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Anisotropy Spectra of the Solvent-Sensitive Fluorophore 4-Dimethylamino-4'-Cyanostilbene in the Presence of Light Quenching[1]

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We examined the emission wavelength-dependent anisotropies of the solvent-sensitive fluorophore 4-dimethylamino-4'-cyanostilbene (DCS) under conditions of light quenching by polarized time-delayed quenching pulses. Illumination on the long-wavelength side of the emission spectrum with time-delayed light pulses resulted in a progressive decrease in the emission anisotropy as the observation wavelength increased toward the stimulating wavelength. The anisotropy changes of DCS were most wavelength dependent when spectral relaxation occurred during the excited-state lifetime. Light quenching of DCS in a low-viscosity solvent revealed no wavelength-dependent anisotropies. Control measurements using a solvent-insensitive fluorophore did not show any wavelength-dependent anisotropy with light quenching. The data for DCS can be explained by a model which allows wavelength-selective quenching of the long-wavelength emission formed by time-dependent spectral relaxation. These results indicate that polarized light quenching can be used to study systems which display multiple emissions and/or time-dependent spectral shifts.

KEY WORDS: Anisotropy; 4-dimethylamino-4'-cyanostilbene; light quenching; time-resolved fluorescence.

INTRODUCTION

Since the pioneering reports of Weber [1,2], fluorescence anisotropy measurements have become extensively utilized in biochemistry [3–5] and clinical diagnostics as so-called fluorescence polarization immunoassays [6–9]. In biochemical applications the time-resolved anisotropy decays are particularly informative [10] because they can reveal the size, shape, and flexibility of biopolymers [11–14].

At present, essentially all measurements of the time-resolved anisotropies have relied on the use of a single polarized excitation pulse, followed by measurement of the time-dependent anisotropy decays. We now describe a new type of anisotropy measurement in which the anisotropy is altered during the excited state lifetimes. To be more specific, the sample is excited with a continuous train of excitation pulses (Scheme I). Following each excitation pulse the intensity and anisotropy decay as usual. However, we now describe the use of a second, longer-wavelength pulse, which is delayed in time relative to the excitation and whose wavelength overlaps the emission spectrum of the fluorophore. As shown in pioneering reports [15–18], illumination with such wavelengths results in stimulated emission along

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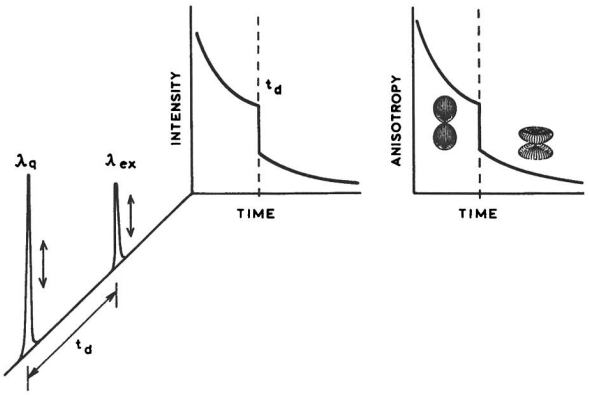
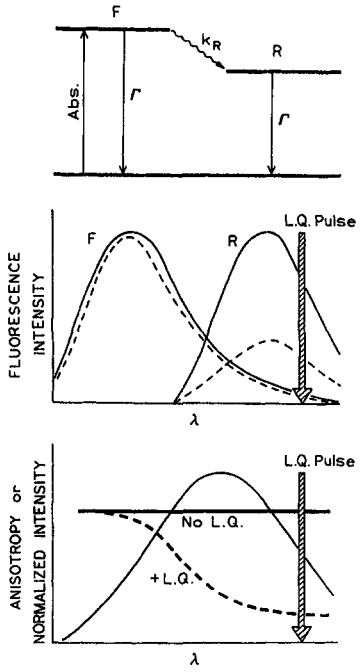


Scheme I. Effect of time-delayed light quenching during the intensity and anisotropy decay. The arrows indicate vertically polarized excitation and quenching.



Scheme II. Schematic description of excited-state spectral relaxation (top) and the effects of light quenching on the emission spectra (middle) and emission anisotropy spectra (bottom).

the axis of the long-wavelength beam. When observed perpendicular to the direction of the long-wavelength beam, the emission appears to be quenched, and hence the term "light quenching" to describe this phenomenon. In recent years we have shown that light quenching can be accomplished with modern Pïcosecond lasers [19,20] and that time-delayed light quenching results in step changes in the intensity and anisotropy decays [21,22].

To avoid a misunderstanding, we note that light quenching is not the same as stimulated emission pumping (SEP) and that these terms are sometimes used to mean different types of experiments. In SEP, one observes the stimulated emission, which is always at the same wavelength in the probe beam. In light quenching, the signal is observed at a right angle to the probe beam. The observed signal is due to the excited-state population which remains in the sample in the presence of the light quenching beam. Occassionally, the term SEP has been used to describe light quenching experiments, the so called "pump-dump" studies of excited-state reactions [23,24]

We now describe the effects of light quenching on the anisotropy spectra of a solvent-sensitive fluorophore, 4-dimethylamino-4'-cyanostilbene (DCS). This probe is known to display emission spectra which are highly sensitive to solvent polarity [25], and the emission spectra display time-dependent spectral shifts at suitable solvent viscosities [26]. This fluorophore can be considered as displaying an excited-state process which shifts the emission to longer wavelengths (Scheme II). Hence, this system can be regarded as a model system for the rapid spectral changes which are generally known to occur in polar solvents [27–29] and in multichromophore systems such as the phycobiliproteins [30,31].

