Photon Counting Data Analysis: Application of the Maximum Likelihood and Related Methods for the Determination of Lifetimes in Mixtures of Rose Bengal and Rhodamine B

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Kalyan Santra, Emily A. Smith, Jacob W. Petrich, and Xueyu Song*

Department of Chemistry, Iowa State University, and U.S. Department of Energy, Ames Laboratory, Ames, Iowa 50011, United States

Supporting Information

ABSTRACT: It is often convenient to know the minimum amount of data needed to obtain a result of desired accuracy and precision. It is a necessity in the case of subdiffraction-limited microscopies, such as stimulated emission depletion (STED) microscopy, owing to the limited sample volumes and the extreme sensitivity of the samples to photobleaching and photodamage. We present a detailed comparison of probability-based techniques (the maximum likelihood method and methods based on the binomial and the Poisson distributions) with residual minimization-based techniques for retrieving the fluorescence decay parameters for various two-fluorophore mixtures, as a function of the total number of photon counts, in time-correlated, single-photon counting experiments. The probability-based techniques proved to be the most robust (insensitive to initial values) in retrieving the target parameters and, in fact, performed equivalently to 2–3 significant figures. This is to be expected, as we demonstrate that the three methods are fundamentally related. Furthermore, methods based on the Poisson and binomial distributions have the desirable feature of providing a bin-by-bin analysis of a single fluorescence decay trace, which thus permits statistics to be acquired using only the one trace not only for the mean and median values of the fluorescence decay parameters but also for the associated standard deviations. These probability-based methods lend themselves well to the analysis of the sparse data sets that are encountered in subdiffraction-limited microscopies.

INTRODUCTION

Time-resolved spectroscopic techniques have a wide range of applications in the physical and biological sciences. Owing to, for example, its ease of use, high sensitivity, large dynamic range, applicability to imaging and subdiffraction-limited microscopies, one of the most widely used techniques is time-correlated, single-photon counting (TCSPC). A major challenge in analyzing the data obtained in these experiments arises from sparse data sets, such as those that may often be encountered in super-resolution microscopies, such as stimulated emission depletion (STED) microscopy. Typically, in a TCSPC experiment, a fluorescence lifetime is determined by acquiring a histogram of arrival time differences between an excitation pulse and the pulse resulting from a detected photon. As we have noted, when a histogram of sufficient quality cannot be obtained to provide a good fit by means of minimizing the residuals (RM) between the experimental data and a given functional form, the maximum likelihood (ML) technique is particularly effective, namely, when the total number of counts is very low. As we have shown in the case of rose bengal, ML retrieved the correct mean lifetime to within 2% of the accepted value with total counts as low as 20; and it retrieved the correct mean lifetime with less than 10% standard deviation with total counts as low as 200.

There are several comparisons of the ML and RM techniques, but most of them have been limited to simulated data. In those cases where the techniques were applied to real experimental data, the comparisons were limited by several factors such as the exclusion of a real instrument response function (IRF), the bin size for the time channels of the histogram, the exclusion of a shift parameter that accounts for the wavelength difference between the instrument response function and the fluorescence signal, and, most importantly, not determining the minimum number of counts at which the respective techniques provide an acceptable result. In our recent work, we addressed all of these issues for a single fluorophore, rose bengal. Here, we extend these efforts by studying mixtures of fluorophores, which is more relevant to the type of data that can be extracted from a STED experiment capable of extracting fluorescence lifetimes. In such experiments, heterogeneity in the lifetimes of the emitting...
fluorophores is expected, and such heterogeneity can provide insight into the processes being probed in the subdiffraction-limited spot under interrogation. To this end, we examined mixtures of the well-characterized dyes, rose bengal (Rb) and rhodamine B (RhB), in methanol. The excited-state lifetime, \( \tau \), of Rb is 0.49 ± 0.01 ns.3 Some reported values are 0.53 ± 0.01 and 0.512 ns,28 with no error estimate. We have measured the excited-state lifetime of RhB to be 2.45 ± 0.01 ns. We have measured the fluorescence decays were collected with a total number of 2250 fluorescence decay profiles were analyzed.

We furthermore examined the performance and utility of other methods related to ML. For example, though analysis of 50 decays gives sufficient statistics to retrieve the two lifetime and amplitude components of the fluorescence decay using the ML method (or the RM method under certain conditions), in a subdiffraction-limited imaging experiment it is usually not practical to perform multiple measurements of the same sample. These other methods are related to ML in that they are based on the binomial and Poisson distributions and have the interesting and useful properties of yielding statistics from only one measurement of the fluorescence decay. In particular, because we know that there is a well-defined probability that a certain number of photons will be accumulated in a given bin of the histogram, we can apply a Poisson distribution or a binomial distribution to the random arrival of photons to estimate the decay constant of the sample by analyzing only one bin. Therefore, photon counts in each bin will furnish a probability that a certain number of photons will be accumulated in a given bin of the histogram. Let 

\[ F(t) = \sum n a_n e^{-\tau_n t} \]

where \( \Sigma a_n = 1 \) and \( a_n \) are the fractions of the \( n \)th species in the sample mixture. In the case of the two-component system of Rb and RhB:

\[ F(t) = a_1 e^{-\tau_1 t} + (1 - a_1) e^{-\tau_2 t} \]

where \( \tau_1 \) and \( \tau_2 \) are the lifetimes of the two species and \( a_1 \) is the fraction of the species with lifetime \( \tau_1 \).

