Optimal Algorithm for Single-Molecule Identification with Time-Correlated Single-Photon Counting

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A pattern-matching procedure for identifying single molecules according to the temporal decay characteristics of the fluorescence is presented and applied to measured single-molecule data of three different dyes. Based on the maximum likelihood principle, this procedure is the best possible identification algorithm if the fluorescence time decays of the different molecular species are exactly known. The presented algorithm is numerically simple and fast and, thus, especially suitable to possible online applications. Exact theoretical results for the misidentification errors of the algorithm are derived and compared with the errors determined from the experimental data.

Introduction

During the past decade, laser-induced fluorescence detection of single molecules in liquids has become a standard laboratory technique with various applications in many fields of research.[1−3] Frequently, it is desirable not only to detect the presence of a fluorescing molecule but also to be able to identify it or to distinguish between different sorts of molecules. The average number of photons that is possible to be detected from an individual molecule is mainly restricted by its photostability. When a molecule is optically excited at room temperature and in solution, there is a nonvanishing chance that it undergoes an irreversible photochemical reaction instead of emitting a fluorescence photon. Thus, the identification of an individual molecule has to be done on the basis of a limited number of detectable fluorescence photons.

In general, photons carry three types of information: energy, polarization, and time of arrival. Measuring the photon energy is the main task of fluorescence spectroscopy. However, the main difficulty in the detection of individual molecules is the necessity of using spectrometers with single-photon-sensitive detectors, an expensive and rarely available piece of equipment.[4,5] A minimal solution is to use only two spectral channels with two single-photon-sensitive detectors for gaining at least some information about the fluorescence spectrum.[6,7]

Polarization measurements are useful if molecules can be distinguished by their rotational diffusion.[8] However, this is only useful if different sorts of molecules have significantly different rotational diffusion constants.

Last but not least, the information contained in the time of arrival of the detected photons can be used. On the micro- and millisecond time scale, the rate of the photon arrivals corresponds to the fluorescence intensity. If the excitation and detection conditions for every detected molecule are equal, the fluorescence intensity yields information about the intrinsic fluorescence brightness of the molecules (absorption cross-section times and fluorescence quantum yield), which can be used to identify single molecules.[9,10] On the pico- and nanosecond time scale, additional information about the molecules' fluorescence can be gained. A fluorescing molecule emits a photon with a (varying) time delay with respect to an exciting laser pulse, because of the finite lifetime of its excited state. By repetition of the pulse-excitation/photon-detection measurement many times, a distribution of the delay times between excitation and fluorescence emission can be built up [time-correlated single-photon counting (TCSPC)[11]]. In the simplest case, this delay-time distribution is a monoexponential curve with a decay time depending on the sort of molecule and its environmental conditions (e.g., solvent pH, solvent polarity, or temperature). These characteristic decay times of different sorts of molecules can be employed for identifying individual molecules in solution[12−17] and at interfaces.[18−20]

In the present paper, an advanced pattern-matching algorithm is presented for single-molecule identification based on their fluorescence decay characteristics. This algorithm is applicable to arbitrary fluorescence decay behavior and does not assume any knowledge of its underlying nature. Thus, no lifetime fitting or similar methods are involved. Moreover, the algorithm is mathematically the best possible for distinguishing molecules by their fluorescence decay behavior. The algorithm is applied to measured single-molecule data in solutions of three different dyes with similar absorption and emission characteristics but slightly different fluorescence decay times (see the Experimental Section). General and exact formulas are derived for the error rates of misidentifying single molecules and are checked against the experimental results.

Theory

In a TCSPC measurement, the times of arrival of the first arriving fluorescence photon with respect to the last exciting laser pulse are measured, and these arrival times are sorted into N discrete time channels with equal but final time width. Thus, a TCSPC curve consists of a discrete set of N integers giving the number of photons falling into each of the N time channels.

