Journal of Fluorescence, Vol. 10, No. 4, 2000

Parameter Recovery in Frequency-Domain Time-Resolved Fluorescence Spectroscopy; Resolution of the Prototropic Forms of 5-Carboxyfluorescein in the Physiological pH Range

Pascal Vallotton[1] and Horst Vogel[1,2]

Received November 2, 1999; revised March 25, 2000; accepted May 28, 2000

In time-resolved fluorescence spectroscopy, the resolution of fluorescence species becomes increasingly difficult as their respective lifetimes get closer. For a biexponential decay, a factor of 1.4 between the two decay times is commonly accepted as the practical resolution limit. The goal of the present contribution is to characterize the fluorescence probe 5-carboxyfluorescein using frequency-domain time-resolved fluorescence spectroscopy (FD-TRFS). To resolve the different prototropic forms of this probe, the limit above had to be overcome. For this purpose, the standard global analysis method was used, and special emphasis was put on the errors associated with the recovered parameters. In particular, a Monte Carlo simulation was performed to estimate these errors and the results of this analysis were compared with those delivered by software packages widely used in the field. The lifetimes of the trianionic and dianionic forms of 5-carboxyfluorescein were 4.01 $\pm$ 0.06 and 3.03 $\pm$ 0.09 ns, respectively, and the pK_a for this acid–base equilibrium was determined to be 6.9 $\pm$ 0.3.

KEY WORDS: Time-resolved; fluorescence spectroscopy; fluorescence lifetimes; Monte Carlo simulation; global analysis.

INTRODUCTION

The power of time-resolved fluorescence spectroscopy as a tool for the investigation of the structure and dynamics of macromolecules, the elucidation of biomolecular interactions, and bioanalytical applications depends critically on the ability to resolve closely spaced lifetimes. In turn, this capacity depends on the signal/noise ratio offered by the instrumentation [1–4], on the amount of experimental data available, and on the techniques used to analyze those data. A factor of 1.4 between the lifetimes of two fluorescent species represents a limit below which errors on the recovered parameters tend to become very important [5]. Hence, in such cases, special care has to be taken with regard to error estimation. In this contribution, we study the photochemistry of the fluorescence probe 5-carboxyfluorescein (Fig. 1) in the physiological pH range, by frequency-domain time-resolved fluorescence spectroscopy (FD-TRFS). The trianionic and dianionic forms of the probe could be resolved, and their lifetimes were shown to differ by less than 25%. We have used the global analysis approach [6], which allowed data collected at different pH values to be analyzed simultaneously, thereby increasing the confidence in the recovered parameters. The 5-carboxyfluorescein probe has found many applications in biology. It has been used to measure intracellular pH [7,8] and to monitor cell viability [9],

[1] Department of Chemistry, Swiss Federal Institute of Technology, LCPPM, CH 1015 Lausanne, Switzerland.
[2] To whom correspondence should be addressed. e-mail: horst.vogel@epfl.ch

1053-0509/00/1200-0325$18.00/0

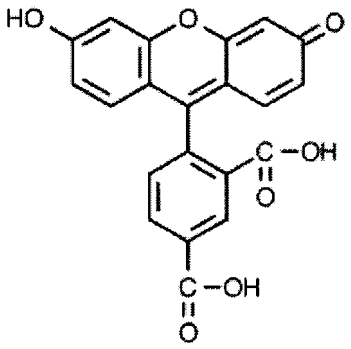

Fig. 1. Chemical formula of 5-carboxyfluorescein. In the physiological pH range, the benzoic carboxylic groups are deprotonated. The trianionic and dianionic forms correspond to the deprotonated and protonated state, respectively, of the xanthene hydroxyl group.

apoptosis [10], and cell adhesion [11], and it can be activated for modification of primary amino groups [12]. Our FD-TRFS experiments permitted us to evaluate the pK_a of the equilibrium between the trianionic and the dianionic forms of the probe and to determine precisely the individual lifetimes of these two species. In comparison to the pK_a of fluorescein, the pK_a found for 5-carboxyfluorescein was significantly higher: a difference that we attribute to electrostatic effects. Confronted with important differences in the errors delivered by standard parameter recovery softwares, we have performed a Monte Carlo simulation to obtain realistic error estimates on the lifetimes and preexponential factors. The noise used in these simulations was generated according to the measured, frequency-dependent instrumental noise, which was characterized in detail. Finally, we have studied the influence on the parameter recovery of either using standard deviations on the phase and demodulation determined experimentally or assuming a priori constant errors, as is done sometimes.

