

Error Analysis of the Rapid Lifetime Determination Method for Double-Exponential Decays and New Windowing Schemes

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The rapid lifetime determination method (RLD) is a mathematical technique for extremely rapid evaluations of lifetimes in exponential decays. It has been applied in luminescence microscopy and single-molecule lifetime evaluation. To date, the primary application has been in single-exponential evaluations. We present extensions of the method to double exponentials. Using Monte Carlo simulations, we assess the performance of both the double-exponential decay with known lifetimes and the double-exponential decay with unknown preexponential factors and lifetimes. Precision is evaluated as a function of the noise level (Poisson statistics), the ratios of the lifetimes, the ratios of their preexponential factors, and the fitting window. Optimum measurement conditions are determined. RLD is shown to work well over a wide range of practical experimental conditions. If the lifetimes are known, the preexponential factors can be determined with good precision even at low total counts (10^4). With unknown preexponential factors and lifetimes, precisions decrease but are still acceptable. A new gating scheme (overlapped gating) is shown to offer improved precision for the case of a single-exponential decay. Theoretical predictions are tested against actual experimental data from a laser-based lifetime instrument.

The rapid lifetime determination (RLD) method is a family of data analysis equations for fitting experimental data that conforms to single- and multiple-exponential decays with or without a baseline contribution.^{1–3} Rather than recording a complete mul-

tipoint decay curve and analyzing the decay by the traditional least-squares methods, the areas under different regions of the decay are used to calculate the decay parameters. The areas over different time intervals of width Δt are obtained by accumulating photon intensity (e.g., gated integrator or gated CCD camera). CCD cameras are particularly suited to the RLD method since they automatically integrate the data over different time intervals. Further, they do not lend themselves readily to recording of multipoint decay curves. Alternatively, the integrals can be computed directly from a multipoint-digitized decay by summing the digitized signal over each interval in software. RLD is much faster than traditional approaches for recording and analyzing decays and does not have problems such as false minimums.

Optimum conditions were determined previously for the RLD method of single-component decays and for single-component decays plus a nonzero baseline using Monte Carlo simulations.³ However, the important case of the double-exponential decay has not been addressed. In addition, we wished to develop more precise methods for the single-exponential case.

To determine for the RLD method the optimum acquisition conditions with differing system parameters, a range of values for each variable must be considered. This is experimentally difficult because of the absence of reliable standard mixtures with different lifetimes and contributions. In addition, such studies would be extremely time-consuming and tedious. To avoid the experimental difficulties, we used theoretical (Monte Carlo) simulations to test a broad range of experimental conditions with different RLD methods.

In the current work, we have developed new integration (gating) schemes and applied them to single- and double-exponential problems. For the double-exponential systems, we examined both the case where the two lifetimes (τ_1 , τ_2) are known, but the preexponential factors (k_1 , k_2) are unknown, and the

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general unconstrained case where the preexponential factors (k_1 , k_2) and lifetimes (τ_1 , τ_2) are unknown. The first case is a common problem where the analyte lifetimes are known and one is determining the relative amounts of the two components, while the second situation occurs where one knows nothing about the components.

EXPERIMENTAL SECTION

Methodology for Single- and Double-Exponential Simulations. Monte Carlo simulations were used to judge the precision and accuracy of the data reduction methods. The parameter values selected to generate data corresponded to expected experimental values. For each set of selected decay parameters, a noise-free data set was generated from the model equation. Noise obeying Poisson statistics was added to the decays, which were then reduced by the appropriate algorithm. This process was repeated a number of times with different noise sets to determine the statistics for the fitting algorithm. The parameters from the reduction were compared with initial parameters used to generate the noise-free data. The calculations were then repeated with different sets of parameters for the modeling equation. Thus, the theoretical calculations allowed testing of the extremes over which the RLD will provide reliable results and direct the design of experiments to achieve a desired accuracy.

Poisson noise was selected since it corresponds to noise that is frequently experimentally encountered (e.g., time-correlated single-photon-counting instrument).⁴ Even analog instruments based on photon detectors can have noise distributions that follow amplitude dependencies similar to Poisson statistics. In all cases, we base our calculation on the total number of photons that would be detected during the entire sample decay from zero time to infinity.