Data Analysis. Modeling the Time-Correlated, Single-Photon Counting Data. When there is more than one emitting species, a multiexponential model can be applied:

■ MATERIALS AND METHODS

Experimental Procedure. Rose bengal (Rb) and rhodamine B (RhB) were obtained from Sigma and Eastman, respectively, and were purified by thin-layer chromatography using silica-gel plates and a solvent system of ethanol, chloroform, and ethyl acetate in a ratio of 25:15:50 by volume. Solvents were used without further purification. The purified dyes were stored in methanol in the dark. Rb absorbs in the region 460–590 nm; RhB, 440–590 nm. Thus, 550 nm was selected as the excitation wavelength. Five sets of samples were prepared so that they had an absorption ratio of Rb:RhB at 550 nm of 100:0, 75:25, 50:50, 25:75, and 0:100 respectively. The net absorbance of each of the five solutions was kept near 0.3 (Figure 1a). Time-resolved data were collected using a homemade, time-correlated, single-photon counting (TCSPC) instrument using a SPC-630 TCSPC module (Becker & Hickl GmbH). A collimated Fianium pulsed laser (Fianium Ltd., Southampton, U.K.) at a 2 MHz repetition rate, was used to excite the sample at 550 nm. The excitation beam was vertically polarized. Emission was detected at the “magic angle” (54.7°) with respect to the excitation using a 590 nm, long-pass filter (Figure 1b). The instrument response function (IRF) was measured by collecting scattered light at 550 nm (without the emission filter) from the pure methanol solvent. The full-width at half-maximum of the instrument function was typically ~120 ps. The TCSPC data were collected in 1024 channels (bins), providing a time resolution of 19.51 ps/channel, and a full-scale time window of 19.98 ns. Nine different data sets consisting of 50 fluorescence decays were collected with a total number of counts of approximately 20, 100, 200, 500, 1000, 3000, 6000, 10000, and 20000, respectively.

Data Analysis. Modeling the Time-Correlated, Single-Photon Counting Data. When there is more than one emitting species, a multiexponential model can be applied:

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where \( \tau_1 \) and \( \tau_2 \) are the lifetimes of the two species and \( a_1 \) is the fraction of the species with lifetime \( \tau_1 \).

Let \( t = \{t_1, t_2, ..., t_{1024}\} \) represent the time axis, where the center of the \( j \)th bin (or channel) is given by \( t_j \) and \( e = 19.51 \) ps is the time width of each bin in the histogram. Let \( C = \{c_{t_1}, c_{t_2}, ..., c_{t_{1024}}\} \) be the set of counts obtained in the 1024 bins. Similarly,

\[ F(t) = a_1 e^{-\tau_1 t} + (1 - a_1) e^{-\tau_2 t} \]

where \( \tau_1 \) and \( \tau_2 \) are the lifetimes of the two species and \( a_1 \) is the fraction of the species with lifetime \( \tau_1 \).
we experimentally measure the instrument response function (IRF) and represent it as I = {I_1, I_2, ..., I_{1024}}, where the I_j are the number of counts in the jth bin.

The probability that a photon is detected in the jth bin, p_j, is proportional to the discrete convolution of the IRF and the model for the fluorescence decay given in eq 2.

\[
p_j \propto \sum_{i=1}^{j-1} I(t_{i+1} - t_i) = \sum_{i=1}^{j-1} I(a_i e^{-(t_{i+1} - t_i)/\tau_1} + (1 - a_i) e^{-(t_{i+1} - t_i)/\tau_2})
\]  

(3)

where \( I \) is given by \( b = j \epsilon \). The parameter \( b \) describes the linear shift between the instrument response function and the convoluted counts generated with the IRF.

The normalization factor in the denominator is independent of the index, \( j \), and, hence, the “dummy index”, \( k \), is inserted while retaining \( I \) as this constant, unknown shift applies for all bins. The denominator is proportional to the total number of convoluted counts generated with the IRF.

Let \( \hat{C} \) represent the number of predicted counts from the multieponential model in the jth bin, taking into account convolution. The number of predicted counts in a given bin is directly proportional to the probability that a photon is detected in that bin: \( \hat{C} \propto p_j \). Thus, we can write the predicted counts as \( C = \{ \hat{C}_1, \hat{C}_2, ..., \hat{C}_{1024} \} \). The area under the decay curves obtained from the observed counts C and the predicted counts \( \hat{C} \) must be conserved during optimization of the fitting parameters. In other words, the total number of predicted counts must be equal to the total number of observed photon counts. The number, therefore, of predicted counts in the jth bin is given by

\[
\hat{C}_j = C_T \frac{\sum_{i=1}^{j-1} I(a_i e^{-(t_{i+1} - t_i)/\tau_1} + (1 - a_i) e^{-(t_{i+1} - t_i)/\tau_2})}{\sum_{i=1}^{1024} \left( \sum_{k=1}^{j-1} I(a_k e^{-(t_{i+1} - t_i)/\tau_1} + (1 - a_k) e^{-(t_{i+1} - t_i)/\tau_2}) \right)}
\]  

(5)

where \( C_T = \sum_{i=1}^{1024} C_i \). It should be noted that in the above equation we allowed the shift parameter, \( \hat{C} \), to assume continuous values. Therefore, we always find an integer, \( j_0 \), such that \( b = j_0 \epsilon + \zeta \), where \( \zeta \) lies between 0 and \( \epsilon \), the time width of the bin. In the case of a single-exponential model, the expressions for the probability, \( p_j \), and the predicted number of counts, \( \hat{C}_j \), are obtained by substituting \( a_1 = 1 \):

\[
p_j = \sum_{i=1}^{j-1} I(e^{-(t_{i+1} - t_i)/\tau_1}) \frac{1}{\sum_{i=1}^{1024} \left( \sum_{k=1}^{j-1} I(e^{-(t_{i+1} - t_i)/\tau_1}) \right)}
\]

\[
\hat{C}_j = C_T \frac{\sum_{i=1}^{j-1} I(e^{-(t_{i+1} - t_i)/\tau_1})}{\sum_{i=1}^{1024} \left( \sum_{k=1}^{j-1} I(e^{-(t_{i+1} - t_i)/\tau_1}) \right)}
\]  

