Biophysical Chemistry, 49 (1994) 223–232
Elsevier Science B.V., Amsterdam

BIOCHE 1818

Light quenching and depolarization of fluorescence observed with laser pulses. A new experimental opportunity in time-resolved fluorescence spectroscopy

Ignacy Gryczyński, Valery Bogdanov and Joseph R. Lakowicz

Center for Fluorescence Spectroscopy, Department of Biological Chemistry, University of Maryland at Baltimore, School of Medicine 108 N. Greene Street, Baltimore, MD 21201 (USA)

(Received 15 June; accepted in revised form 10 September 1993)

Abstract

We report the first time-resolved studies of quenching of fluorescence by light, i.e., "light quenching". The dye 4-(dicyanomethylene)-2-methyl-6-(p-dimethamino)-4H-pyrane (DCM) was excited in the anti-Stokes region from 560–600 nm. At high illumination power the intensities of DCM were sub-linear with incident power. The extent of light quenching was proportional to the emission spectrum at the incident wavelength, as expected for light-stimulated decay from the excited state. The frequency-domain intensity decays indicated the effect was not due to heating or other photochemical effects. Importantly, the decay time was unchanged, as expected for light quenching with a single pulsed laser beam, while the time-zero anisotropy was decreased due to orientation-dependent quenching of the excited state population. Light quenching of fluorescence provides a new method to control the excited state population and orientation of fluorophores, and offers new experimental opportunities for biophysical applications of time-resolved fluorescence.

Keywords: Time-resolved fluorescence: Quenching; DCM; Anisotropy; Fluorophores

1. Introduction

In 1917 Einstein [1] [1] predicted that illumination of atoms in the excited state can stimulate their return to the ground state. This phenomena of "light quenching" can also occur for polyatomic molecules in condensed media [2] and was first observed by Galanin et al. [3]. The phenomena of light quenching was described by the Russian spectroscopists in a series of papers from 1965 to 1984. These experiments used the intense giant pulses from ruby lasers (694 nm) to quench the emission of phthalimide derivatives. These studies showed that light quenching required overlap of the quenching wavelength with the emission spectrum [3], and could induce blue-shifts in the emission spectra of solvent-sensitive fluorophores [4–7], presumably due to the decreased lifetime of the excited state and less time for spectral or solvent relaxation.

An important aspect of light quenching is that it displays the same photoselection rules as does

[1] This paper has been reprinted in: F.S. Barnes, Laser theory (IEEE Press, New York, 1972).

SSDI 0301-4622(93)E0072-D

excitation [8]. Mazurenko [9] observed depolarization at high illumination intensities, apparently due to selective stimulated decay of the vertically polarized component. This phenomena has been the subject of several additional theoretical and experimental studies [10], which often link the extent of spectral relaxation and depolarization [8,9,11]. While these results were interpreted in terms of the decreased lifetimes of the excited states, with one exception [12] there have been no direct time-resolved measurements of light quenching.

To date essentially all studies of light quenching have been performed using the intense giant pulses from Q-switched lasers. We now show that significant light quenching can occur with the lower energy pulses from a cavity-dumped ps dye laser. Observation of light quenching with these commonly available laser sources offers the opportunity to control the lifetime and orientation of the excited state populations, which can have numerous applications in the biophysical applications of time-resolved fluorescence.

2. Theory

2.1. Intuitive description of light quenching

A full description of the theory for light quenching is complex and beyond the scope of this initial report. We present first an intuitive description of time-dependent light quenching and how it can be expected to alter the intensity and anisotropy decays of fluorophores. We then describe several equations which can be used for a more quantitative understanding of light quenching.

One can imagine a variety of light quenching experiments, using either continuous or pulsed illumination, or involving single or dual wavelengths. In scheme 1, we consider pulsed illumination of a sample, in which the excitation and quenching pulses arrive at different times (left), or are coincident in time (right). We will refer to these cases as two- or one-beam experiments, respectively. We assume that the pulse width is much shorter than the fluorescence decay time.

