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Rapid communication

Detection and identification of individual antigen molecules in human serum with pulsed semiconductor lasers

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Abstract. The fluorescence bursts of individual antibody molecules BM-7 (IgG1) labeled with single dye molecules were detected and identified by the characteristic fluorescence lifetimes of the dyes Cy5 (1.5 ns) and JA169 (2.7 ns) directly in neat human serum. Fluorescence excitation was performed by a short-pulse (FWHM < 400 ps, 50 MHz) semiconductor diode laser. The tumor marker mucine (MUC1) was detected in neat human serum by single-molecule events containing both fluorescence lifetimes indicating specific binding of both antibody molecules (Cy5-BM-7 and JA169-BM-7). The sensitivity achieved allows the detection of antigens at concentrations below 10^{-11} M without separation steps.

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Single-molecule detection in condensed phases [1–8] has many important biological applications, including rapid DNA sequencing, medical diagnosis, and forensic analysis. However, because of the difficulties associated with the background signal, e.g. Raman and Rayleigh scattering as well as luminescence from impurities, only purified solvent systems have been used for single-molecule detection experiments until now.

To identify dye molecules in solution either spectral- or time-resolved fluorescence detection methods can be used. Combining an efficient maximum likelihood estimator (MLE)-algorithm and the pattern recognition technique [9] to time-correlated single-photon counting data the identification of single rhodamine 6G and rhodamine B molecules in water was recently demonstrated [10]. These developments have encouraged several research groups to implement this very sensitive technique for biomedical analysis. Especially the screening for specific antibodies or nucleic acid sequences on the single molecule level is of great importance for early-stage diagnostic of tumor development [11]. Unfortunately, no matter how carefully the solvents for single-molecule experiments are purified, the background due to luminescent impurities sets detection limits [12]. Especially in bioanalytical samples containing buffers, enzymes or biological extracts luminescent impurities can decrease the sensitivity or even prevent the definite detection of individual labeled analyte molecules. Additionally, for an application of these sensitive techniques it would be desirable to detect and quantify analyte molecules on the single-molecule level in their natural environment, i.e. in a simplified clinical procedure. Furthermore the technique should not require the separation of bound from free markers and should be insensitive to background sources naturally present in biological samples.

Using fluorescence correlation spectroscopy (FCS) the binding of fluorescently labeled antibodies or antigens has been successfully demonstrated with analyte concentrations in the nanomolar range [13]. The clinical standard antibody tests utilize enzyme-linked immunosorbent assays (ELISA) which achieve a sensitivity of a few nanogram antigen per milliliter corresponding to a concentration of 10^{-9} to 10^{-11} M. In all these methods the analyte molecules have to be amplified after isolation from the serum by binding to specific materials or different washing-steps are required.

Measurements in human serum, e.g. direct detection and monitoring of individual labeled antibodies and their reactions with antigens, are complicated by the fact that besides anorganic ions and hydrophilic organic substances approximately 100 different proteins are present. Therefore human serum samples are strongly luminescent when irradiated in the blue or green region of the spectrum. Hence, detection of single chromophores such as fluorescein or classical rhodamine dyes is prevented in this environment.

Due to the small number of compounds that demonstrate intrinsic fluorescence above 600 nm the use of near-infrared (NIR) fluorescence detection in bioanalytical samples is a desirable alternative to visible fluorescence detection. This fact as well as the availability of diode lasers for this spectral region has prompted current efforts to use NIR dyes for bioanalytical applications. However, there are few chromophores which show sufficient fluorescence quantum yields in the NIR region, especially in aqueous surroundings [14].

In view of these limitations we strove for a compromise between reduced background and high fluorescence quantum yield. Hence, we used newly developed rhodamine dyes [15] together with the commercially available carbocyanine dye Cy5. These dyes are excitable by diode lasers emitting be-

tween 630–650 nm and exhibit a sufficiently high fluorescence quantum yield in aqueous solutions. Very recently we demonstrated [16, 17] diode laser based single-molecule detection of these rhodamine derivatives in ethylene glycol and water applying confocal fluorescence microscopy. Time-resolved measurements in water using a pulsed diode laser and a MLE for the identification showed that 50 photons collected per individual analyte molecule are sufficient to distinguish two labeled mononucleotides with fluorescence lifetimes of 1.9 ns (MR121-dUTP) and 1.0 ns (Cy5-dUTP) with a misclassification of less than 10% [18].

In this communication we present results obtained by applying a new rhodamine derivative JA169 (Fig. 1a) and the carbocyanine dye Cy5 on direct detection and identification of individual labeled antibody molecules (JA169-BM-7, Cy5-BM-7) and their specific binding to multiple epitopes of tumor associated MUC1 molecules [19], even in undiluted human serum samples.