(6)

**Residual Minimization Method (RM).** The traditional method of RM uses the sum of the square of the differences (residuals) between the experimentally obtained counts and the predicted counts to optimize the fit. It is also well-known\(^{5,20,34}\) that minimizing of the weighted square of the residuals provides a better fit than does the unweighted square of the residuals. We, therefore, used the sum of the weighted squares of the residuals and minimized it over the parameters, \( \tau_1, \tau_2, a_1, \) and \( b \), to obtain the optimal values:

\[
S_w = \sum_j w_j (C_j - \hat{C}_j)^2
\]  

where \( w_j \) is the weighting factor. Depending on the choice of \( w_j \), eq 7 can take the following forms of the classical \( \chi^2 \), for example:\(^{9,16,20,23,27,34-36}\)

\[
\chi_p^2 = \text{Pearson's } \chi^2 = \sum_{j=1}^{1024} \frac{(C_j - \hat{C}_j)^2}{\hat{C}_j}
\]  

(8)

or

\[
\chi_N^2 = \text{Neyman's } \chi^2 = \sum_{j=1}^{1024} \frac{(C_j - \hat{C}_j)^2}{\hat{C}_j}
\]  

(9)

The reduced \( \chi^2 \) is obtained by dividing by the number of degrees of freedom:

\[
\chi_{\text{red}}^2 = \frac{1}{n - p} \chi^2
\]  

(10)

where \( n \) is the number of data points and \( p \) is the number of parameters and constraints in the model. For example, in our case we have 1024 data points, two or four parameters (\( \tau_1, \tau_2, a_1, b \)) depending on whether one or two exponentials are used to describe the decay, and one constraint, \( C_T = \hat{C}_T \). This gives \( n - p = 1021 \) or 1019, respectively. For an ideal case, \( \chi_{\text{red}}^2 \) is unity. \( \chi_{\text{red}}^2 < 1 \) implies overfitting of the data. Therefore, the closer \( \chi_{\text{red}}^2 \) is to unity (without being less than unity), the better the fit. The minimization program is run over the parameters to minimize \( \chi_{\text{red}}^2 \).

**Binomial Distribution.** In a time-correlated, single-photon counting experiment, the random events are independent of each other; and each pulse, by experimental design, can only give one photon in any of the 1024 bins. The next photon is detected in a completely different cycle that depends on a pulse identical to but independent of the previous pulse. It can, therefore, be concluded that the successive detection of a photon in any particular bin is independent of the detection of any other photon.

The probability distribution of discrete events, such as occurring in the TCSPC experiment, can be described by several well-known probability distributions. The binomial probability distribution is one example where the probability distribution of the number of successes is described for a series of independent experiments. In each experiment, the probabilities of success or failure are identical.\(^{37}\) (This is also known as a Bernoulli trial.)

Let the probability that a photon is detected (success) in the jth bin be \( p_j \). Depending on whether the fluorescence decay is described by two or one decaying exponentials, the expression for \( p_j \) is given by either eq 4 or eq 6. The probability that the photon is not detected (failure) in the jth bin is given by \( q_j = 1 - p_j \). Let \( c_j \) be the number of photons that is accumulated in jth bin in an experiment, where the total number of counts is \( C_T \). The binomial probability function is thus given by
where the factor on the right in the curved bracket is the binomial coefficient. It is important to note that the binomial probability is independent of all indices except \( j \) and that, therefore, the distribution of the number of photons over all the other channels, \( (C_j - c_j) \), which do not accumulate in the \( j \)th bin, does not affect the binomial probability. These independent but identical binomial probabilities can be maximized with respect to the parameters \((τ_j, a_j, b)\), depending on the model used to describe the fluorescence decay. This procedure thus generates a lifetime value for every channel for one fluorescence decay experiment, from which a histogram of lifetime values can be obtained. From this histogram, the mean and standard deviation of the lifetime parameters can be extracted. Furthermore, we can construct a joint probability distribution to obtain a best possible value of the lifetime corresponding to a single decay curve. The joint probability is given by

\[
p^{\text{binom}}(c_j | C_j) = \prod_{j=1}^{1024} \binom{C_j}{c_j} p_j^{c_j} (1 - p_j)^{C_j - c_j}
\]

(12)

Maximization of the probability \( p^{\text{binom}} \) can be performed over the parameters used to describe the fluorescence decay function.