In the case of pulsed and time-delayed quenching pulse (two-beam experiment), one expects the quenching pulse to create an instantaneous decrease in intensity upon its arrival at the observation point in the sample (middle left). Additionally, the anisotropy is expected to change instantaneously due to polarization-dependent light quenching (lower left). This change in anisotropy is expected because light quenching displays the same photoselection rules as does light absorption [8]. For a fundamental anisotropy (r_0) greater than zero one expects the anisotropy (r) to decrease if the excitation and quenching pulses are parallel ($\updownarrow\updownarrow$), and to increase if these pulses are orthogonally polarized ($\updownarrow\leftrightarrow$). The anisotropy theory for continuous illumination and light quenching has been described by Mazurenko [13], and is now being developed in this laboratory for pulsed excitation [14]. For pulsed illumination and a time-delayed quenching pulse, we expect the mean lifetime of the excited state to decrease and a complex decay of anisotropy.

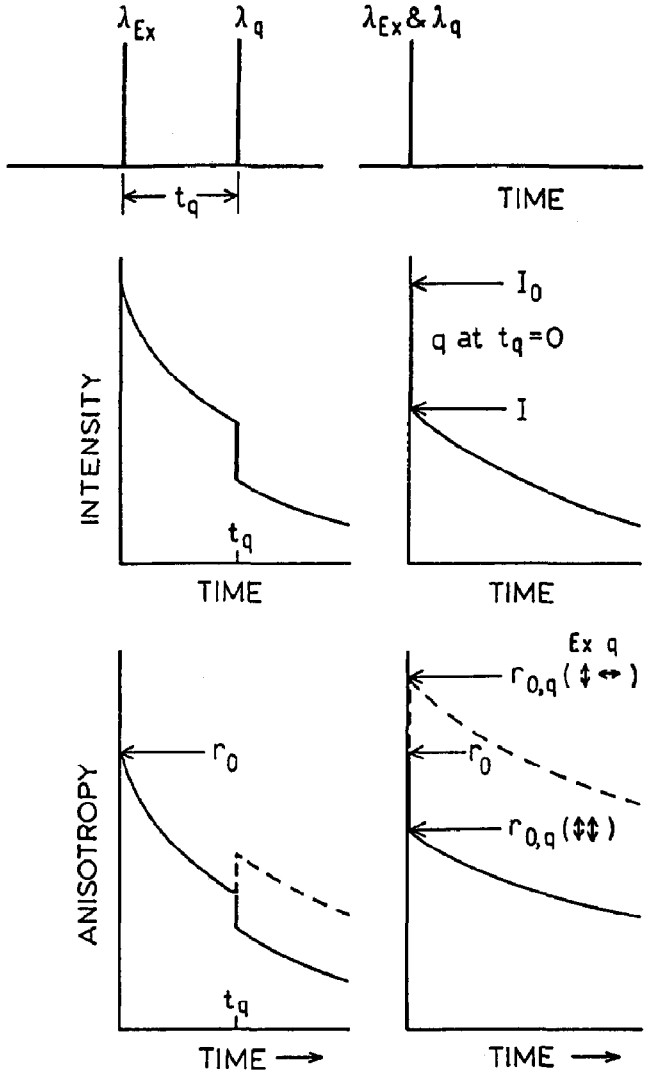
The concept of decreasing the mean lifetime can be better understood by considering a steady-state experiment, in which the emission spectrum and steady-state anisotropy is measured during illumination of the sample by a continuous train of excitation and time-delayed quenching pulses. The quenching pulses selectively deplete the longer-lived portion of the emission. Consequently, one selectively observes that portion of the emission which occurs prior to arrival of the quenching pulse. Hence, the stationary emission displays the properties of a shorter lifetime fluorophores, which can be a blue-shifted (unrelated) emission spectrum or a higher anisotropy.

Two-beam experiments of light quenching are more complex due to the need for precise overlap of the excitation and quenching beams and the precise alignment of an optical delay line. For this reason our initial experiments used a single beam for both excitation and quenching. The expected results are shown in scheme 1 (right). For pulsed illumination, light quenching occurs simultaneously with excitation. Hence, the fluorescence intensity decay is unchanged, except for a decrease in the initial amplitude. Since fluorescence lifetime measurements are not sensitive to

the total intensity, the decay time is expected to be the same, independent of the amount of light quenching.

This case of time-coincident excitation and quenching pulses can be understood by analogy with static quenching of fluorescence. In the case of static quenching the fluorophore-quenching pairs do not emit. The intensity of a statically quenched sample is decreased, but the lifetime is unchanged because the emission results from the unquenched and unperturbed fluorophores. In a similar way, fluorophores which are instantaneously quenched by light do not contribute to the intensity decay, and the lifetime remains unchanged.

