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Detection of single DNA molecules by multicolor quantum-dot end-labeling

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ABSTRACT

Observation of DNA–protein interactions by single molecule fluorescence microscopy is usually performed by using fluorescent DNA binding agents. However, such dyes have been shown to induce cleavage of the DNA molecule and perturb its interactions with proteins. A new method for the detection of surface-attached DNA molecules by fluorescence microscopy is introduced in this paper. Biotin- and/or digoxigenin-modified DNA fragments are covalently linked at both extremities of a DNA molecule via sequence-specific hybridization and ligation. After the modified DNA molecules have been stretched on a glass surface, their ends are visualized by multicolor fluorescence microscopy using conjugated quantum dots (QD). We demonstrate that under carefully selected conditions, the position and orientation of individual DNA molecules can be inferred with good efficiency from the QD fluorescence signals alone. This is achieved by selecting QD pairs that have the distance and direction expected for the combed DNA molecules. Direct observation of single DNA molecules in the absence of DNA staining agent opens new possibilities in the fundamental study of DNA–protein interactions. This work also documents new possibilities regarding the use of QD for nucleic acid detection and analysis.

INTRODUCTION

Over the past several years, single-molecule experiments have provided new insights into the dynamics and function of DNA. The physical properties of this natural polymer have been investigated in detail, and there is a growing interest in understanding its interactions with proteins (1). Single molecule studies of DNA typically involve enzymatic reactions along extended molecules. These molecules are stretched by anchoring one end so that individual DNA segments can be extended and manipulated by various small forces such as electric or dielectric force, viscous drag, surface tension, magnetic force or optical force (2). One method for manipulating DNA is to attach latex or magnetic beads to the molecules. The movement of these beads also allows one to deduce the magnitude of the force exerted on the molecules. Another approach consists of visualizing the entire DNA molecule after staining with a fluorescent dye such as the cyanine dimer YOYO-1 or the groove binding agent SybrGreen. The processes of condensation, denaturation and cleavage of the DNA shorten the molecules sufficiently to be visible, thereby permitting the observation of the action of a protein without visualizing the protein itself. This approach has been used to study chromatin assembly (3), digestion by exonucleases (4) and endonucleases (5), and denaturation induced by DNA helicases (6).

In the absence of DNA staining organic dyes, observation of enzymatic activity is still possible. A helicase activity has been studied by using a combination of a surface immobilization scheme and fluorescence resonance energy transfer between two short end-labeled oligonucleotides (7). Another approach consists in observing the incorporation of fluorescently

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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labeled nucleotides. This has been done in two separate studies for DNA (8) and for RNA (9) polymerases. In both studies, DNA was elongated in a process resembling molecular combing. This commonly used technique extends DNA molecules linearly on a modified glass surface using a receding meniscus (10). This method has proven extremely useful for molecular cytogenetics or DNA replication studies. Nevertheless, the level of overstretching (which can reach 50%) and the way the DNA molecule is attached to the surface are critical factors when combed DNA molecules are used as substrate for DNA binding proteins. We have recently devised a method derived from molecular combing whereby a DNA molecule, one end of which is attached to a surface, is elongated by a water stream. The other end also eventually attaches to the surface (11) resulting in an elongated, but not overstretched molecule, the majority of which is free of the surface.

This study and others emphasized two main drawbacks of the use of DNA staining agents. The first is a decrease of fluorescence over time (photobleaching). This process results in the release of free radicals which induce cleavage of the double-stranded DNA molecule. Although the duration of fluorescence can be extended by reducing light intensity and/or using oxygen radical scavengers, dynamic studies of DNA-protein interactions require high illumination intensity and long observation times to achieve both spatial and temporal resolutions. The second drawback is that the presence of these dyes results in changes in the electrostatic, structural and mechanical properties of DNA which are likely to modify its interaction with proteins. Enzymatic inhibition has been reported for restriction endonucleases (5,12) or exonucleases (4). Moreover, these dyes are flushed away from DNA under sodium and magnesium concentrations consistent with enzymatic activity (13). These limits constrain the use of this labeling method for DNA-protein interaction studies.

Direct observation of a fluorescently labeled protein acting on dye-free DNA has been reported only twice to our knowledge (14,15). In the first study, the movements of an RNA polymerase were examined on a bundle of electrically aligned DNA molecules. Sliding of the proteins along the DNA molecules resulted in the deviation of proteins from bulk flow. In the second study, total internal reflection microscopy was used to observe the interactions of an RNA polymerase with a DNA molecule which was positioned and elongated on an engineered surface by using two optical traps. The complexity of these experimental approaches plausibly explains why they were not further exploited. Visualization of fluorescently labeled proteins interacting with combed DNA molecules would present an interesting alternative if performed in the absence of DNA staining agents.

In this paper, we report a new strategy for localizing single DNA molecules in the absence of DNA-binding organic fluorophores. The DNA molecules are labeled with biotin or digoxigenin at both ends before they are combed onto a glass surface. The detection scheme uses streptavidin- or antibody-conjugated fluorescent quantum dots (QD). The use of two different colored labels provides information regarding the orientation of DNA molecules. Scanning of the surface and selection of spatially concordant QD pairs can be used to detect DNA molecules in the absence of organic dye.

