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Novel approaches to study helicases using magnetic tweezers

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Abstract

Helicases form a universal family of molecular motors that bind and translocate onto nucleic acids. They are involved in essentially every aspect of nucleic acid metabolism: from DNA replication to RNA decay, and thus ensure a large spectrum of functions in the cell, making their study essential. The development of micromanipulation techniques such as magnetic tweezers for the mechanistic study of these enzymes has provided new insights into their behavior and their regulation that were previously unrevealed by bulk assays. These experiments allowed very precise measures of their translocation speed, processivity and polarity. Here, we detail our newest technological advances in magnetic tweezers protocols for high-quality measurements and we describe the new procedures we developed to get a more profound understanding of helicase dynamics, such as their translocation in a force independent manner, their nucleic acid binding kinetics and their interaction with roadblocks.

Keywords: magnetic tweezers; helicases; DNA/RNA hairpins; Click chemistry surfaces; three-way junction; recombinant protein purification; single molecule binding assay; kinetic locking; roadblock interaction.

1. Introduction

Helicases are ubiquitous nanometric molecular motors involved in every step of nucleic acid (NA) metabolism and are present in nearly every living branch. They are NA-dependent NTPases that convert chemical energy from nucleoside triphosphates (NTP, mainly ATP)

binding and hydrolysis to mechanical energy. This induces conformational changes that lead not only to translocation on single-stranded NA (ssNA) or unwinding double-stranded NA (dsNA) as previously thought, but also to remodeling NA-bound protein structures, or simply binding to NA and serving as protein recruitment scaffolds or NA clamps (Gao & Yang, 2020; Linder & Jankowsky, 2011). Initially, the role of helicases had largely been restricted to duplex unwinding and translocation during DNA replication, repair and recombination (Abdelhaleem, 2010; Brosh & Matson, 2020; Mendoza et al., 2016), yet, helicases are also required for RNA metabolism such as splicing, maturation, export, translation and decay (Cordin & Beggs, 2013; Khemici & Linder, 2018; Linder & Fuller-Pace, 2015).

It is estimated that around 1% of eukaryotic genes encode a panoply of RNA and DNA helicases, that are tightly regulated by intra- and intermolecular interactions (Lohman et al., 2008; Patel & Donmez, 2006; Umate et al., 2011; Wu, 2012). Host and viral-encoded helicases play important roles in virus life cycle and infection, and given their vital roles in all cellular aspects, these enzymes are also linked to numerous genetic diseases and cancers. Thus, helicases constitute excellent targets for drug therapies and diagnostic tools (Abdelkrim et al., 2021; Brosh & Matson, 2020; Datta & Brosh, 2018; Dhar et al., 2020; Steimer & Klostermeier, 2012).

The substantial coverage of helicase functions illustrates the importance of studying their mechanisms and regulation. Helicases are classified into six superfamilies (SF1-SF6) differing from their primary sequence, NA preference and polarity, cellular function and structure (Gorbalenya & Koonin, 1993; Singleton et al., 2007). Superfamilies 1 and 2 share the same characteristic sequence motifs in their helicase core and generally act as monomers or dimers, unlike SF3-SF6 helicases that form hexameric toroidal ring-like structures. SF1 and SF2 include helicases with 5'-3' or 3'-5' polarities, and some subfamilies recognize either exclusively DNA, RNA or both NA.

Despite their structural homologies, each helicase dynamic is unique. Indeed, when studying helicase dynamics many questions arise regarding their behavior: does it act locally or does it translocate onto NA and if so, at which speed, processivity (number of consecutive base pairs translocated) and polarity? What is their affinity and how strong is their grip on double-stranded (ds), single-stranded (ss) or duplex NAs? How are their properties coupled to their function? How are they regulated and how do they interact with roadblocks such as NA-binding proteins or NA tertiary structures?

In order to answer these questions, a combination of bulk assays with single-molecule micromanipulation techniques such as optical tweezers and magnetic tweezers have been employed (Bockelmann, 2004; Greenleaf et al., 2007; Neuman et al., 2007; Neuman & Nagy, 2008). Contrary to bulk assays, single-molecule approaches consist in observing in real time the unwinding of individual dsNA by individual helicases. In magnetic tweezers, a NA is tethered on both sides and stretched by applying an external force through a paramagnetic bead subjected to the gradient field of magnets (Figure 1a). The NA's extension is measured by optically tracking the distance between the surface and the bead. When a helicase translocates along the NA, a portion of dsNA is unwound (Figure 1b). The real-time position of the helicase along the NA can then be deduced, and thus its velocity, its processivity, and its directionality can be estimated.

Useful practical handbooks for these methods (Bustamante et al., 2021; Vilfan et al., 2009) as well as several reviews of their application to helicase study have been published (Bianco, 2021a; Hodeib et al., 2016; Manosas et al., 2010; Sun et al., 2010; Yodh et al., 2010). Here, we describe new protocols that we designed to investigate further properties of helicases such as their translocation independently of the applied force, their binding grip, and the impact of roadblocks.

The article is organized as follows: we will first provide some tips for successful synthesis of DNA/RNA hairpins and focus on general progress regarding surface chemistry, NA tethering techniques and *in vitro* purification of recombinant helicases before we detail magnetic tweezers force protocols and the associated data analysis procedures that underlie our most recent advances in helicase properties characterization.

[Insert Figure 1 here]

2. Advances in synthesis, attachment and calibration of nucleic acid hairpins for magnetic tweezer experiments.

As mentioned before, bulk assays and single-molecule micro-manipulations have been largely used for the study of helicases. The latter allow unique measurements on individual molecules, accounting for population diversity in protein batch purifications, contrary to bulk experiments which result in average measurements. Here, we focus on magnetic tweezers experiments for helicase characterization, where a NA hairpin is attached on one side to a glass surface (coverslip) and on the other side to a paramagnetic bead subjected to a force imposed by magnets. The bead's position (Δz , in nanometers) reflects the molecule's extension under the constraint (Figure 1a). Thus, magnetic tweezers are force-clamps while optical tweezers are position clamps. Many DNA/RNA synthesis and attachment strategies have been developed to study single NA mechanics under force (Janissen et al., 2009; Papini et al., 2019; Riener et al., 2003; Walsh et al., 2001; Wildling et al., 2011; Yang et al., 2019) and are now routinely used in the single-molecule community.

All of the helicase experiments that we describe here are based on NA hairpins, self-complementary sequences that unfold at forces between 13 and 20 pN (depending on the NA nature and sequence) and that close between 8 and 14 pN. While short hairpins can now be directly ordered from various providers (up to ~40 base pairs), longer hairpins need to be constructed *in vitro* by attaching tethers and a loop to a dsNA segment. Short synthetic hairpins should be preferred for low-noise experiments while long constructed hairpins can be used when a precision of 2 nm is sufficient. Indeed they allow a better statistical yield, since the larger distance of the bead from the surface reduces the risk of unspecific attachment and allows better identification of well-folded hairpins. As we explain in (Valle-Orero et al., 2022), short hairpins should also be used when the biological question requires

that the analysis of a low-processivity enzyme is performed during the closing of the hairpin. In this case the hairpin should be smaller than the enzyme's processivity.

The NA is generally attached to the bead through streptavidin-biotin interactions, due to their biocompatibility, fast association and extremely high dissociation time at zero force ($\tau_0 = 10^5$ to 10^6 s at neutral pH and room temperature (Chilkoti & Stayton, 1995; Wilchek & Bayer, 1990)). This can be estimated using a simple Arrhenius model that gives τ_F the dissociation time under a force F as function of its value τ_0 at 0 pN force, and the distance Δx to the transition state: $\tau_F = \tau_0 e^{-F\Delta x/kBT}$, $\Delta x \sim 0.2-1$ nm, kBT being the thermal energy (Merkel et al., 1997; Pincet & Husson, 2005; Williams et al., 2000). This dissociation time can even be made virtually infinite for this range of force compared to experimental timescales by inserting two or three biotins in series (Guo et al., 2008).

