

# Analysis of the Scattered Light Component in Distorted Fluorescence Decay Profiles Using a Modified Delta Function Convolution Method

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**An interactive reconvolution method that permits one to recover the decay parameters from fluorescence decay curves distorted with a scattered light component is presented. The algorithm is based on the delta function convolution method, a technique that corrects for the wavelength dependence of the instrument response function. The expected decay parameters were successfully extracted from simulated and experimentally measured, single and double exponential fluorescence decay curves polluted with scattered light. It is shown that when an extra exponential component in the decay function is used to account for the scattered light component, erroneous values of the decay parameters can be obtained.**

The time-correlated single-photon counting technique (TCSPC) is widely used for investigating the time-dependent fluorescence behavior of excited-state molecular species (1, 2). The fluorescence decay profile ( $F$ ) of a sample which results from an impulse function ( $L$ ) must be considered as a convolution of the impulse function, an instrumental response function ( $H$ ), and the true sample response function ( $S$ ) according to eq 1. In this equation,  $t$  is the time,  $\lambda_{ex}$  and

$$F(t, \lambda_{em}) = L(t, \lambda_{ex}) \otimes H(t, \lambda_{em}) \otimes S(t, \lambda_{em}) \quad (1)$$

$\lambda_{em}$  are, respectively, the wavelengths of the excitation light and recovered fluorescence, and  $\otimes$  denotes convolution. But in cases where scattering of the excitation light occurs, eq 1 should be modified to

$$F(t, \lambda_{em}) = L(t, \lambda_{ex}) \otimes H(t, \lambda_{em}) \otimes S(t, \lambda_{em}) + CL(t, \lambda_{ex}) \otimes H(t, \lambda_{ex}) \quad (2)$$

where  $C$  is a measure of the extent of scattered light. This effect is of great importance when analyzing polymeric, biological, and solid samples, since by virtue of their inherent structure they tend to scatter light. The analysis of eq 2, in order to extract  $S$ , has been achieved by Jezeguel et al. (3) and Love et al. (4) using the phase plane method. Another possibility of analysis is the use of moment theory with a moment index displacement as was shown by Isenberg et al. (5-7).

However, the most widely accepted technique for extracting  $S$  is the nonlinear least-squares iterative reconvolution method (8). To perform such an analysis correctly, the impulses response function

$$G(t, \lambda_{em}) = L(t, \lambda_{ex}) \otimes H(t, \lambda_{em}) \quad (3)$$

is required. Experimentally, only the quantity  $G(t, \lambda_{ex}) = L(t, \lambda_{ex}) \otimes H(t, \lambda_{ex})$ , which is generally different than  $G(t, \lambda_{em})$ ,

can be measured. To circumvent this problem, many techniques were used. Namely the zero-shift technique (9), the mimic technique (10), and the delta-function convolution technique (11, 12). Van der Zegel et al. (9) analyzed all these methods and concluded that DFCM is the method of choice. In this case, the decay curve of a standard

$$D_r(t, \lambda_{em}) = G(t, \lambda_{em}) \otimes \tau_r \exp(-t/\tau_r) \quad (4)$$

which is measured under precisely the same conditions as the sample, is introduced directly in eq 1 using the appropriate modified decay of the sample.

In the case of  $S$  being a sum of exponentials

$$S(t) = \sum_{j=1}^k a_j \exp(-t/\tau_j) \quad (5)$$

the modified function  $F_s(t)$  takes the simple form

$$F_s(t) = \sum_{j=1}^k A_j \left[ \delta_0(t) + \left( \frac{1}{\tau_r} - \frac{1}{\tau_j} \right) \exp(-t/\tau_j) \right] \quad (6)$$

where  $A_j$  is equal to the ratio  $a_j/a_r$  and  $\delta_0(t)$  is the Dirac  $\delta$  function at zero. Equation 2 can now be rewritten as

$$F(t, \lambda_{em}) = D_r(t, \lambda_{em}) \otimes F_s(t, \lambda_{em}) + CG(t, \lambda_{ex}) \quad (7)$$

If we measure  $F(t, \lambda_{em})$  (sample),  $D_r(t, \lambda_{em})$  (reference), and  $G(t, \lambda_{ex})$  (lamp), we can then recover the true parameters for  $S$  and the scattering parameter  $C$ .