It should be noted that the fluorophore must display suitable spectral properties to be studied by light quenching. In most laser systems, a limited range of wavelengths is available. In the present experiments, the probe DCS was excited with the UV frequency-doubled output of a dye laser. A favorable property of DCS was its high fundamental anisotropy with UV excitation. The most important property of the probe is spectral overlap with the long-wavelength light quenching beam. The large Stokes' shift displayed by DCS provided such overlap.

In the present experiments DCS was illuminated with both a UV excitation pulse train and a longer-wavelength quenching pulse train. Since the relaxed state overlaps more strongly with the quenching wavelength, one expects selective quenching of the longer-wavelength emission (Scheme II, middle). Since the quenching beam is vertically polarized, and since light quenching displays orientation photoselection similar to absorption [32], the anisotropy is expected to decrease with light quenching (Scheme II, bottom). However, it was not clear whether the decrease in anisotropy would occur uniformly across the emission spectrum or whether the decrease would be localized in some region

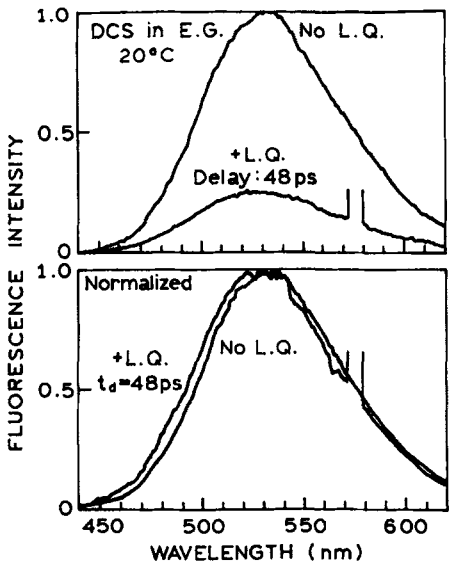


Fig. 1. Effect of time-delayed light quenching on the emission spectra of DCS in ethylene glycol at 20°C.

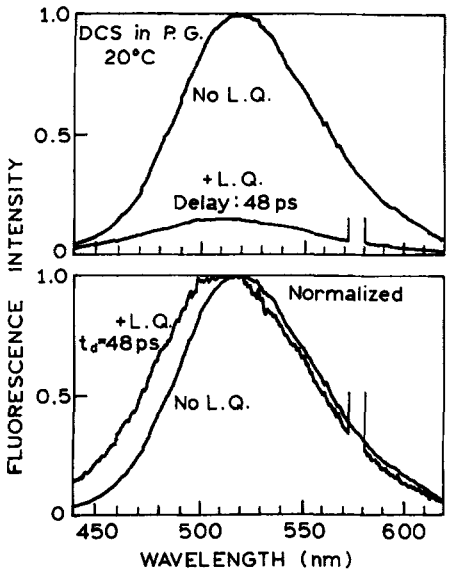


Fig. 2. Light quenching of DCS in propylene glycol at 20°C.

of the spectrum. We note that the present measurements are stationary or steady-state measurements, while the sample is repeatedly illuminated by the excitation and quenching beams.

The observed anisotropy along the emission spectrum may depend on many molecular processes such as multiple relaxations or rotations. Although the complete theory is presented (in the Appendix), we show that relatively simple intuitive model is able to describe qualitatively observed dependences. In this first report we do not attempt to fit the data to a complete model but, rather, describe the phenomenon on an intuitive base.

MATERIALS AND METHODS

The experimental arrangement for light quenching of DCS has been described previously [33], wherein we described spectral shifts of DCS with light quenching. Light quenching with time-delayed pulses requires precise timing between the excitation and the quenching pulses. This was accomplished using the frequency-doubled (285- to 310-nm) and fundamental (570- to 620-nm) outputs of a rhodamine 6G dye laser, synchronously pumped by a mode-locked argon ion laser. The pulse repetition rate near 2 MHz was obtained using a cavity dumper.

The sample containing 4-dimethylamino-4'-cyanostilbene (DCS) is placed in a standard 1 × 1-cm cuvette, and a spatially defined region of the emission was observed through a 200-μm slit. The concentrations of DCS were near 10^{-5} M in ethylene glycol (EG) or propylene glycol (PG), as calculated from the extinction coefficient of 31,000 M^{-1} cm^{-1} at the absorption maxima near 380 nm. To obtain locally intense illumination the two beams were focused to about 20-μm-diameter at the center of the cuvette using a concave mirror with a focal length of 25 mm.

The light-quenching optics were placed in a 10-GHz frequency-domain fluorometer [34,35]. Emission spectra were obtained using an optical fiber to bring the emission to a steady-state fluorometer.

