

Light quenching of pyridine2 fluorescence with time-delayed pulses

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Abstract

We describe the effects of time-delayed long-wavelength pulses on the intensity and anisotropy decays of pyridine2. The sample was exposed to a continuous train of 360 nm excitation pulses and time-delayed 720 nm pulses. The long-wavelength pulses, which overlapped the emission spectrum of pyridine2, resulted in a spatially localized decrease in intensity at the point of beam overlap. The time-delayed quenching pulses caused a stepwise decrease in the intensity and anisotropy decays, as seen by oscillations in the frequency-domain data. The time-resolved anisotropy was shown to decrease below zero (-0.2) following the vertically polarized quenching pulse. The extent of light quenching depended on the time delay between the excitation and quenching pulses, and can be used to measure the decay time. Light quenching and/or multipulse methods may provide a new class of experiments for fluorescence spectroscopy and imaging. © 1997 Elsevier Science B.V.

Keywords: Light quenching; Pyridine2 fluorescence; Time-delayed pulses

1. Introduction

From the origin of fluorescence spectroscopy with G.G. Stokes [1] until about 1990, most fluorescence experiments were performed with relatively simple light sources and one-photon excitation. In recent years, the increasing availability of ps and fs lasers, with high peak intensities, has resulted in the possibility of new classes of experiments involving multiphoton excitation and/or the use of multiple laser beams with different wavelengths. Using pulsed lasers one can accomplish two-photon excitation where the fluorophores interact simultaneously with two photons of the same wavelength [2–10] or with different wavelengths [11–14]. During the past five years, two-photon excitation has been studied in a number of laboratories for investigations on the time-resolved fluorescence of biomolecules [15–18] and for localized excitation in fluorescence microscopy [19–21].

Abbreviations: FD frequency domain; Pyridine2 (1-ethyl-4-(4-*p*-dimethylaminophenyl)-1,3-butadienyl)pyridinium perchlorate

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We recently recognized an additional experimental opportunity of intense laser pulses. One can use laser pulses with wavelengths overlapping the emission spectrum to cause stimulated emission [22]. Our studies of light quenching are an extension of the pioneering efforts of Galanin and other Russian spectroscopists [23–26]. The phenomenon of light quenching is due to stimulated emission of the excited fluorophores. Since the stimulated photons travel parallel with the long-wavelength beam, the intensity observed with right-angle observation is decreased. Hence we refer to this phenomenon of fluorescence quenching by stimulated emission as light quenching. This phenomenon opens up the possibility of manipulating the excited-state population [22] to provide additional information in the time-dependent decays or to allow novel experimental configurations.

In the present paper we describe light quenching of pyridine2. This fluorophore was selected because its absorption and emission wavelengths are available with a titanium:sapphire laser, which is being used in fluorescence microscopy with multiphoton excitation [19–21]. We show that the light quenching is spatially localized at the region of beam overlap, and that the extent of quenching depends on the time delay between the excitation and quenching pulses. The extent of light quenching with various delay times can provide high time resolution limited only by the pulse widths of the excitation and quenching pulses. Importantly, time-delayed light quenching results in oscillations in the frequency-domain intensity and anisotropy decays, which to the best of our knowledge can have no origin other than a stepwise decrease in the excited-state intensity or anisotropy.

2. Materials and methods

The experimental arrangement for light quenching has been described previously [27]. The sample containing pyridine2 is placed in a standard $1 \times 1 \text{ cm}^2$ cuvette, and the emission was observed through a $200 \text{ }\mu\text{m}$ slit. In order to obtain locally intense illumination, the two beams were focused to about $20 \text{ }\mu\text{m}$ at the center of the cuvette using a laser-quality concave mirror with a focal length of 25 mm.

