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Time-resolved identification of individual mononucleotide molecules in aqueous solution with pulsed semiconductor lasers

Markus Sauer†‖, Jutta Arden-Jacob‡, Karl H Drexhage‡, Florian Göbel†, Ulrike Lieberwirth†, Klaus Mühlegger§, Ralph Müller†, Jürgen Wolfrum† and Christoph Zander†‡

† Physikalisch-Chemisches Institut, Universität Heidelberg, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany
‡ Institut für Physikalische Chemie, Universität-Gesamthochschule Siegen, Adolf-Reichwein-Straße 3, 57068 Siegen, Germany
§ Boehringer Mannheim GmbH, Werk Penzberg, Nonnenwald 2, 82377 Penzberg, Germany

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Abstract. We applied a short-pulse diode laser emitting at 640 nm with a repetition rate of 56 MHz in combination with a confocal microscope to study bursts of fluorescence photons from individual differently labeled mononucleotide molecules in water. Two newly synthesized dyes, an oxazine dye (MR121) and a rhodamine dye (JA53), and two commercially available dyes, a carbocyanine dye (Cy5) and a bora-diaza-indacene dye (Bodipy630/650), were used as fluorescent labels. The time-resolved fluorescence signals of individual mononucleotiode molecules in water were analyzed and identified by a maximum likelihood estimator (MLE). Taking only those single molecule transits which contain more than 30 collected photoelectrons, the two labeled mononucleotide molecules, Cy5-dCTP and Bodipy-dUTP, can be identified by time-resolved fluorescence spectroscopy with a probability of correct classification of greater than 99%. Our results show that at least three differently labeled mononucleotide molecules can be identified in a common aqueous solution. We obtain an overall classification probability of 90% for the time-resolved identification of Cy5-dCTP, MR121-dUTP and Bodipy-dUTP molecules via their characteristic fluorescence lifetimes of 1.05 ± 0.33 ns (Cy5-dCTP), 2.07 ± 0.59 ns (MR121-dUTP) and 3.88 ± 1.71 ns (Bodipy-dUTP).

Keywords: single molecule detection and identification, pulsed diode laser excitation, time-resolved fluorescence spectroscopy, single-molecule DNA sequencing

1. Introduction

Ultrasensitive detection has recently become possible with the development of the single molecule detection (SMD) technique, a laser-based method that allows the detection of individual dye molecules in solution. As a dye molecule passes through a focused laser beam, it is repeatedly cycled between the ground electronic state S_0 and the excited electronic state S_1. Dependent on the fluorescence quantum yield, Φ_f of the dye photons are emitted, producing a photon burst. Several instrumental approaches have been used to accomplish SMD, including time-gated detection techniques [1–6], leviated microdroplets [7], confocal microscopy with detection volumes in the femtoliter region [8–11], two-photon excited fluorescence [12–14], near-infrared fluorescence [15] and diode laser excitation in the red region [16–18]. For the detection of single dye molecules in solution it is essential to minimize the background due to scattering and luminescent impurities in the solvent. Thus the ability to detect fluorescence bursts

‖ Corresponding author. E-mail address: sauer@sun0.urz.uni-heidelberg.de

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emitted by individual dye molecules is not as much an issue of sensitive detection as it is of background reduction. The predominant source of background is Rayleigh scattering as well as reflected light, both of which can be efficiently suppressed by suitable optical filters. Since the intensity of Raman scattering is directly proportional to the detection volume, a confocal set-up with a detection volume of only a few femtoliters seems to be ideally suited for SMD techniques. On the other hand, the use of such small detection volumes will require a precise control of analyte molecule trajectories to ensure that all analyte molecules pass through the femtoliter volume and thus are efficiently detected [19].

A very interesting application of the SMD technique is fast DNA sequencing on the single molecule level as proposed by Keller and co-workers [20–23]. The principal idea of this method involves the incorporation of fluorescently labeled mononucleotides in a growing DNA strand, attachment of a single labeled DNA strand to a support (generally latex beads), transfer of the supported DNA into a flowing sample stream [20–23], a detection channel on a microchip [24] or microcapillary [25–28] and detection of the analyte molecules as they are cleaved from the DNA strand by an exonuclease enzyme. The DNA sequence is determined by the order in which labeled nucleotides are detected and identified. Although many problems are associated with the enzymatic incorporation of labeled mononucleotides into a growing DNA strand, different detection and identification strategies have been developed. To identify individual dye molecules in solution either spectral- [3, 24] or time-resolved [17, 18, 29, 30] fluorescence detection methods have been successfully applied. However, only two different dyes have been identified on the single-molecule level in aqueous solution due to their different fluorescence lifetimes [17, 18, 29–31]. On the other hand, the replication of the template with only two differently labeled nucleotides is sufficient to obtain the whole sequence information by changing the two labeled nucleotides in a second experiment. Nevertheless, for bioanalytical multiparameter tests the important question is how many different labels can be identified by time-resolved fluorescence spectroscopy on the single-molecule level in aqueous solvent systems. In this communication we present results on detection and time-resolved identification of individual mononucleotide (dUTP, dCTP) molecules labeled with the carbocyanine dye Cy5, the oxazine dye MR121, the rhodamine dye JA53 and the bora-diaza-indacene dye Bodipy630/650 in water combining a confocal microscope with the technique of time-correlated single photon counting (TCSPC). For excitation of the sample at 640 nm we employed a short-pulse semiconductor diode laser with a repetition rate of 56 MHz. The detection was performed with a single photon avalanche photodiode and a PC interface card for TCSPC.

2. Experimental section

The fluorescent mononucleotide Cy5-dCTP was purchased from Amersham Life Science (Braunschweig, Germany). The synthesis of JA53-, MR121- and Bodipy-dUTP was accomplished by reaction of 5-(3-aminoallyl)-dUTP [32] with the appropriate N-hydroxy-succinimide esters of JA53 and MR121 [33] and Bodipy630/650 (Molecular Probes, Eugene, OR, USA), respectively, as follows.

