

Probing DNA Surface Attachment and Local Environment Using Single Molecule Spectroscopy

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We have employed single molecule fluorescence spectroscopy, using a total internal reflection geometry and wide-angle detection, to study the attachment of singly fluorescently labeled DNA to a silica surface by either a streptavidin–biotin or a covalent linkage. In both cases the DNA is highly monodispersed with no evidence for aggregation. The covalent coupling gave higher signal-to-noise than the streptavidin–biotin linkage and was therefore studied in more detail. Two components in the photobleaching times, corresponding to different states of the tetramethyl rhodamine probe, were observed: a short and long component with populations in the ratio 6.7:1. Only rarely was interconversion between these two states detected during the 30-s observation time of the experiment. Hybridization experiments using a complementary strand of DNA labeled with a different fluorophore gave a low level of colocalized fluorescence, indicating a significant fraction of the surface attached DNA was not available for hybridization. These results are consistent with the surface attached DNA spending significant time collapsed on the surface.

Introduction

In the past few years, the development of detection techniques has enabled the study of single molecules at room temperature in solution,^{1–3} attached to surfaces,^{4–7} and in lipids^{8,9} and gels.^{10,11} Such studies have revealed previously unseen dynamical behavior and the variation or heterogeneity in a particular molecular property. Detailed information of this kind is not obtainable from studies of ensembles of molecules. Detection methods such as scanning confocal microscopy (SCM),¹² scanning near field optical microscopy (SNOM),¹³ and epifluorescence microscopy (EFM)⁹ are now routinely employed in single molecule detection. However, in the case of SCM and SNOM the techniques tend to suffer from slow scan speeds and for EFM it is only possible to image relatively small areas (10 μm \times 10 μm). Consequently studies involving surface-immobilized molecules have generally been performed one molecule at a time. These studies have probed a variety of molecular properties, including conformational dynamics,^{14–18} ligand binding,^{19–21} and enzyme kinetics.^{7,22,23} However, the sample populations studied have tended to be small (typically a few hundred molecules), making it difficult to observe rare events. The experiments described in this report employ surface-specific total internal reflection fluorescence microscopy (TIRFM)^{24–29} to achieve wide field imaging (up to 100 μm \times 100 μm) with single molecule sensitivity. This enables a large number of single molecules to be imaged simultaneously.

The aim of this work is to use single molecule spectroscopy to study the local environment of individual fluorophore labeled DNA molecules, attached to a surface, by studying the fluorescence and photobleaching properties of the fluorophore. We have used two methods of attachment; the streptavidin–biotin linkage, which has been previously studied by Rigler and

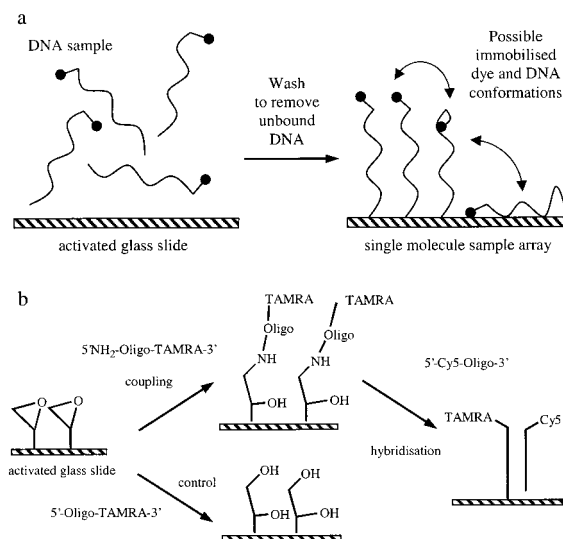


Figure 1. (a) Schematic of the covalent attachment of the DNA to the silica slide. Also shown are some of the possible conformations of the immobilized DNA and the fluorescent probe with respect to the DNA and the surface. (b) Specific chemistry employed in the formation of a covalently attached SMA of DNA.

co-workers^{30–32} and covalent attachment using epoxide–amine coupling. Finally we probed the presentation of the surface attached DNA to other DNA molecules in solution by performing a hybridization experiment. While DNA arrays are now widely used for expression monitoring³³ and genetic analysis, far less is known about the exact presentation and orientation of the DNA on the surface. It may be collapsed flat on the surface or sitting normal to the surface or changing between these two extremes with some characteristic time as schematized in Figure 1a. The presentation of the surface attached DNA to DNA in solution, for hybridization experiments, is an important parameter which needs further investigation and optimization.^{34,35}