## EXPERIMENTAL SECTION

**Materials.** 9-Anthrylmethyl pivalate (AMP) was prepared and purified according to the method of Ng and Guillet (13). Its purity was established by TLC. 9,10-Diphenylanthracene (DPA, Aldrich Gold Label) and 2,5-bis(5-*tert*-butyl-2-benzoxazolyl)thiophene (BBOT, Aldrich, Scintillation Grade) were used as received. Cyclohexane (Caledon, Spectrograde) was stirred over successive aliquots of sulfuric acid and under nitrogen, until the color of the acid remained clear. It was then washed with water, dried, and fractionally distilled under nitrogen, over sodium ribbon. Polystyrene of  $M_n = 900\,000$ ,  $M_n/M_w = 1.10$  was purchased from Pressure Chemical Co.

All samples were prepared in cyclohexane. Solutions of DPA and AMP contained  $1.0 \times 10^{-5}$  M and  $4.0 \times 10^{-5}$  M fluorophore, respectively. The solution of DPA plus AMP was prepared to give final concentrations of ca.  $1.0 \times 10^{-5}$  M DPA and  $4.0 \times 10^{-5}$  M AMP, low enough to avoid reabsorption and energy transfer effects. To each sample was added a small amount of polystyrene (1 mg/mL). When cooled to  $15 \pm 0.5$  °C the samples appear cloudy. The samples were analyzed by TCSPC using front face viewing.

**Instrumentation.** Absorption spectra were recorded on a Hewlett-Packard 8451A diode array UV-vis spectrophotometer. Fluorescence spectra were obtained with a SPEX Fluorolog 2. The decay curves were obtained on a home-built single-photon counting instrument. A coaxial flashlamp (Edinburg Instruments, Model 199F) was used as the excitation source. The excitation light from the lamp was selected by a Jovin-Yvon Model H-20 monochromator and then focused on the sample. After traveling through a SPEX Minimate Model 1670 monochromator, the fluorescence was focused onto the photocathode of a Hamamatsu

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Table I. Simulated Data<sup>a</sup>

curve no.	input parameter					recovered parameters					
	$A_1$	$\tau_1$ , ns	$A_2$	$\tau_2$ , ns	$C$	$A_1$	$\tau_1$ , ns	$A_2$	$\tau_2$ , ns	$C$	$\chi^2$
1	0.673	7.57			0	0.673	7.56			0.001	0.97
2	0.673	7.57			0.219	0.675	7.55			0.218	0.91
3	0.673	7.57			0.797	0.674	7.56			0.797	1.02
4	0.225	2.80			0	0.228	2.79			-0.003	0.95
5	0.225	2.80			0.200	0.227	2.80			0.199	0.95
6	0.225	2.80			0.740	0.223	2.77			0.736	0.98
7	0.201	2.76	0.257	7.66	0	0.198	2.59	0.265	7.57	-0.002	1.10
8	0.201	2.76	0.257	7.66	0.189	0.201	2.70	0.260	7.64	0.186	0.98
9	0.201	2.76	0.257	7.66	0.786	0.200	2.79	0.257	7.63	0.787	1.06

<sup>a</sup> Time scales 0.202 ns/channel (1–3 and 7–9), 0.101 ns/channel (4–6).  $A_1 = a_1/a_r$ ,  $A_2 = a_2/a_r$ .

R928 photomultiplier tube, operated at 1250 V. The dark current was minimized with a thermoelectric cooling system (Photochemical Research Associates, Inc.). The flash lamp was monitored by a Hamamatsu IP28-type phototube. Subsequent output signals (start) were discriminated (ORTEC, Model 436) and fed into a time-to-amplitude converter, TAC (ORTEC, Model 457). Signals originating from the anode of the R928 phototube (stop) were amplified (ORTEC, Model 454), discriminated (ORTEC, Model 463), and then directed to the TAC.

