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Analysis of fluorescence decay by the maximum entropy method: Influence of noise and analysis parameters on the width of the distribution of lifetimes

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Abstract. Maximum entropy method (MEM) was used for resolving multiple decay components in synthetic and experimental time-resolved fluorescence decay. The distribution of lifetimes is unimodal or multimodal and there is a one-to-one correspondence between the results of MEM and discrete exponential analysis. The distribution is symmetric in $\log(\tau)$ space and approximately Gaussian. The width of the distribution is sensitive to several factors related to the experimental or analysis conditions, such as the peak count, discretization in τ space, completeness of decay and the χ^2 stopping criterion. Therefore, the width of the distribution cannot be a useful indicator of the extent of heterogeneity of lifetimes in a sample of a complex biological system.

Keywords. Maximum entropy method; multiple decay components; fluorescence decay; lifetime distributions.

1. Introduction

Time-resolved fluorescence is one of several powerful techniques for studying the structural and dynamical aspects of biological systems such as proteins (Beecham and Brand 1985; Demchenko 1992), membranes (Lentz 1993; Pap *et al* 1994) and even living cells (Fushimi and Verkman 1991). A fluorescent dye molecule is generally used as an extrinsic probe and the perturbation in the fluorescent properties of the probe reveals the structural and dynamical characteristics of the surroundings. In the case of proteins, intrinsic fluorescence from tryptophan is also very useful. A difficult and challenging problem in fluorescence studies in heterogeneous media is in identifying the number of distinct environments in which the fluorophore is distributed. A distinct environment is one whose structure is stable over a time scale longer than the fluorescence lifetime of the probe, typically a few nanoseconds. For example, multiple conformations of a protein, interconverting in a time-scale of microseconds or longer, form a heterogeneous system as far as the fluorescence property is concerned and the fluorescence decay could be multiexponential. Fluorescent probes in living cells or membranes can occupy a number of unique sites and the fluorescence decay is most commonly multi-exponential. There exists also the possibility that the above biological systems can be extensively heterogeneous in structural environments such that a continuous distribution of lifetimes is the best approximation for the fluorescence decay in such complex systems.

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Until recently, the popular method of analysis of time-resolved fluorescence decay data assumes a mathematical equation (multiexponential or otherwise) for the intensity decay. This method then verifies whether the experimental decay is consistent with the equation and thus lends support to a model of excited state dynamics predicting that equation. The models predicting one-, two- or three-exponential equations were generally found appropriate and adequate in most chemical and simple biochemical studies. For obvious reasons, one cannot assume a satisfactory kinetic model for the fluorescence decay in heterogeneous media and biological systems. A method of analysis that does not require the assumption of a specific model is required for these cases. Maximum entropy method (Livesey and Brochon 1987; Brochon 1994) appears to be best suited for this purpose.

The maximum entropy method of analysis of fluorescence decay data assumes that all possible lifetimes in a given range of 10 ps to 10 ns (or some other range appropriate to the problem) have equal probability. The initial assumed distribution plot of probability or amplitude versus lifetime is flat. After the analysis is completed the flat distribution is transformed into a structured distribution which adequately fits the experimental decay data. The structural features of the final distribution are to be interpreted by the experimentalist. MEM analysis of fluorescence decay has been most widely used to resolve the lifetime components in several proteins (Dorovska-Taran *et al* 1993, 1994; Das and Mazumdar 1994, 1995) and in membranes (Prenner *et al* 1993). A multimodal distribution (multiple peaks) indicates multiple lifetime components. Some examples are: pentamodal distribution in bacteriorhodopsin containing eight trytophans (Roy and Periasamy 1995), quadrimodal distribution in horse heart apocytochrome c containing a single Trp protein (Vincent *et al* 1988), bimodal or trimodal distribution in barstar (three tryptophans) and several single Trp proteins (Swaminathan *et al* 1994) etc. Each lifetime resolved component is associated with a width which in principle could be associated with heterogeneity of the local environment. There were attempts to interpret the width of the distribution to indicate environmental heterogeneity (Bismuto *et al* 1991) which was questioned (Swaminathan *et al* 1994b). The purpose of this paper is to examine if one could use the width of the distribution as a quantitative indicator of heterogeneity.