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Experimental Method

Preparation of Streptavidin–Biotin Modified Slides. Spectrosil-2000 fused silica slides (TSL group Ltd, Tyne & Wear) were treated with Decon 90 for 12 h, washed in milli-q water (18.2 M Ω), and dried under N₂. A solution of albumin, bovine-biotinamido-caproyl labeled (Sigma) (2 mg/mL, 50 μ L), was applied and the slide was incubated in a humid environment for 2 h, then washed with water, and dried. A solution of streptavidin (Sigma) (1 mg/mL, 50 μ L) was applied and the slide was incubated for 2 h, then washed, and dried. The tetramethylrhodamine (TAMRA) labeled DNA was prepared by using a 5'-biotin modified DNA of sequence 5'-biotin-TCgCagCCgUCCA (Oswel) (U being a propargyl amino uridine) and TAMRA-succinimidyl ester (Molecular Probes). The labeled material was purified by reverse-phase HPLC, dried, and suspended in milli-q water. The concentration was measured by A260 analysis prior to dilution in water to the appropriate concentration (typically 1–10 pM). The dilute DNA (20 μ L) was applied to the streptavidin slide and incubated for 12 h in a humid environment, then washed with water, and dried.

Preparation of Covalently Modified Slides. A single sequence 20mer deoxyoligonucleotide with a 5'-amino group and a fluorophore (TAMRA) at the 3'-end, with a six-carbon linker, was immobilized onto an epoxy silane modified silica chip as shown in Figure 1b. Oligonucleotide concentrations of 45 pM were found to provide suitably resolvable SMAs at densities equivalent to 10⁷ molecules cm⁻². Spectrosil-2000 slides (TSL) were rinsed in >18.2 M Ω deionized water (milli-q), placed in a bottle containing Decon 90 (an alkaline detergent with nonionic and anionic surfactants), and left for 12 h at room temperature. The slides were rinsed with milli-q, placed in a bottle containing a solution of 1.5% glycidoxypolytrimethoxysilane in milli-q, magnetically stirred for 4 h at room temperature, then rinsed with milli-q, and dried under N₂. The oligonucleotide (Biosearch, San Rafael, CA) (5 μ L, 45 pM) was applied in neat milli-q water and incubated for 12 h at room temperature in a humid atmosphere. The slide was rinsed with milli-q water and dried under N₂. An oligonucleotide of the same sequence and fluorophore but without the 5'-amino group was used as a control. The 5'-cyanine labeled sequence was synthesized by using a Cy5 phosphoramidite and used as supplied (Operon, Ca).

The hybridization experiments were performed as follows: TAMRA labeled 20mer (5'-amino-AACCCTATggACggCTg-CgA-3'-C.TAMRA) coupled slides were treated with a solution of complementary oligonucleotide (5'-Cy5-TCgCagCCgTC-CATAgggTT) (5 μ M, 10 μ L) in PBS (100 mM). After an hour at room temperature, the slide was cooled to 4 °C and left for 24 h. The slide was washed with PBS (100 mM, 1 mL) and rapidly dried under N₂. Following construction of the imaging chamber (see below) the cell was primed with PBS (100 mM, 100 μ L).

Sample Cell. A chamber was constructed on the slide by sealing a coverslip (No. 0, 22 mm x 22 mm, Chance Propper Ltd, UK) over the sample area on two sides with prehardened microscope mounting medium (Eukitt, O. Kindler GmbH & Co., Freiburg) while maintaining a gap of approximately 100 μ m between slide and coverslip. The chamber was flushed 3 times with 100 μ L of milli-q water and allowed to stabilize for 5 min before analyzing on the TIR microscope.

Instrumentation. The surface attached DNA was imaged with the instrument shown in Figure 2. This instrument consists of an inverted optical microscope (Nikon TE200, Japan), two color laser excitation sources, and an Intensified Charge Coupled

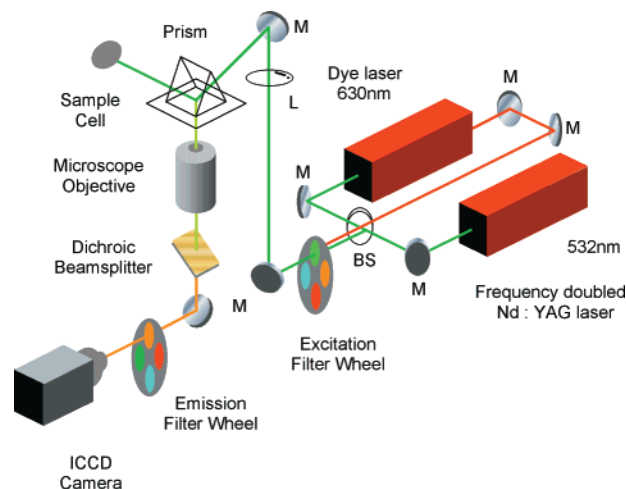


Figure 2. Schematic of the total internal reflection microscope. M = mirror, BS = beam splitter/combiner.