For the surface-binding end, there are two choices of NA tethering: a fast and relatively weak digoxigenin/anti-digoxigenin (DIG/AntiDIG) binding (Figure 2a, b) (fluorescein/anti-fluorescein has also been used, (Bryant et al., 2003)) or a slow (~ 1 h) but strong covalent binding, the most biocompatible and specific being dibenzylcyclooctyne (DBCO)-azide Click chemistry (Eeftens et al., 2015) (Figure 2c, d). DIG/AntiDIG binding can be reinforced by functionalizing the NA with several DIG groups but this is not compatible with high-resolution experiments such as helicase stepping. Indeed, the transient binding/unbinding of single groups at the timescale of a few tens of seconds create spurious steps in the nanometer range that pollute the discrete signal of helicases. For faster attachment using Click chemistry covalent bonds, the surface can be coated with a DBCO-azide bound oligonucleotide to which the NA of interest can be attached with a complementary sequence of more than 30 base pairs (bp). This allows performing the attachment step only once per microfluidic chamber and then rapidly hybridizing hairpins (Figure 2d) (or adaptors, Figure 2c) and removing them by applying denaturing conditions. This solution is tractable for forces smaller than the shearing force of NA duplexes ($\sim 45-60$ pN (Hatch et al., 2008; Li et al., 2021)). For higher forces, the hairpin can also be ligated *in situ* if it is attached through a NA adaptor to the surface oligonucleotide (Figure 2c). The different attachment options are summarized in Figure 2.

If the hairpin is indirectly attached by hybridization, the adaptors should be long enough (>50 bp) so as they close behind the helicase passage, avoiding the molecule's detachment by the helicase. When high spatiotemporal resolution is required, such as for helicase stepping experiments or binding assays based on fluctuating hairpins, the tethers should be integrally made of dsDNA so their higher stiffness increases the spatio-temporal resolution of the experiment.

Table 1 summarizes the optimal choices of hairpin lengths and attachment modes while the following section describes the protocols to follow to synthesize them.

[Insert Figure 2 here]

[Insert Table 1 here]

2.1 Construction of hairpins.

All NA sequences used in this section are referenced in Supplementary Data 1 and their name indicated in italic.

[Insert Figure 3 here]

2.1.1 DNA hairpins.

DNA hairpins are built from dsDNA obtained either by direct synthesis of gene fragments by a suitable provider, PCR or plasmid amplification. For applications where it is important to ensure the absence of epigenetic modifications, the first two methods shall be preferred. The dsDNA is then digested by Esp3I (BsmBI), a type IIS restriction enzyme that cleaves outside its recognition site leaving a 5' overhang, generating two different overhangs with a single cutting enzyme. The digested dsDNA is then ligated to the loop structure and the 5' biotinylated Y-shape adaptor which bears the complementary sequences needed for tethering. The obtained DNA hairpin is then ready to be coupled to the streptavidin-coated beads and hybridized to the Click-bound DNA coated on the surface (Figure 3a).

Materials:

- Plasmid of interest or synthesized gene fragment (*pPS006*).
- Two primers designed for PCR amplification (*PrimerHP1*, *PrimerHP2*) containing the Esp3I recognition site and leaving 5' overhangs compatible with the Y-shape (*OliBiotin* + *OliCompAdaptor*) and the loop (*OliLoop*).
- Q5 PCR kit.
- Wizard® SV Gel and PCR Clean-Up System (Promega) or any other DNA purification kit
- 5' Dual-biotin oligonucleotide (*OliBiotin*, chemically synthesized).
- 5' Phosphorylated oligonucleotide complementary to the adaptor to the surface (*OliCompAdaptor*)
- Hairpin loop (*OliLoop*, chemically synthesized).
- T4 DNA ligase kit.

Protocol:

1. Perform PCR protocol according to the primers' characteristics using Q5 polymerase and gel purify the PCR product.
2. Digest the purified fragment with Esp3I one hour at 37 °C.
3. Purify the digestion by column (no gel purification needed).
4. Ligate 100 nM of the product with 1 µM of Y-shape (*OliBiotin* + *OliCompAdaptor*) and 2µM of *OliLoop* (final concentrations) with T4 DNA Ligase in its buffer in a final volume of 20 µL for one hour at 25°C.
5. Run the ligation product on a 1.5% agarose gel containing EtBr at 100 V in TAE 1X
6. Cut out the bands corresponding to the product using a blue light table.
7. Extract the DNA from the agarose band either by using an extraction kit or by electroelution to maximize elution efficiency (Davis et al., 1986) and resuspend in water.

8. Measure concentration by spectrophotometry.

Tips:

- Primers must be designed to have a melting temperature close to 60 °C and to avoid primer dimers.
- For time saving, digestion and ligation can be coupled in the digestion mix using NEB CutSmart Buffer: after digestion at 37 °C, add 1 mM ATP and 0.5 µL T4 DNA Ligase and incubate 2 hrs at room temperature.
- Other types IIS restriction enzymes such as BsaI can be used.
- Avoid any heating steps during the purification since the melted hairpins may hybridize head-to-tail as dimers, especially when they are highly concentrated.

2.1.2 DIG-tailing DNA hairpins.

The following protocol allows the preparation of hairpins compatible with DIG/AntiDIG attachment in microfluidic chambers (Figure 2c). Briefly, an oligonucleotide complementary to the ss 3' end of the hairpin is hybridized to the hairpin. Its long tail lacking guanosines is polymerized using a Klenow Exo-, a polymerase without strand-displacement activity in presence of DIG-coupled uridines and results in a poly-DIG-tailed hairpin (Figure 3b).

Materials:

- DNA hairpin synthesized as described in section 2.1.1.
- Filling DIG oligonucleotide (Fill-in-Dig-NoG, chemically synthesized) designed to be complementary to the 3' single-stranded end of the DNA hairpin and a long 5'-end flap containing regularly spaced adenines and no guanosines. The 3' of the oligonucleotide may be blocked with a 3' phosphate in order to avoid 3' polymerization.
- Klenow Exo- and NEBuffer2 (NEB).
- dATP, dGTP and dUTP-Digoxigenin (ThermoFisher).

Protocol:

1. Mix 10-100 ng of hairpin with 5 nM of Filling DIG oligonucleotide, 25 nM dATP, dGTP and dUTP-Digoxigenin, 1X NEBuffer 2, with 5U Klenow Exo- in a final volume of 20 µL.

Note. No dCTP nor dUTP are added to the mix.

2. Incubate 1 hour at 37 °C.
3. Run the product in a 1.5% agarose gel containing EtBr, cut the band and purify the hairpin by gel extraction column kit.

2.1.3 DNA fluctuating probe.

Short DNA hairpins (HPKinLock) used for kinetic locking assays (section 3.4.2) were ordered from Eurogentec in order to have a 10 bp hairpin with a 4 bp loop, making a total of 24 bp. For more information refer to (Rieu, Valle-Orero, et al., 2021).

2.1.4 RNA hairpin.

Here, we describe a fast and reliable construction of an RNA hairpin inspired from (Desai et al., 2019). It consists of the *in vitro* transcription of a dsDNA palindromic sequence. Since a palindromic sequence cannot be easily chemically synthesized, it is obtained by the ligation

of two gene blocks, both containing the hairpin sequence. One of the gene blocks contains the T7 promoter for RNA synthesis and the gene block junction corresponds to the tetra-thymidine loop (Figure 3c). This one-step protocol reduces risks of RNase contamination.

Materials:

- Gene block 1 (*GbforRNA1*, chemically synthesized) designed in order to have a T7 promoter in 5' of the 180 bp hairpin sequence followed by a type IIS restriction enzyme recognition site (Bsal) that leaves a tetra-thymidine overhang.
- Gene block 2 (*GbforRNA2*, chemically synthesized) designed to have the Bsal recognition site in 5' of the 180 bp hairpin sequence.
- Bsal-HF enzyme and CutSmart buffer.
- T7 transcription kit.
- Monarch RNA purification kit (NEB, T2040L).
- Murine RNase inhibitor (NEB, M0314L).

Protocol:

1. Mix 2 pmol of each gene block with 0.5 U of the restriction enzyme, 200 U T4 DNA ligase in 1 mM ATP, 1x CutSmart Buffer at a final volume of 12 μ L.
2. Incubate 2 hours at 37 $^{\circ}$ C.
3. Transcribe 2 μ L of ligated palindromic DNA according to the T7 Transcription kit manufacturer's protocol in a final volume of 20 μ L.
4. Purify the product using by column.
5. Elute with 20 μ L RNase free water.
6. Measure concentration by spectrophotometry.
7. Add 1 μ L of the murine RNase inhibitor before storing at -80 $^{\circ}$ C.

Tips:

- Avoiding RNase contamination is key to perform long single-molecule experiments with the RNA hairpin. We recommend using gloves and RNase free water.
- The palindromic DNA and the RNA hairpin can be loaded in a 1.5% Agarose gel with EtBr in order to check their size and integrity.
- Enzymes are not heat-inactivated to avoid RNA/DNA duplexes during renaturation.

