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Detection of Individual Oligonucleotide Pairing by Single-Molecule Microscopy

Werner Trabesinger,† Gerhard J. Schütz, Herrmann J. Gruber, Hansgeorg Schindler, and Thomas Schmidt*

Institute for Biophysics, University of Linz, 4040 Linz, Austria

Hybridization of 20mer probe oligonucleotides to complementary, surface-immobilized target oligonucleotides was visualized on a single-molecule basis by fluorescence microscopy. Coincident determination of the positions of both the target and the probe oligonucleotides using dual-wavelength fluorescence labeling allowed for highly reliable discrimination of specifically bound probe molecules from those being physisorbed. The figures of merit of the assay are characterized by the low probability for false positive (10^{-4}) events and the high speed for detection of up to hundreds of different DNA fragments per second. The probability for false negative events is limited by the biochemical binding probability of short oligonucleotides. The potentials and limitations of this methodology for single-cell single-DNA analysis are discussed.

The importance of DNA-based assays for detection of diseases, genome sequencing, forensics, and environmental control is steadily increasing.[1] DNA-based assays are challenged by the detection of trace amounts of DNA fragments, characterized by a specific sequence in a huge background of other molecules and unspecific DNA. Typically (see Figure 1a), in the first step, the fragments of interest are selected from a heterogeneous sample by immobilization via hybridization to a specific *capture probe* anchored to a surface.[1,2] This allows unspecific fragments to be washed out of the detection volume easily. In the second step, these fragments are hybridized with small detection oligonucleotides (*detection probes*) complementary to a characteristic region within the sequence (e.g., significant for a certain disease).[1] The detection oligonucleotides are reporters for the presence of a particular target sequence in the fragment under study. Preferentially, the short probe sequences are fluorescence labeled (commercially available) and detected using an appropriate fluorescence assay.[1−3] Because of the limited sensitivity of currently available commercial instrumentation, however, the final fluorescence detection asks for DNA amplification (PCR) in combination with enzymatic fluorescence amplification of the target signal. Amplification restricts such methodologies to a minimum of about 100 DNA copies per milliliter of sample.[4]

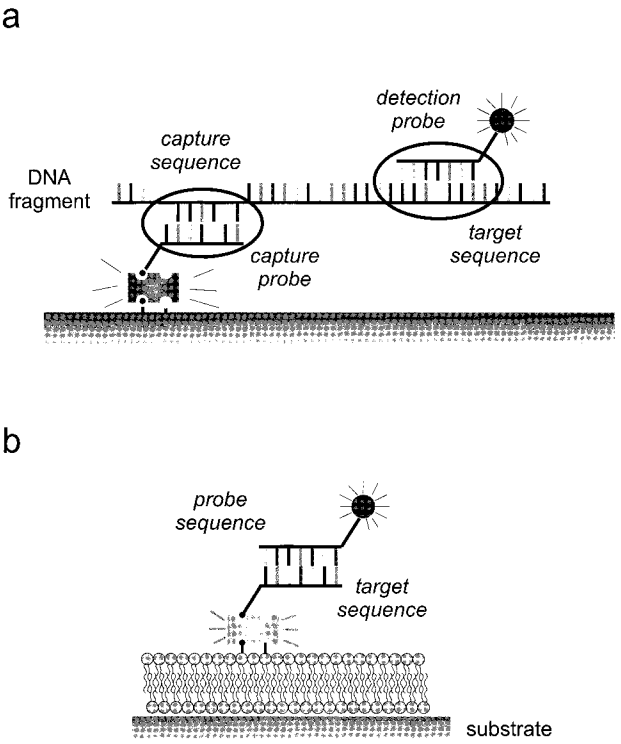

Figure 1. (a) Typical structure of a DNA assay. The DNA (-fragment) is immobilized to a specific, surface-immobilized *capture probe*. Subsequently, the fluorescence-labeled *detection probe* is hybridized to the *target sequence* of interest. (b) Simplified sandwich structure in our experiments. The *target sequence*, a 20mer oligonucleotide, is immobilized via a biotin group to a fluorescence-labeled (λ_{exc} = 514 nm), surface-immobilized streptavidin. Binding of the complementary *probe sequence* is detected as a fluorescence signal from its fluorophore tag (λ_{exc} = 630 nm).

