Chapter 13

A Single-Molecule Approach to Visualize the Unwinding Activity of DNA Helicases

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Abstract

Almost all aspects of DNA metabolism involve separation of double-stranded DNA catalyzed by helicases. Observation and measurement of the dynamics of these events at the single-molecule level provide important mechanistic details of helicase activity and give the opportunity to probe aspects that are not revealed in bulk solution measurements. The assay, presented here, provides information about helicase unwinding rates and processivity. Visualization is achieved by using a fluorescent single-stranded DNA-binding protein (SSB), which allows the time course of individual DNA unwinding events to be observed using total internal reflection fluorescence microscopy. Observation of a prototypical helicase, *Bacillus subtilis* AddAB, shows that the unwinding process consists of bursts of unwinding activity, interspersed with periods of pausing.

Key words: DNA unwinding, Helicase, SSB, Single molecule, TIRF microscopy

1. Introduction

Double-stranded DNA (dsDNA) is the most stable form of DNA, but it must be unwound transiently to form the single-stranded DNA (ssDNA) intermediates required for processes such as DNA replication, repair, and recombination. In each of these processes, duplex unwinding is catalyzed by a class of ubiquitous motor proteins, the DNA helicases. These reactions occur typically in the range 10–1,000 bp/s depending on the specific helicase, and generally the translocation is driven by ATP hydrolysis (1).

Both mechanically and optically based single-molecule methods have been used to determine translocation and unwinding rates, along with force production (2–7). These methods have determined key aspects of helicase mechanisms.

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This chapter describes a method that we developed recently (8) to visualize, in real time, the unwinding of individual double-stranded DNA (dsDNA) molecules by DNA helicases. Single-stranded DNA-binding protein (SSB) is a homotetrameric protein involved in various aspects of DNA metabolism, and we have exploited its high specificity and its rapid and tight binding to ssDNA (9, 10) for use as a probe to detect ssDNA produced by helicase activity. We use fluorescently labeled SSB to give an optical readout of helicase activity, and total internal reflection fluorescence microscopy (TIRFM) combined with a sensitive CCD camera to observe simultaneously many individual DNA unwinding events.

This assay is based on measuring the product formation and so has some advantages over other assays used for measuring dsDNA separation, namely, dye displacement assays. Binding the dye to dsDNA may cause inhibition of the helicase translocation and/or DNA cleavage (11, 12).

Two experimental approaches are described whereby either biotinylated dsDNA or biotinylated helicase is immobilized on an inert surface (Fig. 1) at a low surface density. As the helicase unwinds the DNA substrate, fluorescently labeled SSB molecules accumulate on the growing ssDNA products, which results in localized spots of gradually increasing fluorescence intensity. The fluorescence increase reports the amount of ssDNA product produced by unwinding of individual dsDNA molecules and,

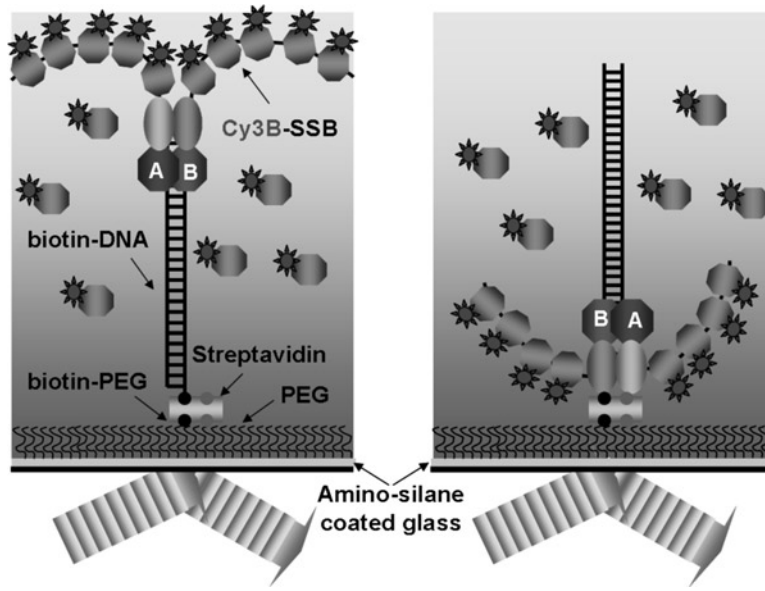

Fig. 1. Experimental scheme of the helicase assay. Biotinylated dsDNA or biotinylated helicase is immobilized on a PEGylated surface coated with streptavidin. DNA unwinding is initiated by addition of ATP in the presence of Cy3B-SSB. As the helicase unwinds its substrate, Cy3B-SSB accumulates on the growing ssDNA products. This results in a localized increase in fluorescence, which is visualized by TIRFM.

hence, provides information on the processivity and unwinding rate of the helicase. The method is compatible with an established bulk solution assay (13), which also utilizes a fluorescent version of SSB to report helicase activity. This is advantageous because the two approaches are complementary. The bulk measurements are quick to perform and can be applied to numerous DNA substrates (linear fragments, forked or gap junctions, partial duplexes, and plasmids of any length). By contrast, the single-molecule assay is best suited to linear DNA fragments, the length of which is limited by the penetration depth of TIRF evanescent wave (~100 nm) to ~2 kb. Because of signal averaging, the bulk methods give very reliable estimates of unwinding rate. However, the single-molecule experiments can reveal new mechanistic details about individual unwinding events that are masked in the bulk measurements.

In this chapter, we present how this method is applied, exemplified by the helicase-nuclease AddAB from *Bacillus subtilis*. AddAB has a role in DNA recombination. It contains a single helicase motor domain in the A subunit and a nuclease domain in each of the A and B subunits. AddAB binds with high affinity to dsDNA blunt ends, unwinds the duplex, and simultaneously degrades the ssDNA product. AddAB is a processive helicase regulated by the specific pentameric sequence 5′-AGCGG-3′ termed Chi_{Bs} (14). In order to avoid the effect of Chi_{Bs} and of DNA degradation, the measurements are done with a nuclease-deficient mutant $AddA^{N}B^{N}$ on DNA substrates devoid of Chi_{Bs} (Chi_{0}). Observation of the helicase activity of $AddA^{N}B^{N}$ with single-molecule resolution reveals bursts of unwinding activity punctuated by prolonged pauses. These details are obscured in conventional bulk phase measurements.