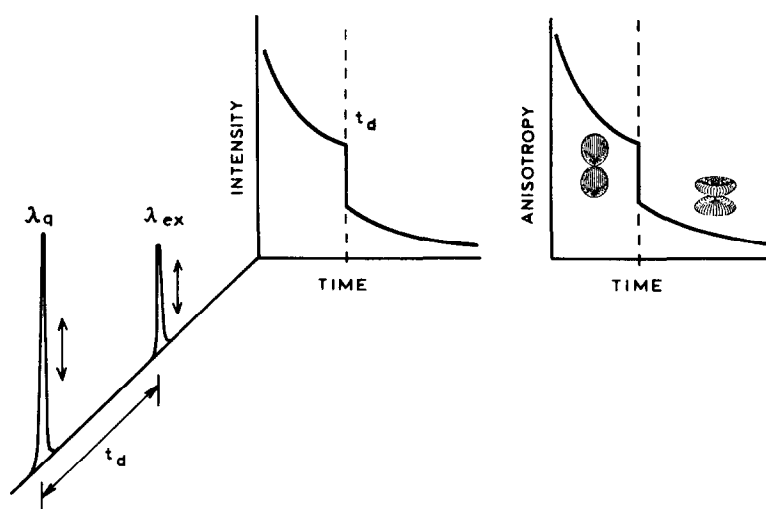
Excitation was provided by the frequency-doubled output of a cavity-dumped pyridine2 dye laser (360 nm). The light quenching beam was the fundamental output of the dye laser (720 nm). The pulse repetition rate near 2 MHz was obtained using a cavity dumper. The pyridine2 dye laser was synchronously pumped by a mode-locked argon ion laser.

For intensity and anisotropy decay measurements, the emission of pyridine2 was observed using a 680 nm interference filter, 10 nm bandpass, combined with cut-off glass filters. The concentration of pyridine2 was near $5 \times 10^{-6} \text{ M}$ in methanol, calculated from the extinction coefficient near $42000 \text{ M}^{-1} \text{ cm}^{-1}$ at the absorption maximum near 500 nm. Precise alignment of the beams was essential for the experiment. The optical arrangement was placed in a 10 GHz frequency-domain fluorimeter [28]. Emission spectra were obtained using an optical fiber to bring the emission to a steady-state fluorimeter. The optical delay between the excitation (360 nm) and quenching (720 nm) pulses were controlled by a hollow retroreflector placed on a precise optical rail.

3. Theory

3.1. Theory for time-resolved two-beam light quenching

We describe a phenomenological theory for light quenching by a time-delayed quenching pulse. In a two-pulse light quenching experiment, the sample is illuminated by two linearly polarized light pulses of different wavelength with delay time t_d (Scheme 1). The first pulse results only in excitation, and the second pulse results only in light quenching. These pulses are called the excitation and quenching pulses, respectively. We assume that the pulse widths are small compared to the excited-state lifetime and the correlation time. If the



Scheme 1. Intuitive description of the effect of a time-delayed quenching pulse on the intensity and the anisotropy decay. The excitation and quenching pulses are vertically polarized along the z -axis.

absorption and emission dipoles of the fluorophore are parallel, then immediately following the excitation pulse the angular distribution of the excited state population is given by

$$n_0(\theta) = NW_{\text{ex}} \sigma_a \cos^2 \theta \quad (1)$$

where N is the number of ground state fluorophores, W_{ex} is the energy density (photons per centimeter squared) of the excitation pulse, θ is the angle between the transition moment and the z -axis (Scheme 1) and σ_a is the cross-section for absorption. The shape of the time-zero distribution, shown in Scheme 1, displays z -axis symmetry and is determined by $\cos^2 \theta$ due to the usual photoselection properties of optical transitions.

Until the quenching pulse arrives, the shape of the distribution relaxes toward a sphere due to the rotational diffusion. Quenching by the delayed pulse is also governed by the same $\cos^2 \theta$ photoselection rule which occurs in excitation. The shape of the excited-state angular distribution after the second pulse is strongly dependent on the mutual orientation of the electric vectors in the excitation and quenching pulses, and on the rate of the rotational diffusion in the sample. A more complete description of these effects on the steady-state intensities and anisotropies will be given elsewhere [29].