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The shape of this histogram is given by the convolution of the fluorescence time decay curve with the so-called instrumental response function of the measurement system. By the employment of suitable fitting algorithms, the fluorescence decay characteristics can be extracted from the measured TCSPC curve, for obtaining, for example, a single decay time in the case of a monoexponential fluorescence time decay. In single-molecule identification based on TCSPC measurements, usually[13,15−18] a fitting algorithm is used for obtaining the decay time for every detected single molecule and then for identifying each molecule on the basis of this decay time value. Such an approach is disadvantageous in several respects: First, the decay-time fitting is time-consuming, which can be problematic when applying the algorithm to online data evaluation, where up to 100 or more molecule detection events/s are feasible.[16] Second, the fitting procedure becomes increasingly complicated for molecules with more complex decays than a simple monoexponential fluorescence decay. Last, the error of the fitting procedure itself adds to the overall error of correctly identifying the detected molecules. In ref 6, molecules were identified by a more suitable lookup table method. Finally, ref 14 uses a maximum likelihood pattern-matching approach for distinguishing between two different molecular species, where the measured TCSPC curves were directly compared with the fluorescence decay curves of the two molecular species.

Here, the maximum likelihood approach is extended to more than two molecular species, and a general theoretical expression for the misidentification error of the pattern-matching algorithm is derived for arbitrary fluorescence decay characteristics of the molecular species. For a given molecular species α, the probability of finding a fluorescence photon within the *n*th time channel of the TCSPC curve shall be denoted by $p_\alpha(n)$. It is assumed that these probabilities are known in advance for all molecular species, e.g., by measuring TCSPC curves on a large ensemble of molecules of each species. Next, for a detected single molecule, the number of photons falling into the *n*th time channel of the TCSPC curve shall be denoted by $m(n)$. The probability of measuring such a TCSPC curve $\{m(n)\}$ from a molecule of the species α is then given by the multinomial distribution

$$P_\alpha\{m(n)\} = M!\prod_{n=1}^{N}\frac{p_\alpha^{m(n)}(n)}{m(n)!} \tag{1}$$

where M is the total number of photons detected from the single molecule. From a mathematical point of view, the *most likely* molecular species that has generated the measured TCSPC curve $\{m(n)\}$ is the one with the highest probability value as calculated by eq 1 (maximum likelihood principle). Thus, for distinguishing between different molecular species, the probabilities $P_\alpha\{m(n)\}$ can be calculated, and the species with the highest value is associated with the detected molecule. It should be emphasized that such a decision method is mathematically the best possible and that no other method can yield better estimates than this maximum likelihood approach, provided that the probabilities $p_\alpha(n)$ are always the same for all molecules of one molecular species. To understand this in more detail, consider for a moment only two molecular species $\alpha = 1$ and 2 and some other identification algorithm distinct from the maximum likelihood method just described. Then there will be specific experimental measurement results $\{m(n)\}$ where that identification algorithm attributes the measurement to, for example, the molecule species 2, although the probability $P_1\{m(n)\}$ is larger than $P_2\{m(n)\}$ for the given $\{m(n)\}$. Thus, in the limit of an infinite number of repeated measurements, the chosen identification algorithm will be, for the specific measurement outcome $\{m(n)\}$, more erroneous (error rate proportional to $P_1\{m(n)\}/[P_1\{m(n)\} + P_2\{m(n)\}]$) than the maximum likelihood algorithm (error rate proportional to $P_2\{m(n)\}/[P_1\{m(n)\} + P_2\{m(n)\}]$). It follows that the optimal identification algorithm must attribute a measured result $\{m(n)\}$ to the molecule with the highest probability value $P_\alpha\{m(n)\}$, which is just the maximum likelihood principle.

The maximum likelihood identification algorithm can be simplified by considering the logarithm of $P_\alpha\{m(n)\}$ instead of the function itself:

$$\ln P_\alpha\{m(n)\} = \sum_{n=1}^{N} m(n)\ln p_\alpha(n) + \ln M! - \sum_{n=1}^{N}\ln[m(n)!] \tag{2}$$

For a given measurement $\{m(n)\}$, the last two terms on the right-hand side (rhs) of the last equation are independent of the value of α. Thus, it is sufficient to compare the values of Q_α defined by

$$Q_\alpha\{m(n)\} = \sum_{n=1}^{N} m(n)\ln p_\alpha(n) \tag{3}$$

If the number of different molecular species is S, then the molecule identification procedure involves SN multiplication of N integers $m(n)$ with the values of the $\ln p_\alpha(n)$ and $S(N-1)$ summations and determination α having the maximum Q_α value.