2. Materials and methods

The picosecond laser time-resolved fluorescence spectrometer has been described earlier (Periasamy *et al* 1988; Bankar *et al* 1989). Tryptophan (Sigma Chemicals, USA) was used without further purification.

2.1 *Simulation of fluorescence decay data* (Demas 1983)

The fluorescence decay function is a convolution of instrument response function and sample-dependent intensity decay function, [(1) below]. The experimental data is also noisy. Simulation of fluorescence decay consists of (i) calculation of the convolution integral numerically, and (ii) addition of noise. The procedures for these operations have been described (Demas 1983; O'Connor and Phillips 1984; Periasamy 1988). Fluorescence decay data with a time resolution of 40 ps/channel were simulated using an experimentally measured excitation function (instrument response function) which had a peak count of 1×10^5 and a full-width-at-half-maximum (FWHM) of 100 ps. Noise-free emission data are calculated by the convolution integral by a numerical approximation method (Grinvald and Steinberg 1974). The emission peak is scaled up

or down by a multiplier to a desired peak count and then Poisson noise is added to each intensity value. Since Poisson noise (which ought to be the proper one in photon counting experiments) and Gaussian noise are similar (Demas 1983) for intensity values above 25 counts, Gaussian noise was used throughout this work. The portion of decay below 25 counts was not used for analysis. Since the peak count in the decay was always greater than 1000 (that is, signal-to-noise greater than 33) the tail region below 25 counts (signal-to-noise ratio of 5 or less) can be neglected.

2.2 *Maximum entropy method*

The general algorithm of maximum entropy method for the application of two-dimensional image reconstruction has been described (Skilling and Bryan 1984). The application of this method for the analysis of fluorescence decay has been described and the advantages for heterogeneous and biological systems have been discussed (Livesey and Brochon 1987). The computer program used for the analysis reported in this paper is written based on the recommendations of the papers cited above. The method is briefly described below.

The experimentally measured fluorescence decay (intensity vs time), $F(t)$ is a convolution of instrument response function, $R(t)$ and intensity decay function, $I(t)$:

$$F(t) = \int_0^t R(s+\delta)\, I(t-s)\mathrm{d}s. \tag{1}$$

δ is the time shift parameter which takes care of experimental or computational artifact which causes an artificial time-shift of the calculated function with respect to experimental data. $I(t)$ is the theoretical intensity decay function, a continuous distribution of lifetimes, as defined below:

$$I(t) = \int_0^{\tau_{max}} \alpha(\tau)\exp(-t/\tau)\mathrm{d}\tau. \tag{2}$$

$\alpha(\tau)$ is the distribution function which requires to be determined. Numerical method of analysis requires the τ space to be discretized in an appropriate manner. In practice τ can vary from a few picoseconds to several nanoseconds in complex systems. Discretization is preferred in the $\log(\tau)$ space, which also makes the initial choice of a flat distribution physically meaningful (Livesey and Brochon 1987). 150 discrete lifetime values uniformly spaced in the $\log(\tau)$ space are computationally manageable for covering the range from 10 ps to 10 ns. With this approximation $I(t)$ is a multi-exponential function,

$$I(t) = \sum_{i=1}^{N} \alpha_i \exp(-t/\tau_i), \tag{3}$$

with the important attribute that α_i $(i = 1, N)$ represent a continuous, smooth function.