We choose not to use error propagation for two reasons. First, it becomes exceptionally complex and cumbersome for the double-exponential case. Second, it is based on infinitesimal errors, which can distort results for the larger errors present in our simulations. Thus, while the plots are noisier, they are more reliable.

All Monte Carlo simulations were performed using Mathcad 7.0 (Waterloo Scientific, Toronto, Canada, 1997). Typically, 100 simulations were performed at each set of conditions to determine the uncertainty in the parameters. The built-in random number generator gives no repeats over $>10^7$ trials, which far exceeds the number involved in our calculations, and it gives a good Gaussian distribution for hundreds of trials. After scaling each decay to give a total area equal to the specified number of photons, noise was added using a Poisson noise generator. Results are displayed as contour plots of fractional percent standard deviation $100 \sigma_x/\bar{x}$, where σ_x is the standard deviation and \bar{x} is the mean of the parameters determined from all simulations.

Equations were derived by analytically evaluating the areas under the different portions of the decay curves. The decay parameters were determined by solving the system of equations. For the overlapping cases, we were only able to get closed form solutions for specific overlaps (0%, 25%, 50%, 75%). However, a solution for any arbitrary overlap eluded us.

Single-Exponential Decays. Noise-free single-exponential decays were generated from

$$I(t) = k \exp(-t/\tau) \quad (1)$$

where k is the preexponential factor, τ is the sample lifetime, and t is time. In the RLD methods, one determines the areas under different regions of the decay and, from appropriate equations (vide infra), calculates k and τ . For the single-exponential decay, only two areas are calculated since there are only two unknowns. The integrated areas D_0 and D_1 are shown in Figure 1a for equal gating and in Figure 1b for the 50% overlapped gating. Obviously there is a continuum of possible displacements. The total counts would equal $k\tau$.

For real data, the data analysis for the RLD method consists of substituting the integrals D_n in the equations below. The D_n 's are obtained by summing the data that has been acquired at equal time intervals δt . For example,

$$D_0 = \sum_{i=1}^{i=n/2} I_i \delta t \quad (2)$$

where I_i is the data point acquired at the i th time ($i \delta t$) and n is the total number of points acquired. In the case of photon counting, the integrals are simply the total counts recorded in the interval Δt . The equations for τ and k are given below with Δt , the time interval of integration. For the contiguous gating, eqs 3 and 4 are the same as used earlier.² Equations 5 and 6 are for the 50% overlapped gating case.

contiguous gating

$$\tau = \Delta t / \ln(D_0/D_1) \quad (3)$$

$$k = D_0^2 \ln(D_0/D_1) / [(D_0 - D_1)\Delta t] \quad (4)$$

overlapped gating

$$\tau = -\Delta t / \ln(D_1^2/D_0^2) \quad (5)$$

$$k = 2D_0^3 \ln(D_1/D_0) / [(D_1^2 - D_0^2)\Delta t] \quad (6)$$

As a check of our Mathcad program, the same experimental parameters were chosen as in the earlier work.² Calculations agreed with the earlier results.

Double-Exponential Decay. Double-exponential decays were computed from

$$I(t) = k_1 \exp(-t/\tau_1) + k_2 \exp(-t/\tau_2) \quad (7)$$

τ_1 will be taken as the shorter lifetime.

There are two different practical applications of the double-exponential decay case: the double-exponential decay (1) with known lifetimes and (2) with no known lifetimes. In both cases, the total number of counts would be equivalent to $k_1\tau_1 + k_2\tau_2$.

Double-Exponential with Known Lifetimes. For the double-exponential case with known lifetimes, there are two required areas since there are only two unknowns, k_1 and k_2 . The k 's are given below by eqs 10 and 11 for contiguous integration integrals and by eqs 12 and 13 for the 50% overlapping integration regions. The integration regions are shown in Figure 1. E_1 and E_2 are constants.

(4) Demas, J. N. *Excited-State Lifetime Measurements*; Academic Press: New York, 1983; p 83.