RESULTS AND DISCUSSION

Emission Spectra Properties of DCS

Prior to describing the effects of light quenching on DCS, it is informative to understand the spectral properties of DCS in polar solutions. Emission spectra of DCS in ethylene glycol are shown in Fig. 1 and in propylene glycol in Fig. 2. At 20°C the emission spectra are shifted to a long wavelength relative to that observed in vitrified solution, in which solvent relaxation occurs more slowly than emission. At −60°C the emission maxima of DCS in ethylene glycol and propylene glycol are near 470 nm (not shown). The emission spectra at 20°C are representative of the solvent relaxed state of DCS. In a previous report [33] we showed that at 20°C spectral relaxation occurs predominantly in less than 5

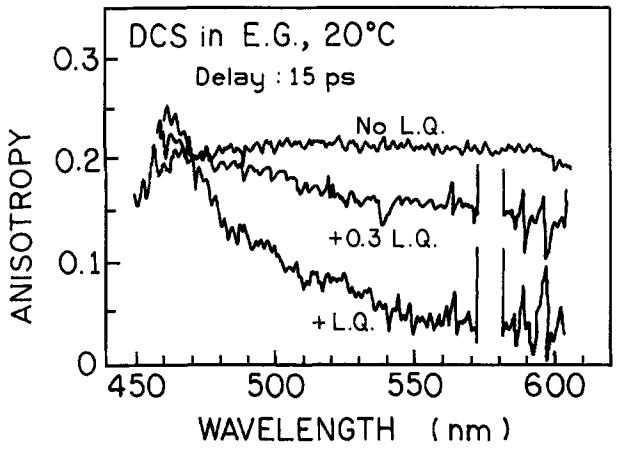


Fig. 3. Emission anisotropy spectrum of DCS in ethylene glycol in the absence (no L.Q.) and presence (+ L.Q.) of light quenching.

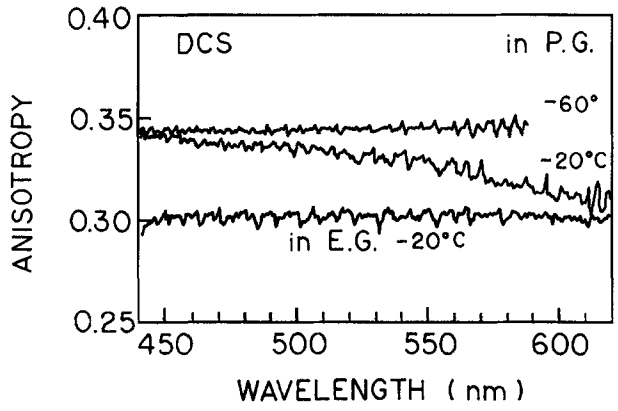


Fig. 4. Emission anisotropy spectra of DCS in propylene glycol at various temperatures.

ps, with a residual relaxation on the 100- to 200-ps time scale. These relaxation times are much shorter than the mean decay time near 1 ns, so that the relaxed state dominates the emission spectrum.

Effects of Light Quenching on the Emission Spectra of DCS

The effects of light quenching on the DCS emission spectra were reported previously [33] but are summarized here to clarify presentation of the anisotropy data. We questioned whether light quenching on the long-wavelength side of the emission would result in selective depletion of the solvent-relaxed state or uniform depletion of DCS at all emission wavelengths. Emission spectra are shown in Fig. 1 for unquenched DCS, and for light quenching at 575 nm with a delay time of 48 ps, both in ethylene glycol at 20°C. The intensity normalized spectra show that the emission spectra are blue shifted by 575-nm light quenching. These results demonstrate that light quenching selectively occurs for the solvent relaxed state whose emission spectrum overlaps with the light quenching wavelength.

Emission spectra for DCS in the more viscous solvent propylene glycol are shown in Fig. 2. One notices that the spectral shift for t_d = 48 ps is severalfold larger than observed in ethylene glycol under similar conditions (Fig. 1). This is consistent with the previous results [33] showing that the spectral relaxation of DCS in propylene glycol is slower than in ethylene glycol.

Effects of Light Quenching on the Emission Anisotropy Spectra of DCS

We next examined the emission anisotropy spectra of DCS. In the absence of light quenching the anisotropy of DCS was found to be essentially independent of emission wavelength (Fig. 3). The constant emission anisotropy is probably the result of rapid spectral relaxation, so that there is only a modest difference in the mean lifetime of the excited state on the blue and red sides of the emission. Wavelength-dependent anisotropies of DCS due to spectral relaxation can be observed in propylene glycol at lower temperatures, where the spectral relaxation time is expected to be comparable to the lifetime (Fig. 4).

Upon illumination at 575 nm the anisotropy spectrum displays a strong dependence on emission wavelength (Fig. 3). The decreasing anisotropy at long wavelengths indicates that light quenching can in fact selectively deplete the relaxed state. The fact that the anisotropy decreases reflects incomplete light quenching of the relaxed state. At long wavelengths the emission is due to that fraction of DCS molecules which have not been quenched by the vertically polarized quenching pulse. These unquenched DCS molecules are not aligned with the vertical axis, so that the anisotropy of the emission is decreased [22]. On the blue side of the emission the anisotropy is unchanged by light quenching, which indicates that the unrelaxed DCS molecules are not quenched by the 575-nm illumination. Although not possible with our present instruments, light quenching on the blue side of the emission should selectively quench the unrelaxed state.

Still more dramatic effects were observed for DCS in propylene glycol. In this case the unquenched anisotropy is higher (Fig. 5), and the wavelength-dependent

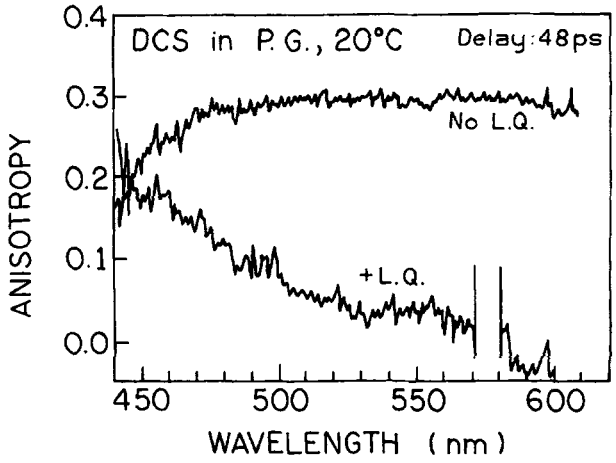


Fig. 5. Emission anisotropy spectrum of DCS in propylene glycol at 20°C, in the absence (no L.Q.) and presence (+ L.Q.) of light quenching.