THEORY

In FD-TRFS, an intensity-modulated light beam interacts with fluorescent molecules. The emitted wave train is retarded by a phase ϕ and its modulation decreases compared to the exciting beam due to the finite lifetime of the excited state of the investigated fluorophore [13]. The phase shift ϕ and the demodulation m, defined as the ratio of the modulation of the fluorescence emission to that of the excitation beam, when represented as a function of the modulation frequency, is the time-resolved spectrum (Fig. 4). For a species with a single lifetime τ, the phase shift ϕ and demodulation m obey

$$\tan(\phi) = \omega\tau \tag{1}$$

$$m = (1 + \omega^2\tau^2)^{-0.5} \tag{2}$$

where ω is the circular frequency of excitation.

The goal of the spectrum analysis is to recover the fluorescence emission $I(t)$ of the sample after it has been illuminated by an infinitely short pulse of light. Under certain assumptions, this impulse response function can be described by

$$I(t) = I_0 \sum_i \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \tag{3}$$

For a diluted, ground-state heterogeneous sample and in the absence of excited-state reactions, the preexponential factors α_i are linked to the concentrations c_i of the fluorescent species indexed by i through

$$\alpha_i = \frac{c_i Q_i \varepsilon_i/\tau_i}{\Sigma c_i Q_i \varepsilon_i/\tau_i} \tag{4}$$

where Q_i is the quantum yield, τ_i is the lifetime of the excited state, and ϵ_i is the extinction coefficient of species i.

A time-resolved spectrum and its corresponding impulse response function form a Fourier transform pair. However, inversion of this transform is highly sensitive to experimental noise. Algorithms relying, for example, on the maximum entropy method [14] or on the method of moments [15] have been applied to cope with this problem. The nonlinear least-squares fitting method used in this article remains by far the most popular approach. It is based on the minimization of the chi-square statistic, which, in the framework of FD-TRFS, is defined by

$$\chi_\nu^2 = \frac{1}{\nu}\left[\sum_{i=1}^{n} \frac{1}{\sigma_{mi}^2}(m_i - M_i)^2 + \sum_{i=1}^{n} \frac{1}{\sigma_{\phi i}^2}(\phi_i - \Phi_i)^2\right] \tag{5}$$

where m_i and ϕ_i denote the experimentally measured demodulation and phase shift, respectively, for the acquisition frequency indexed by i. M_i and Φ_i are the calculated demodulation and phase assuming a trial set of preexponential factors and lifetimes, σ_{mi} and $\sigma_{\phi i}$ are the standard deviations on the modulation and phase, respectively, and ν is the number of degrees of freedom, defined as the number of data points minus the number of estimated parameters.

Provided that the errors follow Gaussian distributions, that the standard deviation appearing in the calculation of the chi-square statistics has been estimated accurately, and that no systematic errors are present, minimization of this statistic recovers the most probable physical parameters [16]. These important conditions, relative to the specific instrumentation used, were investigated in this article.

When recovering physical parameters from noisy experimental data, reducing the number of parameters while keeping the same data set increases the confidence on the recovered parameters. Exploring a phenomenon in more dimensions (pH, temperature, viscosity, wavelength, etc.) has the same effect. This is a consequence of the curvature of the chi-square surface at the global minimum, which can be increased in the direction of the new experimental axis introduced. Implementing these ideas [6] leads to finding physical invariants throughout the experiments while minimizing the chi-square. As an example of this global analysis performed in the present contribution, one can recognize that the lifetimes of individual prototropic forms of 5-carboxyfluorescein should not depend on the pH and therefore link them throughout the experiments done at different pH values.

The Monte Carlo method to estimate errors proceeds by simulating many experiments by starting with the data from one real experiment and adding random perturbations of a magnitude that corresponds to the experimental noise. For each simulated experiment, the desired parameters are then recovered and the standard deviation over these sets is taken as the error. The key assumption of the method is that the physical parameters recovered in the seed experiment should be close enough to the "real" parameters that the estimate of the error obtained in this way reflects the "real" standard error on the recovered parameter [16].