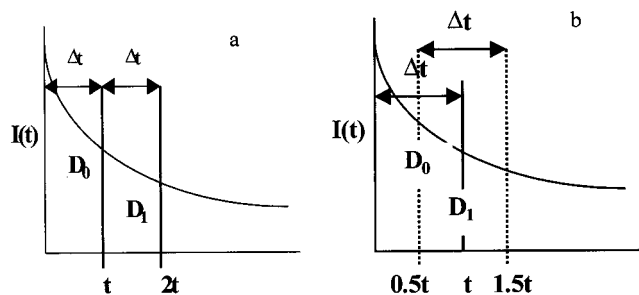


Figure 1. Graph of integrated areas used for the single-exponential decay with contiguous gates (a) and overlapping gates (b).

$$E_1 = \exp(-\Delta t/\tau_1) \quad (8)$$

$$E_2 = \exp(-\Delta t/\tau_2) \quad (9)$$

contiguous gating

$$k_1 = (D_1 - E_2 D_0) \tau_1 / (-E_1 + E_2 E_1 + E_1 - E_1^2) \quad (10)$$

$$k_2 = (D_1 + D_0 E_1) \tau_2 / ((E_1 - E_2)(-1 + E_2)) \quad (11)$$

overlapped gating

$$k_2 = [(D_0 \sqrt{E_1} - D_1) \tau_2] / ((-\sqrt{E_1} + \sqrt{E_2})(-1 + E_2)) \quad (12)$$

$$k_1 = [(-D_1 + \sqrt{E_2} D_0) \tau_1] / (\sqrt{E_1} - (E_1)^{3/2} - \sqrt{E_2} + \sqrt{E_2} E_1) \quad (13)$$

The fractional contributions of the preexponential factors are generally the quantities of interest rather than the absolute values of the k 's. The fractional contributions, f_1 and f_2 are defined:

$$f_1 = k_1 / (k_1 + k_2) \quad (14)$$

$$f_2 = k_2 / (k_1 + k_2) \quad (15)$$

To visualize the interactions of the numerous parameters, we fixed the total counts as well as k_1/k_2 and then varied $\Delta t/\tau_1$ and τ_2/τ_1 to generate an error contour map. For example, τ_2/τ_1 ranged from 1.1 to 5 and $\Delta t/\tau_1$ ranged from 0.2 to 5. This procedure was repeated using several different values for the ratio of preexponential factors and total photons detected. This allowed us to determine best values of $\Delta t/\tau_1$ for different τ_2/τ_1 .

Double-Exponential Decay with Unknown k_1 , k_2 , τ_1 , and τ_2 . Four areas under the curve are required for the unconstrained double-exponential decay since there are four unknowns as seen in Figure 2. The equations are shown below. Equations 22–25 are for the contiguous case and eqs 26–29 are for the 50% overlapping case. O , P , Q , x , y , and $DISC$ are intermediate calculations.

$$R = D_1 D_1 - D_2 D_0 \quad (16)$$

$$P = D_1 D_2 + D_3 D_0 \quad (17)$$

$$Q = D_2 D_2 - D_3 D_1 \quad (18)$$

$$DISC = PP - 4RQ \quad (19)$$

$$x = (-P - \sqrt{DISC}) / (2R) \quad (20)$$

$$y = (-P + \sqrt{DISC}) / (2R) \quad (21)$$

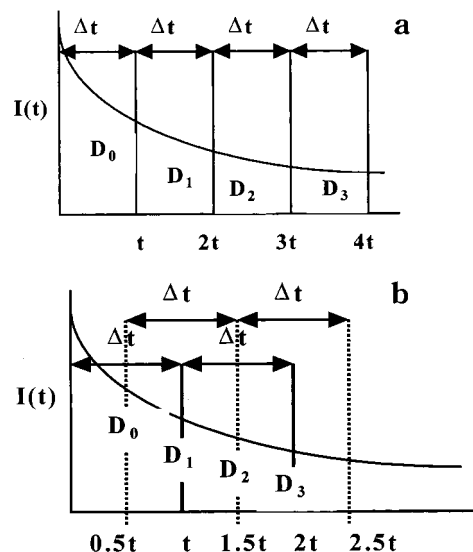


Figure 2. Graph of integrated areas used for the unconstrained double-exponential decay with contiguous gates (a) and overlapping gates (b).