Device (ICCD) camera (Pentamax, Princeton Instruments, NJ). A mode-locked frequency-doubled Nd:YAG laser (76 MHz Antares 76-s, Coherent) was split into two beams to provide up to 100 mW of 532-nm laser light and pump a dye laser (700 series, Coherent) with output powers in excess of 200 mW at 630 nm (DCM, Lambda Physik). The sample chamber was inverted over a x100 oil immersion objective lens and a 60° fused silica dispersion prism optically coupled to the back of the slide through a thin film of glycerol. Laser light was focused with a 20-cm focal length lens at the prism such that at the glass/sample interface it subtends an angle of approximately 68° to the normal of the slide and undergoes total internal reflection (TIR). The critical angle for a glass/water interface is 66°. The footprint of the TIR has a $1/e^2$ diameter of about 300 μ m. Fluorescence produced by excitation of the sample with the surface-specific evanescent wave is collected by the objective, passed through a dichroic beam splitter (560DRLP, Omega Optics), and filtered before imaging onto the ICCD camera. Images were recorded by using synchronized 532 nm excitation with detection at 580 nm (580DF30, Omega) for TAMRA labeled substrates and 630 nm excitation with detection at 670 nm (670DF40, Omega) for Cy5 labeled probes. Exposure times were set between 250 and 500 ms with the ICCD gain at maximum (1 kV). The laser powers at the prism were adjusted to 40 mW at both laser wavelengths. The collection efficiency of objective and filters is approximately 20%, while the 40% quantum efficiency of ICCD and gain of 5.6 counts per photoelectron combine to give an overall detection rate of 0.46 counts per photon.

Analysis Method. The fluorescence from single molecules exhibits distinct spatial and temporal characteristics. We developed a method for counting molecules and assessing the densities of the DNA on the surface using these characteristics. First, fluorescence from a single molecule is confined to a few image pixels and has a narrow intensity profile with a half-width of approximately 0.234 μ m, the width of a single pixel in the experimental configuration. Second, time profiles of the fluorescence exhibit discreet photobleaching steps, which can be quantized by matching the difference in consecutive intensities with criteria that include the threshold level and the standard deviation in the intensity. Background images, which are dominated by thermal noise, show a near-normal distribution of pixel intensities with a mean of 76 cts per 250 ms exposure and standard deviation of 7 cts. A threshold of 6.18 standard deviations from the background mean (the 99.9 percentile for

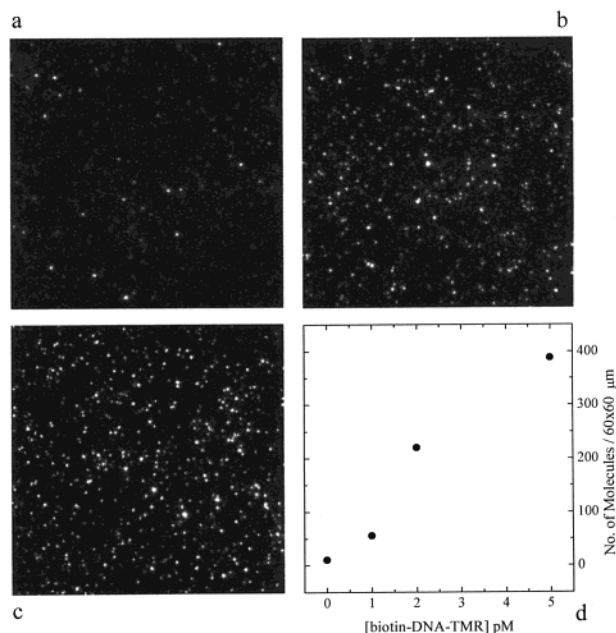


Figure 3. 60 x 60 μm images of biotin/BSA/streptavidin coated fused silica slides prepared with (a) 1 pM, (b) 2 pM, and (c) 5 pM biotinylated 20mer, respectively. (d) The number of molecules dispersed on the surface as a function of initial DNA concentration applied.

a normal distribution) was set to distinguish molecules from the background. The fraction of noisy pixels above threshold calculated from the histogram of the background pixel intensities compared well with 0.1 % expected for a normal distribution. By defining the minimum pixel size of a single molecule as the peak width, the probability of wrongly counting two noisy pixels (false positive) as one or more molecules is only 1 in 10^6 . The threshold set to distinguish fluorescence from noise also determines the probability of missing a weakly fluorescing molecule (false negative). This probability can be estimated from the fraction of the normal distribution of fluorescence intensities from single molecules falling below threshold. For experiments reported here this is approximately 1 in 10^3 . The balance between false positives and negatives can be tuned by varying image integration time and threshold levels. These criteria allow the efficient counting of typically 300–450 molecules per 60 μm x 60 μm on the sample or surface densities of around 0.1 molecules μm^{-2} . Control samples show a small transient population of material nonspecifically bound to the surface at a density of about 0.001 μm^2 or 1% of the typical sample densities.