Output pulses from the TAC were stored in a EG&G ORTEC Model 7100 multichannel analyzer (MCA) operated with 256 channels. Accumulated fluorescence response curves were transferred to a Tektronix Model 4052 computer for analysis. The TAC was calibrated with a ORTEC Model 462 time calibrator.

**Analysis.** Curve fitting computations involved the linearization of the fitting function and a least-squares analysis that minimizes the  $\chi^2$ . The process of minimization is greatly increased by using the Marquart algorithm (14). A recursion formula (15) based in the trapezoid integration rule was used to calculate the convolution integral. To judge the quality of fit of the measured decay profiles to those that are calculated, we consider the reduced chi-squared,  $\chi^2$ , plots of weighted residuals and the autocorrelation function of the residuals with the appropriate weighting factor (9, 12).

## RESULTS AND DISCUSSION

**Simulated Data.** The simulated decay curves,  $F(t_i)$ , were generated by using eq 6. The experimental response curve  $D_r(t_i, \lambda_{em})$  is from a solution of BBOT in 95% ethanol (ca.  $1.0 \times 10^{-5}$  M, a single exponential decay with lifetime 1.47 ns (12)). The scattering component that was added to the undistorted data was from an experimental response function [ $G(t_i, \lambda_{ex})$ , fwhm  $\approx 2.0$  ns] obtained by using a solution of polystyrene (1 mg/mL) in cyclohexane at 15 °C. The number of counts in the maximum channels of  $D_r(t_i, \lambda_{em})$  and  $G(t_i, \lambda_{ex})$  was  $1.0 \times 10^4$  and  $2.0 \times 10^4$  for the data with time scales of 0.101 and 0.202 ns/channel, respectively.

The synthetic noise in channel  $i$  was incorporated according to

$$F_i(\text{noisy}) = F_i + N(F_i)^{1/2} \quad (8)$$

where  $N$  is an array of random numbers normally distributed about the mean value (zero), with standard deviation of unity. These were generated by employing the Box-Muller-Maraglia algorithm (2).

In Table I we compare the input parameters for simulated single and double exponential decays (with and without scattering) with the recovered parameters. The analysis of undistorted simulated decays gave very accurate results for the decay parameters and scattering factor ( $C$ ) very close to zero. Similar accuracy is obtained in the recovery of the decay parameters and scattering factor for distorted decays containing both small and large amounts of the scattering component. We conclude that if actual experimental data are accurate enough and if the amount of scattering is not too high, we ought to be able to recover with this method the

parameters of the true sample response function ( $S$ ) and the scattering component ( $C$ ).

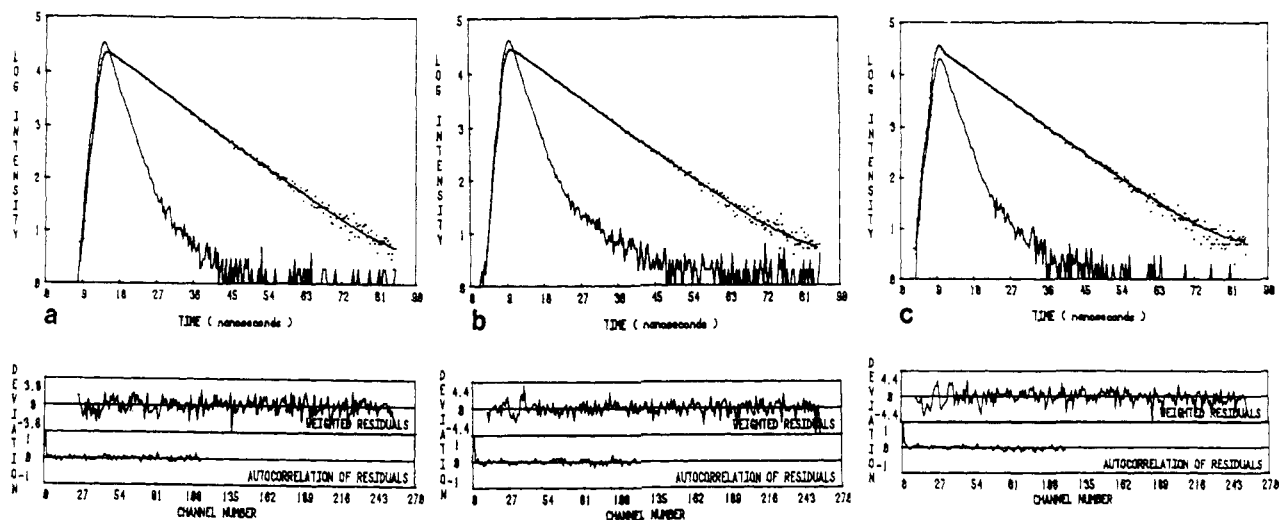
In spite of the results presented above, we felt that it would be informative to analyze the distorted decay curves in Table I by using an additional exponential term to account for the scattered light rather than with the algorithm based on eq 7. When the distorted single and double exponential decays were analyzed in this way, excellent  $\chi^2$  values for all curves were obtained ( $0.90 < \chi^2 < 1.15$ ). Entries 2, 3, 5, and 6 yielded decay parameters ( $A_2, \tau_2$ ) very similar to those in Table I, with short-lived decay components due to scattering of ca. 0.15 ns. Thus, for distorted single exponential decays, the decay parameters are accurately recovered when an additional exponential term is used to fit the scattered light component.