contiguous gating

$$\tau_1 = -\Delta t / \ln(y) \quad (22)$$

$$\tau_2 = -\Delta t / \ln(x) \quad (23)$$

$$k_1 = (xD_0 - D_1)^3 \ln(y) /$$

$$[(xD_0 - xD_1 + D_2 - D_1)(D_0 x^2 - 2xD_1 + D_2) \Delta t] \quad (24)$$

$$k_2 = -R(\ln(yD_1 - D_2) - \ln(D_0 y - D_1)) /$$

$$[(-1 + x)(D_0 x^2 - 2xD_1 + D_2) \Delta t] \quad (25)$$

overlapped gating

$$\tau_1 = -\Delta t / \ln(y^2) \quad (26)$$

$$\tau_2 = -\Delta t / \ln(x^2) \quad (27)$$

$$k_1 = -2(xD_0 - D_1)^4 \ln(y) / [(xD_1 - D_2 + D_0 x - D_1) \times (D_1 x + D_2 + D_0 x - D_1)(D_0 x^2 - 2xD_1 + D_2) \Delta t] \quad (28)$$

$$k_2 = -2R \ln(x) / [(D_0 x^2 - 2xD_1 + D_2)(x^2 - 1) \Delta t] \quad (29)$$

The percent relative standard deviations for all four parameters were calculated as a function of τ_2/τ_1 , $\Delta t/\tau_1$, k_1/k_2 , and the total photons detected. Again, not all four variables could be varied at once, but by assigning two variables a given number and then varying two other variables over a range of experimentally realistic values, the optimum conditions for measuring different parameters could be determined. The same range of parameters was used for this case as was used in the double-exponential decay with known parameters.

Lifetime data were collected for $\text{Ru}(\text{bpy})_3^{2+}$ ($\text{bpy} = 2,2'$ -bipyridine) using a laser-based instrument to compare the simulations with real data. To test several different lifetimes, the lifetime of $\text{Ru}(\text{bpy})_3^{2+}$ was varied by bubbling it in nitrogen, air, and oxygen, which gave lifetimes of 552, 382, and 164 ns, respectively. For each case, four or more different decays were collected as close together in time as possible to ensure the most uniform data set. The measured lifetimes were calculated by a nonlinear

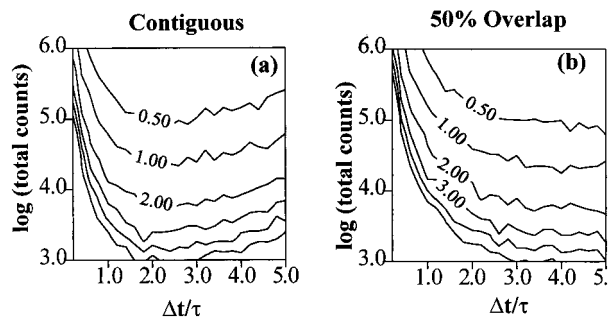


Figure 3. Percent relative standard deviation of τ for the single-exponential decay with contiguous gates (a) and overlapping gates (b) using Monte Carlo Simulations.

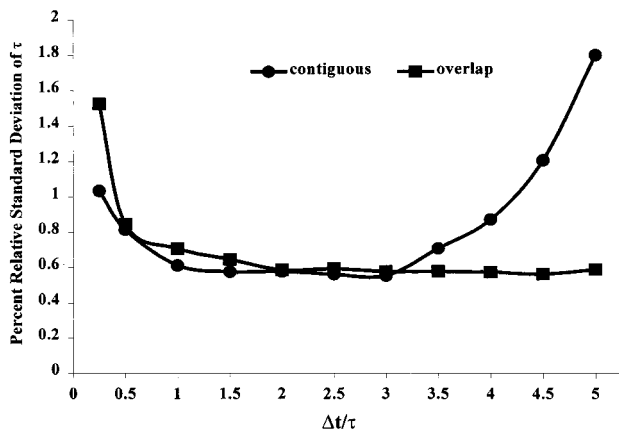


Figure 4. $\text{Ru}(\text{bpy})_3^{2+}$ decay results for single-exponential decay comparing contiguous gates to overlapped gates using the RLD method (nitrogen results).

least-squares method, and the largest relative standard deviation was 0.64%. The averages of all measurements were taken as the actual lifetimes for comparison with the RLD results.