2.2 Surface preparation.

Here, we describe the two main ways to tether hairpins (or adaptors) to the surface: through Click chemistry or DIG/AntiDIG interaction. Click chemistry corresponds to the attachment of DNA oligonucleotides coupled to a DCBO group on an azide-functionalized surface by producing a stable triazole link (Eeftens et al., 2015). The hairpins (or adaptors) can be then indirectly tethered to the DNA-coated surface by hybridization through complementary sequences and washed away with NaOH, rendering reusable and long-lasting surfaces. The two main disadvantages of Click attachment are that hairpins may dehybridize after helicase passage and, when working with ssDNA-binding helicases, the latter are titrated by the surface DNA resulting in protein concentration underestimation. On the other hand, hairpin attachment to the surface through DIG/Anti-DIG antibody is weaker but can be

improved by using poly DIG-tailed hairpins that are directly tethered to anti-DIG antibody coated surfaces (Manosas et al., 2010).

For both preparations a 2.5 mL syringe, a 80 x 120 mm Mylar sheet with two 2 mm holes (50 μm thick, 48-2F-OC, CSHyde), a 35 x 15 mm double-sided channel-containing tape 50 μm thick (ARcare 92712, Adhesive Research), two 10 x 10 mm double-sided tapes with 2 mm holes (ARcare 92712, Adhesive Research), an injection sink and an output connector (homemade in Altuglass) are needed (Figure 4).

[Insert Figure 4 here]

2.2.1 Preparation of ssDNA-coated surfaces through Click chemistry

Materials:

- Azide functionalized coverslip (PolyAn 2D Azide).
- 3'-DBCO oligonucleotide 100 nM (*OiiDBCO*, chemically synthesized, resuspended in 500 mM NaCl, 10 mM PBS).
- Passivation buffer 1X (PB) (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , 2% BSA, 2% Pluronic F-127, 5 mM EDTA, 10 mM NaN_3 pH 7.4).

Protocol:

1. Thoroughly wash the injection sink, the output connector, the Mylar sheet and the double-sided tapes with water then with Ethanol 70%. Rinse the output tube with water to ensure it is not blocked.
2. Stick one face of the double-sided tape directly onto the coverslip without removing the other side's protection (Figure 4).
3. Put a 20-40 μL drop of 3'-DBCO oligonucleotide in the middle of the channel. Incubate at room temperature for 2 hours until dry.
4. Rinse thoroughly the channel with PB then with water.
5. Once everything is dry, stick the Mylar sheet on the other side of the double-sided tape, making sure the holes are aligned with the channel (Figure 4).
6. Stick the two double-sided tapes with 2 mm holes aligned with the holes in the Mylar sheet then stick the input sink and the output connector (Figure 4).

Microfluidic chambers can be used immediately or up to a month if stored at 4 °C and protected from light.

Tips:

- To avoid RNase contamination, do not add BSA in the PB as we noted it often introduces RNases, and beware of dust. Buffers may be filtered, and gloves must be used at all times.
- To wash away hybridized molecules from the surface it is possible to rinse with 15 mM NaOH.
- DNA-binding protein concentrations may be underestimated due to binding to the surface DNA.

- Working with helicases can cause dehybridization of the attached molecule. This can be avoided by adding longer hybridization sequences (>50 bp) that re-hybridize and encircle the helicase after its passage.
- Packs of PolyAn 2D Azide coverslips should be used within a week. If opened, the pack should be stocked at 4 °C, protected from light.
- Never use a sodium azide (NaN₃) containing solution to dilute the DBCO-containing oligonucleotide or it will reduce the yield of the Click-chemistry reaction between the azide surface and the DBCO-labeled DNA.

2.2.2 Preparation of AntiDIG-coated surfaces.

Materials:

- Non-functionalized coverslip (Schott, NEXTERION® Coverslip custom, #1.5H).
- Sigmacote (Sigma-Aldrich).
- Monoclonal mouse Anti-Digoxin IgG (Jackson Immuno Research, 200-002-156).
- Passivation buffer 1X (PB) (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 2% BSA, 2% Pluronic F-127, 5 mM EDTA, 10 mM NaN₃ pH 7.4).

Protocol:

1. Thoroughly wash the injection sink, the output connector, the Mylar sheet and the double-sided tapes with water then with Ethanol 70%. Rinse the output tube with water to ensure it is not blocked.
2. Put a drop of Sigmacote on the coverslip and spread it with a pipette tip. Wait until dry in a covered place to avoid dust from falling on the coverslip.
3. Stick one side of the double-sided tape directly onto the coverslip. Stick the Mylar sheet on the other side. The holes in the Mylar sheet should be aligned in the middle of the tape's channel (Figure 4).
4. Stick the two double-sided tapes with 2 mm holes aligned with the holes in the Mylar sheet then stick the input sink and the output connector. Check if there are any leaks by flowing water through the channel with the syringe (Figure 4).
5. Once the microfluidic chamber is ready, inject 40 µL of the antiDIG in the input and gently flow it through the channel with the syringe in order to cover the whole channel with the antibody. Incubate 3 hrs at room temperature and cover the input sink to avoid evaporation.
6. Rinse the channel with 200 µL PB and let it incubate overnight at 4 °C to saturate unspecific sites.

Note: Microfluidic chambers can be used for up to a couple of weeks if kept with PB at 4 °C.

Tips:

- When sticking the tape, apply slight pressure to ensure proper adhesion of the tape and avoid leaks, but beware of breaking the coverslip.
- Bubbles are an indicator of a leak. If there is a leak, locate it and plug it with glue.
- To avoid evaporation, always cover the input sink with a 1.5 ml Eppendorf tube lid.
- To covalently attach the anti-DIG antibodies to the surface, an epoxy-coated coverslip (Schott, NEXTERION® Coverslip E @1.5H) may be used.

2.3 Bead preparation.

Tethering the hairpin to the streptavidin-coated bead can be achieved either indirectly through a dual-biotin labeled oligonucleotide (chemically synthesized) complementary to the hairpin's end, or directly by biotinylating the hairpin itself. The use of dual-biotin increases the interaction affinity in order to stabilize the hairpin's tethering.

2.3.1 Pre-hybridization of hairpin to adaptors.

Materials:

- Non-biotinylated hairpin (100 nM) produced as described in section A.1.c or d.
- Biotinylated bead adaptor (*OliBiotin*, 100nM, chemically synthesized)
- NaCl 1 M or KCl 1 M.
- Passivation buffer 1X (PB) (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 2% BSA, 2% Pluronic F-127, 5 mM EDTA, 10 mM NaN₃ pH 7.4).

Protocol:

1. Mix 1 µL of the hairpin with 1 µL of the adaptor and 1 µL of 1M salt (NaCl or KCl).
2. Incubate 10 min at room temperature for hybridization.
3. Add 97 µL of PB to dilute the concentration to 1 nM.

The pre-hybridized hairpin can be immediately hybridized to the beads or stored at -20 °C.

Tips:

- Biotinylated adaptors must be added at the same concentration as the hairpin since they cannot be washed after bead hybridization.

2.3.2 Hairpin / bead hybridization.

Materials:

- Dynabeads MyOne T1 (Thermofisher).
- Hairpin (pre-hybridized to adaptor or not) (10 pM).
- Passivation buffer 1X (PB) (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 2% BSA, 2% Pluronic F-127, 5 mM EDTA, 10 mM NaN₃ pH 7.4).
- If needed (Figure 3.1) adaptor between the hairpin and the surface (*OliAdaptor*, 100 nM)

Protocol:

1. Wash 5 µL of beads three times with 200 µL of PB.
2. Resuspend the beads in 19 µL of PB.
3. Add 2 µL of the diluted pre-hybridized hairpin (10 pM), and if needed, 1 µL of *OliAdaptor*.
4. Incubate at least 10 minutes at room temperature in rotation.
5. Wash three times with 200 µL of PB in order to remove unbound NA .
6. Resuspend in 20 µL of PB (final concentration of DNA, 1 pM).
7. Stock beads on a rotator (10 rpm) at room temperature.

Note: Beads can be used for up to several months if sedimentation does not occur. For RNA beads this estimation may be shorter.

Tips:

- The concentration of pre-hybridized hairpin and incubation time may need to be adjusted. Too high attachment to the surface without the possibility to open the hairpin indicates that the beads have more than one NA tethered. On the contrary, low attachment indicates insufficient hairpin tethering to the beads. We sometimes increase the final concentration of hairpins from 1 pM up to 25 pM (stock concentration up to 250 pM).
- Washing away the adaptor oligonucleotide is crucial or free adaptors will saturate the surface. To ensure thorough washing, make sure that less than 5 μ L of liquid are left in the tube between each washing steps.

2.3.3 Bead injection onto the surface.

Materials:

- Beads tethered to the hairpin as prepared in section 2.3.
- Click or DIG surface prepared as described in section 2.2.
- Passivation buffer 1X (PB) (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 2% BSA, 2% Pluronic F-127, 5 mM EDTA, 10 mM NaN₃ pH 7.4).

