacetin (○) on the incident power at 740 and 860 nm from the Ti:sapphire laser. The laser power (*P*) is in milliwatts. The maximum illumination intensity was about 200 kW/cm².


Fig. 3 Effect of a 2.2-fold attenuation of the incident light on the steady-state emission of BBO in toluene (▲, ●) and in triacetin (△, ○). I_0 and I are the emission intensities of BBO without and with the 2-fold attenuation, respectively. The excitation was from the pyridine 2 (△, ▲) or Ti:sapphire laser (○, ●). The maximum illumination intensity was about 200 kW/cm².

and 960 nm, respectively. We also observed identical emission spectra of BBO in triacetin for excitation at 320 and 960 nm (not shown). These emission spectra were obtained using the same solutions of BBO and the same instrument, with the only difference being the excitation wavelength. Similar emission spectra were also observed for excitation at 750 nm (not shown), which results in two-photon excitation. Since BBO does not absorb above 390 nm,[28] it seems unlikely that the emission with 960 nm excitation could result from two-photon excitation, which is not expected to occur above 780 nm. We note that the blue emission of BBO with 960-nm excitation could be readily seen with the naked eye.

We were surprised by the observation of BBO emission with 960-nm excitation, and thus examined the dependence of the emission intensity on laser power. For excitation at the somewhat shorter wavelengths of 860 nm, we found that the emission intensity depended on the cube of the laser power (Figure 2). At 740 nm, the emission intensity of BBO depended on the square of the laser power. This result demonstrated that BBO displayed three-photon excitation at long wavelengths, and that the mode of excitation was dependent upon wave-

length. Similar results were observed for BBO in toluene or in triacetin (Figure 2).

We examined the effect of wavelength on the mode of excitation, that is, two- or three-photon excitation. This was accomplished by measuring the steady-state intensity of BBO with constant power, and with the intensity attenuated by about 2-fold using a neutral density filter. For two- or three-photon excitation, one expects the observed intensity to decrease 4- or 8-fold, respectively. Wavelength-dependent values of the effect of attenuation of the incident light are shown in Figure 3. In this figure, I_0 and I are the intensities of BBO without and with a 2.2-fold attenuation of the incident light. From 700 to 800 nm the emission is attenuated about 5-fold, which is consistent with a quadratic dependence on the incident light, which was attenuated 2.2-fold. Above 800 nm in toluene, the emission decreases 11-fold, which is consistent with a cubic dependence on laser power. A similar dependence on laser power and excitation wavelength was observed in both toluene and triacetin. These data show that the mode of excitation of BBO in toluene and in triacetin changes sharply from two- to three-photon excitation at 800 nm.
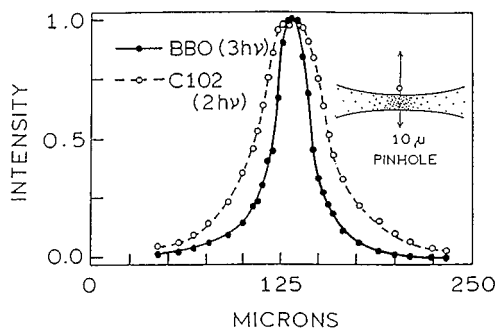
It is known that two-photon excitation results in strongly localized excitation at the focal point of the laser beam due to the quadratic dependence on excitation intensity.[8,9] Hence, a three-photon process should provide greater spatial localization than two-photon excitation due to the cubic dependence on intensity. We examined the spatial distribution
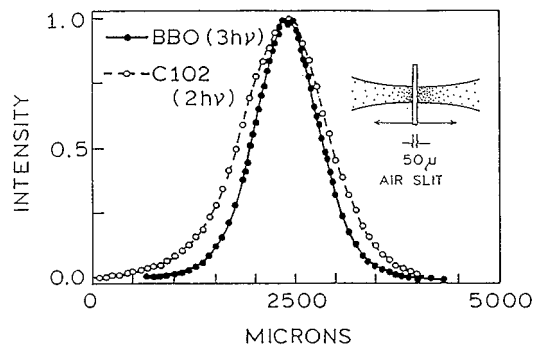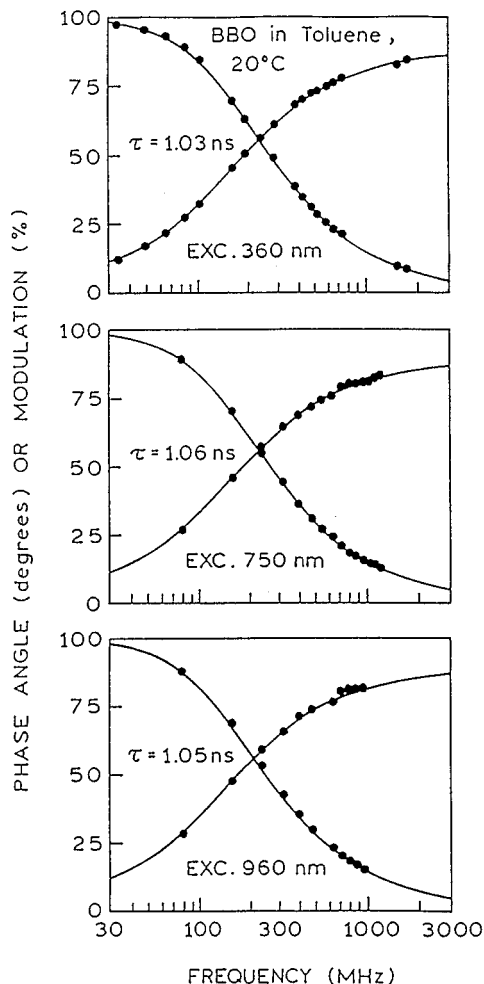
Fig. 4 Spatial distribution of the emission of BBO (●) and coumarin 102 (○) along the laser beam for excitation at 885 nm.