RESULTS AND DISCUSSION

Single-Exponential Decay. Figure 3 is a result of the Monte Carlo simulations for the overlapping (50%) and contiguous gated cases. For $\Delta t/\tau$ less than 2, the contiguous RLD is better than the overlapping case. At high values of $\Delta t/\tau$ (>3), the overlapping RLD is superior and shows continual improvement with increasing $\Delta t/\tau$ even at $\Delta t/\tau = 5$. In the optimum region ($\Delta t/\tau = 2-3$), both methods are comparable; however, in the optimum region, the overlapping RLD can actually use less of the decay curve than the contiguous RLD method. This minimizes the need for data on the less reliable tail of the decay.

Figure 4 shows the results of reducing single-exponential $\text{Ru}(\text{bpy})_3^{2+}$ decays for both the overlapping and contiguous cases. For very small $\Delta t/\tau$, the contiguous gates give smaller relative standard deviation, but the 50% overlapping case has good precision over a much wider range of $\Delta t/\tau$. These experimental results are consistent with the Monte Carlo simulations (Figure 3). Thus, the overlapping case should be used if little is known about the system. The RLD precision for τ (0.6%–1.2%) is comparable to, or only slightly less than, that for nonlinear least-squares fitting (0.6%).

Double-Exponential Decay with Known Lifetimes. Using the Monte Carlo simulations, the percent relative standard deviations for f_1 and f_2 were calculated as functions of τ_2/τ_1 , $\Delta t/\tau_1$

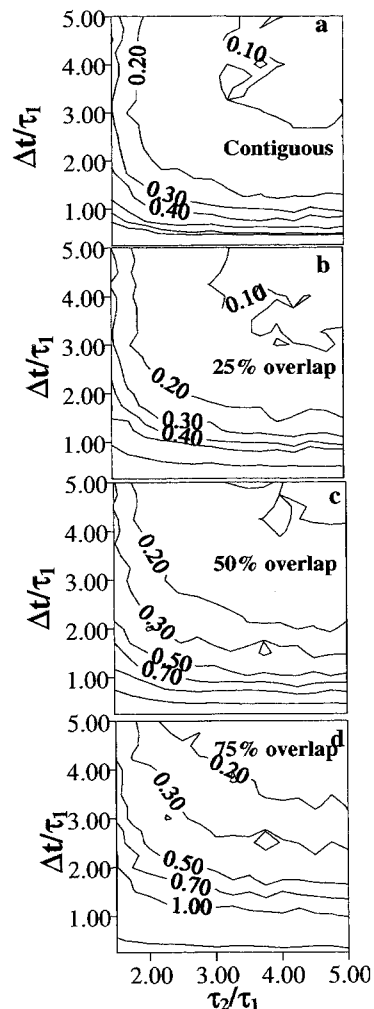


Figure 5. Percent relative standard deviation of f_1 for the double-exponential decay with known lifetimes: (a) contiguous, (b) 25% overlapping, (c) 50% overlapping, and (d) 75% overlapping ($k_1/k_2 = 5$; counts, 10^6).

τ_1 , k_1/k_2 , and the total photons detected. We first consider the question of the optimum gating scheme. Graphs a–d in Figure 5 represent the percent relative standard deviation of f_1 with gate overlaps varying from 0% to 75%. For each graph, k_1/k_2 equals 5, $k_1\tau_1 + k_2\tau_2$ equals 10^6 , $\Delta t/\tau_1$ ranged from 0.25 to 5, and τ_2/τ_1 ranged from 1.5 to 5. The contiguous gating and 25% overlap cases are indistinguishable, but for the 50% and 75% overlap cases, precision clearly decreases. This is also true for other values of k_1/k_2 . However, even at low total counts (10^4), the percent relative standard deviation in the optimum regions is less than 5% for contiguous gating and less than 7% for 50% overlap gating. The shapes of the error curves are similar for the different total counts, but the precision decreases as the total counts decrease. For example, at 10^6 counts the optimum region has a relative standard deviation of 0.55% for $k_1/k_2 = 1$ and at 10^4 the optimum region for the same k_1/k_2 is 5.5%. The optimum region for f_2 is equivalent to the optimum region of f_1 .