10 μmoles of 5-(3-aminoallyl)-dUTP were dissolved in 0.5 ml 0.1 M sodium borate buffer pH 8.5, a solution of 5 μmoles of the NHS ester in 1 ml amine-free dimethylformamide was added and the mixture kept 15 h overnight at ambient temperature. The solvents were subsequently removed under vacuum, the remainder dissolved in 2 ml of distilled water and applied to reversed phase high performance liquid chromatomography (HPLC). Purification was carried out on a RP18-column using a gradient of 0–75% acetonitrile in 0.1 M aqueous triethyl-ammonium acetate (TEAA). Figure 1 shows the molecular structures of the labeled mononucleotides.

All measurements were carried out in pure water. To adjust the dye labeled mononucleotide concentration we used the UV/VIS-spectrometer Lambda 18 (Perkin Elmer). Steady-state fluorescence was measured in standard quartz cuvettes with an LS100 spectrometer (PTI, Canada). Time-resolved data of bulk solutions were measured with the SMD apparatus at concentrations of 10^{-8} M. Stock solutions (10^{-6} M) were stored at $-20\,^{\circ}$C until used.

Figure 2 presents the confocal set-up used in our experiments. A pulsed diode laser (640 nm) served as the excitation source. The pulsing of the diode was performed by a self-matched tunable pulse generator. The laser diode provided pulses of less than 400 ps (FWHM) duration with a repetition rate of 56 MHz. After collimation the beam passed an excitation filter (639DF9; Omega Optics, Brattleboro, USA) and entered an inverted microscope (Axiovert 100TV; Zeiss, Germany) through the back port. It was coupled into an oil-immersion objective (100×, NA = 1.4; Olympus, Tokyo, Japan) by a dichroic beam splitter (645DRLP; Omega Optics, Brattleboro, USA). Measurements were performed with an average laser power of 630 μW at the sample. The fluorescence signal was collected by the same objective, filtered by two bandpass filters (685HQ70, AFAnalysentechnik, Tübingen, Germany) and imaged through the TV outlet on the bottom of the microscope onto a 100 μm pinhole directly in front of the avalanche photodiode (SPAD, AQ 141; EG&G Optoelectronics, Canada).

The signal was amplified and split for a home-built PC card counter working as a multichannel scaler (MCS) with a minimal integration time of 1 μs, and a time-correlated single-photon counting (TCSPC) PC interface card (SPC-330; Becker&Hickl, Berlin, Germany) to acquire time-resolved data. With the PC card SPC-330 the signal was collected in up to 128 histograms with a minimal

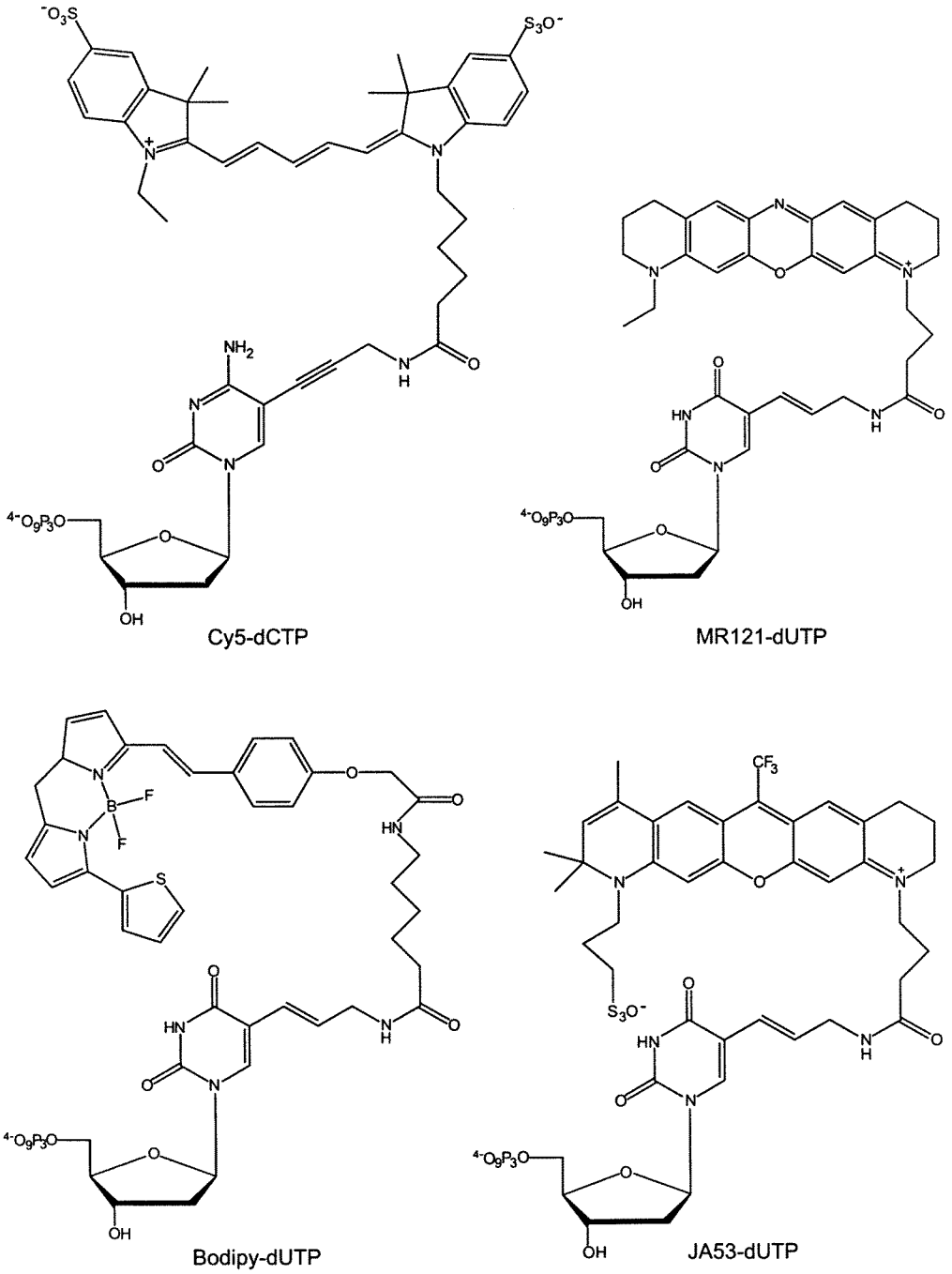

Figure 1. Molecular structures of the labeled mononucleotides Cy5-dCTP, MR121-dUTP, Bodipy-dUTP and JA53-dUTP.

