Published in final edited form as:

J Phys Chem. 1994 September 1; 98(36): 8886–8895. doi:10.1021/j100087a012.

Light Quenching of Fluorescence Using Time-Delayed Laser Pulses As Observed by Frequency-Domain Fluorometry

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Abstract

We describe experimental observations of fluorescence quenching by time-delayed light pulses whose wavelength overlaps the emission spectrum of 4-(dimethylamino)-4'-cyanostilbene (DCS). The relative cross sections for light quenching were proportional to the amplitude of the emission spectra at the light quenching wavelength. The frequency-domain intensity and anisotropy decay measurements showed oscillations resulting from time-delayed light quenching. The amplitude of the oscillations depends upon the amount of light quenching. The frequency of the oscillations depends upon the delay between the excitation and quenching pulses. To the best of our knowledge, only a stepwise decrease in the intensity or anisotropy could produce such oscillations in the frequency-domain data. Light quenching of fluorescence is thus shown to provide a means to control the number and orientation of the excited fluorophores. The use of multiple light pulses for excitation and quenching can have far-reaching applications in the use of time-resolved fluorescence in physical chemistry and biophysics.

Introduction

Modern pulsed laser light sources and high-speed detectors have resulted in remarkable sensitive and resolution of time-dependent processes.^{1–4} With the exception of pump-probe and up-conversion measurements,⁵ most studies of time-resolved fluorescence use the picosecond or femtosecond laser pulses only for excitation but do not take advantage of the opportunities available created by the temporarily intense illumination. This situation is now changing, as seen by the recent reports on the effects of intense illumination on the ground-state fluorophore populations,⁶ theoretical^{7–9} and experimental^{10–16} studies of two-photon-induced fluorescence (TPIF), and the use of TPIF in fluorescence microscopy.^{17, 18}

Two-photon-induced fluorescence (TPIF) requires intense laser pulses to allow two long wavelength photons to be simultaneously absorbed by the fluorophore. The use of intense laser pulses raises the possibility of stimulated emission from the excited-state population (Scheme 1). Since fluorescence is typically observed at a right angle to the direction of excitation, the stimulated portion of the emission is not observed, and the sample appears to

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be quenched, a phenomenon we call “light quenching.” We note that light quenching requires that the quenching wavelength overlap the emission spectra of the fluorophore.¹⁹ Hence, observation of light quenching requires special conditions, such as a fluorophore which can be excited and quenched using a single laser beam at a single wavelength^{20, 21} or the use of two-photon excitation such that the excitation wavelength overlaps the emission spectrum of the fluorophore.²² In the case of one- and two-photon excitation, light quenching is detected by a sublinear or subquadratic dependence of the fluorescence intensity on the incident power, respectively. Light quenching by a single beam of pulses also results in a decrease in the time-zero anisotropy but no change in the fluorescence lifetime or rotational correlation time. The decrease in the time-zero anisotropy is the result of the photoselective nature of light quenching by stimulated emission.^{20, 23} The lifetime and correlation times are unchanged in a single-beam experiment because the excitation and quenching occur within a single picosecond laser pulse, and the time-dependent decays after the quenching pulse are not affected.

In the present report we describe the effects of a time-delayed quenching pulse (Scheme 2) on the intensity and anisotropy decay of a suitable fluorophore. The excitation and quenching wavelengths are selected to overlap with the absorption and emission spectra of the fluorophore, respectively. Suppose the second quenching pulse appears at a time $t = t_d$ following excitation and is adequately intense to cause stimulated emission. Then one expects the excited state population or intensity to display a step decrease at time t_d . Following the step decrease due to the quenching pulse, the fluorophores continue to decay as usual due to spontaneous emission. Additionally, it is known that light quenching displays the same photoselection properties as does light absorption.^{20, 23} Consequently, if the quenching beam is vertically polarized, there is selective quenching of the vertically oriented part of the excited-state population and a step decrease in the anisotropy (Scheme 2).

In the present report we described our first observations of light quenching by time-delayed pulses. Light quenching was observed by a decrease in the steady-state intensity upon illumination with the quenching beam and by the frequency-domain measurements of the intensity and anisotropy decays. Remarkably, the frequency-domain intensity and anisotropy decay data display oscillations, which we show are the result of the step changes in intensity and anisotropy. Light quenching using longer wavelength illumination thus offers a means to control the excited-state population and orientation of fluorophores. In our opinion, this initial observation of light quenching points the way to a new class of fluorescence experiments, using multiple light pulses, to modify the excited-state population during or prior to data acquisition.

Theory

Theory for Time-Resolved Two-Beam Light Quenching.

A complete description of light quenching is beyond the scope of the present report. We now present a phenomenological theory for light quenching by time-delayed quenching pulse. In a two-pulse light quenching experiment the sample is illuminated by two consecutive linearly polarized light pulses of different wavelength with the delay time t_d (Scheme 2). We assume that the first pulse results only in excitation and the second pulse results only in light

quenching. These pulses are thus understood as the excitation and quenching pulses, respectively.