Results and Discussion

Initially samples were prepared with the established chemistry of the streptavidin–biotin linkage as a benchmark for developing procedures for making covalently coupled samples. Figure 3 shows a series of images of samples prepared with (a) 1 pM, (b) 2 pM, and (c) 5 pM biotinylated 20mer. The number of molecules dispersed on the surface shows a near linear dependence on the initial DNA concentration applied. The concentrations required to achieve comparable coupling levels using the amine/epoxide chemistry are approximately 10-fold higher than for the biotin/streptavidin system. Reactions were performed in milli-q water to minimize contaminants and a low reactivity is expected at pH 7. Concentrations of 45 pM 5'-amino 20mer were found to provide suitably resolvable DNA samples at densities equivalent to 10^7 molecules/ cm^2 as defined in Figure 4. At concentrations 10-fold higher than this, the

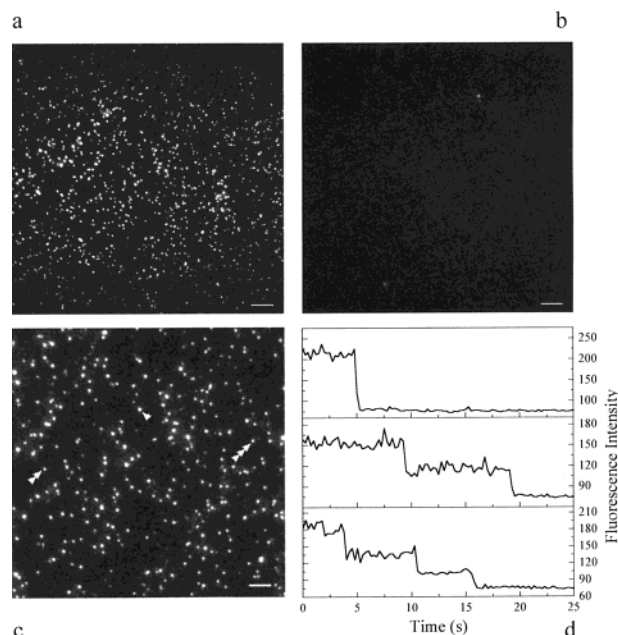


Figure 4. (a) 120 μm x 120 μm images of a typical SMA of TAMRA labeled 5'-amino modified 20mer coupled to an epoxide functionalized glass and (b) a control slide showing near zero nonspecific binding where the background is essentially just the thermal noise of the camera. Exposure time is 250 ms. Scale bars = 10 μm . (c) 60 μm x 60 μm SMA image showing three discrete fluorescent objects (out of 310 objects) containing one, two, and three molecules, as indicated by the number of arrowheads. Scale bar = 5 μm . (d) Photobleaching profiles (100 frames of 250-ms exposures) of the three objects, demonstrating the quantized fluorescence of single molecules.

surface became optically saturated. Saturation in this case is determined by the image magnification and pixel size of the CCD camera. It was also found that the signal levels were approximately 1.6 times more intense from the epoxy coupled surface. The reduction for the protein layers is probably due to two factors. First, the fluorophore molecules are further from the glass surface where the evanescent field is less intense. The field decays exponentially with distance from the interface with a $1/e$ intensity at approximately 100 nm.³⁶ Second, the gradient in the refractive index at the interface, due to the protein layers, will also reduce the field strength. Effects due to the collapse or extension of the DNA at the surface will be minor, since the distance between the fluorophore and surface can only vary between 0 and 7 nm, which will represent a change of less than 7% in the intensity.

An image of a representative region of the 1 cm x 1 cm epoxide coupled DNA sample is shown in Figure 4a. The image shows an overall elliptical fluorescence intensity profile characteristic of the footprint of the laser undergoing TIR. The local variation in intensity between fluorescent signals is due to phenomena such as spectral diffusion, differences in the orientation of the fluorophore, and occasional crossing into a dark state.^{37–39} A control slide treated with a 3'-TAMRA labeled oligonucleotide lacking an amine group gave a greatly reduced fluorescent signal (Figure 4b), indicating the requirement of the amino group for chemical coupling.

To gain insight into the process of surface attachment, the epoxy coupled samples were analyzed to see if there was any evidence for aggregation of the DNA. A characteristic of single molecule fluorescence is that an individual molecule gives a one-step photobleaching profile while two or more molecules show multistep photobleaching. Therefore photobleaching profiles of the molecules on the surface were analyzed. Figure 4c