**Poisson Distribution.** Another well-known probability distribution that describes the occurrence of discrete events is the Poisson distribution.\(^{37}\) The Poisson distribution gives the probability of the occurrence of a certain number of events for a given average number of events in that time interval. The Poisson distribution can be applied if the successive occurrences of the events are independent of each other and the numbers of occurrences are integers. (For our case, we are not interested in the number of events that do not occur.) Because successive photon counts are independent and because a photon count in a bin is an integer, the time-correlated, single-photon counting experiment conforms to the criteria necessary for its being able to be described by a Poisson distribution. Whereas the binomial distribution incorporates the probability that a photon is accumulated (success) or not accumulated (failure) in a given bin directly, the Poisson distribution requires the average number of photons that accumulates in a certain bin to estimate the probability of having a certain number of photons in a given bin in the same time interval. The Poisson distribution is an approximation of the binomial distribution in the limit where the number of trials is relatively large and (or) the probability of success of each trial is very small (which is the case in all of our experiments).\(^{37}\)

For the Poisson distribution to be applied, one must know beforehand that the fluorescence decay is indeed an exponential (or sum of exponentials) because the Poisson distribution employs the mean or the average number of counts in a bin. For example, consider a given decay, where we have a number, \( C_j \), of photons collected over a time period of \( T \). Now, to estimate the average number of photons in a bin within that time period of \( T \), we can simply use the multieponential function, even though the true nature of the probability distribution of the emission may not be known owing to collection of only a small number of photons, because we require only the average number of predicted counts.

Let us assume that we continue collecting the fluorescence decay until it becomes smooth enough to be fit with the usual residual minimization methods. A full decay will have 65535 photons in the peak channel (a 16-bit memory sets the limit of the number of counts to \( 2^{16} - 1 \) in a channel). If this process takes a time period of \( T_m = nT \), then let the total number of photons is \( C_{T_m} \). If the rate of the data acquisition remains constant within the time period, then we have \( C_{T_m} = mC_T \). Now we can apply the multieponential model to estimate the number of predicted counts in a bin:

\[
\hat{c}_m = \frac{\sum_{j=1}^{1024} \binom{C_j}{c_j} p_j^{c_j} (1 - p_j)^{C_j - c_j}}{\sum_{j=1}^{1024} (\binom{C_j}{c_j} p_j^{c_j} (1 - p_j)^{C_j - c_j})}
\]

(13)

The average number of counts in the time period \( T \) is given by

\[
\hat{c}_T = \frac{T}{T_m} \hat{c}_m
\]

\[
= \frac{T}{T_m} C_T \frac{\sum_{j=1}^{1024} \binom{C_j}{c_j} p_j^{c_j} (1 - p_j)^{C_j - c_j}}{\sum_{j=1}^{1024} \binom{C_j}{c_j} p_j^{c_j} (1 - p_j)^{C_j - c_j}}
\]

(14)

Now, the Poisson distribution is given by

\[
P(c_j | \lambda_j) = \frac{\lambda_j^{c_j} e^{-\lambda_j}}{c_j!}
\]

(15)

where \( \lambda_j \) is the average number of success at \( j \)th bin in the same time interval and is given by \( \hat{c}_T = \hat{c}_j \). The important point here is that given the above, we can conclude that each bin follows identical and independent Poisson distributions and that we can maximize the probability of having a number, \( c_j \), of “successes” to obtain the estimated lifetime of the sample at the corresponding time bin. We can define the joint probability distribution of a sequence of counts in a single decay in the same manner as we defined it in the case of the binomial distribution.

\[
P(c_1, c_2, ..., c_{1024}) = \prod_{j=1}^{1024} \frac{\lambda_j^{c_j} e^{-\lambda_j}}{c_j!}
\]

(16)

Maximization of the probability \( P \) can be performed over the parameters, \( \tau_j, a_j, b, \) and \( c_0, c_1, ..., c_{1024} \).

**Maximum Likelihood Method (ML).** Another approach to describe the joint probability distribution is to express it in terms of a multinomial form and to apply the maximum likelihood technique on the resulting distribution function. The total number of having a sequence \( \{c_1, c_2, ..., c_{1024}\} \) subject to the condition, \( C_T = \sum c_j \), follows the multinomial distribution:

\[
Pr(c_1, c_2, ..., c_{1024}) = \frac{C_T!}{c_1! c_2! ... c_{1024}!} \prod_{j=1}^{1024} \left( \frac{p_j}{c_j!} \right)^{c_j}
\]

(17)

We can define a likelihood function as the joint probability density function above: \( L(c_T) = Pr(c_1, c_2, ..., c_{1024}) \). We substitute the expression for the probability as \( p_j = \hat{c}_j / C_j \) to obtain
Following the treatment of Baker and Cousins, we let \( \{ c \} \) represent the true value of \( \{ c' \} \) given by the model. A likelihood ratio, \( \lambda \), can be defined as

\[
\lambda = \frac{L(\tilde{c}, c)}{L(c', c)}
\]  

(19)

According to the likelihood ratio test theorem, the “likelihood \( \chi^2 \)” is defined by

\[
\chi^2 = -2 \ln \lambda
\]  

(20)

which obeys a \( \chi^2 \) distribution as the sample size (or number of total counts) increases.

For the multinomial distribution, we may replace the unknown \( \{ c' \} \) by the experimentally observed \( \{ c \} \). This gives

\[
\lambda = \frac{\prod_{j=1}^{1024} \left( \frac{\tilde{c}_j / C_T}{c_j} \right)^{c_j}}{\prod_{j=1}^{1024} \left( \frac{c_j / C_T}{c_j} \right)^{c_j}}
\]  

(21)

and the “likelihood \( \chi^2 \)” becomes

\[
\chi^2 = -2 \ln \lambda = -2 \ln \prod_{j=1}^{1024} \left( \frac{\tilde{c}_j / c_j}{C_T} \right)^{c_j} = 2 \sum_{j=1}^{1024} c_j \ln \left( \frac{\tilde{c}_j}{c_j} \right)
\]  

(22)

The minimization of the “likelihood \( \chi^2 \)” is done by varying the parameters \( \tau_1, \tau_2, a_1, \) and \( b \).