1 Experimental

In our experiment a pulsed diode laser (637 nm) served as the excitation source. The used system provided pulses of less than 400 ps (FWHM) duration with a repetition rate of 50 MHz. The beam passes an excitation filter (639DF9; Omega Optics, Brattleboro, VT) and entered a conventional microscope (Axiovert 100, Zeiss, Germany) through a back port and was coupled into an oil-immersion objective ($100\times$ NA $= 1.4$; Olympus, Tokyo, Japan) by a dichroic beam splitter (645DRLP; Omega Optics, Brattleboro, VT). Measurements were done with an average laser power of 400 μW at the sample (Fig. 1a). The fluorescence signal was collected by the same objective, filtered by a Raman Edge Cut-off filter (650REFLP, Omega Optics, Brattleboro, VT) and a bandpass filter (675DF50, Omega Optics, Brattleboro, VT) and imaged onto a 150 μm pinhole directly in front of the avalanche photodiode (SPAD; EG&G Optoelectronics, Canada). The signal was amplified and splitted for the PC-adapter counter (CTM-05; Taunton, MA) working as a multi-channel-scaler (MCS) and a 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 integration time of 625 μs each. The raw data are presented without modification by any filtering algorithm.

The instrument response function of the entire system was measured to be 420 ps (FWHM). For preparation of labeled antibodies the dyes JA169 and Cy5 were converted into their N-hydroxy-succinimide-ester. The activated dyes were added to monoclonal antibodies BM-7 in PBS pH 8.3 with a dye/protein ratio of 10:1. Unbound dye was removed by gel filtration (Sephadex G-50). The purification of the conjugates was performed by high-performance liquid chromatography (HPLC). The average number of dye molecules bound to an antibody was estimated spectroscopically. In order to start with a well defined system we used only those antibody fractions which exhibit a dye/protein ratio of 1:1.

The fluorescence lifetimes of bulk solutions were measured with the set-up described above at concentrations of 10^{-8} M (Cy5-BM-7: $\lambda_{abs} = 652$ nm, $\lambda_{em} = 674$ nm, $\tau =$ 1.5 ns; JA169-BM-7: $\lambda_{abs} = 631$ nm, $\lambda_{em} = 654$ nm, $\tau =$ 2.7 ns).