We assume that the pulse widths are small compared to the excited-state lifetime and correlation time. If the absorption and emission dipole 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/cm²) of the excitation pulse, θ is the angle between the transition movement and the z axis (Scheme 1), and σ_a is the cross section for absorption. The shape of the time-zero distribution displays z -axis symmetry and is determined by $\cos^2 \theta$ due to the usual photoselection properties of optical transitions (Scheme 2). Until the quenching pulse arrives, the shape of the distribution relaxes toward a sphere due to the rotational diffusion. If the rotational diffusion correlation time θ is much larger than the delay time t_d , then the distribution at time $t = t_d$ is still described by $\cos^2 \theta$. In the opposite case, when $\theta \ll t_d$, the distribution at time $t = t_d$ becomes practically spherical. As for excitation, quenching by the delayed pulse is also governed by the $\cos^2 \theta$ photoselection rule. This means that 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. Keeping the intensity and wavelength of the pulses fixed, one can obtain different absolute and relative changes in the intensity and anisotropy of the sample after the quenching pulse, depending on the sample's viscosity and the geometry of experimental setup. A more quantitative description of these effects on the steady-state intensities and anisotropies will be given elsewhere.²⁴

The effects of light quenching by a time-delayed pulse can be modeled in simple 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 that the time-delayed pulse results in an instantaneous fractional decrease in intensity described by

$$q = (I_b - I_a)/I_b \quad (2)$$

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

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

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 mean lifetime of the excited state ($\bar{\tau}$) and the mean intensity ($\bar{I}$) are decreased by the time-delayed quenching pulse and are given by

$$\bar{\tau} = \tau \frac{1 - (1 + t_d/\tau)qe^{-t_d/\tau}}{1 - qe^{-t_d/\tau}} \quad (4)$$

$$\bar{I} = \bar{I}_0 \left(1 - qe^{-t_d/\tau}\right) \quad (5)$$

where $\bar{I}_0$ is the mean intensity observed in the absence of light quenching. Hence, the mean lifetime may be shortened by the time-delayed pulse, which in turn can alter the extents of spectral relaxation or rotational diffusion.^{25, 26}

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) can be computed from

$$\phi_\omega = \arctan(N_\omega/D_\omega) \quad (6)$$

$$m_\omega = (1/J)\sqrt{N_\omega^2 + D_\omega^2} \quad (7)$$

where N_ω and D_ω are the sine and cosine transforms of the impulse response function,²⁷

$$N_\omega = \int_0^\infty I(t) \sin(\omega t) dt \quad (8)$$

$$D_\omega = \int_0^\infty I(t) \cos(\omega t) dt \quad (9)$$

For the decay law described by eq 3

$$J = \int_0^\infty I(t) dt \quad (10)$$

$$N_\omega = I_0 \frac{\tau}{1 + \omega^2 \tau^2} \left\{ \omega \tau - [\sin(\omega t_d) + \omega t_d \cos(\omega t_d)] q e^{-t_d/\tau} \right\} \quad (11)$$

$$D_\omega = I_0 \frac{\tau}{1 + \omega^2 \tau^2} \left\{ 1 - [\cos(\omega t_d) - \omega t_d \sin(\omega t_d)] q e^{-t_d/\tau} \right\} \quad (12)$$

$$J = I_0 \tau (1 - q e^{-t_d/\tau}) \quad (13)$$

Equations 6–13 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 values of t_d , q , or τ . At lower and middle modulation frequencies the frequency response predicted by eqs 6 and 7 for $q > 0$ is shifted toward higher frequencies, and for $q < 0$ the frequency response is shifted toward lower frequencies, in both cases compared to the response obtained for $q = 0$. For better understanding this behavior one has to notice that for $q > 0$ the fraction of the long-lived molecules in the sample is decreased, and for $q < 0$ this fraction is increased by the delayed pulse. These fractional changes cause a respective decrease or increase of the average lifetime and also the above shifts of the frequency response.

The step changes in fluorescence intensity caused by the delayed pulse result in oscillations in the frequency response at higher modulation frequencies. In the limit of high frequency the amplitude of the phase oscillations becomes constant. The properties of these oscillations may be analyzed using the approximated formula for ϕ_ω obtained from eqs 6, 11, and 12, with the condition $\omega\tau \gg 1$

$$\phi_\omega = \arctan\left(\frac{1 - \cos(\omega t_d)q_f}{\sin(\omega t_d)q_f}\right) \quad (14)$$

where

$$q_f = qe^{-t_d/\tau} \quad (15)$$

The parameter q_f has a meaning of the fractional decrement of the area under the decay curve, caused by the quenching pulse. Using similar notation as for parameter q (eq 2), the parameter q_f can be also understood as