Protocol:

1. Lift the magnets to remove any magnetic force.
2. Put 200 μ L of PB into the input inlet.
3. Apply a flow of 10 μ L per minute with the microscope pump.
4. Inject 1 μ L of the beads directly onto the cell.
5. When the beads are seen in the field of view, quickly lower the magnets to 10 pN and stop the flow.
7. When the flow is fully stopped, lift the magnets and let the beads sediment for 10 min.
8. Wash the surface with PB at 10 μ L/s until all the beads seen in the screen are attached to the surface.

Tips:

- Before injection, verify that the syringe of the microscope pump is empty. Avoid emptying it during the experiment which induces flows that detach most of the molecules.
- To avoid bubbles, when injecting the beads, do not press until the second stop of the pipette and regularly check that the input sink is not dry when the flow is on.
- The incubation time of sedimented beads may have to be adjusted if too few or too many beads are attached.
- Once the beads are attached, lowering the magnets to a higher force (20 pN) for a few seconds may help the release of untethered beads.

2.4 Bead selection and calibration.

Once the beads are prepared and injected onto the surface, their correct folding must be checked and their position calibrated in order to begin the experimental process.

2.4.1 Selection of properly folded hairpins.

As described in (Manosas et al., 2010), beads tethered by a single NA hairpin must be distinguished from ones either non-specifically bound to the surface, not properly folded in hairpins or bound by two or more molecules. The latter need twice as much force to be opened than a single bound hairpin. Typically, DNA hairpins rapidly open around 15 pN and RNA hairpins at 18 pN, so the double-bound beads will not be opened even at the microscope's maximal force. Improperly folded hairpins will show progressive opening and closing, contrary to properly folded ones.

Protocol:

1. Select a field of view that includes between 30 and 60 beads that are not aggregated.
2. While alternating the force between 18 and 7 pN for example, visualize the beads that show a z-position change between high and low forces.
3. If the z-position variation of the bead corresponds to the size of the hairpin (around 1 nm for 1 bp) and the opening is abrupt, the bead is bound by a properly folded hairpin and should be selected.
4. If the bead doesn't show movement with force variation it should not be selected.

2.4.2 Bead-to-bead force correction using hairpin opening forces.

The force applied on the NA hairpins is crucial for the reproducibility of helicase single-molecule experiments, especially if the helicase's behavior is strongly force-dependent, as seen in the case of the Nsp13 Sars-CoV-2 helicase (Mickolajczyk et al., 2021). Force variation can be calibrated as a function of the distance between the magnets and the microfluidic chamber using the fluctuation of a bead tethered to a relatively long NA (Allemand, 1997; Gosse & Croquette, 2002; Ostrofet et al., 2018; Yu et al., 2014). For closed hairpins, where the bead is closer to the surface, these calibration protocols are not accurate due to the change of viscous drag near the surface (Brenner, 1961) and the random attachment points of the molecule with regard to their magnetic axis (Klaue & Seidel, 2009).

Thus, magnets need to be calibrated with long dsDNA molecules which then serve for subsequent experiments with shorter hairpin molecules. However, an uncertainty remains, due to the variation of magnetization from bead to bead, which depends on the heterogeneity of the magnetic bead preparation. This variation of magnetization induces a multiplicative factor between the true force applied on the bead and the one that is estimated through the calibration which is calculated as follows.

Protocol:

1. The calibration with long molecules is performed on a large sample of different molecules and beads in order to get an accurate average of the force applied on a typical bead of the preparation.
2. Each subsequent experiment with hairpins should start with a few (~20) cycles of opening and closing of the hairpins at a slow magnet speed (typically 0.2 mm/s) (Figure 5a).
3. Draw the extension as a function of force during these opening cycles for each bead (Figure 5b), which follows hysteresis cycles. Then, measure and average the magnet

- position for which the opening and the closing of the hairpin happen. This value is then translated into estimated opening and closing forces using the force calibration of step 1.
4. Draw the distribution of opening forces for the whole assembly of beads (Figure 5b inset). The average of the distribution gives the true opening force of the hairpin in the buffer used and at this magnet speed.
 5. The multiplicative correction factor is assessed independently for each bead and corresponds to the ratio between the estimated opening force of each individual hairpin and the average opening force of the whole sample. For Dynabeads MyOne, it is typically 10% but sometimes reaches 20% (Figure 5b). Taking this factor into account is important to avoid force errors.

[Insert Figure 5 here]

2.4.3 Nanometers to base pairs conversion.

The elasticity of ssDNA is involved in many aspects of the energetic interactions with enzymes. For single-molecule helicase experiments, it also allows the conversion from nanometers (nm) to base pairs (bp). Contrary to dsDNA where this elasticity curve is in complete agreement with the Worm Like Chain model (Bouchiat et al., 1999) and where only two parameters, which have been accurately measured, fully characterize it. ssDNA elasticity is not universal, it depends on sequence and salt (McIntosh et al., 2014) and there are fewer experimental characterizations with somewhat diverging parameters (Jacobson et al., 2017; Viader-Godoy et al., 2021). The measurement of the extension of single molecules is made harder by the propensity of ssDNA to unspecifically attach to the bead or the surface. As a consequence, when the extension of a ssDNA is measured, it is possible that a shorter molecule is being analyzed and thus, less bases than expected are being stretched.

To overcome this difficulty, we use a thorough protocol for the measurement of ssDNA force-extension curves (Figure 6a). This consists of measuring the extension of a hairpin as a function of the force while preventing its closure with a small oligonucleotide complementary to the loop (Figure 6b). This allows removing the contribution of the handles to the total extension and thus getting rid of the surface effects (Figure 6c). The protocol shown in Figure 6 needs to be performed before any single-molecule helicase experiment, in the enzyme's activity buffer, to ensure a thorough estimation of the nm-to-bp conversion coefficient.

[Insert Figure 6 here]

2.5 Fast *in situ* three-way junction assembly by hairpin invasion.

Assembling three-way junctions directly in the microfluidic chamber has been proven useful to study helicases or helicase-like complexes that are able to regress stalled replication forks into Holliday junctions (Amit et al., 2004; Bétous et al., 2013; Bianco & Lu, 2021; Dawid et al., 2004; Manosas et al., 2013; Tan et al., 2017). However, the study of the

processivity of such proteins is limited by the size of the synthetic oligonucleotides used to form the junctions. In order to overcome this limitation, we developed a protocol for the fast and simple assembly of three-way junctions of arbitrary size. The protocol is described in Figure 7a. It consists in using a modified primer (*PrimerSpacer*) to amplify by PCR a segment of dsDNA with a flap. The flap and ds segments are complementary to the hairpin that is tethered between the surface and the bead. Force cycles are applied to the hairpin with the presence in solution of the flapped dsDNA. When the force is lowered below 10 pN, the DNA hairpin can close and the flap of the PCR product can hybridize to the remaining ss region on the handle of the hairpin. Then, the PCR product can invade the tethered hairpin. Indeed, while the hairpin opens and the PCR product hybridizes, the number of hybridized bases stays constant but the bead moves in the direction of the force, which is energetically favored. Thus, the invasion spontaneously occurs, as shown in Figure 7a, resulting in the formation of the three-way junction. Figure 7b shows a typical trace of hairpin invasion and the three way junction resolution by RecG as an example.

[Insert Figure 7 here]

2.6 Recombinant protein production by double affinity purification.

Recombinant proteins used in single-molecule experiments usually go through column chromatography steps (gel filtration, size exclusion, ion exchange...), in order to ensure RNase free, pure and properly folded active proteins (Bianco, 2021b). Yet, due to low yields, this strategy is hard to implement for unstable and lowly expressed proteins bearing unstructured domains. Even if low amounts of protein are needed (10-100 nM) to obtain single events in magnetic tweezers, the double affinity purification strategy is a good compromise between yield and purity. Here, we describe an optimized protocol for this strategy, based on our previous work (Fiorini et al., 2012).

Briefly, coding sequences of interest were cloned into a pET28a vector allowing fusing a Calmodulin Binding Peptide (CBP) sequence in 5' and a hexahistidine sequence in 3' of the expressed recombinant proteins. The plasmids were then transformed into BL21(DE3)-CodonPlus *Escherichia coli* competent bacteria by heat shock and selected on kanamycin containing Luria Broth (LB) plates. Bacteria were then pre-cultivated in LB liquid media containing kanamycin for 4 h at 37 °C and then used for inoculation of 1 L of LB Broth Auto Inducible (LBAI) media containing metals. Bacteria were then left to grow overnight at 18 °C. After bacterial lysis, the double purification was performed first on Nickel-NTA resin then on Calmodulin-coupled resin. Proteins were dialysed against a storage buffer and frozen at -80°C. One advantage of this technique is that, by purifying by both extremities, only full-length proteins are selected and thus, no size exclusion chromatography is needed. This protocol can be adapted for the use of other tags such as GST or Twin-Strep instead of CBP, the fusion of highly structured tags may help the solubility of unstructured proteins.

