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Mechanisms of HCV NS3 Helicase Monitored by Optical Tweezers

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Abstract

As one of the essential enzymes for viral genome replication, the hepatitis C virus NS3 helicase is one of the best characterized RNA helicases to date in understanding the mechanistic cycles in a helicase-catalyzed strand separation reaction. Recently, single-molecule studies on NS3, in particular the use of optical tweezers with sub-base pair spatial resolution, have allowed people to examine the potential elementary steps of NS3 in unwinding the double-stranded RNA fueled by ATP binding and hydrolysis. In this chapter, I detail the essential technical elements involved in conducting a high-resolution optical tweezers study of NS3 helicase, starting from the purification of the recombinant helicase protein from *E. coli* to setting up a high-resolution single-molecule experiment using optical tweezers.

Keywords

RNA helicase; optical tweezers; hepatitis C virus; NS3; single-molecule

1. Introduction

Helicases couple the binding and hydrolysis of nucleoside triphosphate to the separation of complementary strands in nucleic acids (1). This activity is required in almost all aspects of DNA and RNA metabolism such as RNA remodeling (2,3). One of the best characterized non-hexameric helicases is the NS3 protein encoded by the hepatitis C virus (HCV) (4). This helicase is one of the essential enzymes that are required for viral replication life cycle and therefore a potentially important drug target (5,6). Despite extensive biochemical characterization of this protein, the exact biological function of this helicase is still elusive (7). It is required for viral RNA replication (8), and also plays a role in viral particle assembly (9). Based on the protein sequence, NS3 is a representative member of the Superfamily 2 helicases (10), with structural resemblance to other helicase proteins despite the overall low sequence identity beyond helicase motifs (11). This protein is shown to possess both DNA and RNA helicase activities *in vitro* (12). Although oligomerization of NS3 appears to be important for the processive RNA helicase activity (13), several studies have shown that the monomeric form of the NS3 protein is an active helicase (14–16).

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Therefore NS3 has become a simple model system to understand mechanisms of helicase activities in general.

The advent of single-molecule techniques in recent years has revolutionized the study of proteins and nucleic acids at mechanistic level. In a typical ensemble experiment, observations are made through averaging of many different molecules. In single-molecule experiments, the intermediates in a biochemical or biophysical process can be directly monitored, which often yields information that is hidden in conventional ensemble experiments. Helicase-catalyzed nucleic acids unwinding reaction is composed of multiple biochemical and biophysical steps; with the binding and hydrolysis of ATP, the helicase protein moves along the nucleic acids substrate and meanwhile releases the nascent single-stranded nucleic acids. Proper design of a single-molecule experiment allows one to directly ‘visualize’ this process in real time, and derive detailed mechanistic information about the entire process. We have developed a high-resolution single-molecule assay for NS3 helicase based on optical tweezers (17) (Fig. 1). Compared to other single-molecule techniques that have also been applied to NS3 helicase such as single-molecule fluorescence resonance energy transfer (smFRET) (18), optical tweezers has the unique advantage to directly monitor the number of nucleotides that are released by the enzyme with high spatial resolution, and to examine the sensitivity of the unwinding reaction to mechanical force (19). By using an optical tweezers instrument that we recently developed (20), we were able to monitor the NS3-catalyzed unwinding reaction down to sub-base pair resolution.

To set up a high-resolution optical tweezers assay for NS3 requires the NS3 protein, nucleic acids substrates, polystyrene beads coupled with functional proteins for attachment of the substrates to beads, microfluidic chambers and the instrument. While this chapter focuses on the technical aspects of conducting single-molecule assays using a home-built instrument (20), the readers are encouraged to consult the excellent chapter on how to construct a high-resolution optical tweezers instrument (21), and the website on how to build a miniature version of optical tweezers that is capable of high-resolution measurements (<http://tweezerslab.unipr.it>).

2. Materials

Prepare all solutions using ultrapure water (ddH₂O, prepared by purifying deionized water to attain a resistivity of 18.2 M Ω -cm at 25°C) unless otherwise noted. All buffers should be filtered through 0.2 μ m (pore size) filters for long-term storage.

2.1 Overexpression and Purification of HCV NS3 Helicase

1. 2 $\times$ LB broth: 20 g tryptone, 10 g yeast extract and 10 g NaCl. Fill to 1 L with singly-distilled water and sterilize by autoclaving.
2. 1000 $\times$ ampicillin stock solution at 50 mg/mL in water. Sterilize with a 0.2 μ m filter unit and store at –20°C.
3. 1000 $\times$ kanamycin stock solution at 30 mg/mL in water. Sterilize with a 0.2 μ m filter unit and store at –20°C.

4. M15[pREP4] cells (Qiagen) harboring a pQE40 vector (Qiagen) that encodes the full-length HCV NS3 protein from genotype 1a (GenBank AF009606) (14,22).
5. 1 M ZnCl₂.
6. Chicken egg lysozyme.
7. Lysis buffer: 50 mM Tris-HCl, 0.2 M NaCl, 15% (v/v) glycerol, 20% (w/v) sucrose, and 2 mM 2-mercaptoethanol. Adjust pH to 8.0 at 4°C using HCl and then store at 4°C.
8. Ni-NTA agarose.
9. Buffer A for Ni-NTA column: 25 mM HEPES, 0.5 M NaCl, 20% (v/v) glycerol, 10 mM 2-mercaptoethanol. Adjust pH to 8.0 at 4°C using NaOH and then store at 4°C.
10. Buffer B for Ni-NTA column: 25 mM HEPES, 0.5 M NaCl, 20% (v/v) glycerol, 10 mM 2-mercaptoethanol, 20 mM imidazole. Adjust pH to 8.0 at 4°C using NaOH and then store at 4°C.
11. Buffer C for Ni-NTA column: 25 mM HEPES, 0.5 M NaCl, 20% (v/v) glycerol, 10 mM 2-mercaptoethanol, 500 mM imidazole. Adjust pH to 8.0 at 4°C using NaOH and then store at 4°C.
12. Buffer D for S200 gel filtration column: 25 mM HEPES, 0.5 M NaCl, 20% (v/v) glycerol, 2 mM DTT. Adjust pH to 8.0 at 4°C using NaOH and then store at 4°C.
13. Buffer for NS3 concentration measurement: 20 mM sodium phosphate buffer, 6 M guanidinium chloride. Adjust pH to 6.5 at 25°C using either sodium phosphate monobasic or sodium phosphate dibasic and then store at room temperature.
14. Ultrasonic processor (e.g., S-4000 processor from Misonix, Farmingdale, NY).
15. Empty flow-through chromatography column (e.g., Polyprep column from Biorad).
16. HiLoad Superdex 200 16/60 column (GE Healthcare).
17. Automated liquid chromatography system (e.g., FPLC system).
18. Precast 4–15% gradient gels and electrophoresis system for polyacrylamide gel electrophoresis (PAGE).

2.2 Making RNA Unwinding Substrates

Resuspend the oligodeoxynucleotides listed in subheadings 1 to 10 in ddH₂O to a concentration of 100 μM.