$$q_f = (I_b - I_a)/I_0 \quad (16)$$

In our analysis we will limit ourselves to the cases when $-1 \leq q_f \leq 1$, which involve all cases of light quenching ($0 \leq q_f \leq 1$) and the cases of small to moderate enhancement of emission ($-1 \leq q_f \leq 0$). The phase shift ϕ_ω given by eq 14 reaches its extreme (e) values (minimum or maximum) for frequencies $f_e = \omega_e/(2\pi)$ fulfilling the condition

$$\cos(\omega_e t_d) = q_f \quad (17)$$

Using this condition, one can show that for $\omega\tau \gg 1$ the minima and maxima of the phase angle ϕ_ω are placed at frequencies given by the following equations

$$f_n^{\min} = \frac{1}{t_d} \left[n + \frac{q_f \arccos(q_f)}{|q_f| 2\pi} \right] \quad (18)$$

$$f_n^{\max} = \frac{1}{t_d} \left[n - \frac{q_f \arccos(q_f)}{|q_f| 2\pi} \right] \quad (19)$$

where n are positive integers. It is seen from the above equations that the frequency difference, Δf , between two consecutive minima or maxima is given by

$$\Delta f = 1/t_d \quad (20)$$

After substitution of eq 17 into eq 14, one obtains the following expression for the phase values at the minima and maxima of the oscillations

$$\phi_{\omega}^{\min} = \arctan\left(\frac{\sqrt{1 - q_f^2}}{|q_f|}\right) \quad (21)$$

$$\phi_{\omega}^{\max} = \pi - \phi_{\omega}^{\min} \quad (22)$$

The amplitude of the phase oscillations is given by

$$A_{\phi} = \pi/2 - \phi_{\omega}^{\min} \quad (23)$$

One can see from eqs 21–23 that for $-1 \leq q_f \leq 1$ the maximum value of the amplitude A_{ϕ} is $\pi/2$. In this case, the oscillating phase angles span the range from 0 to π .

The frequency-domain anisotropy data in the presence of a time-delayed quenching beam can be predicted in a similar manner as 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 2). 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

$$r(t) = \begin{cases} r_0 e^{-t/\theta} & \text{for } 0 \leq t \leq t_d \\ \left(r_0 + \Delta r e^{t_d/\theta}\right) e^{-t/\theta} & \text{for } t \geq t_d \end{cases} \quad (24)$$

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

$$I_{\parallel}(t) = \begin{cases} \frac{1}{3} I_0 e^{-t/\tau} [1 + 2r_0 e^{-t/\theta}] & \text{for } 0 < t \leq t_d \\ \frac{1}{3} (1 - q) I_0 e^{-t/\tau} \left[1 + 2 \left(r_0 e^{-t_d/\theta} + \Delta r \right) e^{-(t-t_d)/\theta} \right] & \text{for } t > t_d \end{cases} \quad (25)$$

$$I_{\perp}(t) = \begin{cases} \frac{1}{3}I_0 e^{-t/\tau} [1 + r_0 e^{-t/\theta}] & \text{for } 0 < t \leq t_d \\ \frac{1}{3}(1-q)I_0 e^{-t/\tau} \left[1 - \left(r_0 e^{-t_d/\theta} + \Delta r \right) e^{-(t-t_d)/\theta} \right] & \text{for } t > t_d \end{cases} \quad (26)$$

In eqs 25 and 26 $I_{\parallel}(t)$ 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 Δ_{ω} between the perpendicular and parallel components of the modulated emission and the ratio Λ_{ω} of the ac amplitudes of the components can be calculated from eqs 25 and 26 and the well-known expressions for differential polarization phase and modulation data.^{28–30}

$$\Delta_{\omega} = \arctan\left(\frac{D_{\parallel}N_{\perp} - N_{\parallel}D_{\perp}}{N_{\parallel}N_{\perp} + D_{\parallel}D_{\perp}}\right) \quad (27)$$

$$\Lambda_{\omega} = \frac{\sqrt{N_{\parallel}^2 + D_{\parallel}^2}}{\sqrt{N_{\perp}^2 + D_{\perp}^2}} \quad (28)$$

where the quantities $N_{\parallel}$, $N_{\perp}$, $D_{\parallel}$, and $D_{\perp}$ are defined by

$$N_{\parallel} = \int_0^{\infty} I_{\parallel}(t) \sin(\omega t) dt \quad (29)$$

$$D_{\parallel} = \int_0^{\infty} I_{\parallel}(t) \cos(\omega t) dt \quad (30)$$