It is important to recognize that the multinomial form given in eq 17 and the “likelihood \( \chi^2 \)” form given in eq 22, popularized by Baker and Cousins and used by several others, are formally identical to each other. Maximization of the probability in eq 17 is equivalent to minimization of \( \chi^2 \) in eq 22.

Furthermore, we note that all the probability-based methods are equivalent under certain assumptions. It has already been pointed out in the previous section that the Poisson distribution is related to the binomial distribution in the limit where the number of trials is relatively large and (or) the probability of success of each trial is very small. The joint Poisson probability distribution given in eq 16 can be written as

\[
P(c_1, c_2, ..., c_{1024}) = \prod_{j=1}^{1024} \frac{c_j^{\tilde{c}_j} e^{-c_j}}{\tilde{c}_j!}
\]  

(23)

because \( \tilde{c}_j = \tilde{c} \). This equation can be transformed to

\[
\ln P(c_1, c_2, ..., c_{1024}) = \ln \left( \prod_{j=1}^{1024} \frac{c_j^{\tilde{c}_j} e^{-c_j}}{\tilde{c}_j!} \right)
\]  

(24)

\[
= \sum_{j=1}^{1024} \tilde{c}_j \ln \tilde{c}_j - \sum_{j=1}^{1024} \tilde{c}_j - \sum_{j=1}^{1024} \ln c_j!
\]

Under the assumption that the total number of predicted counts is equal to the total number of observed photon counts (\( \sum \tilde{c}_j = \sum c_j = C_T \)), we have

\[
\ln P(c_1, c_2, ..., c_{1024}) = \sum_{j=1}^{1024} \tilde{c}_j \ln \tilde{c}_j - C_T - \sum_{j=1}^{1024} \ln c_j!
\]  

(25)

Now, because \( \tilde{c}_j = \tilde{c} \), eq 25 can be written as

\[
\ln P(c_1, c_2, ..., c_{1024}) = \sum_{j=1}^{1024} c_j \ln p_j + \beta_l
\]  

(26)

where \( \beta_l \) is independent of the parameters \( \tau_1, \tau_2, a_1, \) and \( b \) and thus remains constant during optimization. Furthermore, from eq 17, it can also be shown that

Figure 2. Representative fluorescence decay for a given number of total counts (as indicated in each panel) for a 50:50 Rb-RhB mixture. Experimental data are given by the black traces; the fits, by the red curves; and the instrument response functions (IRFs), by the blue traces.
Figure 3. Histograms of the (a) lifetime of rose bengal ($\tau_1$), (b) lifetime of rhodamine B ($\tau_2$), and (c) the amplitude of the lifetime of the short lifetime of rose bengal ($a_1$) estimated by ML (red), Poisson (green), binomial (blue), RM-Pearson (magenta), RM-Neyman (orange), and SPCI (cyan) methods for the total counts of 200, 6000, and 20000 in the Rb:RhB 50:50 data sets. The bins for all of the histograms are 10 ps wide. The vertical dark gray dashed lines give the target values $\tau_1 = 0.49$ ns, $\tau_2 = 2.45$ ns, and $a_1 = 0.44$ in (a), (b), and (c) respectively.
Table 1. Rose Bengal (τ_r) Mean Lifetime ± Standard Deviation (ns) for a Rb:RhB 50:50 Mixture

<table>
<thead>
<tr>
<th>total counts</th>
<th>ML</th>
<th>Poisson</th>
<th>Binomial</th>
<th>RM-Pearson</th>
<th>RM-Neyman</th>
<th>SPCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.5 ± 0.5</td>
<td>0.5 ± 0.5</td>
<td>0.5 ± 0.5</td>
<td>0.3 ± 0.4</td>
<td>0.4 ± 0.3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td>0.6 ± 0.5</td>
<td>0.6 ± 0.5</td>
<td>0.6 ± 0.5</td>
<td>0.2 ± 0.2</td>
<td>1.1 ± 0.6</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>200</td>
<td>0.6 ± 0.5</td>
<td>0.7 ± 0.5</td>
<td>0.7 ± 0.5</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>500</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>1.4 ± 1.0</td>
</tr>
<tr>
<td>1000</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>0.43 ± 0.08</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>3000</td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.87 ± 0.08</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>6000</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>10000</td>
<td>0.48 ± 0.06</td>
<td>0.48 ± 0.06</td>
<td>0.48 ± 0.06</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>20000</td>
<td>0.47 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>0.6 ± 0.1</td>
<td>0.47 ± 0.05</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

Table 2. Rhodamine B (τ_r) Mean Lifetime ± Standard Deviation (ns) for a Rb:RhB 50:50 Mixture