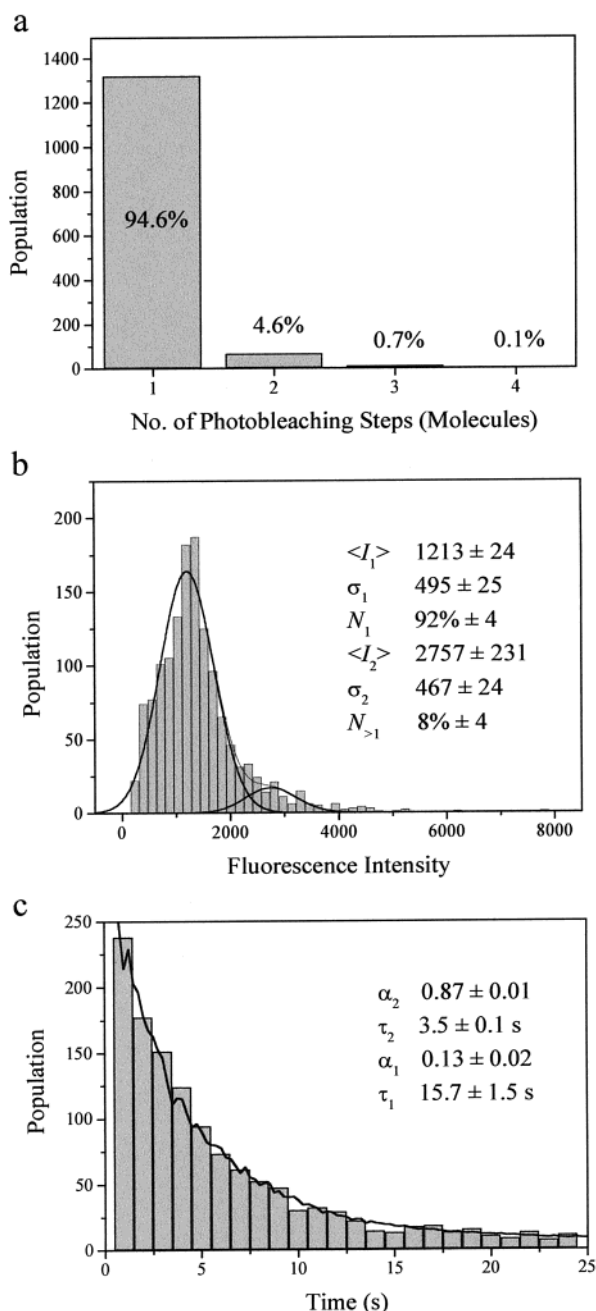


Figure 5. (a) Histogram of the populations of single molecules and multimolecules determined by analysis of the number of photobleaching steps in each fluorescent object. In this case the intensity trajectories of 1405 objects were analyzed. The fraction of single molecules was found to be 94%, with only 6% containing two or more molecules. (b) Histogram of the intensity of 1405 fluorescent objects matching threshold criteria. The distribution was fit with two normal profiles using a nonlinear Levenberg–Marquardt least-squares routine. The populations show 92% of objects with single molecule intensities and 8% with twice the intensity. (c) Histogram showing the photobleaching times of a population of 1318 single molecules. For comparison the photobleaching profile of a saturated surface of the same oligonucleotide substrate is superimposed.

shows a typical $60 \mu\text{m} \times 60 \mu\text{m}$ image of part of the SMA with the positions of fluorescent objects which contain one, two, and three molecules marked by the respective number of arrowheads. The fluorescence trajectory of these molecules as a function of time is shown in Figure 4d. A histogram of the number of steps in the photobleaching profiles of over 1400 molecules is presented in Figure 5a, showing the fraction of two or more molecules to be only 6% under the covalent coupling conditions.

This shows that minimal aggregation of DNA takes place during surface attachment. The sample was further characterized by examining the distribution of the fluorescence intensities of molecules over an image. Presented in Figure 5b is the histogram of the total intensity of all the fluorescent objects on the surface. It is expected from the above photobleaching profile analysis that the distribution will be made up of a large population of single molecule intensities and a significantly smaller population of objects containing two or more molecules. A binormal best fit to the data is shown for guidance, with populations of 92% single molecules and 8% higher intensity objects. An estimate of the fluorescence from a single molecule of TMR⁸ ($I_f = \phi_f \sigma \lambda I_L / hc$) gives around 1000 cts per 250 ms under the experimental laser power densities and detection efficiency. The smaller population has a mean intensity close to twice that of the single molecule distribution, and the number of signals exhibiting intensities from two or more molecules agrees well with that obtained from analysis of the photobleaching profiles. It is important to recognize, however, that the broad width of these two distributions is due in part to the fact that a single fluorophore can exist in states of different brightness as discussed below.⁴⁰ This means that the intensity from a single molecule can exceed that expected from two molecules and visa versa, as seen in the intensity profiles of Figure 4d. The analysis presented does support the observation of a population of single molecules in excess of 90%, demonstrating a high level of monodispersed molecules on the surface.