An important issue is to obtain information about the reliability of such an identification procedure. This can be done by calculating the error rates of misidentifying a detected molecule of the species α to be a molecule of the species β. If $R_{\beta\alpha}(Q_\beta - Q_\alpha|M)$ denotes the probability density distribution of the values of $Q_\beta - Q_\alpha$ for an experiment where a molecule of species α is present and exactly M photons were detected, then the misidentification error $\mathrm{err}_{\beta\alpha}(M)$ is given by the integral

$$\mathrm{err}_{\beta\alpha}(M) = \int_0^\infty R_{\beta\alpha}(x|M)\,\mathrm{d}x \tag{4}$$

(misidentification corresponds to $Q_\beta > Q_\alpha$). The probability density distribution $R_{\beta\alpha}$ itself can be found exactly by[21]

$$R_{\beta\alpha}(x|M) = \sum_{\substack{\{m(n)\}\\ \sum_{m(n)=M}}} \delta\left(x - \sum_{j=1}^{N} m(j)\ln\frac{p_\beta(j)}{p_\alpha(j)}\right) M!\prod_{n=1}^{N}\left(\frac{p_\alpha^{m(n)}(n)}{m(n)!}\right) \tag{5}$$

where δ denotes Dirac's delta function, which is nonzero only when x equals $Q_\beta - Q_\alpha$ for a given set $\{m(n)\}$, and the product is the probability of the occurrence of such a set $\{m(n)\}$ for a molecule of species α. Abbreviating the $\ln p_\beta(j)/p_\alpha(j)$ term by $r_{\beta\alpha}(j)$ and replacing the delta function by its integral representation

$$\delta(x) = \int_{-\infty}^{\infty}\frac{\mathrm{d}k}{2\pi}e^{ikx} \tag{6}$$

one has

$$R_{\beta\alpha}(x|M) = \sum_{\substack{\{m(n)\}\\ \sum_{m(n)=M}}} \int_{-\infty}^{\infty}\frac{\mathrm{d}k}{2\pi}e^{ikx}M!\prod_{n=1}^{N}\left(\frac{[p_\alpha(n)\exp(-ikr_{\beta\alpha}(n))]^{m(n)}}{m(n)!}\right) \tag{7}$$

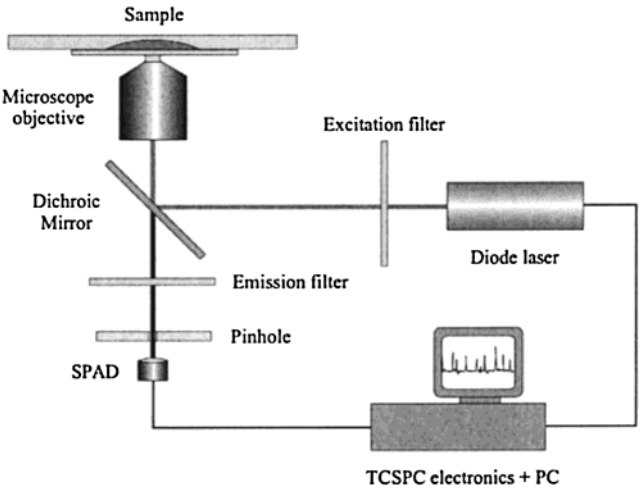

Figure 1. Schematic diagram of the experimental setup.

Interchanging integration and summation and carrying out the summation over $\{m(n)\}$ yield the compact result

$$R_{\beta\alpha}(x|M) = \int_{-\infty}^{\infty} \frac{dk}{2\pi} e^{ikx} \langle \exp(-ikr_{\beta\alpha}) \rangle_{\alpha}^{M} \qquad (8)$$

where $\langle \cdot \rangle_{\alpha}$ stands for the averaging $\langle f \rangle_{\alpha} \equiv \sum_{n=1}^{N} p_{\alpha}(n)\, f(n)$. The rhs of the last equation can easily be calculated numerically, and subsequent integration according to eq 4 yields the desired misidentification error. These results can be generalized to the case of a distribution $h(M)$ of detected photons per molecule, where $h(M)$ denotes the normalized frequency of encountering a single-molecule detection event with M photons. Then, the probability density distribution for $Q_{\beta} - Q_{\alpha}$ is given by the weighted sum

$$R_{\beta\alpha}(Q_{\beta} - Q_{\alpha}) = \sum_{M} h(M)\, R_{\beta\alpha}(Q_{\beta} - Q_{\alpha}|M) \qquad (9)$$

with $R_{\beta\alpha}(Q_{\beta} - Q_{\alpha}|M)$ as calculated by eq 8.