MATERIALS AND METHODS

DNA preparation

The following primers were synthesized by Eurogentec (Seraing, Belgium): 5'-CGCTTGGTCTCTGGGACGGTAT-CAGCTCACTCAAAG-3' (fwB), 5'-CGCTTGGTCTCTCTGGCGGTATCAGCTCACTCAAAG-3' (fwD) and 5'-GGC-GATAAGTCGTGTCTTAC-3' (rv). Primer fwB carries a biotin at the 5' end and primer fwD carries a digoxigenin. Their concentration was calculated using a nearest-neighbor model for absorption coefficients. DNA fragments containing multiple biotin or digoxigenin were synthesized by PCR by mixing the fwD or fwB, respectively primer and the rv primer (1.6 μ M each) in 50 μ l of Taq buffer (Promega) with 2 mM $MgCl_2$; 50 μ M of dATP, dCTP and dGTP each; 33 μ M of dTTP; 17 μ M of biotin-dUTP or digoxigenin-dUTP, respectively (Roche); 10 pg/ μ l of pBluescript SK+ as a template; and 0.1 U/ μ l of Taq polymerase (Promega). After 30 cycles of amplification in three stages (30 s at 94°C, 30 s at 61°C and 1 min at 72°C, increasing the last stage by 10 s per cycle) and a concluding extension of 10 min at 72°C, primers and unincorporated dNTP were removed using PCR purification kits (Qiagen). Then the PCR products were digested overnight at 50°C with 50 U BsaI (New England Biolabs) in a total volume of 50 μ l. The modified extremities and the non-digested fragments were removed using anti-digoxigenin- or streptavidin-coated magnetic beads (Dynabeads) for the biotin- and digoxigenin-modified fragments, respectively. The labeled fragments were then ethanol precipitated and quantified on an agarose gel containing ethidium bromide. The number of biotin or digoxigenin incorporated into the DNA fragment should be 75 based on a best efficiency calculation. This number was corroborated by measurements obtained with a dot-blot assay, as described in (16). Briefly, different concentrations of the DNA fragments that contained biotin or digoxigenin were spotted on a nylon membrane (Hybond N+, Amersham). Biotin or digoxigenin were detected with streptavidin or anti-digoxigenin linked to alkaline phosphatase (Roche), respectively. Alkaline phosphatase was then detected by a colorimetric assay using nitro-blue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche). The number of incorporated modified nucleotides was evaluated by comparison with various dilutions of a standard sample of known concentration, which consisted of either an oligonucleotide or the same DNA fragment carrying a single biotin or digoxigenin attached to its 5' end. This assay provided a number of incorporated modified nucleotides between 50 and 100. A fragment that contained fewer biotin was also prepared using the same protocol except that the PCR was carried out in the presence of 50 μ M dTTP and 2 μ M biotin-dUTP, i.e. the solution contained nine times less biotin. The dot blot assay provided evidence for 5–10 incorporations in this case. Another biotin-containing fragment was synthesized by using the fwB primer, i.e. in order to produce a biotin-modified fragment that has the same cohesive end as the digoxigenin-modified fragment. In this case, the modified extremity was removed using a PCR purification kit (Qiagen).

Labeled fragments were ligated to the 6.3 kb DNA by incubating 25 ng (7.5 nM) of labeled fragments with 100 ng of 6.3 kb DNA (2.5 nM) and 100 U of T4 DNA ligase (New England Biolabs) in 10 μ l of the recommended buffer

overnight at 20°C. Removal of excess labeled fragments was performed using the previously described spermidine precipitation procedure (16). Briefly, the ligation mix was diluted to a final volume of 50 μ l in a buffer containing 40 mM Tris, pH 8.0, 80 mM NaCl and 16 mM MgCl₂. Then, 50 μ l of 40 mM ice-cold spermidine were added. The sample was incubated for 30 min at room temperature and centrifuged for 15 min at 14 000 r.p.m. The resulting pellet was rinsed with 100 μ l of a 1:1 v:v mix of isopropanol and a solution containing 600 mM NaCl, 20 mM MgCl₂ and 50 mM EDTA. It was resuspended in 10 μ l of TE supplemented with 1.5 μ M YOYO-1 (Molecular Probes) at a ratio of 1–10 bp.

Combing and detection of DNA molecules

DNA combing was carried out on hydrophobic glass coverslips at low pH. Coverslips were first cleaned using a Plasma Cleaner (Harrick Scientific, USA). They were then spin-coated with polystyrene (5% w:v in toluene) and baked at 100°C for at least 1 h. Ten nanograms of YOYO-1 stained DNA (at a dye to bp ratio of 1–10) were added in a reservoir containing 3 ml of 50 mM MES, pH 5.5, 1 mM EDTA. Combing was achieved by dipping a coverslip into the reservoir for 5 min and then slowly removing. At appropriate pH, DNA molecules in the reservoir bind to the coverslip's surface by one of their extremities. As the coverslip is pulled up out of the reservoir, the DNA molecules are aligned and uniformly overstretched (by ~50%) by the receding meniscus (10).