1. top-left : 5' (phos)-
AATTCTCATGCAGGACAGTCGGAATTATTTATTTATTTATTTATTTATT
TTACCGCCCGCCCGCCCGCCCGCCCGCCCGCCGCGATGCGGGCGGGCGGGC
G- 3';
2. top-right: 5' (phos)-
GGCGGGCGGGCGGGCGGTAAAATAAAATAAAATAAAATAAATAAATA
GGAGCTCAGCTATCAGA- 3';

3. bottom-left: 5' (phos)-
GGGCGGCGGGCGGGCGGGCGGGCGGGCGGTAAAATAAAATAAAATAAA
TAAATAAATAATTCCGACTGTCCTGCATGAG- 3';
4. bottom-right: 5' (phos)-
AGCTTCTGATAGCTGAGCTCCTATTATTTATTTATTTATTTATTTATTTT
ACCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCATGC-3';
5. T7 promoter top strand: 5' (phos)-
GTTATAATACGACTCACTATAGGGACTGGTGAGT-3';
6. T7 promoter bottom strand: 5' (phos)-
ACTCACCAGTCCCTATAGTGAGTCGTATTATAACTGCA-3';
7. A-forward: 5'-GGAATTCCGACTGGTGAGTACTCAACCAAGTC-3';
8. A-reverse: 5'-TCCGACTGTCCTGCATGAG-3';
9. B-forward: 5'-CTATCAGAAGCTTTAATGCGG-3';
10. B-reverse-Biotin: 5' (Biotin)-GCATTAGGAAGCAGCCCAGTAGTAGG-3'.
11. DNA oligo annealing buffer: 10 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl₂. Adjust pH to 7.5 at 25°C with HCl and store at 4°C.
12. pBR322 plasmid vector (New England Biolabs).
13. EcorI, HindIII, SphI, StyI, ScaI, and EcoNI restriction enzymes.
14. DNA Quick ligation kit (New England Biolabs).
15. Commercial kit for mini preparations of DNA (e.g., QIAprep spin miniprep kit from Qiagen).
16. Commercial kit for gel purification of DNA (e.g., QIAquick gel extraction kit from Qiagen).
17. Commercial kit for DNA maxi preparations of DNA (e.g., Qiagen maxi prep kit from Qiagen).
18. Commercial kit for DNA ligation (e.g., NEB quick ligation kit).
19. Commercial T7 transcription kit (e.g., Megascript T7 Transcription kit from Ambion).
20. 5× MOPS buffer: 250 mM MOPS, 5 mM EDTA. Adjust pH to 7.0 at 20°C with NaOH, filter through a 0.2 µm filter unit and store at room temperature in the dark.
21. Formaldehyde loading buffer: mix 40 µl of 5× MOPS buffer with 35.8 µl of formaldehyde (37% solution, ~12.3 M). Volume can be scaled based on needs.
22. LiCl precipitation solution: 7.5 M lithium chloride, 50 mM EDTA.
23. DEPC-treated ddH₂O.

24. Phosphate buffer for RNA concentration measurement: 10 mM sodium phosphate buffer, 100 mM NaCl. Adjust pH to 7.0 at 25°C using either sodium phosphate monobasic or sodium phosphate dibasic and then store at 4°C.
25. 5 U/μl Taq DNA polymerase.
26. RNA/DNA annealing buffer: mix 400 μl of formamide with 1 μl of 0.5 M EDTA pH 8.0 (1 mM final), 20 μl of 1 M PIPES pH 6.3 (40 mM final), and 40 μl of 5 M NaCl (0.4 M final). Volume can be scaled based on needs.
27. RNA ladder (Invitrogen).
28. Precast 4–15% gradient PAGE gels and electrophoresis system.
29. SYBR Green I Nucleic Acid Gel Stain (Invitrogen).
30. SYBR Green II Nucleic Acid Gel Stain (Invitrogen).
31. dATP.
32. Digoxigenin-11-dUTP.
33. 5 U/μL Klenow exo^- polymerase.
34. 0.5 M EDTA.
35. 70% ethanol.
36. 100% ethanol.
37. DH5α competent cells.
38. Agarose, electrophoresis grade.
39. Centrifugal filter devices (e.g., Microcon filters from Millipore).
40. PCR thermocycler.
41. Typhoon Multi-mode Scanner (GE Healthcare).

2.3 Biochemical Characterization of NS3 Activity in Bulk

The bulk activity of NS3 can be conveniently monitored using FRET-based assay in a steady-state fluorometer or stopped-flow spectrophotometer.

1. Single-stranded RNA T10: 5'GCCUCGCUGC-Cy3-3', dissolved in autoclaved ddH₂O to a concentration of ~100 μM checked by absorbance at 260 nm (A_{260}).
2. Single-stranded RNA B20: 5'Cy5-GCAGCGAGGCAGAGGAGCAG-3', dissolved in autoclaved ddH₂O to a concentration ~100 μM (checked by A_{260}).
3. 5×RNA oligo annealing buffer: 50 mM MOPS, 150 mM NaCl. Adjust pH to 6.5 at 25°C and then store at 4°C.
4. Helicase Buffer: 20 mM MOPS, 30 mM NaCl, 0.9% V/V glycerol, 0.75 mM MgCl₂, 0.1% (v/v) Tween 20. Adjust pH to 6.5 at 22°C using ammonia.
5. 1 M DTT stock solution. Aliquot and store at –20°C.

6. ATP stock solution. Aliquot and store at -20°C .
7. 5 μM NS3 helicase.
8. A fluorometer that allows kinetic measurement of steady-state fluorescence of Cy3, with excitation at 515 nm and emission at 570 nm (23).

2.4 Coupling Antibodies to Polystyrene Beads

1. Activation buffer: 0.1 M MES, 0.5 M NaCl. Adjust the pH to 6.0 at 22°C with NaOH.
2. Antibody reconstitution buffer (PBS1): 0.019 M NaH_2PO_4 , 0.081 M Na_2HPO_4 , 0.14 M NaCl, 2.7 mM KCl.
3. Antibody crosslinking buffer: 0.1 M Na_2HPO_4 , 0.1 M NaCl (or other non-amine containing buffer with pH 7–9).
4. Bead storage buffer (PBS2): 0.039 M NaH_2PO_4 , 0.061 M Na_2HPO_4 , 0.14 M NaCl, 2.7 mM KCl.
5. 2% (w/v) sodium azide solution.
6. Carboxyl-polystyrene particles, 1.26 micron average diameter (Spherotech).
7. Recombinant Protein G.
8. N-Hydroxysulfosuccinimide (Sulfo-NHS).
9. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDC).
10. 2-mercaptoethanol.
11. Dimethylpimelimidate dihydrochloride (DMP)
12. Sheep polyclonal anti-Digoxigenin antibody.
13. Ultrasonic processor equipped with a cup horn.

2.5 Cleaning and Modification of Coverslips for Making Microfluidic Chambers

1. N-(2-Aminoethyl)-3-Aminopropyltrimethoxysilane (see Note 1).
2. Methoxy-poly (ethylene glycol)-succinimidyl valerate, Average MW 5,000 (mPEG-SVA from Laysan Bio, Inc.) (see Note 2).
3. 0.1 M NaHCO_3 solution.
4. 98% sulfuric acid.
5. 30% Hydrogen Peroxide solution.
6. 100% Methanol.