2. Materials

2.1. Surface Chemistry

1. Vectabond (Vector Laboratories, Peterborough, UK).
2. Meo-PEG-COO-Su, MW 5,000 (Iris Biotech GmbH, Marktredwitz, Germany) (see Note 1).
3. Biotin-PEG-COO-Su, MW 5,000 (Iris Biotech GmbH, Marktredwitz, Germany) (see Note 1).
4. Glass coverslips (22 mm × 50 mm, No. 1).
5. Microscope Slides (25 × 75 × 1 mm).
6. Glass spacers (3 mm × 40 mm, No. 1).
7. Stainless-steel Coverslip Staining Rack (Raymond A Lamb Ltd., East Sussex, UK).
8. Glass Trough with Glass Lid, (Raymond A Lamb Ltd., East Sussex, UK).
9. UV-curing, Loctite 350 Adhesive, (RS Components, UK).

10. Streptavidin is dissolved at 1 mg/ml in TB buffer (see Subheading 2.4), aliquoted and stored at −20°C.
11. Cy3-Streptavidin, stored as 1 mg/ml solution at 4°C.
12. Biotin, 200 μg/ml solution in distilled H_2O stored in aliquots at −20°C.

2.2. PCR

1. Template: a suitable DNA sequence that could be used as substrate for the helicase under study. The DNA fragment should contain sequences required for the loading of the helicase or the complex containing the helicase, such as the *ori*D sequence in the case of PcrA·RepD (15). Also, the template could contain regulatory sequences, such as Chi sequences in the case of AddAB (14) and RecBCD (16). In this assay, the template used for $AddA^NB^N$ was the pSP73-JY0 plasmid, which contains a 5.27-kb region of the *Escherichia coli* K12W311 genome devoid of Chi_{Bs} sequences (8).
2. Primers: suitably designed depending on the PCR template. To generate biotinylated DNA fragments of various lengths, a single 5′-Biotin-TEG (see Note 2) forward primer is used in combination with a number of nonbiotinylated reverse primers which are designed to anneal at different sites along the DNA template. The same primer pairs, but without the biotin-TEG tag, could be used to generate nonbiotinylated fragments, which could be used for negative control experiments and for studying the activity of immobilized helicase.
3. Phusion® Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Herts, UK) (see Note 3).
4. Agarose, for analysis of PCR products by electrophoresis.
5. 50× Tris-acetate-EDTA (TAE) stock. 1× TAE is used for preparation of agarose gels and as a running buffer during electrophoresis.
6. Ethidium bromide, 10 mg/ml of Ethidium bromide solution.
7. 6× Blue/Orange Loading Dye.
8. 1 kb Plus DNA Ladder (Invitrogen, UK).
9. Mini-Sub cell GT electrophoresis system and a PowerPac Basic power supply (Bio-Rad, Hertfordshire, UK).
10. UV transilluminator.
11. QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK).
12. Ethanol precipitation reagents: Ethanol, 3 M CH_3COONa, pH 5.2, Ethanol (70% v/v).
13. UV spectrophotometer.
14. DCC-SSB (see Subheading 3.5) for assessing the presence of ssDNA contaminants into the PCR preparation.
15. Fluorescence Spectrophotometer.

2.3. ssDNA Probe

Cy3B-labeled, W88C mutant of the single-stranded DNA-binding protein (SSB) from *E. coli*, termed Cy3B-SSB (8) (see Notes 4 and 5).

2.4. Helicase Assay

1. 100 mM ATP solution in 25 mM Tris–HCl, pH 7 (see Note 6).
2. TB Buffer: 25 mM Tris–HCl, pH 7.5, 10 mM NaCl.
3. H Buffer: 25 mM Tris–HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$.
4. Cy3B-SSB: 1.25 μM Cy3B-SSB in H buffer containing 5 μM Bovine Serum Albumin (BSA) to prevent SSB from sticking to surfaces.
5. Oxygen scavenger system: consisting of 10 mg/ml glucose, 50 μg/ml glucose oxidase, 400 μg/ml catalase, 500 μg/ml BSA, 1 mM DTT, 1 mM ascorbic acid, and 1 mM methyl viologen (see Note 7).
6. ATP regeneration system, consisting of 100 μg/ml creatine phosphokinase and 500 μg/ml creatine phosphate. These components are added to the reaction buffer when low ATP concentrations are used.

2.5. TIRF Microscopy

This assay was performed using a custom-made, objective-type TIRFM (8).

1. 532 nm, 50 mW, solid-state laser (Suwtech 532-50 with 1500 LDC controller, SP3-Plus) was used for excitation.
2. An electron-multiplying charge-coupled device (EMCCD) camera with high quantum efficiency, dynamic range, and signal-to-noise ratio (e.g., iXon897 BV, Andor) was used for imaging.

3. Methods

This assay is potentially applicable for the study of many different DNA helicases. In most cases, no modification of the helicase is required. However, several features of the helicase should be taken into consideration:

1. DNA Substrate. In order to be immobilized on the surface, the DNA substrates need to be end-modified with biotin. This can be achieved by PCR or a ligase reaction (see Subheading 3.5) or possibly by other methods such as annealing to a biotinylated oligonucleotide. Some helicases, however, use specific DNA substrates (whole plasmids, forked or Gap junctions, partial duplexes etc), the generation or immobilization of which could not always be feasible or straightforward.
2. Processivity. At high salt concentration, the assay has a theoretical resolution of 65 bases (65-base binding mode, see

Subheading 3.1). Therefore, to monitor an unwinding time course, the helicase should have a processivity of at least few hundred base pairs.

3. Unwinding Rate. A relatively slow helicase (unwinding rate of a few bases per second) would require long imaging times, during which the observed time-course could be limited by photobleaching. On the contrary, in the case of a rapid helicase (unwinding rate of hundreds of base pairs per second), the observed unwinding rate could be limited by SSB binding. All of these characteristics can be assessed using solution studies (17–19).
4. Additional Functions. Besides catalyzing unwinding, many DNA helicases have additional functions, such as nuclease activity. In this chapter, the assay is exemplified using the activity of the nuclease-inactive mutant $AddA^{D1172A}B^{D961A}$ ($AddA^{N}B^{N}$).