integration time of 625 μs each. The instrument response function of the entire system was measured to be 420 ps (FWHM). The raw data are shown without modification by any filtering algorithm. The solutions for single-molecule experiments were prepared by diluting the stock solutions containing labeled mononucleotides (10^{-6} M) with the appropriate amount of bidistilled water down to the required concentration of 10^{-11} M. The samples were transferred onto a microscope slide with a small depression and covered by a cover glass. Poisson statistics predicts, for a concentration of 10^{-11} M, that the number of molecules fluctuates predominantly between zero and one in the applied detection volume of approximately 2 femtoliters. The probability ($p_{n=1}$) to detect a single molecule during an experiment depends on the specific sample concentration C (molecules/detection volume) and the relative measuring time T_R (integration time/diffusion time) according to the

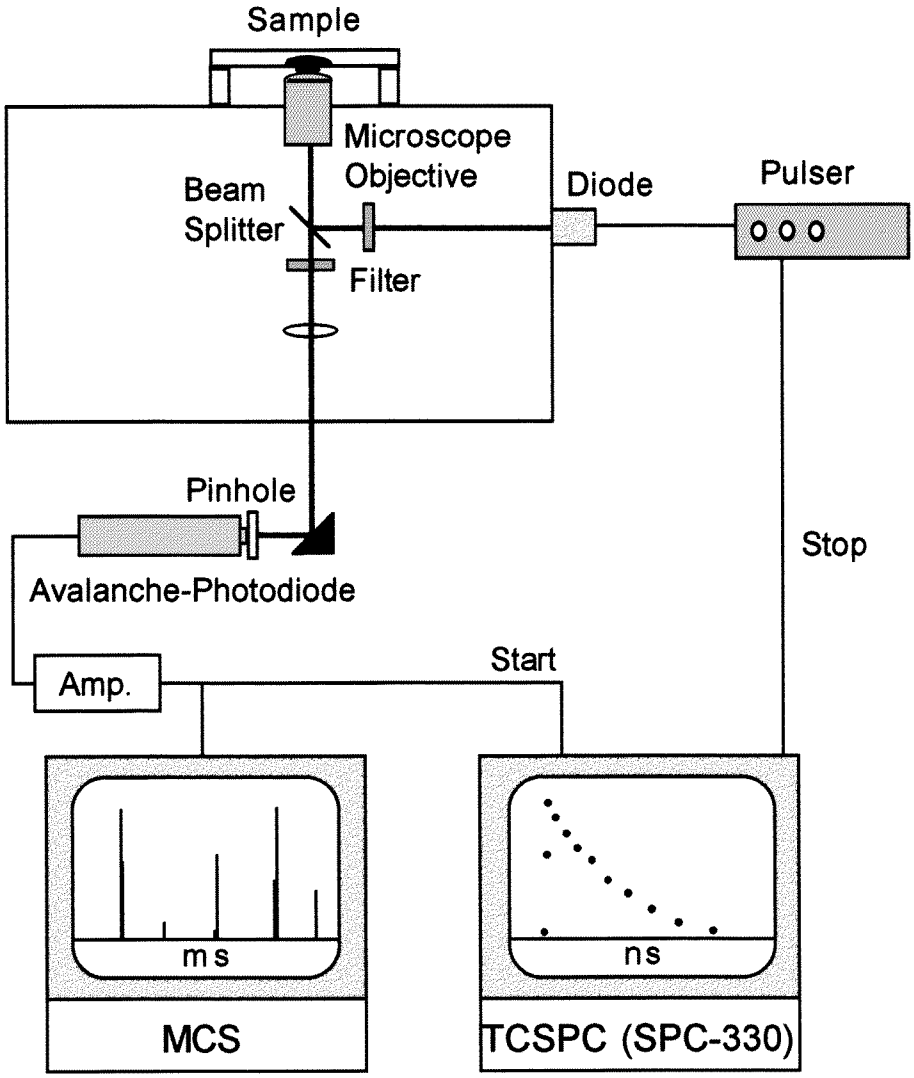

Figure 2. A schematic diagram of the optical and electronic SMD apparatus. The MCS system produces a trace of the accumulated photons per bin (1 μs–50 ms). The SPC-330 system manages the time-resolved data acquisition with 625 μs accumulation time for each fluorescence decay. The complete electronics for TCSPC are housed in a personal computer (PC).

expression:

$$p_{n=1} = C(1 + T_R)\ \exp(-C(1 + T_R)).$$

With a characteristic diffusion time of a labeled mononucleotide molecule through the detection volume of approximately 300 μs and an integration time of 625 μs, we calculate the probability of detecting a single mononucleotide molecule in a 10^{-11} M solution of $p_{n=1} = 0.035$. Hence, we can be sure to observe mainly single events during our time-resolved measurements. On the other hand, we should detect about 20 single molecule events per second using a mononucleotide concentration of 10^{-11} M. The calculated data are in good agreement with the measured burst numbers for all four differently labeled mononucleotide molecules.