¹Amino silane expires in 6 months upon arrival. Store at room temperature. Every time after use, place the bottle into a desiccator, with the cap loose, draw vacuum first and then fill with N_2 or argon. Tighten the cap, wrap the cap with parafilm for storage.

²Upon arrival, aliquot mPEG-SVA in 0.1 g in screw cap tubes, place tubes in a jar bottomed with drierite and store in -80°C freezer. Every time before use, take the jar out of -80°C freezer to fully equilibrate to room temperature before opening.

7. Glacial acetic acid.
8. Glass coverslips (24mm×60mm×0.17mm).
9. Vertical staining dishes (8 slots).
10. Tabletop sonic bath cleaner.
11. Laser Engraver (e.g., VersaLASER Model VLS2.30 from Universal Laser Systems).

2.6 Microfluidic Chamber Assemblies

1. 100% Ethanol.
2. Ablefilm 561-1-003 (Henkel Corporation).
3. 0.040 mm ID glass micropipette (King Precision Glass, Inc).
4. Precleaned plain microscope slides.
5. Flat top hot plate.
6. Ø1/2" × 2" Stainless Steel Optical Post (Thorlabs), two required
7. Lead Brick ASTM-B749 Grade B Purity 2" × 4" × 8" (Amazon).
8. Chamber frame (made by various machine shops): see Fig. 2 for schematic drawings with dimensions.

2.7 Single-Molecule Optical Tweezers Assay

1. Streptavidin coated polystyrene particles, 1.26 micron average diameter (SpheroTech).
2. Anti-digoxigenin coated polystyrene particles (see Section 3.4).
3. Standard Buffer: 10 mM Tris-HCl, 100 mM NaCl. Adjust pH to 7.0 at 22°C using HCl and then store at 4°C.
4. Nuclease-free BSA.
5. Helicase Buffer (see Section 2.3, subheading 4).
6. DNA-RNA hybrid substrates (See Section 3.2).
7. 1 M DTT stock solution. Aliquot and store at −20°C.
8. ATP stock solution. Aliquot and store at −20°C.
9. 5 µM NS3 helicase.
10. Intramedic PE Tubing, 0.011 in. ID (PE10).
11. Intramedic PE Tubing, 0.023 in. ID (PE50).
12. 1mL BD tuberculin Syringes.
13. 10 mL BD tuberculin Syringes.

14. BD PrecisionGlide Single-use Needles, 23G.
15. BD PrecisionGlide Single-use Needles, 30G.
16. Ultrasonic processor.
17. Home-built high-resolution optical tweezers: the core components include a tapered amplifier diode laser (SYS-420-830-1000, Sacher LaserTechnik, LLC, Germany) that operates at 830 nm; two Nikon water-immersion objectives (60× CFI Plan APO VC, N.A.=1.2) directly mounted on an optical table, one for formation of optical traps and one for collection of photons that exit the optical trap; two dual-axis position sensing diodes for measurement of laser deflection; a multifunctional and multichannel data acquisition device (National Instruments) and a broad-band vibrational suppression optical table (6'×4').

3. Methods

3.1 Overexpression and Purification of HCV NS3 Helicase

The following protocol was adapted from Beran et al. (22) with modifications.

1. Inoculate 40 ml of LB broth supplemented with 50 µg/ml of ampicillin and 30 µg/ml of kanamycin with a single colony of M15[pREP4] harboring the pQE40-wtNS3 plasmid and incubate overnight at 37°C (See Note 3).
2. Prepare four flasks, each containing 1L of 2×LB broth supplemented with 150 µg/ml Ampicillin and 90 µg/ml of kanamycin, and add 10 ml of saturated overnight culture to each flask.
3. Incubate with shaking at 37°C until optical density at 600 nm (OD₆₀₀) reaches 0.8. Change the incubator temperature to 16°C. Allow 1 hour for the incubator to cool. Then, add 1 ml of 1 M IPTG and 100 µl of 1 M ZnCl₂ to each liter of culture and continue to incubate with shaking overnight at 16°C.
4. Centrifuge the cultures at 4,000 g for 10 minutes at 4°C. Discard the supernatant. The cell pellet can be frozen at –80°C until further use or can be directly lysed as follows for NS3 protein purification.
5. Resuspend cell paste in lysis buffer (3 mL per gram of cell paste), make sure there are no clumps in the suspension, and add lysozyme to a final concentration of 0.4 mg/ml. Incubate under stirring for 1 h in a cold room.
6. Adjust NaCl concentration to 0.5M final by adding 5 M NaCl drop-by-drop. Sonicate with 50% duty cycle at a power setting of 1 for 6 min (settings may depend on the type of sonicator used). Centrifuge at 13,000 g for 30 min at 4°C and recover supernatant.

³Potentially due to the toxicity of the NS3 protease within the full-length NS3 protein, the plasmid encoding the full-length NS3 protein is toxic to *E. coli* cells without suppression of its background expression. Thus, we have used the M15(pREP4) from Qiagen for cloning and protein expression, in which pREP4 is the plasmid that constitutively expresses the lac repressor protein encoded by the lac I gene.

7. To equilibrate Ni-NTA beads, dispatch Ni-NTA slurry (1 mL per 10 g of cell paste) into empty gravity-flow columns (one column per mL of bead slurry). Then, add 10 ml buffer A to each column and drain by gravity. When buffer A is almost gone, add another 10 ml of buffer A, drain by gravity, and repeat this one more time. The beads are then ready to use.
8. Resuspend the beads from each column in 1 ml of buffer A, pool beads, and mix with cleared cell lysate. Incubate the mixture in a 125 ml Erlenmeyer flask in cold room, with gentle stirring for 2 hours.
9. Load the batch binding mixture into empty gravity-flow column(s). Wash the column(s) once with 10 column volume (CV) of Buffer A and then four CV of Buffer B.
10. Elute the protein from the column(s) using Buffer C. If necessary, use a centrifugal filter unit to reduce the volume of the protein-containing fraction to less than 1mL.
11. Install the HiLoad Superdex 200 16/60 column on the automated chromatography system and equilibrate it with 2 CV of Buffer D at a flow rate of 0.4 ml/min. Load the eluted protein from Ni-NTA agarose (1 ml maximum) onto the column and then collect ninety-five 1.2 ml fractions (See Note 4).
12. Analyze fractions by standard 4–15% gradient SDS-PAGE. Pool NS3 fractions of highest purity and determine protein concentration by using absorbance at 280 nm (See Note 5). Flash freeze the protein in liquid nitrogen and store in –80°C freezer (See Note 6). Typically around 1 mg of pure NS3 protein is obtained from 1 L culture.

3.2 Making RNA Unwinding Substrates

To generate the RNA hairpin substrates (Fig. 1) for use in optical tweezers, we first prepare a linear DNA amplicon containing a T7 RNA polymerase (RNAP) promoter using standard PCR procedures. This DNA amplicon serves as a DNA template to produce the RNA transcripts that will be annealed to two complementary DNA pieces for making the DNA-RNA hybrid (Fig. 1). While the strategy to make the DNA template with a generic hairpin sequence is straightforward, the strategy to make DNA template with homopolymeric AT or GC sequences is not (see Note 7). This type of substrates can be used to assess the sequence dependence of NS3 unwinding activity. They are also used for internal calibration of worm-like-chain parameters of single-stranded RNA (17), which are essential to convert the change in RNA extension to the number of nucleotides. Below is the alternative strategy that the author has developed to make this type of DNA template.

