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Time-Resolved Fluorescence Intensity and Anisotropy Decays of 2,5-Diphenyloxazole by Two-Photon Excitation and Frequency-Domain Fluorometry

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Abstract

We report the first time-resolved fluorescence measurements of the intensity and anisotropy decays resulting from two-photon excitation. A 10-GHz frequency-domain fluorometer (*Rev. Sci. Instrum* **1990**, *61*, 2331), equipped with two focal lenses and an emission monochromator, was used for steady-state and time-resolved measurements of PPO fluorescence. The emission spectra and the intensity decays observed with single- and two-photon excitation were essentially identical. The steady-state limiting anisotropy r_0 of PPO in glycerol at -5°C measured for two-photon excitation is significantly higher than that observed for one-photon excitation. The r_0 value of 0.54 for two-photon excitation is well in excess of the theoretical maximum of 0.4 for single-photon excitation. A similar value of $r_0 \approx 0.50$ was obtained from the frequency-domain anisotropy data with two-photon excitation of PPO in methanol, butanol, and propylene glycol at 20°C . These higher values of r_0 indicate that two-photon excitation results in a more highly oriented photoselected population, which can increase the resolution of rotational correlation times and/or complex anisotropy decays. The anisotropy resolution can still be increased by using global analysis of anisotropy decays measured with single- and two-photon excitation.

Introduction

Advances in laser technology have opened new possibilities in optical spectroscopy. In particular, cavity-dumped and synchronously pumped dye lasers are now commonly used light sources in many fluorescence laboratories. With such equipment, the time-resolved fluorescence measurements of organic and biochemical compounds are routinely performed on the nano- and picosecond time scales.^{1–4} These picosecond (ps) laser systems provide pulses with very high peak power (10^3 – 10^4 W). Such powerful laser light sources, including Q-switched YAGs, excimer-pumped dye lasers^{5–7} as well as cavity-dumped dye lasers, are suitable for two-photon excitation. Two-photon spectroscopy has already been used in analytical chemistry for chromatographic detection^{8,9} and for probing optically dense media.

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¹⁰ Recently, several authors reported detection of two-photon absorption phenomena in extremely dilute solutions.^{6,11,12} The polarization and symmetry parameters of solutes excited by two photons were described by Wirth and co-workers.^{13,14} Two-photon spectroscopy has also been applied for steady-state measurements of biomolecules.¹⁵ In particular, Callis and co-workers used two-photon excitation to study the 1L_a and one 1L_b excited states of indole derivatives.¹⁶ Birge and co-workers used two-photon spectroscopy to investigate the symmetry of the lowest excited state of visual pigments.^{17,18} In fluorescence microscopy, two-photon excitation was successfully applied by Webb and colleagues for confocal imaging.¹⁹ According to our best knowledge, there have been no time-resolved studies of the emission resulting from two-photon excitation.

In this paper we present the first measurements of the time-resolved emission resulting from two-photon excitation. In particular, we compared the frequency-domain intensity and anisotropy decays of 2,5-diphenyloxazole (PPO) resulting from single and two-photon excitation. These experiments revealed a unique property of the excited-state population resulting from two-photon excitation, this being the stronger photoselection of the initially excited orientational distribution (Scheme I). The advantage of two-photon excitation for anisotropy decay measurements will be discussed.

Theory

Anisotropy for One- and Two-Photon Excitation.

It is well known that two-photon absorption has different selection rules than one-photon absorption.^{18,20–22} However, we are unaware of a description of the anisotropy²³ values expected for two-photon excitation with linearly polarized light. We now show for randomly oriented molecules with a single transition, that photoselection by two-photon absorption can excite a population of molecules with a higher orientation than for one-photon absorption.