<table>
<thead>
<tr>
<th>total counts</th>
<th>ML</th>
<th>Poisson</th>
<th>Binomial</th>
<th>RM-Pearson</th>
<th>RM-Neyman</th>
<th>SPCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>2.7 ± 0.7</td>
<td>2.6 ± 0.8</td>
<td>2.6 ± 0.7</td>
<td>3.1 ± 0.6</td>
<td>2.1 ± 0.6</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td>2.6 ± 0.5</td>
<td>2.6 ± 0.5</td>
<td>2.6 ± 0.5</td>
<td>3.48 ± 0.08</td>
<td>1.6 ± 0.2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>200</td>
<td>2.7 ± 0.5</td>
<td>2.7 ± 0.5</td>
<td>2.7 ± 0.5</td>
<td>3.48 ± 0.08</td>
<td>2.3 ± 0.2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>500</td>
<td>2.4 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>3.48 ± 0.07</td>
<td>3.47 ± 0.08</td>
<td>6 ± 7</td>
</tr>
<tr>
<td>1000</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>3.48 ± 0.07</td>
<td>3.5 ± 0</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>3000</td>
<td>2.4 ± 0</td>
<td>2.4 ± 0</td>
<td>2.4 ± 0</td>
<td>3.4 ± 0.1</td>
<td>3.5 ± 0</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>6000</td>
<td>2.39 ± 0.06</td>
<td>2.39 ± 0.06</td>
<td>2.39 ± 0.06</td>
<td>3.1 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>10000</td>
<td>2.39 ± 0.04</td>
<td>2.39 ± 0.04</td>
<td>2.39 ± 0.04</td>
<td>2.9 ± 0.2</td>
<td>2.7 ± 0.5</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>20000</td>
<td>2.38 ± 0.03</td>
<td>2.38 ± 0.03</td>
<td>2.38 ± 0.03</td>
<td>2.61 ± 0.06</td>
<td>2.28 ± 0.04</td>
<td>2.45 ± 0.08</td>
</tr>
</tbody>
</table>

Table 3. Mean Value of the Amplitude (a_j) of the Component of Rose Bengal Emission ± Standard Deviation for a Rb:RhB 50:50 Mixture

<table>
<thead>
<tr>
<th>total counts</th>
<th>ML</th>
<th>Poisson</th>
<th>Binomial</th>
<th>RM-Pearson</th>
<th>RM-Neyman</th>
<th>SPCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.4 ± 0.4</td>
<td>0.999 ± 0.009</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>200</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.4 ± 0.3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>500</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>1000</td>
<td>0.49 ± 0.09</td>
<td>0.49 ± 0.09</td>
<td>0.48 ± 0.09</td>
<td>0.58 ± 0.05</td>
<td>0.64 ± 0.05</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>3000</td>
<td>0.45 ± 0.06</td>
<td>0.45 ± 0.05</td>
<td>0.45 ± 0.05</td>
<td>0.64 ± 0.04</td>
<td>0.72 ± 0.02</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>6000</td>
<td>0.44 ± 0.03</td>
<td>0.44 ± 0.03</td>
<td>0.44 ± 0.03</td>
<td>0.61 ± 0.06</td>
<td>0.76 ± 0.05</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>10000</td>
<td>0.44 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.57 ± 0.05</td>
<td>0.56 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>20000</td>
<td>0.44 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td>0.42 ± 0.05</td>
</tr>
</tbody>
</table>

$$\ln P(c_1, c_2, ..., c_{1024}) = \ln C_T! + \sum_{j=1}^{1024} c_j \ln p_j - \sum_{j=1}^{1024} \ln c_j!$$

$$= \sum_{j=1}^{1024} c_j \ln p_j + \beta_2$$

(27)

where $\beta_2$ is another constant independent of the parameters $\tau_r$, $\tau_p$, $a$, and $b$. Therefore, the maximization of the probability given in eq 26 and 27 will be at the same point in the parameter space. In the ensuing discussion, for simplicity and economy, we shall, however, primarily discuss ML as representative of the probability-based methods unless otherwise noted.

**Computational Methods.** The RM, ML, binomial, and Poisson analyses described above are performed using codes written in MATLAB that were run on a machine equipped with a quad-core Intel Core i7 processor and 16 Gigabytes of memory. We employ the GlobalSearch toolbox, which uses the “fmincon” solver to minimize the objective function in the respective cases. In each calculation, a global minimum was found. In the case of a single-component system, we have two parameters, $\tau_r$ and $b$. For a two-component system, there are four parameters: $\tau_r$, $\tau_p$, $a$, and $b$. With our in-house routines, we experimented with different initial values in the following ranges for $\tau_r$, $\tau_p$, $a$, and $b$: 0.01–1.5 ns, 1.5–3.5 ns, 0.0–1.0, and ±0.1 to ±0.1 ns, respectively. Within the specified ranges, we always retrieved the same fit results through the third decimal place. Because the binomial and the Poisson distributions can be defined for individual channels in a single fluorescence data trace by eq 11 and 15, we have estimated the parameters for given traces for each individual channel and subsequently constructed histograms of the parameter values to obtain statistics for those values. For purposes of illustration, we have arbitrarily chosen three individual fluorescence decays from total-count data sets for a 50:50 mixture for 200, 6000, and 20000 total counts. (Experiments for all the mixtures for all the total counts numbers were performed, and a large selection of the results are presented in the Supporting Information.) Finally, for comparison, the data were also analyzed with the proprietary SPCEImage software v. 4.9.7 (SPCl), provided by Becker & Hickl GmbH.