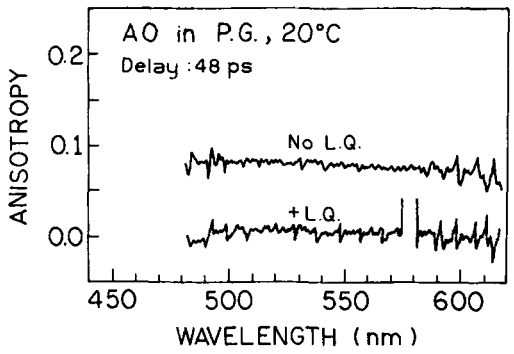


Fig. 6. Emission anisotropy spectrum of acridine orange in propylene glycol in the absence (no L.Q.) and presence (+ L.Q.) of light quenching.

decrease in anisotropy is larger. And, finally, control experiments on the solvent-insensitive fluorophore acridine orange show a decrease in anisotropy due to light quenching (Fig. 6). However, the anisotropy remains constant across its emission spectrum independent of the extent of light quenching.

These changes in emission anisotropy were observed only when spectral relaxation occurred during the excited-state lifetime. If the solvent was changed to methanol, so that relaxation was complete at 20°C, then the anisotropy remained constant across the emission spectrum (not shown).

Simplified Model for the Effect of Light Quenching on the Emission Anisotropy Spectra

We show that observed changes in emission anisotropy can be qualitatively described by simple kinetic model. A more general theory for solvent relaxation in the presence of light quenching is provided in the Appendix. The simplified model presented below is a specific case of the general theory with rigorous assumption.

We consider the two-state model—an initially excited state (F) and a relaxed state (R) which is populated with rate constant k_R (Scheme II). The normalized emission spectra of each state are given by $I_F(\lambda)$ and $I_R(\lambda)$, and the normalized total emission spectrum is given by $I_T(\lambda)$. For simplicity we assume that all species decay to the ground state with radiative rate constant Γ (Scheme II). We also assume that the emission transition moments have the same orientation in the F and R states and that the fluorophore displays the same steady-state anisotropy in each state (F and R). The latter assumption is supported by the constant anisotropy observed across the emission spectra of DCS in the absence of light quenching (Figs. 3 and 5). Finally, we assume that light quenching acts only on the relaxed state (R), i.e., the initially excited state (F) is not depopulated by the quenching pulse.

To calculate the wavelength-dependent anisotropies we use the additivity of the anisotropies based on the fractional intensities [36,37],

$$r(\lambda) = \sum_i f_i(\lambda)\, r_i \qquad (1)$$

In this expression $f_i(\lambda)$ are the fractional intensities of each species at the wavelength λ emitting with an anisotropy r_i. In the case of light quenching of a solvent-sensitive fluorophore, the emission originates from several species as summarized below.

N_F is the number of molecules emitting from the F state with an anisotropy $r = r_F$.

N_{Rb} is the number of molecules emitting from the R state prior to arrival of the quenching pulse with an anisotropy $r = r_R$. In this simple model we assume $r = r_F = r_R$.

N_{Rq} is the number of molecules emitting from the R state which remain after the quenching pulse. The anisotropy of this population is changed by polarized light quenching to $r_{Rq} = r + \Delta r$.

N_{Ra} is the number of molecules emitting from the R state after the quenching pulse due to replenishment of the R state by solvent relaxation. This population displays the original anisotropy $r = r_R$.

Using these definitions the fractional intensities for each type of emission is given by

$$f_i(\lambda) = \frac{N_i I_i(\lambda)}{\sum_i N_i I_i(\lambda)} = \frac{N_i I_i(\lambda)}{I_T(\lambda)} \quad (2)$$

where $I_i(\lambda)$ refers to the shape of the emission spectrum corresponding to the desired species (F or R). The subscript i can be one of those listed above. The wavelength-dependent anisotropy is thus given by

$$r(\lambda) = [f_F(\lambda) + f_{Rb}(\lambda) + f_{Ra}(\lambda)]\, r + f_{Rq}(\lambda)\,(r + \Delta r) \quad (3)$$

Hence the emission anisotropy spectrum can be calculated from knowledge of the fractional emission from each species and the change in anisotropy (Δr) due to light quenching.