Consider a molecule with its directions A and E of the absorption and emission oscillators fixed in the molecule (Figure 1). The electric vector ϵ of the exciting light is oriented along the z -axis. $I_{\parallel}$ and $I_{\perp}$ designate the components of the total fluorescence intensity ($I = I_{\parallel} + 2I_{\perp}$) parallel and perpendicular to ϵ , respectively, as observed along the x -axis. The angles ω' , ω'' , β , and δ obey the relation

$$\cos \omega'' = \cos \omega' \cos \beta + \sin \omega' \sin \beta \cos \delta \quad (1)$$

On squaring (1) and averaging over the azimuthal angle δ ($\langle \cos \delta \rangle = 0$, $\langle \cos^2 \delta \rangle = 1/2$) we obtain for a given absorption oscillator direction ω' a mean emission oscillator value

$$\langle \cos^2 \omega'' \rangle = \cos^2 \omega' \cos^2 \beta + \frac{1}{2} \sin^2 \omega' \sin^2 \beta = \left(\frac{3}{2} \cos^2 \omega' - \frac{1}{2} \right) \left(\cos^2 \beta - \frac{1}{3} \right) + \frac{2}{6} \quad (2)$$

where β is constant for a given transition in the molecule.

Averaging over the population of excited molecules gives the mean ($\langle \cos^2 \omega'' \rangle$) which is connected with the emission anisotropy $r(\omega'')$ ²⁴

$$r(\omega'') = \frac{3}{2} \frac{I_{\parallel}}{I} - \frac{1}{2} = \frac{3}{2} \langle \cos^2 \omega'' \rangle - \frac{1}{2} \quad (3)$$

Substitution of the averaged eq 2 into (3) yields

$$r(\beta, \omega') = \left(\frac{3}{2} \langle \cos^2 \omega' \rangle - \frac{1}{2} \right) \left(\frac{3}{2} \cos^2 \beta - \frac{1}{2} \right) \quad (4)$$

where

$$\langle \cos^2 \omega' \rangle = \frac{\int_0^{\pi/2} \cos^2 \omega' f(\omega') d\omega'}{\int_0^{\pi/2} f(\omega') d\omega'} \quad (5)$$

and $f(\omega') d\omega'$ is the directional distribution in the excited state owing to the polarized absorption.

The distribution in the excited state following one-photon excitation is given by

$$f_1(\omega') = \cos^2 \omega' \sin \omega' \quad (6)$$

Using eqs 4 and 5, one can find $\langle \cos^2 \omega' \rangle = 3/5$ and

$$r(\beta) = \frac{2}{5} \left(\frac{3}{2} \cos^2 \beta - \frac{1}{2} \right) \quad (7)$$

known as a Perrin equation. Thus the maximum value of r_0 for one-photon absorption is $2/5$ for $\beta = 0$ and the minimum value is $-1/5$ for $\beta = 90^\circ$.

In two-photon absorption phenomena, the two photons simultaneously interact with and are absorbed by the fluorophore. For the purpose of explaining the anisotropy results, we assumed the second photon interacts only with the ensemble of molecules which were preselected by the first photon. If the only nonzero elements in the two-photon transition tensor²⁰ are the diagonal elements, and one element is significantly larger than the other, then the orientation distribution in the excited state followed by two-photon absorption is given by

$$f_2(\omega') = \cos^2 \omega' f_1(\omega') = \cos^4 \omega' \sin \omega' \quad (8)$$

Using eqs 4 and 5, one can find $\langle \cos^2 \omega' \rangle = 5/7$ and

$$r(\beta, \omega') = \frac{4}{7} \left(\frac{3}{2} \cos^2 \beta - \frac{1}{2} \right) \quad (9)$$

Hence, the limiting value of r_0 for two-photon excitation is equal to $4/7$. For three-photon excitation $r_0 = 7/9$, etc. Additional values of r_0 for other values of β are summarized in Table I.

Fluorescence Intensities Resulting from One- and Two-Photon Excitation.

For single-photon excitation with low absorption, the fluorescence intensity F_1 is given by²⁵

$$F_1 = K\phi P_1 \sigma b C \quad (10)$$

where K is a collection of experimental parameters, ϕ is the quantum yield, P_1 is the laser power at the wavelength for single-photon excitation, σ is the absorption cross section in cm^2 , b is the effective path length, and C is the concentration of fluorophore.

