Accurate Single Molecule FRET Efficiency Determination for Surface Immobilized DNA Using Maximum Likelihood Calculated Lifetimes

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Received: October 4, 2006; In Final Form: January 12, 2007

Single molecule fluorescent lifetime trajectories of surface immobilized double-stranded DNA coupled with a tetramethylrhodmaine and Cy5 FRET pair were directly measured using time-tagged single-photon counting and scanning confocal microscopy. A modified maximum likelihood estimator (MLE) was developed to compensate for localized background fluorescence and instrument response. With this algorithm, we were able to robustly extract fluorescent lifetimes from their respective decays with as few as 20 photons. Fluorescent lifetimes extracted using an MLE were found to be highly dependent on background fluorescence. We show that appropriate factors are required to extract true lifetime trajectories from single fluorophores.

Time-resolved detection of single fluorophores using the principles of time-correlated single-photon counting (TCSPC) and confocal scanning microscopy has become increasingly popular in the past few years.[1−6] The fluorescence lifetime of individual dyes is an intrinsic property of the molecule, affected only by its chemical environment. In contrast, the *detected* photon intensity is an extrinsic quantity, which depends on many experimental factors, such as excitation intensity, collection efficiency, and position of the dye with respect to the excitation beam, all of which do not report on the actual dye properties. Consequently, a quantitative comparison between single-molecule (SM) intensity information and bulk measurements is non-trivial. On the other hand, SM lifetime data can be directly compared with bulk (or ensemble-averaged) measurements, and especially in conjunction with SM intensity measurements, it can reveal information hidden or imbedded in SM intensity-based experiments, such as static versus dynamic quenching and molecular heterogeneity.[7]

The most common experimental approach for SM lifetime is performed by observing diffusing single fluorophores through a detection probe volume defined by the confocal volume (commonly known as SM lifetime burst analysis). This technique has the advantage that large SM statistics can be quickly acquired, due to the rapid diffusion of the molecules through the confocal volume. However, it is limited by the relatively short residence time of the molecule within the probe volume, typically no more than ~1 ms, which precludes the detection of processes with slower timescales. Alternatively, SMs can be immobilized on a surface allowing long measurements (tens of seconds, limited only by photobleaching) of individual molecules to be performed. This approach offers an essential dimension for probing biomolecular dynamics on time scales highly relevant for many biomolecular processes.

The fundamental signal in the TCSPC experiment is the time delay between the excitation laser pulse and a single photon emitted by the fluorophore. This signal, however, contains a few artifacts: first, the time delay is convolved with the instrument response function (IRF) of the measuring apparatus. Second, the signal may contain photon contributions from background fluorescence as well as scattering, which contaminate the pure fluorescence lifetime of the probed molecule. A number of numerical methods have been developed to solve these problems. In particular, Maus and co-workers have recently described a method for IRF deconvolution as well as background scattering determination, applied to burst analysis of diffusing molecules. In this paper, we extend these methods for the case of immobilized SM in conjunction with SM fluorescence resonance energy transfer (sm-FRET). We show that similar to the intensity-based sm-FRET measurements[8] the background fluorescence and background scattering may vary from molecule to molecule and therefore need to be treated independently. To this end, we develop a simple numerical approach to perform time-resolved SM lifetime determination, which takes into account scattering and fluorescence background on a per molecule basis. We demonstrate our method by measuring SM time-resolved lifetime from donor−acceptor FRET pairs conjugated to double-stranded DNA (dsDNA), which serves as a rigid scaffold for the dye. We find that our method is extremely robust and can provide reliable lifetime results with as few as 20 photons. Time-resolved single molecule FRET of immobilized molecules using lifetime probing opens the door to accurately measure the dynamics of individual molecules and to probe distances and distributions of nucleic acids and proteins at the single molecule level.[9−12]