In order to reduce non-specific interactions of QD with the surface during subsequent incubations, the coverslips were incubated for 10 min with blocking reagent (Roche) 1.5 mg/ml in 5 mM MES, pH 5.5, 1 mM EDTA. Coverslips were then washed twice with washing solution (borate 40 mM, pH 8.3). For the digoxigenin labeling scheme, the coverslips were first incubated for 10 min with mouse anti-digoxigenin (Roche) 200 ng/ml in revelation solution (40 mM borate, pH 8.3, 100 mM NaCl and 300 μ g/ml blocking reagent), then washed. The coverslips were then incubated for 10 min with 2 nM QD in revelation solution, using either a QD 605 (number indicates emission wavelength) streptavidin conjugate for biotin labeling, or a QD 655 goat F(ab')₂ anti-mouse IgG conjugate for digoxigenin labeling, or a mix of QD 565 streptavidin conjugate and QD 655 goat F(ab')₂ anti-mouse IgG conjugate (2 nM each) for the dual color labeling scheme. Coverslips were washed five times at the end of the process. The QD were purchased from Quantum Dot Corp. (USA). For each of these steps, the revelation solution (100 μ l) or the washing solution (200 μ l) was pipetted down onto the coverslip and removed by aspiration after the incubation time.

Fluorescence microscopy and image analysis

The coverslips were scanned using an inverted microscope (Olympus IX70) equipped with a 60 \times water-immersion objective (NA = 1.2). The light source was a mercury lamp. After passing through an excitation filter 475AF40, the light was directed onto the sample using a dichroic mirror 505DRLP (Omegafilters). Stained DNA and QD were detected separately with different band-pass filters: 535DF45 for YOYO-1, 540DF27 for QD 565 streptavidin conjugate, 595DF60 for QD 605 streptavidin conjugate and 645DF75 for QD 655 anti-mouse conjugate. Simultaneous observation of DNA

and QD was performed with a 510ALP long-pass filter. Images were captured by a Coolsnap camera (Roper Scientific) and processed with the Metaview software application. Each pixel of the CCD camera chip corresponds to a 215 \times 215 nm square on the sample.

An image of stained DNA was taken in order to localize combed DNA molecules on the surface (exposure time: 1 s). Due to the intermittent fluorescence of QD ('blinking'), a single image of the coverslip was not sufficient to localize all the QD as some of them could be in a dark state during the shot. An important feature of QD is that there is no characteristic blink duration. Therefore, the probability that the QD will emit light during >1 s in a 60 s timeframe is very high (17). We recorded a 60-frame movie (exposure time for each frame: 1 s), which was used to generate two types of processed images. The first is a QD maximum image where each pixel intensity is shown at the maximum intensity displayed in the 60-frame image stack. In the second, the fluorescence signal was averaged. The first image ensures equivalent size for all QD and is used for convenient visualization when overlaying the DNA image and the QD image, as in Figure 2. The second is used for the automated analysis described below.

Images were further analyzed using a program written in MATLAB (MathWorks). In order to localize both QD and DNA, we used a method based on cross-correlation between the fluorescence image and a specific correlation template. The template for QD detection was a two-dimensional isotropic Gaussian function which corresponds to the point spread function of our optical system. This template is justified in that the fluorescent source that we aimed to detect was made of one or a few QD attached to a 0.5 kb DNA fragment. This length corresponds to a maximum distance of 250 nm between two adjacent fluorophores, which is smaller than the optical resolution (~300 nm) of our system. When the QD images were cross-correlated with the QD template, local maxima above a user-fixed signal-to-noise detection threshold were localized. A least-squares Gaussian fit was performed to obtain the localization of the center of the fluorescent spot (18). The DNA detection template was elongated parallel to the combing direction, with a length equivalent to a combed DNA molecule and a Gaussian cross-section. DNA images were also cross-correlated with the DNA template. Local maxima above a user-fixed signal-to-noise detection threshold were localized and associated to the middle of combed DNA molecules. Then, for each maximum, a region-growing algorithm was applied to locate the entire molecule and subsequently its ends. Since DNA molecules may break during the combing process, only combed DNA molecules with apparent lengths ranging from 1.5 to 4.0 μ m were taken into account in further analysis (expected length of a 7.3 kb combed plasmid is ~3 μ m). The labeling was considered to be successful when a QD was localized at <1.0 μ m of a DNA extremity.

RESULTS

Labeling strategy

Our ultimate goal was to label both ends of combed DNA molecules in order to locate these molecules by fluorescence microscopy in the absence of DNA staining agents as well as

to determine their orientation on the coverslips. To do so, two different molecular entities detectable by fluorescence microscopy must be attached to both extremities of the DNA molecule. Specific recognition of DNA extremities can be achieved by ligation of DNA fragments with different cohesive ends. The fluorescent dyes that are used for detection of the modified extremities should be tightly linked to the DNA and produce bright fluorescent signals at two different wavelengths. QD are nanometer-sized, bright fluorescent probes which show exceptional photostability (no bleaching over extended periods of time) and have large excitation and narrow emission spectra, thereby allowing multicolor imaging by using only one excitation wavelength. They are commercially available in a water-soluble form at various emission wavelengths conjugated with biologically active molecules such as streptavidin, biotin and antibodies. Therefore, we chose to incorporate biotin or digoxigenin on both ends of the DNA molecule. We used streptavidin QD for biotin detection and a two layer scheme for digoxigenin detection involving one layer of antidigoxigenin antibodies and one layer of QD coated with a secondary antibody.