The number of molecules in each state can be found from the excited-state model in Scheme II. The solution to this irreversible model [38–40] is given by

$$N_F(t) = N_0\, e^{-(\Gamma + k_R)t} \quad (4)$$

$$N_R(t) = N_0\,(e^{-\Gamma t} - e^{-(\Gamma + k_R)t}) \quad (5)$$

where N_0 is the number of excited molecules at $t = 0$, which is also the number of initially excited F-state molecules. Now we calculate the fractions of excited molecules emitting fluorescence with anisotropy r, and the fraction which emits fluorescence with anisotropy $r + \Delta r$ [see Eq. (3)]. The number of molecules emitting from the F state is independent of light quenching because we are assuming that the F state is not quenched. The total number of molecules emitting from the F state is given by

$$N_F = N_0 \frac{\Gamma}{\Gamma + k_R} \quad (6)$$

Assume that at the time t_d the quenching pulse appears. The population of the R state will be $N_R(t_d)$ at this moment and will be decreased by the quenching pulse to the value N_{Rq} given by

$$N_{Rq} = (1-q)\, N_R(t_d) \quad (7)$$

This remaining portion of excited molecules will emit fluorescence with anisotropy $r_{Rq} = r + \Delta r$. The parameter q describes the relative change caused by the quenching pulse in the relaxed state population at time t_d. This parameter can be expressed as

$$q = \frac{N_R(t_d) - N_{Rq}}{N_R(t_d)} \quad (8)$$

The number of R-state molecules quenched by the quenching pulse is given by

$$N_q = N_R(t_d) - N_{Rq} \quad (9)$$

Now we calculate the number of molecules, N_R, emitting fluorescence from state R with no change in anisotropy:

$$N_R = N_{Ra} + N_{Rb} = N_0 - N_F - N_q - N_{Rq} = N_0 - N_F - N_R(t_d) \quad (10)$$

According to Eq. (3), the observed anisotropy is given by

$$r(\lambda) = \frac{N_F I_F(\lambda)}{I_T(\lambda)}\, r + \frac{N_R I_R(\lambda)}{I_T(\lambda)}\, r + \frac{N_{Rq} I_R(\lambda)}{I_T(\lambda)}\,(r + \Delta r) \quad (11)$$

where

$$I_T(\lambda) = N_F I_F(\lambda) + N_R I_R(\lambda) + N_{Rq} I_R(\lambda) \quad (12)$$

We used this model to calculate the emission anisotropy spectra expected in the presence of light quenching. For this calculation we assumed that the emission spectra were Gaussians centered at 460 and 530 nm, for the F and R state, respectively, with a half-width of 3000 cm^{-1} on the wavenumber scale. We used a time delay t_d = 50 ps and assumed that the anisotropy of the R state changed by $\Delta r = -0.2$, -0.4, and -0.6 due to the quenching pulse. The value of Γ was 10^9 s^{-1}, corresponding to a lifetime of 1 ns in the absence of relaxation. The value of k_R was 5×10^{10} s^{-1}, corresponding to a relaxation time of 20 ps. These values were used to determine the fractional intensities from each population, allowing calculations of r(λ) using Eq. (3).

Emission anisotropy spectra calculated with the parameter values given above are shown in Fig. 7. These anisotropy spectra roughly correspond to the experimental observation on DCS in the two solvents. Our simplified model describes qualitatively the anisotropy dependence within the emission spectrum in presence of light quenching. Similar results of wavelength-dependent anistropies can be expected for a mixture of two or more fluorophores. However, one fluorophoe is present in the present experiments, and DCS surely displays spectral relaxation.

Of course, the actual situation is more complex, and a complete theory must take into account rotational diffusion and its effects on the extent of quenching by polarized light.

DISCUSSION

Can measurements of the emission wavelength-dependent anisotropy resulting from light quenching be useful in biophysical and biochemical studies of molecules? The steady-state measurements described above contain information about ultrafast processes occurring within the lifetime of the fluorophore. The simplified intuitive model allows one to describe qualitatively the phenomena and properly estimate the time scale of physical processes which occur after fluorophore excitation. The more complete theory in the Appendix is more complex and contains many variable parameters. However, we wish to point out that the steady-state anisotropy measurements can be performed with extremely high accuracy [41]. Also, such measurements can be analyzed globally with spectral shift data, which may significantly increase the resolution of the spectral and kinetic parameters. In fact, fluorescence depletion by stimulated emission has already been successfully used by several groups to study pico- and femtosecond dynamics of chemical reactions [42–45]. We expect growing interest in light quenching of biological fluorophores, which should be possible with the increasing availability of pulse amplifiers covering the UV visible spectrum.

APPENDIX

Steady-State Anisotropy of a Solvent-Sensitive Fluorophore in the Presence of Two-Pulse Light Quenching

We now describe how the emission anisotropy spectra with light quenching can be explained by preferential light quenching of the solvent relaxed state. In this general model we consider a mechanism with no specific limitations. We consider a two-state model where, initially there is the excited state (F), which may relax to the ground state with a rate constant Γ or to the relaxed state (R) with rate constants k_{Rj}. We assume the same initial anisotropy r_0 only for the *R* and *F*states. We assume that light quenching acts on both states and change anisotropy by Δr_R and Δr_F for relaxed and unrelaxed, respectively.

The fluorescence decays $I_F(\lambda, t)$ and $I_R(\lambda, t)$ of the F and R states at any wavelength λ can be written as the products of the amplitude factors $I_F(t)$, $I_R(t)$ and the shape factors $f_F(\lambda)$, $f_R(\lambda)$

$$I_F(\lambda, t) = I_F(t)f_F(\lambda) \tag{A1}$$

$$I_R(\lambda,t) = I_R(t)f_R(\lambda) \tag{A2}$$

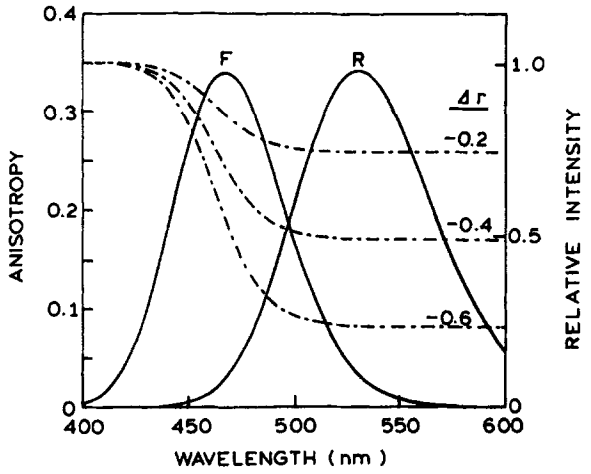


Fig. 7. Emission anisotropy spectra calculated using Eq. (3), with the parameter values given in the text.