To probe the local environment of the fluorophore, the photobleaching lifetimes of over 1300 single surface-attached DNA molecules was analyzed. Here analysis on the 94% population of single molecules reveals a distribution of photobleaching times in excellent agreement with the corresponding macroscopic measurement on a surface saturated with the labeled oligonucleotide (Figure 5c). Both single molecule and ensemble profiles show biexponential behavior with a short (3.5 ± 0.1 s) and long (15.7 ± 1.5 s) bleaching time, as determined by a least-squares fit. The respective populations, 87% and 13%, compare well with the populations of the components of the fluorescence lifetime of the TAMRA labeled oligonucleotide measured at a surface.⁴¹ Power dependent studies showed the photobleaching time to be inversely proportional to the laser intensity over a power range between 30 and 60 mW. The results indicate that a large population of molecules spend a longer period in the excited state on each fluorescence cycle, raising the probability of reaction with surrounding labile molecules such as singlet oxygen. A smaller population of fluorophores exists in an environment where the quenching of the excited-state lifetime by 3–4-fold reduces the probability of reaction and raises the survival time by the same degree. Rigler and co-workers have previously studied the photobleaching lifetimes of 102 surface attached DNA molecules, molecule by molecule, using epi-fluorescence.³² Analysis of the death numbers and photobleaching times revealed two characteristic survival times of 1.7 and 8.1 s with a ratio of 4.7, which agrees well with the ratio of 4.5 in our experiment. The absolute values depend on the excitation rates, which for our experiment was approximately one-third of that used by Rigler et al. In addition we observed directly the switching of fluorescence between at least two states. Presented in Figure 6a is the typical fluorescence trajectory of a molecule undergoing photobleaching, along with the trajectories of five molecules (Figure 6b–f) which exhibit interconversion between a number of fluorescent states. The former represents about 98% of the single molecules analyzed. The important features observed in

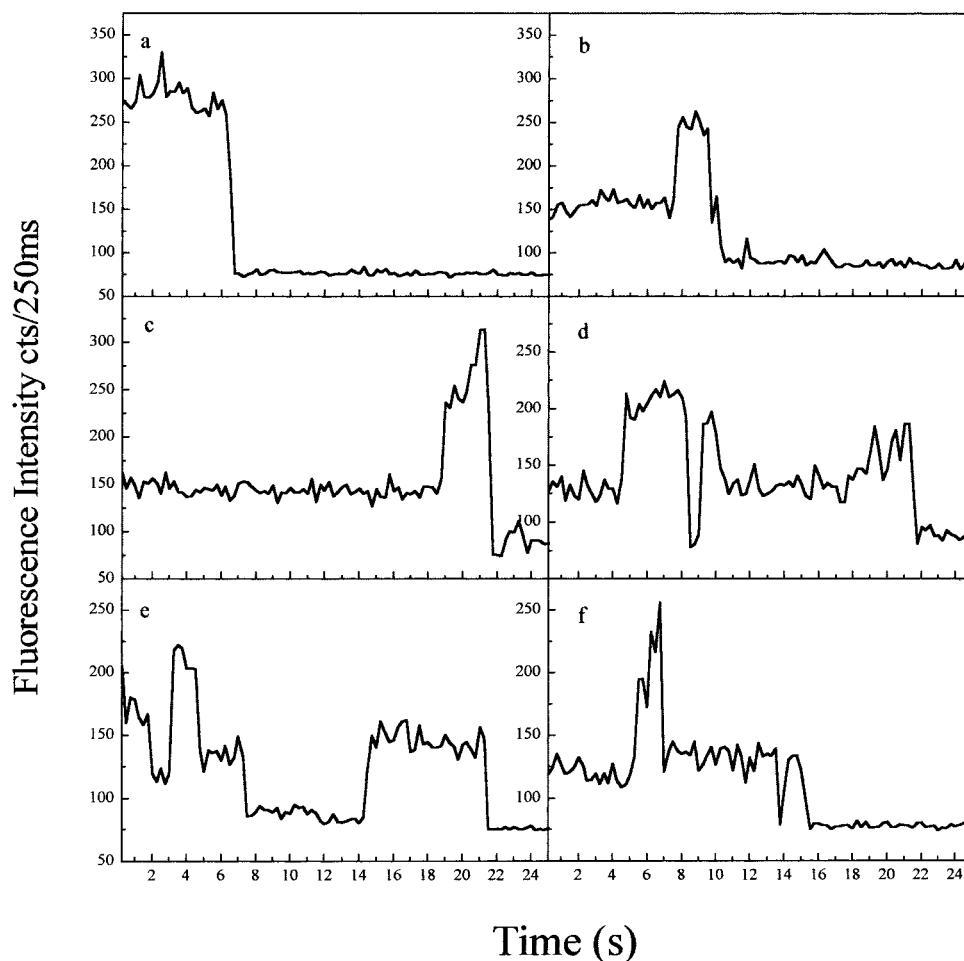


Figure 6. Single molecule fluorescence intensity trajectories of TAMRA labeled 5'-amino-DNA coupled to an epoxide surface. (a) Typical time profile of the intensity of a single molecule undergoing constant fluorescence cycling before photobleaching. (b and c) Intensity trajectories of a molecule existing in a long-lived low fluorescence state before switching to a short-lived high fluorescence state which photobleaches in a single step. (d to f) Examples of molecules which undergo interconversion between (I) a low fluorescence state, (II) a high fluorescence state, and (III) a reversible dark state before photobleaching.