Class IIS restriction enzymes, such as BsaI or BsmBI, cleave outside their recognition site and therefore can generate various overhangs. Digestion of the pET11-parC plasmid with BsmBI produced a 6.3 kb fragment with two different cohesive ends (Figure 1). Short fragments of DNA (0.5 kb) with a

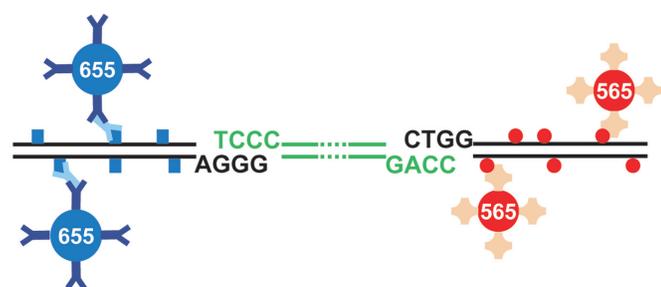


Figure 1. Description of the dual color labeling scheme used in this study. A DNA restriction fragment (shown in green) is ligated to short DNA fragments (shown in black) that are modified with biotin (red circles) or digoxigenin (blue squares), which are revealed using streptavidin QD 565 or one layer of mouse anti-digoxigenin (light blue) followed by anti-mouse QD 655.

cohesive end compatible with one extremity of this restriction fragment were obtained by cleavage of a DNA made by PCR at a BsaI site. This site was introduced close to one extremity by using appropriate primer sequences. The sequences of the overhangs were chosen in order to permit only the hybridizations that lead to the expected construct (Figure 1). Biotin- and digoxigenin-containing fragments were obtained by including biotin-dUTP or digoxigenin-dUTP in the PCR. Our protocol led to incorporation of ~ 75 modified deoxynucleotides in each fragment. Efficient ligation of the gel-purified restriction fragment to one or two 0.5 kb modified DNA fragments, giving DNA molecules of ~ 6.8 or 7.3 kb, was confirmed by gel electrophoresis (data not shown). The yield exceeded 95% for the construct that required two ligations.

Observation of combed DNA molecules by fluorescence microscopy

Modified DNA molecules were then stained with YOYO-1, combed on hydrophobic surface and observed by fluorescence microscopy. Stained DNA molecules are detected by the green fluorescence of the DNA binding dye, while the extremities are detected by red or far red fluorescence of QD. Green images revealed DNA molecules with an apparent length of $\sim 3 \mu\text{m}$ (Figure 2). This is compatible with the length of the combed DNA molecule (6.8 or 7.3 kb). The mean density of DNA molecules was $\sim 10^3/\text{mm}^2$. Because QD blink, red images were acquired every second for one minute and the series was processed as described in the experimental section. Then, the red and green images were superimposed (Figure 2).

We first modified only one end of the DNA molecule by ligating a biotin-containing fragment, using streptavidin QD for revelation. The red images revealed bright monodisperse fluorescent dots, some of which colocalize with the extremities of DNA molecules (Figure 2A). The red dots which did not colocalize with DNA were attributed to non-specific adsorption. Reducing this adsorption was one of the key steps in our experiments. The hydrophobic surface was uniformly covered with QD conjugates after incubation in the absence of treatment (Figure S1, see Supplementary Material). In order to reduce these interactions, coverslips were incubated with a solution of a commercial blocking reagent (Roche) prior to labeling. This reagent, whose principal ingredient is casein, was also added to the solution of QD during incubation.

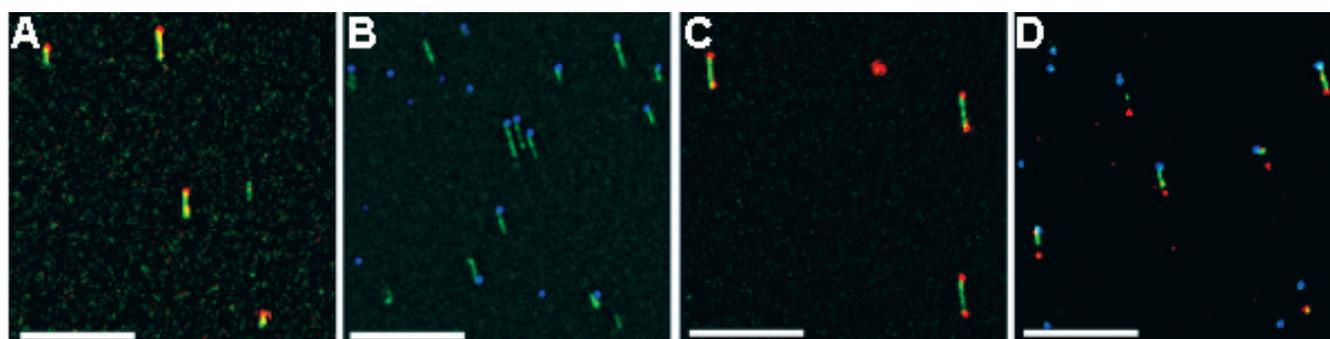


Figure 2. Combed DNA detection with QD. DNA molecules were modified with biotin or digoxigenin at one or both ends, stained with YOYO-1 (green) and combed. Biotin was detected with streptavidin QD 565 (D) or 605 (A and C) (shown in red) and digoxigenin was detected with mouse anti-digoxigenin and anti-mouse QD 655 (shown in blue). Four different labeling schemes were performed: biotin at one end (A), digoxigenin at one end (B), biotin at both ends (C), biotin at one end and digoxigenin at the other end (D). The QD images were processed from a 60 s long image movie (60 images), each pixel intensity is the maximum value of this pixel in the image stack. DNA image and QD maximum were then overlaid. The horizontal bar represents $10 \mu\text{m}$.