$\alpha_i (i = 1, N)$ have to satisfy the condition that the experimental data are correctly fitted. That is, the intensity calculated by (1), $(F_c(t_i))$, and the experimental value, $(F_e(t_i))$, satisfy the χ^2 statistic.

$$\chi^2 = (1/M) \sum_{i=1}^{M} \{F_c(t_i) - F_e(t_i)\}^2/\sigma_1^2 \sim 1{\cdot}0, \tag{4}$$

where σ_i is the standard deviation for the ith data point and M is the number of data points, usually varying from 200–500. It is possible that the good fit criterion of $\chi^2 \sim 1{\cdot}0$

could be obtained for many different distributions of $\alpha(\tau)$. The optimum distribution is the one which fits the data adequately ($\chi^2 \sim 1{\cdot}0$) and maximizes the value of the Shannon–Jaynes entropy function S, as defined below.

$$S = -\sum p_i \log p_i, \tag{5}$$

where $p_i = \alpha_i / \Sigma \alpha_i$. If there is prior knowledge about the distribution (m_i) then (5) is modified as follows.

$$S = -\sum p_i \log(p_i/m_i). \tag{6}$$

If the χ^2 criterion is satisfied for many distributions then the maximum entropy criterion selects that distribution which contains the minimum number of peaks (or structures) in the distribution and the maximum width for each peak. This is in agreement with the logic that the outcome of analysis ought to be consistent with the information content in the data and the data are not overinterpreted. There are other definitions for entropy (also called regularizing function) in the literature (Narayan and Nityananda 1986; Press *et al* 1992) which were found to work just as well as (5).

The important features of the general algorithm (Skilling and Bryan 1984) implemented in the MEM analysis of fluorescence decay are as follows. The initial distribution of lifetimes is assumed to be flat and the distribution is improved in successive iterations: $\alpha_i(\text{new}) = \alpha_i(\text{old}) + x\Delta\alpha_i$. $\Delta\alpha_i$ in each iteration is determined by the optimization procedure which uses three search directions constructed using $\nabla\chi^2$, ∇S and $\nabla\nabla\chi^2$. The multiplication factor x for the search direction which minimises χ^2 and maximises S is determined by the 'α-chop and p-chop' technique (Skilling and Bryan 1984) to achieve an aimed χ^2 value which is higher than the lowest possible. Care is taken to avoid negative values for α_i, by using only a fraction of x and/or by equating negative values to zero (which tends to increase χ^2). Successive iterations give distributions which minimize the χ^2 and the analysis is terminated when $\chi^2 \leqslant 1{\cdot}0$ or when χ^2 and $\alpha(\tau)$ do not change in successive iterations.

3. Results and discussion

Figure 1 shows the distribution of lifetimes at various iteration numbers obtained while the MEM analysis is in progress. The fluorescence decay used in the analysis is for a sample of tryptophan in water ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 360$ nm, peak count of 2×10^4 at 37·8 ps/ch). The analysis is begun (iteration 0) with a flat distribution for $\alpha(\tau) = 0{\cdot}002$. The starting value for the flat distribution is not important but it is desirable to choose a value that is expected to produce a total integrated count (when convolved with the instrument response function) which is of the same order as the experimental one. A broad distribution appears after iteration 1 with a χ^2 value of 177·0. In successive iteractions the peak at $\sim 2{\cdot}5$ ns becomes prominent and narrower. At the same time the distribution in the 0·1–1·0 ns region remains broad but gets narrower in successive iterations. After 90 iterations the distribution obtained gives a χ^2 of 1·06. The distribution and χ^2 are unchanged afterwards. Thus, MEM analysis gives a bimodal distribution of lifetimes for the fluorescence of tryptophan.