To our surprise, analysis of entries 8 and 9 (with a sum of three exponential terms) yielded completely wrong results in spite of the excellent fits and the additional parameter. It seems that the errors are due to correlation (16) between the short component and the component due to scattering. It is clear then that analysis of distorted double exponential decays should be done by using the algorithm based on eq 6.

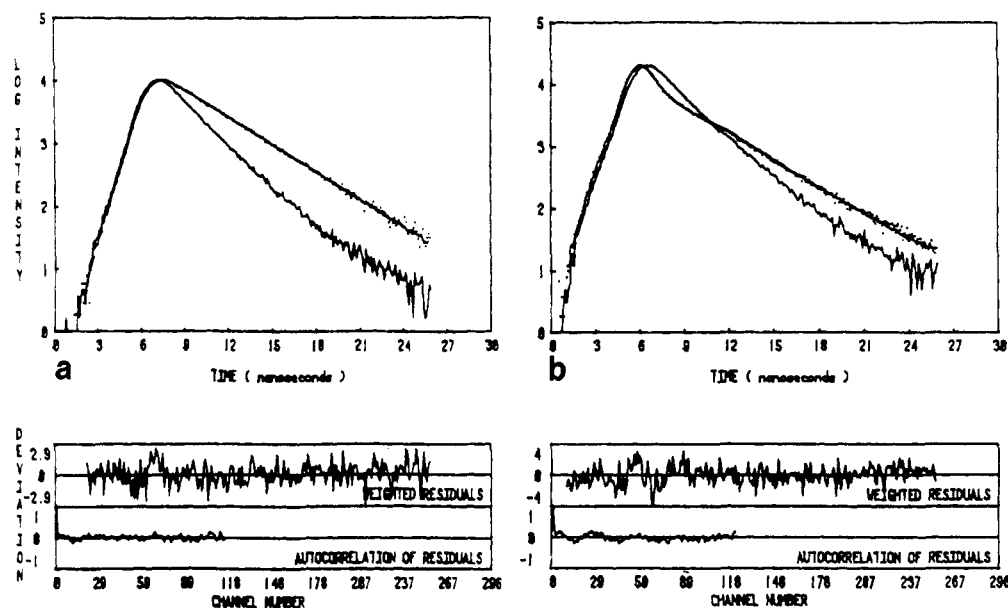
**Experimental Data.** The decay curve for the samples,  $F(t_i, \lambda_{em})$ , and for the reference solution,  $D_r(t_i, \lambda_{em})$ , were collected at the same wavelengths of excitation and of emission with the front face geometry, at 15 °C. The scatter decay,  $G(t_i, \lambda_{ex})$ , was recovered by using a turbid solution of polystyrene in cyclohexane at 15 °C, with the same geometry, at the excitation wavelength.

