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Light Quenching of Tetraphenylbutadiene Fluorescence Observed During Two-Photon Excitation

Ignacy Gryczynski,[1] Valery Bogdanov,[2] and Joseph R. Lakowicz[1]

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We observed the steady-state and time-resolved emission of tetraphenylbutadiene (TPB) when excited by simultaneous absorption of two photons (514 to 610 nm). The intensity initially increased quadratically with laser power, as expected for a two-photon process. At higher laser powers the intensity increases in TPB were subquadratic. The intensity and anisotropy decay times of TPB were unchanged under the locally intense illumination. Importantly, the time zero anisotropy of TPB was decreased under conditions where the intensity was subquadratic. Furthermore, the subquadratic dependence on incident power was not observed for two-photon excitation of 2,5-diphenyloxazole (PPO), for which the incident wavelength does not overlap with the emission spectrum. These results are consistent with stimulated emission (light quenching) of TPB at high laser intensities. The phenomenon of light quenching may be important for other fluorophores used in biochemical research, particularly for the high local intensities used for two-photon excitation.

KEY WORDS: Tetraphenylbutadiene; light quenching; two-photon excitation; 2,5-diphenyloxazole.

INTRODUCTION

The increasing availability of high-power picosecond (ps) and femtosecond (fs) lasers has resulted in a renewed interest in two-photon processes, in particular, simultaneous absorption of two identical long-wavelength photons to result in excitation of the first singlet state. Two-photon excitation of fluorescence can be readily accomplished using the cavity-dumped output of high-repetition rate dye lasers, as has been demonstrated for a variety of fluorophores including PPO[3] [1], aromatic amino acids [2,3], proteins [4], the membrane probe diphenylhexatriene (DPH) [5], the nucleic acid probe 4′,6-diamidino-2-phenylindole (DAPI) [6], and the calcium probe Indo-1 [7]. It appears that the use of two-photon excitation (TPE) of fluorescence, in combination with measurement of the time-resolved emission, can provide improved resolution of anisotropy decays due to the higher time zero anisotropies observed for some fluorophores [1,5] and new information about overlapping electronic states [2]. These uses of TPE are distinct from earlier studies [8–11] which used TPE to determine the symmetry of the electronic configuration. However, there have been recent attempts [12] to link these earlier observations [8–11] in fluid solutions with the frozen-solution anisotropy spectra of fluorophores [12].

An important characteristic of TPE is that the local fluorescence intensity depends on the square of the local power, as expected for a process which requires simultaneous interaction of the fluorophore with two-photons. This property of TPE has been exploited to provide confocal excitation in fluorescence microscopy [13,14]. In

[1] Center for Fluorescence Spectroscopy and Department of Biological Chemistry, University of Maryland School of Medicine, 108 North Greene Street, Baltimore, Maryland 21201.

[2] Vavilov State Optical Institute, St. Petersburg, Russia.

[3] **Abbreviations used:** bis-MSB, *p*-bis(*O*-methylstyryl)benzene; DAPI, 4′,6-diamidino-2-phenylindole; DPH, diphenylhexatriene; FD, frequency domain; FLIM, fluorescence lifetime imaging; OPE, one-photon excitation; PPO, 2,5-diphenyloxazole; R6G, rhodamine 6G; TPB, tetraphenylbutadiene; TPE, two-photon excitation.

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this application of TPE, only those fluorophores in a thin focal plane are exposed to the high intensities needed for TPE. The fluorophores outside the focal plane are not excited or photobleached. These measurements require the locally and instantaneously intense excitation available from the fs dye lasers [13,14].

An additional emerging technology which uses ps laser sources and fluorescence microscopy is fluorescence lifetime imaging (FLIM). The FLIM method provides contrast in fluorescence image based on the fluorescence lifetimes at each pixel in the detector [15,16]. Furthermore, FLIM methods are being combined with TPE [17] to obtain improved spatial resolution as well as lifetime-based contrast.

For the reasons described above it has become important to understand the behavior of fluorophores under locally intense excitation. While the phenomenon of saturation has recently been considered [18], there has been no consideration of the phenomena of stimulated emission, which can occur if the incident wavelength overlaps the emission spectrum of the fluorophore [19]. Since the stimulated fraction of the emission is collinear with the incident light, this portion is not observed using standard right-angle observations. Hence, the observed intensity is decreased, and we refer to the process as "light quenching."