Experiment

The three rhodamine derivatives JF9,[19] JA167,[19] and JA53[15] were purified on a RP18 column using a gradient of 0−75% acetonitrile in 0.1 M aqueous triethylammonium acetate. All measurements were carried out in water containing 20% glycerol at room temperature. A schematic diagram of the optical and electronic setup for TCSPC at the single-molecule level is shown in Figure 1. For efficient excitation of the three dyes, a short-pulse diode laser emitting at 635 nm with a repetition rate of 64 MHz was used (PDL800; PicoQuant, Berlin, Germany). This laser provides light pulses with a duration of ∼100 ps full width at half-maximum. The collimated laser beam passed an excitation filter (639DF10; Omega Optical, Brattleboro, VT) and entered an inverse microscope (Axiovert 100TV; Zeiss, Germany) through the back port. The beam was coupled into an oil-immersion objective (100×; 1.4 NA; Nikon, Japan) by a dichroic beam splitter (640DRLP; Omega Optical, Brattleboro, VT) and focused into the sample. Solutions for single-molecule experiments (10^{-11} M) were prepared by diluting stock solutions with the appropriate amount of solvent. The samples were transferred onto a microscope slide with a small depression and covered by a cover slip. The average laser power at the sample was adjusted to be 300 μW. The fluorescence signal of individual dye molecules passing the detection volume of ∼1 fL was collected by the same objective, filtered by two band-pass filters (680HQ65; AF Analysentechnik, Tübingen, Germany and 675RDF50; Omega Optical, Brattleboro, VT), and imaged onto a 100 μm pinhole oriented directly in front of an avalanche photodiode (AQ141; EG&G Optoelectronics, Canada). The detector signal was registered by a PC plug-in card for TCSPC (SPC-430; Becker & Hickl, Berlin, Germany). With this card, a minimum collection (integration) time of 150 μs/decay curve at 64 channels is possible.[22] From the data of the TCSPC card, multichannel-scalar (MCS) traces were generated by adding up all photons of a decay curve to a bin of the MCS trace (Figure 2). With our experimental setup, an average background level of ∼1 kHz was measured. Hence, we calculated signal-to-background ratios of more than 200 for the most intense peaks in our experiments.

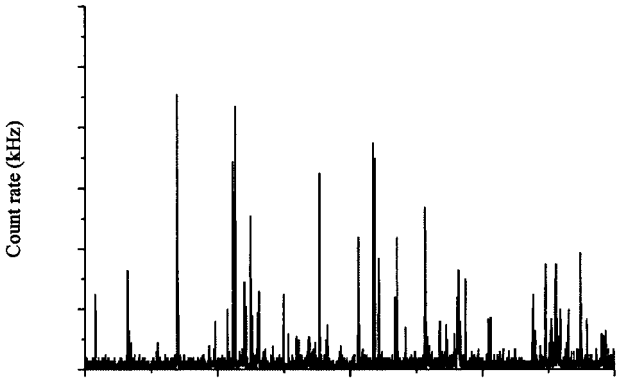

Figure 2. Time-dependent fluorescence signals observed from a 10^{-11} M solution of JF9 in water containing 20% glycerol. Data were binned into 400 μs time intervals. Average laser power at the sample was 300 μW.

Results

Single-molecule fluorescence decay data were recorded on pure solutions of three different rhodamine derivatives—JF9, JA53, and JA167—in water containing 20% glycerol. Every 400 μs, a complete TCSPC curve was measured (64 time channels, width of channel equal to 0.2 ns) and stored. Single-molecule bursts were extracted from these raw data in the following way: For every 400 μs time bin, the complete number of detected photons was summed. The resulting photon count track was smoothed with a moving average of 2 ms width. A single-molecule burst was defined as a couple of subsequent time bins where the smoothed photon count rate raised over a threshold of 3 times the average background rate (3 kHz). Bursts with less than 25 photons were discarded. For each determined single-molecule burst, the photon numbers within each TCSPC channel were summed up over the duration of the burst to yield a single TCSPC curve associated with each burst. In this way, a total of 5872 bursts for JF9, 4816 bursts for JA53, and 4609 bursts for JA167 were determined and used in the subsequent analysis.