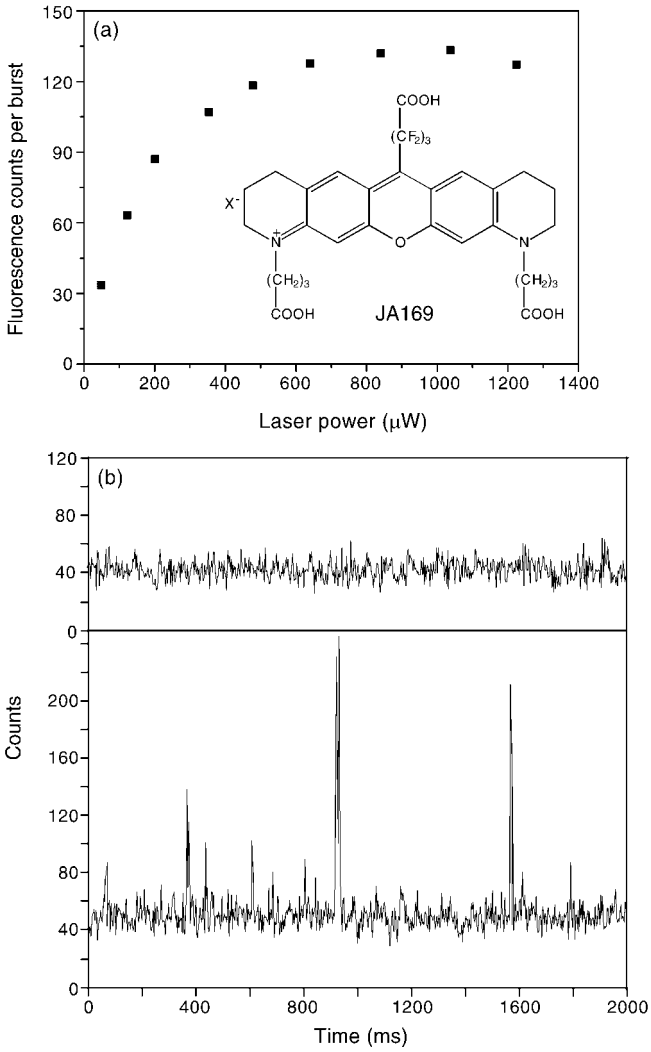

Fig. 1. a Fluorescence counts per single-molecule burst as a function of the average laser power at the sample for JA169 labeled BM-7 antibody molecules 5×10^{-11} M in 1% BSA-PBS (pH 7.4). The measured fluorescence count rates are averaged values of 50 detection events. To obtain as much fluorescence photons as possible without photodestruction of the individual labeled antibody molecules all measurements were done with 400 μW. **b** Fluorescence signals observed from a 10^{-11} M solution of JA169 labeled antibody molecules BM-7 in neat human serum at an average excitation power of 400 μW at the sample (2 ms integration time per bin) using the pulsed diode laser. In the upper section the MCS trace of neat human serum is shown

The solutions for single-molecule experiments (150 μ) were prepared by diluting a stock solution containing labeled antibodies (10^{-6} M) with the appropriate amount of human serum or 1% BSA-PBS buffer (pH 7.4) containing different concentrations of mucine down to the required concentration of 10^{-11} M or 5×10^{-11} M, respectively. The samples were transferred onto a microscope slide with a small depression and covered by a cover glass (0.17 mm thick). The serum samples were prepared by standard procedure for serum preparation for tumor marker analysis and stored at $-20\,^{\circ}$C until use. Unless stated otherwise all measurements were done in neat human serum.

2 Results and discussion

As indicated by the MCS-trace in the upper part of Fig. 1b human plasma exhibits a nearly burstfree background with count rates of approximately 20 kHz if the excitation is performed at 637 nm, i.e. there is a quasi continuum of photons which arise from relatively high concentrations of weakly luminescent or quenched compounds. In the lower part of Figure 1b the time-dependent fluorescence signals of individual BM-7 antibody molecules labeled with JA169 in human serum are shown. The mean molecular transit time through the detection volume of approximately 20 femtoliter was estimated to be 3–4 ms for both, the Cy5 and JA169 labeled antibodies. During this time we go up to 400 photocounts per individual antibody molecule with an average excitation energy of 400 μW at the sample. This result clearly demonstrates that even in neat human serum a real-time detection of individual antibody molecules is possible if the appropriate label in combination with a pulsed diode laser emitting at 637 nm is used.

In Fig. 2a a typical time-resolved photon burst of a 10^{-11} M solution of Cy5 labeled antibody molecules BM-7 in neat human serum is shown. Using an integration time of 2 ms per decay and an antibody concentration of 10^{-11} M the probability for finding more than one event in a measured decay is negligible. As can be judged by comparing the MCS-trace in Figure 1b with the number of background events collected per 2 ms in Fig. 2a the signal-to-background ratio is further increased by a detection window of only 13 ns. We achieved a signal-to-background ratio of over 10 in the time-resolved experiments (integration time 2 ms). In order to identify the individual labeled antibody molecules in neat human serum the luminescence decay of the pure serum has to be taken into account. Three different human serum samples were measured and exhibit a multiexponential fluorescence decay which can be described by a biexponential model with a short lifetime τ_1 of 700 ps and a longer lifetime τ_2 between 3.2 and 3.8 ns. The relative amplitudes of the components vary with different patient serums ($a_1 = 85$–95%, $a_2 = 5$–15%). Hence, we considered the luminescence decay of human serum by a biexponential fit $I(t) = I(0)[a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)]$ with a constant short lifetime of $\tau_1 = 700$ ps and a variable lifetime τ_2 to describe the measured fluorescence decays. Prior to lifetime determination the measured data were deconvoluted from the scatter function. By applying this technique an identification of differently labeled individual antibody molecules is possible due to the different values of the lifetime τ_2 which describes the fluorescence lifetime of the attached label, even in neat human serum (Fig. 2b). The histogram of Fig. 2b was obtained from separate experiments in human serum containing only one class of labeled antibodies (Cy5-BM-7 or JA169-BM-7). Taking only those decays for the construction of the histogram where $a_2\tau_2$ was greater than 0.7, the error of classification in this experiment was less than 10%. If we fit the data of Fig. 2b to a Gaussian we get mean fluorescence lifetimes of 1.6 ns and 2.8 ns which are in good agreement with the data measured in bulk aqueous solutions of 1.5 ns and 2.7 ns for Cy5 and JA169 labeled antibody molecules, respectively.

These results demonstrate the advantage of time-resolved diode laser based single-molecule detection techniques in combination with suitable dye molecules. Due to the reduced background at 637 nm excitation and the fluorescence lifetimes of the dyes of a few nanoseconds a definite detection and identification of individual molecules is possible, even in biological systems such as human serum.