* To whom correspondence should be addressed. Tel.: (+43) 732 2468 9265. Fax: (+43) 732 2468 822. E-mail: thomas.schmidt@jk.uni-linz.ac.at.

† Present address: Institute for Physical Chemistry, ETH Zürich, 8042, Switzerland.

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Increasing the sensitivity of fluorescence detection to the limit of an individual fluorophore would allow for an assay on a single-cell, single-DNA (-fragment) basis without any additional amplification.

The detection of single fluorophores has become possible with state-of-the-art detectors and detection schemes.[5−9] These ultrasensitive fluorescence detection schemes have been successfully applied to a variety of issues in (bio-)chemistry and biosciences, covering such diverse areas as capillary electrophoresis,[10−13] DNA sequencing,[14] molecular mobility studies,[15−17] studies of ligand and protein colocalization,[18−20] and structural rearrangements of proteins[21] and of oligonucleotides.[22,23] Recently, the observations of single-oligonucleotide hybridization in solution by correlation spectroscopy[24] and optical single-molecule fluorescence burst detection in a sheath flow[25] have been reported. Here we show the applicability of single-molecule imaging for detection of small amounts of oligonucleotides immobilized on functionalized surfaces, a configuration used in standard DNA assays. This report is focused on quantitative characterization of the optical detection methodology. For that we chose a simplified version of the general DNA detection scheme (Figure 1b). A biotinylated *target sequence* with a length of 20 bases was immobilized on a streptavidin-coated membrane. This approach ensures reliable assembly of samples with clearly separated target oligonucleotides. Hybridization with the complementary *probe sequence*, being tagged with a single fluorophore, was directly observed on a molecule-by-molecule basis. Combined observation of both target and probe oligonucleotides by dual-wavelength fluorescence imaging allows discrimination of specifically from unspecifically bound probe oligonucleotides. This ability renders the assay highly specific and quantitative.

MATERIALS AND METHODS

Materials. Glass cover slips were coated with phospholipid membranes of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholin (DMPC, Sigma) containing 10^{-6} mol % biotinylated phospholipid (biotin-X-DMPE, Molecular Probes, Eugene, OR) by standard Langmuir−Blodgett technique. Incubation for 2 min with 100 nM tetramethylrhodamine (TMR, λ_{exc}= 514 nm)-labeled streptavidin molecules (S-870, Molecular Probes) yielded a "biotin-reactive" surface with a homogeneous distribution of streptavidin molecules (i.e. biotin binding sites) at a density below 0.1 μm^{-2}. On average, each streptavidin molecule carried 5.3 tetramethylrhodamine fluorophores. Subsequently, the biotin-labeled *target sequences* (5′ biotin-CATAGCACTATAGAACTCTG 3′; Boehringer Mannheim, Penzberg, Germany) were immobilized via a biotin−streptavidin link onto the coated glass substrates by incubation for 10 min at a concentration of 10 nM. At these concentrations, virtually all biotin binding sites on the surface were occupied (K_D = 100 fM[26]).[26] After extensive washing (flushing for 5 min with phosphate-buffered saline), the samples were incubated at room temperature for 10 min with the fluorescence-labeled *probe sequence* (5′ Cy5-CAGAGTTCTATAGTGCTATG 3′, λ_{exc}= 630 nm; MWG Biotech, Ebersberg, Germany) at a concentration of 10 nM. A final washing step largely removed unbound probe sequences from the sample.

Microscopy. The experimental setup used was slightly extended compared to that previously described in detail.[16] A second illumination path consisting of a dye laser, and an acousto-optic modulator was added and its optical axis overlapped with that of the Ar^+ laser illumination path. Areas of 9.6 × 9.6 μm^2 of the samples were observed while the sample was illuminated alternatingly for 5 ms at an intensity of 12 ± 3 kW/cm^2 either with 514-nm light from an Ar^+ laser (C306, Coherent) or with 630-nm light from a dye laser (CR599, Coherent; dye, DCM) using a ×100 objective (PlanNeofluar, NA = 1.3, Zeiss) in an epi-fluorescence microscope (Axiovert 135TV, Zeiss). A $\lambda/4$ plate assured that the excitation light was circularly polarized. The fluorescence was effectively separated from scattered light by appropriate filter combinations (custom-made dichroic and emission filters, Omega). Crosstalk between both wavelength channels was less than 5%.