To determine the probability functions $p_{\alpha}(n)$, the TCSPC curves of all detected bursts for a given species α were summed up and normalized. This is equivalent to recording a TCSPC curve on a large ensemble of molecules (bulk measurements). The results for the three dyes used are shown in Figure 3. Using the such determined functions $p_{\alpha}(n)$, for every detected molecule, the three values Q_{α}, $\alpha \in \{\text{JF9, JA167, JA53}\}$, were calculated as described in the theoretical section. For every molecular species α, the two distributions of the values of $Q_{\beta} - Q_{\alpha}$, $\beta \neq \alpha$, were calculated by histogramming the $Q_{\beta} - Q_{\alpha}$ values. The six mutual error rates $\mathrm{err}_{\beta\alpha}(M)$ as functions of the number M of detected photons were calculated from the experimental distributions of the values of $Q_{\beta} - Q_{\alpha}$: If, for a molecule of species α, the value of $Q_{\beta} - Q_{\alpha}$, $\beta \neq \alpha$, was larger

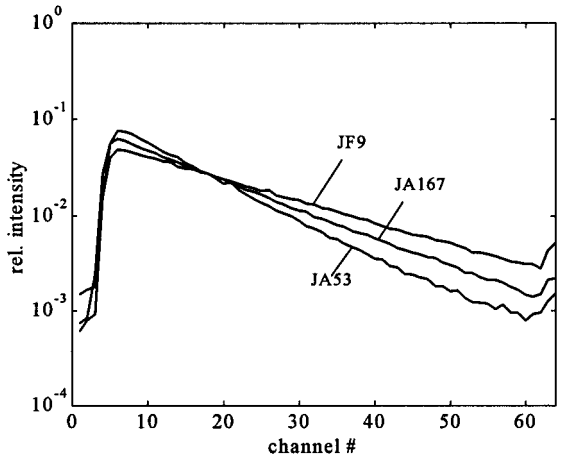

Figure 3. Plots of the normalized TCSPC curves as determined from the accumulated single-molecule data for the three dyes used. This curve served as the matching functions $p_\alpha(n)$ in the maximum likelihood single-molecule identification.

than zero, a misidentification occurred. Summing up the number of all misidentifications for molecule detection events with M detected photons and dividing this sum by the total number of molecules of species α with M detected photons give the experimentally determined error rate $\mathrm{err}_{\beta\alpha}(M)$. The theoretical error rates were calculated according to eqs 4 and 8. The results are plotted in Figure 4, together with the burst size distributions (frequencies of having a single-molecule event with a given number of detected photons).

Also, the theoretical distributions $\sum_M h_\alpha(M)\, R_{\beta\alpha}(Q_\beta - Q_\alpha|M)$ were calculated according to eq 9, with the $h_\alpha(M)$ denoting here the normalized frequencies of single-molecule events with M photons within the measured data of species α (see also Figure 4B). The comparison between the experimentally determined and the theoretically calculated distributions is shown in Figure 5.

Discussion and Conclusion

The error rates plotted in Figure 4 show a good correspondence between experimental and theoretical results. The relatively high values of the error rates are due to the fact that the fluorescence decay characteristics of the three dyes are not significantly different (see Figure 3) and the numbers of photons per molecule are rather low (25−105). Larger differences in the fluorescence decay and higher numbers of detected photons per molecule can dramatically decrease errors of misidentification (because of the nearly exponential decrease of the error rate with increasing number of photons), making fluorescence decay measurements an interesting candidate for single-molecule sensitive multivariate chemical analysis. A comparison with the error rates as reported in refs 13 and 15−18, where the more conventional method of lifetime fitting with subsequent identification was used, shows that the maximum likelihood method yields ca. 2−3 times smaller error rates (for similar lifetime differences and number of photons per molecule). With respect to the lookup table method as reported in ref 6, it should be noted that the maximum likelihood and the lookup table method