3.1. Selection of the ssDNA Biosensor Experimental Conditions

The helicase assay monitors DNA unwinding by measuring the SSB binding on the growing ssDNA; therefore, the selection of the SSB concentration and the buffer conditions are critical. These will determine the maximum theoretical observed rate (limited by the first-order SSB binding rate to the ssDNA) that can be measured by the assay and, therefore, its suitability to study specific helicases. The binding stoichiometry of SSB to ssDNA greatly depends on salt concentration and protein-to-DNA ratio: at high salt conditions and low protein-to-DNA ratio binding predominantly occurs with a stoichiometry of ~65 bases per SSB tetramer, whereas low salt concentrations and high protein-to-DNA ratio favors binding with a stoichiometry of ~35 bases per tetramer (9). Given the nature of this assay, the binding stoichiometry and kinetics of SSB on ssDNA determine the limitations of the assay and, therefore, should be carefully taken into account. In addition, the effect of SSB on helicase activity (see Note 8) could also affect the interpretation of the data.

1. Select buffer conditions to ensure a single SSB binding mode. The buffer conditions contribute to the binding mode of SSB to ssDNA. Therefore, it is important to select conditions that ensure one predominant mode (20). Buffer conditions should also be compatible with the helicase activity, since salt concentration could have big effect on the processivity of the helicase (13). Selecting the optimum buffer conditions for the helicase is advisable. If a different buffer has to be used, then the helicase should be characterized under these conditions (13).
2. Determine the affinity and confirm the binding mode of Cy3B-SSB in the helicase assay buffer. This is achieved by performing a titration of ssDNA to the Cy3B-SSB. This methodology is outside the scope of this review, but this is described by Kunzelmann et al. (20).

3. Select SSB concentration to ensure rapid, high-affinity binding. It is advisable to use at least tenfold higher concentration than the K_d measured under similar conditions. Additionally, SSB binding should occur faster than the unwinding rate of the helicase ($r_{helicase}$). At given buffer conditions and SSB concentration ([SSB]), the binding rate ($k_{binding}$) of SSB to ssDNA should be: $k_{binding}\ (\mathrm{SSB/s}) = k_{on} \times [\mathrm{SSB}]$. For a given binding mode at these conditions (y bases/SSB, $y = 65$ or 35 bases), the maximum rate (r_{max}) that can be observed is calculated from: $r_{max}\ (\mathrm{bp/s}) = k_{on} \times [\mathrm{SSB}] \times y$. The r_{max} should be significantly greater than $r_{helicase}$.

For example, the assay performed at high ionic strength and at 25 nM SSB-Cy3B results in the following (8). The 65-base binding should predominate (20), and the observable maximum unwinding rate was calculated to be ~600 bp/s ($= 36 \times 10^7\ \mathrm{M^{-1}\,s^{-1}} \times 25\ \mathrm{nM} \times 65$ bp).

3.2. Selection of the Optimum Surface Chemistry

The single-molecule analysis of DNA helicases in vitro requires the surface immobilization of either the helicase or its DNA substrate. The immobilization strategy should ensure the specific tethering of the helicase or the DNA on the surface, preserve the functionality of the immobilized molecule, and prevent the nonspecific absorption of other components of the assay. To this end, several different approaches have been described (21, 22). The specific tethering of the molecule of interest is usually achieved via the high-affinity interaction of streptavidin with biotin functionalized glass surfaces. Passivation of the surface by coating with nonspecific blocking proteins (such as BSA or casein), the linear or star-shaped polymer polyethylene glycol (PEG), or lipid bilayers ensures that helicase activity is maintained and that there is minimum background fluorescence caused by the nonspecific binding of the SSB probe. The selection of the immobilization strategy greatly depends on the components of the assay and has to be optimized for a specific application. The optimum surface chemistry for this helicase assay is based on the use of aminosilane coated surfaces coupled to aminoreactive linear PEG (succinimidyl ester PEG), a fraction of which is end-modified with biotin. This surface allows the specific immobilization of DNA and helicase molecules and abolishes the nonspecific absorption of both DNA and Cy3B-SSB to the underlying surface (Fig. 2).

3.2.1. Plasma Cleaning

Commercially available coverslips, although usually precleaned, are covered with organic contaminants, which could interfere with the immobilization process and could introduce fluorescent background, which reduces data quality. Such contaminants can be removed through treatment with harsh solvents (chemical etching). These include concentrated KOH or HCl in water or ethanol, or a

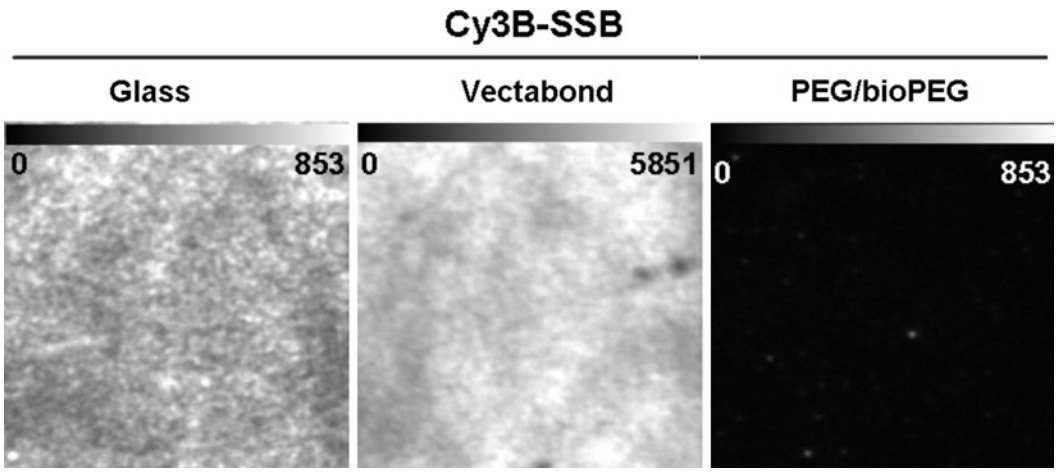

Fig. 2. PEGylation prevents the nonspecific absorption of Cy3B-SSB on the underlying surface. Glass coverslips following plasma cleaning, Vectabond treatment, and PEGylation were incubated with 25 nM Cy3B-SSB. Surface fluorescence was observed using TIRFM.

mixture of sulfuric acid and hydrogen peroxide in water (23). Contaminants can also be removed by exposing the coverslips to gas plasma discharge. There is no need to use specific gas such as argon. Air plasma is sufficient for this application. If a commercial plasma cleaner is available, plasma cleaning is recommended because it is simpler and less time-consuming.

1. Place coverslips into the stainless-steel rack.
2. Place the rack at the center of plasma cleaner chamber, close the chamber, and start the vacuum pump.
3. Wait until the vacuum gage indicates that the pressure of the chamber has reached approximately 2 Torr.
4. Turn the power on and set the level to high until you observe a purplish glow.
5. Expose the coverslips to the plasma discharge for 5 min.
6. Turn power off, open the valve to let air in, and remove the coverslips.
7. Store coverslips in a closed container at room temperature.