Substitution of the decay intensities (25) and (26) into eqs 29 and 30 yields

$$N_{\parallel} = \frac{1}{3}I_0(n_1 + 2n_2) \quad (31)$$

$$D_{\parallel} = \frac{1}{3}I_0(d_1 + 2d_2) \quad (32)$$

$$N_{\perp} = \frac{1}{3}I_0(n_1 - n_2) \quad (33)$$

$$D_{\perp} = \frac{1}{3}I_0(d_1 - d_2) \quad (34)$$

where the quantities n_i and d_i are expressed as

$$n_1 = \frac{\tau}{1 + \omega^2 \tau^2} \left\{ \omega \tau - [\sin(\omega t_d) + \omega \tau \cos(\omega t_d)] q e^{-t_d/\tau} \right\} \quad (35)$$

$$n_2 = \frac{H}{1 + \omega^2 H^2} \left\{ r_0 \omega H - [\sin(\omega t_d) + \omega H \cos(\omega t_d)] \text{Re}^{-t_d/H} \right\} \quad (36)$$

$$d_1 = \frac{\tau}{1 + \omega^2 \tau^2} \left\{ 1 - [\cos(\omega t_d) - \omega \tau \sin(\omega t_d)] q e^{-t_d/\tau} \right\} \quad (37)$$

$$d_2 = \frac{H}{1 + \omega^2 H^2} \left\{ r_0 - [\cos(\omega t_d) - \omega H \sin(\omega t_d)] \text{Re}^{-t_d/H} \right\} \quad (38)$$

with

$$\frac{1}{H} = \frac{1}{\tau} + \frac{1}{\theta} \quad (39)$$

$$R = r_0 - (1 - q) \left(r_0 + \Delta r e^{t_d/\theta} \right) \quad (40)$$

Equations 27, 28, and 31–40 allow for calculation of phase difference ω and the ratio Λ_ω of the ac amplitudes, which are dependent on the modulation frequency ω , time delay of the quenching pulse t_d , lifetime τ , correlation time θ , time-zero anisotropy r_0 , relative change in intensity q , and the absolute change in anisotropy r . As for the intensity responses, the calculated anisotropy frequency responses also show oscillations at higher frequencies. The analysis of this behavior in the case of anisotropy is more difficult than in the case of intensity. However, one can see from the simulations that the interval Δt between the two consecutive minima or maxima is also equal to $1/t_d$.

The above phenomenological description of the frequency-domain anisotropy responses may be significantly improved. It is evident that the parameters q and r are related. At fixed values of the other parameters, a given change in intensity is associated with strictly determined change in anisotropy. Introducing this relation between q and r into the theory will lower the number of the unknown parameters. This relationship will be presented elsewhere.²⁴

Materials and Methods

Excitation was provided by the frequency-doubled output of a cavity-dumped rhodamine 6G dye laser (285–310 nm). The light quenching beam was the fundamental output of the dye laser (570–620 nm). The pulse repetition rate near 8 MHz was obtained using a cavity dumper. The R6G dye laser was synchronously pumped by a mode-locked argon ion laser.

The experimental arrangement for light quenching is shown in Figure 1. The sample containing 4-(dimethylamino)-4'-cyanostilbene (DCS) is placed in a standard $1 \times 1 \text{ cm}^2$ cuvette, and the emission was observed through a $200 \mu\text{m}$ slit. In order to obtain locally intense illumination, the two beams were focused to about $20 \mu\text{m}$ at the center of the cuvette using a laser-quality concave mirror, M_4 , with a focal length of 25 mm. The beams were

combined prior to this mirror using an antireflection-coated (580–620 nm) optical plate (OP), with the quenching beam passing through the plate and the excitation beam reflecting from the plate. For intensity and anisotropy decay measurements the emission of DCS was observed using a 520 or 540 nm interference filter, 10 ns band-pass, combined with cutoff glass filters. The concentrations of DCS were near 10^{-5} M in all solvents, calculated from the extinction coefficient near $31\,000\text{ M}^{-1}\text{ cm}^{-1}$ at the absorption maxima near 380 nm. Precise alignment of the beams was essential for the experiment. Further alignment was done by mirrors M_3 and M_4 mounted in high precision mirror holders. This optical arrangement was placed in a 10 GHz frequency-domain fluorometer.³¹ Emission spectra were obtained using an optical fiber to bring the emission to a steady-state fluorometer. The optical delay (DL) between the excitation (UV) and quenching (VIS) pulses was controlled by Hollow retroreflector placed on a precise optical rail.

The relative cross sections for light quenching (σ_{1q}) were found using Stern–Volmer plots

$$I_0/I = 1 + cP\sigma_{1q} \quad (41)$$

where I_0 and I are the intensities in the absence and presence of the quenching beam, c is a constant, and P is the laser power. The relationship (eq 41) is only approximate.²⁴

Results

Probe Selection for Light Quenching.