MATERIALS AND METHODS

Time-Resolved Fluorescence Spectroscopy

Time-resolved spectra were acquired on a ISS K2 multifrequency cross-correlation phase and modulation fluorimeter (ISS Inc., Champaign, IL). Data at each frequency were collected using the default settings as defined by the instrument manufacturer. Quartz cuvettes of $1 \times 1\text{-cm}^2$ cross section (Hellma GmbH & Co.) were used. The emission polarizer was set at the magic angle (54.7°), followed by a 530 nm long-pass filter (Omega Optical, Inc.). Vertically polarized excitation light at 488 nm was provided by a Sabre argon ion laser (Coherent Inc., Santa Clara, CA).

The time-resolved spectra were analyzed with the Globals Unlimited and Miniglobal softwares (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana–Champaign) as well as with the CFS software (Center for Fluorescence Spectroscopy, University of Maryland, Baltimore).

Monte Carlo Error Estimation

To get independent error estimates on the recovered physical parameters describing the time-resolved decay of 5-carboxyfluorescein as a function of pH, Monte Carlo simulations were performed as follows. One thousand copies of the original experimental data set (each consisting of six spectra from pH 5.4 to pH 8.7 to be analyzed globally) were generated by the addition of noise. We used the Splus 4.5 software (MathSoft, Inc.) to produce the necessary 120,000 random phase and demodulation errors from Gaussian distributions of the appropriate experimentally determined width (see Results). These errors were inserted in the files of the spectra by using routines written in Visual Basic 5 (Microsoft) and using the macro processing facilities of Word 7.0 (Microsoft) for minor corrections. The 1000 copies were then treated with the Globals Unlimited software using its batch processing capabilities to recover the relevant parameters from each simulated experiment. Result files produced by this software were parsed with Visual Basic 5 routines written for this purpose and the recovered parameter sets were analyzed with Splus 4.5 to calculate the standard errors.

Recently, the CFS software was made available to us as a freeware. Its built-in Monte Carlo error estimation capabilities were used for comparison.

Chemicals and Samples

5-Carboxyfluorescein (Molecular Probes Inc.), 10 n*M*, in 0.01 *M* NaOH was used as the lifetime reference solution. This solution displays a single lifetime of 4.0 $\pm$ 0.1 ns when measured versus a diluted scattering glycogen solution.

A 10 m*M* citric acid–sodium citrate buffer was used to prepare the solutions of pH 5.4 and 6.0. A 10 m*M* Hepes buffer was used for the solutions of pH 6.6, 7.2, 7.8, and 8.7. No pH adjustments were performed, to minimize possible artifacts caused by variation of ionic strengths. All solutions were degassed with helium prior to measurements.

RESULTS

Preliminary Noise Analysis

The recovery of physical parameters from time-resolved spectra demands first that the standard errors of the phase and demodulation are known at each modulation frequency. Indeed, these errors enter into the expres-

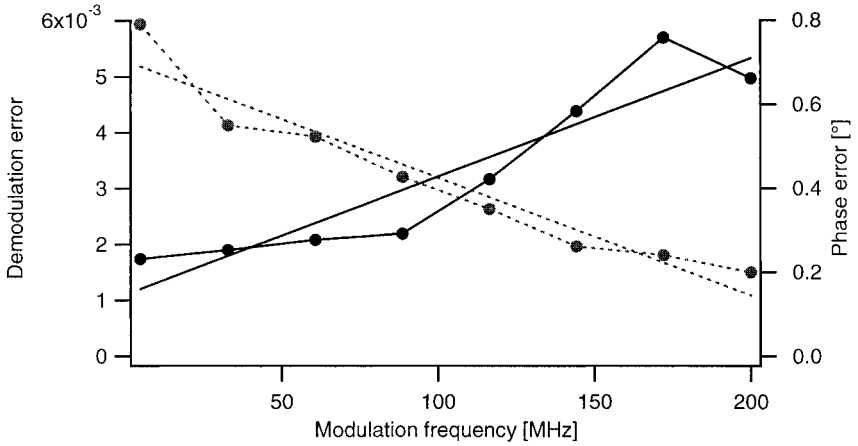

Fig. 2. Standard deviation of the phase (solid line) and demodulation (dashed line) as a function of the modulation frequency of the excitation light. The noise on the phase and demodulation were fitted by straight lines to evaluate the noise at other acquisition frequencies.