3.2.2. Surface Functionalization

The plasma-cleaned surfaces are functionalized with the aminosilane reagent Vectabond, which yields a dense coating of primary amines, which can react with the succinimidyl ester PEG. Surface coating is performed as recommended by the manufacturer.

1. Immerse one rack of plasma-cleaned coverslips, laid down, in the first glass trough containing 200 ml of acetone and incubate for 5 min. Coverslips tend to stick to each other. Therefore, using forceps, separate coverslips so that their whole surface is exposed to the acetone.

2. Using forceps, transfer the rack into the second trough containing 1/50 dilution of Vectabond in acetone (200 ml final volume) and incubate for 5 min. Coverslips tend to stick to each other. Using forceps, separate them so their whole surface is treated with the Vectabond.
3. Transfer the rack on a tissue paper so that the excess of Vectabond is removed and immediately immerse into the third trough containing distilled water. Using forceps, move the rack in/out of the water ~10 times, replace the water, and wash for ~10 times again.
4. Lay the rack down on a tissue to remove the excess water, separate the coverslips, and store the rack upright in a tip-box on the bench. To accelerate the drying process, store the racks under vacuum in the desiccator. Once the coverslips have dried, proceed to the PEGylation. Otherwise, the Vectabond-coated coverslips can be stored at room temperature for up to 3 months.

3.2.3. Surface PEGylation

PEGylation refers to the covalent attachment of succinimidyl PEG to the exposed amines of the Vectabond-treated coverslips. The conjugated polymer forms a dense brush which blocks the nonspecific absorption of components to the highly adhesive Vectabond-coated surface.

1. Prepare 500 ml of 100 mM $NaHCO_3$, pH 8.3. No pH adjustment is usually required. It is recommended to prepare the buffer just before use because its pH tends to change over time.
2. Dissolve 1 g aliquot of Meo-PEG-NHS (25 % w/w) and 60 mg of Biotin-PEG-COO-Su (1.5 % w/w) in 4 ml of 100 mM $NaHCO_3$. Mix well until the solution is clear. The 4 ml PEG/Biotin-PEG solution is sufficient for coating 26 coverslips.
3. Coat a large rectangular container (e.g., 500 cm^2 cell culture dish) with parafilm. Cover all four sides of the dish with wet tissues, to create a humid environment and prevent the coverslips from drying.
4. Place 13 Vectabond-treated coverslips on the parafilm, apply 300 μl of PEG/Biotin-PEG solution and place a second coverslip on top. The second coverslip is placed slowly so that the solution is evenly spread without formation of bubbles.
5. Close the lid and incubate the coverslip sandwiches for 3 h at room temperature, in the dark (see Note 9).
6. Fill three small beakers with 100 mM $NaHCO_3$ and three with distilled water (dH_2O) and place them in a row.
7. Using forceps, disassemble the sandwiches one by one (so that coverslips do not dry) and remove excess PEG solution on each coverslip by resting its edge on a tissue. Wash the coverslip

by immersing it ~10 times first into each of the $NaHCO_3$ and then the dH_2O beakers. Replace $NaHCO_3$ solution and dH_2O after washing six coverslips (see Note 10).

8. Place the washed coverslips on a rack. *Be careful! The coverslips are coated only on one side*. Therefore, it is crucial to always place them facing the same side of the rack. It could be helpful to put a mark on the rack indicating the coated side.
9. Store the coverslips in a desiccator under vacuum, in the dark, for maximum 3 weeks. Storage in the cold could prolong their life span (see Note 11).

3.3. Flow-Cell Construction

The PEGylated coverslips are used to construct two-channel cruciform flow-cells. This maximizes the use of the expensive PEGylation reagents and allows direct side-by-side comparison between two experimental conditions, avoiding the complication of surface variability.

1. Channel preparation (this step can be done while the coverslips are being coated with the PEG). Using UV-glue, glue three coverglass spacers (No. 1 coverslip cut to 3 mm×40 mm), spaced by ~5 mm, with the long axis orthogonal to the axis of the microscope slide, to form two parallel channels. Use the right amount of glue so that the area underneath the spacer is covered without the glue overflowing. With the forceps press on the spacers so that the glue forms a uniform layer between the spacer and the slide and eliminate air bubbles. Allow the UV-glue to set by incubating the slides in a UV light box for 10 min.
2. Flow-cell assembly: Glue a PEGylated coverslip, with the coated side facing inward, onto the three spacers to generate two parallel channels that run across the width of the microscope slide. Again, it is critical to use the right amount of glue, which is sufficient to create a uniform layer without overflowing and sealing the channels. With the forceps, lightly press on the coverslip, to ensure that it is completely horizontal and eliminate any air bubbles. Allow the UV-glue to set by incubating the slides in a UV light box for 10 min. The PEGylated coverslip should protrude by about 12 mm on either side of the slide. These protrusions facilitate the addition of experimental solutions to the channel: during sample preparation, the slide is tilted slightly and new solution is added to the upper side of the channel, whereas old solution is removed from the lower side of the channel, using the capillary action of a tissue paper. The volume required to fill in one channel is 20–30 μl.
3. Storage: Lay the PEGylated flow-cells flat on plastic containers (e.g., tissue culture dishes) and store them at room temperature in the dark, desiccated under vacuum, for maximum 3 weeks. Storage at 4°C or even –20°C could prolong their life span (see Note 11).

3.4. Assessing the Functionality of PEGylated Flow-Cells

It is important to assess the efficiency and uniformity of PEGylation as well as the functionality of each batch of PEGylated surfaces. A simple and easy way is to incubate the PEGylated surfaces with Cy3-Streptavidin in the absence and presence of biotin. The two-channel flow-cells allow comparison of the two conditions on a single surface.

1. Dilute 1/50 the 1 mg/ml Cy3-Streptavidin stock in TB buffer to obtain 50 µl of 20 µg/ml (= 0.33 µM) solution.
2. Repeat the same dilution of Cy3-Streptavidin in the presence of 2 µl of 200 µg/ml free biotin (= 33 µM, final concentration). This 100-fold excess of free biotin ensures saturation of the streptavidin binding sites.
3. Incubate for 15 min at room temperature.
4. Apply one solution on each channel of the PEGylated flow-cell.
5. Incubate for 15 min at room temperature.
6. Wash each channel six times using 50 µl TB buffer.
7. Visualize fluorescence using TIRF. When comparing signal intensities, use the same setting of Look-Up-Table (LUT). An example of successful PEGylation is shown in Fig. 3 (see Note 12).