The fluorescence was collected on a liquid-nitrogen-cooled slow-scan CCD camera system (AT200, Photometrics, equipped with a TK512CB chip, Tektronix, 512 × 512 pixel, pixel size 27 × 27 μm^2) and stored on a PC. Averaging seven frames for each wavelength results in a total illumination time of 35 ms, which made it possible to detect 4000 ± 1100 counts from individual Cy5 fluorophores and 5100 ± 1800 counts from individual fluorescence-labeled streptavidin molecules. At a typical background noise level of 24 counts rms, the signal-to-background noise ratio for single-molecule detection was S/B > 170. Subsequent data analysis determined the lateral position of each signal to within 27 nm by fitting the fluorescence intensity profile to a two-dimensional Gaussian surface.[27] Thus, the distance between any two fluorescence signals was determined with an accuracy

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of $2^{1/2} \times 27 = 38$ nm. Systematical errors due to mechanical instabilities of the setup and chromatic aberrations were less than 17 nm over the whole field of view ($9.6 \times 9.6\ \mu m^2$), as determined by imaging immobilized, heavily stained dual-color fluorescent latex beads.

RESULTS AND DISCUSSION

The sample configuration is sketched in Figure 1b. The biotinylated *target sequence* was immobilized via biotin−streptavidin linkage to a phospholipid membrane on a glass substrate. We have chosen this sandwich-type structure because the surface density of the fluorescence-labeled streptavidin molecules can be reliably controlled. The density of streptavidin molecules was kept below $\sim 0.1\ \mu m^{-2}$ so that clustering could be excluded as observed in previous single-molecule microscopy studies.[19] After hybridization of the target sequence with the Cy5-labeled *probe sequence* (Figure 1), two differently colored fluorescence signals were observed. Both signals were easily distinguished using a dual-wavelength approach. Selective excitation of the tetramethylrhodamine labels (by means of an Ar^+ laser tuned to 514 nm) yields the positions of single, immobilized streptavidin molecules, which is equivalent to locations of target oligonucleotides. Consecutive excitation of the Cy5 fluorescence labels (by means of a dye laser tuned to 630 nm) made it possible to determine the positions of probe oligonucleotides. Hybrids of probe and target sequences are characterized by elementary fluorescence signals for both excitations at identical positions, i.e., within the accuracy of our experiment of 38 nm.

Figure 2 exemplifies different stages of the assay. A $9.6 \times 9.6\ \mu m^2$ area of the sample was imaged while the sample was illuminated for 35 ms by 514-nm (left column, TMR-target excitation) and by 630-nm light (right column, Cy5-probe excitation). Figure 2a,b shows images of a lipid membrane on the glass substrate before addition of fluorescence-labeled streptavidin. As expected, neither target nor probe molecules were observed. The intensity fluctuations of both images are characterized by the background noise of 24 counts rms. After incubation with the streptavidin/probe−oligonucleotide complex, eight target oligonucleotides were observed (Figure 2c). As a control, no fluorescence signals were identified for excitation at 630 nm (Figure 2d). In the final image sequence (Figure 2e,f), obtained after incubation with the labeled probe oligonucleotide, both target and probe molecules were observed. Thus, the assay makes it possible to directly count, i.e., "*digitally quantitate*",[19,28] all bound probe sequences.

One major problem of binding assays is that of unspecific binding. Visual inspection of Figure 2e,f showed that one probe oligonucleotide (Figure 2f, circle) did bind to a target oligonucleotide (Figure 2e, circle), while the other probe in Figure 2f exhibited no counterpart in Figure 2e. In a quantitative analysis of Figure 2e,f, the positions of probe (Figure 2f) and target oligonucleotides (Figure 2e) were determined by fitting a two-dimensional Gaussian surface to the respective fluorescence signals. Given the signal-to-background ratio of the experiments presented, the position of each molecule was determined to an accuracy of 27 nm,[29] yielding a minimal detectable distance of 38 nm. Both molecules marked in Figure 2e,f were closer (estimated distance 16 nm), thus representing a nucleotide-pairing event. The second probe oligonucleotide in Figure 2f has no target sequence as counterpart in Figure 2e. Its nearest-target sequence had a distance of 977 nm. Presumably, it was physically adhered to the substrate. That example shows the potential of the single-molecule dual-wavelength approach: the high lateral accuracy of the applied methodology makes it possible to clearly identify specific binding events.