Fluorescence of tryptophan in aqueous solutions is one of the extensively studied systems and hence it was chosen to illustrate the application of MEM. The fluorescence decay is generally agreed to be biexponential (Beechem and Brand 1985). The exact

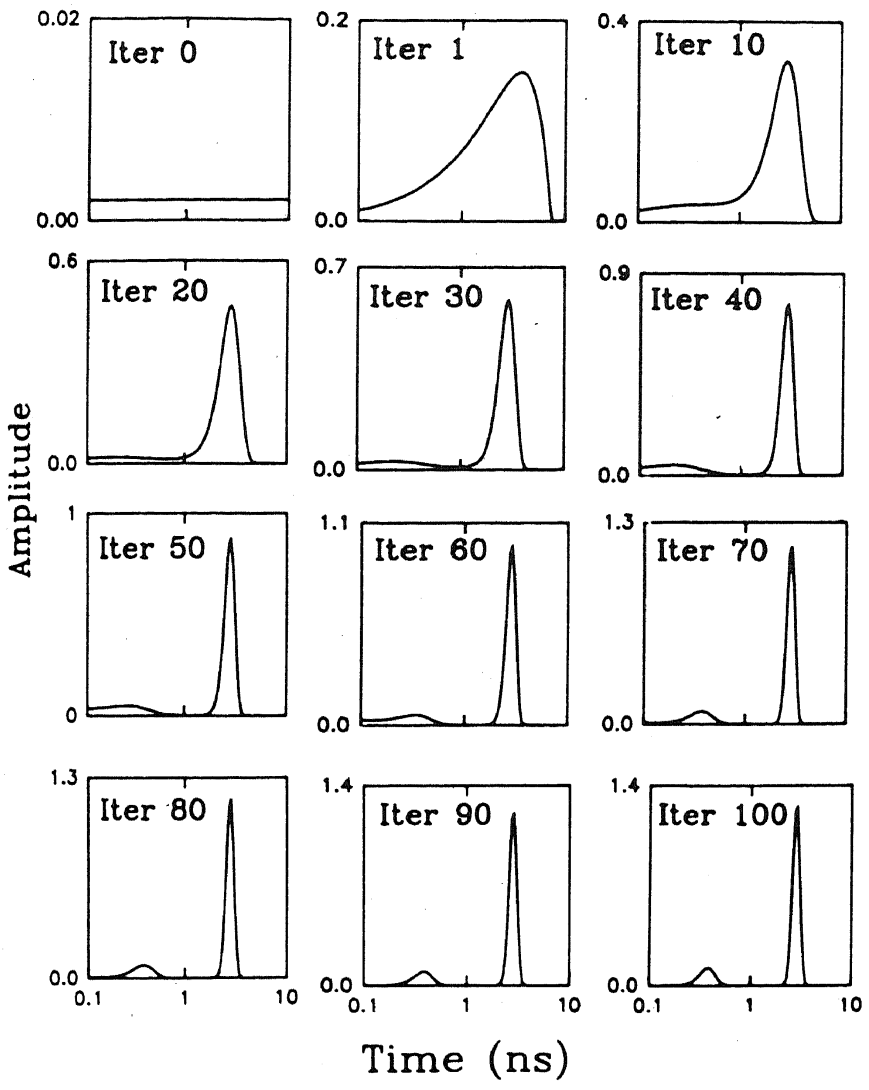

Figure 1. Progress of MEM analysis of the experimentally measured fluorescence decay of tryptophan in water is shown. The values of χ^2 for the iteration numbers shown in the figure are as follows: (1) 177, (10) 11·4, (20) 2·7, (30) 1·66, (40) 1·19, (50) 1·13, (60) 1·10, (70) 1·07, (80) 1·07, (90) 1·06, (100) 1·06.

values of the two lifetimes depend upon the sample condition, especially temperature, because the lifetime of the indole chromophore is sensitive to temperature (Joshi *et al* 1990). The bimodal distribution is therefore consistent with the two lifetimes of tryptophan.