⁴NS3 elutes primarily between fractions 60 and 70. Usually the earliest fractions (*i.e.* 60–65) contain the purest NS3.

⁵The NS3 full-length protein we studied contains 23 Tyr and 6 Trp residues, with a theoretical molecular weight of 69,719.63 Dalton. We use an extinction coefficient of $63,580 \text{ M}^{-1}\text{cm}^{-1}$ in 6 M Guanidinium Chloride buffered with sodium phosphate (see **Section 2.1**) to determine the protein concentration. The typical concentration of the protein is around 5 μM .

⁶Aliquot the protein in small aliquots such as 5 μl to avoid repeated flash freezing. We don't use proteins that have been frozen more than twice.

⁷The DNA template containing 30 base pairs of AT and 30 base pairs of GC cannot be correctly amplified by Taq DNA polymerase in a PCR reaction.

1. Mix 2.5µl of Top-left and 2.5µl of bottom-left oligonucleotide stocks with 45 µl of DNA annealing buffer in a PCR microtube. Mix 2.5µl of Top-right and 2.5µl of bottom-right oligonucleotide stocks with 45 µl of DNA annealing buffer in a second tube. Using a PCR thermocycler, heat both samples at 95°C for 4 min, then cool down to room temperature at a rate of 5 min per °C.
2. Collect 25 µl from each of the annealing reactions above and mix them. Heat the mixture to 56°C for 4 min and cool down to room temperature at a rate of 5 min per °C. The resulting annealing product is named 'LR'. Upon annealing, a SphI restriction site is formed on LR.
3. Use restriction digest with SphI to check an aliquot of the annealed product LR (200 ng) on 4–15% native gradient PAGE. Stain the gel with SYBR green I and image it with the Typhoon scanner. The correctly annealed product should yield smaller fragments after digestion. Determine concentration of LR based on the band intensity in comparison to a reference ladder.
4. Digest the pBR322 plasmid with the EcoRI and HindIII enzymes following manufacturer's instructions. Purify the digested vector on a 1% agarose gel and ligate it to LR at a molar ratio of 1:2. Transform the ligation mixture into DH5α competent cells, following manufacturer's instructions, and spread 100–200 µL of cells on a LB-agar plate containing 50 µg/mL of ampicillin. Incubate overnight at 37°C.
5. Pick up at least two single colonies and use each one to inoculate 2 ml of LB broth containing 50 µg/ml Ampicillin. Grow overnight at 37°C and then use 1.5 ml of each culture for a plasmid mini preparation with a commercial kit, following manufacturer's instructions.
6. Use both single and double restriction digests with the SphI and StyI enzymes to check the presence of the expected fragment insertion in the resulting plasmids (see Note 8).
7. Use the appropriate remaining 0.5 mL culture(s) to amplify the correct plasmid (pWCRNA5) using a commercial maxi preparation kit, following manufacturer's instructions. Typically, around 100 µg of pWCRNA5 is obtained from 1 L culture.
8. To verify the sequence of pWCRNA5, digest 3 µg of plasmid with SphI and StyI and then purify the two DNA fragments with sizes around 616bp and 3078bp on an agarose gel. Recover DNA with a commercial kit (often, mini prep kits can also be used for DNA recovery from agarose gels) and send the recovered DNA to a sequencing facility/company (see Note 9).
9. To prepare the linear DNA template for *in vitro* transcription, digest 20 µg of pWCRNA5 with ScaI and EcoNI at 37°C for 16 hours in a warm room (see Note

⁸There are two SphI sites and a single StyI site in the constructed plasmid. Double digestion by SphI and StyI should generate three bands around the following size: 616, 801, and 3078 bp.

⁹Due to the homopolymeric sequences of AT and GC in pWCRNA5, DNA Polymerases fail to read through the entire hairpin sequence in a sequencing reaction. Thus the plasmid needs to be digested with restriction enzymes to cut the hairpin sequence in half before sequencing. Otherwise, the entire hairpin sequence cannot be fully confirmed.

10). Gel-purify the resulting 1276 bp fragment as described in step 8. Concentrate the DNA using a centrifugal filter device (or ethanol precipitation) to at least 60 ng/ μ l.

10. Anneal T7 promoter top and bottom strand oligonucleotides using the annealing protocol in Step 1. The resulting duplex contains a *Psi*I site and half of a *Sca*I digestion site (blunt end).
11. Blunt-end ligate the 1276-bp DNA fragment and the T7 promoter duplex at a molar ratio of 1:20 using a commercial ligation kit.
12. After ligation, add *Psi*I to the ligation mixture and incubate overnight at 37°C to clean up the ligation reaction.
13. Purify the digested DNA onto a 1.5% agarose gel as described in step 8. The concentration of the purified DNA may need to be concentrated to at least 60 ng/ μ l (see step 9).
14. Prepare the RNA hairpin transcript (Fig. 1) using a commercial *in vitro* T7 transcription kit and the DNA template from step 13. Assemble the transcription reaction strictly following the manufacturer's instructions (see Note 11).
15. To verify the transcription products, prepare a 1.5% denaturing agarose gel containing 37% (v/v) formaldehyde and 1 $\times$ MOPS buffer. Pre-run the gel at 100 V for 10 min (see Note 12). Meanwhile, prepare samples for loading by mixing 2 μ l of RNA mixture (transcription or RNA ladder) with 3.8 μ l of formaldehyde loading buffer and 5 μ l of 1 $\times$ MOPS buffer (See Note 13). Heat both samples at 70°C for 15 min before loading onto the gel. Run gel at 70 V for 1 h (see Note 12). Stain the gel with SYBR green II and image it with a Typhoon Scanner. Check that a strong band at the correct size is present in the transcription sample.
16. Purify the transcription product by precipitation with LiCl. First, centrifuge the transcription reaction mixture at 13,000 g for 10 min at room temperature. Transfer supernatant to a new microtube and add 1.5 volume of LiCl precipitation solution. Mix thoroughly and incubate for at least 30 min at -20°C. Centrifuge at 13,000 g for 15 min at 4°C to pellet RNA. Carefully remove the supernatant, wash the pellet once with 1 ml 70% ethanol, and then centrifuge again for 15 min at 4°C. Carefully remove supernatant and resuspend the pellet in 20 μ l of DEPC-treated ddH₂O. determine RNA concentration from absorbance at 260 nm.
17. To prepare DNA handle A, prepare several 100 μ L mixtures containing 0.2 mM dNTPs, 0.5 μ M of Primer A-forward and 0.5 μ M of Primer A-reverse

¹⁰Restriction enzyme digestion overnight should be carried out in a warm room or an incubator with the entire tube situated at 37°C to prevent condensation of water and change of buffer composition during digestion.

¹¹With the Megascript T7 Transcription kit, we typically assemble 40 μ l reactions for each RNA sample: vortex 10 $\times$ reaction buffer until all the contents are completely in solution, keep the reaction buffer at room temperature while assembling the reactions. The order of addition is as follows: nuclease-free water; NTP mix: 16 μ l; 10 $\times$ reaction buffer: 4 μ l; linear DNA template: up to 2 μ g; Enzyme mix: 4 μ l, 40 μ l total.