sion [Eq. (5)] of the chi-square which is minimized during the parameter recovery. To determine these errors experimentally, we measured the time-resolved spectrum of a reference fluorescein solution in 21 independent experiments. We think that the standard deviations of the phase and the demodulation at each frequency taken over these independent experiments are the errors that should be used by the recovery algorithm. Time-resolved spectra were acquired from 5 to 200 MHz at eight equidistant frequencies and the variances on the phase and demodulation were calculated for each modulation frequency (Fig. 2) using Excel (Microsoft). These errors were interpolated by straight lines to provide estimates of the errors when other modulation frequencies are used.

To investigate whether the results produced by our instrument have a Gaussian character (this is implicitly assumed by the recovery algorithm), the data produced in the 21 independent experiments were reduced. This was done to improve the statistics which would not have been significant if, for example, 21 phases for a single frequency only had been tested for normality. Hence, the mean of the phase (demodulation) at each frequency was subtracted and the result was divided by the corresponding frequency-dependent standard deviation for each data point. The histograms for these combined, reduced data sets were compared with the normal distribution as shown in Fig. 3. Reduced chi-squares for the phase and demodulation data sets when tested against this distribution were 0.61 and 2.56, respectively.

In the 21 independent experiments described above, the sample cuvette contained a fluorescein solution. The reference cuvette contained the same solution. Because the lifetime of the reference solution (4.0 ns) is used as an input by the acquisition software to determine the lifetime of the sample, the latter should have a mathematical expectation value of 4.0 ns. Based on this and on Eqs. (1) and (2), we computed a phase and demodulation ratio at each frequency. Significant deviations of the experimentally determined phase and demodulation values from these calculated values represent systematic instrumental errors that must be subtracted before recovering the lifetimes. However, here, systematic errors were always found to be below the noise level, and such a correction was not necessary.

Resolution by FD-TRFS of the 5-Carboxyfluorescein Prototropic Forms

To resolve the individual prototropic forms of 5-carboxyfluorescein by FD-TRFS, six time-resolved spectra (10 equidistant frequencies ranging from 10 to 200 MHz) were acquired for six pH values, ranging from 5.4 to 8.7. These spectra were analyzed globally assuming that only two prototropic forms were present. The concentrations of the two species, but not the lifetimes of the individual forms, are expected to change with the pH value. Hence, we linked the lifetime of each prototropic form across the experiments, leaving the preexponential factors free to vary individually. The standard deviations used during the chi-square minimization were the errors determined experimentally earlier. The global chi-square was 0.36 and local chi-squares for each experiment are listed in Table I. The spectrum obtained at a pH value of 5.4 is shown as a representative result in Fig. 4, with its associated global fit and the residues. The global lifetime of the trianionic form of carboxyfluorescein was 4.01 ns and that of the dianionic form was 3.03 ns.

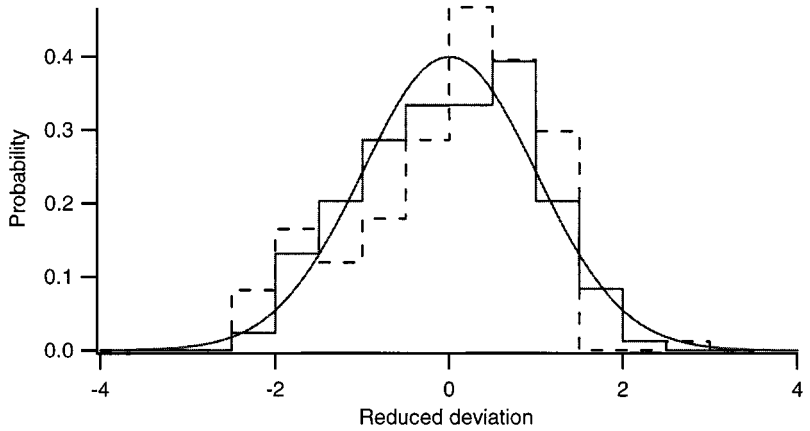

Fig. 3. Reduced noise (see text for details) for the phase (solid line) and demodulation (dashed line) data compared to the normal distribution showing the approximate Gaussian character of the data. Reduced chi-squares for the phase and demodulation when tested against this distribution were 0.61 and 2.56, respectively.