The probe DCS was selected for these initial light quenching experiments because of its favorable spectral properties compared to our available laser sources. Absorption and emission spectra are shown in Figure 2. DCS displays an emission maxima near 540 nm, with a tail to over 600 nm. This allows use of the R6G dye laser to quench the emission by illumination at 580–620 nm. The emission can be observed at 540 nm using a monochromator or interference filter without interference from the longer wavelength quenching beam. The large Stokes' shift of DCS^{32–38} facilitates these observations by allowing excitation with the frequency-doubled output of the R6G laser (290–310 nm) and light quenching with the fundamental output of this same laser.

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 R6G dye laser for excitation and quenching of DCS, respectively (Figure 1). Additionally, DCS displays a high fundamental anisotropy for 300 nm excitation, as seen by the steady-state anisotropy spectrum in glycerol (Figure 2, lower panel). This is desirable for the present experiments where we wish to observe the decrease in anisotropy due to light quenching. In the present experiment we also want the quenching pulse to arrive during the anisotropy decay. This condition is met in ethylene glycol, where the steady-state anisotropy is about one-half of the fundamental anisotropy. The lifetime and correlation times of DCS are nearly equal in this solvent.

Intensity and Anisotropy Decay of DCS without Light Quenching.

An additional advantage of DCS is that it displays a single-exponential decay time in a range of solvents of different viscosity.³⁴ Frequency-domain intensity decay data are shown in Figure 3. The lifetime of DCS depends on the solvent, ranging from 463 ps in methanol to 1.13 ns in ethylene glycol. The decay is closely approximated by a single exponential, as can be seen from the best-fit single-exponential curve (Figure 3) and the acceptable values of χ_R^2 (Table 1). This simple decay should facilitate detection of light quenching as deviations from the single-exponential model.

The final favorable feature of DCS is that the anisotropy decay appears to be dominantly due to a single correlation time. This behavior can be seen from the frequency-domain anisotropy data (Figure 4). In ethylene glycol the correlation time near 1.67 ns is comparable to the lifetime of 1.13 ns. Hence, the effects of light quenching are thus expected to be seen as deviations from the single-exponential anisotropy (Figure 4) decay.

Steady-State Measurements of Light Quenching.

We first examined whether the steady-state intensity of DCS could be decreased by illumination with 590 nm light (Figure 5). For these measurements the sample was illuminated with the 8 MHz pulse train of excitation pulses or the combined pulse train of both excitation and quenching pulses. We then measure the steady-state intensity in a spectrofluorometer. Observation of light quenching requires careful overlap of the two beams, which is determined by the decrease in intensity at 540 nm. Simultaneous illumination at 295 and 590 nm results in a substantial decrease in intensity (Figure 5). 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, but there may be a small blue shift with light quenching. We recognize that the presence or absence of spectral shifts with light quenching may provide information on the rates of solvent relaxation or the extents of homogeneous or inhomogeneous line broadening.

The extent of light quenching is expected to be proportional to the amplitude of the emission spectrum.³⁵ Hence, we examined the wavelength dependence of this phenomenon, as shown in the Stern–Volmer type representation of the data (Figure 6). The extent of light quenching increases with increasing laser power. At a given power for the quenching beam the extent of quenching increases with decreasing wavelength. The slopes of these lines are proportional to the cross sections for light quenching, as shown in Figure 2 (open circles). The light quenching cross sections follow the emission spectrum of DCS, as expected for the phenomenon of light quenching. While these plots appear to be linear (Figure 6), we know this is only approximately correct.²⁴

Frequency-Domain Simulations of Light Quenching.

The data shown in Figures 5 and 6 demonstrate that light quenching has occurred but do not reveal the form of the intensity or anisotropy decay. Prior to the frequency-domain (FD) measurements we questioned how the step decreases in intensity would affect the FD data. Simulated time-domain data are shown in Figure 7 for an assumed lifetime of 1.0 ns. The simulations were performed for increasing amounts of light quenching at a time delay of 0.5

ns (top) and for the same amount of light quenching with increasing time delays (bottom). Simulated frequency responses are shown in Figures 8 and 9. Remarkably, a step decrease in the intensity is expected to result in oscillations in the FD data (Figure 8). As the amount of light quenching increases, the amplitude of the oscillations increases. In addition to these distinctive oscillations, light quenching can result in phase angles larger than 90° . Importantly, the simulated data show that it is possible to distinguish light quenching from a step increase in the intensity, which could be due to two-photon absorption of the long-wavelength light or detection of scattered light as fluorescence. In this case, the oscillations initially increase (---) over the unquenched frequency response (Figure 8, ... in lowest panel). These simulations indicate that it should be possible to detect light quenching from the frequency-domain data and that time-delayed light quenching can be distinguished from additional excitation or scattered light.

The frequency of the oscillations depends upon the time delay between excitation and quenching (t_d). As the time delay increases, the oscillations display a higher frequency (Figure 9). As the time delay increases, the amplitude of the oscillation decreases. This is because we kept $q = 0.5$ for the simulations in Figure 9. At longer delay times there is less signal to quench because of the assumed 1.0 ns decay time.