of the BBO emission when excited at 885 nm (Figure 4). For comparison we also examined the spatial distribution of the emission from coumarin 102 (Scheme 2), which displays two-photon excitation at this same wavelength of 885 nm. Both BBO and coumarin 102 were dissolved in toluene. The excited volume was substantially smaller for BBO than for coumarin 102, both along the direction of the incident beam (Figure 4) and across the focused beam (Figure 5). Since these spatial profiles were obtained at the same excitation wavelength, the difference in size can only be due to the nature of the excitation process. Also, the smaller excited volume with three-photon excitation of BBO could be visually seen by looking at the emission of BBO or coumarin 102. These results strongly support our conclusions that BBO in toluene undergoes three-photon excitation at wavelengths above 800 nm.

We questioned whether the intensity decays of BBO were altered by the mode of excitation. For one-photon excitation at 360 nm, BBO displays a single exponential intensity decay with a lifetime of 1.06 ns in toluene. Identical intensity decays of BBO were observed for two-photon excitation at 750 nm and three-photon excitation at 950 nm (Figure 6). In triacetin, BBO also displayed the same single expo-

Fig. 6 Frequency-domain intensity decay of BBO in toluene for excitation at 360, 750, and 960 nm.

nential decay time of 1.13 ns for excitation at 360, 750, and 950 nm (Table 2). Hence, the intensity decays of BBO do not depend on the mode of excitation.

It is known that the anisotropy of organic fluorophores can be different for one- and two-photon excitation.[18–24] This difference is due primarily to the more highly oriented excited-state population, and higher time-zero anisotropy (r_{0i}) which occurs for two-photon excitation of colinear transitions. Hence, we examined the frequency domain anisotropy decays of BBO in toluene and triacetin for excitation at 960 nm (Figure 7). In toluene the maximum differential phase angle is 43.7 deg, which is above the maximum value of 41.8 deg for two-photon excitation with colinear oscillators (Table 1). Triacetin, which is a more viscous solvent, resulted in higher modulated anisotropies. In this case the modulated anisotropy of BBO is as high as 0.625, which is larger than the two-photon maximum of 0.57 (Table 1).

