A simple DNA stretching method for fluorescence imaging of single DNA molecules

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ABSTRACT

Stretching or aligning DNA molecules onto a surface by means of molecular combing techniques is one of the critical steps in single DNA molecule analysis. However, many of the current studies have focused on λ-DNA, or other large DNA molecules. There are very few studies on stretching methodologies for DNA molecules generated via PCR (typically smaller than 20 kb). Here we describe a simple method of stretching DNA molecules up to 18 kb in size on a modified glass surface. The very low background fluorescence allows efficient detection of single fluorescent dye labels incorporated into the stretched DNA molecules.

INTRODUCTION

Much progress has been made over the last decade in the manipulation of single DNA molecules, providing a way to study the physical properties (1), DNA–protein interactions (2) and genomic organization of DNA molecules in more detail (3). Recently, we reviewed the steps required for a direct molecular haplotyping method based on single molecule fluorescence microscopy (4). In this approach, alleles of several SNPs contained in long-range PCR products are labeled specifically with two different fluorescent probes and the double-stranded DNA backbone is labeled with a third fluorescent dye. The labeled PCR products are stretched on a glass cover slip and the linear molecules are imaged with multicolor total internal reflection fluorescence (TIRF) microscopy. By determining the colors and positions of the fluorescent labels with respect to the backbone, the haplotype can be inferred, in a manner similar to reading a barcode. In this and other applications, the ability to stretch DNA molecules into linear form is required for good visualization of the DNA backbone.

Currently, two general approaches to DNA stretching are in common use. Either the DNA is stretched in solution as it flows through a microfluidic channel (5–7) or it is stretched on a solid surface (8,9). In the microfluidic setting, the flow is generally too fast to allow for accurate measurements of individual fluorescent labels on the backbone and too disruptive to preserve interactions between protein (and most labels) and DNA. Therefore, most applications involving manipulation of single DNA molecules are performed on a solid surface. Typically, the DNA molecules are attached to the solid support on one end and are extended by various weak forces (e.g., electric force, surface tension, or optical force) (10). One particular method, DNA combing (8), has found many applications in the field of genomics. In this approach, the end of a DNA molecule is first anchored to a hydrophobic surface (typically modified glass) by adsorption. The anchored DNA molecules can be stretched in a number of ways. For example, stretching has been done by a receding meniscus (11), evaporation (12), or by nitrogen gas flow (13).

In order for the DNA fragment to be anchored to the glass surface, the surface has to be modified to make it hydrophobic, either by chemical modification or by polymer coating (14). Furthermore, the glass surface must be clean (so that there are no spurious fluorescent signals) and modified (for DNA attachment) with reagents that do not interfere with protein–DNA interactions during solid phase enzymatic reactions.

In this study, we report a simple DNA stretching method utilizing a modified glass surface with very low background fluorescence. We show that this strategy allows for the detection of single fluorescent dye labels on stretched DNA molecules, as well as the detection of stretched DNA molecules without the need of backbone staining.

MATERIALS AND METHODS

DNA preparation

Oligonucleotide primers synthesized by Integrated DNA Technologies (Coralville, IA) had the following sequences: 4 kb long-range PCR forward primer: CTGAGCCAGGTAC-CACCATTGTAAG, reverse primer: AGAAAGTAGAG-CATTTGGGGCTCTG; 6.7 kb long-range PCR forward primer: TGTTGACCCAGGGAACAAGATCTAA, reverse

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primer: GACTCCACAGTCAGTCTCCAGGTTC; 9.3 kb long-range PCR forward primer: CACCCTTTCCATAGGG-AGGAATG, reverse primer: GAGTCATGATGGGATTC-CTGTGG; 12.5 kb long-range PCR forward primer: TTGT-CTTGGAAACTCAGCCTTGC, reverse primer: CAGCTGT-CCAGCACCAGCTTC; and 18 kb long-range PCR forward primer: CCTTCACTGTCTGCCTAACTCCTTCGTGTGTT-CC, reverse primer: GCAGGGGTGCTGCAGAACTCTGA-GCTGTACTTCC.

For Figures 1 and 3, the DNA was amplified from genomic DNA with primers that bear the same sequence as the 9.3 kb primers used above. However, they were modified with a fluorophore conjugated at the 5′ terminus (Integrated DNA Technologies, Coralville, IA). For Figure 1, the forward primer was labeled with Cy5, and the reverse primer was labeled with Cy3. For Figure 3, both the forward and the reverse primers were labeled with Cy3.