**Single Exponential Decays.** In Figure 1 are shown the experimental decay curves of DPA (0.332 ns/channel) containing various amounts of scattered light. Also presented are the reference compound decay profiles, the best fit curves obtained from iterative reconvolution, and plots of the weighted residuals and autocorrelation of the weighted residuals. In Figure 1a, the sample is excited at 372 nm and the emission collected at 415 nm. Analysis of this curve yielded a lifetime 7.63 ns and a very low negative scattering component ( $C = -0.02$ ). In fact, when this sample was fit with a single exponential decay function (no scattering term), precisely the same decay time is obtained. Moreover, the decay time obtained from both analyses is in good agreement with the previously published value of 7.68 ns (17) for DPA in cyclohexane.

Figure 1, parts b and c, shows decays obtained for DPA samples excited at 400 nm and fluorescence signals recovered at 415 nm and 410 nm, respectively. As expected, we observe a larger scattering component for sample c, measured with a smaller difference between wavelengths for excitation and emission. The fits for these samples are both reasonable as indicated by the  $\chi^2$  values ( $\chi^2(b) = 1.14$  and  $\chi^2(c) = 1.46$ ), plots of the weighted residuals, and the autocorrelation functions. The parameters obtained are  $\tau = 7.66$  ns,  $A = 0.364$ , and  $C = 0.132$  for curve b and  $\tau = 7.57$  ns,  $A = 0.669$ , and  $C = 0.800$



**Figure 1.** Experimental fluorescence decay curves of a sample of DPA and polystyrene in cyclohexane at 15 °C (0.332 ns/channel) collected with excitation/emission wavelengths of 372/415 nm (a), 400/415 nm (b), and 400/410 (c), analyzed with an exponential component and a scattering component (eq 7).



**Figure 2.** Experimental fluorescence decay curves (0.101 ns/channel) from a solution of AMP and polystyrene in cyclohexane at 15 °C.  $\lambda_{ex}$  385 nm,  $\lambda_{em}$  410 nm (a);  $\lambda_{em}$  395 nm (b).

for curve c. Notice the good agreement between the decay times for these samples and for the DPA sample (a) collected under conditions where no scattering component is detected.

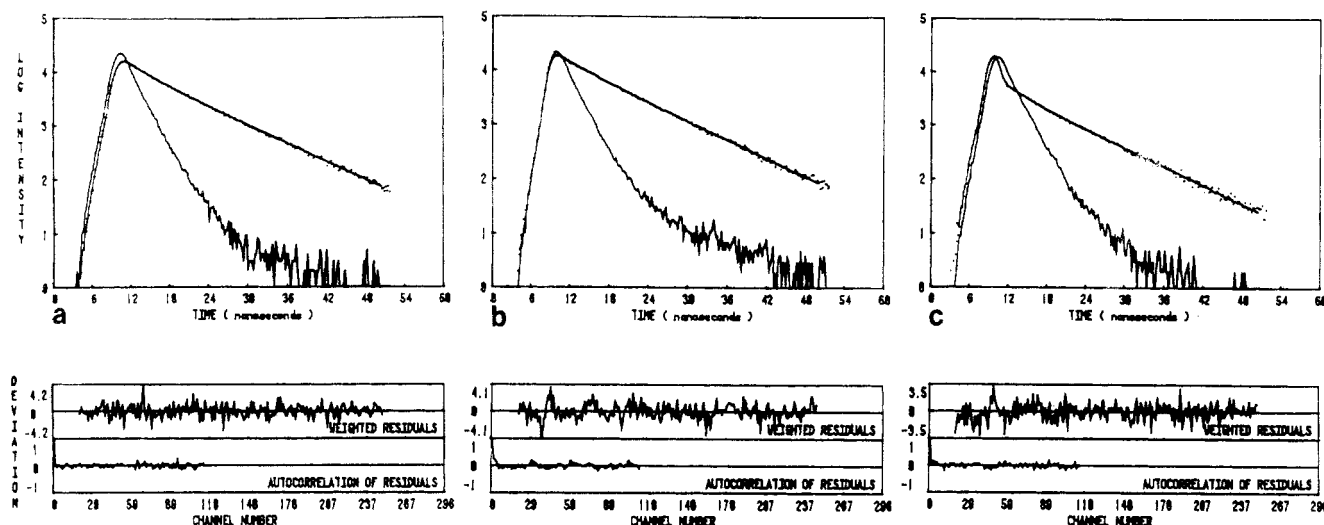
The small deviations in the weighted residuals in the vicinity of the maximum of the reference response curves in Figure 1a,b are cause for concern, however. We believe these deviations to be due to instabilities (drift) in our lamp source since it took us approximately 2 h to collect sequentially the sample, reference, and lamp decay curves. The analysis of curves b and c with double exponential decay laws gives very good fits with the lifetimes of the long components identical with the value obtained by using the algorithm based on eq 7, and a short component with a subnanosecond lifetime (0.2–0.3 ns).