For two-photon excitation with low absorption, the fluorescence intensity F_2 is given by

$$F_2 = K(\phi/2)P_2^2 \delta b C / A \quad (11)$$

where P_2 is the incident laser power, δ is the two-photon “Cross section” in $\text{cm}^4 \text{s}/(\text{photon molecule})$, and A is the area of the excitation beam. Consequently, for two-photon excitation, the fluorescence intensity is expected to depend on the squared laser power.

Materials and Methods

One- and two-photon experiments were performed using a 10-GHz frequency-domain fluorometer described elsewhere in detail.²⁶ For two-photon excitation we used the fundamental output from a cavity-dumped rhodamine 6G (R6G) dye laser, which was synchronously pumped by a mode-locked argon ion laser. The pulse repetition rate of R6G dye laser was 3.975 MHz and half-width of the pulse was near 6 ps. The average power of this laser beam was 120–150 mW, resulting in peak powers near 7000 W. The excitation beam was focused by using a 5 cm focal length lens. A similar lens was used for the collection of the fluorescence. Assuming the beam is focused to 10^{-4} cm^2 , the peak power is estimated to be about $7 \times 10^7 \text{ W/cm}^2$. For the one-photon experiments we used $0.5 \text{ cm} \times 0.5 \text{ cm}$ cuvettes with excitation and emission near a corner positioned at the center of a $1 \text{ cm} \times 1 \text{ cm}$ cuvette holder. For the two-photon experiments we used $1.0 \text{ cm} \times 0.5 \text{ cm}$ cuvettes, with the long axis aligned with the incident light and with the focal point positioned about 0.5 cm from the surface facing the incident light. The position of the cuvette was adjusted so that excitation laser beam crossed the solution near the observation window. Such a position practically eliminated trivial reabsorption, which could occur for a longer path length. For one-photon excitation, we used the frequency-doubled output of the same dye laser. Emission spectra were obtained using a monochromator with a bandwidth of 10 nm. The time-resolved and limiting anisotropy values were measured without the monochromator using glass cutoff filters, Corning 7–59 and 7–51. Emission spectra and frequency-domain intensity decays were measured under “magic angle” conditions. The concentration of PPO was 10^{-4} M in all solutions, except for emission spectra measurements where we used $2 \times 10^{-4} \text{ M}$. PPO (spectroscopic grade) was from Fisher and was used without further purification. All solvents were HPLC or spectroscopic grade and did not show any significant signal under our experimental conditions.

Frequency-Domain Intensity and Anisotropy Decay Analysis.

The intensity decays were recovered from the frequency-domain data in terms of the multiexponential model

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (12)$$

where α_i are the preexponential factors and τ_i are the decay times. For a single-exponential decay the phase and modulation are related to the decay time (τ) by

$$\tan \phi_\omega = \omega \tau \quad (13)$$

$$m_\omega = (1 + \omega^2 \tau^2)^{-1/2} \quad (14)$$

For a multiexponential decay, the expected phase and modulation values can be calculated²⁷ from the sine and cosine transforms of $I(t)$

$$N_\omega = \sum_i \frac{\alpha_i \omega \tau_i^2}{(1 + \omega^2 \tau_i^2)} / \sum_i \alpha_i \tau_i \quad (15)$$

$$D_\omega = \sum_i \frac{\alpha_i \tau_i}{(1 + \omega^2 \tau_i^2)} / \sum_i \alpha_i \tau_i \quad (16)$$

where ω is the angular modulation frequency ($2\pi \times$ the modulation frequency in hertz). The calculated (c) frequency-dependent values of the phase angle ($\phi_{c\omega}$) and the modulation ($m_{c\omega}$) are given by

$$\tan \phi_{c\omega} = N_\omega / D_\omega \quad (17)$$

$$m_{c\omega} = [N_\omega^2 + D_\omega^2]^{1/2} \quad (18)$$

The parameters (α_i and τ_i) are varied to yield the best fit between the data and the calculated values, as indicated by a minimum value for the goodness-of-fit parameter χ_R^2

$$\chi_R^2 = \frac{1}{\nu} \sum_\omega \left[\frac{\phi_\omega - \phi_{\omega c}}{\delta \phi} \right]^2 + \frac{1}{\nu} \sum_\omega \left[\frac{m_\omega - m_{\omega c}}{\delta m} \right]^2 \quad (19)$$

where ν is the number of degrees of freedom and $\delta \phi$ and δm are the uncertainties in the phase and modulation values, respectively. The subscript c is used to indicate calculated values for assumed values of α_i and τ_i .