It is of interest to determine how the precision varies as a function of the total number of counts and k_1/k_2 . For a given k_1/k_2 , we find that standard deviation in both f_1 and f_2 is inversely proportional to the square root of the total counts. Figure 6 is a plot of percent relative standard deviation for total counts ranging

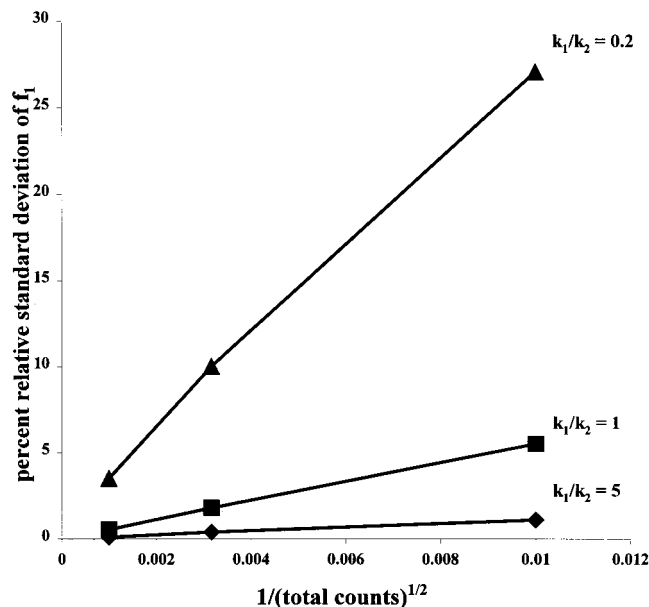


Figure 6. Percent relative standard deviation of the fractional contribution of k_1 for double-exponential decay for differing values of k_1/k_2 and total counts.

from 10^4 to 10^6 and k_1/k_2 with values of 5, 1, or 0.2. At the higher counts, the precision is excellent. For example, with $k_1/k_2 = 5$, the percent relative standard deviation in f_1 is 0.1% at 10^6 counts and 1.1% at the low 10^4 total counts. The inverse square root dependence is reasonable since it corresponds to the relative noise level in the data. This observation allows one to estimate the noise level for varying total counts and k_1/k_2 's. f_2 obeys a similar count dependence.

The relative precision in f_1 and f_2 depends on their contributions. The larger the fractional contribution the better the precision. For example, the precision in f_1 is roughly linearly dependent on $1/(k_1/k_2)$ at all total counts tested while the precision in f_2 is linearly dependent on k_1/k_2 .

Double-Exponential Decay with No Known Parameters.

Graphs a–h in Figure 7 represent the percent relative standard deviation from a representative set of Monte Carlo simulations. Because the number of parameters is greater, results for the fitted parameters of the unconstrained double-exponential decay were less precise than for the double decay with known lifetimes. However, the precision of the RLD method is quite acceptable for a variety of measurements at reasonable photon levels. The precision of each parameter is shown for both contiguous and 50% overlapped gating with $k_1/k_2 = 5$ and 10^6 total photons. Comparing the best regions for each parameter, the overlapping RLD has comparable or superior performance to the contiguous RLD method. In addition, the regions of comparable error tend to be larger for the overlapping method than for the contiguous RLD. Thus, the overlapping RLD places less stringent conditions on the measurement conditions. If there is interest in a specific parameter, these curves can be used to select the optimum region for measuring that parameter.

Frequently all four parameters are of interest. To judge the optimum conditions for simultaneously measuring all parameters, we calculated an error surface for the average error for all parameters. This was done by pointwise averaging all of the data of the four parameters in Figure 7 with each of the two methods.