In the present report we describe light quenching of tetraphenylbutadiene (TPB), when excited by simultaneous absorption of two photons at wavelengths ranging from 514 to 610 nm, which overlap the emission spectrum of TPB.

THEORY

A complete description of the theory for light quenching, and particularly its dependence on light polarization and probe orientation, is beyond the scope of the current report and will be presented elsewhere [20]. We consider here the case of excitation using a single laser beam. For simplicity we assume that the continuous train of ps pulses produces the same effect as a constant beam of comparable peak power, but we know this approximation is not precisely correct [21].

Light Quenching for One-Photon Excitation (OPE)

The expressions which describe the extent of light quenching can be derived in a manner similar to the well-known Stern–Volmer equation for collisional quenching. In the presence of light quenching the excited-state population $N^*(t)$ is given by

$$\frac{dN^*(t)}{dt} = N\sigma_{a1}P - N^*(t)\left[\frac{1}{\tau} + \sigma_{1q}P\right] \quad (1)$$

where N is the ground-state concentration, $N^*(t)$ the excited-state population, σ_{a1} the cross section for one-photon absorption, τ the unquenched fluorescence lifetime, P the laser power density (photons/cm^2 s), and σ_{1q} the cross section for stimulated emission, "light quenching," at the incident wavelength. For simplicity we assume that the quenching is not dependent on the orientation of the excited molecules. In the presence of a constant quenching beam the derivative can be set equal to zero, and the steady-state fluorescence intensity is given by

$$I = kN\tau\frac{\sigma_{a1}P}{1 + \tau\sigma_{1q}P} \quad (2)$$

where k is a constant. For a rectangular quenching pulse where the pulse width (t_p) is much less than the lifetime ($t_p << \tau$) and weak quenching ($t_p\sigma_{1q}P < 1$), Eq. (2) becomes

$$I = kN\tau Rt_p\frac{\sigma_{a1}P}{1 + t_p^e\sigma_{1q}P} \quad (3)$$

where R is the pulse repetition rate and t_p^e the effective quenching pulse width ($t_p^e = 0.5t_p$ for a rectangular pulse in a one-beam experiment).

The amount of quenching can be characterized by I_0/I, where I_0 is the intensity expected in the absence of light quenching and I the intensity with light quenching. Setting $\sigma_{1q} = 0$ yields $I_0 = kN\tau\delta_{a1}Pt_pR$ and hence

$$Q = \frac{I_0}{I} = (1 + t_p^e\sigma_{1q}P) \quad (4)$$

We note that I_0 is not a directly observable quantity in a single-beam experiment where excitation and quenching are provided by the same laser beam. In the absence of quenching, I_0 is proportional to the incident power P. Hence, relative values of the cross section for quenching (σ_{1q}) can be found from the slope of

$$\frac{P}{I} = k_1(1 + t_p^e\sigma_{1q}P) \quad (5)$$

where k_1 is an arbitrary constant. We note that the decay times are unchanged during single-beam light quenching [22].

Light Quenching with TPE

The one-photon theory described above is easily extended to the case of TPE. The excited-state popula-

tion is given by

$$\frac{dN^*(t)}{dt} = N\sigma_{a2}P^2 - N^*(t)\left[\frac{1}{\tau} + \sigma_{lq}P\right] \quad (6)$$

where σ_{a2} is the cross section for two-photon absorption. Using the steady-state assumption and assuming $t_p << \tau$, one obtains

$$Q = \frac{I_0}{I} = 1 + t_p^e \sigma_{lq} P \quad (7)$$

Since the unquenched intensity is proportional to P^2, the relative cross section for quenching can be found from

$$\frac{P^2}{I} = k_2 (1 + t_p^e \sigma_{lq} P) \quad (8)$$

where k_2 is an arbitrary constant.

Cross Section for Light Quenching

Light quenching requires that the emission spectrum of the fluorophore overlap with the wavelength of the quenching beam [19]. This cross section can be approximated by

$$\sigma_{lq} = \frac{K}{\tau_N} \frac{I(\bar{\nu})}{\int I(\bar{\nu})\, d\bar{\nu}} = K\Gamma \frac{I(\bar{\nu})}{\int I(\bar{\nu})\, d\bar{\nu}} \quad (9)$$

where Γ is the intrinsic rate of emission ($\Gamma = 1/\tau_N$), τ_N is the natural lifetime, and $I(\bar{\nu})$ is the emission spectrum [23]. A similar expression was presented previously.