Recovery of the anisotropy decay from the frequency-domain data is best understood by consideration of the polarization-dependent intensity decays. Suppose the sample is excited with a δ -function pulse of vertically polarized light. The decays of the parallel ($\parallel$) and perpendicular ($\perp$) components of the emission are given by

$$I_{\parallel}(t) = \frac{1}{3}I(t)[1 + 2r(t)] \quad (20)$$

$$I_{\perp}(t) = \frac{1}{3}I(t)[1 - r(t)] \quad (21)$$

where $r(t)$ is the time-resolved anisotropy and the total (rotation free) intensity decay is given by

$$I(t) = I_{\parallel}(t) + 2I_{\perp}(t) \quad (22)$$

Generally, $r(t)$ can be described as a multiexponential decay,

$$r(t) = \sum_j r_{0j} e^{-t/\theta_j} \quad (23)$$

where θ_j are the individual correlation times and r_{0j} the amplitude associated with the j th correlation time. One expects $r_{0j} = r_0$, where r_0 is the limiting anisotropy in the absence of rotational diffusion.

In the time-domain one measures the time-dependent decays of the polarized components of the emission (eqs 20 and 21). These decays are used to determine the anisotropy decay law which is most consistent with the data. The experimental procedures and the form of the data are different for the frequency-domain measurements of the anisotropy decays. The sample is excited with amplitude-modulated light, which is vertically polarized. The emission is observed through a polarizer, which is rotated between the parallel and the perpendicular orientations. There are two observable quantities which characterize the anisotropy decay. These are the phase shift ϕ_{ω} at the circular modulation frequency ω , between the perpendicular ($\phi_{\perp}$) and parallel ($\phi_{\parallel}$) components of the emission,

$$\Delta_{\omega} = \phi_{\perp} - \phi_{\parallel} \quad (24)$$

and the ratio

$$\Lambda_{\omega} = m_{\parallel}/m_{\perp} \quad (25)$$

of the parallel ($m_{\parallel}$) and the perpendicular ($m_{\perp}$) components of the modulated emission. To avoid confusion we stress that Λ_{ω} is the ratio of the modulated amplitudes for each polarization, not the ratio of the modulation of each polarized component. The physical meaning of the ratio Λ_{ω} is made clearer by use of a slightly different form. We use the frequency-dependent anisotropy (r_{ω}) which is defined by

$$r_{\omega} = \frac{\Lambda_{\omega} - 1}{\Lambda_{\omega} + 2} \quad (26)$$

At low modulation frequencies r_{ω} tends toward the steady-state anisotropy, and at high frequencies r_{ω} tend toward the limiting anisotropy in the absence of rotational diffusion (r_0). The calculated value ($r_{c\omega}$ and $\Lambda_{c\omega}$) can be obtained from the sine and cosine transforms of the individual polarized decays²⁸

$$N_k = \int_0^{\infty} I_k(t) \sin \omega t \, dt \quad (27)$$

$$D_k = \int_0^{\infty} I_k(t) \cos \omega t \, dt \quad (28)$$

where the subscript k indicates the orientation or parallel ($\parallel$) or perpendicular ($\perp$). The expected values of r_{ω} ($r_{c\omega}$) and Λ_{ω} ($\Lambda_{c\omega}$) can be calculated from the sine and cosine transforms of the polarized decays (eqs 20 and 21). The calculated values of r_{ω} and Λ_{ω} are given by