Long-range PCR was performed with the Eppendorf TripleMaster PCR System (Westbury, NY), which includes the TripleMaster PCR Polymerase mix and 10× tuning buffer with Mg^{2+}. The 10 mM dNTP mix was from Invitrogen (Carlsbad, CA). Long-range PCR was performed in an MJ PTC-225 Peltier Thermal Cycler (Bio-Rad, Hercules, CA). All PCRs were conducted according to the manufacturer's instructions. Two different master mixes were prepared on ice in separate sterile microcentrifuge tubes. Master mix 1 contained 4 µl each of the forward and reverse PCR primer at 5 µM, 10 µl of molecular biology grade water and 2 µl of DNA template (250 ng). Master mix 2 comprised 22.1 µl molecular biology grade water, 5 µl of 10× tuning buffer with Mg^{2+}, 2.5 µl dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP) and 0.4 µl TripleMaster polymerase mix (0.04 U/µl). Immediately prior to cycling, the two master mixes were combined and gently mixed by pipetting up and down several times. Then the solution was placed into a thermal cycler preheated to 93°C and incubated at 93°C for 3 min to denature the DNA template, followed by 10 cycles of amplification. Each cycle included template denaturation at 93°C for 15 s, followed by primer annealing at 65°C for 30 s, and extension at 68°C for 15 min. A second round of amplification included 17 cycles of 93°C for 15 s, followed by 30 s

at 65°C, then 15 min at 68°C (+20 s/cycle). The PCR product was stored at 4°C or at −20°C (long term) until further use. PCR products were visualized on a 0.4% high-melt agarose gel in TAE buffer. Long-range PCR products internally tagged with fluorescent dyes were generated using the same protocol, but including 1 µM Cy5-labeled dATP.

Preparation of the glass coverslips

This protocol was adopted from published reports with some modifications (15,16). Briefly, Fisher premium coverslips (22 × 30 mm) were sonicated in 2% MICRO-90 soap (Cole-Parmer, Vernon Hills, IL) for 20 min and then cleaned by boiling in RCA solution (6:4:1 high-purity H_2O/30% NH_4OH/30% H_2O_2) for 1 h. Poly(allylamine) (PAll) and Poly(acrylic acid) (PAcr) (Sigma-Aldrich, St Louis, MO) were dissolved at 2 mg/ml in high-purity water. The solutions were adjusted to pH 8.0 using either HCl (for PAll) or NaOH (for PAcr). The polyelectrolyte solutions were then passed through a 0.22 µm filter. The RCA-cleaned coverslips were immersed in the positive (+, PAll) and the negative (−, PAcr) polyelectrolytes according to the scheme +/wash/−/wash/+/wash. Each polyelectrolyte incubation was 30 min on a shaker at 150 r.p.m. at room temperature (25°C); each wash step involved three rinses with high-purity water. The polyelectrolyte-coated coverslips were stored in high-purity water at room temperature.

DNA combing

Combing by capillary flow. DNA mounting was performed by a procedure similar to that published by the Schwartz group (17). Column-purified DNA was diluted to ~100 pM in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Glass slides were blown clean of dust with nitrogen then passed through a propane torch flame to remove impurities and moisture. The purified DNA was pre-stained with 300 pM of YOYO-1 iodide (Molecular Probes, Eugene, OR), which is an intercalating dye that stains the DNA backbone and makes it possible for visualization. A modified coverslip was placed on the glass slide, and 8 µl of the DNA diluted in the imaging buffer (see below) was pipetted onto the edge. The solution was drawn under the coverslip by capillary action. The strong capillary flow caused the DNA fragments to be stretched and aligned on the coverslip surface. The coverslip was then sealed with clear nail polish (950 Extra Life Top Coat, Revlon).

The imaging buffer consisted of 300 pM YOYO-1 iodide and 20% 2-mercaptoethanol in sterile TE buffer. 2-Mercaptoethanol is a strong reducing agent that prevents photobleaching of the YOYO-1 and Cy3 dyes by scavenging oxygen from the solution.