Comparison of Figures 8 and 9 suggests that the parameters t_D and q have distinct effects on the data and that both parameters should be easily resolvable from a least-squares analysis of the data. These analyses are summarized in Table 1 for t_d values ranging from 0.1 to 2 ns and for q values from 0.1 to 0.75. In all cases, we were able to recover both t_d and q with little uncertainty. The simulations indicate that it will be possible to detect even small amounts of light quenching. For instance, 10% and 25% quenching ($q = 0.1$ and 0.25) results in χ_R^2 values which are elevated 18–118-fold, respectively, when fit to the single-exponential model. These oscillating decays cannot be fit by the multiexponential model, which does not result in a significant reduction in χ_R^2 (Table 1). Also, it was not necessary to fix any of the parameters in the analysis, and fixing a parameter (τ , t_d , or q) appeared to have no effect on the parameter confidence intervals. This indicates that the values of τ , t_d , and q are not strongly correlated.

To further examine the achievable resolution for the light quenching parameters, we examined the χ_R^2 surfaces. The values of q and t_d are each easily determined since the value of χ_R^2 increases rapidly if q is held fixed at a value different from its true value (Figure 10). Additionally, we examined the χ_R^2 surfaces for each parameter (τ , t_d , or q) as the other parameters were held fixed during the analysis (Figure 11). The χ_R^2 surfaces remained well-defined. Importantly, there was no significant effect of holding any of the parameters constant during this analysis. This result confirmed that the parameters are nearly uncorrelated. This is a pleasant result in consideration of the strongly correlated parameters which are usually found in multiexponential and nonexponential intensity decays.

We also simulated the effects of time-delayed light quenching on the frequency-domain anisotropy data. A step decrease in the time-resolved anisotropy (Figure 12, top) results in oscillations in the frequency-domain anisotropy data (bottom). It is interesting to notice that

the oscillations result in differential phase angles below zero and modulated anisotropies larger than 0.4.

Frequency-Domain Measurements of Light Quenching.

To the best of our knowledge, oscillations in frequency-domain data have not previously been observed for light quenching or for any other process. We are unaware of any process, other than a step decrease in intensity or anisotropy, which could result in such unusual data. Hence, we decided to look for the predicted oscillations as an unambiguous demonstration of light quenching by a time-delayed light pulse. Frequency-domain data of DCS in methanol are shown in Figure 13. Upon illumination with the long-wavelength pulses the intensity decay displays a profound change in shape. The extent of the changes in the frequency response can be seen by comparison of these data for light- quenched DCS (—●—) with the single-exponential frequency response observed upon blocking the quenching beam (···). It is obviously impossible to fit these data to the single-exponential model (---), resulting in $\chi_R^2 = 1190$ for $t_d = 36$ ps (Table 2). If the time delay is increased to 260 ps, the shape of the FD data changes, with an apparent increase in the oscillation frequency. Similar results were found for DCS in ethylene glycol (Figure 14). In the presence of light quenching, the phase angles can exceed 90° . In all cases, the FD data returned to the single-exponential shape (···) upon blocking the quenching beam (Figures 13 and 14).

Least-squares analyses of the intensity decay data for DCS with and without light quenching are summarized in Table 2. In all cases, we recovered the expected values of the delay time t_d , the extent of quenching q , and the unquenched lifetime τ . Fixing any of the parameters did not have a significant effect on the parameter values or confidence intervals. These results suggest that it will be readily possible to perform quantitative measurements of light quenching. Importantly, use of the light quenching model (eqs 2 and 3) results in a good fit to the experimental data, as seen from the solid lines in Figures 13 and 14. The lack of correlation between the parameters is shown by the χ_R^2 surfaces (Figure 15). The three parameters are all closely defined by the data, and fixing any of the parameters did not alter the range of values consistent with the data.

We also measured the frequency-domain anisotropy decay of DCS in ethylene glycol (Figure 16). As expected from the simulations (Figure 12), the data display remarkable oscillations in the presence of a time-delayed quenching pulse. Additionally, some of the differential phase angles are less than zero (Figure 16). Least squares analysis of these data using eqs 24 and 28 resulted in a good match (—) to the data (●) and recovery of the expected time delay (Table 3). The data could not be explained by a single correlation time model (---).

Discussion

The first observation of light quenching may have been made by Galanin in 1969 in his early studies of two-photon excitation,³⁶ and this phenomenon was anticipated by Einstein as stimulated emission in 1917.³⁷ In a series of measurements the Russian spectroscopists have studied light quenching and its effect on the emission spectra and depolarization of fluorophores.^{38–40} These measurements were performed with long laser pulses resulting in

essentially steady-state light quenching. With one exception,⁴¹ there have been no direct measurements of changes in the intensity decays of fluorophores under pulsed illumination. In their pioneering study, Lessing et al.⁴¹ observed an altered intensity decay for rhodamine under intense illumination at the ruby fundamental wavelength of 694 nm.