A somewhat different result is expected for the anisotropy decay. Assuming $r_0 > 0$ and vertically polarized illumination, one expects selective quenching of the population aligned along the vertical axis. The correlation time and rate of



Scheme 1. Predicted effects of time-delayed (left) and coincident (right) light quenching pulses on the intensity (middle) and anisotropy decays (bottom) of a fluorophore.

anisotropy decay is expected to remain unchanged. Hence, the hallmark of light quenching for single beam pulsed illumination is a constant lifetime and rotational correlation time, but a decrease in the time-zero anisotropy.

2.2. Simplified description of light quenching

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

$$\frac{dN^*(t)}{dt} = N\sigma_a P - N^*(t)\left(\frac{1}{\tau} + \sigma_{lq} P\right), \quad (1)$$

where N is the ground state concentration, $N^*(t)$ is the excited state population, σ_a the cross section for absorption, τ the fluorescence lifetime, P the laser power and σ_{lq} the cross section for stimulated emission. The excited state population in the absence of light quenching is described by eq. (1) with σ_{lq} equal to zero.

Consider the case of continuous illumination, in which case the derivative in eq. (1) can be set equal to zero. In this case the intensities in the absence (I_0) and presence (I) of light quenching are given by

$$I_0 = k\sigma_a \tau NP, \quad (2)$$

$$I = k\frac{\sigma_a \tau NP}{1 + \sigma_{lq}\tau P}, \quad (3)$$

where k is an instrumental constant. Hence, the extent of quenching is given by

$$\frac{I_0}{I} = 1 + \sigma_{lq}\tau P. \quad (4)$$

We can now consider the effect of replacing continuous illumination with pulsed illumination, with a pulse width t_p, and $t_p < \tau$. In this case the quenching light can act only during the pulse itself, rather than during the entire decay (τ). Hence, use of pulsed quenching results in replacement of τ by t_p,

$$\frac{I_0}{I} = 1 + \sigma_{lq} t_p P, \quad (5)$$

where the power P is in appropriate units. A more complete description may require the use of the pulse repetition rate and power per pulse, but in any event the extent of quenching (I_0/I) is expected to show Stern–Volmer-like behavior, where the concentration of quencher is replaced by the density of quenching photons. Use of eq. (5) requires knowledge of I_0. These values, at each value of the incident laser power, are estimated by linear extrapolation from low-power region of the experimental intensity data.

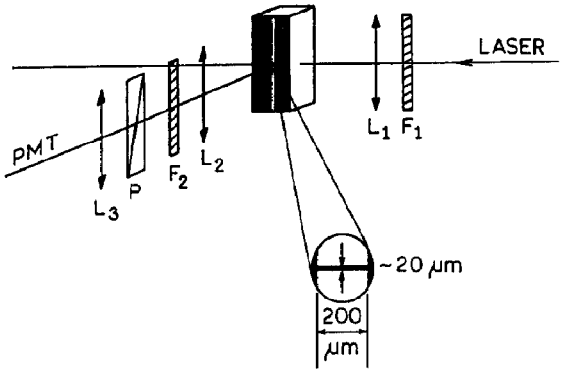
As discussed above, light quenching is expected to decrease the time-zero anisotropy. A complete description of this effect is complex. Briefly, consideration of orientation effects results in modification of eq. (1) to be

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

where θ is the angle between the colinear absorption and emission moments and the electric vector of the incident light. The quenching light selectivity depletes that portion of the excited fluorophores which are aligned parallel to the electric vector of the incident light. A decrease in this component results in a decrease in the anisotropy.

3. Materials and methods

The light source in these experiments was the cavity-dumped output of a rhodamine 6G (R6G) dye laser, which was synchronously pumped by the 514 nm output of a mode-locked Argon ion laser. The pulse width was near 5 ps, with a repetition rate of 3.795 MHz. Hence, an incident power of 50 mW corresponds to an approximate peak power of 2.5 kW. This light was focused



Scheme 2. Sample and focusing configuration used for light quenching studies of DCM.

(L_1) to a spot size of about 20 μm in diameter (scheme 2), resulting in a maximum intensity of about 1.0×10^9 W/cm^2. Without focusing, the spot size was about 0.2 cm in diameter.