the switching are the discreet changes in intensity between a low fluorescent state, a high fluorescent state, and, in some cases, a nonfluorescent dark state. The increase from low to high fluorescence is approximately 3–4-fold after taking into account the background. The change correlates well with that expected from the ratio of the two fluorescence lifetimes ($\tau_1/\tau_2 = 3.7$) of the normal and quenched states.⁴¹ In general, molecules entering the higher fluorescent state finally undergo photobleaching in a single step, so it is highly unlikely that two molecules (within a pixel area) are contributing to this state. It is evident from the examples shown in Figure 6 that the low fluorescent state will tend to contribute to the long component in the distribution of bleaching times, while the high fluorescent state appears much more labile with a tendency toward short survival times. The findings are consistent with a simple model that contains at least three states, similar to that proposed by Seidel and co-workers.⁴² In our model the TAMRA probe may be (a) internalized in the DNA base stacking, where it will be protected from oxidation by a reduced singlet state lifetime (possible guanine quenching^{32,42}), (b) exposed to an aqueous environment, where the increased excited-state lifetime raises the probability of oxidation and photobleaching, or (c) in a reversible dark state. In the case of DNA in solution the interconversion between these states was on the millisecond time scale.^{42,43} In our experiment less than 2% of the molecules observed undergo slow transitions between the three states, while the majority remain either in the low or the high fluorescent state the entire time. The

population of those molecules exhibiting switching behavior was considered too small to obtain accurate measurements of the relaxation times of the three states. However, values ranging from 1 to 6 s for the high fluorescent state and 6–15 s for the low fluorescent state are evident from Figure 5, while the dark state appears to vary from very short 0.5–1 s intervals up to 8 s. Transitions to the dark state were not reported by Rigler and co-workers and may reflect the difference in the surface attachment chemistry or DNA length used, since the fluorophore and linker length are the same. The authors used a 217 kbse biotinylated DNA bound to a streptavidin protein coated surface, the length allowing greater extension into solution, the protein prohibiting direct interaction of the fluorophore with the substrate. In our experiment the 21mer DNA is directly attached to the surface through only a six carbon atom linker. Our results therefore suggest that the fluorophore is interacting with the surface, leading to very slow interconversion between internal and external states and the formation of a dark state. Since the fluorophore is attached to the DNA at a point furthest away from the surface, it also seems that some of the DNA is also interacting with the surface and is possibly collapsed onto it. Behavior of this kind has been investigated extensively by Weiss and co-workers using polarization spectroscopy on single DNA molecules collapsed onto glass under air and tethered DNA under water. The authors showed that even under liquid, at low laser power densities as used in our experiments, spontaneous dipole rotational jumps typically occur every 11 s and suggested

this may be due to thermally activated conformational changes.^{38,44,45} In the case of DNA in solution, the available thermal energy can be channelled entirely into conformational changes resulting in the fast millisecond dynamics as observed by Seidel et al.⁴² At a surface, thermal energy will be partitioned between conformational change and desorption processes, depleting the available energy required to surmount any one activation barrier and hence reducing the rate of conversion between states. Experiments performed over a controlled temperature range will provide greater insight to the number and magnitude of activation barriers separating the different fluorescent states observed.

To determine if the molecules are arrayed in a biologically functional manner, hybridization experiments were carried out. To date there has only been one single molecule study of DNA hybridization by Schmidt and co-workers, where target DNA was anchored to a lipid bilayer via multifluor labeled streptavidin.²¹ In our work a covalent SMA of the TAMRA labeled oligonucleotide was incubated with its 5'-Cy5 labeled 20mer complement (Figure 1b) and a dual color excitation/detection scheme employed to spectrally resolve the two fluorescent labels. A combination of 532 nm excitation and 580 nm detection was used in the case of TAMRA and 630 nm excitation with 670 nm detection for the Cy5 complementary label. The detection light path was also extended to include an automated filter wheel, which reduced the projected imaged area on the ICCD to 90 μm x 90 μm . In this configuration a pixel width corresponds to 0.176 μm . Figure 7a shows a resultant overlay of colored raw images of the TAMRA labeled 20mer SMA (yellow) and Cy5 labeled complement (red), which are presented in parts b and c of Figure 7, respectively. The overlay allows identification of colocalized TAMRA and Cy5 labels (orange signals). To emphasize the excellent signal-to-noise ratios ($>30:1$) that can be achieved in these experiments Figure 7e,d displays 3D projections of a small region of the SMA where two hybridization events have occurred. Discrete single molecule, fluorescent peaks are clearly observed on both channels. Significantly, the two Cy5 signals from the complementary target DNA only appear where there is a corresponding TAMRA signal from the surface attached probe DNA. To locate the center of the fluorescence peaks, we simply determine the expectation values of the peak coordinates $\langle r(x,y) \rangle = \Sigma(r)r(x,y)/\Sigma(r)$, from which we obtain an average two-channel peak separation of 68 ± 43 nm. The probability of two molecules being randomly located within this distance is only 1 in 700 or approximately 0.2%.^{46,47} In fact, of the total number of TAMRA and Cy5 fluorescence peaks, the number colocalized within the subpixel separation is some 20 times higher than expected in the absence of hybridization. At present the levels of hybridization are low, 5% of the visible TAMRA molecules and 21% of the visible Cy5. The TAMRA labeled DNA in this case is covalently bound to the glass slide, while the Cy5 labeled complement, if not hybridized, will only constitute a small physisorbed fraction after flushing, such that the number of TAMRA molecules detected should always be in excess. The results suggest that the immobilized DNA has limited accessibility to the DNA in solution and is consistent with it being collapsed on the surface. Application of single molecule assays of this type clearly requires the development of attachment strategies that allow optimal presentation of probe molecules at the surface to target molecules in solution.