The effects of light quenching by a time-delayed pulse can be modeled in phenomenological terms. Assume that the intensity and anisotropy decay with a lifetime τ and a correlation time θ , respectively, and that the anisotropy in the absence of rotational diffusion is given by r_0 . Assume also that the time-delayed pulse results in an instantaneous fractional decrease in intensity described by

$$q = \frac{I_b - I_a}{I_b} \quad (2)$$

where I_b and I_a are the intensities immediately before and after the quenching pulse. The fluorescence intensity decay then has the form

$$I(t) = \begin{cases} I_0 e^{-t/\tau} & \text{for } 0 \leq t \leq t_d \\ I_0(1 - q) e^{-t/\tau} & \text{for } t > t_d \end{cases} \quad (3)$$

The apparent lifetime τ is thus expected to be unchanged if one examines only the time-dependent decay before or after the quenching pulse. However, the intensity decay is no longer an exponential decay, but shows a step

decrease at $t = t_d$. The mean decay time, including times before and after the quenching pulse, is decreased by light quenching.

In the present paper we use frequency-domain methods to measure the time-dependent decays. For any decay law the phase angle (ϕ_ω) and modulation (m_ω) at each modulation frequency (ω ; rad s⁻¹) can be computed from

$$\phi_\omega = \arctan \left(\frac{N_\omega}{D_\omega} \right) \quad (4)$$

$$m_\omega = \frac{1}{J} \sqrt{N_\omega^2 + D_\omega^2} \quad (5)$$

where N_ω and D_ω are the sine and cosine transforms of the impulse response function [30]

$$N_\omega = \int_0^\infty I(t) \sin(\omega t) dt \quad (6)$$

$$D_\omega = \int_0^\infty I(t) \cos(\omega t) dt \quad (7)$$

$$J = \int_0^\infty I(t) dt \quad (8)$$

For the decay law described by Eq. (3)

$$N_\omega = I_0 \frac{\tau}{1 + \omega^2 \tau^2} \left\{ \omega \tau - [\sin(\omega t_d) + \omega \tau \cos(\omega t_d)] q e^{-t_d/\tau} \right\} \quad (9)$$

$$D_\omega = I_0 \frac{\tau}{1 + \omega^2 \tau^2} \left\{ 1 - [\cos(\omega t_d) - \omega \tau \sin(\omega t_d)] q e^{-t_d/\tau} \right\} \quad (10)$$

$$J = I_0 \tau (1 - q e^{-t_d/\tau}) \quad (11)$$

Eq. (4)–(11) predict the phase and modulation of the emission measured relative to the first excitation pulse. These expressions can be used to predict the frequency response for any value of t_d , q or τ . At lower and middle modulation frequencies, the frequency response predicted by Eq. (4) and (5) for $q > 0$ is shifted toward higher frequencies, reflecting the decrease in the average time a fluorophore spends in the excited state. At higher modulation frequencies, the step changes in fluorescence intensity caused by the delayed pulse result in oscillations in the frequency response [27].

The frequency-domain anisotropy data in the presence of a time-delayed quenching beam can be predicted in a similar manner to that for the intensity data. The time-delayed pulse results in a photoselective decrease in the excited-state population. If this pulse is oriented along the z -axis (Scheme 1), then those fluorophores whose transition moments are similarly aligned will be preferentially quenched, resulting in a decrease in the anisotropy (Scheme 1, far right). Assume that the anisotropy prior to any rotational motion is r_0 , and that the rotational diffusion is isotropic with a correlation time Θ . The anisotropy decay then has the form

$$r(t) = \begin{cases} r_0 e^{-t/\Theta} & \text{for } 0 \leq t \leq t_d \\ (r_0 + \Delta r) e^{-t/\Theta} & \text{for } t > t_d \end{cases} \quad (12)$$

where $\Delta r = r_a - r_b$ is the change in anisotropy from the values immediately before (r_b) and after (r_a) the quenching pulse.