Table I. Local (Reduced) Chi-Square Values Relative to the Global Analysis Biexponential Fit Describing the Time-Resolved Decay of 5-Carboxyfluorescein as a Function of the pH Value

	pH					
	8.7	7.8	7.2	6.6	6	5.4
χ^2_ν	0.42	0.22	0.15	0.39	0.90	0.33

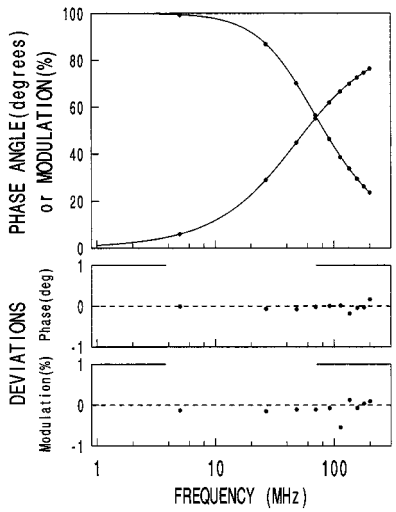

Fig. 4. Time-resolved spectrum of 5-carboxyfluorescein measured at a pH value of 5.4. Similar results were obtained for the measurements performed at pH values of 6.0, 6.6, 7.2, 7.8 and 8.7. The fit and the residues (bottom) result from a biexponential decay fitted by global analysis.

The preexponential factors were dependent on the pH value and those of the trianionic form are reported in Fig. 5. To determine the influence of the errors used during the chi-square minimization, we have compared the parameter recovery both with frequency-independent errors (0.2° for the phase and 0.004 for the demodulation) and with the experimentally determined errors. The results are shown in Fig. 5, and to our eyes, the differences are not sufficient in this case to exclude the use of these frequency-independent errors. Figure 5 shows a titration curve which was fitted based on the Henderson–Hasselbach equation,

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[A^-]}{[HA]} \tag{6}$$

where $[A^-]$ is the concentration of the trianionic and $[HA]$ is the concentration of the dianionic form of 5-carboxyfluorescein. The values [17] of the quantum yield and extinction coefficient for the dianionic (0.93 and 87,700 M^{-1} cm^{-1}, respectively) and monoanionic (0.36 and 16,425 M^{-1} cm^{-1}, respectively) form of fluorescein were used in Eq. (4) to calculate the theoretical preexponential factors for a given pK_a value. Based on this, we could fit the data, and a pK_a value of 6.9 ± 0.3 for the trianion-dianion equilibrium was obtained.

Errors relative to the recovered parameters were then investigated. We first used our implementation of the Monte Carlo method described under Materials and Methods. As a typical result of these simulations, the two recovered lifetimes obtained from each simulated experiment are shown in Fig. 6. The standard deviations on these recovered parameters as well as the errors determined

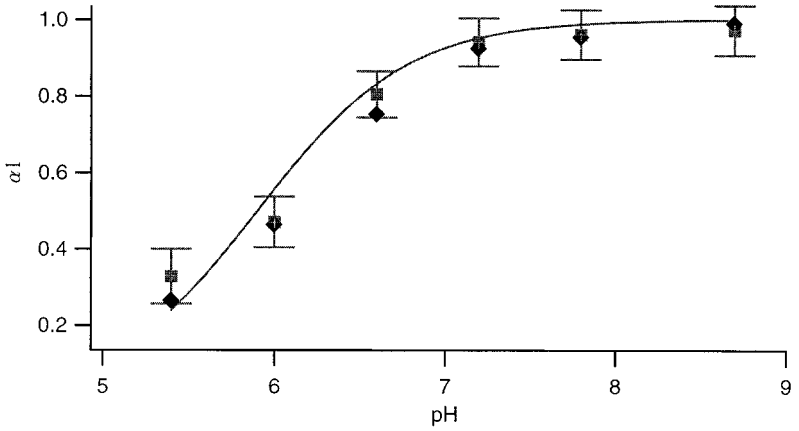

Fig. 5. Preexponential factors for the trianionic form of 5-carboxyfluorescein as a function of the pH of the solution recovered using frequency-dependent noise (■) with associated Monte Carlo error bars. The same parameter recovery was performed for comparison with frequency-independent noise (♦). The pK_a of the trianion–dianion transition was 6.9 ± 0.3.

by other methods and softwares are listed in Table II. Obviously, the differences in these errors are important. However, it can be noted that the errors that we have determined agree well with those determined by the CFS recovery software using the Monte Carlo option.