The fluorescence decay of tryptophan in water for which the bimodal distribution was obtained was also fitted to a two-exponential function. The lifetimes (and amplitudes) were 2·80 ns (0·82) and 0·42 ns (0·18) and the χ^2 value was 1·09. These results ought to be consistent with the distribution of lifetimes obtained by MEM. This is indeed the case, qualitatively and quantitatively. Figure 2A shows the result by MEM and figure 2B, the result by discrete exponential fit. The width of the lines in figure 2B indicates the standard deviation in the lifetimes as determined from the diagonal elements of error matrix (Yguerabide and Yguerabide 1984; Bevington and Robinson 1992). For quantitative comparison, the average lifetime and amplitude for each of the

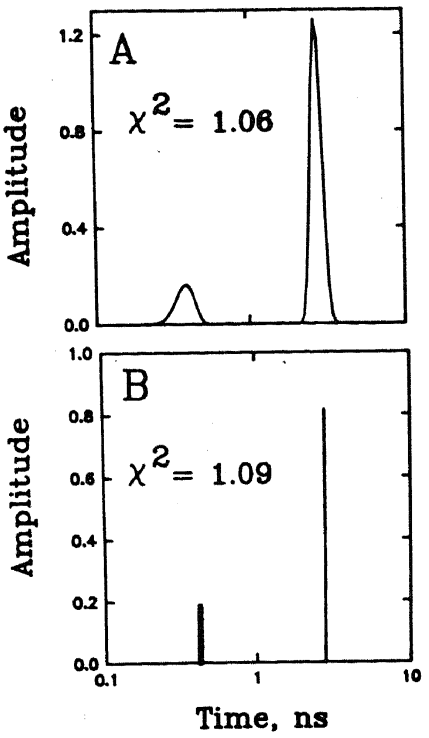

Figure 2. The results of the analysis of the fluorescence decay of tryptophan in water. (A) MEM. (B) Discrete two-exponential analysis.

two peaks in the bimodal distribution have to be calculated. For peak 1, $\langle\tau_1\rangle$ and $\langle\alpha_1\rangle$ are given by

$$\langle\tau_1\rangle = \sum\alpha_j\tau_j \Big/ \sum\alpha_j, \quad j=1, M_1, \tag{7}$$

$$\langle\alpha_1\rangle = \sum_{j=1}^{M_1}\alpha_j \Big/ \sum_{j=1}^{M}\alpha_j, \tag{8}$$

where M_1 is number of lifetimes represented in peak 1, and M the number of lifetimes represented in the distribution. Similar equations are used for $\langle\tau_2\rangle$ and $\langle\alpha_2\rangle$. The calculated values are $\langle\tau_1\rangle = 2{\cdot}78$ ns, $\langle\alpha_1\rangle = 0{\cdot}82$, $\langle\tau_2\rangle = 0{\cdot}38$ ns, $\langle\alpha_2\rangle = 0{\cdot}18$, which agree excellently with the values obtained in the discrete exponential fit.

The results described above and the discussion indicate that if the fluorescence decay is multiexponential, a multimodal distribution can be expected in MEM analysis and vice versa. In a complex system of unknown fluorescence dynamics it is not possible to know *a priori* the number of exponentials required to fit the data. On the other hand, MEM analysis could give a single or multimodal distribution as demanded by the data. Thus, MEM is a superior method for the analysis of fluorescence decay of complex systems.

MEM analysis produces a multimodal distribution of lifetimes if the fluorescence decay is multiexponential. If the decay is a single exponential the distribution is unimodal (a distribution with a single peak), and if biexponential the distribution is bimodal. The shape of the distribution function is symmetrical in the log(τ) space and the distribution is best approximated to a Gaussian function. Multimodal distributions

can be approximated as a superposition of multiple Gaussian distributions (Roy and Periasamy 1995). However, there is no theoretical justification for the Gaussian function. The widths of the distributions reported in this paper are based on Gaussian fits. An important question which needs to be addressed is whether the shape and width of the distribution obtained in MEM analysis have physical meaning. For example, there are reports in the literature where distribution width was used as a quantitative indicator of heterogeneity of environments (Alcala *et al* 1987; Bismuto *et al* 1991). This aspect is examined using computer simulated data.