The excitation was polarized vertically, as occurs from the output of our dye laser. The emission was observed through a Corning 2-59 filter, which transmits light above 650 nm. For intensity measurements the emission polarizer was 54.7° from the vertical. Control measurements using solvents without DCM gave signals less than 0.5% of the DCM emission, for all polarization conditions and excitation (quenching) wavelengths. DCM was obtained from Exciton laser grade and used without further purification. Concentration of DCM in solutions was about 10^{-5} M/l.

All intensity, anisotropy and frequency-domain (FD) data were obtained using the instrumentation described previously [15–17]. The detector was a R928 PMT, which is sensitive to the long wavelength emission of DCM. The average and peak power of the incident light were varied by insertion of neutral density filters (F_1) into the excitation beam (scheme 2). In order to determine if sample heating altered the data, the total power was decreased without changing the instantaneous power. This was accomplished by the use of a low-speed mechanical light chopper in the excitation beam, by which the average intensity was decreased 100-fold. The samples (except glycerol) were stirred during the measurements, and we observed no effects of illumination time on the intensity or anisotropy values. The signals were stable upon continuous illumination at our experimental conditions.

4. Results

To date, light quenching has only been observed using the intense giant pulse from Q-switched lasers. However, modern time-resolved measurements are presently performed using ps or fs pulses from dye lasers. Hence, we questioned whether significant amounts of light quenching were observable using the output of a

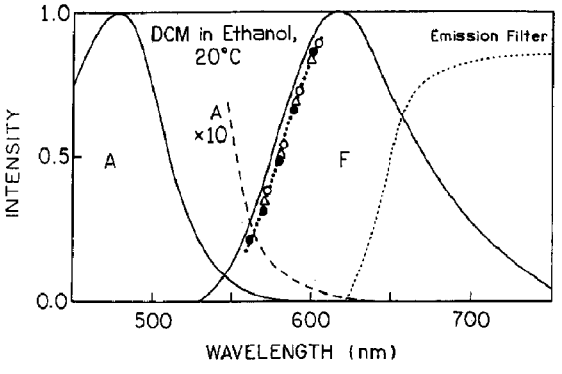


Fig. 1. Absorption and emission spectra of DCM in ethanol. The symbols on the emission spectrum show the relative cross section of light quenching (eq. (1)), at the indicated wavelength, in propylene glycol (●), iso-butanol (○) and ethanol (△). The right side dashed line shows the transmission spectrum of the filter used to isolate the emission. The relational cross section is calculated using $(Q-1)/P$.

amounts of light or depletion of the ground state DCM population.

The amount of light quenching is expected to depend on the extent of overlap of the incident light with the fluorescence emission spectrum. More specifically, the cross section for quenching is expected to be proportional to the emission spectra $I(\bar{\nu})$ and inversely proportional to the natural (radiative) lifetime (τ_N) by

$$\delta_{lq} = \frac{k'}{\tau_N} \frac{I(\bar{\nu})}{\int I(\bar{\nu})\, d\bar{\nu}}, \quad (7)$$

where k' is a constant. This suggests that the amounts of quenching should scale as the emission spectrum.

The wavelength-dependent quenching constants are shown in fig. 1, along with the DCM emission spectrum. The amount of quenching clearly follows the DCM emission in all three solvents. To the best of our knowledge, these data represent the first measurement of the light quenching spectrum of a fluorophore. It should be noted that this observation of a quenching spectrum corresponding to the emission spectrum indicates that this spectrum already exists within the 5 ps duration of the excitation pulse. More explicitly, this result suggests that the excited state of DCM has already relaxed from the Frank–Condon state to the solvent relaxed state during the laser pulse. This result is consistent with experimental observations [23] of ultrafast relaxation of the excited state of DCM in ethylene glycol. However, in the present experiments with long-wavelength excitation, the results are also consistent with excitation of the relaxed state by the red-edge effect [20–22], and more detailed studies are needed over a wider range of wavelengths to demonstrate this assertion. In any event, light quenching within the single beam ps pulses may provide a method to study ultrafast relaxation processes.

As a final confirmation of our observation of light quenching we examined the frequency-domain (FD) intensity and anisotropy decays of DCM for low and high power illumination (fig. 4). As discussed in section 2.1 (scheme 1), light quenching is expected to decrease the steady-state intensity and anisotropy, but not change the decay time or rotational correlation time. While the fluorescence quantum yield of DCM is decreased by high power illumination, its decay time is not changed (fig. 4, top). The inserts show the intensity decays reconstructed from the FD data, and the amounts of light quenching shown in fig. 3.