Single molecule decays were measured using a high throughput scanning confocal microscope similar to that described by Sabanayagam and co-workers.[8,13] In brief, a modified Zeiss Axiovert 200 microscope integrated with a DC stage and a piezo-driven nanopositioner (Physik Instrumente) was used for all measurements. An 80 MHz femtosecond Ti:Sapphire laser (Tsunami, Spectra Physics) operating at 1000 nm was frequency doubled using a lithium triborate crystal, with the resultant excitation wavelength at 500 nm. The laser was attenuated with a polarizer to reduce the power at the sample to 10 μW, and its polarization was made circular using a quarter wave plate. The

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10.1021/jp066530k CCC: $37.00 © xxxx American Chemical Society
Published on Web 02/27/2007 PAGE EST: 4.6

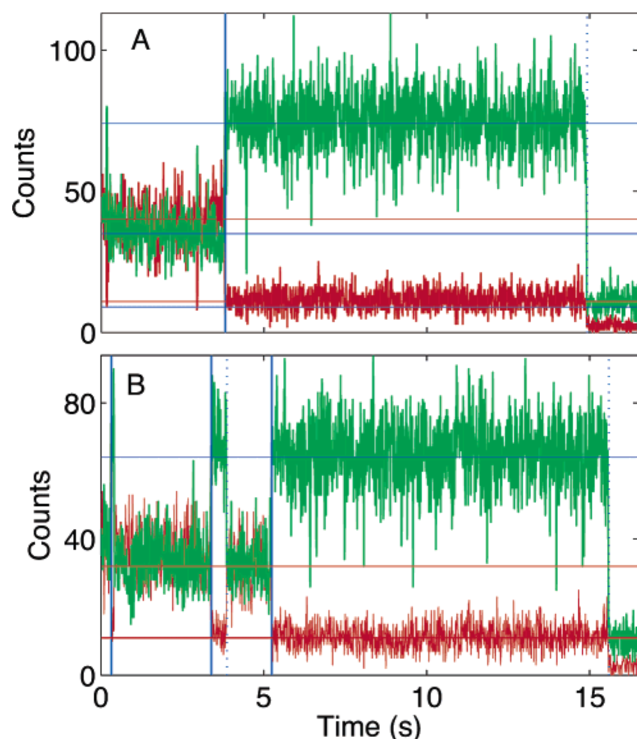

Figure 1. (A and B) Two typical time traces of individual DNA molecules labeled with TMR and Cy5. The fluorophores are separated by 14 nucleotides. The red and green trajectories correspond to Cy5 (acceptor) and TMR (donor), respectively. The transition and average levels are denoted by vertical and horizontal lines, respectively.

beam was expanded to fill the back aperture of the 63× (N.A. 1.45) oil immersion microscope objective producing a diffraction-limited spot at the focal point. Fluorescence from the sample was collected by the same microscope objective, passed through a dichroic beam splitter, and focused onto a 100 μm pinhole using the internal microscope lens. The pinhole image was spectrally split using another dichroic mirror centered at 640 nm. Silicon avalanche photodiode detectors (Perkin-Elmer AQR 14) were used to collect fluorescence in the red channel (650−750 nm) and green channel (505−635 nm), respectively. Using the appropriate emission filters, fluorescence cross talk between channels was reduced to 16%. The signal outputs of the detectors were coupled into a TimeHarp 200 (PicoQuant, GmbH) TCSPC board and a multichannel router for the acquisition of time-tagged photon arrival times (TTPAT). In TTPAT, each photon registered is given a macroscopic arrival time with 50 ns resolution as well as a microscopic arrival time with 37 ps resolution.