The influence of noise content in the fluorescence decay data on the width of the MEM distribution was investigated. For this purpose, fluorescence decays were simulated for a lifetime of 3 ns at 40 ps/ch with different peak counts: 1×10^3, 1×10^4, 2×10^4, 5×10^4 and 1×10^5. The total time span in each decay is 20 ns (500 data points). The signal-to-noise ratio increases with the peak count (see § 2 for details), as follows: 31·6, 100, 141·4, 223·6 and 316·2 respectively, for the five decays at the peak. The decays were analysed by MEM which gave a unimodal distribution of lifetimes. The plot of the full width at half maximum of the distribution in the $\log(\tau)$ space versus peak count is shown in figure 3. The width was highest for the decay with a peak count of 1000 and decreased with increasing peak count. However, the width seems to reach a plateau value at higher peak counts instead of decreasing continuously to zero. This indicates that there are other factors, besides signal-to-noise ratio, which influence the width. These are examined below.

The maximum entropy method uses 50 lifetimes for a decade variation of lifetimes (for example, 1 ns to 10 ns) which are uniformly spaced in the $\log(\tau)$ space. The lifetime of 3·0 ns for which the fluorescence decay was simulated is not represented as one of the 50 τ_i. The lifetimes (closest to 3·0 ns) which are represented are 2·947 ns and 3·089 ns. For this analysis condition, the possibility exists that the width of the MEM distribution may be dependent on the value of the lifetime, whether it coincides with a τ_i or not. This was examined by simulating fluorescence decays (peak count of 1×10^4) for

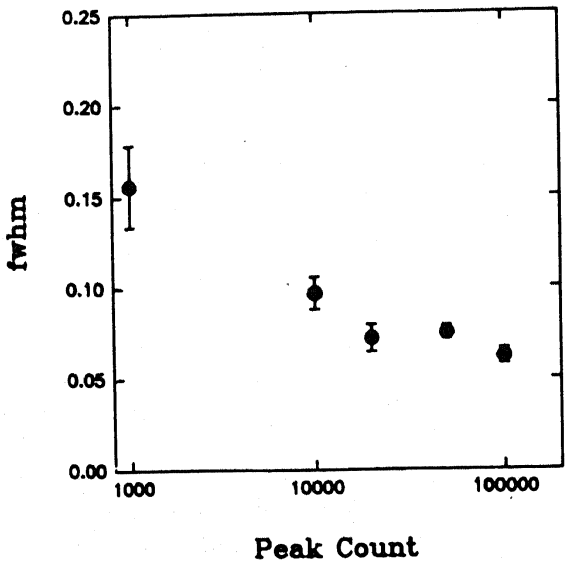

Figure 3. The dependence of the width (FWHM) in log τ space for the unimodal distribution of lifetimes on the peak count of the fluorescence decay. Each point is an average of 10 data sets simulated for a lifetime of 3 ns. The error bar is the standard deviation.

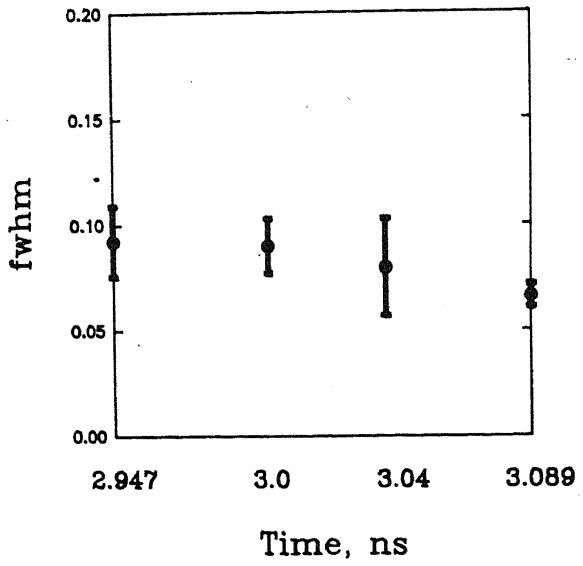

Figure 4. The dependence of the width of unimodal distribution on the lifetime used for the simulation of data. See text for details and discussion.

different lifetimes in the range between 2·947 ns and 3·089 ns. The widths of the unimodal distribution obtained in the MEM analysis are plotted in figure 4. The results show that the width is not substantially influenced by the lifetime discretization procedure in MEM analysis.