Alternatively, the efficiency of PEGylation can be assessed by the addition of any fluorescently labeled component of the assay (e.g., Cy3B-SSB). Apply 25 nM Cy3B-SSB (the working concentration for the assay) on the PEGylated surface and observe the background fluorescence (Fig. 4). This gives information about the blocking capacity of the surface, but not about its functionality, i.e. the presence of biotin on the surface. Unsuccessful or partial PEGylation results in patchy fluorescence background.

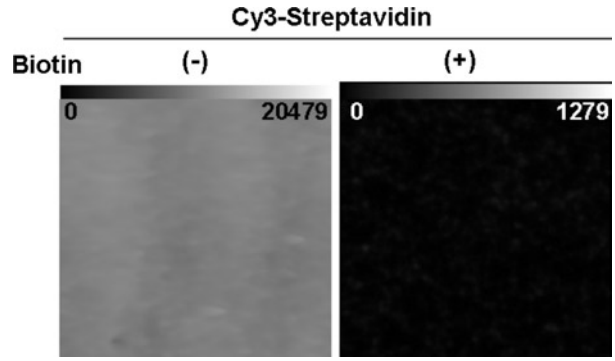

Fig. 3. Example of functional PEGylated surfaces. Freshly made PEGylated surfaces were incubated with 20 µg/ml Cy3-Streptavidin in the presence and absence of 100-fold excess of free biotin. Saturation of the binding sites of Cy3-Streptavidin with biotin abolishes coating of the PEG/Biotin-PEG surface with Streptavidin, which confirms the presence of Biotin on the surface. Also, the uniformity of the coating indicates complete coverage of the surface with PEG.

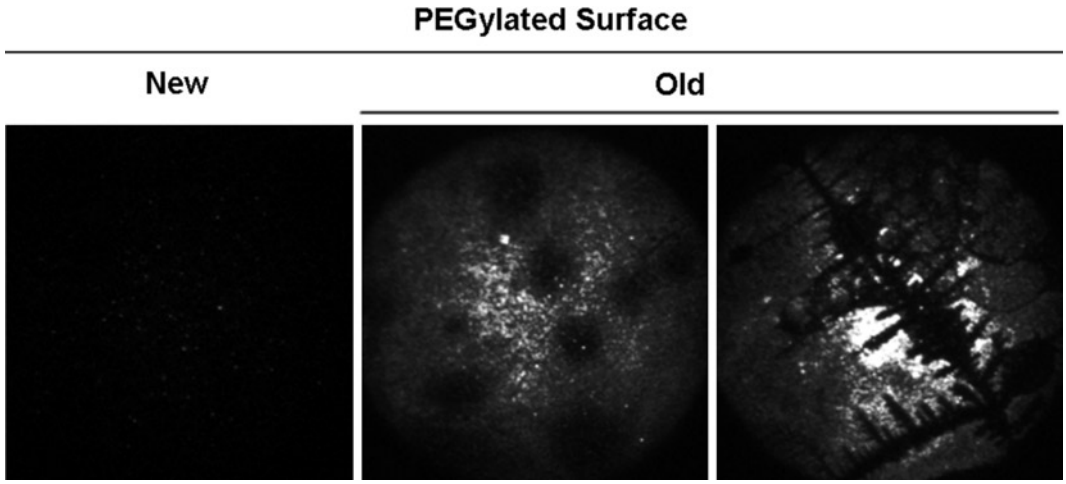

Fig. 4. Examples of degradation of the PEG coating. Freshly made PEGylated coverslips were incubated with 25 nM Cy3B-SSB and surface fluorescence was observed using TIRFM. Prolonged storage of the coated surfaces results in degradation of the coating, leading to patchy fluorescence background. Old surfaces are also characterized by extensive crystals.

3.5. Preparation of DNA Substrates

PCR is a simple and quick way to generate biotinylated and nonbiotinylated dsDNA fragments of various lengths. It can be applied on any sequence and requires only selection of various primer pairs that are suitably spaced on the DNA template (see Subheading 2.2). Although very versatile, PCR could present some disadvantages (see Note 13). Alternatively, DNA substrates can be generated by restriction enzyme digestion (see Note 14).

3.5.1. PCR Reaction and DNA Purification

The protocol below is suitable for the generation of DNA fragments up to 3 kb (see Note 15) and assumes the use of Phusion Hot Start polymerase and the Bio-Rad's Mini-Sub cell GT electrophoresis system. The high purity and the length homogeneity of the DNA fragments are critical for obtaining high-quality data. It is, therefore, highly recommended to purify the PCR products by agarose gel extraction. This allows the detection of PCR side-products and their separation from the main product. Subsequent ethanol precipitation ensures further improvement of DNA purity.

1. Following the instructions of the manufacturer, prepare 6 × 50 μl PCR reaction mix (final volume of 300 μl) and aliquot it into six PCR tubes.
2. Select the cycling conditions, as recommended by the manufacturer, and perform the reaction.
3. Spin down the reactions and pull them together in one tube.
4. Mix the 300 μl PCR reactions with 60 μl of 6× Orange/blue loading dye.
5. Prepare one 1 % (w/v) agarose gel in 1× TAE containing 1 μg/ml ethidium bromide. 100 ml agarose solution is sufficient for a

single gel (see Note 15). Before casting the gel, unify all but one the wells of a 1.5-mm comb into a single long well using tape.

6. Load the DNA ladder and the PCR reaction mix and run the gel in 1× TAE at 100 V for approximately 1–2 h, until a good separation of the ladder is achieved (see Note 15).
7. Expose the gel to UV light and, using a scalpel, extract the band that has migrated at the correct size. A successful PCR should result in a single band (see Note 16). Minimize the exposure of the DNA to the UV. Prolonged exposure will damage the DNA and may affect the activity of the helicase.
8. Extract the DNA from the gel using the QIAquick Gel Extraction Kit, as recommended by the manufacturer.
9. Mix the eluted DNA with two volumes of ethanol and the required volume of 3 M CH_3COONa to achieve a final concentration of 0.3 M CH_3COONa. Mix well.
10. Store the mixture for at least 1 h at –20°C (the mixture could also be stored at –20°C overnight).
11. Centrifuge for 15 min at 14,000 × *g* and 4°C and discard the supernatant.
12. Wash with 70 % (v/v) ethanol and centrifuge for 5 min at 14,000 × *g* and 4°C.
13. Discard supernatant and air-dry at room temperature.
14. Resuspend in 150–200 µl 10 mM Tris–HCl, pH 8.5.
15. Measure the concentration of the DNA using a UV spectrophotometer. The above procedure should result in a concentration of approximately 50 ng/µl.