Biotinilated dsDNA (57 bp) was internally labeled with Cy5 (acceptor) and tetramethylrhodamine (TMR, donor), according to the procedure described elsewhere.[8,13] All measurements were performed in 10 mM TRIS buffer. The fluorophores were conjugated to the two DNA strands and separated by 14 basepairs (~4.9 nm). This distance is comparable to the Förster radius of the FRET pair (~5.3 nm) measured in bulk. Bulk lifetime (50 nM) for unquenched TMR was determined to be 2.4 ns in Tris buffer. Quenching due to FRET resulted in a lifetime of 1.0 ns for the donor dye, TMR. The average bulk lifetime of Cy5 undergoing FRET was determined to be 1.1 ns. Cy5 excited with a 637 nm laser line (PicoQuant LDH-P−C-635B) exhibits a biexponential decay with lifetimes of 0.83 and 1.67 ns and relative amplitudes of 0.4 and 0.6 respectively, yielding an averaged lifetime of 1.3 ns.

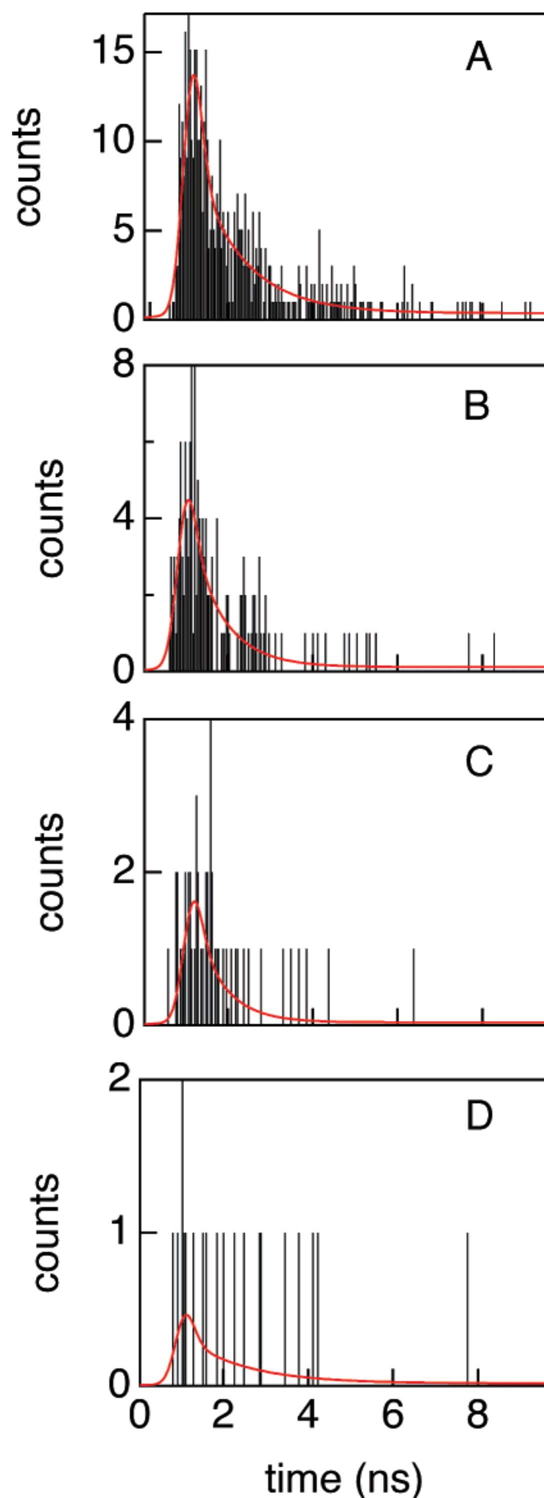

Figure 2. Fluorescent lifetime decays and fits of TMR using a maximum likelihood estimator fitting algorithm with background and scattering subtraction for the accumulation of 500, 150, 50, and 20 photons (A−D, respectively). The fits yield fluorescence lifetime values of 1.06, 0.96, 0.97, and 1.2 ns with 9%, 16%, 28%, and 44% error at 95% confidence for A−D, respectively.