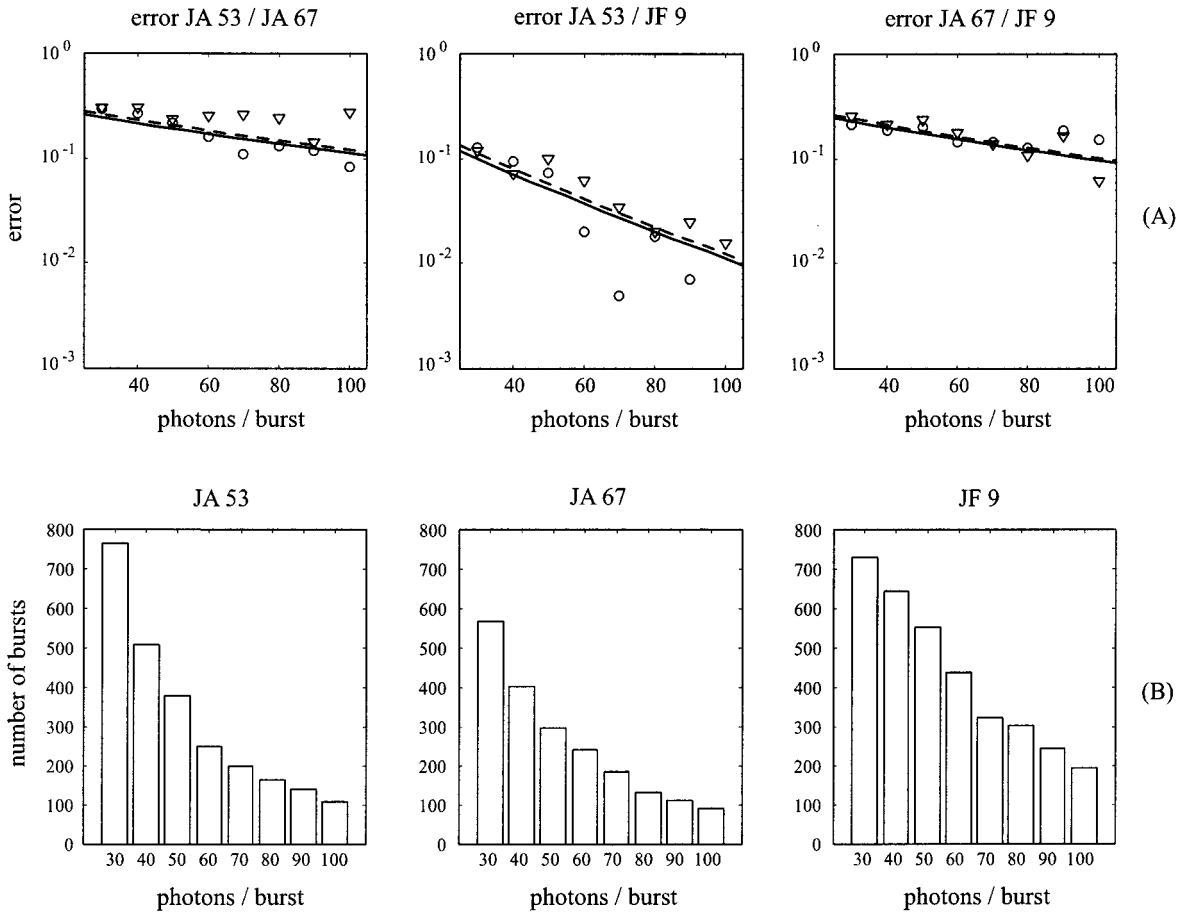

Figure 4. (A) Misidentification errors for the three pairs of dyes. Solid and dashed lines are the theoretical calculated errors $\mathrm{err}_{\beta\alpha}$ and $\mathrm{err}_{\alpha\beta}$, respectively, where β corresponds to the first dye in the plot title and α to the second. Circles and triangles are the experimentally determined errors for $\mathrm{err}_{\beta\alpha}$ and $\mathrm{err}_{\alpha\beta}$, respectively. (B) Burst size distribution histograms showing the number of detected bursts with different numbers of photons.