DISCUSSION

As recognized by others [3] the instrumental errors for the phase shift and demodulation ratio should be determined for every instrument at each modulation frequency. It is apparent that assuming a constant phase and demodulation error throughout the frequency range (0.2° for the phase and 0.004 for the demodulation ratio are commonly used in the literature) does not adequately describe the characteristics of our instrument (Fig. 2). However, as can be seen from our results, choosing to use a priori errors or experimentally determined errors has little influence, in our situation at least, on the parameter recovery.

The acquisition software of our instrument also provides, for each spectrum and at each frequency, a measured error on the phase and demodulation ratio. We did not use these values, as we found that they strongly underestimate the variance as measured with our method. We attribute this discrepancy to the limited time during which data at a specific frequency are acquired to deliver

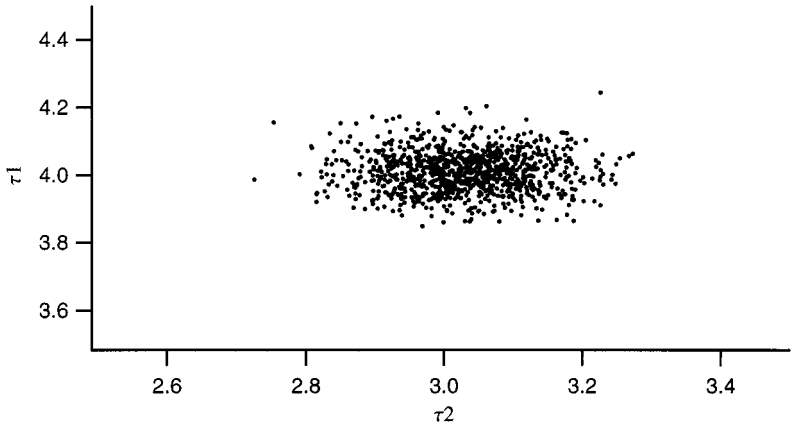

Fig. 6. Typical results of the Monte Carlo simulation showing the two recovered lifetimes τ_1 [ns] and τ_2 [ns] for each simulated experiment. The averages ± standard deviations were 4.01 ± 0.06 and 3.03 ± 0.09 ns for the trianionic and dianionic carboxyfluorescein, respectively. Similar graphs (not shown) were obtained for the preexponential factors.

Table II. Errors of the Lifetimes and Preexponential Factors Describing the Time-Resolved Decay of 5-Carboxyfluorescein as a Function of the pH Value[a]

	Method						
Errors Software	MC Exp. CFS	MC Fixed CFS	*F* test Exp. CFS	*F* test Fixed CFS	*F* test Exp. Mini	*F* test Fixed Mini	MC Exp. This article
τ_1	0.11	0.07	0.02	0.03	0.24	0.16	0.12
τ_2	0.16	0.10	0.06	0.06	0.42	0.35	0.18
α_1, pH = 5.4	0.11	0.06	0.03	0.03	0.33	0.49	0.13
α_1, pH = 6.0	0.11	0.07	0.03	0.03	0.30	0.38	0.13
α_1, pH = 6.6	0.11	0.07	0.03	0.03	0.18	0.25	0.13
α_1, pH = 7.2	0.11	0.08	0.03	0.03	0.16	0.21	0.12
α_1, pH = 7.8	0.11	0.08	0.03	0.04	NN	NN	0.13
α_1, pH = 8.7	0.13	0.10	0.04	0.05	NN	NN	0.14

[a] Several methods, softwares, and choices of the errors on the phase and demodulation were used to determine the errors in the recovered parameters. NN means that the Miniglobal software did not provide a value at the corresponding pH. MC, Monte Carlo.

the estimate of the error, making slowly varying noise invisible. In contrast, these instrumental drifts are represented in our estimation of the error as they result from independent experiments, well separated in time. Interestingly, Gratton *et al.* [3] have observed the same phenomenon for their instrument.