It should be noted that all prior studies of light quenching used giant pulses from ruby lasers. Perhaps the most important conclusion for our experiments is that significant light quenching can be observed with modern high repetition rate lasers, which are widely used for research in physical chemistry and biophysics. It is important to note that light quenching is not the same as polarized photobleaching, in which case part of the fluorophore population is destroyed by the bleaching pulse at the absorption wavelength. Also, in the case of light quenching, changes in the anisotropy can occur without depletion of the ground state.⁴² For light quenching, the fluorophore will typically be illuminated at a nonabsorbed wavelength, and the effects we describe do not require that any fluorophores be destroyed.

Light quenching of fluorescence can provide a new method to control the excited-state population and orientation of fluorophores. This means that not only is the total excited-state population altered by the quenching pulse but also that selectively oriented parts of the excited-state population are quenched. Consequently, depending on the polarization of the quenching light, the polarization of the emission can be altered from 1.0 to -1.0 ,²⁴ resulting in a high degree of orientation of the excited-state population. Additionally, we now know that light quenching can be used to break the z -axis symmetry which is common in optical spectroscopy. The ability to measure time-dependent light quenching can result in a new class of fluorescence experiments in which the sample is excited with one pulse, and the excited-state population is modified by the quenching pulse(s) prior to measurement. Such multipulse experiments may find use in studies of the rotational diffusion of asymmetric biomolecules and may be even more informative in studies of macroscopically oriented systems.

Acknowledgment.

This work was supported by Grants BIR-9319032 and MCB-8804931 from the National Science Foundation, with support for instrumentation from the National Institutes of Health.

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Figure 1.

Experimental arrangement for the two-pulse light quenching experiment. L are lenses, P are polarizers, D are diaphragms, M are mirrors, R are polarization rotators, and F are optical filters. The delay line (DL) is placed in the excitation beam. OP is a coated optical plate (dichroic mirror), and PD is a reference photodiode, used in time-resolved measurements.

Figure 2.

Absorption and emission spectra of DCS in methanol (top) and ethylene glycol (EG, bottom). The open circles (O) show the relative cross section for light quenching in methanol. The dashed (---) and dotted (···) lines (lower panel) show the steady-state anisotropy spectrum in ethylene glycol and glycerol, respectively. The star (*) is the $r(0)$ value recovered from the frequency-domain anisotropy decays.

Figure 3.
Frequency-domain intensity decay of DCS in methanol (top) and ethylene glycol (EG, bottom).

Figure 4. Frequency-domain anisotropy decay of DCS in ethylene glycol (●) and methanol (○).

Figure 5. Emission spectra of DCS in the absence of light quenching (LQ 0%) and with light quenching. The peak near 590 nm is the scattered quenching light.

Figure 6.
Wavelength-dependent light quenching at DCS in methanol.

Figure 7.
Simulated time-dependent intensity decays for increasing amounts of light quenching (top) and increasing delay times (bottom).

Figure 8. Effect of light quenching on the frequency-domain intensity decays for increasing amounts of light quenching. The dashed line in bottom panel shows the effect of excitation by the time-delayed pulse. The unquenched frequency responses are shown as dotted lines.

Figure 9.
Effect of light quenching on the frequency-domain intensity decays for increasing time delays. The dotted lines show the unquenched frequency responses.

Figure 10.
 χ_R^2 surfaces for the extent of light quenching for different values of a q (top) and t_d (bottom).

Figure 11.

χ_R^2 surfaces for the unquenched lifetime (top), the delay time (middle), and the extent of quenching (bottom). The dashed lines indicate the χ_R^2 surface when the indicated parameter is held fixed during the analysis.

Figure 12. Simulated time-domain (top) and frequency-domain (bottom) anisotropy decays in the presence of time-delayed light quenching. The dotted lines (···) show the data expected without light quenching.

Figure 13.

Frequency-domain measurements of time-delayed light quenching of DCS in methanol. The dashed lines show the best single-exponential fit, and the dotted lines show the unquenched frequency response.

Figure 14. Frequency-domain measurements of time-delayed light quenching of DCS in ethylene glycol. The dashed lines show the best single-exponential fit, and the dotted lines show the unquenched frequency response.

Figure 15.

χ^2_R surfaces for the intensity decay of DCS with light quenching. The dashed lines show the χ^2_R surface when the indicated parameter is held fixed during the analysis.

Figure 16.

Frequency-domain anisotropy decays of DCS in the absence (top) and presence (bottom) of a time-delayed quenching pulse. The best fit to a single correlation time is shown by the dashed line.

SCHEME 1:
Intuitive Description of Light Quenching

SCHEME 2: Intuitive Description of Two-Pulse Light Quenching^a

^a Both the excitation and time-delayed (t_d) quenching beam are vertically polarized. The quenching pulse causes a step decrease in the intensity and anisotropy.