2.6.1 Protein overexpression.

1. Transform 100 µL of BL21(DE3)-CodonPlus competent *Escherichia coli* bacteria (Agilent) with 500 ng of plasmid by 2 min at 42 °C heat shock. Add 1 mL liquid LB

media and incubate 1 hour at 37 °C. Pellet the cells by 4 min centrifugation at 3500 g. Resuspend the pellet in 50 µL LB media, plate in kanamycin containing LB plates and leave to grow overnight at 37 °C.

Tip: Other competent bacteria designed for protein overexpression such as BL21(DE3)-Rosetta strain may be tested depending on the expressed proteins.

2. Resuspend 2-3 colonies in 25 mL LB liquid media containing kanamycin in a 250 mL Erlenmeyer flask and incubate at 37 °C, 200 rpm for 4 hours.
3. Inoculate the culture in 1 L of LBAI containing metals (Formedium) added with kanamycin and chloramphenicol in a 5 L Erlenmeyer flask and incubate at 18 °C, 200 rpm overnight.

Tip: Other culture media may be used depending on the expressed proteins. For non-inducible media, induction is performed by addition of 0.2-0.5 mM of IPTG when optic density reaches the exponential phase. Although we highly recommend the use of auto-inducible media (Luria Broth or Terrific Broth), with or without metals, protein yields may vary upon culture conditions.

4. Pellet the culture by 10 min, 4000 g centrifugation at 4°C.

Tip: Pellets can be stored at -20 °C. However, we recommend using fresh cultures for purification.

2.6.2 First affinity purification.

All following steps must be performed at 4°C.

1. Resuspend the pellet in 25 mL of Lysis buffer (1.5X PBS, 1 mM MgAc₂, 0.1% NP-40, 20 mM imidazole, 10% glycerol, 2 mM DTT).
2. Add 1X of protease inhibitors (Apoprotinine, Leupeptine, PMSF, Pepstatin).
3. Sonicate in a metal beaker at 30% amplitude and 1 second pulse for 4 min.

Tip: So temperature does not exceed 15 °C, the beaker can be placed on a mix of dry ice and ice.

4. Pellet the bacterial debris for 30 min at 18,000 rpm.
5. Retrieve the supernatant and mix in a Falcon tube with 1 mL of Nickel-NTA Agarose slurry (Qiagen) equilibrated with Lysis Buffer. Incubate in a rotator for 1 hour at 4 °C.
6. Centrifuge at 4,000 rpm for 5 min at 4 °C to pellet the beads.
7. Remove the supernatant (Flow Through) and wash with 25 mL of Lysis Buffer.
8. Transfer into a Poly-Prep chromatography column (Bio-Rad) pre-equilibrated with 1 mL of Lysis Buffer.
9. Wash the column with 5 mL of Lysis Buffer then with 5 mL of Wash Buffer (1.5X PBS, 250 mM NaCl, 1 mM MgAc₂, 0.1% NP-40, 50 mM imidazole, 10% glycerol, 2 mM DTT) then again with 5 mL of Lysis Buffer.
10. Close the column and incubate the resin with 1 mL of Elution Buffer (1.5X PBS, 1 mM MgAc₂, 0.1% NP-40, 250 mM imidazole, 10% glycerol, 2 mM DTT) for 10 minutes at 4°C.
11. Elute successively with 1 mL of Elution Buffer and measure protein concentration by Bradford Assay.

12. Pool the fractions with highest concentration and dialyse overnight against 1 L of Calmodulin Binding Buffer (CBB) (10 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM MgAc₂, 4 mM CaCl₂, 0.05% NP-40, 10% glycerol, 2 mM DTT) at 4 °C.

2.6.3 Second affinity purification.

1. Retrieve the dialysed fractions and mix with 500 µL of Calmodulin Sepharose 4B resin (50% Slurry, GE Healthcare) washed with CBB. Incubate in a rotor for 2 hours at 4°C.
2. Transfer into a Poly-Prep chromatography column (Bio-Rad) pre-equilibrated with 1 mL of CBB.
3. Wash with 5 mL of CBB.
4. Close the column and incubate with 500 µL of Calmodulin Elution Buffer (10 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM MgAc₂, 0.05% NP-40, 20 mM EGTA, 10% glycerol, 2 mM DTT) for 10 min at 4 °C.
5. Elute successively with 500 µL of Calmodulin Elution Buffer and measure protein concentration by Bradford Assay.
6. Pool the highest concentrated fractions and dialyse overnight against 1 L of Storage Buffer (1.5X PBS, 1 mM MgAc₂, 10% glycerol, 2 mM DTT).
7. Retrieve the dialysed proteins and aliquot them in small volumes (10-100 µL, depending on the final protein concentration). Store at -80 °C.

Tips:

- 10µL of Supernatant, Pellet, Flow Through Histidine, Wash Histidine, Elution Histidine, Dialysis Histidine, Flow Through Calmodulin, Elution Calmodulin and Dialysis Calmodulin may be kept for loading a 10% SDS-PAGE gel, revealed by Coomassie Blue coloring in order to visualize the different purification steps.
- Reducing agents must be added to the buffers extemporaneously.
- Buffers may have to be adapted for each purified protein depending on their stability. Some proteins may need more or less salt, no glycerol, or different pH.
- If the protein precipitates during dialysis, centrifuge and measure the concentration again.
- For higher reproducibility, aliquots should be only thawed once before each experiment. Leftovers should be discarded after thawing.

3. Advanced protocols for helicase characterization using magnetic tweezers.

In previous reviews (Hodeib et al., 2016; Manosas et al., 2010), we described classical magnetic tweezers protocols for helicase studies and explained how the processivity, velocity and directionality can be extracted by dynamically measuring the extension of a hairpin unwound by the helicase. Here, we will focus on recent experimental advances we developed for measuring other helicase properties.

First, we will describe two assays for measuring translocation of a helicase in a force-independent manner: the peeling assay and the bubble assay. Then, we will focus on the binding capacities of helicases by describing a qualitative binding assay using force cycles and a more quantitative assay using a fluctuating short hairpin. The latter allows measuring binding kinetics independently of their unwinding activity and in a fluorescence-free manner. Lastly, we will focus on the impact of roadblocks, either NA-binding proteins or NA tertiary structures, on the translocation of helicases by embedding the roadblock inside a hairpin and monitoring the time they take to either resolve or bypass the obstacle.

3.1 Mitigating the impact of force on helicase translocation.

In classical magnetic tweezer assays, a constant force is applied. Typically, in order to observe a good quality signal, the applied force lies between 6 pN and 12/18 pN (DNA/RNA). At higher forces, the hairpin spontaneously opens, preventing the observation of helicase unwinding activity and at lower forces, the signal-to-noise ratio is too low. This assay draws criticism concerning passive (force-dependent activity) and active (force-independent activity) helicases. Indeed, if the helicase is passive (such as polymerase-coupled helicases) it is possible that at zero force, no activity would be measured. Active helicases, on the other hand, which would display activity even at zero force, may also be influenced by the applied force. Hence, the observed activity in these assays may differ from the helicase's behavior as measured in bulk assays. In order to go beyond this assumption, unwinding activity can be probed in other configurations. Here, we propose two assays to mitigate the impact of force on helicase assays in magnetic tweezers: the peeling assay and the bubble assay.

3.2 Peeling assay

The peeling assay consists of hybridizing a lateral oligonucleotide on the side of the main hairpin, on which the helicase translocates (Figure 8a). In this configuration, an increasing force hinders the unwinding, instead of favoring it since the bead moves opposite to the force when the enzyme unwinds the hairpin. The unwinding helicase activity is thus in an energetic context closer to zero-force bulk experiments. It is noteworthy that this configuration induces a specific biochemical context in which the helicase is confronted with two forks, which might affect its translocation. Figure 8b (inset, 1235 s) shows an example of peeling assay of the catalytic core of *E.coli* RecQ, RecQ- Δ C. Note that after the oligonucleotide is peeled, the helicase is able to translocate directly onto the hairpin as in classical constant-force experiments (1425 – 1432 s).