With this treatment, the observed density of streptavidin QD was $\sim 3 \times 10^3/\text{mm}^2$. Images were analyzed in order to estimate the efficiency of the labeling procedure using a program created for that purpose (see Material and Methods). About 60% of the DNA molecules displayed a label at one of their extremities (Figure 3, sample A). We noted a preferential orientation of the modified combed DNA. The upper DNA end in Figure 2 is the one that binds to the surface first during the combing process. Ninety percent of the QD signals were located on this end of the DNA molecules, suggesting that the biotin-modified end attaches more frequently to the surface than the non-modified end. To test whether a large number of biotin was required to ensure efficient labeling, we performed the same experiment with a DNA molecule which contains ~ 10 times fewer biotin-modified nucleotides at the extremity. This reduced the labeling efficiency from 60 to 30% (Figure 3, sample A*).

We then performed the same experiment with a digoxigenin-modified DNA molecule (Figure 2B). The molecules were revealed using mouse anti-digoxigenin antibodies and then anti-mouse IgG coated QD. A higher non-specific adsorption was observed with this protocol, and the mean density of anti-mouse QD was $\sim 2 \times 10^4/\text{mm}^2$. This can be attributed to the supplementary incubation step with mouse anti-digoxigenin antibodies, which may non-specifically bind to the surface and therefore lead to additional adsorption of QD. The labeling efficiency was the same as for the biotin-modified DNA (Figure 3, sample B). QD were detected at both ends of the DNA for very few combed molecules (1% of the biotin-modified DNA and 7% of the digoxigenin-modified

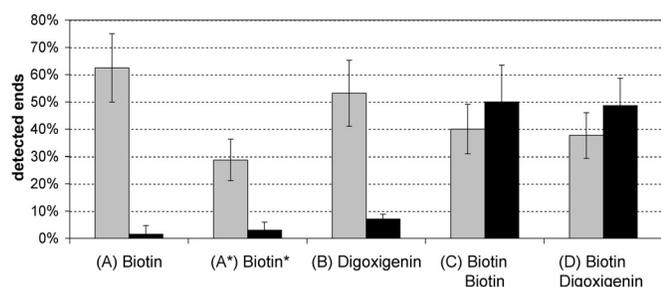


Figure 3. Efficacy of QD DNA detection. DNA was labeled as described in Figure 2, (A*) is a DNA molecule labeled with ten times less biotin than in (A). DNA and QD images were analyzed using a home-made software (see Materials and Methods). The proportion of DNA molecules with one label (gray) or two labels (black) is reported. The error bar is the SD between all fields of view.

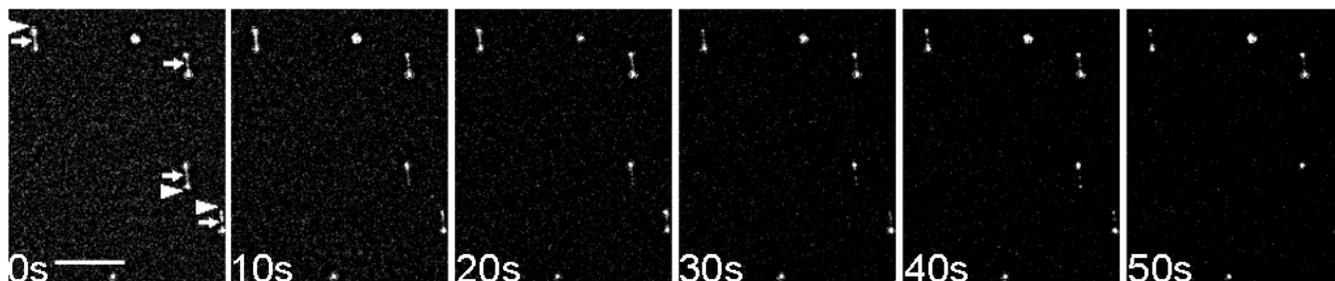


Figure 4. One-minute long observation of labeled DNA. Combed DNA molecules with two biotin-modified extremities were stained with YOYO-1 and visualized using streptavidin QD 605. A 1 min movie was recorded using a long pass filter. Images are shown every 10 s. YOYO-1 stained DNA molecules (arrows) tend to disappear and cannot be visualized after a few tens of seconds. Blinking QD are indicated by triangles. The horizontal bar represents 10 μm .

DNA). This may be due to non-specifically adsorbed QD closer to a DNA end than the chosen localization distance criterion of 1 μm . These values are in agreement with the false-positive detection values expected from the measured QD densities.