¹²We use a mini gel tank with electrode separation of ~ 16 cm. The voltage applied during the gel running should be adjusted based on this distance.

¹³Based on the typical transcription yield, 1 μ l transcription mixture should be enough to give an intense band in a denaturing agarose gel.

oligonucleotides, 0.4ng/ μ L of pWCRNA5 plasmid, and 5 U/ μ L of Taq DNA Polymerase in 1 $\times$ supplied polymerase buffer. Perform PCR amplification using an initial denaturation at 94°C for 3 min and 34 cycles of denaturation at 94°C for 45 s, annealing at 51°C for 40 s, and extension at 72°C for 90 s, followed by incubation for 5min at 72°C. Purify the PCR product (handle A) using a commercial kit.

18. Digest handle A with EcoRI at 37°C overnight to produce 3'-recessed end. Then, inactivate the enzyme by incubation at 65°C for 20 min. Withdraw a small reaction aliquot (~1 μ l) to check digestion on an agarose gel.
19. Mix 200 μ L of the above sample with 0.7 μ L of 10 mM dATP, 7.3 μ l of 1 mM Dig-dUTP, 5 μ l of ddH₂O, 2 μ l of 10 $\times$ Klenow buffer, and 5 μ l of Klenow exo-polymerase (fill-in reaction). Incubate the mixture at room temperature or 22°C for 2 ~ 3 hours. Stop the reaction by first adding 4.5 μ l of 0.5 M EDTA, then heating at 75°C for 20 min to inactivate the enzyme. Withdraw a small reaction aliquot (~1 μ l) to check reaction on an agarose gel.
20. Remove excess Dig-dUTP by using a commercial PCR purification kit and determine the concentration of DNA from absorbance at 260 nm.
21. To prepare DNA handle B, repeat Step 17 using the B-forward and B-reverse-Biotin oligonucleotides as primers. In this way, a single biotin will be incorporated at the 5' end of the DNA handle B.
22. Set up the annealing reaction between the RNA hairpin transcript (from step 16) and DNA handles A and B (from steps 20 and 21) on ice as follows: mix 1 μ g of handle A, 1 μ g of handle B, 80 μ l of RNA/DNA annealing buffer, and 10 μ g of RNA transcript in a total volume of 100 μ l (See Note 14). Set up the following annealing protocol in a PCR machine: 85°C for 10min, 62°C for 2hr, 52°C for 2hr, 0.1°C/sec to 10°C, and then 4°C forever.
23. After annealing of the RNA/DNA hybrid molecules, add 3 volumes of 100% ethanol, mix by inverting tubes 5–6 times or pipetting gently up and down for 6–7 times, and incubate at –80°C for at least 2 h. then, centrifuge at 13,000 g for 10 min at 4°C and carefully remove supernatant. A small brownish or close-to-white pellet should be visible at the bottom of the tube.
24. Wash the pellet by adding 50 μ l of 70% ethanol, centrifuge for another 10 min at 13,000 g and 4°C and carefully remove supernatant. Dissolve the pellet in 20 μ l of DEPC-treated ddH₂O (the pellet should easily dissolve upon tapping the outside of the tube gently).
25. Check the annealed substrate by electrophoresis on a 1.5% native agarose gel for 90 min at 70 V. Stain the gel with SYBR green I and image it with the Typhoon scanner.

¹⁴This order of addition is very important, first DNA handles, then RNA/DNA annealing buffer, pipette up and down to mix DNA handles with annealing buffer and add RNA last. This order will reduce the chances of RNA being degraded by trace nucleases that may be present in handle preparations, and thus ensures a reproducible RNA/DNA annealing condition.

3.3 Biochemical Characterization of NS3 Activity in Bulk

The helicase activity of purified NS3 helicase can be conveniently monitored using FRET in a fluorometer (24).

1. Prepare the RNA duplex substrate (designated as 10–10 RNA) by mixing the two complementary single-stranded RNA strands T10 and B20 (both at 20 μ M final concentration) in 100 μ L of 1 $\times$ RNA oligo annealing buffer. In a thermocycler, heat the mixture at 95°C for 4 min, and then cool it down to room temperature at a rate of 5 min per °C.
2. Prepare a solution of 10 nM 10–10 RNA substrate in 1 $\times$ helicase buffer supplemented with 2 mM ATP and 2mM DTT.
3. Prepare 100 μ L of 20 nM NS3 protein in helicase buffer supplemented with 2 mM DTT (see Note 15).
4. Mix 50 μ L of the 10 nM RNA substrate solution (step 2) with 50 μ L of the NS3 protein solution and immediately transfer 50 μ L of the mixture into a clean fluorescence cuvette. Using a fluorometer, record fluorescence of Cy3 for 15 min (see Note 16).
5. Repeat Steps 3 and 4 altering conditions to obtain final NS3 concentrations of 20, 30, 40, 60, 80, and 100 nM.
6. Using the fluorescence time courses, plot the unwinding reaction rates and amplitudes as a function of NS3 concentration.

3.4 Coupling Antibodies to Polystyrene Beads

1. Spin down 200 μ L of carboxyl-polystyrene bead slurry at 3000 g for 6 min. Resuspend beads in 900 μ L of activation buffer. Repeat this step two more times.
2. Dissolve 22 mg of sulfo-NHS in 1 ml ddH₂O and immediately transfer this 1 ml solution to the bottle containing 10 mg EDC. Vortex to dissolve. Immediately mix 100 μ L of this cross-linker mixture with the 900 μ L bead slurry in activation buffer. Tumble on a nutator for 15 min at room temperature.
3. Add 2.8 μ L of 2-mercaptoethanol to a final concentration of 40 mM to quench the cross-linking reaction.
4. Spin down beads at 3000 g for 6 min and resuspend in 800 μ L of PBS1. Repeat this step two more times.
5. Dissolve 1 mg of protein G in 200 μ L of PBS1. Mix with the 800 μ L bead slurry by inverting tubes and continue tumbling on a nutator at room temperature for 2 h.
6. Spin down beads at 3000 g for 6 min and resuspend in 1 ml of antibody crosslinking buffer. Repeat this step two more times.

¹⁵Account for the amount of NaCl brought by the storage buffer of NS3 (NS3 is stored in a buffer with 0.5 M NaCl) to obtain a final NaCl concentration of 30 mM.

¹⁶Cy3 fluorescence is monitored with an excitation wavelength of 515 nm and an emission filter at 570 nm.

7. To prepare beads for crosslinking with antibody, sonicate the beads in a cup horn (5 min at a power setting of 1 with the Ultrasonic processor S-4000).
8. Dissolve 200 µg of anti-Digoxigenin antibody in 200 µl of PBS1. Mix 60 µl of dissolved antibody with the 1 ml bead slurry in crosslinking buffer and tumble on a nutator for 15 min at room temperature. The remaining antibody solution may be split in aliquots and flash frozen for future use.
9. Dissolve 50 mg of DMP in 1 ml of antibody crosslinking buffer (See Note 17). Add 30 µl of this DMP solution to the beads-antibody mix and tumble on a nutator for 1 h at room temperature.
10. Stop the crosslinking reaction by adding 60 µl of 1 M Tris pH 6.8. Continue tumbling for 20 min at room temperature.
11. Exchange beads into storage buffer PBS2 by centrifugation and resuspension, as described in Step 6. Add concentrated sodium azide solution directly to the bead slurry to reach a final concentration of 0.02 % azide. Mix well and store at 4°C.