**RESULTS AND DISCUSSION**

**Complete Fluorescence Decay Analyses.** Each of the fluorescence decays was analyzed by the RM-Pearson (eq 8), RM-Neyman (eq 9), ML (eq 22), binomial (eq 12), and the Poisson (eq 16) methods. For purposes of comparison, the
commercial software (SPCI) was also used. Figure 2 presents the sample decay traces for Rb:RhB 50:50 along with the fit obtained with the ML method. Histograms of the lifetime parameters ($\tau_1$, $\tau_2$, and $a_1$) for the 50:50 mixture obtained using all the methods are given in Figure 3a–c. The vertical dotted dark gray line in each panel represents the target value for the parameter. The results of the mean and the standard deviation for $\tau_1$, $\tau_2$, and $a_1$ computed from the different methods are summarized in Table 4–6, respectively for the 50:50 mixture. Table 4–6 present a concise summary of the results for all of the mixtures for all of the techniques employed at which a minimum number of total counts provided mean values within $\sim$10% of the target values with standard deviations of $\sim$20% of the target value. These results indicate that the probability-based methods (ML, Poisson and binomial) are very effective in recovering the target fluorescence decay parameters. These three methods yield very similar results (indeed, identical through the second or third decimal place), as might be expected, given their similarity. A few salient points can be noted. When data for the mixtures are analyzed using the probability-based methods, the lower limit of the number of total counts where one retrieves the target mean with $\sim$20% standard deviation is higher than that of pure compound (for which the total standard deviation is about 20) in general. For the lifetime of rose bengal ($\tau_1$), the mean target lifetime can be retrieved to less than 20% of standard deviation with a total number of counts as low as 6000 in the case of the 50:50 mixture. For the lifetime of rhodamine B ($\tau_2$), the mean target lifetime can be retrieved to about 20% of the standard deviation with only 100 total counts for the same mixture. The amplitude of the rose bengal lifetime ($a_1$) can be obtained with the same degree of precision with only 1000 total counts for the same mixture. The minimum number of total counts required to estimate the lifetime of rose bengal increases as the fraction of rhodamine B increases. For example, to retrieve the target lifetime of rose bengal ($\tau_1$) with a standard deviation of $\sim$20% SD even with 20000 counts, a result is nevertheless still reported.

Table 4. Rose Bengal Lifetime ($\tau_1$)$^a$

<table>
<thead>
<tr>
<th>sets</th>
<th>ML</th>
<th></th>
<th></th>
<th>RM-Pearson</th>
<th></th>
<th></th>
<th></th>
<th>RM-Neyman</th>
<th></th>
<th></th>
<th></th>
<th>SPCI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 100:0</td>
<td>0.5 ± 0.1</td>
<td>20</td>
<td>0.54 ± 0.02</td>
<td>6000</td>
<td>0.53 ± 0.06</td>
<td>500</td>
<td>0.48 ± 0.04</td>
<td>500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 75:25</td>
<td>0.5 ± 0.1</td>
<td>1000</td>
<td>0.53 ± 0.03</td>
<td>20000</td>
<td>0.49 ± 0.03</td>
<td>20000</td>
<td>0.52 ± 0.06</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 50:50</td>
<td>0.5 ± 0.1</td>
<td>6000</td>
<td>0.6 ± 0.1</td>
<td>20000</td>
<td>0.47 ± 0.05</td>
<td>20000</td>
<td>0.9 ± 0.1</td>
<td>20000</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 25:75</td>
<td>0.5 ± 0.1</td>
<td>10000</td>
<td>1.0 ± 0.3</td>
<td>20000</td>
<td>0.5 ± 0.3</td>
<td>20000</td>
<td>1.9 ± 0.1</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 0:100</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The total number of counts required for a given method to obtain a mean value within $\sim$10% of the target value ($\tau_1 = 0.49$ ns) with a standard deviation of $\sim$20%. In those cases where the results are not within $\sim$10% of the mean with $\sim$20% SD even with 20000 counts, a result is nevertheless still reported.

Table 5. Rhodamine B Lifetime ($\tau_2$)$^a$

<table>
<thead>
<tr>
<th>sets</th>
<th>ML</th>
<th></th>
<th></th>
<th>RM-Pearson</th>
<th></th>
<th></th>
<th></th>
<th>RM-Neyman</th>
<th></th>
<th></th>
<th></th>
<th>SPCI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td>lifetime (ns)</td>
<td>min total counts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 100:0</td>
<td>2.5 ± 0.5</td>
<td>100</td>
<td>2.61 ± 0.04</td>
<td>20000</td>
<td>2.4 ± 0.1</td>
<td>10000</td>
<td>2.4 ± 0.2</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 75:25</td>
<td>2.6 ± 0.5</td>
<td>100</td>
<td>2.61 ± 0.06</td>
<td>20000</td>
<td>2.7 ± 0.5</td>
<td>10000</td>
<td>2.45 ± 0.08</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 50:50</td>
<td>2.7 ± 0.5</td>
<td>100</td>
<td>2.8 ± 0.1</td>
<td>20000</td>
<td>2.36 ± 0.09</td>
<td>20000</td>
<td>2.9 ± 0.1</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 25:75</td>
<td>2.4 ± 0.5</td>
<td>20</td>
<td>2.74 ± 0.03</td>
<td>6000</td>
<td>2.48 ± 0.09</td>
<td>3000</td>
<td>2.4 ± 0.5</td>
<td>10000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 0:100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The number of total counts required for a given method to obtain a mean value within $\sim$10% of the target value ($\tau_2 = 2.45$ ns) with a standard deviation of $\sim$20%.

Table 6. Amplitude of the Rose Bengal Contribution to the Fluorescence Decay ($a_1$)$^a$