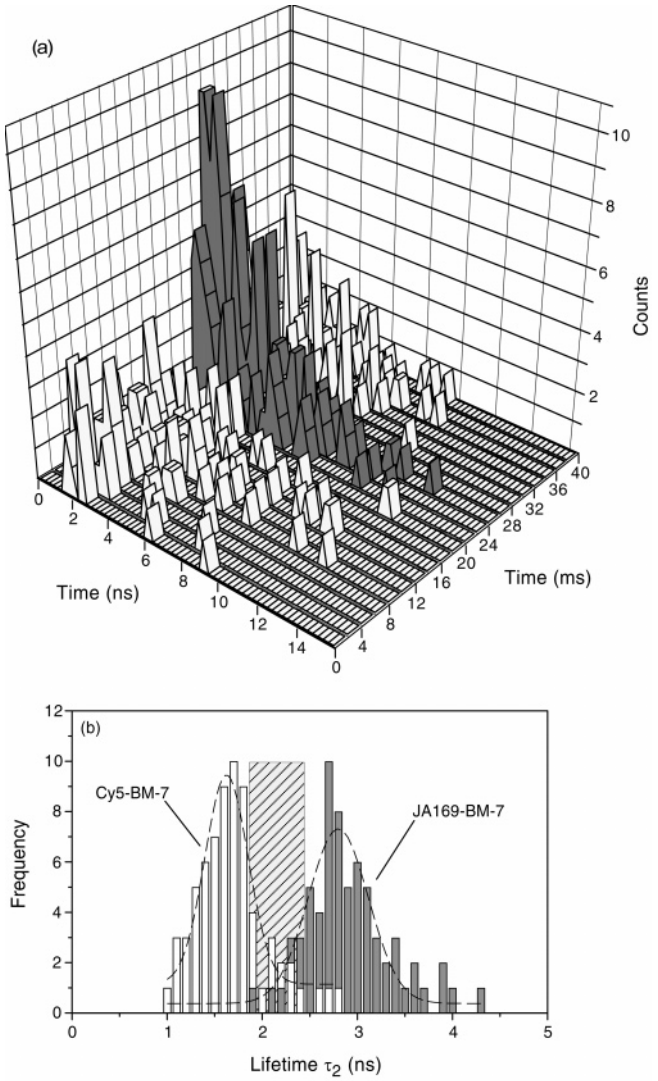

Fig. 2. a Fluorescence decay curve of an individual Cy5 labeled BM-7 antibody molecule (10^{-11} M) in neat human serum with 2 ms integration time per decay using the PC-card SPC 330 (250 ps/channel). The 3D plot shows the time-resolved signals measured during the 2 ms integration time intervals. During the first 20 ms after start of the experiment the decay curves of pure human serum are shown. The passage of a Cy5 labeled BM-7 antibody molecule between 20 and 26 ms is clearly indicated by the measured fluorescence decay. **b** Histogram of the lifetimes τ_2 (ns) of individual BM-7 antibody molecules labeled either with Cy5 or JA169 measured in human serum. The product of the fractional intensity a_2 and the lifetime τ_2 reaches values greater than 0.7 only if a labeled antibody molecule passes the detection volume. For pure human serum $a_2\tau_2$ was determined to be always smaller than 0.4 upon excitation at 637 nm. More than 60 data sets of both Cy5 and JA169 labeled BM-7 antibody molecules were analyzed. The average number of photons used for the calculation was in both cases approximately 120

However, to screen for the early-stage development of breast and ovarian tumors the specific binding of labeled BM-7 antibody molecules has to be monitored on the single-molecule level. The complete monoclonal antibody system for detection of the high molecular weight glycoprotein mucine (MUC1) is [19, 20]. It consists of the antibody BM-7

(IgG1) which is directed against multiple epitopes of tumor-associated MUC1 molecules. MUC1, which shows increased concentrations in serum in the patients with breast or ovarian cancer, has a molecular weight between 360.000 and 430 000 Daltons and multiple epitopes for BM-7 antibodies. The defined normal value of healthy persons is in the range of 20 Units per ml, which is equal to a concentration of 3×10^{-10} M (1 Unit corresponds to 6 ng/ml). The conventional clinical method for tumor marker determination uses an ELISA test which exhibits a sensitivity limit of 7.5×10^{-11} M (5 Unit/ml) mucine. Hence, for an early-stage monitoring of tumor development it would be desirable to develop a fast method without several washing-steps which exceeds the sensitivity of the ELISA test by a factor of 10–100. To be able to detect a mucine concentration in the range of 10^{-12}–10^{-13} M in neat human serum we used the time-resolved fluorescence information by detecting the fluorescence decay of individual analyte molecules. As can be seen in Fig. 2b single antibody molecules Cy5-BM-7 and JA169-BM-7 can be identified due to the characteristic fluorescence lifetime of the attached dyes. As can be seen from the marked area in Fig. 2b only about 20% of the measured single-molecule events exhibit a fluorescence lifetime τ_2 between 1.9 ns and 2.4 ns. On the other hand, a single mucine molecule can bind Cy5 labeled and JA169 labeled antibody molecule BM-7 simultaneously. Hence, the time-resolved fluorescence detection of this individual antigen exhibits a fluorescence lifetime τ_2 which contains both lifetimes 1.5 ns and 2.7 ns. The biexponential fit results in a lifetime τ_2 of 1.9–2.4 ns (marked area in Fig. 2b).