$$\Delta_{c\omega} = \arctan \left[\frac{D_{\parallel} N_{\perp} - N_{\parallel} D_{\perp}}{N_{\parallel} N_{\perp} + D_{\parallel} D_{\perp}} \right] \quad (29)$$

$$\Lambda_{c\omega} = \left[\frac{N_{\parallel}^2 + D_{\parallel}^2}{N_{\perp}^2 + D_{\perp}^2} \right]^{1/2} \quad (30)$$

where the N_i and D_i are calculated at each frequency. The parameters describing the anisotropy decay are obtained by minimizing the squared deviations between measured and calculated values, using

$$\chi_R^2 = \frac{1}{\nu} \sum_{\omega} \left(\frac{\Delta_{\omega} - \Delta_{c\omega}}{\delta \Delta} \right)^2 + \frac{1}{\nu} \sum_{\omega} \left(\frac{\Lambda_{\omega} - \Lambda_{c\omega}}{\delta \Lambda} \right)^2 \quad (31)$$

where δ and $\delta \Lambda$ are the uncertainties in the differential phase and modulation ratio, respectively.

Results

Emission Spectra.

The fluorescence emission spectra of PPO in methanol are presented in Figure 2. Essentially the same spectra were obtained using one-photon (dashed line) or two-photon (solid lines) excitation. The small shift to the longer wavelengths in the case of two-photon excitation is probably not significant. The 2-fold reduction of the average power of the excitation beam results in 4-fold decrease of fluorescence intensity (Figure 2, insert), whereas, for one-

photon excitation, such reduction of excitation gives only 2-fold decrease of fluorescence (not shown).

Limiting Anisotropy.

We usually perform frequency-domain measurements without lenses so as to avoid locally intense excitation. Therefore, we first measured the steady-state anisotropy of PPO in glycerol at $-5\text{ }^{\circ}\text{C}$ using one-photon excitation with and without lenses. Under these conditions (glycerol at $-5\text{ }^{\circ}\text{C}$), the solution is sufficiently viscous so that the PPO cannot rotate significantly during the lifetime of the excited state. Hence, the measured anisotropy is a measure of the fundamental anisotropy r_0 . We obtained identical values of r_0 in both measurements after correction for the g factor,²⁹ which were 1.01 and 1.05 without and with lenses, respectively. Hence, focusing of the laser beam did not alter the single-photon values of r_0 . Comparison of the one- and two-photon anisotropy values is shown in Figure 3. In both cases, the dependence on wavelength is weak, but the values of r_0 are extremely different. The maximal observed value of r_0 for one-photon excitation is about 0.35, whereas for two-photon excitation, it is about 0.54. The horizontally dashed lines in Figure 3 indicate the theoretical maximal values of r_0 for collinear absorption and emission oscillators, which are $2/5$ and $4/7$ for one- and two-photon excitation, respectively. Higher value of r_0 is expected to be a significant advantage of two-photon excitation for the resolution of complex anisotropy decays, as has been observed previously for various values of r_0 obtained using one-photon excitation.³⁰

Intensity Decays.

The frequency-domain intensity data for PPO in 1-butanol are presented in Figure 4. For both, one-photon (Figure 4, top) and two-photon (Figure 4, bottom) excitation, we observed single-exponential decays. The double-exponential analysis did not result in a decrease in χ_R^2 . Essentially, the same lifetimes were measured for both modes of excitation, about 1.4–1.5 ns (Table II). However, in all three solvents, the lifetimes measured with two-photon excitation were a few percent smaller than those measured for one-photon excitation. This could be the influence of a strong electric field of the excitation beam, but the differences are too small to claim such a dependence.

Anisotropy Decays.

The frequency-domain anisotropy data are not expected to be identical for one- and two-photon excitation. Due to the higher value of r_0 for two-photon excitation, we expect rather distinct data between the two excitation modes. Figures 5 and 6 present the frequency-domain anisotropy data for PPO obtained using one- or two-photon excitation, respectively. For two-photon excitation, the differential phases and the modulated anisotropies are larger than those observed for one-photon excitation. The recovered correlation times (Table III) are independent of the mode of excitation and are well fitted by a single correlation time.