For single molecule experiments, biotinilated DNA was specifically bound to streptavidin molecules on a BSA (bovine serum albumin)-coated fused silica coverslip.[8,13] During acquisition, an O_2 scavenging system[14] was pumped into the fluidic channel at a flow rate of 5 μL/min. This minimized photobleaching and triplet state blinking. Examples of typical time trace trajectories for single DNA molecules are shown in Figure

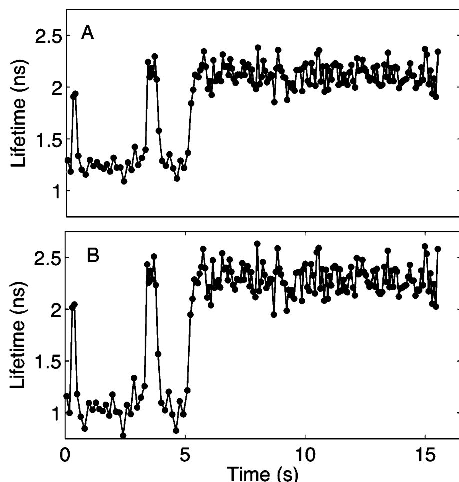

Figure 3. Fluorescent lifetime trajectories of the TMR from Figure 1b using an MLE algorithm. (A) Without background correction. (B) With background subtraction. The laser intensity in both cases was 10 μW, and each lifetime was determined using 500 photons.

1A,B. These trajectories were obtained by down sampling the TTPAT macroscopic time data to a bin time resolution of 10 ms. In both examples shown, the acceptor photobleaches before the donor. In Figure 1A, the TMR−Cy5 FRET pair undergoes energy transfer from 0−3.8 s, after which the acceptor photobleaches resulting in an increased count rate of the donor. This is followed by complete photobleaching of the donor at 14.9 s. In Figure 1B, the trajectory is analogous with the exception that dark Cy5 states are observed before complete photobleaching. Similar dark states have recently been reported for a TMR−Cy5 FRET pair and can be attributed to radical formation.[14]

A fluorescence lifetime decay curve is a convolution of the IRF with the true fluorescence decay. The IRF was experimentally determined by measuring the system response to Auramine-O. This dye exhibits a picosecond lifetime,[15] which is >2 orders of magnitude faster than the detector's response time (350 ps). Experimental decays must be deconvolved from the IRF to extract the true fluorescence lifetimes. The most common approach is to use a least-squares minimization.[16] However, this approach breaks down when the total number of photon counts is low (<2000), as is often the case in SM lifetime. Alternatively, a maximum likelihood estimator (MLE) method can be used to cope with this limitation.[17,18] The MLE (γ_i) is defined by eq 1

$$\gamma_j = \sum_1^k n_i \log\left(\frac{n_i}{Np_i(j)}\right) \quad (1)$$

where n_i is the number of photon counts in channel i, k is the number of channels (or bins) for each fluorescence decay (~300 for the TimeHarp 200 board with a laser operating at 80 MHz), and $p_i(j)$ is the probability that a group of photons will fall in channel i if the particles have a lifetime j. $N = \sum_i^k n_i$ is the total number of photons in a given decay. For a fluorophore with a monoexponential decay, eq 1 can be shown to have the following form:[19]

$$\frac{\omega}{1 - e^{-\omega/\tau}} - \frac{k\omega}{e^{k\omega/\tau} - 1} - \frac{1}{N}\sum_{i=1}^k i\omega n_i = 0 \quad (2)$$