Protocol:

1. Using a microfluidic chamber (prepared as described in section A), run a force cycle for successive hairpin openings and closings (for example 2 s at 18 pN, 4 s at 8 pN) and start the recording of the beads' positions.
2. Remove the buffer from the microfluidic chamber inlet with a pipette. Rinse the inlet three times with 200 μ L of reaction buffer (no ATP, no enzyme).
3. Flow 160 μ L of reaction buffer at a constant speed of 10 μ L/min to rinse the microfluidic channel.
4. Flow 100 μ L of reaction buffer + 100 nM of complementary oligonucleotide.
5. Wait for the oligonucleotide to be hybridized to all hairpins. This results in a partial blocking of the hairpins at 7 pN in opening/closing cycles.
6. Repeat steps 2-3 to remove the excess oligonucleotides.
7. Stop the force cycles and measure the beads' positions at constant medium force ($F = 8$ pN).
8. Add the buffer mixed with the enzyme and 1 mM ATP to the inlet of the chamber. Flow at a constant speed of 5 μ L/min.
9. Record data during the desired period of time.

Tips:

- Start the thermalization of your setup early enough before the recording, at the desired temperature to avoid slow measurement drifts due to temperature changes.
- In addition to adding reducing agents such as TCEP or DTT to the solution, you may degas the buffer to minimize protein oxidation: leave the tube containing the buffer without ATP nor enzyme in a vacuum desiccator at room temperature for 10 minutes.
- Between each rinsing step, carefully remove most of the buffer from the inlet. Only a few μ L should remain in the inlet so that each rinsing step allows the dilution of the previous buffer by a factor ~ 100 .
- When preparing the enzyme mix, gently stir with the pipette tip. Avoid the insertion of air and bubble formation when mixing in order to avoid the buffer's fast re-oxygenation as well as the concentration of proteins at air-water interfaces.

Data analysis:

1. Find the unwinding events in the data: they consist of a progressive decrease of the bead extension followed by an abrupt rezipping of the hairpin once the full oligonucleotide has been removed.
2. Measure the speed of the enzyme in nm/s for each event by dividing the total loss of extension during the progressive phase by the duration of this phase.
3. Convert the speed from nm/s to bp/s. The conversion factor is $l_{ssNA}(F) + l_{dsNA}(F)$, since one unwound base results in removing one ds base and one ss base from the extended molecule.

[Insert Figure 8 here]

3.3 Bubble assay

This assay is performed using a long hairpin (>1 kb) that is able to re-hybridize after the helicase's passage and thus encircle the helicase in a "bubble". By periodically changing the force as shown in Figure 9a, the hairpin is open at high force (> 15 pN), then at medium

force (~ 7-8 pN), it starts to close. When the enzyme is bound, the hairpin will only partially close until the enzyme's position. By imposing a low force (3-4 pN), the hairpin completely closes and encircles the helicase (if it's not ejected, see part 3.4.1). At this step, no force is applied inside the hairpin, meaning that the helicase's behavior while it is encircled is measured at zero force. By comparing the bead's position over successive cycles, it is possible to extrapolate the helicase's translocation while encircled. Figure 9.b, illustrates an example of peeling assay on the human helicase core of Upf1.

Protocol:

1. Follow steps 1-3 from protocol B1.
2. Stop the force cycles and choose the force cycles for the experiment. For example 2 s at 15 pN, 5s at 8 pN, 5 s at 3 pN.
3. Add the buffer mixed with the enzyme and 1 mM ATP to the inlet of the chamber. Flow at a constant speed of 5 μ L/min.
4. When the inlet is almost empty, repeat step 3.
5. Record data during the desired period of time.

Data analysis:

1. Measure the binding rate k_{on} of the enzyme to the substrate by drawing the distribution of times elapsed between the injection of the enzyme and the first blockage or the first unwinding event. These times will typically follow exponential distribution, of parameter $\tau = 1/k_{on}$.
2. If the binding rate is large compared to the time spent at high force, where the presence of the enzyme cannot be probed, decrease the enzyme concentration to ensure that a signal observed in two successive cycles is the same enzyme.
3. Compute the probability of encircling the enzyme during re-hybridization by dividing the number of events where the enzyme is present both before and after the re-hybridization step by the total number of events where the enzyme is present before the re-hybridization step.

[Insert Figure 9 here]

3.4 Estimating NA binding dynamics of helicases.

3.4.1 Qualitative single-molecule binding assay (SMBA).

One important aspect of helicase dynamics is their binding to NA and their residence time. Standard single-molecule unwinding assays allow measuring the dissociation time of the helicase from its NA substrate in the presence of ATP, when the enzyme translocates. However, in order to decouple this dissociation from the conformational changes induced by ATP hydrolysis, it is sometimes useful to be able to measure and compare the grip of the helicase on its NA substrate in absence of ATP.

In Kanaan et al., 2018, we described an ATP-free qualitative single-molecule binding assay (SMBA, Figure 10b, c), to estimate helicase binding to NA and their residence time. In this assay, the protein is injected without ATP allowing binding but not translocation. Force cycles are then applied to test the persistence of the helicase despite the mechanical constraints of a closing hairpin. First, a high force opening the hairpin (>15 pN for DNA and

>18 pN for RNA) is applied for a couple of seconds, the helicase is thus able to bind randomly anywhere in the single-stranded regions. Then, a medium force (~ 7-8 pN) induces the hairpin's closure. The closing fork pressing against the bound helicase can have two outcomes: if the helicase's grip is strong enough, the hairpin is blocked at the helicase's position, else, the helicase is ejected and the hairpin completely closes (Figure 10a). The ejection time can be almost immediate or last a few tenths of seconds. Thus, this step is held for 20-30s or more. Figure 10b and c show a qualitative comparison of the grip of two different catalytic cores from Superfamily 1 helicases : yeast UPF1 and yeast IGHMBP2 (Kanaan et al., 2018).

Protocol:

1. Follow steps 1-3 from protocol 3.1.
2. Stop the force cycles and choose the force cycles for the experiment : 1-3 s at 15-18 pN, 20-30 s at 7-8 pN.
3. Add the buffer mixed with the enzyme without ATP to the inlet of the chamber. Flow at a constant speed of 5 μ L/min.
4. When the inlet is almost empty, repeat step 3.
5. Record data during the desired period of time.

Tips:

- In order to avoid multiple helicases binding to the same substrate, the lowest concentration possible is injected and can vary from 1 to 20 nM, depending on protein activity.

Data analysis:

1. The binding time corresponds to the inverse of the ejection rate. Sum the blocking time of the closing hairpin at intermediate force and divide it by the binding events during the recording.
2. Relative errors are calculated by computing the square root of the number of unbinding events.

[Insert Figure 10 here]

3.4.2 Quantitative binding kinetics using kinetic locking assay.

In some cases, the association of a helicase with its substrate does not cause changes of NA extension that are sufficiently large to be measured with single-molecule micromanipulation techniques. Measurement of helicases' binding kinetics is thus often performed with bulk stopped-flow assays (Kocsis et al., 2014). However, such bulk assays involve complex fitting procedures, and may require to label the proteins and/or to introduce chemical competitors to evaluate dissociation kinetics.

We recently showed (Rieu, Valle-Orero, et al., 2021) that it is possible to use a fluctuating hairpin to perform single-molecule measurements of helicases' binding kinetics at a few tens of milliseconds timescales. Indeed, small hairpins (10-15 bases) fluctuate rapidly when they are pulled at the force that equilibrates the free energy level of the open and closed states ($F_{1/2}$) (Woodside et al., 2006). When the helicase binds to the hairpin, it keeps the hairpin open and prevents its fluctuations (Figure 11a). The association and dissociation rates of the enzymes from the substrates can be extracted from the duration and the frequencies of

these long pauses. Figure 11b and c show the binding kinetics of the catalytic core of RecQ helicase in this configuration (Rieu, Valle-Orero, et al., 2021).

Protocol:

1. Using a microfluidic chamber (prepared as described in section 2, run a force ramp (5 s per force level, steps of 0.5 pN).
2. Find beads which fluctuate rapidly between the open and closed states. Change the force to $F_{1/2}$, the force where the duration of the closed and open states are equal.
3. Increase the camera frequency up to a few kHz.
4. Rinse the microfluidic chamber with the reaction buffer as explained in previous protocols.
5. Add the buffer mixed with the enzyme to the inlet of the chamber. Flow at a constant speed of 5 $\mu\text{L}/\text{min}$.
6. Record data during the desired period of time.
7. Repeat step 5 with increasing helicase concentrations.