Another factor which affects the width of the MEM distribution of lifetimes is the stopping criterion which is used to terminate the analysis. An optimum value of 1·0 for the χ^2 is used as the stopping criterion in MEM analysis (Livesey and Brochon 1987). That is, the distribution obtained when χ^2 reaches this optimum value is taken to be the final result. Attempts to minimize the χ^2 value below the optimum value may lead to over-determination of the distribution, not warranted by the data. On the other hand, χ^2 value itself is statistical and only a probabilistic interpretation of χ^2 is meaningful. For example, for a data set with 200 points of random noise (a typical number of data points in most fluorescence decay experiments) the probability for $\chi^2 > 1{\cdot}0$ is 0·5, for $0{\cdot}9 < \chi^2 < 1{\cdot}1$ is $\sim 0{\cdot}7$, and for $0{\cdot}8 < \chi^2 < 1{\cdot}2$ is $\sim 0{\cdot}96$ (Bevington and Robinson 1992). In an individual experiment the χ^2 value could therefore be in the range of 0·8 to 1·2. The effect of the termination criterion of $\chi^2 = 1{\cdot}0$ is the possibility of either overestimating (the width will be narrower if the true $\chi^2 > 1{\cdot}0$) or underestimating (the width will be broader if true $\chi^2 < 1{\cdot}0$) the distribution. It is therefore necessary to examine the effects of the χ^2-stopping criterion on the width of the distribution of lifetimes obtained in MEM analysis.

Fluorescence decays were simulated for a single exponential decay of lifetime 3 ns in which the random noise added gave a χ^2 of 0·9. Figure 5 shows the distributions obtained when the χ^2 values were 1·22, 1·09, 1·01 and 0·9. The termination criterion of 1·0 does lead to a significantly broader width than the width warranted in this example for which the true χ^2 was 0·9. In computer-simulated data it was possible to know the true χ^2 of the noise. However, in real experimental data the value of true χ^2 will never be known and thus the width of the distribution cannot be used for quantitative interpretation of heterogeneity of lifetimes.

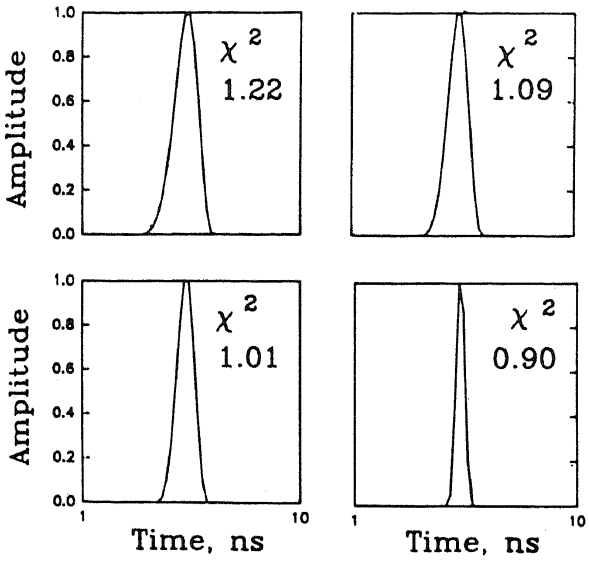

Figure 5. The distribution of lifetimes obtained in MEM analysis when the analysis was terminated with the value of χ^2 in the 'acceptable' range of 0·8–1·2. See text for details and discussion.