Fig. 5 Spatial distribution of the emission of BBO (●) and coumarin 102 (○) across the focused laser beam for excitation at 885 nm. The emission image was enlarged 3-fold, so that the effective pinhole size is about 3.5 μ.

Table 2 Intensity decay times and rotational correlation times of BBO for one-, two-, and three-photon excitation.

| Solvent	n^a	λ_{ex} (nm)	τ (ns)	r_{0i}	θ_i (ns)
Toluene	1	360	1.06	0.372	0.133
	2	750	1.03	0.523	0.134
	3	960	1.05	0.651	0.126
Triacetin	1	360	1.13	0.360	3.31
	2	750	1.13	0.503	3.29
	3	960	1.15	0.649	3.34

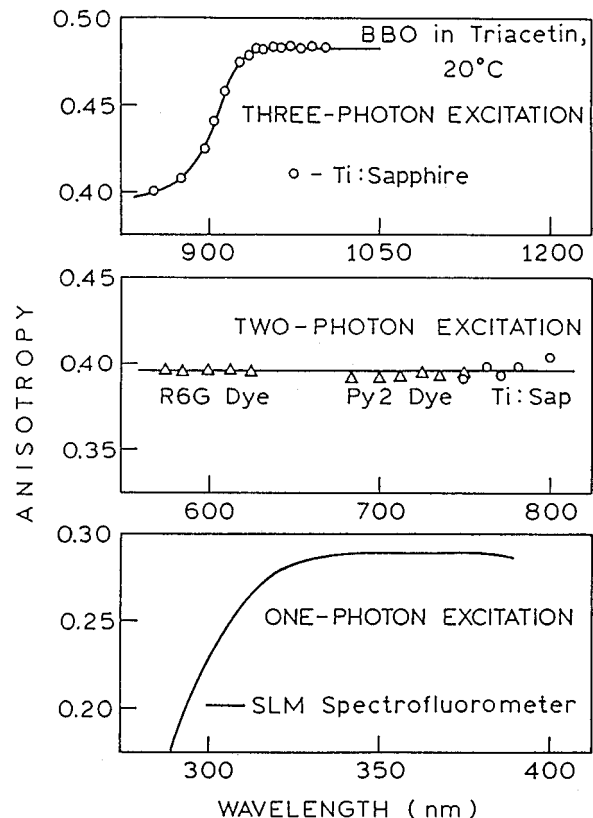^{a} One-, two-, or three-photon excitation.

These frequency domain anisotropy data were used to recover the time-zero anisotropies and rotational correlation times (Table 2). The correlation times were identical for one-, two-, and three-photon excitation of BBO in toluene (0.13 ns) and for one-, two-, and three-photon excitation in triacetin (3.3 ns). This similarity of the rotational correlation times, independent of the mode of excitation, suggests that the sample is not being heated by the focused light used for two- or three-photon excitation (Table 2). The time-zero anisotropies (r_{0i}) are distinct for each mode of excitation. The average ratio of $r_{02}/r_{01}=1.40$, which is close to the predicted value of 1.43 for colinear oscillators. The average ratio of $r_{03}/r_{01}=1.78$, which is somewhat larger than the predicted ratio of 1.67, but within experimental error given the uncertainties in the r_{0i} values. The r_{0i} values recovered for excitation at 360, 750, and 960 nm are thus consistent with one-, two-, and three-photon excitation of BBO.