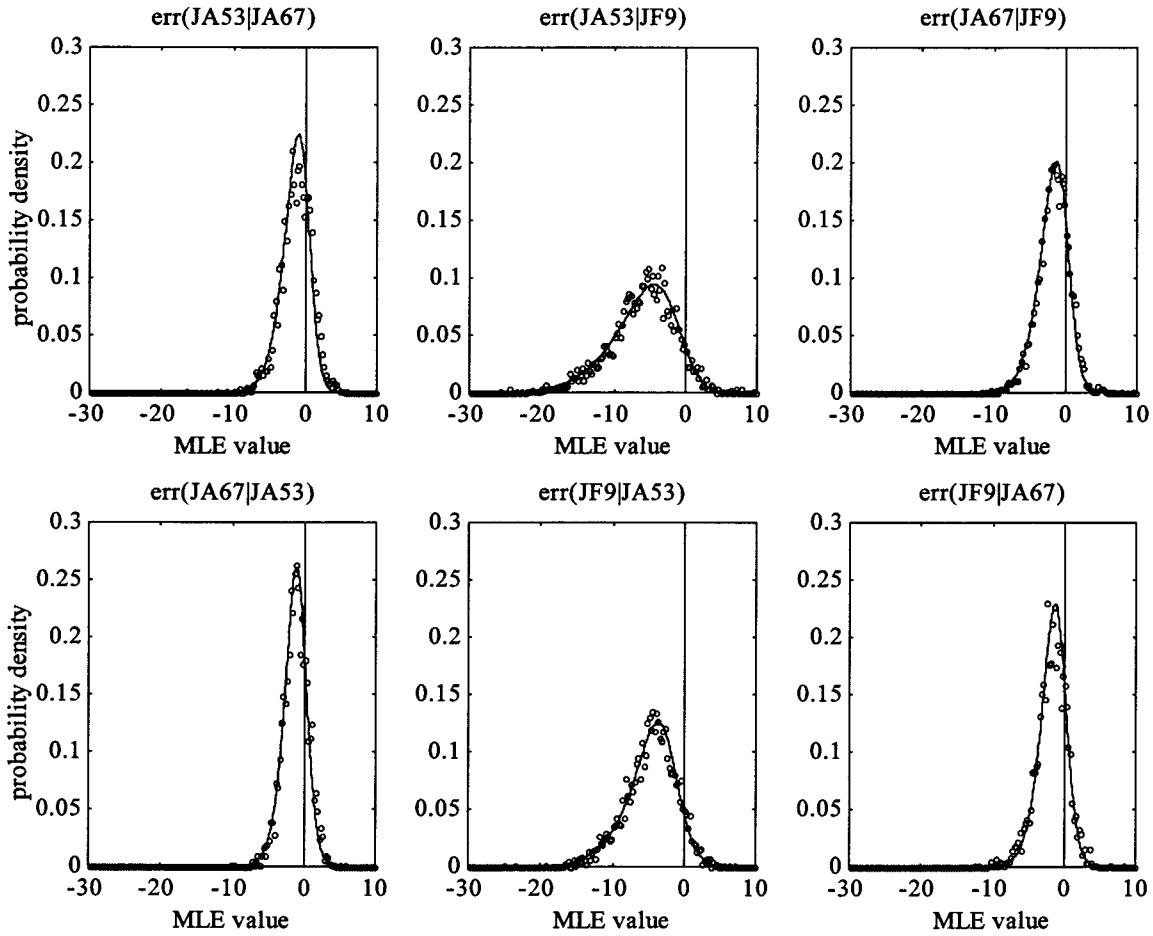

Figure 5. Calculated and experimental distributions of the $Q_\beta - Q_\alpha$ values (maximum likelihood estimator values), where β corresponds to the first dye in the plot title and α to the second. All values of $Q_\beta - Q_\alpha$ larger than zero correspond to misidentifications.

are completely equivalent if the lookup table is set up according to the maximum likelihood principle.

An important assumption for a successful application of the maximum likelihood method for single-molecule identification was that all molecules of one molecular species behave, in a statistical sense, similarly. This means that there are no subpopulations of molecules with different probability distributions $p_\alpha(n)$ (e.g., in their dependence on the number of detected photons per molecule), which could make the application of a maximum likelihood estimator using a single, averaged probability function $p_\alpha(n)$ dramatically erroneous. To check this assumption, we determined not only the overall error rates but also the full probability distributions of the variables $Q_\beta - Q_\alpha$ (Figure 5) and compared these curves with the calculated theoretical curves. Any presence of a more complicated fluorescence decay behavior (i.e., the presence of different subpopulations of molecules having different decay behavior) in the experimental data would inadvertently lead to deviations of the experimentally derived distributions from the theoretically calculated ones. The excellent correspondence of experimental and theoretical results is an independent confirmation that the maximum likelihood estimator is indeed the optimal method for the given molecule-identification problem.

In summary, we have presented a fast and general algorithm for identifying single molecules based on their measured TCSPC fluorescence decay data. Derived from the maximum likelihood principle, the algorithm is the best one possible for an identification, provided that the fluorescence decay characteristics of a given species of molecules do not vary from molecule to molecule. It should be emphasized that for the determination of the pattern functions $p_\alpha(n)$ no explicit knowledge about the monoexponential character of the fluorescence decay or any explicit values of the decay times of the dyes was used. For the presented identification algorithm, general and exact expressions were derived for the errors of misidentification.

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