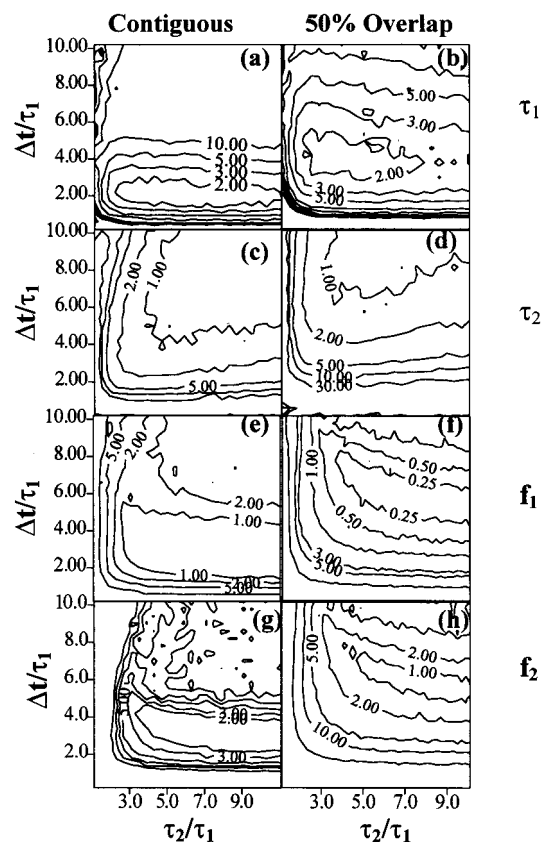


Figure 7. Percent relative standard deviation for the unconstrained double-exponential decay of (a, b) τ_1 , (c, d) τ_2 , (e, f) f_1 , and (g, h) f_2 . The left column represents simulations for the contiguous case and the right column the overlapping case.

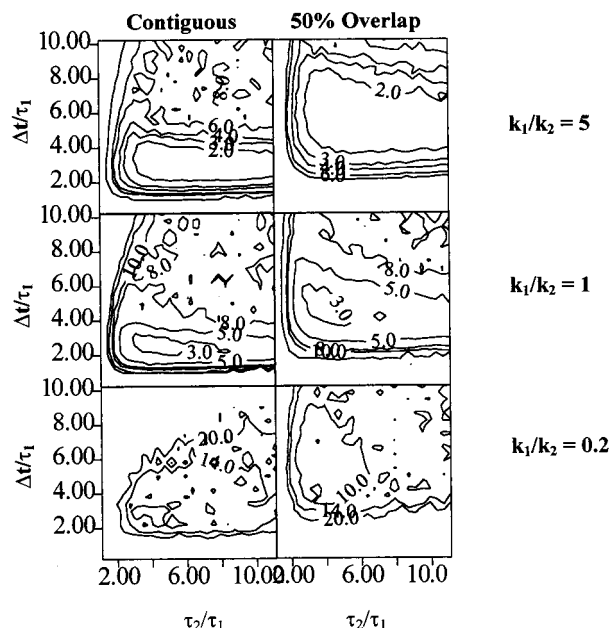


Figure 8. Percent relative standard deviation for unconstrained double-exponential decay for all parameters, comparing the contiguous gates (left column) with the overlapping gates (right column) for differing values of k_1/k_2 (counts, 10^6).

The result is shown in Figure 8 for several values of k_1/k_2 . While in the optimum regions, both contiguous and overlapping RLD yield comparable precisions, the 50% overlapped gating has a much

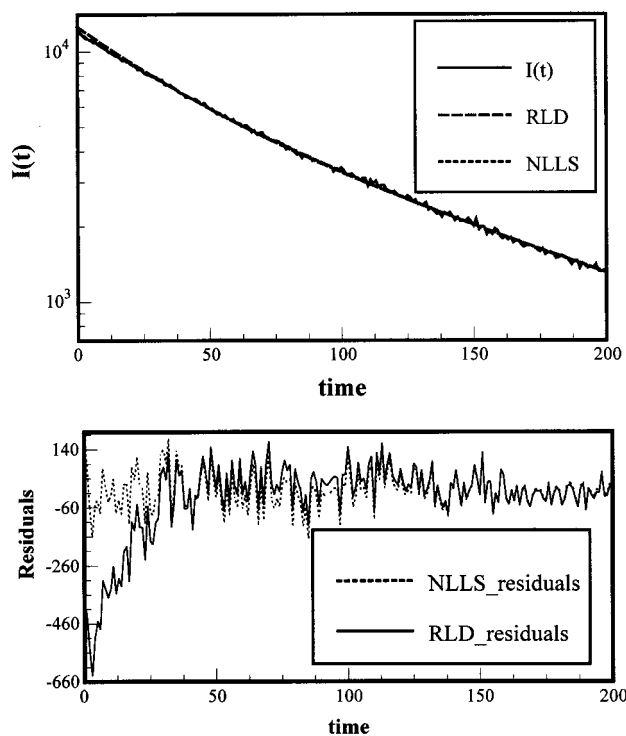


Figure 9. Plots for nonlinear least-squares method and unconstrained RLD for a synthetic double-exponential decay. Details in text.

larger high precision valley. Therefore, the overlap RLD will be much more forgiving about the selection of acquisition parameters.