Combing by force flow. A coated coverslip was placed over the glass slide with one edge touching the slide at an angle of 20°, and 7 µl of DNA in imaging buffer was pipetted onto the interface between the slide and the coverslip. By dropping the coverslip onto the slide, the DNA solution was pushed between the coverslip and the slide, resulting in a strong fluid flow, which caused the DNA fragments to be stretched and aligned on the coverslip surface.
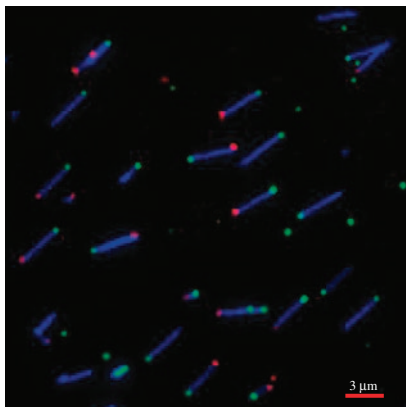

Figure 1. Single DNA molecules labeled at both ends A composite image of three color-channels of the 9.3 kb long-range PCR product labeled with Cy3 at one end and Cy5 at another. The DNA backbone was stained with YOYO-1.

Single molecule fluorescence microscopy

The TIRF microscope was set up as described in (18) with some modifications. The imaging platform utilized the Olympus (Olympus America Inc., Melville, NY) IX-71 microscope with a 100× SAPO objective (Olympus SApo 100×/1.40 oil). YOYO-1, the DNA backbone dye, was excited using 488 nm wide-field excitation from a mercury lamp mounted on the rear port of the microscope. Cy3 (green) and Cy5 (red), the end and internal labels, were excited using a 543 or 628 nm helium–neon laser, respectively (JDS Uniphase, San Jose, CA). The two laser beams were combined by a dichroic mirror and expanded to a 7 mm diameter. The 543 and 628 nm excitation emissions were focused via total internal reflection through a translation stage. The emitted photons were collected through two separate filter cubes HQ510LP for the blue dye YOYO-1 and bandpass filters (Z543–633M) for Cy3 and Cy5 (Chroma Technology Corp., Rockingham, VT). The blue and the bandpass emission filters were aligned to eliminate pixel shift during filter cubes switching. The signal was then magnified 1.6× and detected by a back-illuminated, thermoelectric-cooled, frame-transfer charge-coupled device (CCD) detector BV887 (Andor, Ireland). The DNA backbone image and the individual label images were integrated for 1 s.

Data analysis

Calculating DNA length with backbone staining. Each DNA fragment was constructed using the following procedure: (i) contiguous pixels with intensity significantly greater than the background were extracted. (ii) Each pixel by itself was a point spread function (PSF) and could easily fit into a 2D Gaussian distribution. Therefore, a 'strip' of contiguous pixels could be viewed as a series of Gaussian curves, with the peak of each curve (the centroid) representing the brightest area of each pixel. (iii) Each DNA backbone was constructed by drawing a contour line through the centroids. (iv) Finally, the length of each DNA fragment was defined as the length (in pixels) of each contour line.

Observing DNA fragments without backbone staining. The single fluorescent signal of each dye molecule in the green (for Cy3) and the red (for Cy5) channels was identified and extracted by fitting it to a 2D Gaussian distribution. The signals of the two channels were superimposed, and false colors were assigned to form a composite image. The DNA fragments were identified as multiple red (Cy5) spots falling between two green (Cy3) end labels. The DNA length was calculated as the distance (in pixels) between the two end labels.

RESULTS

Observing single DNA molecules with fluorescent end-labels

The polyelectrolyte multilayer (PEM)-modified surface (16) has been shown to have extremely low fluorescence background and is therefore suitable for fluorescence single molecule detection. The low background noise is achieved by electrostatic repulsion of the multilayer charged polymer.

We reasoned that if the outermost polymer layer was positively charged, the negatively charged DNA molecule could be anchored and stretched on the modified, low fluorescence background surface.

In the first set of experiments, 9.3 kb PCR products were labeled at both ends with Cy3- and Cy5-labeled PCR primers. The DNA backbone was stained with YOYO-1. Figure 1 is a composite of images obtained from three color-channels; blue lines represent the YOYO-1 backbone stain; red spots the Cy5 dyes; and green spots the Cy3 dyes. About 20 DNA molecules are seen in this microscopic field and most of the molecules are fully stretched. Furthermore, the DNA fragments are clearly separated from each other and individual molecules can be imaged and analyzed without interference. Most of the green and red fluorescent signals coincide with the ends of the DNA backbone, indicating that these are the end labels of Cy3 and Cy5 molecules. The detection of single fluorescence Cy3 and Cy5 was very efficient, with ~60% of the DNA molecules exhibiting both red and green labeling. Even though the sample used in this experiment was not purified, only a few bright fluorescent spots were observed outside DNA backbones, confirming that the background noise was remarkably low.