It is important to notice that the values of r_0 recovered from the frequency-domain analysis are higher for two-photon excitation than for one-photon excitation (Table III). These values are in good agreement with the steady-state values observed in glycerol (Figure 3). The fact that the values in Table III are somewhat smaller than in Figure 3 is easily explained by

some small fraction of the anisotropy decay which is not recovered from the frequency-domain data, particularly for short correlation times.²⁸

Further support for the similarity of the correlation times and the differences between the one- and two-photon τ_0 values is provided by the χ_R^2 surfaces (Figures 7 and 8). These surfaces are constructed by holding, in turn, each parameter fixed at a given value, followed by minimization of χ_R^2 by variation of the other parameter. The procedure accounts for all possible correlations between the parameters and reveals the range of values consistent with the data. For the correlation times (Figure 7), the χ_R^2 minima are the same for one- and two-photon excitation. In contrast, the χ_R^2 minima are clearly distinct for one- and two-photon excitation (Figure 8). In this case the χ_R^2 surfaces were calculated from a global analysis of the frequency-domain anisotropy data in all three solvents, using a single value of τ_0 but different correlation times (Table IV). There is little doubt from the steady-state (Figure 3) and frequency-domain data (Figure 8) that the τ_0 values of PPO are significantly larger for two-photon excitation. The fact that the τ_0 values for one- and two-photon excitation are experimentally distinct is further supported by attempts to fit the one-photon data to the τ_0 values recovered from the two-photon data, and vice versa (Table III). These attempts resulted in substantial increases in χ_R^2 , demonstrating that the one-photon data are not consistent with the two-photon τ_0 , and vice versa. The lack of sensitivity to the values of τ_0 for PPO in methanol is expected due to the short correlation time.²⁸ In the other solvent χ_R^2 increases manyfold if the alternative value of τ_0 is used in the analysis (Table III).

The similarity of the correlation times observed for one- and two-photon excitation (Tables III and IV) led us to perform a global analysis using the one- and two-photon data with a single correlation time but different values of τ_0 . One such analysis is shown in Figure 9 for PPO in propylene glycol. In this analysis, the correlation time for the one- and two-photon data were taken as the global parameter, that is, identical for the one- and two-photon data, and the one- and two-photon τ_0 values were allowed to differ (nonglobal). The consistency of both the one- and two-photon data sets to this model is illustrated in Figure 9, in which the theoretical curves for one correlation time and two τ_0 values pass directly through the data points. Similarly, good global fits to the one- and two-photon anisotropy data were observed in all three solvents, as can be judged from the acceptable values of χ_R^2 (Table V).

Discussion

What are the advantages of two-photon excitation for time-resolved fluorescence studies of simple solutions and macromolecules? Since essentially the same lifetime and emission spectra were observed by one- and two-photon excitation, there appears to be little additional information on the intensity decays. However, it is too early to conclude that all emission spectra will remain invariant for one- and two-photon excitation. It is possible that the emission spectra can differ due to either selective excitation of solvated species or other interactions of the fluorophore with the environment, particularly those which alter the two-photon absorption spectra.¹⁸ It should be remembered that the two-photon cross sections are distinct for various fluorophores, which may allow selective excitation of fluorophores in two-photon processes which is not possible with one-photon excitation. Such a possibility is suggested by the observation that the two-photon cross section of indole is several-fold

larger than the two-photon cross section of analogous molecules.³³ Additionally, two-photon excitation can allow experimentation in media which are optically dense at the excitation wavelength, as has been suggested previously.¹⁰ Furthermore, two-photon excitation can result in decreased autofluorescence and/or scattering background from the sample resulting either from the absence of two-photon-induced autofluorescence or from the spatially limited excitation and emission volumes. And finally, as pointed out by Webb and co-workers,¹⁹ two-photon excitation allows spatially localized excitation of UV-absorbing fluorophores using microscopes where the UV transmission is typically poor and where excitation of fluorescence outside the focal plan leads to decreased contrast.