Following δ-pulse excitation the fluorescence decays $I_F(t)$, $I_R(t)$ are then given by

$$I_F(t) = I_{F0}\sum_{j=1}^{n} \xi_j e^{-\gamma_j t} \tag{A3}$$

$$I_R(t) = I_{F0}\,(e^{-\Gamma t} - \sum_{j=1}^{n} \xi_j e^{-\gamma_j t}) \tag{A4}$$

where ξ_j are normalized amplitudes of relaxation rates, $\gamma_j = \Gamma + k_{Rj}$ and I_{F0} is the total fluorescence intensity of the unrelaxed species at time $t = 0$, and $\tau = \Gamma^{-1}$ is the lifetime in the absence of spectral relaxation. We assumed that the relaxation is irreversible. For $n = 1$ Eqs. (A3) and (A4) reduce to the respective solutions for homogeneous relaxation, that is, a single spectral relaxation time.

In the presence of LQ the intensity decays (A3) and (A4) display an instantaneous decrease in intensity at the arrival time of the quenching pulse at time $t = t_d$.

$$I_F(t) = \begin{cases} I_{F0}\sum_{j=1}^{n} \xi_j e^{-\gamma_j t} & \text{for} \quad 0 \le t \le t_d \\ I_{F0}\,(1 - q_F)\sum_{j=1}^{n} \xi_j e^{-\gamma_j t} & \text{for} \quad t > t_d \end{cases} \tag{A5}$$

$$I_R(t) = \begin{cases} I_{F0}\left[e^{-\Gamma t} - \sum_{j=1}^{n} \xi_j e^{-\gamma_j t}\right] & \text{for} \quad 0 \le t \le t_d \\ I_{F0}\left[(1 - q_E)e^{-\Gamma t} - (1 - q_F)\sum_{j=1}^{n} \xi_j e^{-\gamma_j t}\right] & \text{for} \quad t > t_d \end{cases} \tag{A6}$$

where

$$q_E = q_R + (q_F - q_R)\sum_{j=1}^{n} \xi_j e^{-k_{Rj} t_d} \tag{A7}$$

The parameters q_F, q_R, and q_E describe the relative changes caused by the quenching pulse in the excited F and R populations and in the entire (E) excited fluorophore population, respectively. These parameters can be expressed as $q_k = (n_{kb} - n_{ka})/n_{kb}$, where n_{kb} and n_{ka} are the respective numbers of excited molecules immediately before (b) and after (a) the quenching pulse, and k is F, R, or E. The fluorescence decay given by the second line of Eq. (A6) takes into account the possibility of repopulation of the R state after its quenching by light, due to the relaxation from the usually less quenched F state. Notice that Eqs. (A3) and (A4), describing the fluorescence decays in the absence of light quenching, may be easily obtained from Eqs. (A5) and (A6) by setting $q_F = q_R = 0$.

At a given observation wavelength λ, the steady-state anisotropy is a time-averaged value of the time-dependent anisotropy, weighted by the intensity decay law

$$r(\lambda) = \frac{\int_{\infty}^{0} r(\lambda,t)I(\lambda,t)dt}{\int_{\infty}^{0} I(\lambda,t)dt} \tag{A8}$$

Based on Eq. (A1) one can find that the anisotropy $r(\lambda)$ observed for the solvent-sensitive fluorophore in the presence of light quenching is given by

$$r(\lambda) = \frac{r_F J_F(\lambda) + r_R J_R(\lambda)}{J_T(\lambda)} \tag{A9}$$

where $J_F(\lambda)$ and $J_R(\lambda)$ are steady-state fluorescence spectra of the fluorophore resulting from states F and R, respectively, and $J_T(\lambda)$ is the total emission,

$$J_T(\lambda) = J_F(\lambda) + J_R(\lambda) \tag{A10}$$

According to Eq. (A8),

$$r_F = \frac{\int_{\infty}^{0} r_F(t)I_F(t)dt}{J_F} \tag{A11}$$

and

$$r_R = \frac{\int_{\infty}^{0} r_R(t)I_R(t)dt}{J_R} \tag{A12}$$

The steady-state anisotropies r_F and r_R can be calculated using Eqs. (A11) and (A12) and a model of light quenching. This model assumes an instantaneous decrease at $t = t_d$ in the fluorescence decays of the F and R state, the same correlation time for the F and R states, and no effect of spectral relaxation on the directions of the transition moment. The instantaneous decrease in intensity is associated with an instantaneous change in anisotropy described by $\Delta r_k = r_{ka} - r_{kb}$, where r_{kb} and r_{ka} are the anisotropies immediately before and after the quenching pulse, and k refers to the F or R state. The fluorescence anisotropy decay of the F species then has the form

$$r_F(t) = \begin{cases} r_0 e^{-t/\theta} & \text{for} \quad 0 \le t \le t_d \\ (r_0 + \Delta r_F e^{t_d/\theta}) e^{-t_d/\theta} & \text{for} \quad t > t_d \end{cases} \tag{A13}$$

where θ is the correlation time describing the rotational diffusion of fluorophore.