Stretching of long-range PCR products of different sizes

In our recently proposed direct molecular haplotyping method, deposition of linear long-range PCR products onto a solid surface is one of the critical steps (4). It is well established that longer DNA molecules (>40 kb) can be easily and consistently stretched on the solid surface. Few studies have been done with shorter DNA molecules (<20 kb), which is the upper limit of current long-range PCR techniques.

To systematically investigate the stretching capability of DNA molecules, long-range PCR products of 4, 6.7, 9.3, 12.5 and 18 kb were stretched on the polymer-coated coverslip. Typical results with the corresponding length distributions observed are shown in Figure 2 and Table 1. In general, for DNA up to 12.5 kb, there were two populations: the first population included fragments which measured <10 pixels (or 1 μm), suggesting that these were either unstretched or broken DNA fragments. The second population consisted of fully-stretched linear DNA molecules. For example, the average length of 4 kb fragments was 12 pixels (1.2 μm); that of 6.7 kb fragments was 21 pixels (2.1 μm); 9.3 kb was 37 pixels (3.7 μm); 12.5 kb was 56 pixels (5.6 μm); and 18 kb was 72 pixels (7.2 μm) (the effective size of one pixel is 100 nm). These numbers suggest that the DNA molecules were slightly longer than the theoretical length of fully-stretched double-stranded DNA molecules, i.e. 0.34 nm per base pair. In addition, the proportion of fully-stretched DNA increased as the amplicon size increased (Table 1). Nearly 70% of the 18 kb DNA molecules were fully-stretched, compared to only 38% of the 4 kb DNA molecules. In between the unstretched and the fully-stretched molecules, there was also a significant number of DNA molecules of other lengths. These could be either broken DNA molecules, DNA molecules folded over onto themselves, or partially-stretched DNA.

For DNA molecules as short as 4 kb, stretching quality can be improved with stronger flow induced by dropping the
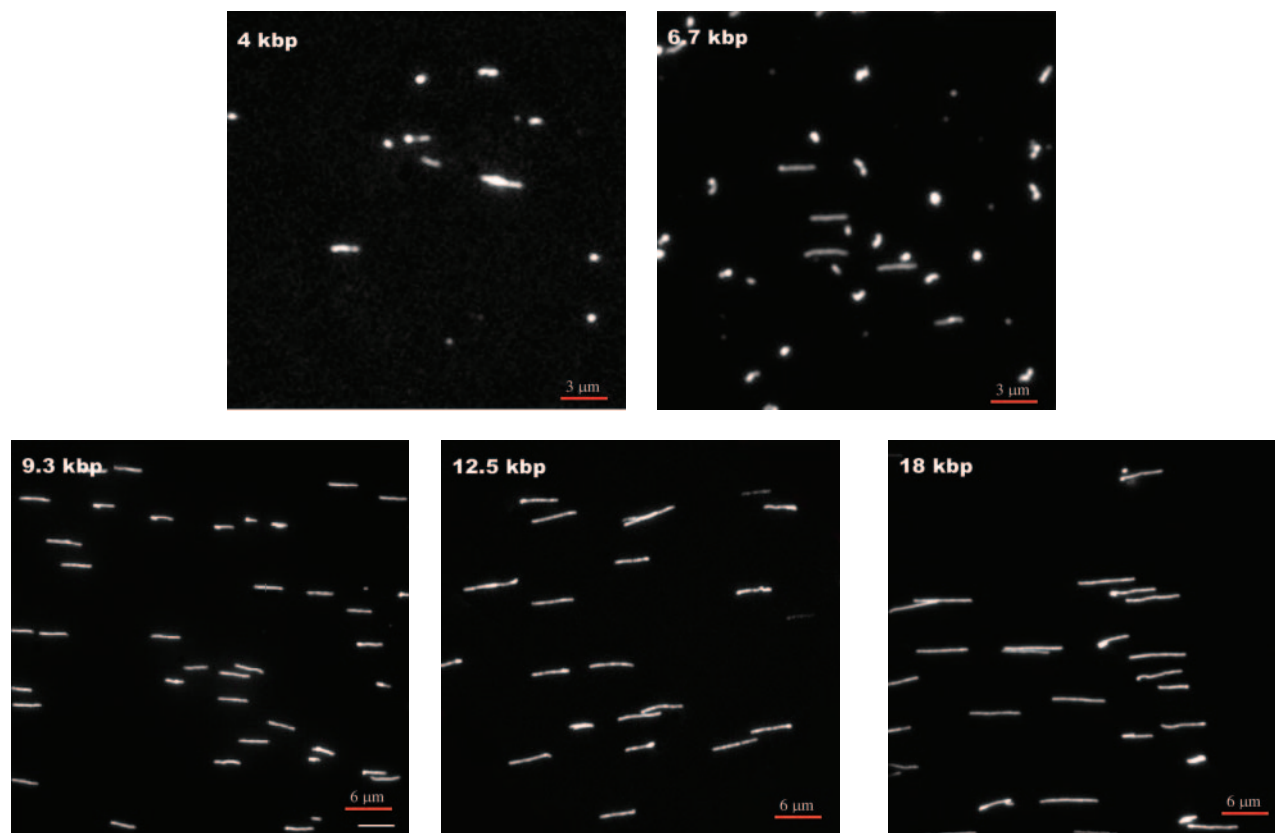
Figure 2. Stretching DNA of various sizes.