3.5 Cleaning and Modification of Coverslips for Making Microfluidic Chambers

NS3 helicase is known to undergo self-assembly in solution. To focus our study on the monomeric form of NS3, it is necessary to control the concentration of this protein inside the microfluidic chamber. However, at low NS3 concentrations, the protein is lost quickly through nonspecific sticking to the coverslip surface (17). Coating of the glass coverslip surface with polyethylene glycol (PEG) prevents nonspecific adsorption of NS3 and has been crucial in experiments with very low concentrations of NS3. All the steps should be carried out in a fume hood unless otherwise noted. All the staining dishes should be well cleaned, rinsed with ddH₂O and dried before use.

1. Use the laser engraver to drill 6 holes in three coverslips. The location of the 6 holes should match with the drawings in Fig. 2.
2. Place 6 coverslips in a 100-ml vertical staining dish that contains 8 slots, submerge the coverslips in acetone and sonicate for 30 min in the tabletop sonic bath cleaner (see Note 18).
3. While acetone cleaning is on, take a 500-ml beaker, fill with singly-distilled H₂O to 210 ml, and place a precleaned staining dish into the beaker so that the dish stands half-way into the water. Place the beaker together with the dish into a microwave, heat for 2 min or until the water inside the beaker reaches ~80 °C. Now place the beaker together with the dish on a hot plate to maintain a temperature of 70–80°C.

¹⁷Prepare a fresh DMP solution immediately before use. A 10–30 fold molar excess of DMP crosslinker over primary amine-containing molecules is recommended. DMP powder should be stored desiccated at 4°C. The storage bottle containing DMP should be warmed up in the dark before opening the bottle. If properly stored, the DMP stock should be good for at least one year.

¹⁸This step is for removal of dirt and grease from coverslips. We typically place in the staining dish 3 coverslips without holes and 3 coverslips with holes so that we can make a total of 3 chambers out of these coverslips. When handling these coverslips, use Teflon-coated forceps, which are chemically inert in the various treatment solutions. Do not place 8 coverslips in the dish because coverslips will stick tightly to the wall if placed in the two slots on the sides.

4. At the end of acetone cleaning, prepare a 3rd staining dish, fill it with ddH₂O, then use Teflon-coated forceps to take the coverslips out of the 1st acetone dish and place in the 3rd dish filled with ddH₂O. Now go to a sink with a ddH₂O source nearby, pour off water of the dish. Repeat this four more times by filling the dish with ddH₂O and then pouring off. Next, go back to the hood, leave the coverslips in the dish, and let them drain to almost dry.
5. While coverslips are drained, prepare 'piranha' bath. Use a glass pipette or glass graduated cylinder to transfer 60 ml of 98% sulfuric acid into a clean 250-ml beaker. Then, transfer 20 ml of hydrogen peroxide solution into the beaker drop by drop. Swirl the beaker GENTLY to mix.
6. Use the Teflon-coated forceps to place the dry coverslips into the empty staining dish in the water bath that has been set at 70–80 °C (from Step 3), and then pour in the freshly prepared piranha solution (see Note 19).
7. Leave the dish open inside the hood, maintaining a temperature around 70–80°C for 2 h. *Label the beaker properly so that people are aware of this solution and ongoing treatment.*
8. Take the dish out of the water bath, transfer the piranha solution into a designated waste bottle, and then immediately refill the dish with ddH₂O and pour it off. Repeat this ddH₂O rinse four more times and, then, fill the dish with 100% methanol. The coverslips are ready for the silane coating step.
9. Rinse a 250-ml triangle bottle with methanol, fill it with 100 ml methanol, add 300 µl of glacial acetic acid, and mix by swirling. Then, fill a clean staining dish with methanol. Transfer the coverslips into this clean dish using forceps, and then add and pour off methanol to completely get rid of residual water on coverslips.
10. Immediately add 1 ml of silane to the triangle bottle and swirl for 8 sec. Add 5 ml ddH₂O using a transfer pipette, swirl for 8 sec to mix, and immediately pour the silane solution into the dish with coverslips, coating for 20 min at room temperature. At 10 min, sonicate for 1 min in the tabletop sonic bath cleaner, then take the dish out and continue for the rest of the 10 min coating procedure.
11. Pour off the silane solution, fill the staining dish again with methanol and rinse with methanol for two more time. Leave coverslips in the dish to air dry in the hood (this only takes several minutes). Now ready for curing of silane, i.e., a covalent linkage to be formed between the silane and the coverslip substrate with concomitant loss of water.
12. Place a clean piece of foil on a hot plate, then place the dry coverslips on the foil, cover the foil and coverslips with a Pyrex tray, and bake the coverslips on the hot plate at 120°C for 20 min. Then, let the coverslips cool down to room temperature.

¹⁹Make sure all the coverslips are completely dry and free of any tiny water spots before placing them into the piranha bath. Use Kimwipe to remove excess water on the coverslip by spotting but not wiping.

13. Dissolve 0.1 g of mPEG-SVA-5000 in 500 μ l of 0.1 M NaHCO₃ solution (see Note 20). Place the cooled coverslips without holes in a humidity box (see Note 21). Spot ~160 μ l of the mPEG solution on each coverslip, and then place coverslips with holes right on top so that the mPEG solution is sandwiched between the two coverslips (see Note 22). The humidity box should be level so that PEG solution will not leak out with time. Close the humidity box, cover with foil to protect the content from light, and incubate for 4 h at room temperature.
14. Rinse the coverslips with copious amounts of ddH₂O and then place coverslips with the coated surface facing up on a piece of foil inside a petri dish (see Note 23). Place the dish in a desiccator, withdraw vacuum to dryness and store the coverslips in this desiccator at room temperature until use (see Note 24).

3.6 Microfluidic Chamber Assemblies

1. Use 100% ethanol to clean up a designated bench surface for making chambers (See Note 25).
2. Using a clean razor blade to cut out a piece of Ablefilm (see Note 26), following the pattern shown on Fig. 2C. Also use the razor blade to carefully cut the glass tubing to 7-mm long pieces (See Note 27).
3. Place a piece of PEG-coated coverslip without holes on the working surface, with the coated surface facing up, place the cut Ablefilm on top of the coverslip, and then use clean forceps to place two pieces of glass tubing on the Ablefilm at the locations shown in Fig. 2C.
4. Place a piece of PEG-coated coverslip with holes on top of the Ablefilm, and make sure that the coated surface is facing down, and holes are aligned with holes on the Ablefilm. Place a clean microscope slide on top of the coverslip sandwich. Use two thumbs to apply pressure on the cover slide, from low to high pressure, which serves to temporarily fix the chamber assembly, and ensure the two glass tubes are in place inside the chamber without movement.
5. Place a second clean microscope slide on the flat surface of a hot plate that is set at 120°C (see Note 28). Transfer the coverslip sandwich to the top of the second slide,

²⁰Precise adjustment of the pH for NaHCO₃ solution is not necessary, but it is best to make the solution fresh because the pH of the solution will slowly increase with time. We typically store the solution at 4°C for at most one month, and then discard it thereafter. A pH between 8 and 9 is very important for the silane crosslinking reaction.