We then observed DNA molecules that were modified at both ends, either with biotin alone (Figure 2C) or with biotin at one end and digoxigenin at the other (Figure 2D). In the second case, two different QD conjugates were used at the same time, thereby allowing a 2-color detection scheme. The proportion of molecules with signals at both ends was $\sim 50\%$ in each case. Forty percent of the molecules were labeled at only one end (Figure 3, samples C and D), half of these displayed a QD signal at 565 nm and the other half at 655 nm. We noticed that DNA molecules modified with both biotin and digoxigenin anchored to the surface by the digoxigenin end more than nine times out of ten.

We noted, as expected, that the fluorescence of the DNA staining agent decreased over time when irradiated at 475 nm. Figure 4 shows images taken every 10 s during a 1 min movie of a DNA molecule with two biotin-modified extremities. The use of a long pass filter allowed us to observe the emission of both the DNA staining dye and the QD 605 at the same time. The bleaching of the DNA staining agent as well as the blinking of QD can be clearly observed on these images (see also the movie provided as Supplementary Material, Figure S2).

QD-mediated DNA detection

We then investigated if the QD images could be used to localize the DNA molecules using only their labeled fluorescent extremities. The fluorescent pairs that were separated by $<10 \mu\text{m}$ were selected and their separation length and orientation with respect to the combing direction were recorded and analyzed. For the double-biotin labeling, the distribution of distances showed a peak between 2.5 and 3.0 μm , and the distribution of orientations showed a peak close to 0° (Figure 5A and B). If we restrict the set of molecules to those that have an orientation between -10° and $+10^\circ$, the peak for the distribution of distances can be more clearly distinguished (Figure 5D). Similarly, when the distance between fluorescent pairs is restricted to 1.5–4.0 μm , the orientation distribution peak becomes more apparent (Figure 5C). The biotin–digoxigenin modified samples were submitted to the same analysis. Because of a higher non-specific binding of antimouse-QD to the surface (see above), the histogram background was higher and no peak could be detected in