The fluorescence lifetime determination of single-molecule events was realized by a monoexponential maximum likelihood estimator (MLE) using the following relation [34]:

$$1 + (\mathrm{e}^{T/\tau} - 1)^{-1} - m(\mathrm{e}^{mT/\tau} - 1)^{-1} = N^{-1} \sum_{i=1}^{m} i N_i \quad (1)$$

where T is the width of each channel, m the number of utilized time channels, N the number of photon counts taken into account, and N_i the number of photon counts in time channel i. The parameters used to determine the lifetimes are $m = 48$, $T = 0.195$ ns.

3. Results and discussion

For the identification of different fluorescent dyes or labeled analyte molecules it is not necessary to collect as many photons as are necessary for an exact lifetime measurement

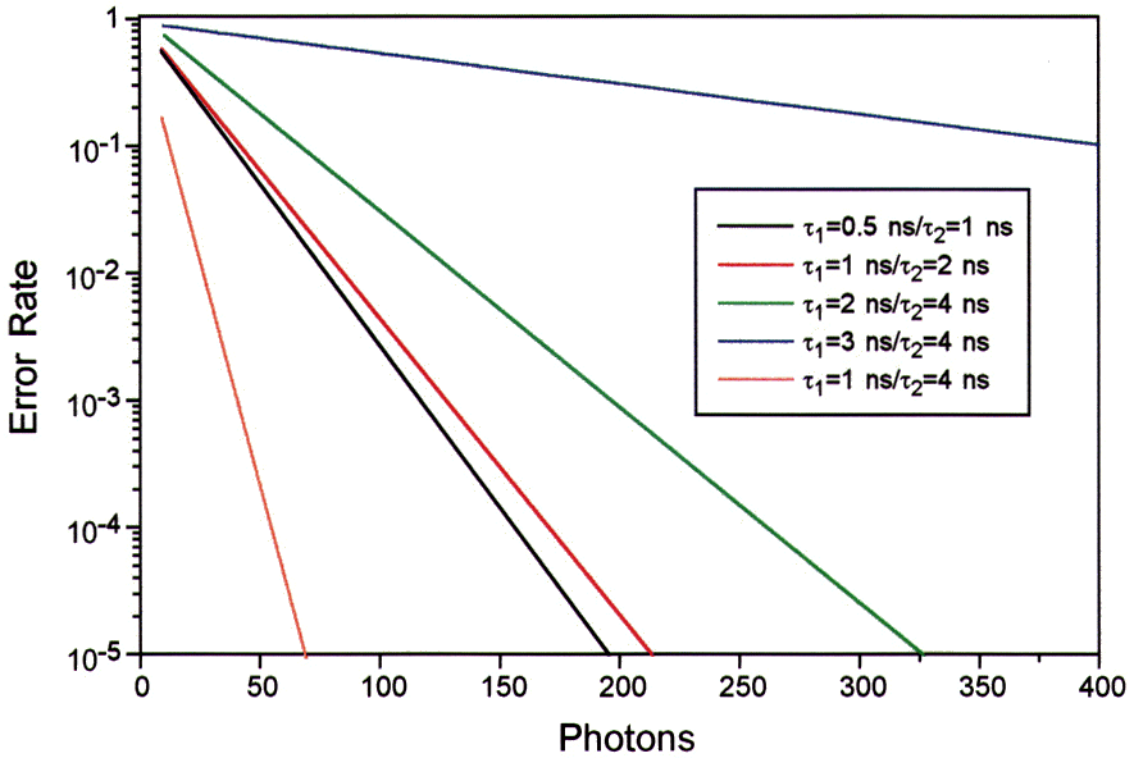

Figure 3. The theoretical prediction of the error rate calculated with equation (2) for the identification of two dye molecules with different fluorescence lifetimes as a function of the number of detected photons. The following experimental parameters were used for the calculation: time window 9.4 ns, 48 channels, 0.195 ns/channel.

with the time-correlated single-photon counting (TCSPC) technique. Instead a much smaller number of photons is sufficient [35–38]. The discrimination is achieved by comparing the raw data with the expected fluorescence decays measured in concentrated solutions with high precision. From a statistical point of view, one is confronted with a problem of classification. An optimal way of classification has already been developed in the framework of information theory and is described in detail elsewhere [36, 37]. For an estimation of how many photons must be collected, if two or more labels with different fluorescence lifetimes are to be distinguished, Cramérs inequality can be used [36]. By this relation an upper threshold for the probability of erroneous classification of a molecule of type j as a molecule of type h is given (equation (2)), where $p_i(j)$ are the probabilities of finding a photon in channel i ($p_i(j) = \exp(-iT/\tau_j)/\sum \exp(-iT/\tau_j)$). m gives the number of utilized time channels and N the total number of acquired photons:

$$P_N(h/j) \leq \left[\min_{0<\nu<1} \sum_{i=1}^{m} p_i(h)^{\nu} p_i(j)^{1-\nu} \right]^N. \qquad (2)$$

For example, at a given number of 50 collected photons per single molecule burst within an applied time window of 9.4 ns (48 channels at 0.195 ns) and lifetimes of $\tau_1 = 2.0$ ns and $\tau_2 = 4.0$ ns, $P_{50}(h/j)$ is smaller than 20%. Figure 3 shows the theoretically predicted error rate in the classification of two dyes with different fluorescence lifetimes as a function of the number N of detected photons. Collecting 50 photons per burst, an identification of individual mononucleotide molecules should be possible with low error rates if the appropriate fluorescent labels are used, i.e. labels with lifetimes of for example $\tau_1 = 0.5$ ns and $\tau_2 = 1$ ns, $\tau_1 = 1$ ns and $\tau_2 = 2$ ns or $\tau_1 = 2$ ns and $\tau_2 = 4$ ns, respectively (figure 3). It should be noted that due to the fixed time window in our experiments $P_N(h/j)$ is not exclusively a function of the ratio τ_1/τ_2. Therefore, using 48 channels with a channel width of 0.195 ns, the error rate in the classification of two shorter fluorescence lifetimes (1 ns and 2 ns) should be smaller than that for the classification of two dyes with longer lifetimes (2 ns and 4 ns). As expected, the error rate drastically decreases if the difference in fluorescence lifetime increases (1 ns and 4 ns in figure 3). Table 1 shows the spectroscopic characteristics of the labeled mononucleotide molecules Cy5-dCTP, MR121-dUTP, Bodipy-dUTP and JA53-dUTP in water. Due to their similar absorption and emission characteristics but unequivocal fluorescence lifetimes they are well suited for time-resolved single molecule identification using pulsed diode laser excitation at 640 nm. From figure 3 it can be seen that at least three of the differently labeled