3.5.2. Assessing the Level of ssDNA Contamination

This measurement will require the use of a fluorescence spectrophotometer and a different fluorescent SSB, DCC-SSB (13). DCC-SSB exhibits a sixfold increase in fluorescence when bound to DNA; therefore, it is a sensitive detector of ssDNA contamination.

1. Prepare a 25 nM DCC-SSB solution in the helicase buffer. Presence of 5 µM BSA will prevent DDC-SSB from sticking to the sides of the tube or cuvette. Calibrate the signal from DCC-SSB with a standard ssDNA solution of known concentrations of ssDNA binding sites (for more details see ref. 13, 20).
2. Add an excess of the DNA fragment (in terms of binding sites) to a fresh DCC-SSB solution. Measure the fluorescence change. Use the calibration to determine the amount of ssDNA contamination (see Note 17).

3.6. Intensity Calibration

The TIRFM approach used here involves excitation of the surface-immobilized specimen by an exponentially decaying evanescent field. Quantitation requires that the relationship between the

optical signal intensity and the amount of ssDNA·Cy3B-SSB is established. It is important to define the maximum DNA length below which fluorescence intensity has a linear dependence on DNA length. This sets an upper limit in the length of DNA substrates that can be used with this assay and, therefore, the processivity that can be measured. The relationship between intensity and DNA length can be used as an empirical calibration to quantify the amount of SSB bound and, therefore, the length of ssDNA product as well as to correlate changes in the unwinding rate with sequence repeats along the DNA substrate.

3.6.1. Generation of Biotinylated Cy3B-SSB·ssDNA Complexes

This can be achieved either using helicase activity (see Note 18) or by heat denaturation (see Note 19) of biotinylated dsDNA of various lengths in the presence of excess Cy3B-SSB (see Note 20).

1. Helicase activity: Mix 70 pM of biotinylated dsDNA of various lengths with 10 nM $AddA^NB^N$ and 1 mM ATP in H Buffer. Incubate each sample for 5 min at room temperature (see Note 21).
2. Heat denaturation: In a PCR tube prepare 10 µl of 700 pM dsDNA of various lengths in denaturation buffer (50 mM Tris–HCl pH 7.5, 10 % (w/v) sucrose, 2 mM $(CH_3COO)_2Mg$, and 2 mM DTT). Using a thermocycler, heat-denature the dsDNA fragments at 95°C for 10 min. Place the different samples immediately on ice to avoid reannealing of the duplex and mix with 90 µl of ice-cold Cy3B-SSB in H buffer to achieve a final concentration of 25 nM Cy3B-SSB. Keep on ice for 30 min (see Note 21).

3.6.2. Surface Immobilization and Visualization

1. Apply 30 µl of 20 µg/ml streptavidin in TB buffer on each channel of the PEGylated flow-cell and incubate for 15 min at room temperature.
2. Wash six times using 50 µl of TB buffer to remove excess protein.
3. Immobilize the products of helicase activity or heat denaturation and incubate for 15 min at room temperature.
4. Wash with 50 µl of H Buffer containing 25 nM Cy3B-SSB and supplemented with the Oxygen Scavenger system.
5. Visualize the immobilized complexes by TIRFM. Using the same acquisition parameters (laser power and camera exposure time) as for the helicase assay, record the intensity of immobilized Cy3B-SSB·DNA complexes.

3.6.3. Fluorescence Intensity as a Function of DNA Length

Using an image processing software (such as the custom-written software freely available at http://www.nimr.mrc.ac.uk/gmimpro, ImageJ, or other) obtain the intensity distribution of the immobilized complexes. Then, plot the average or Gaussian mean of the

intensity distribution against the DNA length. When developing this assay, the calibration curve revealed that the dependency of the mean intensity on the DNA length was linear for substrates up to 2 kb.

3.7. Helicase Assay

3.7.1. AddA^{N}B^N Activity on Immobilized dsDNA

1. Apply 30 µl of 20 µg/ml streptavidin in TB buffer on each channel of the PEGylated flow-cell and incubate for 15 min at room temperature.
2. Using the capillary action of a tissue paper placed at the bottom of the channel, wash six times using 50 µl of TB buffer to remove excess protein.
3. Flow in 50 µl of 200–500 pM of biotinylated dsDNA in TB buffer and incubate for 15 min at room temperature (see Note 22).
4. Wash six times using 50 µl of TB buffer to remove unbound dsDNA.
5. Flow in 50 µl of 10 nM AddA^{N}B^N in H buffer containing 25 nM Cy3B-SSB, the oxygen scavenger system and the ATP regeneration system, if required. Incubate for 5 min at room temperature, to allow the helicase to bind its substrate (see Note 23). Alternatively, flow in 1 mM ATP in the above buffer (see Note 24).
6. Place the flow-cell on the microscope and record the background fluorescence of the surface (see Note 25).
7. Initiate dsDNA unwinding by flowing in 50 µl of 1 mM ATP in the same buffer as in step 5 (see Note 26). Alternatively, initiate unwinding by 10 nM AddA^{N}B^N.
8. Record dsDNA unwinding using the optimum acquisition rate (frames per second) so that the events are completed within the duration of the record with maximum temporal resolution.

3.7.2. Activity of Surface-Immobilized AddA^{N}B^N

1. Coat the PEGylated flow-cell with streptavidin as described above.
2. Flow in 50 µl of 2.5 nM biotag-AddA^{N}B^N in TB buffer and incubate for 15 min at room temperature.
3. Wash six times using 50 µl of TB buffer to remove unbound helicase.
4. Flow in 50 µl of 1 nM nonbiotinylated dsDNA in H buffer containing 25 nM Cy3B-SSB, the oxygen scavenger system and the ATP regeneration system, if required. Incubate for 5 min at room temperature, to allow the helicase to bind its substrate.
5. Initiate unwinding by flowing in 50 µl of 1 mM ATP in the same buffer as in the previous step.