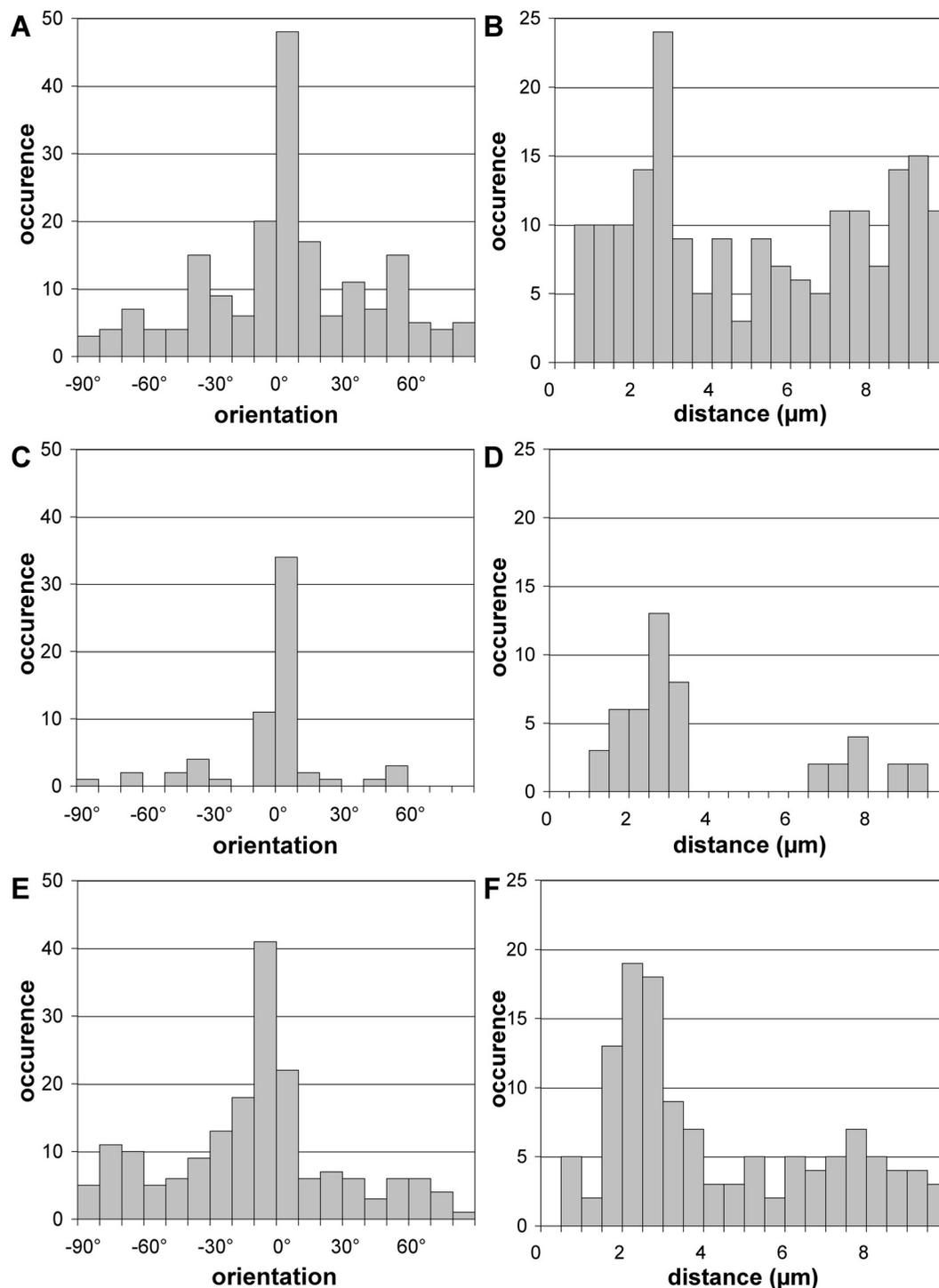


Figure 5. Preferential layout of QD pairs. DNA molecules were modified with biotin at both ends (A–D) or biotin at one end and digoxigenin at the other end (E and F) and combed. Biotin was detected with streptavidin QD 565 or 605 and digoxigenin was detected with mouse anti-digoxigenin and anti-mouse QD 655, respectively. QD images were analyzed using a program written in MATLAB. The angle between a QD pair and the combing direction (A, C and E) and the distance between both QD of the pair (B, D and F) were measured. Results are reported for QD 605 pairs (A, B, C and D) and for pairs involving one QD 565 and one QD 655 (E and F). In C and E, only QD pairs for which the distance is between 1.5 and 4.0 μm are represented. In D and F, only QD pairs for which the angle is between -10° and $+10^\circ$ are represented.

the absence of correlation (data not shown). Nevertheless, when the biotin–digoxigenin data were analyzed in the same manner as the biotin data, the histograms showed peaks corresponding to approximately the same values (Figure 5E and F).

The criteria established above (i.e. a length between 1.5 and 4.0 μm and an angle between -10° and $+10^\circ$) were applied in combination in order to select sets of QD pairs to be analyzed for the presence of DNA. The average distance for these pairs

was $2.6 \pm 0.5 \mu\text{m}$ for the double-biotin detection scheme and of $2.6 \pm 0.6 \mu\text{m}$ for the biotin–digoxigenin scheme. The average orientation was $3 \pm 4^\circ$ for the double-biotin detection scheme and $-1 \pm 5^\circ$ for the biotin–digoxigenin scheme. DNA molecules could be identified by the green emission of YOYO-1 between the two QD in 43 out of 45 cases for the double-biotin labeling (96%), and in 120 out of 165 cases for the biotin–digoxigenin labeling (73%).

We calculated the probability P that a QD pair that satisfies the above-mentioned criteria is actually associated with a DNA molecule. A detected pair can either be two QD at both ends of a DNA molecule or two QD randomly meeting these criteria. P is therefore given by

$$P = \frac{Y \cdot D_{\text{DNA}}}{D_{\text{pairs}} + Y \cdot D_{\text{DNA}}}$$

where Y is the efficiency for labeling a DNA molecule at both ends, D_{DNA} is the density of DNA molecules and D_{pairs} is the density of random QD pairs. When only one type of label is present, for example streptavidin QD, the density D_{pairs} of QD pairs that satisfy specific distance (between r_1 and r_2) and orientation (between a_1 and a_2) criteria by chance is given by

$$D_{\text{pairs}} = \frac{D_b^2}{2} \times \pi(r_2^2 - r_1^2) \times \frac{(a_2 - a_1)}{180}$$

where D_b is the total density of streptavidin QD. The formula changes to

$$D_{\text{pairs}} = D_b \times D_d \times \pi(r_2^2 - r_1^2) \times \frac{(a_2 - a_1)}{180}$$

when two types of different QD were used, where D_d is the total density of anti-mouse QD. D_{pairs} represents here only pairs composed of one streptavidin QD and one antimouse QD. Using our values ($r_1 = 1.5 \mu\text{m}$, $r_2 = 4.0 \mu\text{m}$, $a_1 = -10^\circ$, $a_2 = 10^\circ$, $D_b = 3.10^3/\text{mm}^2$ and $D_d = 2.10^4/\text{mm}^2$), we found D_{pairs} values of 21 and 288 pairs/ mm^2 for the double-biotin and the biotin–digoxigenin scheme, respectively. Taking a value of 50% for Y , as documented by the experiments described above, we can deduce the value of P , which is 96% for the double-biotin labeling scheme and 63% for the biotin–digoxigenin labeling scheme. These values are compatible with the experimental values obtained from our images.

It should be noted that these values depend on both density and length of the DNA molecules, and on the nonspecific adsorption of QD. For example, the value of P for the double-biotin labeling scheme will decrease from 96 to 69% if the length of the DNA is increased by a factor of 8, which corresponds to the length of phage lambda DNA (48.5 kb). The density of DNA we used and the length of these DNA molecules, in combination with the small number of non-specifically bound QD, made for a high probability that QD pairs that satisfy our specific distance and orientation criteria were in reality associated with a DNA molecule. This probability is close to 1 for the biotin labeling scheme and remains over 0.6 for the biotin–digoxigenin labeling scheme, which provides additional information about the orientation of the molecule.