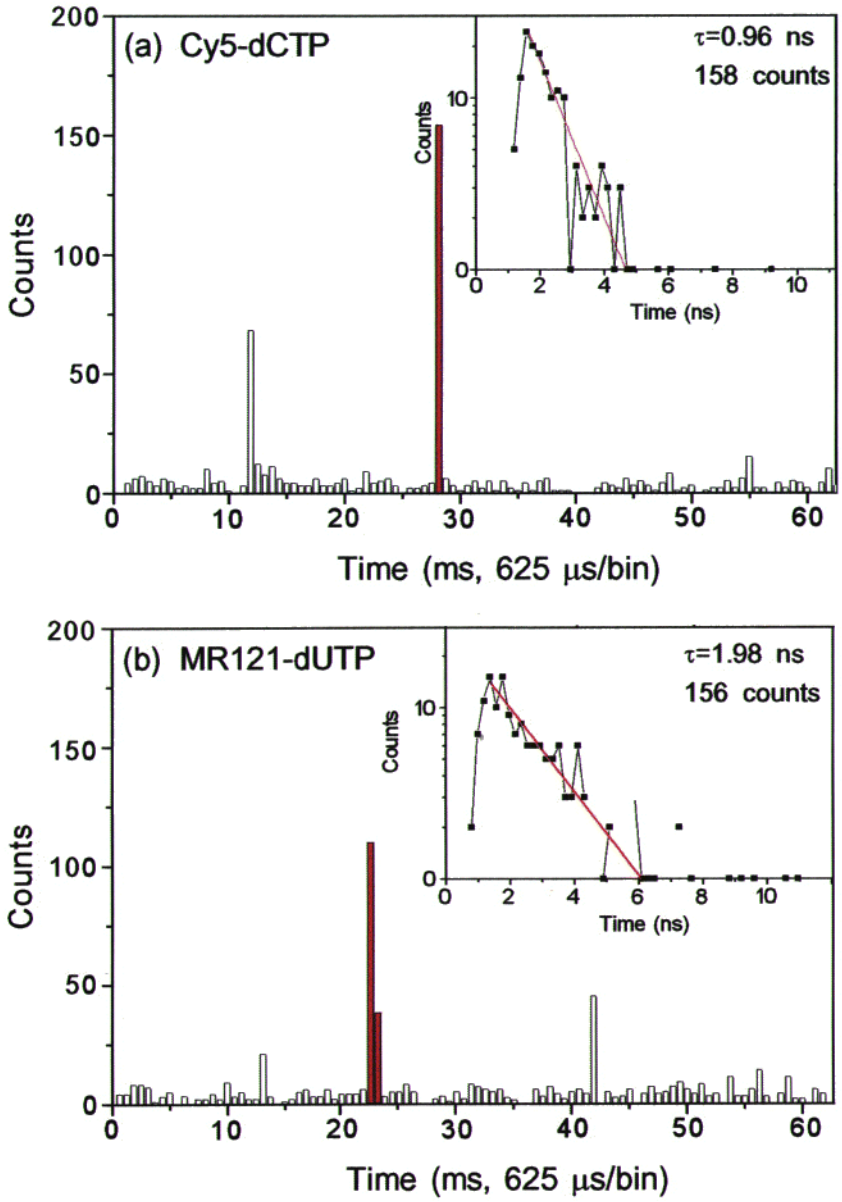

Figure 4. Fluorescence signals observed from an aqueous 10^{-11} M solution of (a) Cy5-dCTP, (b) MR121-dUTP, (c) Bodipy-dUTP and (d) JA53-dUTP recorded with the SMD apparatus and the SPC-330 card with integration times of 625 μs and an excitation energy of 0.63 mW. The insets show the time-resolved fluorescence decays (0.195 ns/channel) recorded during single molecule bursts (marked bins) and the corresponding fluorescence lifetime calculated with the MLE estimator.

mononucleotides should be distinguishable on the single molecule level if the detection efficiency of the used SMD apparatus is high enough.

In figure 4 the time-dependent fluorescence signals (625 μs/bin) observed from 10^{-11} M aqueous solutions of differently labeled mononucleotide molecules, with burst rates of up to 150 photons in 625 μs (240 kHz), are shown. With our experimental set-up an average background level of 1.1 kHz was obtained in pure water, arising mainly from Raman scattered photons passing the emission filter simultaneously with the excitation pulse. On the basis of this background, we calculate signal-to-background ratios of more than 200 for the most intense peaks in our time-resolved experiments. From MCS traces measured with shorter integration times we calculated characteristic diffusion times of approximately 300 μs for single mononucleotide molecules passing the center of our detection volume, i.e. for the higher bursts, independent of the attached fluorescent dye. Hence, with a repetition rate of 56 MHz and a fluorescence quantum yield of Cy5 in water of 28% [39] theoretically up to 4700 photons are available from an individual labeled mononucleotide molecule Cy5-dCTP during the diffusion time through the detection volume. Considering burst sizes of up to 200