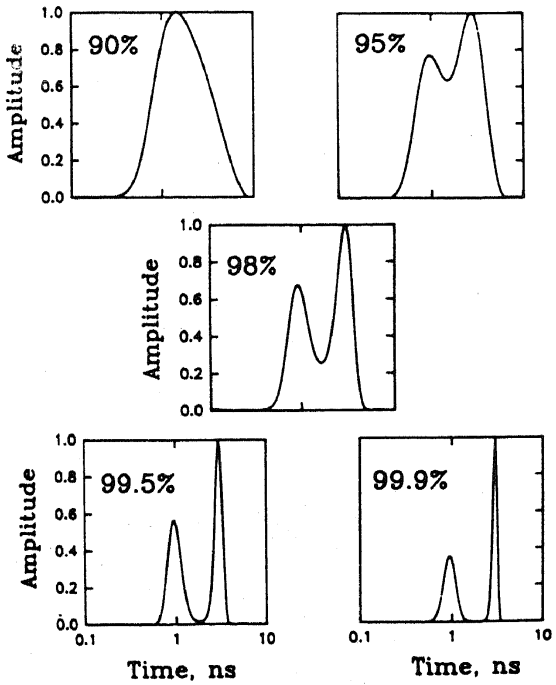

Figure 6. The fluorescence lifetime distributions obtained by MEM analysis for decays simulated at various levels of completeness as indicated in the figure. The temporal resolution (time per channel) is 10, 15, 20, 30 and 40 ps respectively. The peak count is $1{\cdot}5 \times 10^4$ in all cases. The simulation parameters are $\tau_1 = 1$ ns, $\tau_2 = 3$ ns, and $\alpha_1 = 0{\cdot}5$ and $\alpha_2 = 0{\cdot}5$.

Yet another factor which affects the width of the distribution is the completeness of the decay which restricts the information content in the data. Ideally, one would like to use a fluorescence decay which is complete (that is, the intensity in the tail end is

comparable to the background level) for determining lifetime parameters or distributions. However, it is common practice to use a fluorescence decay which is not complete. This is especially so in cases where the decay half-life is around 10–20 ns. One of the reasons for this practice is the appearance of a second emission decay either due to a multi-dynode photomultiplier (not an MCP PMT) or due to the leakage of a laser pulse in a cavity dumped picosecond laser (O'Connor and Phillips 1984). Incomplete decays, do not cause any problem if the decay is a single exponential. In case of multiexponential decay, incomplete decays will result in poor estimates of the lifetimes, especially the longer components. One would therefore expect that incomplete decays would also affect the distribution of lifetimes in MEM analysis.

In order to evaluate this aspect quantitatively fluorescence decays were simulated for biexponential functions with lifetimes of 1·0 and 3·0 ns, for an amplitude of 0·5 each. The simulated decay data were complete to the extent of 90, 95, 98, 99·5 and 99·9% with a peak count of 15 000 consisting of ~ 400 points in each. The random noise added to each of the decays was chosen to give a χ^2 between 0·98 and 1·02. The MEM analysis gave distributions ($\chi^2 = 1{\cdot}0$) which are shown in figure 6. The decay which was complete (> 99·5%) resulted in a well-resolved bimodal distribution as it should be. The decay which was only 90% complete was not able to resolve the two peaks and gave a unimodal distribution. The resolvabilities and widths of the distribution peaks were dependent on the extent of completeness of the decay. This illustrates the importance of using a complete decay for MEM analysis.

The results and discussion thus far clearly indicate that the width of a distribution recovered in the MEM analysis depends upon the information content in the data. Noisier the decay data, wider will be the distribution. This is precisely the result one should expect in MEM analysis where χ^2 is minimised to an optimum value of 1·0 and an entropy function is simultaneously maximised. The result will be the broadest distribution possible which is compatible with the noise or information content in the data. An immediate consequence of the above results is that the lifetime distribution width cannot be used to quantify the real heterogeneity of lifetimes in a sample until one quantifies the contribution of inherent noise in the decay to the width.

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