Light Quenching and Anisotropy

For collinear absorption and emission oscillators, it is known that light quenching by continuous illumination with vertically polarized light results in a decreased anisotropy [25,26]. This effect is easily understood as selective quenching of those fluorophores whose emission oscillators are aligned with the incident light. We recently demonstrated for OPE of the laser dye DCM [4-(dicyanomethylene)-2-methyl-6-(*p*-dimethamino)-4H-pyrane] that this effect is due to a decrease in the time 0 anisotropy [$r(0)$] and that the rotational correlation time is not changed [22].

MATERIALS AND METHODS

All intensity, anisotropy, and frequency-domain (FD) data were obtained using the instrumentation described previously [27–29]. The detector was a red-sensitive version of a R2566 from Hamamatsu, a microchannel plate PMT with 6-μm channels, which provides FD data up to 10 GHz [29]. FD intensity decays were measured using a fluorophore which displays a known lifetime [30]. In this case we used *p*-bis(*O*-methylstyryl)benzene (bis-MSB), which displays the same 1.63-ns lifetime in cyclohexane for OPE and TPE [31]. The average and peak power of the incident light were varied by insertion of neutral density filters into the excitation beam. To determine if sample heating altered the data, the total power was decreased without changing the instantaneous power. This was accomplished by the use of a low-speed mechanical light chopper in the excitation beam, by which the average intensity was decreased up to 20-fold. Except for mineral oil at 5°C, the samples were stirred during the measurements. We observed no effects of illumination time on the intensity or anisotropy values. The signals were stable upon continuous illumination at our experimental conditions.

The excitation was polarized vertically, as occurs from the output of our argon ion and dye lasers. The emission was observed through a 460-nm (10-nm-bandpass) interference filter. For intensity measurements the emission polarizer was 54.7° from the vertical. Control measurements using solvents without TPB gave signals less than 0.5% of the TPB emission, for all polarization conditions and excitation (quenching) wavelengths. TPB was obtained from Aldrich, scintillation grade, and used without further purification. The concentrations of TPB in hexadecane and mineral oil were near 5×10^{-5} *M*, and bis-MSB in cyclohexane was 3×10^{-5} *M*.

For TPE and light quenching, at 514 nm we used the mode-locked output of our argon ion laser, 76 MHz, and $t_p = 120$ ps. The 1-W output at 514 nm was reduced 20-fold, to an average power of 50 mW, using a low-speed mechanical chopper. For TPE and light quenching at long wavelengths we used the cavity-dumped output of our R6G dye laser. The pulse width t_p was near 5 ps, with a repetition rate of 3.795 MHz. Hence, an incident power of 50 mW corresponds to an approximate peak power of 2.5 kW. This light was focused to a spot size of about 20 μm in diameter, resulting in a maximum intensity of about 1.0×10^9 W/cm^2. For both light sources the peak power was reduced by inserting neutral density filters into the excitation beam.

The light quenching experiments were performed using PPO (10^{-4} *M*) as a reference compound for TPE [1]. Like TPB, PPO is also excited by two photons but is not quenched by light within our experimental conditions and accuracy. The fluorescence signals of the TPB sample and PPO reference were matched when laser

power was attenuated 16-fold using a neutral density filter.

RESULTS

Light quenching requires intense illumination and, until recently [22], has been observed only using the intense giant pulses from ruby or Q-switched lasers [19,24,32,33]. Observation of light quenching with our less intense high-repetition rate laser requires careful selection of the fluorophore based on its spectral properties. We selected TPB because of its high two-photon cross section and its emission spectrum, which overlaps with the wavelength used for excitation and quenching (514 to 610 nm; Fig. 1). Since the two-photon absorption is low, we do not expect significant depletion of the ground state or other undesirable thermal or photochemical effects. Additionally, TPB displays a relatively short natural lifetime, near 2 ns, which results in a larger cross section for quenching relative to molecules with longer decay times [Eq. (9)].