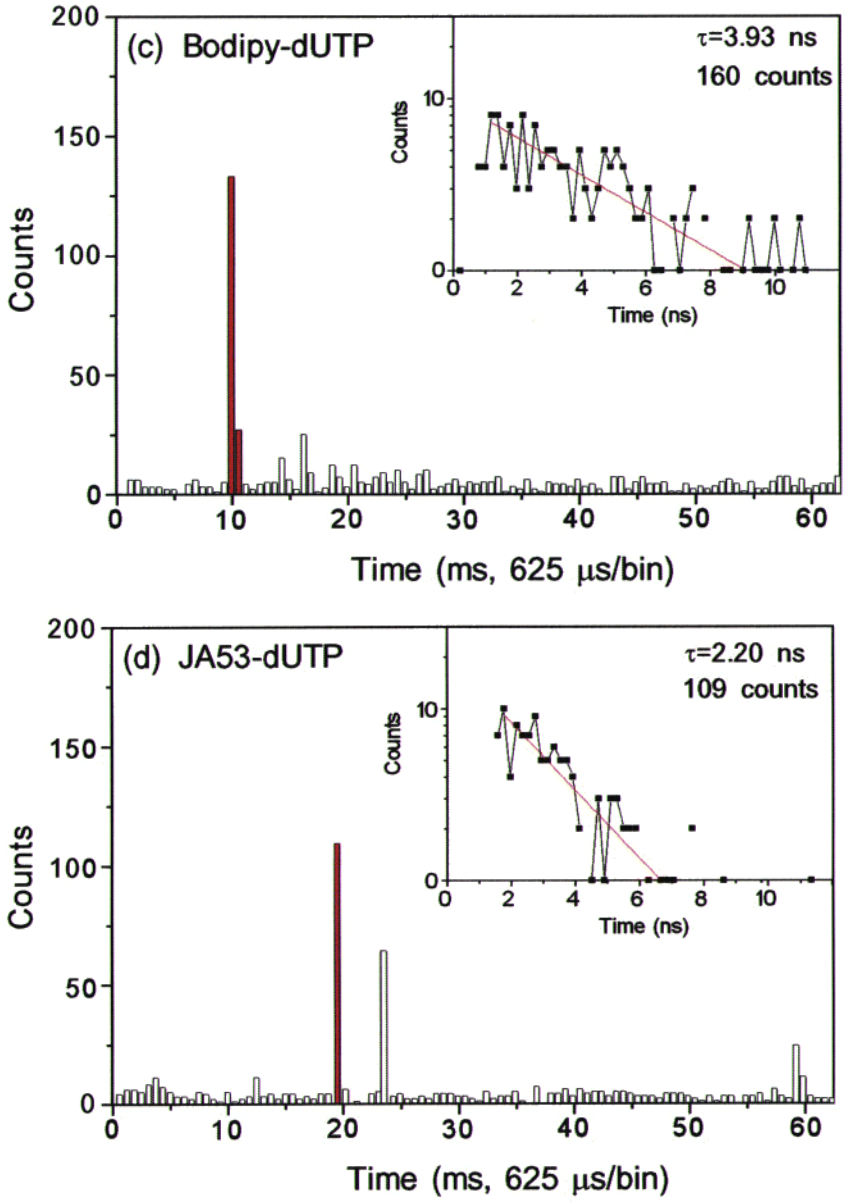

Figure 4. (Continued)

counts, we calculate an overall detection efficiency of about 4% for the experimental set-up. At this point it should be noted that the molecules diffuse in and out of the open detection volume due to their Brownian motion. Therefore, different burst sizes are detected dependent on their trajectory.

We measured more than 250 time-resolved decays for individual mononucleotide molecules on different solutions containing either Cy5-dCTP, MR121-dUTP, Bodipy-dUTP or JA53-dUTP molecules and calculated the lifetimes using the MLE (equation (1)). As can be seen in figure 5 the differently labeled mononucleotide molecules exhibit similar burst size distributions. At this point it should be pointed out that the carbocyanine dye Cy5 exhibits the smallest fluorescence quantum yield but the highest extinction coefficient at 640 nm among the four used dyes. Therefore, similar burst sizes are obtainable.

For further analysis we used only those bursts which contain more than 30 collected photons. Unfortunately, the resulting distribution of measured fluorescence lifetimes (figure 5(c)) is relatively broad in the case of Bodipy-dUTP. This behavior proved to be independent of additional purification steps. Due to the relatively hydrophobic nature of the dye structure, Bodipy-dUTP molecules tend to adsorb on the glass surface of the cover slide in aqueous solvents, thereby changing the fluorescence lifetime. We assume that the relatively broad distribution of measured fluorescence lifetimes for single Bodipy-dUTP molecules results from