As one would expect, precision suffers badly as the two lifetimes approach each other. To obtain reasonable overall precisions, τ_2/τ_1 should be at least 2 and 3–4 would be best. For larger τ_2/τ_1 's, little additional precision is gained. For $\Delta t/\tau_1 > 2$, the precision reaches a minimum and is best for $k_1/k_2 > 1$.

Of course, least-squares methods will always give better fits to complete decay curves than those generated by RLD using a few integration regions. We demonstrated this by fitting a 512-point synthetic double-exponential decay curve with nonlinear least squares and by the unconstrained double-exponential RLD. The total counts for the decay were 10^6 , $k_2 = k_1 = 6000$, and $\tau_2/\tau_1 = 3$ with $\tau_1 = 42.5$. Poisson noise was added to the decay before fitting. We used $t = 0-511$. The k 's and τ 's were 6160, 5833, 43.8, and 129 by least squares and 6332, 6218, 38.2, and 127 by RLD. Plots of the best fits calculated by both methods are essentially indistinguishable except at the earliest times (Figure 9). When the computational times were compared, it was found that the RLD was submillisecond and the nonlinear least squares required several seconds with PSIPLOT. The differences can be better revealed by the residuals plots, which are also shown in Figure 9. As expected, least squares gives a better fit, but the RLD fit is comparable while being based on vastly fewer points and simpler calculations.

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CONCLUSIONS

We have evaluated the precision of the RLD method for single-exponential decays utilizing a new windowing (overlapping gates) scheme and double-exponential decays with both known lifetimes and unknown preexponentials and lifetimes as a function of different measurement conditions and decay parameters (e.g., total counts, k_1/k_2 , $\Delta t/\tau_1$, τ_2/τ_1 , gating schemes).

For a single-exponential decay, the new overlapping windowing scheme gives precision that is comparable to the earlier contiguous windowing. However, overlapping gating is more forgiving of different measurement conditions, which is a major advantage when an unknown system is measured (e.g., chromatography).

For the double-exponential decay with known lifetimes, the precision of the fractional contributions is excellent at higher total counts and usable even at counts as low as 10^4 . For the simulations and Poisson statistics, contiguous gating gave better results than overlapping gates. Precision can easily be estimated since it depends simply on the total number of counts and k_2/k_1 .

For the double-exponential decay with unknown k_1 , k_2 , τ_2 , and τ_1 , the 50% overlapping gate is superior. In the optimum regions, precision is comparable between the overlap and contiguous gating. However, the optimum areas are larger for overlapping gating, either for any individual parameter or for all parameters taken as a whole.

Our results show that the new gating scheme for single-exponential and double-exponential decays provides a simple and extremely rapid method of lifetime evaluation. While not as precise as the least-squares method, precision is still very good, and not all instrumentation lends itself to recording the complete multi-point decay curves required of least-squares methods (e.g., CCD cameras). Another disadvantage of RLD is that it gives no warning of more complex decays.² It should be applied to systems that have already been fully characterized as to their kinetics. We are currently experimentally applying the method to double exponential systems.

The ultimate goal of this research is to use the RLD method to assess the contributions of the lifetime and the preexponential factors of double-exponential decays in biological systems and polymer-supported sensors using luminescence microscopy. Lifetime measurements would allow the quantitative determination of intracellular factors such as $[H^+]$, $[Ca^{2+}]$, $[O_2]$, membrane potential, temperature, polarity of the probe environment, and alterations in the conformation and interactions of macromolecules.^{5–7}

ACKNOWLEDGMENT

The authors thank the National Science Foundation for their support (REU Grant CHE 93-22225 and Grants CHE 94-19074, CHE 97-26999, and DBI-9604709) and an award from the Academic Enhancement Program of the University of Virginia.

Received for review September 22, 1998. Accepted December 9, 1998.

AC981050D