For $0 \le t \le t_d$, the fluorescence anisotropy of the relaxed species is the same as that of the unrelaxed species until the arrival of the quenching pulse and is described by the first line of Eq. (A13). For $t > t_d$ the time dependence of the fluorescence anisotropy of the R species is more complex. For these times the fluorescence emission of the R species consists of two parts, which, in general, have different anisotropies. The first part of the emission is from those R molecules which were present in the sample at time $t + t_d$. The intensity decay, I_{R1}, and anisotropy decay, r_{R1}, of these molecules is described by the equations

$$I_{R1}(t) = I_{F0}(1 - q_R)\left(1 - \sum_{j=1}^{n} \xi_j e^{-k_{Rj}t_d}\right) e^{-\Gamma t} \tag{A14}$$

$$r_{R1}(t) = (r_0 + \Delta r_R e^{td/\theta})\, e^{-t/\theta} \tag{A15}$$

The second part of the emission is generated by those R molecules which are created by the process of relaxation after $t = t_d$. The intensity decay, I_{R2}, of these molecules is given by

$$I_{R2}(t) = I_{F0}(1 - q_F) \sum_{j=1}^{n} \xi_j (e^{-k_{Rj}t_d} - e^{k_{Rj}t}) e^{-\Gamma t} \tag{A16}$$

and their anisotropy decay, r_{R2}, is described by the second line of Eq. A13,

$$r_{R2}(t) = (r_0 + \Delta r_F e^{td/\theta})\, e^{-t/\theta} \tag{A17}$$

In summary, we can write

$$r_R(t) = \begin{cases} r_0 e^{-t/\theta} & \text{for} \quad 0 \le t \le t_d \\ \left(r_0 + \dfrac{\Delta r_R I_{R1}(t) + \Delta r_F I_{R2}(t)}{I_{R1}(t) + I_{R2}(t)} e^{t_d/\theta}\right) e^{-t/\theta} & \text{for} \quad t > t_d \end{cases} \tag{A18}$$

After introducing Eqs. (A5) and (A13) into Eq. (A11), one obtains

$$r_F = \frac{\sum_{j=1}^{n} \xi_j h_j^{-1} [r_0 (1 - q_F e^{-h_j t_d}) + \Delta r_F (1 - q_F) e^{-\gamma_j t_d}]}{\sum_{j=1}^{n} \xi_j \gamma_j^{-1} (1 - q_F e^{-\gamma_j t_d})} \quad \text{(A19)}$$

where $h_j = \gamma_j + \theta^{-1}$. Similar calculations, using Eqs. (A6), (A18), and (A12), yield

$$r_R = \frac{R_b + R_{a1} + R_{a2}}{J_{OR}} \quad \text{(A20)}$$

where $J_{OR} = J_R/I_{F0}$, and

$$R_b = r_0 \left[H^{-1} (1 - e^{-Ht_d}) - \sum_{j=1}^{n} \xi_j h_j^{-1} (1 - e^{-h_j t_d}) \right] \quad \text{(A21)}$$

$$R_{a1} = (r_0 + \Delta r_R e^{t_d/\theta}) (1 - q_R) H^{-1} e^{-Ht_d} \left[1 - \sum_{j=1}^{n} \xi_j e^{-h_j t_d} \right] \quad \text{(A22)}$$

$$R_{a2} = (r_0 + \Delta r_F e^{t_d/\theta}) (1 - q_F) \sum_{j=1}^{n} \xi_j [H^{-1} e^{-(h_j + H)t_d} - h_j^{-1} e^{-h_j t_d}] \quad \text{(A23)}$$

with $H = \Gamma + \theta^{-1}$.

Using Eq. (A9) and Eqs. (A18)–(A23) one can predict the dependence of anisotropy within the emission spectrum of fluorophore in the presence of relaxation and light quenching.

The simplified intuitive model used in the text can be obtained from the more complete description presented above. Equation (A13) becomes $r_F = r_0 = r$ when rotations are neglected and results as the first term in Eq. (11). Equation (18), upon simplified assumptions, gives the second and third terms in Eq. (11). Hence, the intuitive description of anisotropy dependence is consistent with the more general approach.