Table 1. A summary of results from the DNA length analysis

DNA fragment size (kbp)	Theoretical length (µm)	Observed length (µm)	% Difference between the observed and the theoretical lengths	% of DNA molecules of length within the difference (n = no. of molecules with sizes >1 µm)
4	1.36	1.2	12	38 (n = 532)
6.7	2.28	2.5	10	29 (n = 162)
9.3	3.16	3.7	17	52 (n = 383)
12.5	4.25	5.6	32	76 (n = 499)
18	6.12	7.0	14	69 (n = 426)

coverslips onto the slides. With this modified procedure, the average length of fully stretched DNA was ∼16 pixels (=1.6 µm, data not shown), indicating some degree of over-stretching of the DNA backbone. However, this method has adverse effects on DNA molecules longer than 10 kb, and can shear these molecules into smaller fragments.

Detecting single DNA molecules without backbone staining

We next investigated the possibility of detecting single DNA molecules without staining the DNA backbone. In this experiment, both ends of the DNA molecules were labeled using Cy3-labeled PCR primers, and the internal backbones were labeled during PCR by adding a mixture of labeled Cy5-labeled nucleotides and unlabeled nucleotides. The ratio of labeled dNTP and native dNTP was controlled, so that, on average, only a few labeled nucleotides were introduced into the backbone for 9.3 kb DNA molecules. Using this approach, DNA molecules are expected to appear as Cy3 (green) spots marking both ends flanking Cy5 (red) spots dotted in between (Figure 3). Most patterns in Figure 3 feature red (Cy5) spots flanked by green (Cy3) dyes (as shown by the red arrows), indicating that these are individual DNA molecules. A couple of patterns lack Cy5-red spots in the middle (as shown by the yellow arrows); these could still be DNA molecules, but there are either no Cy5 labels present on the backbone or the labels are not detected. Another pattern (as shown by the cyan arrows) lacks one of the green Cy3 end labels to show the full contour of the DNA molecules. Green label pairs separated by at least 0.5 µm and with red spots in-between were chosen to calculate the DNA contour length. The average length of the DNA molecules was 2.5 µm for the 9.3 kb amplicons, which is shorter than the 3.5 µm length of fully-stretched DNA molecules in the presence of backbone staining agent (YOYO-1). This showed that a backbone staining agent such as YOYO-1 may enhance the rigidity of the DNA molecule and facilitate stretching to full length. To confirm that the above pattern (dotted red spots with two green spots marking the ends) truly corresponds to individual DNA molecules, we subsequently stained the DNA molecules with YOYO-1 and observed the coincidence of all three channels. 100% of DNA molecules measuring between 1 and 3 µm that could
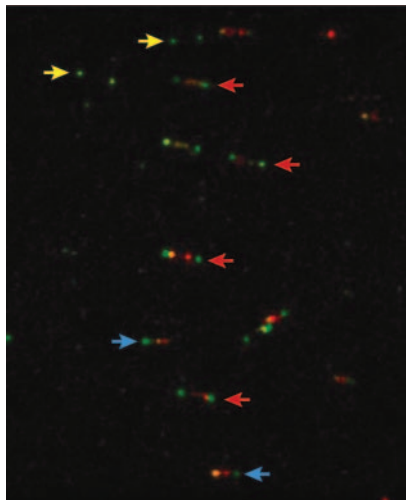
Figure 3. Observing single DNA molecules without backbone staining 9.3 kbp DNA molecules were labeled at both ends by use of Cy3-labeled primers, and a small amount of Cy5-labeled dATP was introduced in conjunction with native dNTPs during the PCR steps. Red arrows indicate individual DNA molecules with green end-labels and red labels in between the two green labels. Yellows arrows indicate individual DNA molecules with green end-labels, but without the red internal labels. Cyan arrows indicate individual DNA molecules with one green end-label undetected. The results showed that single DNA molecules can be observed without the need of a backbone stain.