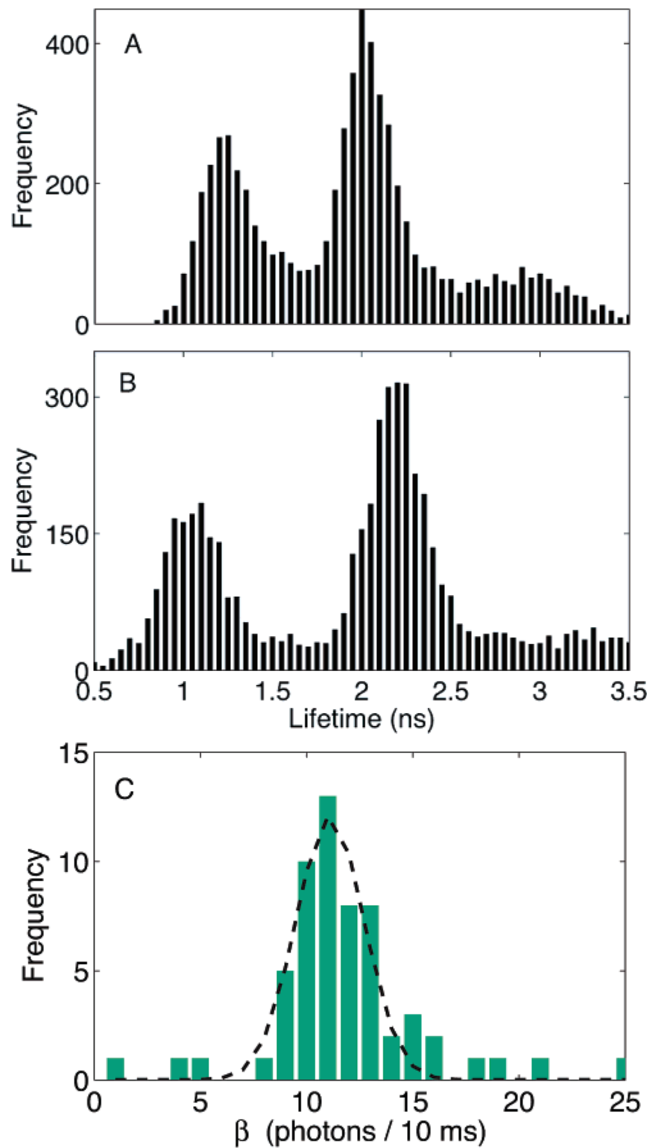

Figure 4. Histogram from the accumulation of 61 independent single molecule lifetime trajectories for TMR using 500 photons per lifetime. (A) Without background correction: TMR has a FRET lifetime of 1.25 ns and a non-FRET lifetime of 2 ns. (B) With background subtraction: TMR has a FRET lifetime of 1.0 ns and a non-FRET lifetime of 2.3 ns, which is in good agreement with the bulk lifetime values. (C) Histogram of the mean background intensity of 63 TMR molecules (green) with a Gaussian fit (black).

where the fluorescent lifetime is defined by τ and ω is the bin resolution. Although this is perhaps the most common approach in determining lifetimes with few photons, the IRF as well as background fluorescence has not been taken into account. Selection of start and end bins within the decay profile is also somewhat arbitrary in nature and, if incorrectly chosen, can also result in additional errors. Recently, Maus et al. have used a modified $p_i(j)$ probability function, which incorporates IRF convolution as well as a background scattering parameter.[20] Their scattering background was determined by sampling 128 decays of a blank and subtracting these contributions. Here, we extend this approach by taking into account background fluorescence as well as scattering for each and every single molecule trajectory (eqs 3 and 4 below). Our approach results in better determination of lifetimes for immobilized molecules, where the local background of each molecule varies. For a