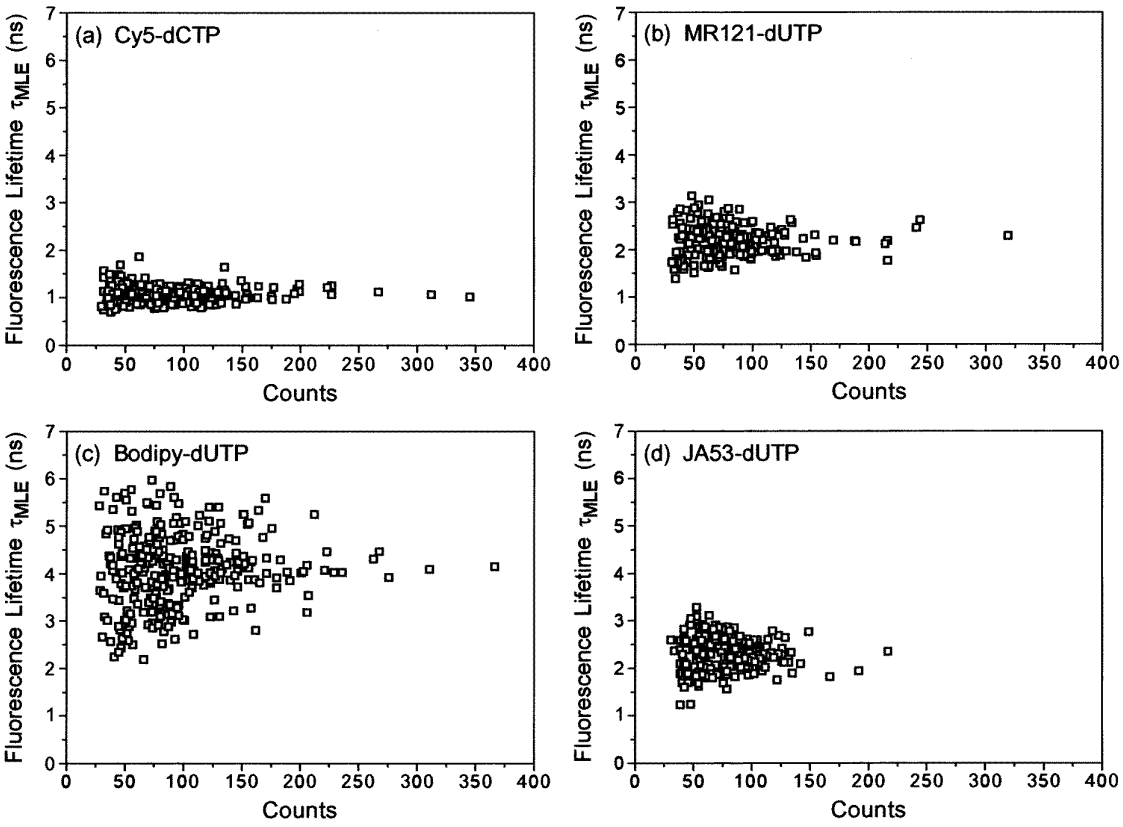

Figure 5. Fluorescence lifetimes obtained by the MLE algorithm for single (a) Cy5-dCTP, (b) MR121-dUTP, (c) Bodipy-dUTP and (d) JA53-dUTP molecules in water against number of detected photons per burst.

Table 1. Spectroscopic characteristics of the conjugates (10^{-6} M) at 25 °C in water. The fluorescence lifetimes τ_{bulk} of bulk solutions (10^{-8} M) were measured with the SMD apparatus. The lifetimes τ_{MLE} for single molecule events were calculated using equation (1). The standard deviation σ_{exp} is derived from the distribution of the lifetimes τ_{MLE} obtained from separate experiments containing only one class of mononucleotide molecules. The standard deviation σ^*_{exp} is derived from the distribution of the lifetimes τ^*_{MLE} obtained from a 1:1:1 mixture. Only those single molecule events with a minimum of 30 collected photons per transit were used for the calculation of the fluorescence lifetimes τ_{MLE} and τ^*_{MLE}.

	$\lambda_{abs,max}$ (nm)	$\lambda_{em,max}$ (nm)	τ_{bulk} (ns)	τ_{MLE} (ns)	σ_{exp} (ns)	τ^*_{MLE} (ns)	σ^*_{exp} (ns)
Cy5-dCTP	651	670	1.04	1.05	0.33	1.05	0.33
MR121-dUTP	668	682	2.10	2.07	0.59	2.04	0.66
Bodipy-dUTP	633	650	3.87	3.88	1.71	3.75	1.65
JA53-dUTP	652	673	2.21	2.24	0.63	—	—

different distances of individual mononucleotide molecules from the glass surface.

To calculate the experimental standard deviation σ_{exp} the obtained fluorescence lifetimes were plotted against their frequency. The resulting histograms (figure 6(a)) were fitted by three Gaussians. Figure 6(a) demonstrates that three differently labeled mononucleotide molecules can be identified on the single molecule level in water with more than 30 photons per burst due to their characteristic fluorescence lifetimes of 1.05±0.33 ns (Cy5-dCTP), 2.07± 0.59 ns (MR121-dUTP) and 3.78±1.71 ns (Bodipy-dUTP). In agreement with predictions, the relative error in the estimated fluorescence lifetimes exceeds the ideal $N^{-1/2}$ limit that holds for N counts, free of background, recorded over an infinite time window. By forming the convolution of the normalized Gaussians a classification probability of 91% is calculated (2% for misclassification of Cy5-dCTP and MR121-dUTP, and 7% for misclassification of MR121-dUTP and Bodipy-dUTP). This result is slightly better than estimated by equation (2) (figure 3). On the other hand, using only the two mononucleotide molecules whose lifetimes differ most, i.e. Cy5-dCTP and Bodipy-dUTP, the