As a control molecule we chose PPO because of its known large cross section for TPE [1]. The emission of PPO does not overlap with the excitation (quenching) wavelength, so that light quenching is not expected during TPE of PPO. The fluorescence intensities of TPB and PPO were adjusted to be equal in the absence of light quenching, that is, when the incident light was attenuated 16-fold by a neutral density filter. The fluorescence intensities of PPO for increasing incident power are shown in Fig. 2. As expected, the PPO intensity depends quadratically on the laser power (– – x – –). In contrast, the fluorescence intensities of TPB display a less-than-quadratic dependence (—●—). We believe that this difference between PPO and TPB is due to the longer-wavelength emission of TPB and its overlap with the incident wavelength.

The relative cross sections for quenching can be obtained from the "quadratic" Stern–Volmer plots [Eq. (8)]. In this plot (Fig. 3) the data for PPO appear as a horizontal line, due to its pure quadratic dependence on the incident power and the absence of light quenching. Our interpretation of the subquadratic TPB intensities as being due to light quenching is supported by the quenching observed at longer wavelengths (Fig. 2). Progressively less quenching is seen at 514, 575, 595, and 615 nm (Fig. 2), which corresponds to decreasing overlap with the emission spectrum of TPB (Fig. 1). In fact, the slopes of the "quadratic" Stern–Volmer plots (relative cross section for quenching) closely follows the emission

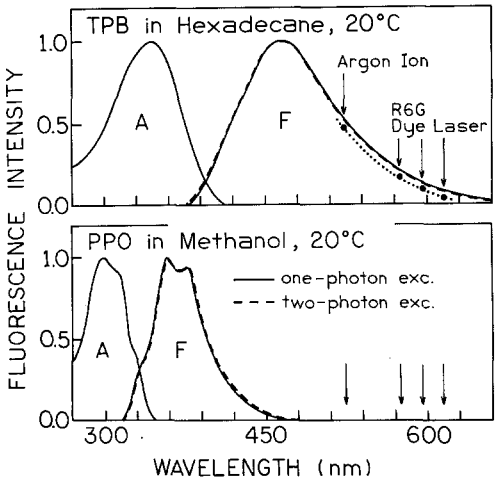

Fig. 1. Absorption (A) and emission (F) spectra of TPB in hexadecane (top) and PPO in methanol (bottom). In each case the solid line shows the emission spectrum for OPE (360 nm for TPB and 290 nm for PPO), and the dashed line the emission spectrum for TPE at 575 nm. The filled circles show the relative cross section for quenching, obtained from Fig. 3.

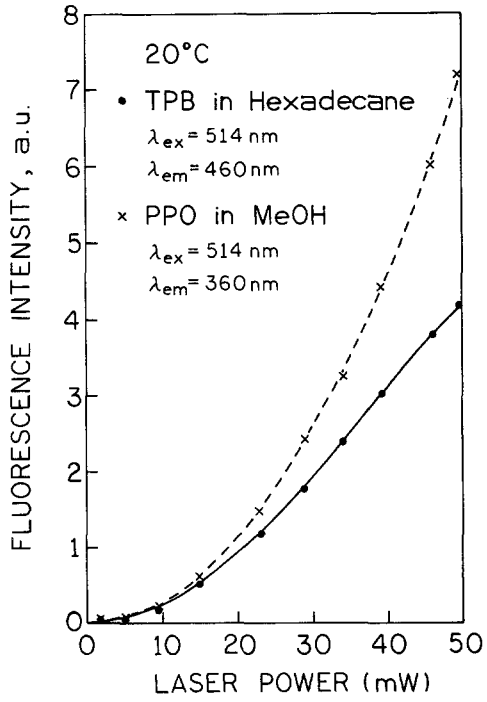

Fig. 2. Fluorescence intensity of PPO and TPB for increasing laser power. The incident light was focused and/or attenuated as described under **Materials and Methods**. The dashed line is the square of the laser power normalized to the intensity of PPO at low incident power.