Tips:

1. Since the fluctuations only happen in a limited range of force ($\sim 0.5\text{-}1$ pN) and beads have some variability in their magnetization ($\sim 10\text{-}20$ %), it is hard to find many beads that fluctuate at the same magnet position, especially at high acquisition frequencies where the field of view is limited by the camera's transfer frequency. However, since the fluctuations are fast, one or two beads are usually sufficient to sample several hundreds of events.
2. It is important to unambiguously distinguish between the two states of the hairpins (change of 10 nm at the millisecond timescale). A magnetic tweezer setup with high-spatio-temporal resolution is crucial (Rieu, Vieille, et al. 2021; Dulin et al. 2015; Huhle et al. 2015; Kim et Saleh 2009). A signal of poor quality may be improved by removing the spurious correlations between the axial and transverse movements of the beads, as described in the supplementary materials of Rieu *et al.* 2021 (Rieu, Vieille, et al. 2021).
3. The temperature of the sample must be precisely controlled (~ 0.01 °C) since the kinetics of the hairpin fluctuations are sensitive to it.
4. Maintaining a constant concentration of helicase is critical to assess the association time. For this reason, keeping a constant flow during the experiments is important. If the enzyme has a strong affinity to DNA, we recommend not using DNA-coated azide surfaces but other attachment chemistry, such as DIG, to avoid biasing the local concentration of helicase close to the surface.

Data analysis:

Analysis of the freely fluctuating hairpin (no helicase):

1. Compute the standard deviation σ of the measured position in both closed states and open states. At 1 kHz of acquisition frequency and a 10 bp hairpin with 120 bp dsDNA handles, they typically lie between 1 and 2 nm.
2. Automatically detect the transitions between the closed and open states. A criterion of $3\text{-}4$ σ ($\sim 6\text{-}8$ nm) can be used by detecting a jump if the difference of position measured between two successive frames is larger than 3 σ . To avoid biases caused by the finite velocity of the bead during the transition, sum up all differences

- of position among successive frames as long as the movement is monotonous (series of frames with the position of the bead either increasing or decreasing).
3. Compute the times spent in the closed states (difference between two successive closing and opening events) and the times spent in the open states (difference between two successive closing and opening events).
 4. Draw the histograms of multiple (> 100) closed and open times (the bin size should be a multiple of the acquisition frequency to avoid spurious irregularities). Fit the histograms by an exponential distribution and infer the typical times spent in the close and open states.
 5. Average the closing and opening times by chunks of N points ($N > 100$) and draw the averages as a function of the acquisition time. Check that the averages are constant within the statistical error (square root of N). If an important drift is observed over time, this might be due to a flow that is not constant or a change of temperature. Wait until this parameter is stabilized and restart the measurement.

Derivation of the binding kinetics:

1. When the helicase is present in solution and can bind to the substrate, the association and dissociation processes of the helicase of rates k_{on} and k_{off} adds up to the internal fluctuating rates of the hairpins. This results in the distribution of the open times of the hairpin changing from single exponential to double exponential. By fitting the time spent in the open times at the different concentrations of helicases as described in (Rieu, Valle-Orero, et al., 2021), one can deduce k_{on} and k_{off} .
2. Noteworthy, if k_{off} is much smaller (~ 10 fold) than the spontaneous closing rate of the hairpin, k_{off} will be very close to the inverse of the long-time of the double-exponential distribution (either the hairpin closes spontaneously - short time - or it stays open due to the presence of the helicase - a long time.). In this case, the distributions are particularly easy to interpret.

[Insert Figure 11 here]

3.4.3 Helicase interaction with roadblocks.

We have previously described how some helicases are capable of remodeling ribonucleoprotein complexes (Fiorini et al., 2015) by covering a ssDNA hairpin with NA-binding proteins and analyzing the progression of the helicase in this context. In *Fiorini et al., 2015*, we demonstrated that the human UPF1 helicase core is able to translocate onto a Gp32-covered DNA at a normal rate, by remodeling NA-protein interactions. Recently, we developed an encerclement-based protocol to embed roadblocks, such as tertiary NA structures, in the NA hairpin (Tran et al., 2021) shown in Figure 12a. The dynamics of a helicase interaction with such roadblocks as well as their probability of removing or bypassing them can be studied by injecting the enzyme and analyzing its stalling at the roadblock's position. The time spent at this position can be calculated in order to analyze how the helicase interacts with the roadblock. This protocol has been recently used to study the interaction of the *Saccharomyces cerevisiae* helicase Pif1 with G-quadruplexes (Valle-Orero et al., 2022). Figure 12b shows a schematic trace of the roadblock embedding and a helicase's translocation in this configuration.

Protocols:

1. Follow steps 1-3 from protocol 3.1.
2. Add the roadblock in solution and observe its binding/formation at the 7-8 pN phase (it will cause a transient blockage of the closing of the hairpin). Check that the roadblock is not expelled during the low force phase and is stable when encircled in the hairpin.
3. Rinse the chamber with the reaction buffer.
4. Stop the force cycles.
5. Apply a high force to open the hairpin, then go to 7 pN to close the hairpin until the roadblock position and finally to your working force at which the helicase activity is analyzed.
6. Apply a low force (2-4 pN) to encircle the roadblock.
7. From the low force, go to the working force, where the hairpin should be closed.
8. Add the buffer mixed with the enzyme to the inlet of the chamber. Flow at a constant speed of 5 $\mu\text{L}/\text{min}$.
9. Record data during the desired period of time.

Data analysis:

1. Detect unwinding events corresponding to molecules whose extensions increase.
2. Check whether the helicase stalls at the roadblock position by comparing the time spent by the helicase at the roadblock position at the working force (checked at step 6 of the protocol) with the average time spent at any other position during unwinding.
3. If the helicase stalls and then resumes translocation, draw the distribution of the stalling duration over multiple events. It will typically follow an exponential distribution, whose characteristic time will be the “bypass time”, i.e. the time needed for the helicase to go through the roadblock.
4. Check whether the helicase removes the roadblock. For this purpose, choosing a working force that prevents the full closing of the hairpin in the presence of the roadblock is recommended. In the absence of a roadblock, the hairpin will close completely after the passage of the helicase. If the roadblock remained and was just bypassed by the enzyme, the hairpin will only partially close after the passage of the helicase.

[Insert Figure 12 here]

4. Discussion

The various elements that we have described demonstrate the wealth of possibilities that have emerged to study helicases in a single molecule configuration. This panel of approaches is in part the result of the richness of NA structures (ssDNA, dsDNA, three-way or four-way junction). The only limitation seems to be our imagination. As a result, depending upon the question investigated, it is possible to choose among various possibilities to either achieve the strongest signal of the simplest experimental design. What we have described for magnetic tweezers can also be used in most situations with optical tweezers.

We have not discussed nanopore-related technologies applied to study helicases, although they offer very interesting features: thanks to their very high spatial resolution, single-molecule measurements of helicase stepping are extremely promising (Caldwell & Spies, 2017; Craig et al., 2019; Laszlo et al., 2017). Their main advantage is the ability to study protein translocation on hairpin-free ssNA while measuring rates of translocation at a nucleotide resolution. Micromanipulation techniques require a hairpin that converts translocation into a change of extension and recent studies have achieved the accuracy of a single base (Cheng et al., 2011; Qi et al., 2013; Rieu, Vieille, et al., 2021), but they are not yet as precise as nanopores.

Altogether, the single molecule approaches described here nicely complement bulk biological tools to study helicases' activities which are not simple to characterize since their unwinding effect can easily be masked by the re-hybridization of NA if nothing is done to prevent it.

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Tables

Specific aim of the helicase experiment	Preferred hairpin lengths	Preferred attachment mode
Good statistics of velocity and processivity	200 bp ~ several kbp	Hybridized to ssDNA coated on the surface through azide-DBCO covalent binding.
High spatio-temporal resolution	10-100 bp	Fully hybridized to ssDNA coated on the surface (no ssDNA left after hybridization). Avoid DIG-antiDIG since it can produce spurious steps in the data.
Good control of helicase concentration	Not relevant	DIG-antiDIG attachment of the hairpin to the surface or direct covalent bond of the hairpin to the surface (Eeftens et al., 2015) (to avoid DNA-coating effects on local concentration of enzyme).

Screening of a large number of different sequences (sequence effect on helicase translocation)	10-50 bp: library of synthetic hairpins can be directly bought from providers, preventing time-consuming lab constructions.	Hybridized to ssDNA coated on the surface through azide-DBCO covalent binding. (no need of functionalization of the hairpin and reusable surface for each sequence.)
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Table 1 : Preferred hairpin lengths and attachment methods as a function of the main objective behind the single-molecule helicase experiment.

Figure legends

Figure 1: Standard setup for the single-molecule characterization of helicases with magnetic tweezers.

(a) A nucleic acid hairpin is tethered between a coverslip and a superparamagnetic bead. Magnets create a magnetic field gradient applying a force on the molecule through the bead. (Image created with BiorRender.com) (b) Progression of a helicase (in orange) through the tethered hairpin at constant force ($f = 8$ pN) and bead position variation.