An example of the above experiments is summarized in Fig. 5. The assay should be repeated with different DNA lengths and a variety

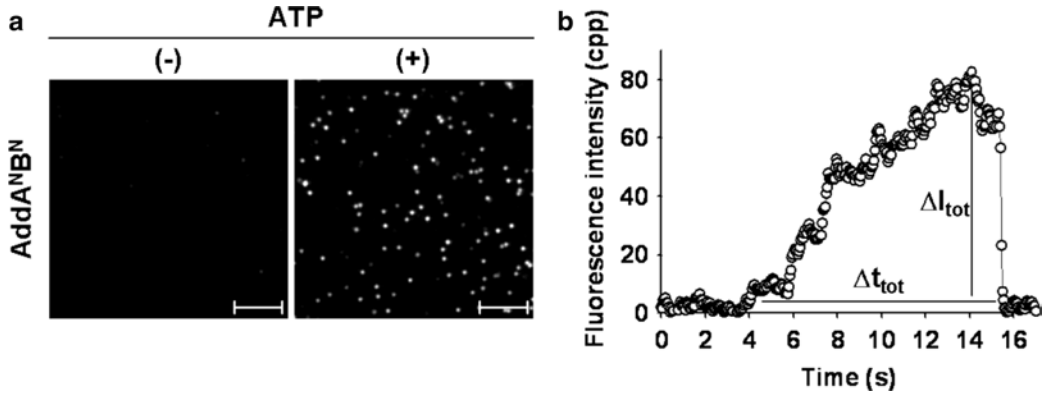

Fig. 5. Monitoring the unwinding of multiple dsDNA molecules by AddA^{N}B^{N} in real time. Biotinylated 1.5-kb Chi$_0$ dsDNA fragments were immobilized on the PEGylated surface through biotin–streptavidin interaction and incubated with the nuclease inactive mutant AddA^{N}B^{N}. (**a**) Addition of 1 mM ATP resulted in the appearance of multiple fluorescence spots of increasing intensity, each corresponding to the unwinding of a single dsDNA molecule. Scale bar, 5 μm. (**b**) Representative example of an intensity time-course that reflects the helicase activity of AddA^{N}B^{N} is shown here. The single-step decrease in intensity at the end of the time-course represents the simultaneous release of the two ssDNA products due to the disruption of the biotin–streptavidin interaction by AddA^{N}B^{N}.

of helicase and ATP concentrations (see Note 27). It is also important to perform the assay with double the Cy3B-SSB concentration to test that the observed rate is not limited by SSB binding (see Note 28).

3.8. Data Analysis

In this assay, the data have the form of time-courses of increasing intensity, which can be obtained by image processing of the video recordings. Analysis of the image sequence can be achieved with any computer software capable of the following:

1. Identifying all the fluorescent spots of increasing intensity present.
2. Discarding spots of constant or instantaneously increasing intensity (see Note 29).
3. Determining the intensity as a function of time.

Such custom-written software is freely available at http://www.nimr.mrc.ac.uk/gmimpro/

Each time-course is characterized by two parameters, the total increase in intensity (ΔI_{tot}) and total duration of unwinding (Δt_{tot}). Therefore, the next step in the analysis is to plot the distribution of these two parameters at different experimental conditions. These distributions will provide information about features of the helicase such as processivity, unwinding rate, and heterogeneity in the population. They will also provide validation of the experiments and allow comparison with bulk measurements.

Finally, the intensity time-courses should be analyzed for the presence of unwinding intermediates, such as pausing and bursts of

activity. This can be achieved using custom-written software that can do the following:

1. Smooth the intensity data by running average or median filter using the appropriate window size to remove noise (see Note 30).
2. Calculate the first derivative as a function of time.
3. Use a threshold to separate pause phases from unwinding phases (see Note 31).

4. Notes

1. These PEG derivatives are sensitive to humidity, so extensive handling in air could degrade them. It is recommended to purchase PEG in single-use aliquots.
2. 5′-Biotin primers (labeled with Biotin Phosphoramidite) could also be used. However, the labeling with Biotin-TEG (BiotinTEG Phosphoramidite) provides an additional linker of ten atoms between the biotin and the DNA. The long linker ensures that the biotin is freely available to bind to the streptavidin-coated surface.
3. The choice of the polymerase is crucial for obtaining high-quality data. Some polymerases tend to dissociate during amplification, generating dsDNA products containing ssDNA overhangs. Such overhangs would bind SSB and, therefore, result in background fluorescence, even in the absence of helicase. The performance of the polymerase can vary between PCR reactions. All such prematurely terminated side-products cannot be separated by agarose gel electrophoresis. Among the polymerases tested, the Phusion Hot Start gave the minimum, if any, detectable ssDNA contamination. The purity of the PCR products can be assessed as described in Subheading 3.5.
4. Cy3B-SSB was purified and labeled as previously described (13) so that each SSB subunit contains one Cy3B. Cy3B-SSB should be stored at micromolar to millimolar concentrations at −80°C. It is recommended to avoid freeze–thaw cycles. Cy3B-SSB binds ssDNA tightly and rapidly.
5. Among several fluorescently labeled SSB mutants, Cy3B-SSB was selected as the best because it combined the fast association kinetics and high affinity for ssDNA with good optical properties, such as high quantum yield, high photostability, and long excitation and emission wavelengths.
6. It is recommended to adjust the pH of the ATP solution at pH 7, which ensures higher stability. Measure the exact concentration

of ATP in solution by measuring the absorbance at 254 nm using a UV spectrometer (extinction coefficient at 254 nm = 15.4 cm^{-1} mM^{-1}). Store at −20°C and avoid multiple freeze–thaw cycles.

7. A fluorescence-based assay requires conditions that ensure photostability of the fluorescent label and minimum photobleaching. This is usually achieved by a combination of oxygen removal and quenching of the triplet state by means of reducing agents (24). The oxygen scavenger system should be optimized for the specific fluorophore used. For this assay, minimum photobleaching was achieved with the glucose oxidase–catalase oxygen scavenger supplemented with mixture of reducing (ascorbic acid) and oxidizing (methyl viologen) agents (25). With a laser power of 0.5 mW, the photobleaching rate was ~0.009 s^{-1}.
8. The fact that SSB is a natural component of DNA metabolism is an advantage of this assay, as it allows simulation of the native conditions of DNA unwinding. However, there are several examples of SSB interacting with, and regulating the activity of various DNA helicases (26–28). It is, therefore, important to assess and be aware of the effect of SSB on the helicase under investigation, especially when SSB has not previously been used in the characterization of the helicase. This can be easily achieved by bulk measurements of the helicase activity in the presence and absence of SSB (29).
9. The reaction results in the gradual formation of small air bubbles. This can be used as an indicator to the fact that the reaction is occurring.
10. Do not wash or scrub the coverslip surface excessively, since the coating is not very stable and could wash away.
11. If the surfaces are stored for more than 3 weeks, their functionality should be tested before use (see Subheading 3.4). Old surfaces tend to lose the PEG coating exposing the underlying Vectabond layer. This results in high fluorescent background when fluorescent molecules are applied on the surface. Also, extensive crystals can be observed (Fig. 4).
12. This experiment tests the presence of free biotins on the surface and also can reveal an unsuccessful or partial PEGylation. If the coating has failed, high fluorescent background will be observed in both channels. This background corresponds to the nonspecific absorption of Cy3-Streptavidin to the underlying surface. In the case of partial or inefficient coating, patches of high fluorescence against a dark background will be observed on the (+) biotin channel.
13. PCR can generate various side-products, such as fragments of different sizes as well as incomplete products containing ssDNA overhangs (see Subheading 2.2). The selection of the polymerase