TABLE 1:

Light Quenching Analysis of Simulated Intensity Decay Data

expected				found			
τ (ns)	t_d (ns)	q	τ (ns)	t_d (ns)	q	χ^2_R	
1.00	0.10	0.50	$\langle 1 \rangle^a$		$\langle 0 \rangle$	938.5	
			0.58(0.06)		$\langle 0 \rangle$	713.9	
1.00	0.25	0.50	$\langle 1 \rangle$	0.10(0.01)	0.50(0.01)	0.9	
			1.00(0.01) ^b		$\langle 0 \rangle$	498.7	
			0.59(0.05)		$\langle 0 \rangle$	293.5	
1.00	1.00	0.50	$\langle 1 \rangle$	0.50(0.01)	0.50(0.01)	0.8	
			1.00(0.01)		$\langle 0 \rangle$	197.3	
			0.77(0.03)		$\langle 0 \rangle$	126.5	
1.00	2.00	0.50	$\langle 1 \rangle$	1.00(0.01)	0.50(0.01)	0.9	
			1.00(0.01)		$\langle 0 \rangle$	29.7	
			0.93(0.02)		$\langle 0 \rangle$	24.2	
1.00	0.50	0.19	$\langle 1 \rangle$	2.00(0.01)	0.50(0.01)	1.1	
			1.00(0.01)		$\langle 0 \rangle$	18.0	
			0.93(0.02)		$\langle 0 \rangle$	12.7	
1.00	0.50	0.25	$\langle 1 \rangle$	0.49(0.01)	0.10(0.01)	1.1(11.9) ^c	
			1.00(0.01)		$\langle 0 \rangle$	117.7	
			0.82(0.03)		$\langle 0 \rangle$	77.8	
1.00	0.50	1.00(0.01)	0.50(0.01)	0.25(0.01)	1.1		
			1.00(0.01)		$\langle 0 \rangle$	1239.2	
			0.48(0.04)		$\langle 0 \rangle$	643.6	
1.00	0.50	0.75	$\langle 1 \rangle$	0.50(0.01)	0.75(0.01)	0.9(643.6) ^c	
			1.00(0.01)		$\langle 0 \rangle$		

^aThe value of the parameter in brackets was kept constant during the analyses.

^bStandard deviations from the least-squares analysis.

^cBest two-exponential fit.

TABLE 2:

DCS Intensity Decay Analysis in Absence and Presence of Light Quenching

solvent	expected			found			X_R^2
	τ (PS)	t_d (PS)	LQ	τ (ns)	t_d (ns)	q	
methanol	463		no	463(1) ^a		$\langle 0 \rangle^b$	1.2
				465(3)	4(2)	0.002(0.01)	1.1
	463	36	yes	154		$\langle 0 \rangle$	1189.7
				$\langle 463 \rangle$	$\langle 36 \rangle$	0.72	1.0
				$\langle 463 \rangle$	35(1)	0.73(0.01)	1.0
				456(3)	33(2)	0.73(0.01)	0.9
	463	260	yes	314		$\langle 0 \rangle$	255.0
				$\langle 463 \rangle$	$\langle 260 \rangle$	0.54	1.2
ethylene glycol				$\langle 463 \rangle$	268(2)	0.54(0.1)	1.0
				461(3)	269(2)	0.54(0.1)	0.9
	1130		no	1130(3)		$\langle 0 \rangle$	1.7
				1180(6)	284(5)	0.05(0.01)	1.3
	1130	570	yes	546		$\langle 0 \rangle$	388.6
				$\langle 1130 \rangle$	$\langle 570 \rangle$	0.66(0.01)	1.9
				$\langle 1130 \rangle$	573(2)	0.66(0.01)	1.9
				1060(4)	570(2)	0.63(0.01)	1.4

^aStandard deviations from the least-squares analysis.^bThe value of the parameter in brackets was kept constant during the analyses.

TABLE 3:
Anisotropy Decay Analysis of Simulated Data and DCS in the Presence of Light Quenching

sample	expected			found		
	r_0	θ (ns)	r	r_0	θ (ns)	r
simulations	0.4	1.0	-0.20	0.39	0.84	$\langle 0 \rangle^a$
						χ_R^2
						953.5
$\tau = 1$ ns				$0.40(0.01)^b$	$0.99(0.01)$	-0.20(0.01)
$q = 0.5$						0.9
$t_d = 0.25$ ns						
DCS in EG	0.32^c	1.67^c		0.31	1.86	$\langle 0 \rangle$
				$0.33(0.01)$	$1.67(0.05)$	-0.22(0.01)
$\tau = 1.113$ ns						8.8
$q = 0.6$						
$t_d = 0.57$ ns						

^aThe value of the parameter in brackets was kept constant during the analysis.

^bStandard deviations from the least-squares analysis.

^cThe parameters found in the absence of light quenching (Figure 4).