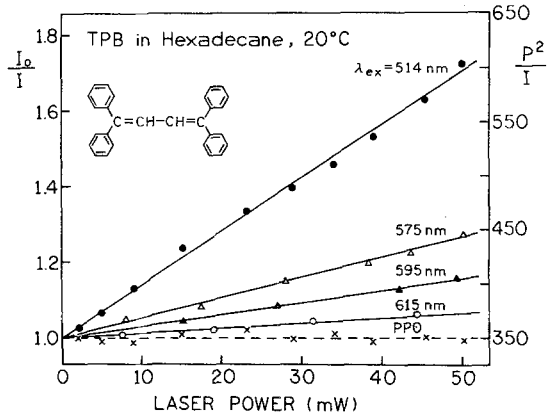

Fig. 3. "Quadratic" Stern–Volmer light quenching plots for PPO (– – –) and TPB (—). The excitation and quenching wavelengths are indicated.

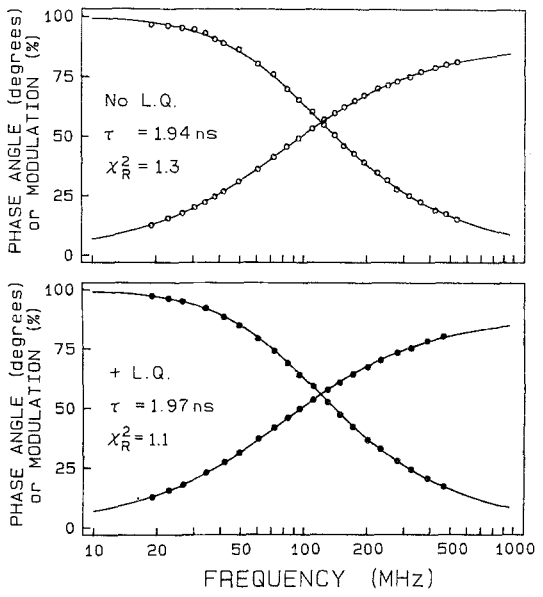

Fig. 4. FD intensity decay data for TPB in hexadecane at 20°C, in the absence (top) and presence (bottom) of light quenching. The incident laser power at 575 nm was 3 (top) and 50 mW (bottom). The solid line shows the best single exponential fit to the data.

spectrum, as expected for the phenomena of stimulated emission. We note that comparable results (Figs. 1–3) were obtained for TPB in mineral oil.

High-intensity illumination can result in a variety of undesirable photochemical processes, such as sample heating and photobleaching of the fluorophore. While effects are not expected to be substantial for the weak two-photon absorption, we nonetheless measured the extent of light quenching with lower average power. The peak power was kept constant, while the average power was decreased, by the use of a low-speed mechanical chopper in the excitation beam. The same power-dependent intensities (Fig. 2) were observed when the average laser power was attenuated 20-fold with the low-speed light chopper. Also, the intensities were stable during illumination, both at the highest laser powers used in these experiments and for the periodically chopped excitation. These results strongly suggest that the subquadratic intensities observed for TPB were not due to photochemical effects in the sample.

To exclude further the possibility of photochemical effects as the origin of the observed light quenching of TPB, we examined its fluorescence intensity and anisotropy decays. These time-dependent decays are likely to be sensitive to the occurrence of light-dependent changes of the fluorophore. Hence, we examined the intensity decays of TPB in hexadecane (Fig. 4) and mineral oil (Fig. 5). In hexadecane the lifetimes remained single exponentials at low- and high-intensity illumination. The intensity decay of TPB in mineral oil at 5°C was found to be multiexponential in the absence of light quenching (Fig. 5, top). However, essentially the same frequency–response (Fig. 5, bottom) and intensity decay parameters

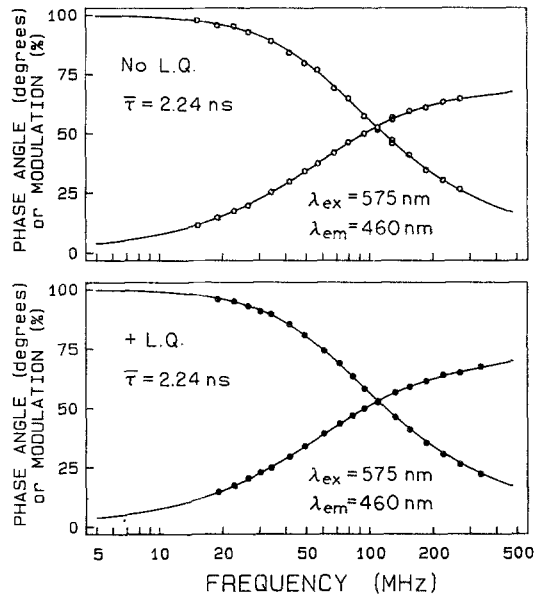

Fig. 5. FD intensity decay data for TPB in mineral oil at 5°C, in the absence (top) and presence (bottom) of light quenching (see the legend to Fig. 4). The solid lines shown the best double-exponential fits to the data (Table I).