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REFERENCES

1. G. Weber (1952) *Biochem. J.* **51**, 145–155.
2. G. Weber (1952) *Biochem. J.* **51**, 155–167.
3. R. F. Steiner (Ed.) (1983) in *Excited States of Biopolymers*, Plenum Press, New York, pp. 117–162.
4. R. F. Steiner (1991) in J. R. Lakowicz (Ed.), *Topics in Fluorescence Spectroscopy, Vol. 2. Principles*, Plenum Press, New York, pp. 1–52.
5. R. B. Cundall and R. E. Dale (Eds.) (1983) *Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology*, Plenum Press, New York, p. 785.
6. R. D. Spencer, F. B. Toldeo, B. T. Williams, and N. Yoss (1973) *Clin. Chem.* **19**, 838–844.
7. E. Haber and J. C. Bennett (1962) *Proc. Natl. Acad. Sci. USA* **48**, 1935–1942.
8. S. A. Levison, W. B. Dandiker and D. Murayama (1977) *Env. Sci. Technol.* **11**, 292–297.
9. S. D. Stroupe (1981) *Clin. Chem.* **27**, 1575–1579.
10. D. M. Jameson and T. L. Hazlett (1991) in T. G. Dewey (Ed.), *Biophysical and Biochemical Aspects of Fluorescence Spectroscopy*, Plenum Press, New York, pp. 105–133.
11. G. G. Belford, R. L. Belford and G. Weber (1972) *Proc. Natl. Acad. Sci. USA* **69** (6), 1392–1393.
12. E. W. Small and I. Isenberg (1977) *Biopolymers* **16**, 1907–1928.
13. T. J. Chuang and K. B. Eisenthal (1972) *J. Chem. Phys.* **57**, 5094–5097.
14. M. Ehrenberg and R. Rigler (1972) *Chem. Phys. Lett.* **14**, 539–544.
15. M. D. Galanin, B. P. Kirsanov and Z. A. Chizhkova (1969) *Soviet Phys. JETP Lett.* **9** (9), 502–507.
16. A. N. Rubinov, V. I. Tomin and V. A. Zhivnov (1973) *Opt. Spectrosc. (USSR)* **35** (4), 451–452.
17. O. P. Girin (1987) *Izvest. Akad. Nauk (USSR)* **42** (3), 550–553.
18. N. G. Bakhshiev, E. S. Voropai, V. A. Gaisenok, O. P. Girin and A. M. Sarzhevskii (1981) *Opt. Spectrosc. (USSR)* **50** (6), 614–618.
19. J. R. Lakowicz, I. Gryczynski, V. Bogdanov and J. Kuśba (1994) *J. Phys. Chem.* **98**, 334–342.
20. I. Gryczynski, V. Bogdanov and J. R. Lakowicz (1994) *Biophys. Chem.* **49**, 223–232.
21. I. Gryczynski, J. Kuśba, V. Bogdanov and J. R. Lakowicz (1994) *J. Phys. Chem.* **98**, 8886–8895.
22. J. R. Lakowicz, I. Gryczynski, J. Kuśba and V. Bogdanov (1994) *Photochem. Photobiol.* **60**, 546–562.
23. J. Sepiol (1990) *Chem. Phys. Lett.* **175** (5), 419–424.
24. I. P. Dzugan, I. Schmidt and I. I. Aarisma (1986) *Chem. Phys. Lett.* **127** (4), 333–342.
25. R. Lapouyade, K. Czeschka, W. Majenz, W. Rettig, E. Gilabert and C. Rulliere (1992) *J. Phys. Chem.* **96**, 9643–9650.
26. A. Safarzadeh-Amiri (1986) *Chem. Phys. Lett.* **125** (3), 272–278.
27. E. Gorlach, H. Gygax, P. Lubini and U. P. Wild (1995) *Chem. Phys.* **194**, 185–193.
28. T. O. Harju, A. H. Huizer and C. A. G. O. Varma (1995) *Chem. Phys.* **200**, 215–224.
29. A. Mokhtari, A. Chebira and J. Chesnoy (1990) *J. Opt. Soc. Am. B* **7** (8), 1551–1557.
30. D. Wong, F. Pellegrino, R. R. Alfano and B. A. Zilinskas (1981) *Photochem. Photobiol.* **33**, 651–662.
31. I. Yamazaki, M. Mimuro, T. Murao, Yamazaki, K. Yoshihara and Y. Fujita, (1984) *Photochem. Photobiol.* **39**, 233–240.
32. Y. T. Mazurenko, V. V. Danilov and S. I. Vorontsova (1973) *Opt. Spectrosc. (USSR)* **35** (1), 107–108.
33. I. Gryczynski, J. Kuśba, Z. Gryczynski, H. Malak and J. R. Lakowicz (1996) *J. Phys. Chem.* **100** (24), 10135–10144.
34. G. Laczko, J. R. Lakowicz, I. Gryczynski, Z. Gryczynski and H. Malak (1990) *Rev. Sci. Instrum.* **61**, 2331–2337.
35. J. R. Lakowicz and I. Gryczynski (1991) in J. R. Lakowicz (Ed.), *Topics in Fluorescence Spectroscopy, Vol. 1. Techniques*, Plenum Press, New York, pp. 293–355.
36. A. Jablonski (1960) *Bull. Acad. Pol. Sci.* **8**, 259–264.
37. G. Weber (1952) *Biochem. J.* **51**, 145–155.
38. J. R. Laws and L. Brand (1979) *J. Phys. Chem.* **83**, 795–802.
39. A. Gafni and L. Brand (1978) *Chem. Phys. Lett.* **58**, 346–350.
40. J. R. Lakowicz and A. Balter (1982) *Biophys. Chem.* **16**, 223–240.
41. A. Kawski, I. Gryczynski, K. Nowaczyk, P. Bojarski, and J. Lichacz (1991) *Z. Naturforsch.* **46a**, 1043–1048.
42. M. J. Cote, J. F. Kauffman, P. G. Smith, and J. D. McDonald (1989) *J. Chem. Phys.* **90**, 2865–2873.
43. V. E. J. Hauser (1992) *Arab. J. Sci. Eng.* **17**, 209–219.
44. J. L. Herek, S. Pedersen, L. Banares, and A. H. Zewail (1992) *J. Chem. Phys.* **97**, 9046–9061.
45. J. S. Baskin, L. Banares, S. Pedersen, and A. H. Zewail (1996) *J. Phys. Chem.* **100**, 11920–11933.