be identified by blue YOYO-1 emission could also be detected by the red–green patterning (red spots in-between green spots at the ends) without backbone staining (data not shown).

DISCUSSION

DNA combing techniques (stretching long DNA molecules into linear form) have been widely used in genomic and genetic studies, such as optical mapping (19), fiber-fluorescence *in situ* hybridization (20), genetic disease screening and molecular diagnostics (21,22). Most DNA molecules used in these studies are longer than 40 kb, and the resolution is normally no better than 3–5 kb, since the markers along the stretched DNA molecules have to be labeled with multiple dyes to enhance the detection sensitivity. Improved resolution is needed for the fine localization of genetic markers (4) as well as for the study of precise DNA–protein interactions. With the rapid development of ultrasensitive instruments and new microscopy techniques, especially the single-molecule fluorescence detection methods, one can achieve the resolution of a few bases (18). We demonstrate here that DNA combing on a modified glass surface can consistently produce fully-stretched DNA molecules for fluorescent imaging.

Construction of PEM by sequential deposition of polyanions and polycations have been described for years (23,24). The PEM serves as an excellent platform for surface chemistry and has been used to study single-molecule enzymology (15,16). Other groups have also tried aligning long DNA molecules on a coated surface, see Ref. (25) for example. In this study, we have shown that DNA molecules as short as 4 kb can be stretched into linear form on positively-charged PEM-modified glass coverslips. The PEM-modified

glass coverslips show such low background fluorescence that single fluorescent molecules at both ends of the DNA fragment can be efficiently detected.

The percentage of fully-stretched DNA molecules depends on the length of the DNA molecules. We found that with DNA combing by capillary flow, the longer the DNA molecules, the easier it was to achieve a complete stretching. For example, over 40% of the 10 kb DNA molecules could be routinely stretched with capillary flow, while only 20% of the 4 kb molecules were fully stretched using the same method. For shorter DNA fragments, the stretching quality can be improved with the stronger flow induced by dropping the coverslips onto the slides. However, this approach shears longer DNA fragments into shorter pieces and is therefore not suitable for stretching longer molecules.

Direct observations of the DNA backbone normally requires backbone staining fluorescent dyes. Direct detection of combed, end-labeled DNA has not been performed until recently. Crut and colleagues (26) reported a method of detecting single DNA molecules without backbone staining using quantum-dot end-labeling. This labeling scheme requires incorporation of multiple hapten (biotin or digoxigenin) molecules at both DNA ends, and involves complicated quantum-dot staining and washing steps. These could potentially interfere with the interaction of DNA molecules with DNA-binding proteins. In our labeling scheme, both the end labels and the internal labels were introduced during PCR amplification, therefore avoiding the harsh washing steps. The overall number of internal labels can be controlled by varying the proportion of labeled dNTPs in the PCR mix. After purification, the labeled DNA molecules can be directly mounted onto the polymer-coated surface without the need for further staining. This also eliminates non-specific adsorption of dye molecules onto the glass coverslips.

In conclusion, we have developed a simple and reproducible method of stretching short to medium length DNA on a modified glass surface with very low background. Our method provides a good platform for the study of single DNA molecules and their interactions with proteins by TIRF imaging.

ACKNOWLEDGEMENTS

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