As mentioned before, the minimal detectable distance between the position of a target and that of a probe oligonucleotide is given by $d_{min} = 38$ nm. This high spatial accuracy makes the assignment of false positives, i.e., probe detection without target colocalization, improbable. For a surface density of target oligonucleotides $n < 0.1\ \mu m^{-2}$, valid for the experiments presented, the probability of accidentally colocalized signals closer than d_{min} is $p_{FP} = 1 - \exp(-\pi n d_{min}^2) \approx \pi n d_{min}^2 < 5 \times 10^{-4}$.[30] False negative results, on the other hand, will occur in cases where probe oligonucleotides are present but not detected. This is governed by two factors: the biochemical binding probability and the optical detection efficiency. The first, equivalent for all assays, is controlled by probe concentration, temperature, buffer conditions, and time of incubation and amounts to $p_b = 1-50\%$ (5% in the current study). The second factor is specific for the method of detection. In the assay presented, it is given by photobleaching of the fluorophores attached to the probe oligonucleotide. At the illumination intensities used, the mean photobleaching time is $\tau_b = 30 \pm 5$ ms.[31] For detection at sufficient signal-to-background ratio ($S/B > 5$), the fluorophore has to be observed for at least $t_{min} = 1$ ms. Given these experimental constraints, the probability for false negative results due to photobleaching is $p^{(p)}_{FN} = 1 - \exp(-\tau_{min}/t_b) = 3 \times 10^{-2}$. Thus, the total probability for a false negative result, p_{FN}, is primarily determined by the biochemical binding probability, p_b, which is identical for all assays. It should be further noted that the parallel approach as presented here allows for screening at high speed. An area of 100 μm^2 and a surface density of 0.1 oligonucleotide/μm^2 is analyzed simultaneously within the illumination time of 30 ms, corresponding to a screening rate of $\sim$300 oligonucleotides/s.

In total, we have analyzed three samples at 310 different positions. In our study, 1334 target sequences and 289 probe sequences were observed. Of the probe sequences, 5% (i.e., 16 observations) were colocalized with a target sequence. The latter value was expected given the incubation time of 10 min (see Materials) compared to that commonly used ($\sim$1 h) in hybridization assays.[2] The high stability of our analysis is demonstrated by the probability for correct colocalization assignment of even all 289 probe oligonucleotides of $(1 - p_{FP})^{289} = 0.87$.

In the current report, we have demonstrated the feasibility of a hybridization-type fluorescence assay on the level of individual fluorescence-labeled oligonucleotides. Implementation of this approach into standard biochemical assays makes it possible to omit the typical DNA amplification in combination with enzymatic