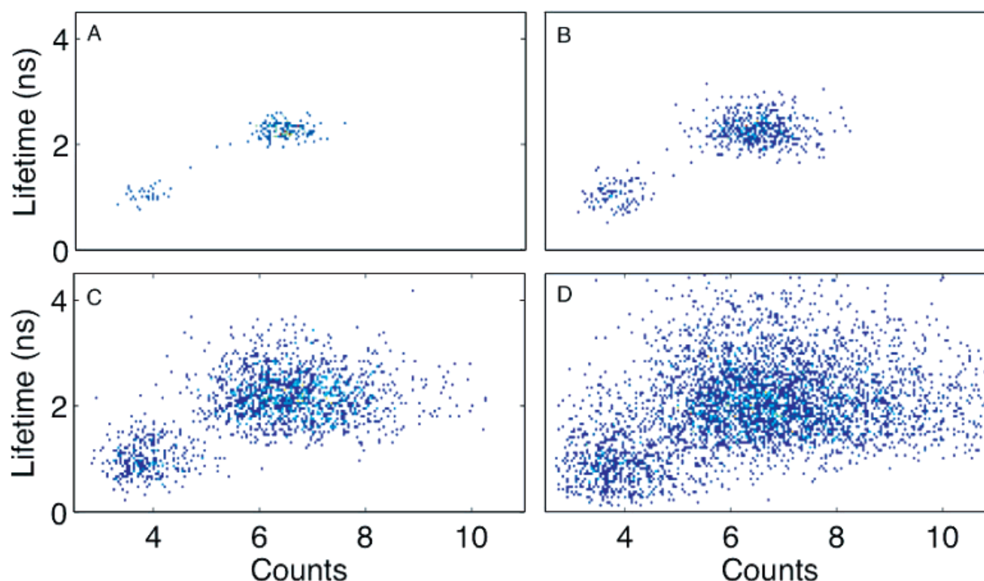

Figure 5. Scatter plots of lifetime versus counts for TMR using a total of (A) 500, (B) 150, (C) 50, and (D) 20 photons per data point. The TMR populations having a mean of 1 and 2.3 ns correspond to the FRET and unquenched lifetime of TMR, respectively.

monoexponential decay, the MLE convolved with an IRF is given by

$$\frac{\omega}{1-e^{-\omega/\tau}} - \frac{k\omega}{e^{k\omega/\tau}-1} - \frac{1}{N}\sum_{i=1}^{k} i\omega n_i\left(i - \sum_{j=1}^{i} jr_j e^{j\omega/\tau} / \sum_{j=1}^{i} r_j e^{jw/\tau}\right) = 0 \quad (3)$$

The IRF contribution to the decay corresponds to r_j in each bin j. The fluorescence lifetime, τ, can then be extracted by using a minimization routine such as a Levenberg−Marquardt algorithm. The lifetime must be further corrected for background fluorescence not associated with the IRF, yielding

$$\tau' = \frac{\tau\alpha - \beta\tau_{bkg}}{\alpha - \beta} \quad (4)$$

where τ_{bkg} is the background fluorescent lifetime, for each molecule, after photobleaching occurred using eq 3. α is the mean fluorescence intensity for both high and low FRET, and β is the mean background fluorescence intensity. These values are extracted for each and every single molecule trajectory.

In Figure 2, we display four typical fluorescence lifetime decays (donor channel) taken from a small part of the single molecule trace shown in Figure 1B. The photon arrival histograms of 500, 150, 50, and 20 photons (Figure 2A−D, respectively) are shown with their corresponding background and scattering subtraction MLE fit according to eqs 3 and 4. The fluorescence lifetime values obtained using this procedure were in the range 0.9−1.2 ns with 9%, 16%, 28%, and 44% error (A−D), calculated at the 95% confidence level using counting statistics. Decreasing or increasing the time resolution has negligible effect on the quality of fit because the MLE probability algorithm is used (as opposed to a least-squares approach) in fitting the data. Even with less than 20 photons, the MLE remains stable; however, as the fits are ultimately governed by counting statistics, the errors become significant.