For calculation of the anisotropy decay it is necessary to use the decays of the parallel and perpendicular components of the fluorescence emission. These components are given by

$$I_{\parallel}(t) = \begin{cases} \frac{1}{3}I_0 e^{-t/\tau} (1 + 2r_0 e^{-t/\theta}) & \text{for } 0 \leq t \leq t_d \\ \frac{1}{3}(1-q)I_0 e^{-t/\tau} [1 + 2(r_0 e^{-t_d/\theta} + \Delta r) e^{-(t-t_d)/\theta}] & \text{for } t > t_d \end{cases} \quad (13)$$

$$I_{\perp}(t) = \begin{cases} \frac{1}{3}I_0 e^{-t/\tau} (1 - r_0 e^{-t/\theta}) & \text{for } 0 \leq t \leq t_d \\ \frac{1}{3}(1-q)I_0 e^{-t/\tau} [1 - (r_0 e^{-t_d/\theta} + \Delta r) e^{-(t-t_d)/\theta}] & \text{for } t > t_d \end{cases} \quad (14)$$

In Eq. (13) and (14), $I_{\parallel}(t)$ and $I_{\perp}(t)$ denote intensities which are polarized parallel or perpendicular to the polarization direction of the excitation pulse, respectively. The frequency-dependent phase difference $\Delta\omega$ between the perpendicular and parallel components of the modulated emission and the ratio A_{ω} of the a.c. amplitudes of the components can be calculated from Eq. (13) and (14), and the well known expressions for differential polarization phase and modulation data [31]. These complete expressions for Eq. (13) and (14) are presented elsewhere [27]. As for the intensity responses, the calculated anisotropy frequency responses also show oscillations at higher frequencies.

4. Results

4.1. Selection of pyridine2 for light quenching

While our experiments were performed with a ps dye laser, the probe pyridine2 was selected for its compatibility with the fundamental and frequency-doubled outputs of a titanium:sapphire laser. Absorption and emission spectra of pyridine2 are shown in Fig. 1. Pyridine2 displays an emission maxima near 720 nm, with a tail to over 850 nm. This allows use of the pyridine2 dye laser to quench the emission by illumination at 720 nm. These wavelengths are also readily available from the fundamental and frequency-doubled outputs of titanium:sapphire lasers. Light quenching requires precise timing of the quenching beam relative to the excitation beam. The requirement is met by using the frequency-doubled and fundamental outputs of the dye laser for excitation and quenching, respectively. The emission can be observed above or below this wavelength using a monochromator or interference filter without interference from the longer-wavelength quenching beam.

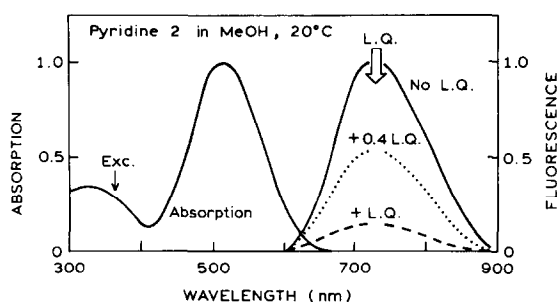


Fig. 1. Absorption and emission spectra of pyridine2. Emission spectra are shown without (—) and with increasing amounts of light quenching.

4.2. Steady-state measurements of light quenching

We first examined whether the steady-state intensity of pyridine2 could be decreased by illumination at 720 nm (Fig. 1). For these measurements the sample was illuminated with the pulse train of 360 nm excitation pulses, or the combined pulse train of both 360 nm excitation and 720 nm quenching pulses. We then measure the steady-state intensity in a spectrofluorimeter. Observation of light quenching requires careful overlap of the two beams, which is determined by the decrease in intensity at 720 nm. Simultaneous illumination at 360 and 720 nm results in a decrease in intensity which is proportional to the intensity of the quenching beam (Fig. 1). This effect was completely and immediately reversible upon blocking the quenching beam. The emission spectra are nearly identical in the absence and presence of light quenching. We recognize that the presence or absence of spectral shifts with light quenching may provide information on the rates of solvent relaxation, and such shifts have been observed [32]. In other studies of light quenching of other fluorophores we showed that the extent of quenching was proportional to the amplitude of the emission spectrum at the quenching wavelength.