<table>
<thead>
<tr>
<th>sets</th>
<th>ML</th>
<th></th>
<th></th>
<th>RM-Pearson</th>
<th></th>
<th></th>
<th></th>
<th>RM-Neyman</th>
<th></th>
<th></th>
<th></th>
<th>SPCI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fraction of $\tau_1$</td>
<td>min total counts</td>
<td>fraction of $\tau_1$</td>
<td>min total counts</td>
<td>fraction of $\tau_1$</td>
<td>min total counts</td>
<td>fraction of $\tau_1$</td>
<td>min total counts</td>
<td>fraction of $\tau_1$</td>
<td>min total counts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 100:0</td>
<td>0.7 ± 0.1</td>
<td>200</td>
<td>0.75 ± 0.01</td>
<td>10000</td>
<td>0.72 ± 0.03</td>
<td>10000</td>
<td>0.70 ± 0.03</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 75:25</td>
<td>0.49 ± 0.09</td>
<td>1000</td>
<td>0.50 ± 0.02</td>
<td>20000</td>
<td>0.42 ± 0.02</td>
<td>20000</td>
<td>0.42 ± 0.05</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb-RhB 50:50</td>
<td>0.23 ± 0.04</td>
<td>10000</td>
<td>0.38 ± 0.08</td>
<td>20000</td>
<td>0.23 ± 0.08</td>
<td>20000</td>
<td>0.65 ± 0.07</td>
<td>20000</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Rb-RhB 25:75</td>
<td>0.22 ± 0.04</td>
<td>10000</td>
<td>0.38 ± 0.08</td>
<td>20000</td>
<td>0.23 ± 0.08</td>
<td>20000</td>
<td>0.65 ± 0.07</td>
<td>20000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The total number of counts required for a given method to obtain a mean value within $\sim$10% of the target value ($a_1 = 0.68, 0.44, and 0.22$ for Rb:RhB 75:25, Rb:RhB 50:50, and Rb:RhB 25:75, respectively) with a standard deviation of $\sim$20%. DOI: 10.1021/acs.jpca.6b10728 J. Phys. Chem. A 2017, 121, 122–132
of 20000 total counts in the case of mixtures. The extent to which this shortening occurs depends roughly on the concentration of the other component. This observation has been confirmed from an independent experiment where the decay traces are collected to the highest quality supported by the memory.

With regard to the relative merits of the techniques, the residual minimization methods (RM-Pearson and RM-Neyman) proved to be markedly inferior to the ML and probability-based methods in retrieving the fluorescence lifetime parameters (Figures 3 and Tables 1–3). In this context, we also note that the commercial software (SPCI), which is also based on a residual minimization method, has its own peculiarities. Some of these are summarized here. Except for the pure rose bengal data sets, one needs at least 500 total counts for the software even to initiate the analysis. In the case of pure rose bengal, one needs at least 200 total counts. In almost all cases, SPCI retrieves significantly different target values with larger standard deviations than all of the other methods, especially for mixtures where the total number of counts is less than 20000 (Tables 1–3). And even with 20000 total counts for the 50:50 mixture, SPCI grossly overestimates the lifetime of rose bengal as 0.9 ns. Because SPCI is propriety, we are unable to obtain the source code to discern the origins of this behavior.

**Bin-by-Bin Analyses of a Single Fluorescence Decay Trace To Yield Statistics.** As noted above, the probability distribution for the number of photon counts in each individual bin can be obtained using the binomial (eq 11) and the Poisson (eq 15) probability distributions. This property permits the analysis of a single fluorescence decay trace, bin-by-bin, and of constructing frequency histograms of the various fluorescence parameters.

![Figure 4](https://i.imgur.com/3Q5Q5Q5.png)

Figure 4. Histograms of the frequencies of obtaining values of the fluorescence decay parameters for $\tau_1$, $\tau_2$, and $a_1$ presented in (a), (b), and (c), respectively. The histograms are obtained from a bin-by-bin analysis using the Poisson distribution of a representative, single fluorescence decay trace from a 50:50 mixture of Rb and RhB with total counts of 200, 6000, and 20000. The histograms are fit to Gaussians using the values of the mean and standard deviation obtained from them.
decay parameters. From the histograms, the mean, median, and standard deviations of the parameters can be obtained. To demonstrate this, we have arbitrarily chosen three individual fluorescence decay traces from the sets of experiments with total counts 200, 6000, and 20000, respectively. Each trace has been analyzed by using the Poisson and the binomial methods, which have been applied to all five Rb:RhB mixtures examined (Supporting Information). For purposes of illustration, the histograms obtained using the Poisson distribution method are presented in Figure 4 for the Rb:RhB 50:50 mixture. A normalized Gaussian line (red) has been overlaid in each histogram using the calculated mean and standard derivation of \( \tau_i, \tau_o, \) or \( a_i \). As one might expect, the distribution becomes narrower and more well-defined as we progress from 200 to 20000 total counts.

■ CONCLUSIONS

We have presented a detailed comparison of probability-based methods (ML, binomial and the Poisson) with residual minimization-based methods (RM-Pearson, RM-Neyman, and SPCI) to retrieve the fluorescence decay parameters for various two-component mixtures in time-correlated, single-photon counting experiments. The maximum likelihood (ML) proved to be the most robust way to retrieve the target parameters. All the probability-based methods, however, have performed equivalently to 2–3 significant figures. This is to be expected, as the three methods are all fundamentally related. ML consistently outperforms the RM methods. In some cases, RM-based methods did not converge to the expected values for a given number of total counts. RM-Pearson tends to overestimate parameters whereas RM-Neyman tends to underestimate them, both giving larger standard deviations than ML. We have discussed a bin-by-bin analysis of a single fluorescence decay trace and have shown that it is possible to retrieve not only their mean and median values but also the associated standard deviations by constructing frequency histograms from the analysis of the fluorescence decay at each bin. In conclusion, the ML technique or a bin-by-bin analysis provide robust methods (insensitive to initial conditions) of analyzing time-correlated, single-photon counting data for sparse data sets, and in the case of bin-by-bin analysis, providing statistics from one fluorescence decay. These methods lend themselves well to the sparse data sets that can be encountered in subdiffraction-limited microscopies, such as STED.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpca.6b0728.

Notes

The authors declare no competing financial interest.

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