and optimization of the PCR conditions are critical to minimize the presence such contaminants. In addition, extensive purification and assessment of the quality of the product is essential to obtain a high-quality DNA substrate.

14. Restriction enzyme digestion can overcome some of the limitations of PCR. In that case, the digested fragments can be labeled with biotin using terminal transferase (30).
15. PCR can be used for the generation of very long fragments up to 48 kb. Phusion Hot Start polymerase is suitable for amplification up to 20 kb, whereas for longer fragments a special polymerase, such as the EXL DNA polymerase (Stratagene), should be used. In addition, the percentage of agarose, the voltage, and the duration of the electrophoresis have to be optimized. For instance, PCR products above 10 kb should be separated in a 0.4% agarose gel and electrophoresis should be performed for 18 h at 25 V.
16. If PCR side products are present, modify the conditions of the PCR. Usually, more stringent conditions (higher annealing temperature, shorter denaturation etc) could eliminate the side products. However, if this cannot be avoided, run the gel for longer time to achieve good separation of the main product from the additional bands.
17. It is important to use at least a tenfold excess of SSB binding sites over DCC-SSB. This excess will give the greatest sensitivity because background fluorescence from unbound DCC-SSB will be minimal. The greater sensitivity allows for a greater accuracy when determining the ssDNA contamination. The formula to calculate the concentration of binding sites is as follows: [dsDNA molar concentration]×[dsDNA length in nucleotides per strand]×[2 strands]/[65 nucleotides per SSB].
18. When helicase activity is used to generate the ssDNA·Cy3B-SSB complexes, it is important to know whether unwinding occurs from both ends of the substrate. In this case, the two unwound single strands can be held together by the opposite moving helicases, and therefore, the intensity of the immobilized complex will be double of the one expected for a single strand. Unidirectional unwinding is preferable and could be achieved either by using a helicase which requires a specific sequence to be loaded on the DNA or by preincubating the dsDNA with streptavidin. The latter option, however, can be applied to helicases that do not disrupt the biotin–streptavidin interaction (8).
19. Heat denaturation, when successful, generates single strands labeled with Cy3B-SSB. These complexes should have half the intensity of the final products of the helicase assay. However, depending on the sequence, denaturation is not always complete and should be optimized.

20. Excess of Cy3B-SSB tetramers is crucial to achieve saturation of the ssDNA products with SSB and prevent reannealing. As a rule of thumb, the concentration of SSB and dsDNA should ensure a ratio of 1 SSB per 20 bases of ssDNA. The assay is performed at 25 nM Cy3B-SSB. Therefore, the required molar concentration of dsDNA is calculated on the basis of the longer dsDNA fragment as follows: [dsDNA length]×[2 strands]/[20 bases per SSB].
21. Both reactions generate 140 pM Cy3B-SSB·ssDNA products which, when immobilized, should provide the optimum surface density of fluorescent spots. If reactions are performed with higher dsDNA concentrations, serial dilution should be performed so that the immobilized products appear as discrete fluorescent spots.
22. Serial dilutions should be performed to achieve the optimum immobilization density so that the unwinding events appear as discrete fluorescent spots. The optimum dsDNA concentration could be defined by labeling the immobilized dsDNA with a dimeric cyanine dsDNA specific dye (Invitrogen), such as YoYo-1, PoPo-1, etc.
23. Depending on the helicase, it may be advantageous to incubate the coverslips at a specific temperature and/or to use a microscope enclosed in an incubator. This can improve the efficiency of helicase loading and activity.
24. This option is recommended in the case of helicases whose affinity for DNA is enhanced by ATP. Also, it is advisable when low ATP concentrations are used, because it allows the ATP concentration to be equilibrated before initiation of the reaction.
25. Until the helicase reaction is initiated, only a few, if any, fluorescent spots of constant intensity should be observed. These would correspond to ssDNA contaminants or Cy3B-SSB aggregates nonspecifically absorbed on the surface.
26. $AddA^{N}B^{N}$ is added to this solution to ensure that the helicase is not washed out when ATP is added. This may not be necessary, if the helicase has high affinity for the dsDNA.
27. These measurements allow characterization of the helicase and also serve as controls for the assay. The total increase in intensity and duration of unwinding should increase with the DNA length. Moreover, the duration of unwinding will increase with decreasing ATP concentration, whereas the total increase in intensity should be ATP independent. Furthermore, change in helicase concentration could result in a change in the number of observed events. Changes in unwinding rate may be observed if the helicase forms oligomers or the helicase has a low processivity. If these points are not observed in the assay, then it is likely there is a problem in the conditions.

28. This control will confirm that unwinding is not limited by the rate of SSB binding. If the observed rate of unwinding and the maximum increase in intensity both increase with SSB concentration, then this would indicate that SSB binding is rate limiting. However, it should be noted that, depending on the range of SSB concentration used, change in the maximum increase in intensity could also be attributed to the appearance of a 35-base binding.
29. Spots of constant intensity correspond to background spots such as Cy3B-SSB aggregates or immobilized incomplete PCR products containing single-stranded sections (see Subheading 3.5). The spots of instantaneous intensity increase are the result of free Cy3B-SSB·ssDNA complexes colliding with the surface. These can be caused, for example, by the release of ssDNA from the surface by helicases that disrupt the biotin–streptavidin interaction.
30. The window size will have to be optimized depending on the amount of noise in the data. It is important not to remove features from the unwinding trace.
31. The threshold will have to be optimized. It is important to apply one set of conditions to the whole dataset. Owing to noise and fluctuations in the signal, it may not be possible to have ideal conditions for every trace.

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