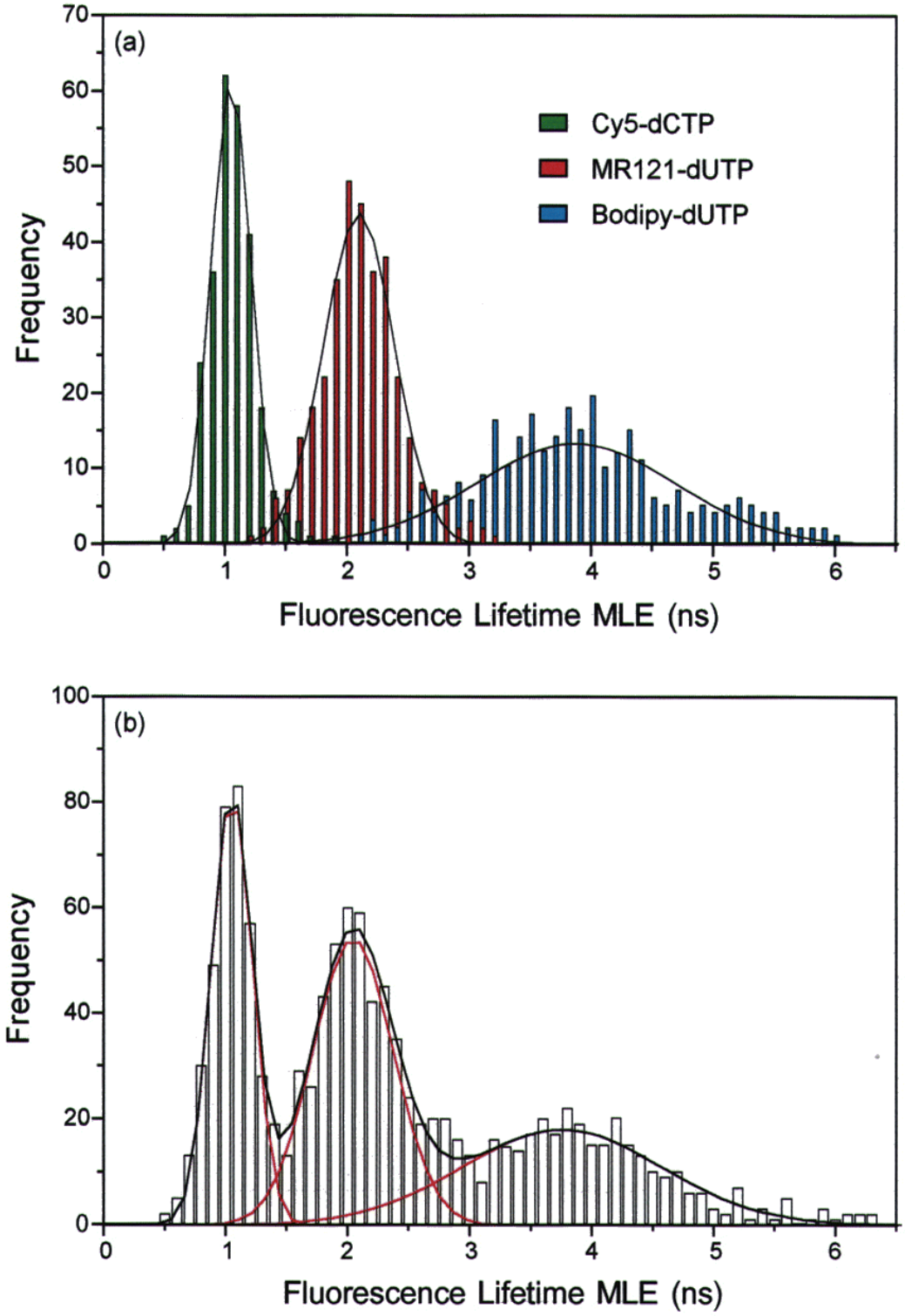

Figure 6. (a) A histogram of the determined lifetimes of single Cy5-dCTP, MR121-dUTP and Bodipy-dUTP molecules in water obtained from separate experiments containing only one class of labeled mononucleotide molecules and the corresponding Gaussian fit. More than 250 data sets of single molecule events were analyzed for each labeled mononucleotide. The fluorescence lifetimes measured on single molecules of 1.05 ± 0.33 ns (Cy5-dCTP), 2.07 ± 0.59 ns (MR121-dUTP), and 3.88 ± 1.71 ns (Bodipy-dUTP) are in good agreement with the fluorescence lifetimes measured in bulk solutions (table 1). (b) A histogram of 1108 measured fluorescence lifetimes of a 1:1:1 mixture of Cy5-dCTP, MR121-dUTP and Bodipy-dUTP molecules 10^{-11} M in water with a minimum of 30 collected photons per single molecule transit and the corresponding Gaussian fits. The fluorescence lifetimes measured on single molecules are 1.05 ± 0.33 ns (Cy5-dCTP), 2.04 ± 0.66 ns (MR121-dUTP) and 3.75 ± 1.65 ns (Bodipy-dUTP).

classification probability is higher than 99%. Hence, single-molecule DNA sequencing is possible with an error rate in the classification of the two mononucleotide molecules of less than 1 error in 100 detected nucleotides. As mentioned above the identification error strongly depends on the number of measured photons and the difference in lifetime (equation (2)).

Additionally, we measured a 1:1:1 mixture (10^{-11} M) of the three differently labeled mononucleotide molecules Cy5-dCTP, MR121-dUTP and Bodipy-dUTP in water. The obtained fluorescence lifetimes (τ_{MLE}) of more than 1000 detected bursts are given as a histogram in figure 6(b). The parameters of the calculated Gaussian fits (peak 1: $\tau = 1.05 \pm 0.33$ ns, peak 2: $\tau = 2.04 \pm 0.66$, peak 3: $\tau = 3.75 \pm 1.65$ ns) are similar to the parameters obtained from separate experiments (compare figure 6(a) with the data in table 1). These results demonstrate that the time-resolved identification of three differently labeled individual analyte molecules is possible with a low error rate (classification probability of approximately 90%), even in a mixture.

In addition, experiments in mixtures reveal information about the composition of the solution. The ratio of the molecules in the sample N_M should be proportional to the peak frequency P ($N_M = (2\pi)^{1/2}\sigma \exp P$) with a deviation of $\Delta N_M = (N_M)^{1/2}$. In our experiment we found a ratio of (65 ± 8):(91 ± 10):(74 ± 9) for Cy5-dCTP, MR121-dUTP and Bodipy-dUTP. For the conjugates MR121-dUTP and Bodipy-dUTP the results are in good agreement with the ratio of the concentrations of the stock solutions. In the case of Cy5-dCTP there is a deviation from the expected value. However, any deviation may well be caused by the dilution steps.

4. Conclusion

We have demonstrated that confocal fluorescence microscopy in combination with pulsed diode laser excitation at 640 nm provides a simple and sensitive tool for detection and identification of individual fluorescently labeled analyte molecules in aqueous solvents. Because of the reduced background on excitation above 600 nm, signal-to-background levels higher than 100 are easily attainable. Two mononucleotide molecules were identified with a classification probability of greater than 99%. The identification of three mononucleotides Cy5-dCTP, MR121-dUTP and Bodipy-dUTP on the basis of the fluorescence lifetimes appears to be the first single molecule identification of more than two different molecules in a mixture. The results presented here demonstrate new possibilities for time-resolved single strand DNA sequencing and other important bioanalytical applications. Excitation in the red region in combination with suited dye molecules offers advantages such as significant background reduction, which means easy sample handling, and allows the construction of an all-solid-state instrument for single molecule detection and identification.

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