²¹A regular pipette tip box can be used as a humidity box. Fill the inside of the tip box with ddH₂O, and place coverslips on top of the inner plastic support.

²²Make sure there are no bubbles when placing the coverslips with holes on top of the coverslip with holes. This is best achieved by laying the coverslip on its shorter edge, and slowly lowering it down.

²³Do not place coverslips directly inside a petri dish, it will get stuck! A piece of foil is necessary.

²⁴Because PEG chain will slowly react with oxygen in the air, leading to chain break, it is important to store the coated coverslips under vacuum.

²⁵We typically use the surface of a high density polyethylene (7" by 12" in size) for making chambers, which is easy to clean and convenient to work on.

²⁶After surface coating by PEG, the coverslip is resistant to sealing by melted parafilm without leaking. In contrast, this special brand of Ablefilm works very well in making a microfluidic chamber without leaking.

²⁷Because the glass tubing is very thin, cut the tubing by poking the end to the sticky surface of a Scotch tape. After cutting, the 7-mm piece naturally gets stuck on the Scotch tape, which can be picked up later using forceps.

²⁸For both coverslips, their surface should be carefully cleaned with ethanol and examined to make sure there are no dust particles. The presence of any dust particle may crack the coverslips when even gentle pressure is applied.

and replace the first slide on top of the coverslip sandwich. Use two stainless posts, and apply pressure evenly across the entire surface of the slide for ~ 1 min. Remove the top slide, adding 10 μ l ddH₂O into each of the three channels in the microfluidic chamber.

6. After water has completely vaporized, replace the top slide on the chamber, and now apply a lead brick on top of the slide-chamber assembly for 30 min (see Note 29). The chamber is now ready for use.
7. Before tweezers experiments, the chamber needs to be installed on a chamber frame, with tubes connected for injection of beads and reagents. Place the assembled chamber on a chamber frame (see Fig. 2B), with holes on the chamber aligned with holes on the chamber frame. Use two Plexiglas bars and 4–40 screws to secure the chamber on the chamber frame. The tubes are then connected to the chamber inlets and outlets through hollow 8–32 set screws.

3.7 Single-Molecule Optical Tweezers Assay

A photo of the home-built high-resolution optical tweezers (20) is shown in Fig. 3. The instrument should be housed in a room with its own temperature control.

1. Before mounting of the microfluidic chamber, make sure all the inlets and outlets for fluid have been tested to function properly, no leakage should occur. All the channels should be filled with ddH₂O and no bubbles should be present throughout all the channels.
2. Mount the microfluidic chamber onto the chamber stage (See Note 30). Clamp down the chamber post and ensure the chamber is sitting vertically in between the two objectives as judged by the eye (see Note 31).
3. Using the LabView user interface, move the chamber stage to engage the chamber with the back objective. When the distance between the front nose of the back objective and the left surface of the chamber is around 50 μ m, stop the movement. Now engage the front objective with the chamber manually by turning the objective mount micrometer, until the distance between the front nose of the front objective and the right surface of the chamber is around 50 μ m, stop the movement.
4. Using a PE50 tubing connected to a 10-ml syringe through a 23G needle, deliver singly distilled water in between the chamber and objectives to make water immersions (see Note 32).

²⁹The lead brick is necessary to apply sufficient pressure so that the chamber will seal without leakage. The lead brick is very heavy. Carefully apply a slow but steady motion when placing the brick on the chamber assembly.

³⁰The mounting of the microfluidic chamber is highly critical to the success of the high-resolution optical tweezers experiments. For a well-aligned optical tweezers (20), the back objective will never be moved, which is to ensure the precision of the back-focal-plane (BFP) interferometry measurement. The position of the front objective is also uniquely defined given a well-aligned system, which ensures the delivery of a collimated beam out of the back objective in the absence of any trapping, i.e., the distance between the front noses of the two objectives is the sum of their working distances. The microfluidic chamber sits roughly at the midpoint between the two objectives, whose location is also well defined because the chamber should align with the front focal plane of the back objective so that a clear focused image of the chamber can be formed by the back objective. One should always keep these constraints in mind during the alignment of the microfluidic chamber.

³¹Judgment by the eye is sufficient for this step, because the chamber tilt will be precisely aligned in Step 7.

5. Switch on the LED for bright field illumination, go to the computer, and start to focus the chamber by moving the motion stage along the axial direction so that a clear image of the chamber can be formed on the TV monitor. This procedure is best done by first focusing on an interfacial region between the water inside the middle channel and the surrounding Ablefilm from either the top or the bottom channel. A clear image of the Ablefilm indicates a chamber that is close to focus.
6. Move the chamber along x- and y-axis so that one of the glass tubes inside the chamber is now in the field of view (FOV). Focus the chamber to get a clear image of the tube. Now the chamber is in focus.
7. Save the z-location of the chamber. Move the chamber along x- and y-axis so that the very center of the middle channel is now in the FOV. Go inside the tweezers room; align the front objective by moving the objective stage micrometer so that the laser exiting the back objective is collimated. This is best done by installing a reflecting mirror at 45 degree angle to intercept the laser exiting the back objective. This allows the projection of the laser over a long distance and, based on the spot size of the laser on the IR detector, to determine if the laser has reached collimation. Remove the reflecting mirror.
8. The tilt of the chamber is also very important, and needs to be aligned at this stage. This is done by projecting the laser spot onto a regular CCD camera after it exits the back objective. The presence of any asymmetry in the laser spot image indicates a tilt of the chamber. Rotate the chamber mounting screw around the axis of the chamber mounting post to correct for this tilt, until a perfect round-shape image of the laser is obtained. Note that after adjustment of the chamber tilt, the chamber location will now deviate slightly from being focused by the back objective, and thus needs to be refocused. Repeat steps 5 and 6 to refocus the chamber. In LabView, record the locations for both glass tubes (dispensers) together with an experimental region that is upstream of both dispenser exits by 1.5 mm. The chamber is now aligned for single-molecule experiments.
9. Prepare beads suspension. For both streptavidin coated polystyrene particles and home-made anti-digoxigenin coated polystyrene particles (see **section 3.4**, step 11), make 1:20 dilution in standard buffer for each beads. Then, respecting the order of addition, mix 9 μ l of standard buffer (SB), 1 μ l of 20 mg/ml BSA, and 1 μ l of bead dilution in a 1.7 ml microtube. Stir the solution using pipette tip while adding the reagents. Cap the tube and sonicate the beads slurry in a cup horn (for 5 min at a power setting of 1 with the Ultrasonic processor S-4000).
10. Prepare a 1:10 dilution of the RNA/DNA hybrid substrate (see **section 3.2**, step 25) with SB. Add 1 μ l of the diluted substrate to anti-digoxigenin coated beads. Stir the pipette tip while injecting the substrates (see Note 33). Incubate the mixture on ice

³²We use singly distilled water for making water immersion objectives as we found ddH₂O leads to discoloration and corrosion of the objective front nose.