In Figure 2 are fluorescence decay profiles (0.101 ns/channel) from AMP in cyclohexane with (a) and without (b) a component due to scattered light. The excitation wavelength for both samples was 385 nm and the emission wavelengths were 410 and 395 nm, respectively. The parameters obtained for curve a are  $\tau = 2.80$  ns,  $A = 0.758$ ,  $C = -0.028$ , and  $\chi^2 = 1.23$ . For curve b,  $\tau = 2.82$  ns,  $A = 0.223$ ,  $C = 0.746$ , and  $\chi^2 = 1.55$  were obtained. It is remarkable that the scattering algorithm (eq 6) fits curve b as well as it does; notice that the

component due to scattering is approximately 3.5 times as strong as the fluorescence and, also, the decay time for AMP is not much greater than that of the reference compound itself (BBOT,  $\tau = 1.47$  ns). Analysis of curve b using a sum of two exponential terms yielded lifetimes of 0.27 and 2.81 ns, and a  $\chi^2$  value of 1.12.

**Double Exponential Decays.** In Figure 3 are presented the experimental fluorescence decay curves from a mixture of DPA, AMP, and polystyrene in cyclohexane at 15 °C (0.202 ns/channel). The excitation/emission wavelengths in nanometers for curves a, b, and c are 365/410, 388/406, and 388/400, respectively.

Each of the sample fluorescence decay profiles in Figure 3 was well fit with a double exponential function and a scattering component as indicated by the plots of the weighted residuals and the respective autocorrelation functions. The decay parameters for these profiles are listed in Table II. For curve a in Figure 3, the decay times obtained are very close to the expected values of 7.63 and 2.80 ns for DPA and AMP, respectively, and the small negative scattering factor (−0.044) indicates that there is no scattered light impurity. Notice the large amounts of scattered light that pollute curves b and c, however; the ratios,  $C/(A_1 + A_2)$ , are equal to 0.39 and 4.34,



**Figure 3.** Experimental fluorescence decay curves from a mixture of DPA, AMP, and polystyrene in cyclohexane at 15 °C (0.202 ns/channel). The excitation/emission wavelengths in nanometers for curves a, b, and c are 365/410, 388/406, and 388/400, respectively.

**Table II.** Decay Parameters from a Deaerated Cyclohexane Solution of DPA, AMP, and Polystyrene at 15 °C Run under Various Conditions

curve <sup>a</sup>	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	τ <sub>1</sub> /ns	τ <sub>2</sub> /ns	τ <sub>3</sub> /ns	C	χ <sup>2</sup>
a	0.257	0.201		7.66	2.76		-0.044	1.06
b	0.333	0.155		7.57	2.87		0.189	1.45
b'	0.348	0.296	1.54	7.47	2.11	0.15		1.10
c	0.119	0.062		7.53	2.24		0.786	1.29
c'	0.118	0.073	6.93	7.58	1.64	0.15		0.75

<sup>a</sup> Curves a, b, and c are results from analysis using a sum of two exponential terms and a scattering component (eq 7). Curves b' and c' were analyzed with a triple exponential decay function.

respectively. The data in Table II show that when the contribution to the decay from scattered light is not too large (for curve b it is still larger than the A factor of the component due to AMP), reasonably accurate lifetimes values are recovered.