Figure 3 shows a titration of labeled antibody molecules with mucine monitored by time-resolved fluorescence detection of single-molecule events at different mucine concentrations in 1% BSA-PBS pH 7.4. Each data point was calculated from approximately 100 single-molecule events collected during 2 minutes. With decreasing mucine concentration the portion of the single-molecule events which contain both fluorescence lifetimes 1.5 and 2.7 ns (fluorescence lifetime τ_2 between 1.9 ns and 2.4 ns) increases drastically if the mucine concentration is lower than the antibody concentration. The maximum of the titration curve in Fig. 3 is located at 0.1 Units mucine per ml corresponding to a concentration of 1.5×10^{-12} M. This represents a sensitivity nearly two orders of magnitude higher than the clinically used ELISA test. At this mucine concentration each mucine molecule has bound approximately 33 antibody molecules. Further reduction of the mucine concentration results in a smaller portion of biexponential single-molecule events because the epitopes of the tumor-associated MUC1 molecules are saturated by BM-7 antibody molecules. The circles in Fig. 3 represent values observed on serum samples from different patients with a mammary carcinom.

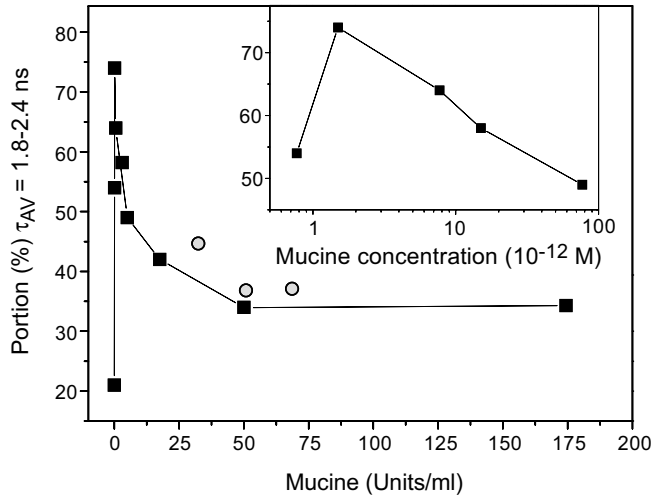

Fig. 3. The rectangles show the titration of a 5×10^{-11} M mixed solution of Cy5-BM-7 and JA169-BM-7 molecules in 1% BSA-PBS (pH 7.4) with mucine. The portion (%) of the measured fluorescence lifetimes τ_2 between 1.9 ns and 2.4 ns versus the mucine concentration shows its maximum at 1.5×10^{-12} M. The circles represent measured values in serum samples from different patients with mammary carcinomes. The amount of mucine in the serum samples was also measured with the ELISA technique. The inset shows an expanded view of the titration curve (0.05–5 Units/ml) and demonstrates the increased sensitivity of our technique compared to the detection limit of the conventional ELISA test of approximately 10^{-10} M MUC1 (corresponding to 5 Units/ml). For each rectangle or circle approximately 100 single-molecule events were measured

The antibody BM-7 used presently in clinical ELISA tests reacts with both, tumor specific and non-specific epitopes of MUC1 molecules. Therefore, each serum sample exhibits mucine concentrations of at least 20 Units per ml serum and an early-stage diagnosis of tumor development is very difficult.

However, new antibodies are under development which react only with tumor specific epitopes of underglycosilated MUC1 molecules which are not present in serum samples of healthy persons. As shown above (Fig. 3) our technique exceeds the sensitivity of the conventional ELISA and allows the detection of tumor specific MUC1 antigen molecules already at much lower concentrations. Hence, an early-stage development of breast and ovarian cancer in neat human serum can be monitored. Besides the early-stage detection of tumor development these results may have important implications in early diagnostic of infections and sensitive analysis of cytokines directly in human serum.

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