In our opinion, a significant advantage of two-photon excitation is the higher limiting anisotropies for two-photon excitation. The resolution of anisotropy decays, particularly multiexponential anisotropy decays, is significantly increased for higher values of r_0 . This is illustrated in Figure 10, which shows simulated frequency-domain anisotropy data for a single-exponential anisotropy decay with a 1-ns correlation time. The differential phase angles and modulated anisotropy all increase roughly in proportion to the value of r_0 . The χ_R^2 surfaces for this correlation time, reveal a substantially increased resolution as the value of r_0 increases (Figure 11). Furthermore, it is possible that the r_0 values differ for one- and two-photon excitation due to different cross sections for overlapping states, as already observed for indole and similar molecules.³³ In such cases, the one- and two-photon anisotropy data could reflect differing amounts of transitions with distinct absorption and emission dipoles, and thereby emphasize rotational motions of the fluorophore about different molecular axes. In such cases, global analysis of the anisotropy data could provide improved resolution of the motions of interest in both simple solutions and for probes bound to macromolecules.

In conclusion, we have demonstrated that two-photon excitation is easily accomplished using the picosecond dye laser sources currently used for time- and frequency-domain fluorimeters. Consequently, time-dependent decays resulting from two-photon excitation are easily observed in many laboratories. We demonstrated one advantage of two-photon excitation, this being increased orientation of the population excited with linearly polarized light. Other advantages and/or possibilities must await further experimentation.

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Figure 1.

Geometry of the system. The exciting light ϵ is polarized in the z direction. The fluorescence is observed along x . The vectors $\mathbf{A}$ and $\mathbf{E}$ represent the direction of the absorption and emission transition moments. δ is the angle between the planes formed by the z , A and E , A directions. The meaning of the angles ω' , ω'' , α_1 , and α_2 is evident from the figure.

Figure 2. Fluorescence emission spectra of PPO in methanol obtained with one (---) and two-photon (—) excitation. The insert shows the dependence of two-photon fluorescence intensity on the intensity of excitation light.

Figure 3. Steady-state anisotropy spectrum of PPO in glycerol at -5°C . The horizontally dashed lines indicate maximal values of r_0 , $2/5$ for one-photon and $4/7$ for two-photon excitation.

Figure 4. Frequency-domain intensity data for PPO in 1-butanol at 20 °C obtained using one- (top) or two-photon (bottom) excitation.

Figure 5. Frequency-domain anisotropy decays data for PPO in methanol, 1-butanol, and propylene glycol obtained with one-photon excitation.

Figure 6. Frequency-domain anisotropy decays data for PPO in methanol, 1-butanol, and propylene glycol obtained with two-photon excitation.

Figure 7.
 χ^2_R surfaces obtained for the correlation times.

Figure 8. $\chi^2_{R_N}$ surfaces for the r_0 values. The $\chi^2_{R_N}$ values are from a global analysis of the data in three solvents, to a single value of r_0 .

Figure 9.
Global analysis of anisotropy decays for PPO in propylene glycol.

Figure 10.

Simulated frequency-domain anisotropy data. For the simulation used here $\tau_F = 1 \text{ ns}$, $\theta = 1 \text{ ns}$, $r_0 = 0.1, 0.3$, and 0.5 , and the uncertainties $\delta = 0.2^\circ$ and $\delta\Lambda = 0.005$.

Figure 11.

χ_R^2 correlation times surfaces obtained for the simulated data. The values of r_0 was allowed to vary when the data (Figure 10) was force-fit to the correlation times shown in the x -axis.