DISCUSSION

The aim of this study was to investigate new labeling strategies for DNA detection by optical microscopy in the absence of any DNA staining agent, in order to facilitate further dynamic studies of DNA–protein interactions at the single molecule level. For this purpose, biotin and/or digoxigenin fragments were ligated to the ends of linear DNA molecules. The modified DNA molecules were then combed on a glass surface and observed by fluorescence microscopy. To the best of our knowledge, this is the first time that the ends of DNA molecules have been detected by fluorescence microscopy using fluorescent QD in a 2-color mode. We have demonstrated that the detection of single DNA molecules is possible by using only the QD images. QD pairs for which distance and orientation were characteristic of labeled DNA molecules can be used as an indicator for the presence of DNA molecules with a low error rate. Image analysis and QD pair selection can be automated using software.

The incorporation of multiple biotin or digoxigenin molecules at each extremity was apparently necessary for efficient detection. These molecules were introduced in the form of modified deoxynucleotides incorporated into short DNA fragments that are produced by PCR and ligated at both ends of the DNA to be studied. The ligation yield exceeds 95%. Non-fluorescent haptens were used instead of fluorescent nucleotides, in order to provide a more versatile approach. Under our experimental conditions, the labeling efficiency for one end was 60% for both labeling strategies, i.e. using biotin and streptavidin QD or digoxigenin, mouse antidigoxigenin and antimouse QD. This efficiency dropped by 50% when the DNA fragment contained ten times less biotin. As QD are likely to always be detected, these yields probably reflect inefficient binding of biotin and digoxigenin by the modified QD. The proximity of the surface might disturb the expected specific interactions, or many biotin or digoxigenin molecules might stick to the surface during the combing process. A preferential attachment of the combed DNA molecules by their modified extremity supports this hypothesis. Moreover, records of single spot fluorescence temporal evolution suggest that only one or very few QD are responsible for the fluorescent signal (see Supplementary Material and Figure S3). The number of modified nucleotides is therefore a critical parameter for efficient labeling to take place.

Organic DNA staining agents interfere with DNA–protein interactions. In addition, these staining agents tend to bleach which leads to DNA cleavage. Moreover, the presence of fluorescence along the entire DNA molecule results in low signal to noise ratio when the emission of other fluorophores is imaged in the vicinity of DNA. We labeled DNA molecules with QD, which display specific optical properties that are not shared by organic fluorophores or fluorescent microspheres and are particularly valuable for single-molecule visualization. For example, they have recently been used to follow the diffusion of glycine receptors in the membrane of neurones (19). They produce localized and long-standing fluorescence signals with sharp emission spectra. QD that have different emission wavelengths can be excited by the same wavelength. It is therefore easy to combine two different wavelengths for DNA extremities and a third for a protein. Our labeling strategy can be used in the presence of physiologically relevant

ionic conditions. Two problems are encountered when one wants to use QD on glass surfaces. The first is that QD blink. This problem was circumvented by acquiring 60 QD images at 1 s intervals. The second is non-specific adsorption onto surfaces. This problem was solved by using an appropriate buffer for the pretreatment of the coverslips as well as during the incubation step. More non-specific adsorption of QD was observed with the anti-mouse QD used for digoxigenin detection. This resulted in a higher probability of erroneous QD-mediated DNA detection. At the date of the experiments, anti-digoxigenin QD conjugate was not yet available. As anti-digoxigenin conjugated QD are currently being commercialized, the protocol could now be simplified and non-specific adsorption would probably be lower.

The development of ultrasensitive instrumentation and new microscopy techniques opens the way to dynamic studies of interacting biomacromolecules by fluorescence microscopy, which will be particularly interesting for DNA studies and its related machinery. The detection scheme presented here will likely be helpful to future investigators. For example, it may be possible to use it in experiments where DNA is elongated by a flow (3), and preliminary experiments suggest that it can be applied to DNA molecules that are attached to a surface by only their extremities (11).

The detection of double-stranded DNA by fluorescence microscopy requires specific labeling strategies. Specific sequences on stretched single DNA molecules have been probed by using fluorescently labeled DNA binding proteins (20,21). We have recently implemented a method of labeling short internal DNA sequences using a triplex forming oligonucleotide ligated to a fragment containing multiple fluorophores (22). In the present paper, QD are used for the detection of biotin or digoxigenin moieties that have been ligated to the ends of DNA molecules. QD have so far been used in only a few studies for detection of nucleic acids. Oligonucleotide probes were conjugated directly to QD or linked to streptavidin-QD upon biotin functionalization, and these conjugates were used for DNA detection in fluorescence in situ hybridization (23,24) or DNA chips (25,26) experiments. In all these cases, the target sequences were present in multiple copies. The detection of short unique sequences of DNA at the single molecule level still represents an experimental challenge. In our approach, the ends of a DNA molecule were detected with a 60% success rate using QD. This efficiency required the incorporation of a large number of biotin or digoxigenin moieties, only a few of which were recognized by QD.

In conclusion, the method we propose for the detection of DNA molecules paves the way towards new dynamic studies of single DNA molecules and their interactions with proteins. QD fluorescent probes appear to be a valuable alternative to the organic dyes that are commonly used to stain DNA. They permit not only long-standing observation by fluorescence microscopy, but also a 2-color determination of the orientation of single DNA molecules. This study also provides useful experimental data regarding the possible use of QD for fluorescence detection of short DNA sequences.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR online.

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