Conclusions

We have shown that by performing global analysis on a large number of surface attached molecules, using single molecule

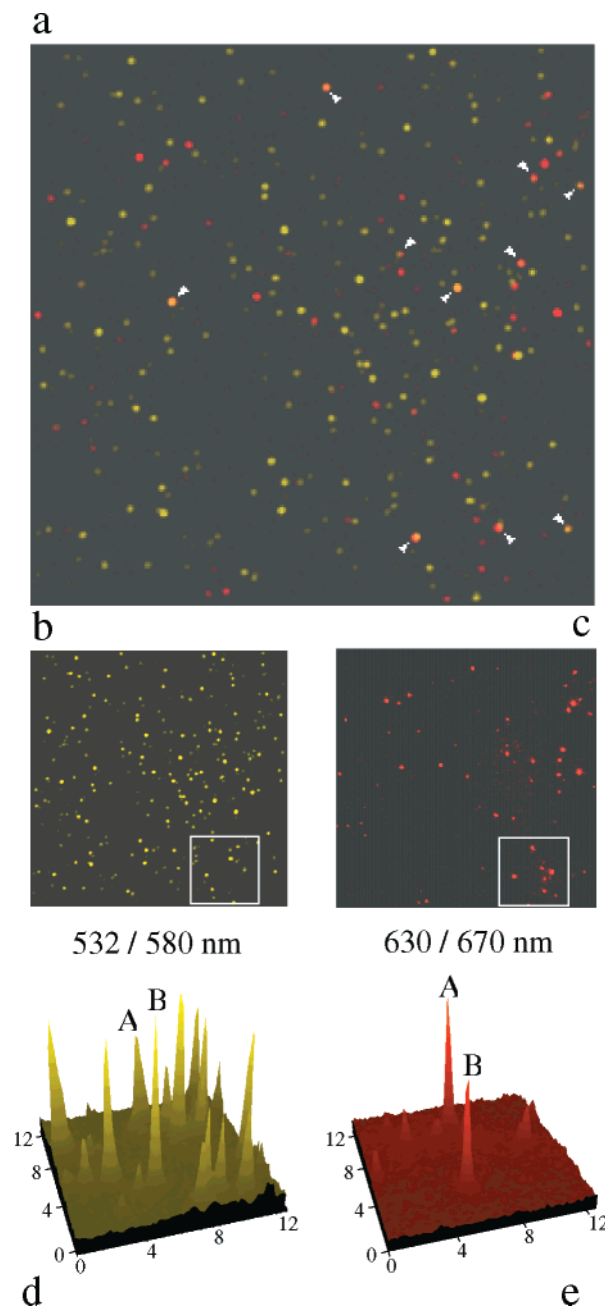


Figure 7. 45 μm x 45 μm images of a hybridization experiment. (a) TAMRA (yellow) and Cy5 (red) channels overlaid to show colocalization (orange) of hybridized strands (arrowed). (b) TAMRA labeled 20mer coupled to a glass slide under 532-nm excitation and 580-nm detection. (c) Cy5 labeled complement excited with 630 nm and detected at 670 nm. The number of surface-bound TAMRA labeled oligonucleotides is $N_{\text{TAMRA}} = 203$, the number of Cy5 labeled complements $N_{\text{Cy5}} = 47$, and the number colocalized is $N_{\text{Colocal}} = 10$. (d) 3D representations of a subsection (12 μm x 12 μm) of the hybridization experiment showing the distinct single molecule peaks. TAMRA channel (532/580 nm) showing a small cluster of single 20mer oligonucleotides. (e) The same area on the Cy5 channel (630/670 nm) showing only two complementary strands. The peaks A and B indicate those molecules that are colocalized with maxima separated by less than a single pixel.

fluorescence, it is possible to gain information about both the local environment of the fluorophore and the availability of the DNA to molecules in solution. The majority of the molecules are monodispersed with no evidence of aggregation. Photobleaching studies on a large number of individual, covalently immobilized DNA molecules showed that the fluorophore exists

in three states with very little interconversion between these during the 30-s observation time of the experiment, in marked contrast to DNA in solution. The fluorophore therefore appears to be interacting with the surface. In addition, it was found that only a small fraction of the immobilized DNA is available for hybridization with DNA in solution. These results are consistent with the surface attached DNA spending significant time collapsed on the surface.

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