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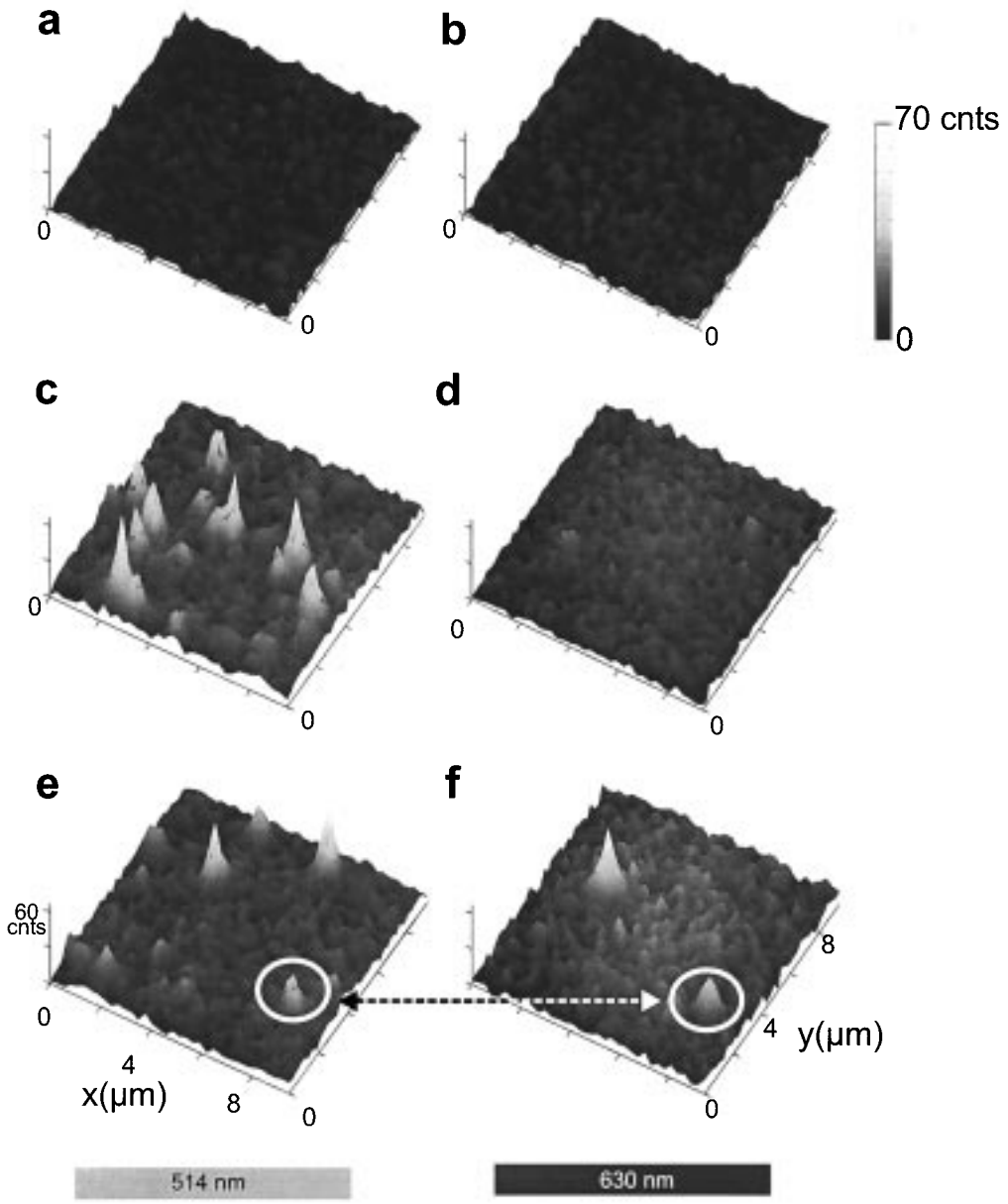

Figure 2. (a,b) Fluorescence signals from a pure phospholipid membrane when excited in the green (a, 514 nm) and red (b, 630 nm), respectively. (c,d) After incubation with the streptavidin/target sequence complex, eight signals are clearly distinguished from the background for 514-nm excitation (c), whereas no signal was observed for 630-nm excitation (d). (e,f) Subsequent incubation with the fluorescence-labeled probe sequence results in additional signals for 630-mm excitation (f). The two oligonucleotide fluorescence signals marked by circles (e,f) were colocalized and thus reflect a successful hybridization event.

fluorescence amplification step(s). Two further advances are to be taken (cf. Figure 1a). The first is implementation of immobilized *capture probes* to bind the DNA (-fragments). This can be achieved by biotin−avidin linkage (as in the current report) or, preferentially, via direct chemical linkage onto the support. These *capture probes* have to carry a fluorophore (the DNA-fragments are unlabeled). The second step is utilization of standard commercially available substrates. Extension of the assay as proposed here to conventional substrates such as wells, filters, and gels, all being optimized for a high degree of specific binding, appears straightforward. It has been shown before that single-molecule detection on such substrates, when prebleached before use, can be achieved.[32] The virtue of a surface-based assay in comparison to volume-type assays [24,25] is twofold. First, as the DNA (-fragments) are selectively immobilized, nonspecific DNA can be largely removed from the detection area, leading to a largely reduced background. Second, the hybridization probe sequences to the DNA (-fragment) are facilitated since the adjacent surface increases the binding probability due to a restriction of dimensionality.[33] In addition, combination of this ultrasensitive fluorescence detection scheme with novel (bio-)chemical approaches as the array technology,[34] fluorescence energy transfer,[35] or extension

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to multiple-wavelength multiple-probe assays[36] may also open new perspectives in high-throughput screening. We think that the technique presented, as being easy to implement into standard assays, has the potential of becoming a standard working ground for easy and fast DNA assays in the future. As it relies on very small detection probes tagged with a single fluorphore, one might speculate that it will be a tool for characterization of short translocations or even point mutations, which are difficult to identify using today's technology.

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