The lower half of fig. 4 shows the FD anisotropy decay. The correlation times are identical for low- and high-intensity illumination, which argues strongly against the presence of any heat-dependent effects of the observed light quenching. Heating of the sample is expected to decrease the rotational correlation time. Importantly, the time-zero anisotropy ($r(0)$) is decreased for high laser power, as predicted for co-incident excitation and quenching pulses (scheme 1).

Table 1

Intensity and anisotropy decay parameters for DCM

Solvent	Low laser power (no lq)					High laser power (+ lq)				
	intensity decays		anisotropy decays			intensity decays		anisotropy decays		
	τ (ns)	χ^2_R	r_0	θ (ns)	χ^2_R	τ (ns)	χ^2_R	r_0	θ (ns)	χ^2_R
propylene glycol	2.10	1.4 (1.4) [a]	0.371	5.08	1.3 (1.3)	2.18	1.5 (1.0)	0.256	5.05	1.6 (1.6)
iso-butanol	2.01	2.6 (2.1)	–	–	–	2.03	2.5 (2.2)	–	–	–
ethanol	1.81	1.5 (1.2)	–	–	–	1.87	1.9 (1.6)	–	–	–

[a] χ^2_R values obtained for two-exponential fits.

The intensity and anisotropy decay data are summarized in table 1. If the DCM were being degraded by the intense laser pulses, one would expect the intensity or anisotropy decay to become multi-exponential. This is not the case, as both the intensity and anisotropy decay analysis is not improved by the use of the two decay times or two correlation times, respectively. These experiments provide strong evidence of light quenching of DCM by high-intensity illumination.

5. Discussion

The phenomenon of fluorescence quenching has been widely used to study the structure and dynamics of biological molecules [24–27]. These studies have been used to reveal the rates of macromolecule permeation by quenchers [28,29], and the accessibility of macromolecule-bound fluorophores to polar and non-polar quenchers [26–28]. The use of collisional quenching requires significant concentrations of dissolved quenchers, which may absorb the incident light or perturb the structure of the macromolecules. An important aspect of light quenching is that the effect is easily and immediately reversible by alternating or blocking of the quenching beam.

An alternative use of collisional quenching has been its use to alter the mean lifetime of the excited state. By performing steady-state measurements with various mean lifetimes, i.e. lifetime-resolved measurements, one can determine rotational correlation times [30–32] and the rates of spectral relaxation of solvent-sensitive fluorophores [33,34]. The use of light quenching as a reversible and non-perturbing quencher can facilitate the expanded use of lifetime-resolved methods.

One may question whether the modest amounts of quenching observed in the present report are adequate for lifetime-resolved measurements. It is apparent that more powerful lasers will continue to become available. With presently available Q-switched lasers one can readily predict quenching of 100-fold. Consequently, an unquenched lifetime of 1 ns can be reduced to 10 ps, which can provide resolution of ps processes from the steady-state measurements of the anisotropy or emission spectra. Because of the high sensitivity of fluorescence detection, and probably that background auto-fluorescence will also be quenched by the light pulses, a 100-fold decrease in intensity is well within present experimental capability.

In our opinion, the preferred use of light quenching will involve two-beam experiments. It is apparent that ps or fs pulses can quench fluorescence and can also serve as an instantaneous perturbation of the excited state population and orientation. Importantly, we now know that light quenching can be used to increase the anisotropy to values much greater than the usual limit of 0.4, and can be used to break the z axis symmetry which is usually present in studies of non-oriented samples [14]. Such experiments, when coupled with time-resolved measurements of the intensity and anisotropy decays, will provide information on the events following the ps or fs quenching pulse, such as time-dependent spectral shifts or rotational diffusion. Light quenching allows a new class of experiments in which time-delayed quenching pulses are used to control and/or modify the excited state population and orientation. Such experiments are presently in progress in this laboratory.

6. Acknowledgement

This work was supported by grants from the National Science Foundation (MCB-8809931 and BIR-9319032), with support for instrumentation from the National Institutes of Health (RR-08119 and RR-07510).

7. References

1 A. Einstein, Phys. Z. 18 (1917) 121.
2 O. Suelto and D.C. Hanna, Principles of lasers (Plenum Press, New York, 1989).
3 M.D. Galanin, R.P. Kirsanov and Z.A. Chizhikova, Soviet Phys. JETP Letters 9 (1969) 502–507.
4 A.N. Rubinov, V.I. Tomin and V.A. Zhivnov, Opt. Spectry. (USSR) 35 (1973) 451–452.
5 O.P. Girin, Izvestiya Akademii Nauk (USSR) 42 (1978) 550–553 (pp. 86–89 English translation).