The protonation of 5-carboxyfluorescein was studied by FD-TRFS as a function of the pH value. Data could be fitted assuming only the trianionic and dianionic forms to be present. This is surprising at first sight since there are already three possible protonated forms for the dianionic 5-carboxyfluorescein (Fig. 1). However, it has been shown for fluorescein that the pK_a's of the xanthene and benzoic group differ by at least two orders so that the species bearing a protonated benzoic group represent only minor components. It is reasonable to expect that 5-carboxyfluorescein behaves similarly. Considering that the fit could be done with a two-species model, our results also support this view.

A pK_a value of 6.3 has been published for the dianion–monoanion equilibrium of fluorescein [17], which is significantly lower than the pK_a value of 6.9 that we have determined by FD-TRFS for the corresponding 5-carboxyfluorescein trianion–dianion equilibrium. We explain this difference by the greater electrostatic attraction felt by the interacting proton due to the extra negative charge on the benzoic ring of 5-carboxyfluorescein compared to that of fluorescein. An estimation of this increase can be made by considering the change in free energy due to this interaction. Evaluating the distance between the xanthene hydroxyl group and the 5-carboxyl group on the benzoic ring as 1 nm, the electrostatic potential energy between the two charged groups can be calculated, corresponding to an increase in the pK_a value of 0.3 unit. The thermodynamic equation linking the Gibbs free energy with the pK_a of an acid–base couple: $\Delta G = -2.303RT\ln pK_a$ was used here [18]. This calculated value of the pK_a shift is smaller than the experimentally determined one. In our estimation above, we have used the dielectric constant of water to estimate the electrostatic energy, whereas, in reality, the polarizability of fluorescein modifies the effective dielectric constant between the two charges. Estimating this dielectric constant theoretically is a task that would require evaluating the polarizability of fluorescein, a molecular dynamic simulation to place the water molecules around the chromophore taking into account exclusion effects, and, finally, computing an average. We did not address this calculation here but suspect that this effect is the cause of the discrepancy.

In deriving the pK_a of the trianion–dianion transition for 5-carboxyfluorescein, we assumed that the values of the quantum yield and extinction coefficient were equal to those of fluorescein. The protonation state of the xanthene group is the main determinant of the absorption properties. Indeed, the value reported by the manufacturer for the extinction coefficient of the trianionic carboxyfluorescein is only slightly below (81,000 $cm^{-1}\ M^{-1}$) the value corresponding to the dianionic form of fluorescein (89,000 $cm^{-1}\ M^{-1}$). Concerning the quantum yield, we observe that lifetimes are certainly the most sensitive parameters to physicochemical changes. Considering that our lifetime values agree closely with those, 4.1 and 3.0 ns, measured by Sjöback *et al.* [19] for fluorescein, we expect the quantum yield of 5-carboxyfluorescein not to differ much from that of fluorescein, thus justifying our assumption.

If errors are estimated accurately and if a chosen model is effectively able to describe a data set, chi-square values should be close to one. Here they were significantly smaller (Table I). Errors are dependent on many parameters such as the time given for the instrument to stabilize before performing the measurement, the intensity of the light collected by the detector, or the gain of the latter. Hence, they can vary from one day to the next, but according to our experience, the ratio of the errors at two frequencies does not change. Multiplying all errors by a constant factor has no effect on the parameter recovery, and hence, we are not concerned by the small chi-square values observed. However, the latter lose their absolute character in deciding on the validity of a model.

We have determined the errors on the lifetimes and preexponential factors using several methods and several softwares. The important differences found in the magnitude of these errors deserves some comments. First, comparing the results provided by our implementation of the Monte Carlo method with those provided by the Monte Carlo method as it is implemented by the CFS software (both using the experimentally determined errors), we find that they agree well. The small differences that are observed are due to the difference in the definition of the errors. In the CFS software, the 68.2% confidence interval contains 68.2% of the recovered values from the simulated experiments. In our implementation of the method, the error reported is the standard deviation on the recovered parameter. The interval limited by plus or minus the standard deviation must contain exactly 68.2% of the data only if the distribution is normal.