One would expect that analysis of curve b in Figure 3 with a triple exponential decay function would yield good results; we obtain, however, an incorrect value of 2.11 ns for the lifetime of AMP in spite of the good fit. This is an excellent example of the correlation effect discussed above, at the end of the simulated-data section. Also it supports our conviction that nonexponential decays containing a scattered light component cannot be analyzed properly using algorithms based on eq 1; scattering algorithms should be employed.

In curve c, there is a tremendous scattered light component. In spite of this, we find that the long component lifetime (DPA) is close to the expected value. The short component decay time is in error ( $\tau = 2.24$  ns); however simulated data presented above indicate that, for this sample, we are beyond the limit for which the scattering algorithm can provide accurate results ( $C/A_2 = 12.7$ ). Nevertheless, this analysis is clearly superior to a sum of three exponential terms, which recovers  $\tau$  (AMP) = 1.64 ns.

### CONCLUSION

The distortion of the scattered light in experimental fluorescence decay curves is well taken into account using an iterative reconvolution fitting method. This is possible due to the use of the delta function convolution method (DFCM), a technique which corrects for the wavelength dependence of the instrumental response function and allows the analysis from the initial rise portion of the decay profiles.

Our results show that it is practically impossible to discriminate between the effect of scattered light and a subnanosecond component in experimental fluorescence decay measurements. However, by changing the configuration of the TCSPC system in such a way so that greater or lesser

amounts of scattered light would be expected, one ought to be able to establish whether or not scattering effects contribute to the measured fluorescence decay.

Flash-lamp drift during decay measurement is very important, particularly when a large amount of scattered light is present. Drift effects explain the small deviations present in plots of the weighted residuals for our experimental fluorescence decay data. It does not influence significantly the recovery of expected decay parameters, however.

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### LITERATURE CITED

- (1) O'Connor, D. V.; Phillips, D. *Time-Correlated Single Photon Counting*; Academic: New York, 1984.
- (2) Demas, J. N. *Excited State Lifetime Measurements*; Academic: New York, 1983. Kobler, Z. S.; Barkley, M. D. *Anal. Biochem.* **1986**, *152*, 6.
- (3) Jezeguel, J. Y.; Bouchy, M.; Andre, J. C. *Anal. Chem.* **1982**, *54*, 219.
- (4) Love, J. C.; Demas, J. N. *Anal. Chem.* **1984**, *56*, 82.
- (5) Isenberg, I. *J. Chem. Phys.* **1973**, *59*, 5696.
- (6) Small, E. W.; Isenberg, I. *J. Chem. Phys.* **1972**, *66*, 3347.
- (7) Small, E. W.; Isenberg, I. *Biopolymers* **1976**, *15*, 1093.
- (8) O'Connor, D. V.; Ware, W. R.; Andre, J. C. *J. Phys. Chem.* **1979**, *83*, 1333.
- (9) Zegel, M. Van der; Boens, N.; Daenies, D.; DeSchryver, F. C. *Chem. Phys.* **1986**, *101*, 311.
- (10) James, D. R.; Dimmer, D. R. M.; Verrall, R. E.; Steer, R. P. *Rev. Sci. Instrum.* **1983**, *54*, 1121.
- (11) Gauduchon, P.; Wahl, Ph. *Biophys. Chem.* **1978**, *8*, 78.
- (12) Zuker, M.; Szabo, A. G.; Bramall, L.; Krajcarski, D. T.; Selinger, B. *Rev. Sci. Instrum.* **1985**, *56*, 14.
- (13) Ng, D.; Guillet, J. E. *Macromolecules* **1982**, *15*, 724.
- (14) Marquardt, D. W. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431.
- (15) Grinvald, A.; Steinberg, I. Z. *Anal. Biochem.* **1974**, *59*, 583.
- (16) Grinvald, A. *Anal. Biochem.* **1976**, *75*, 260.
- (17) Meech, S. R.; Phillips, D. J. *Photochem.* **1983**, *23*, 193.

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