4.3. Spatial distribution of quenching

In a previous review [22] we compared the features of collisional quenching and light quenching. One distinctive feature of light quenching is localization of the quenching effect to the focal point of the quenching beam. This feature is demonstrated experimentally in Fig. 2, in which we measured the local intensity of pyridine2 along the excitation beam. In the absence of the 720 nm quenching beam the intensity is constant, as is expected for a dilute (5×10^{-6} M) solution with an optical density near 0.02 at the excitation wavelength. Upon illumination with the quenching beam the intensity of pyridine2 is decreased, but only at the focal point and region of beam overlap (Fig. 2). This unusual spatial distribution can only be the result of localized quenching by the intense 720 nm illumination. This effect has already been used in fluorescence microscopy to provide an amplitude- modulated signal due to two overlapping laser beams [33,34].

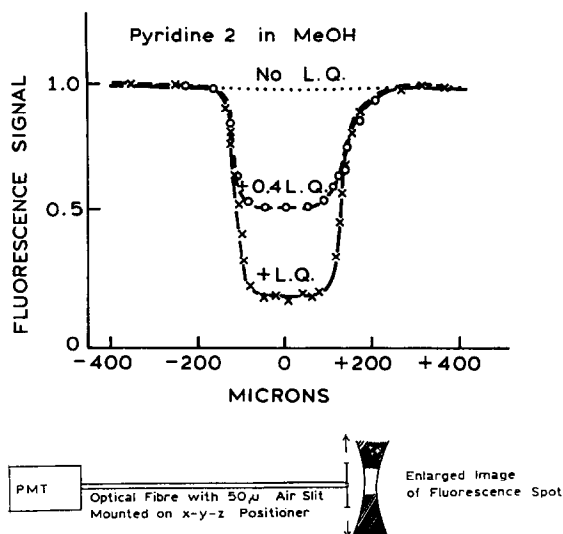


Fig. 2. Spatial distribution of the emission of pyridine2 with light quenching.

4.4. Frequency-domain measurements of light quenching

The intuitive description of light quenching (Scheme 1) predicts stepwise changes in the intensity and anisotropy decays upon arrival of the quenching pulse. Such step changes are predicted to cause oscillations in the frequency-domain intensity decays [27]. To the best of our knowledge, no other process other than a step change can result in such oscillations. Hence, observation of an oscillating frequency response is an unambiguous demonstration of light quenching by a time-delayed light pulse.

Frequency-domain intensity decay data for pyridine2 in methanol are shown in Fig. 3. In the absence of the time-delayed quenching pulse ($-\cdot-$) the FD data show the usual monotonic changes in phase and modulation with increasing frequency. These data are consistent with a single exponential intensity decay of pyridine2 with a decay time of 301 ps (Table 1).

Illumination of the sample with the long-wavelength pulses causes a remarkable change in shape. The frequency response ($-\circ-$) now shows oscillations in the phase angle and somewhat less visible oscillations in these modulations. It is impossible to fit these data to the single exponential model (Table 1). If the time delay is changed, the shape of the FD data changes, with an apparent change in the oscillation frequency (not shown). In the presence of light quenching, the phase angles can exceed 90° , which is not possible for a single exponential decay. The FD data returned to the single exponential shape upon blocking the quenching beam.

The oscillating FD data can be analyzed in terms of the model which accounts for the step changes due to the quenching pulse. Least-squares analysis of the intensity decay data for pyridine2 with and without light quenching are summarized in Table 1. In all cases, we recovered the expected value of the delay time t_d , and the unquenched lifetime τ , and reasonable values for the extent of quenching q . Importantly, use of the light quenching model (Eq. (2) and (3)) results in a good fit to the experimental data, as seen from the dashed lines in Fig. 3. These results suggest that it will be readily possible to perform quantitative measurements of light quenching, and that the time delay and extent of quenching can be reliably recovered from the FD data.