Concerning the errors determined with the *F* test by the Miniglobal and the CFS software, one can see that they differ much from the Monte Carlo errors and differ still more between each other. We have no explanation for these discrepancies. However, in light of the results presented here and because we find the Monte Carlo method conceptually satisfying, we will rely mostly on this approach in the future to determine errors.

CONCLUSION

FD-TRFS was applied to the resolution of the prototropic forms of 5-carboxyfluorescein. The lifetimes of the trianion and dianion forms were 4.01 $\pm$ 0.06 and 3.03 $\pm$ 0.09 ns, respectively. The pK_a of the trianion–dianion equilibrium was 6.9 $\pm$ 0.3. Interestingly, the recovery did not depend appreciably on whether we were using frequency-dependent or frequency-independent errors. We determined the errors on the recovered parameters with a Monte Carlo simulation where the characteristics of the added noise were studied experimentally. Except for the higher pK_a value for the acid–base equilibrium of 5-carboxyfluorescein compared to that of fluorescein, the properties of both probes are remarkably similar. We believe that the results presented here will be of importance for probing the microenvironment of biological macromolecules, in particular, in the field of fluorescence lifetime imaging.

ACKNOWLEDGMENTS

We are grateful to Pr. E. Gratton and Dr. R. Hovius for reading the manuscript and making useful suggestions. This work was supported by the Swiss National Science Foundation (project 31-57023.99) and internal grants of the EPFL (Microtechnique 96).

REFERENCES

1. J. R. Alcala, E. Gratton, and F. G. Prendergast (1987) *Biophys. J.* **51**, 587–596.
2. R. E. Dalbey, J. Weiel, W. J. Perkins, and R. G. Yount (1984) *J. Biochem. Biophys. Methods* **9**, 251–266.
3. E. Gratton, M. Limkeman, J. R. Lakowicz, B. P. Maliwal, H. Cherek, and G. Laczko (1984) *Biophys. J.* **46**, 479–486.
4. J. R. Lakowicz, R. Jayaweera, H. Szmacinski, and W. Wiczk (1990) *Anal. Chem.* **62**, 2005–2012.
5. J. R. Lakowicz, G. Laczko, H. Cherek, E. Gratton, and M. Limkeman (1984) *Biophys. J.* **46**, 463–477.
6. J. M. Beechem and L. Brand (1986) *Photochem. Photobiol.* **44**, 323–329.
7. S. Mordon, J. M. Devoisselle, and V. Maunoury (1994) *Photochem. Photobiol.* **60**, 274–279.
8. R. Sanders, A. Draaijer, H. C. Gerritsen, P. M. Houpt, and Y. K. Levine (1995) *Anal. Biochem.* **227**, 302–308.
9. L. Cavarec, A. Quillet-Mary, D. Fradelizi, and H. Conjeaud (1990) *J. Immunol. Methods* **130**, 251–261.
10. K. H. Elstein and R. M. Zucker (1994) *Exp. Cell Res.* **211**, 322–331.
11. L. S. De Clerck, C. H. Bridts, A. M. Mertens, M. M. Moens, and W. J. Stevens (1994) *J. Immunol. Methods* **172**, 115–124.
12. R. P. Haugland (1996) *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes, Eugene, OR.
13. J. R. Lakowicz. (1999) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
14. J. M. Shaver and L. B. McGown (1996) *Anal. Chem.* **68**, 611–620.
15. Z. Bajzer, A. C. Myers, S. S. Sedarous, and F. G. Prendergast (1989) *Biophys. J.* **56**, 79–93.
16. W. H. Press, S. A. Teukolsky, W. T. Vetterling, and B. P. Flannery (1993) *Numerical Recipes in C: The Art of Scientific Computing*, Cambridge University Press, Cambridge.
17. N. Klonis and W. H. Sawyer (1996) *J. Fluoresc.* **6**, 147–157.
18. P. W. Atkins (1990) *Physical Chemistry*, Oxford University Press, New York.
19. R. Sjöback, J. Nygren, and M. Kubista (1995) *Spectrochim. Acta A* **51**, L7–L21.