were observed in the presence and absence of light quenching (Table I). Given the sensitivity of the mul-

Table I. Intensity Decay Parameters of TPB Fluorescence Induced by TPE at 20°C

Solvent	Light quenching	n^a	α_i	f_i	τ_i (ns)	χ^2_R
Hexadecane	–	1	1	1	1.94	1.3[b]
		2	0.001	0.000	0.11	
			0.999	1.000	1.94	1.3
	+	1	1	1	1.97	1.1
		2	0.046	0.025	1.10	
			0.954	0.975	2.01	0.9
Mineral oil	–	1	1	1	2.03	178.6
		2	0.434	0.065	0.22	
			0.566	0.935	2.39	1.8
	+	1	1	1	2.00	224.3
		2	0.388	0.073	0.30	
			0.612	0.927	2.39	1.0

[a]Number of components in the multiexponential fit.
[b]The uncertainties in phase and modulation were taken as 0.2° and 0.005, respectively.

tiexponential decay parameters to the fluorophore and its precise local environment, these results strongly support the absence of photochemical damage or local heating of TPB under our experimental conditions.

For the single-beam, single-wavelength experiments described in this report, the light quenching must occur during the excitation pulse. Consequently, the emission occurs after the pulse, and the rotational correlation time is expected to remain the same, irrespective of light quenching. In fact the correlation time remained unchanged for low- and high-intensity illumination of TPB in hexadecane (Fig. 6) and mineral oil (Fig. 7). A decrease in the correlation time is expected if the temperature of the illuminated volume is increased by the incident light. The consistency of the lifetimes and correlation times, and the absence of additional components in these decays, strongly suggest that the TPB molecule and its local environment are not altered by the intense illumination. This result supports our interpretation of the quenching data as being due to light quenching.

Examination of Figs. 6 and 7 reveals that differential polarized phase angles (top panels) and modulated anisotropies (bottom panels) are uniformly smaller in the presence of light quenching (Table II). This experimental result indicates that the time 0 anisotropy [$r(0)$] is smaller in the presence of light quenching. This decrease in $r(0)$ is the result of the photoselection rules for light quenching [25], which are the same as for one-photon absorption. Since the incident light is vertically polarized, this light also selectively quenches those molecules

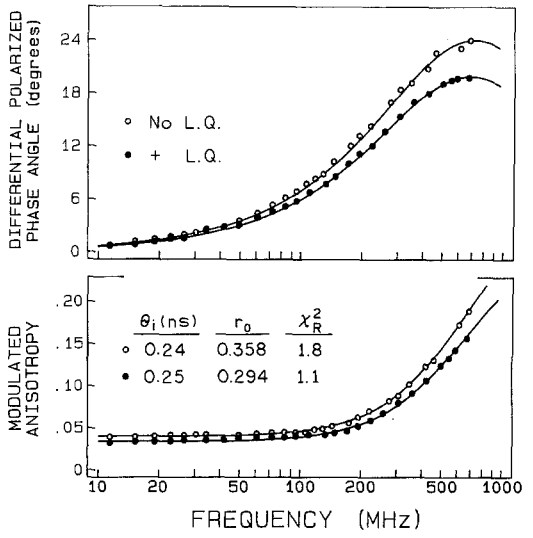

Fig. 6. FD anisotropy decay of TPB in hexadecane at 20°C. See the legend to Fig. 4 for details. The solid lines show the best single correlation time fit to the data (Table I).

whose transition moments are aligned along the vertical axis, assuming that the fundamental anisotropy (r_0) is near 0.4.