4.5. Anisotropy decays with light quenching

Time-delayed light quenching, with a vertically polarized quenching beam, is expected to result in a step decrease in the anisotropy decay. Such changes can be detected from frequency-domain measurements of the anisotropy decay. In the absence of light quenching the FD anisotropy decay reveals a single correlation time of 97 ps. Such an anisotropy decay is characterized by a Lorentzian-like distribution of phase angles and a modulated anisotropy which increases monotonically with increasing modulation frequency (Fig. 4). Illumina-

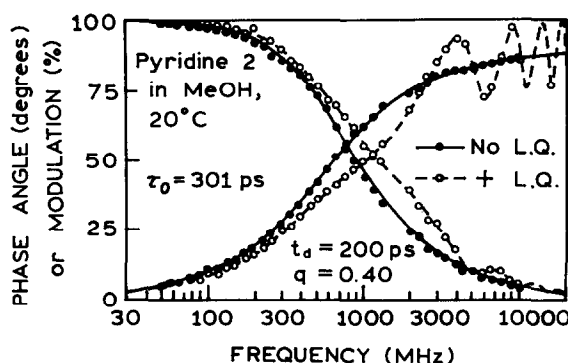


Fig. 3. Frequency-domain intensity decays of pyridine2 ($-\cdot-$) without and ($-\circ-$) with a time-delayed light quenching pulse.

Table 1
Pyridine intensity decay analysis^a in the absence and presence of light quenching

Expected			Found			
τ (ps)	t_d (ps)	L.Q.	τ (ps)	t_d (ps)	q	χ_R^2
301	–	No	301	–	$\langle 0 \rangle^b$	2.9 ^c
301	200	Yes	$\langle 301 \rangle$	$\langle 200 \rangle$	$\langle 0 \rangle$	302.6
301	200	Yes	229	200	$\langle 0 \rangle$	153.7
301	200	Yes	$\langle 301 \rangle$	$\langle 200 \rangle$	0.40	2.7
301	200	Yes	299	201	0.39	2.7

^a In methanol at 20°C.

^b The values of the parameter in angular brackets $\langle \rangle$ was kept constant during the analyses.

^c The uncertainties were $\delta\phi = 0.3^\circ$ and $\delta_m = 0.007$ [30].

tion with the time-delayed 720 nm pulse results in dramatically different FD anisotropy data (Fig. 5). In the presence of the light quenching pulses the FD anisotropy data display oscillations in both the differential phase angles and modulation.

The data in Fig. 5 can be used to recover the time delay and magnitude of the change in anisotropy due to the quenching pulse. This analysis (Table 2) indicates that the anisotropy change is -0.25 , and that the anisotropy becomes negative upon arrival of the quenching pulse. The recovered parameters were used to reconstruct the time-domain anisotropy decay shown as an insert in Fig. 5.

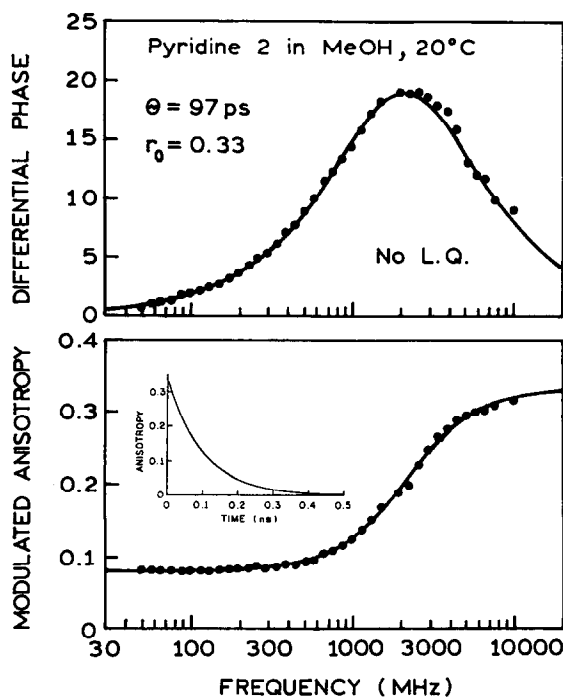


Fig. 4. Frequency-domain anisotropy decay of pyridine2 in methanol without light quenching.