An example of typical lifetime trajectories correlating to the burst scan shown in Figure 1B using the 350 ps resolution of the TTPAT data is shown in Figure 3. This is shown using an MLE algorithm without full background correction using eq 3 (Figure 3A). In this scan, only a cross talk correction factor was added. In Figure 3B, full background correction was implemented using eqs 3 and 4. In all cases, each individual lifetime was computed on a total of 500 unique photons and continuously performed for the entire trajectory. When comparing the bulk lifetimes with the lifetime trajectories, it is obvious that there is a clear discrepancy with the values in Figure 3A. The FRET lifetime of TMR has a mean lifetime of 1.25 ns, and the non-FRET lifetime has a value of 2 ns. The background lifetime was determined to be 1.5 ns in essence perturbing the overall lifetimes by decreasing the lifetime of the unquenched state while increasing the lifetime of the quenched state. Utilizing full background correction, the means are in much closer agreement with the bulk lifetime values (2.3 ns for the unquenched state and 1.0 ns for the quenched state). The calculated FRET lifetime also using eqs 3 and 4 for Cy5 was 1.0 ns. There is also a significant improvement when comparing the lifetime trajectories using an IRF deconvolution routine versus the extraction of lifetimes without deconvolution using simply eq 2. Although the means in both lifetime trajectories for a given excited transitional state are approximately the same, the spread or standard deviations of the average lifetime is greatly increased when measured without an IRF. This effect is a direct consequence of using the entire decay path (12.5 ns), with IRF deconvolution, as opposed to running the analysis routine by simply cropping out the IRF dominant section of the decays.

Summing single molecule lifetime trajectories over an accumulation of 61 independent scans produced lifetime histograms as shown in Figure 4A,B for uncorrected and corrected lifetimes of TMR, respectively. All lifetimes were determined with 500 photons, and background correction was performed as described above. In the uncorrected system, there is significant overlap (>25%) between the FRET and non-FRET states of TMR resulting in a significant error associated with the assignment of events. In the corrected histograms, however, this overlap error is significantly reduced. It is interesting to note that in both histograms there appears to be a third lifetime component greater than 2.5 ns. This correlates with a very bright

TMR state and shows up in approximately 2% of all the intensity and lifetime trajectories.

In Figure 4C, a histogram of β values determined from 63 SM trajectories is shown (green channel only).[21] β was calculated from the wavelet filtered intensity trajectories and thus varies as the fluorophore is quenched via FRET or bleached. The mean background lifetime, τ_{bkg}, was determined by extracting the lifetime from all photons associated with the minimum horizontal transition at the time the acceptor, Cy5, is photobleached. Because the value of β varies from molecule to molecule and molecules are uncorrelated, we expect a normal distribution. This observation is supported by our data, which we fit using a Gaussian function, yielding an average β value of 11 ± 4 (fwhm) photons/10 ms. The spread in the values of β underlines the importance in applying the correction factors independently for each trajectory.

In Figure 5, scatter plots of lifetimes versus photon counts are shown for a single molecule using a total of 20, 50, 150, and 500 photons for each lifetime. The lifetime distribution for Cy5 is omitted for clarity. As the number of photons is decreased, the standard deviation steadily increases, as expected; however, even at 20 photons, the 2 lifetime populations of TMR are clearly distinguishable. In fact, it was possible to determine the MLE lifetime with fewer than 20 photons (10 μs acquisition time) before losing stability in the fitting algorithm.

In summary, we have demonstrated that IRF deconvolution and MLE fitting with background and scattering subtraction yield more stable and reliable results for time-resolved lifetime extraction of single molecule trajectories. We have demonstrated our method using a TMR−Cy5 FRET pair internally positioned in dsDNA. Background fluorescence signal as well as deconvolution reduces errors associated with the extraction of lifetime data. The analysis routine takes less than 1 s to run per molecule on a standard PC, which makes this routine real-time capable. The analysis approach utilizing lifetime trajectories also appears to be a useful tool for studying FRET systems in general and can be used with as little as 20 photons. We are currently using such tools to study DNA dynamics with high temporal resolution.

Acknowledgment. We acknowledge discussions and contributions from Dr. J. Mathé and critical remarks from Dr. E. Atas and Mr. N. DiFiori. A.M. acknowledges financial support from the National Science Foundation grant No. PHY-0417067 and National Institute of Health grant GM 072893.

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(21) For example, in Figure 1a, the green channel has $\beta = 9$ photons/10 ms and the red channel has a β of 12 photons/10 ms before TMR is fully photobleached.