6 N.G. Bakhshiev, E.S. Voropai, V.A. Gaisenok, O.P. Girin and A.M. Sarzhevskii, Opt. Spectry. (USSR) 50 (1981) 614–618.
7 N.G. Bakhshiev and O.P. Girin, Opt. Spectry. (USSR) 52 (1982) 7–8.
8 A.I. Butko, E.S. Voropai, V.A. Gaisenok, V.A. Saechnikov and A.M. Sarzhevskii, Opt. Spectry. (USSR) 52 (1982) 153–156.
9 Y.T. Mazurenko, V.V. Danilov and S.I. Vorontsova, Opt. Spectry. (USSR) 35 (1973) 107, 108.
10 A.I. Butko, E.S. Voropai, I.I. Zholnerevic, V.A. Saechnikov and A.M. Sarzhevskii, Izvestiya Akademii Nauk (USSR) 42 (1978) 626–630.
11 E.S. Voropai, V.A. Gaisenok, A.A. Kirsanov, V.A. Saechnikov and A.M. Sarzhevskii, Opt. Spectry. (USSR) 57 (1984) 140–142.
12 H.E. Lessing, E. Lippert and W. Rapp, Chem. Phys. Letters 7 (1970) 247–253.
13 Y.T. Mazurenko, Opt. Spectry. 35 (1973) 137–139.
14 J.R. Lakowicz, I. Gryczynski, J. Kuśba and V. Bogdanov, manuscript in preparation.
15 J.R. Lakowicz and B.P. Maliwal, Biophys. Chem. 21 (1985) 61–78.
16 J.R. Lakowicz, G. Laczko and I. Gryczynski, Rev. Sci. Instr. 57 (1986) 2499–2506.
17 G. Laczko, J.R. Lakowicz, I. Gryczynski, Z. Gryczynski and H. Malak, Rev. Sci. Instr. 61 (1990) 2331–2337.
18 I. Gryczyński, V. Bogdanov and J.R. Lakowicz, unpublished observation.
19 V.V. Danilov, Y.T. Mazurenko and S.I. Vorontsova, Opt. Commun. 9 (1973) 283–286.
20 A.P. Demchenko, in: Topics in fluorescence spectroscopy, Vol. 3, ed. J.R. Lakowicz (Plenum Press, New York 1992) pp. 65–111.
21 M.J.E. Morgenthaler, S. Meech and K. Yoshihara, Chem. Letters 197 (1992) 537–541.
22 A.N. Rubinov and V.I. Tomin, Opt. Spectry. 29 (1970) 578–580.
23 D.C. Easter and A.P. Baronavski, Chem. Phys. Letters 201 (1993) 153–158.
24 M.R. Eftink, in: Topics in fluorescence spectroscopy, Vol. 2, ed. J.R. Lakowicz (Plenum Press, New York 1991) pp. 53–126.
25 J.M. Vanderkooi, S.W. Englander, S. Papp, W.W. Wright and C.S. Owen, Proc. Natl. Acad. Sci. 87 (1990) 5099–5103.
26 S.S. Lehrer, Biochemistry 10 (1971) 3254–3263.
27 D.B. Calhoun, S.W. Englander, W.W. Wright and J.M. Vanderkooi Biochemistry 27 (1988) 8466–8474.
28 J.R. Lakowicz and G. Weber, Biochemistry 12 (1973) 4171–4179.
29 E.B. Abuin and E.A. Lissi, Progr. React. Kinetics 16 (1991) 1–33.
30 J.R. Lakowicz, B. Maliwal, H. Cherek and A. Balter, Biochemistry 22 (1983) 1741–1752.
31 J.R. Lakowicz and B. Maliwal, J. Biol. Chem. 258 (1983) 4794–4801.
32 J.R. Lakowicz, F.G. Prendergast and D. Hogen, Biochemistry 18 (1979) 520–527.
33 J.R. Lakowicz and F.G. Prendergast, J. Biol. Chem. 254 (1979) 1771–1774.
34 J.R. Lakowicz and D. Hogen, Biochemistry 20 (1981) 1366–1373.