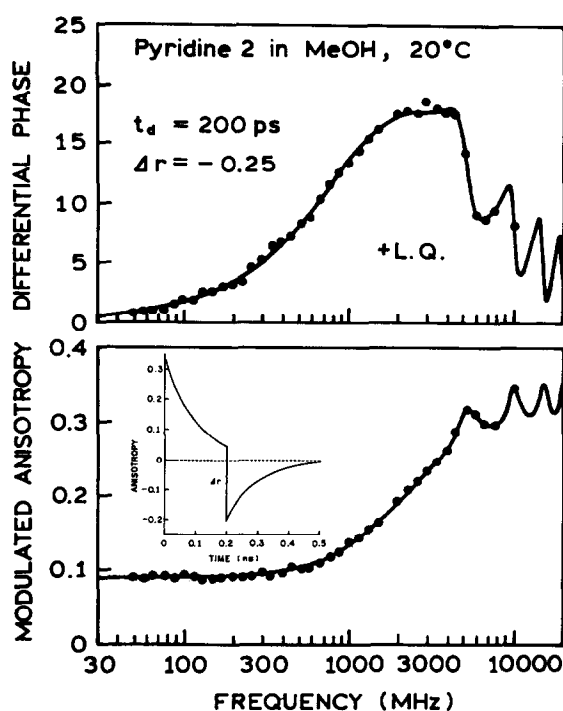


Fig. 5. Frequency-domain anisotropy decay of pyridine2 with a time-delayed quenching pulse. The excitation and quenching pulses were vertically polarized.

4.6. Intensity decay measurements by light quenching

Another opportunity of light quenching is the ability to measure intensity decays without the limitations due to the finite time response of the detectors. Consider a sample which is simultaneously exposed to a pulse train for excitation and a second pulse train of quenching pulses at the same repetition rate, and that one observes the steady-state intensity. The basic idea is that the extent of light quenching will depend on the number of fluorophores which are in the excited state and thus available for light quenching. Hence, one expects light quenching to be maximal when the light quenching pulse arrives immediately after the excitation pulse, and no

Table 2
Anisotropy decay analysis of pyridine2^a in the absence and presence of light quenching

Expected			Found			
θ (ps)	t_d (ps)	L.Q.	θ (ps)	t_d (ns)	q	χ_R^2
97	–	No	97	–	$\langle 0 \rangle$ ^b	3.5 ^c
97	200	Yes	$\langle 97 \rangle$	$\langle 200 \rangle$	$\langle 0 \rangle$	38.8
97	200	Yes	101	200	$\langle 0 \rangle$	36.7
97	200	Yes	$\langle 97 \rangle$	$\langle 200 \rangle$	0.40	2.8
97	200	Yes	100	200	0.46	1.7

^a In methanol at 20°C.

^b The values of the parameter in parentheses $\langle \rangle$ was kept constant during the analyses.

^c The uncertainties were $\delta\Delta = 0.3^\circ$ and $\delta A = 0.007$ [31].

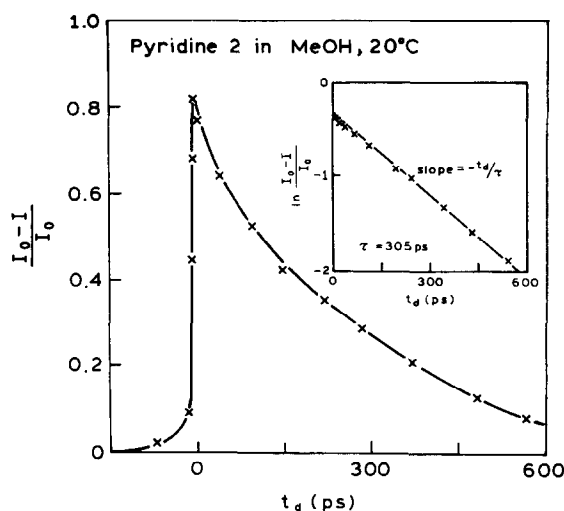


Fig. 6. Effect of the time delay on the extent of light quenching of pyridine2.

light quenching is expected if the quenching pulse arrives after the excited state has completed its spontaneous decay. Hence, the effect of various time delays t_d on the extent of quenching should reveal the decay time of the fluorophore.