It is of interest to examine the excitation anisotropy spectrum of BBO. We measured the steady-state anisotropies of BBO in triacetin at 20 °C, where the rotational correlation time is about 3-fold longer than the decay time. These steady-state anisotropy values are shown in Figure 8, in which the individual panels separate the data where BBO displayed one-, two-, or three-photon excitation. For
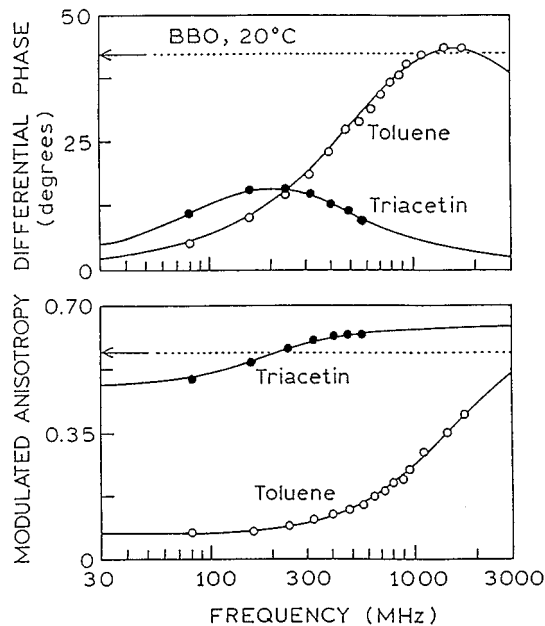
Fig. 7 Frequency domain anisotropy decays of BBO in toluene and triacetin. The dashed lines show the upper limits of the differential phase angles and modulated anisotropies for two-photon excitation.

Fig. 8 Steady state anisotropies of BBO in triacetin for one-, two-, and three-photon excitation.

one-photon excitation, BBO displays a constant anisotropy throughout the S_0 to S_1 absorption band, with smaller anisotropies for excitation below 325 nm. This behavior is typical of numerous organic fluorophores. For two-photon excitation of BBO from 580 to 800 nm, the anisotropy is constant, suggesting a single transition throughout this range of wavelengths. The ratio of the anisotropies for one- and two-photon excitation is about 1.36, which is comparable to that expected for colinear transitions (Table 1).

Surprising behavior was observed for the anisotropy spectrum of BBO for three-photon excitation. For wavelengths above 900 nm the anisotropy is constant, and the ratio of r_{03}/r_{01} is about 1.65, comparable to the expected value (Table 1). However, the anisotropy decreases for excitation below 900 nm, even though the emission intensity is still dependent on the cube of the laser power. Hence, the anisotropy spectrum observed for three-photon excitation is analogous to that observed for one-photon excitation. These results suggest that more than one electronic transition can occur for three-photon excitation, but that a single transition occurs for two-photon excitation. Additional experimentation is needed to determine whether multiple transitions will be common in three-photon excitation.

5 DISCUSSION

The results presented here demonstrate that three-photon excitation can be observed with the fundamental output of Ti:sapphire lasers which provide intense femtosecond pulses in the near-infrared region of the spectrum. The data for BBO indicate that the mode of excitation can be strongly dependent on excitation wavelength and that the anisotropy spectra can be simple or complex. It should be noted that the concentration of BBO was moderately dilute, $2 \times 10^{-5} M$, which is comparable to that used for one-photon excitation. In addition, we have recently shown that the calcium-sensitive fluorophore Indo-1 can be excited by three photons at 885 nm,[32] and that tryptophan could be excited by three photons at 840 nm.[33] Thus the availability of modern fs lasers will allow photophysical studies of fluorophores with multiphoton excitation.

An additional advantage of three-photon excitation is the smaller excited volumes. By comparing the emission from cuvettes containing fluorophores that display two- or three-photon excitation, we showed that the emission is seen from a smaller area for three-photon excitation. In addition to supporting our conclusions of three-photon excitation of BBO, these spatial profiles suggest that higher spatial resolution can be obtained by three-photon excitation in laser scanning microscopy. Such work is now in progress in our laboratories.[34]

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