SCHEME 1:

Intuitive Representation of the Photoselected Population Resulting from One- and Two-Photon Excitation

TABLE I:

Relationship between Angular Displacement of the Absorption and Emission Dipoles (β) and r_0 for P_0 ^a for Various Kinds of Excitation

β , deg	one-photon excitn		two-photon excitn		three-photon excitn	
	r_0	P_0	r_0	P_0	r_0	P_0
0	0.40	0.50	0.57	0.67	0.78	0.84
45	0.10	0.14	0.14	0.20	0.19	0.27
54.7	0.00	0.00	0.00	0.00	0.00	0.00
90	-0.20	-0.33	-0.29	-0.50	-0.39	-0.72

^aThe polarization is related to the anisotropy by $P_0 = 3r_0/(2 + r_0)$. The values of r_0 and P_0 are for polarized excitation.²⁹

TABLE II:

Intensity Decay Analysis of PPO Fluorescence at 20 °C

solvent	one-photon excitn			two-photon excitn		
	$\tau,^a$ ns	χ_R^2	$\chi_R^2(1 \text{ exp})^b / \chi_R^2(2 \text{ exp})$	$\tau,^a$ ns	χ_R^2	$\chi_R^2(1 \text{ exp})^b / \chi_R^2(2 \text{ exp})$
methanol	1.51	1.13	0.98	1.44	1.46	1.04
1-butanol	1.44	1.30	1.00	1.41	2.25	0.99
propylene glycol	1.46	1.34	1.04	1.45	2.10	1.01

^aThe preexponential factor was fixed ($\alpha = 1$) in these analyses.

^bThe ratio of the χ_R^2 values for analysis of the FD intensity decay data in terms of a single- and double-exponential decay.

TABLE III:

Anisotropy Decay Analysis of PPO Fluorescence at 20 °C

solvent	one-photon excitn				two-photon excitn			
	r_0	θ , ns	χ_R^2	$\chi_R^2(1 \text{ exp})/\chi_R^2(2 \text{ exp})$	r_0	θ , ns	χ_R^2	$\chi_R^2(1 \text{ exp})/\chi_R^2(2 \text{ exp})$
methanol	0.333 (0.099) ^a	0.035 (0.011)	1.43	0.98 ^c	0.493 (0.065)	0.038 (0.005)	0.93	0.99
1-butanol	$\langle 0.493 \rangle^b$ 0.331 (0.0062)	0.024 0.130 (0.002)	1.44 0.89	1.00	$\langle 0.333 \rangle$ 0.493 (0.0053)	0.057 0.136 (0.002)	1.23 1.37	0.98
propylene glycol	$\langle 0.492 \rangle$ 0.349 (0.0004)	0.082 2.51 (0.012)	8.60 1.43	1.01	$\langle 0.331 \rangle$ 0.506 (0.0004)	0.212 2.60 (0.009)	32.5 2.10	1.01
	$\langle 0.506 \rangle$	0.796	3273.6		$\langle 0.349 \rangle$	91.2	8255.5	

^a Asymptotic standard errors obtained from least-squares analysis.^{31,32}^b The angular brackets $\langle \rangle$ indicate these values were held fixed during the analysis.^c The ratio of the χ_R^2 values for analysis of the FD intensity decay data in terms of one and two correlation times.

TABLE IV:

Global r_0 Analysis of the PPO Anisotropy Decays

solvent	one-photon excitn		two-photon excitn	
	θ , ns	χ_R^2	θ , ns	χ_R^2
methanol	0.034		0.037	
	(0.001) ^a		(0.001)	
1-butanol	0.122		0.132	
	(0.001)		(0.001)	
propylene glycol	2.51	1.28	2.60	1.50
	(0.011)		(0.008)	
	$r_0(\text{global})$	0.349	$r_0(\text{global})$	0.506
		(0.001)		(0.001)

^a Asymptotic standard errors obtained from least-squares analysis.^{31,32}

TABLE V:

Global Correlation Time Analysis of the PPO Anisotropy Decays

solvent	r_0		θ , ns	χ_R^2
	one photon	two photon		
methanol	0.315	0.500	0.037	1.13
	(0.042) ^a	(0.063)	(0.005)	
1-butanol	0.323	0.499	0.134	1.18
	(0.0034)	(0.0051)	(0.002)	
propylene glycol	0.348	0.507	2.58	2.21
	(0.0003)	(0.0003)	(0.008)	

^a Asymptotic standard errors obtained from least-squares analysis.^{31,32}