The effect of the delay time on the steady-state intensity of pyridine2 is shown in Fig. 6. The intensity changes are shown as $(I_0 - I)/I_0$ where I_0 and I are the intensities in the absence and presence of the light quenching pulses (Fig. 6). The extent of quenching, as measured by $(I_0 - I)/I_0$, appears to decrease exponentially with time. In fact, one can show [22] that

$$\frac{I_0 - I}{I_0} = q \exp(-t_d/\tau) \quad (15)$$

A plot of $\ln[(I_0 - I)/I_0]$ versus delay time t_d yields the decay time of 305 ps, which is in excellent agreement with the directly measured value of 301 ps (Fig. 3).

5. Discussion

What are the potential applications of light quenching in biochemical research? One application is illustrated by the measurement of decay times by varying the delay time of the quenching pulses (Fig. 6). While the decay time of pyridine2 is readily measurable with a high-speed detector, many rapid processes are difficult to resolve with direct detection. The use of steady-state measurements with various delay times circumvents the need for high-speed detection, and the time resolution becomes limited only by the pulse width of the excitation and quenching pulses. Hence, one can expect light quenching with various time delays to be valuable in studies of phycobiliproteins [35,36] and visual pigments in which excitation results in rapid energy transfer and/or photochemical isomerization. Time-delayed light quenching may also be valuable in studies of rapid spectral relaxation in fluid solvents [37–39] or intensity oscillations due to coherence phenomena or optical dephasing [40–43].

One advantage of light quenching is derived from the use of two laser beams and spatial localization of the quenched fluorophores (Fig. 2). The volume element exposed to the quenching beam can be positioned as

desired within the sample. This capability has already been productively used in fluorescence microscopy to provide the equivalent of confocal conditions at the point of laser beam intersection [33,34].

Another advantage of light quenching is that it can be effective under conditions when collisional quenching is not practical. In contrast to collisional quenching, light quenching can occur in viscous media, and the effect is immediately reversible upon blocking the quenching beam. Light quenching may be possible for fluorophores which are buried within macromolecules and thus shielded from the solvent.

It is also possible that additional information will become available by experiments with multiple light pulses. For instance, we have shown (Fig. 5) that polarized light quenching can change the orientation of the excited-state population. Depending upon the experimental conditions, the polarization of the excited state can be changed from -1.0 to $+1.0$ [22]. For example, if for some reason, the studied system has a zero or very low anisotropy, the use of light quenching with a polarized quenching beam can induce anisotropy. Hence, such system can be further studied by time-resolved fluorescence. It also seems possible that measurements of the anisotropy decays under such conditions will reveal additional information on the rotational motions of non-spherical molecules. The quenching with perpendicular polarization can create an asymmetric distribution of excited dipoles [45]. Anisotropy studies of such systems require a redefinition of the anisotropy, that is the anisotropy in systems without z -axis symmetry should be treated as a vector [29].

Can light quenching be used for biomolecules such as tryptophan? The application of light quenching to proteins is limited by the availability of appropriate wavelengths and pulse power. One-pulse light quenching (where excitation and quenching occur within the same pulse) has been already applied to study a mixture of single tryptophan proteins [46]. Two-pulse light quenching was also used to remove unwanted fluorescence from a dissolved fluorophore in the presence of the same fluorophore bound to human serum albumin. Hence, using suitable wavelengths and pulse energies most biomolecules can be studied with light quenching.

One can question whether strong light pulses can lead to photobleaching. In light quenching experiments the concentration of fluorophore is low, the excitation is (in contrast to other pump–probe methods) weak as in conventional fluorescence measurements, and the quenching pulses are not absorbed (except for multiphoton transitions). In fact, stimulated emission returns the molecules to the ground state, so photoprocesses in the excited state are less effective when light quenching is used.

And finally we note that technological advances in commercially available lasers result in the exotic experiments described above being readily accomplished in many laboratories. For instance, two-photon excitation has recently been reported with a semiconductor laser [44], so that one can imagine light quenching being performed with multiwavelength solid-state light sources. Light quenching can result in a new class of fluorescence experiments in which the excited-state population is prepared for measurement by a series of excitation and/or quenching pulses.

Acknowledgements

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