Figure 2: Different configurations for tethering a NA hairpin between a surface and a magnetic bead in magnetic tweezers.

(a, b) DIG-AntiDIG hairpin tethering. The surface is first coated with Anti-DIG antibodies and the adaptor (a) or hairpin (b) are labeled with multiple DIG groups. The hairpin may also be indirectly (a) or directly (b) tethered to the bead through dual-biotinylation. (c, d) Click chemistry hairpin tethering. The azide surface is first coated with a DBCO-labeled ssDNA that is complementary to the adaptor (c) or the hairpin (d). The hairpin may also be directly (c) or indirectly (d) tethered to the bead through dual-biotinylation. In configuration (c), a ligation of the hairpin and the surface oligonucleotide can be performed *in situ* to covalently attach the hairpin to the surface.

Figure 3: Representation of the biochemical steps for hairpin construction.

(a) Construction of a DNA hairpin. (b) DIG-tailing of a DNA hairpin for DIG/AntiDIG surfaces. (c) Construction of an RNA hairpin.

Figure 4: Assembly of a microfluidic chamber for magnetic tweezers experiments.

Double-sided tapes allow the assembly of the microfluidic chamber between a coverslip and a Mylar sheet, bearing a channel through which a constant flow passes. The buffers and enzymes are injected through the input and a syringe connects the output to an outside pump. (Image created with BiorRender.com)

Figure 5 : Force adjustment protocol to correct for the bead-to-bead magnetic heterogeneity.

(a) Force extension curve over several force cycles of a 1 kb hairpin tethered to a Dynabead MyOne microbead. The red (blue) points represent the points with the highest time derivative of the extension change during the opening (closing). The average of the abscissa of these points over several cycles gives the estimated (i.e. before the bead-to-bead correction) opening (closing) force for that bead. **(b)** The estimated opening forces are compared for all beads tracked during a particular experiment. They follow a Gaussian distribution (inset), here of average 15 pN and standard deviation 1.6 pN. The average of the force applied over all beads gives the true opening force F_{true} (dashed orange line) and allows deducing the corrective multiplicative factor for each bead. Buffer : 70 mM KCl, 5 mM MgCl_2 , 20 mM Tris-HCl pH 7.6.

Figure 6: Protocol for nanometer to base pair conversion.

(a) Sketch of the force protocol and the DNA behavior. **(b)** Superposition of curves representing the extension of a 2129 bp hairpin versus force in a series of cycles with force sweeping from 2 pN to 22 pN. Light gray curves with dots showcase the raw data with typically 100 cycles. The molecule is placed in a 50 mM NaCl buffer in the presence of 100 nM of a 12 bp oligonucleotide complementary to the apex region of the hairpin. The blue curve from (1) to (2) is the average extension of the closed hairpin which reflects the elasticity of the handles used to attach the hairpin to the bead and the surface. The blue curve from (2) to (3) represents the averaged opening behavior of the hairpin. The green curve from (4) to (8) pN corresponds to the elastic behavior of ssDNA, while the red curve represents the same behavior until 2 pN. The red curve corresponds to the situation where the oligonucleotide is hybridized and prevents the hairpin from refolding before the force is low enough to encircle the oligonucleotide or to eject it . **(c)** The final elasticity curve extracted from the curve in (a). The extension shown in the blue averaged curve from (1) to (2) was subtracted from the red averaged curve and normalized by the number of base pairs in the hairpin (2129 bp). This curve thus corresponds to the extension of two ssDNA nucleotides that were fitted with the Langevin model used for Freely Jointed Chain (Smith et al., 1992).

Figure 7: Protocol for a fast *in situ* assembly of arbitrary long three-way junctions onto a tethered hairpin.

(a) Sketch of the experiment. **(b)** Corresponding experimental traces of RecG. Force cycles (1) - (2) are applied to repeatedly open and close a hairpin in presence of 18 nM of a flapped 600 bp dsDNA complementary to the hairpin, until the dsDNA binds to the hairpin (3) and spontaneously invades the hairpin, at time 510 s, to form a three-way junction (4) - (5). The substrate is then ready to study fork regression by an enzyme such as RecG (1750 s) (Manosas et al., 2013). 5 nM RecG, 25 mM TrisOAc (pH 7.50), 150 mM KOAc, 5 mM MgOAc, 1 mM dithiothreitol and 1 mM ATP.

Figure 8: Peeling assay: an alternative experiment to mitigate the impact of the force on helicase translocation.

(a) Sketch of the experiment. **(b)** Corresponding experimental traces of RecQ- Δ C. Force cycles (1) - (2) are applied to repeatedly open and close a hairpin in presence of 100 nM of a 3' flapped ssDNA complementary to the hairpin, until the ssDNA binds to the hairpin (3). The hairpin is then closed (4) and RecQ- Δ C injected at a concentration of 100 pM (5). The helicase binds to the flap and unwinds the oligonucleotide, resulting in the progressive closing of the hairpin (6 and inset of the trace), until it completely removes the oligonucleotide at 1235 s with an unwinding velocity of 97 ± 10 bp/s (7). At 1425s, another enzyme binds and unwinds the hairpin in the standard configuration with an unwinding velocity of 95 ± 0.3 bp/s (8-9). Tris-HCl (pH 7.60), 50 mM NaCl, 3 mM MgCl₂, 1 mM DTT and 1 mM ATP.

Figure 9: Bubble assay: an alternative protocol to measure helicase progression inside a dsDNA at zero force.

(a) Sketch of the experiment. **(b)** Corresponding experimental traces of human UPF1 (hUPF1) helicase core. Force cycles are applied to repeatedly open (1), close (3) and encircle the helicase (4). The translocation velocity of the helicase stays approximately constant over the whole experiment (blue dashed line, linear fit of steps (3) where the position of the bead depends on the position of the helicase). Thus, hUPF1 keeps translocating roughly at the same speed when it is encircled. Note that the time it takes for hUPF1 to be encircled increases with the encircling force. Buffer : 20 mM Tris HCl pH 7.5, 75 mM KOAc, 3 mM MgCl₂, 1% BSA, 1 mM DTT, 1 mM ATP, 30 nM yUPF1-HD.

Figure 10: Sketch of the Single Molecule Binding Assay (SMBA) protocol allowing qualitative estimation of a helicase's grip onto nucleic acids.

(a) Sketch of the experiment. **(b,c)** Corresponding experimental traces reproduced from (Kanaan et al., 2018), in agreement with the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>). The numbers corresponding to the hairpin state in panel (a) were added to the traces. Yeast UPF1 catalytic helicase core (yUpf1 HD) and IGHMBP2 helicase domain were subjected to force cycles without the addition of ATP. yUPF1 HD blocks the hairpins closure with its tight grip, unlike IGHMBP2 HD that slides and is ejected by the hairpins closure.

Figure 11: Single-molecule measurement of the binding kinetics of a helicase without ATP through kinetic locking.

(a) Sketch of the experiment. A rapidly fluctuating 10 bp hairpin allows measuring short binding times of RecQ- Δ C through the alteration of the folding/unfolding rates induced by the association of the helicase. **(b)** Corresponding force-extension curve of RecQ- Δ C. When the helicase binds to the hairpin, it blocks it in its open state for a longer time (red). **(c)** Distributions of open times as a function of the concentration of helicases. Fitting to double-exponential distributions allows retrieving the association and dissociation kinetic rates. **(b,c)**

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Figure 12: Protocol for the single-molecule observation of a helicase's interaction with a roadblock.

(a) Sketch of the experiment. **(b)** Typical extension traces and interpretation. (1) A hairpin is opened by applying a high force on the magnetic bead (20 pN). (2) The roadblock is formed. It can consist in the deposition of a NA-binding protein present in solution or in the formation of a NA-tertiary structure. (3) At a force of ~ 8 pN, the hairpin can only partially close due to the presence of the roadblock. This allows probing the presence of the roadblock. (4) The force is then lowered to a force (generally 2-5 pN) allowing encircling the roadblock inside a double-stranded hairpin. (5) The force is then increased to the working force of the helicase. The helicase is injected in solution. While the helicase unwinds the hairpin, the extension of the molecule increases. If the roadblock prevents the translocation of the helicase, a pause is observed in this increase of extension. If the helicase stalls and passes through the roadblock, one records the bypass time (in purple), which probability distribution is the main quantitative parameter provided by the assay. After the passage of the helicase, either the roadblock is still present (blue) or it has been removed (green). If it is still here, provided that the working force is too high to allow the encirclement of the roadblock, the hairpin will only partially close. Measuring several events thus allows accessing the probability of resolution.