To demonstrate further the decrease in anisotropy due to light quenching, we examined the steady-state anisotropy of TPB in mineral oil at 5°C. As a control we examined PPO in glycerol at 5°C, where the aniso-

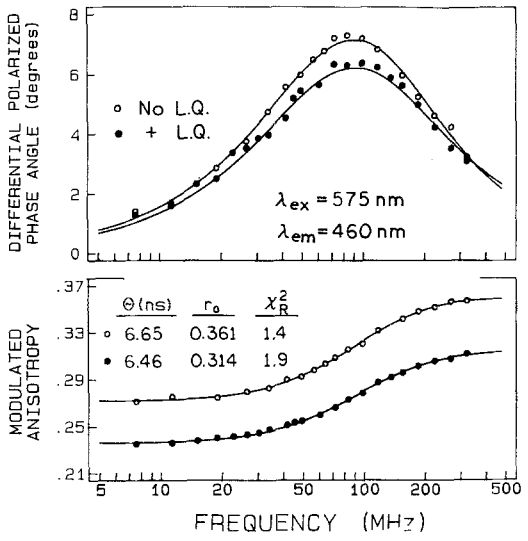

Fig. 7. FD anisotropy data of TPB in mineral oil at 5°C. See the legend to Fig. 4 for details. The solid line shows the best single correlation time fit to the data (Table II).

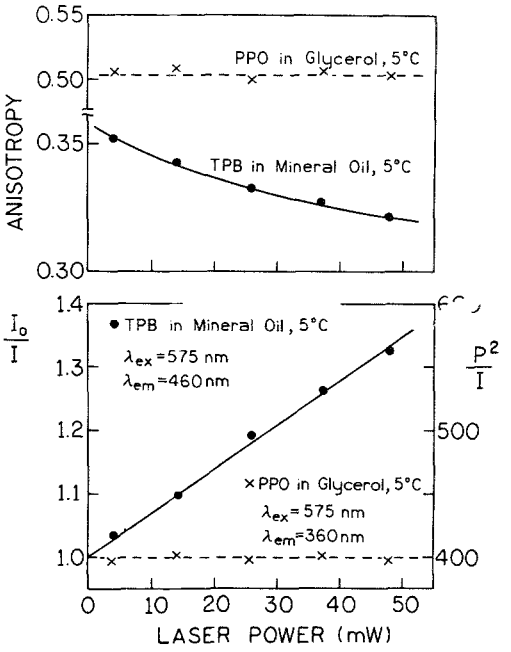

Fig. 8. Dependence of the steady-state anisotropy of TPB and PPO on the extent of light quenching.

Table II. Anisotropy Decay Parameters of TPB Fluorescence Induced by TPE at 20°C

Solvent	Light quenching	n^a	$r_0 g_i$	θ_i (ns)	χ^2_R
Hexadecane	−	1	0.358	0.242	1.8[b]
		2	0.028	0.022	
			0.342	0.251	1.8
	+	1	0.294	0.253	1.0
		2	0.024	0.018	
			0.271	0.269	0.9
Mineral oil	−	1	0.361	6.65	1.4
		2	0.034	2.67	
			0.328	7.43	1.2
	+	1	0.314	6.46	1.9
		2	0.017	0.87	
			0.301	7.09	1.1

[a]Number of components in the multiexponential fit.
[b]The uncertainties in the differential polarized phase angle and modulated anisotropy were taken as 0.2° and 0.005, respectively.

tropy is near the frozen solution value for TPE [1]. In the case of PPO in glycerol, where there is no light quenching (Fig. 8, bottom; – – x – –), the anisotropy is unchanged with laser power (Fig. 8, top; – – x – –). In contrast, the anisotropy of TPB decreases (–•–) with increasing laser power and light quenching. Evidently, under conditions used for TPE, some fluorophores display power-dependent values of the anisotropy due to orientation-dependent light quenching. This effect must be considered in any quantitative interpretation of the anisotropy value resulting from TPE.

DISCUSSION

Light quenching of fluorescence offers new opportunities for the use of fluorescence methods for study of the orientation and dynamics of fluorophores. The fact that light quenching displays the same photoselection as light absorption suggests that this phenomenon can be used to alter the orientational distribution of the excited-state population. While such modifications are possible using a single beam, the most promising opportunities involve separate excitation and quenching beams, possibly impinging on the sample along different axes. The fact that the intensity decays remained unchanged with significant amounts of light quenching suggests that the excited-state population can be modified by the quenching pulse without photodamage to the sample.

An important aspect of light quenching is that the effect is easily and rapidly reversible by blocking of the quenching beam. Hence, even small amounts of light

quenching will be detectable using lock-in techniques with frequency-selective detection.

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