³³The exact dilution of the RNA/DNA hybrid substrates needs to be determined experimentally. The optimal dilution of the substrates should yield less than one tether per bead on average. Dilute the substrate further if you can get molecules for each pair of beads trapped.

for 20 min, then dilute with 1 ml of helicase buffer (HB), pipetting up and down while adding the buffer. For the streptavidin coated beads, also dilute with 1 ml HB, pipette up and down while injecting the buffer to mix. Both beads are ready to use.

11. Respecting the order of addition, mix 1 ml of HB, 2 μ l of 1 M DTT, ATP (to a final concentration of 1 mM or a desired concentration), NS3 helicase (to a final concentration of 0.2 nM) in a 1.7 mL microtube (see Note 34). For helicase, make sure to pipette at least 1 μ l for volume accuracy; if the helicase stock is too concentrated, dilute the protein by 10 fold using HB and then take the volume needed. Cap the tube, mix by gently inverting the tube for 5 to 6 times. The NS3 helicase solution is ready for injection into the microfluidic chamber.
12. Use a 1 ml BD syringe, 30G needle, and PE10 tubing to deliver the NS3 helicase solution into the middle channel of the microfluidic chamber. Use 1 ml BD syringes, 23G needles, and PE50 tubing to deliver both polystyrene beads (see Step 10), with streptavidin beads into the bottom channel and anti-digoxigenin coated beads into the top channel. Make sure no bubbles are introduced to any of the channels (see Note 35).
13. Leave the instrument room and close the door to the instrument room. All subsequent steps are controlled from the desktop computer outside of the instrument room (see Note 36).
14. With the steerable trap on (trap A) but the static trap off (trap B), command the chamber stage motion controller so that the opening of the bottom dispenser is in the FOV, and capture a single streptavidin coated bead. Ensure that trap A and B are separated by their maximum vertical distance ($\sim 8 \mu$ m).
15. Turn on trap B, and command the chamber stage motion controller so that the opening of the top dispenser is in the FOV, and capture a single anti-digoxigenin coated bead. Then, command the chamber stage motion controller to drive both beads to the experimental region, which is at least 1.5 mm upstream from both dispenser openings.
16. After the chamber movement is settled for 5 s, with both beads at their maximum distance, record laser deflection data from both traps for 10 s, which will be used for construction of power spectrum and measurement of trap stiffness (see Note 37).

³⁴For optical tweezers using 1064 nm laser, it is essential to include oxygen scavenger system in the NS3 solution to reduce the oxidative damage of RNA mediated by reactive oxygen species generated as a result of 1064 nm laser excitation. The use of 830 nm laser for optical trapping eliminates the production of reactive oxygen species and the associated photo damage (20). As a matter of fact, we never use oxygen scavenger system in our experiments.

³⁵Deliver reagents either manually or through a syringe pump. Syringe pump has the advantage to offer remote control of sample injection, which is desired for high-resolution experiments, limiting human perturbations of the instrument room.

³⁶For high-resolution experiments, it is essential to minimize perturbation to the instrument during the data collection. To this end, the investigator should sit in front the computer outside of the instrument room. The temperature inside the instrument room should be kept constant with a standard deviation of 0.2°C or less.

³⁷To measure traps stiffness based on the calibration of the power spectrum, the diameter of the beads needs to be known accurately. We found that the real bead size always deviates from the specifications given by the manufacturer by 10 to 40%, and thus a direct measurement of the bead size using electron microscopy is necessary.

17. Steer trap A to engage contact between the streptavidin coated bead and the anti-digoxigenin coated bead. Upon visual contact of the two beads (as observed on the TV monitor), incubate the two beads together for ~ 10 s, and then move trap A away from trap B while monitoring the laser deflection signals along y-axis for both traps. If a tether is already formed between the two beads, upon separation, the laser deflection from both traps should change in an anti-correlated manner which indicates the development of force on the molecule. Repeat this step until a tether is formed between the two beads.
18. Upon tether formation, perform force-extension measurement by moving the streptavidin coated bead at a speed of 10 nm/s away from the anti-digoxigenin coated bead. Only those single molecules of hairpin that yield force-extension curves quantitatively consistent with expected values (17) are selected for further unwinding experiments with NS3.
19. With a single-molecule substrate in between the two beads, hold the molecule at various forces below the hairpin rupture forces by holding the nanopositioning stage at a constant voltage for trap A. Record laser deflection signals for both traps as a function of time at 2.5 kHz. The activity of NS3 helicase will show up as stepwise decrease in the force on the molecule together with a concomitant stepwise increase in the extension of the substrate (See Note 38).
20. Analyze the data using established formulism and detect the individual steps by using the published Generalized Least Squares analysis method (25) (See Note 39).

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³⁸Control experiments are absolutely necessary. This is done by simply omitting NS3 helicase from solution, which also allows one to get a baseline for the noise of the instrument.

³⁹To calculate the extension of the molecule in between the two beads, the voltage setting of the nanopositioning stage at which the steerable trap A overlaps spatially with the static trap B needs to be measured. This spatial overlap between two traps can be judged by trapping a single bead using two traps that alternate in time. The centroid of the bead image should remain constant while the traps are being switched. To convert the extension change of a molecule in nm to the number of nucleotides released by the helicase, the worm-like-chain parameters for single-stranded RNA are needed. We found that the best way to obtain these parameters is to directly measure these parameters under exactly the same solution conditions as helicase reactions so that parameters are transferrable without major assumptions (17).

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Figure 1.

Experimental design and attachment of the RNA substrate to the dual-trap optical tweezers to monitor HCV NS3 helicase activity at single-molecule level with sub-base pair spatial resolution on a sub-second time scale. The RNA (black) and DNA (orange) hybrid molecule with a single RNA hairpin in the middle was attached to two polystyrene beads through biotin-streptavidin, digoxigenin and anti-digoxigenin interactions. The two beads are held by optical traps. The displacement of the beads as a result of NS3 unwinding reaction is measured to report the number of nucleotides released by NS3. There is a NS3 loading site

3' to the hairpin for the initiation of NS3 helicase reaction. The four subdomains in NS3 are colored in green, yellow, pink, and blue, respectively. Not to scale (Modified from (17)). Reprinted with permission from AAAS).

Figure 2.

Schematic drawings of the microfluidic chamber frame and chamber gasket. Dimensions are in inches. **(A)** The cross-section view of the Plexiglas bar to fix the microfluidic chamber onto the chamber frame. Two bars and four 4–40 screws are needed. **(B)** Chamber frame schematic. The bigger holes are for 8–32 set screws. The set screw was drilled at the center so that rubber tubing can be threaded through for making connections with the inlets and outlets of the microfluidic chamber. The smaller holes are for 4–40 screws to fix the chamber using Plexiglas bars. **(C)** The schematic of the chamber gasket, which is cut from the Ablefilm. The locations of the inlets and outlets match exactly with the locations of the bigger holes in the chamber frame schematic (Panel B).

Figure 3.

A photo of the home-built high-resolution optical tweezers instrument using an 830-nm tapered amplifier diode laser (20). The key components are labeled in the figure. The microfluidic chamber, the front objective, the back objective, the chamber stage, the LED and the TV are as described in **Section 3.7**. The front objective is used to form the dual traps in the microfluidic chamber. The back objective is used to collect laser exiting the optical traps for BFP interferometry measurement and also to image the microfluidic chamber through the LED illumination. The image of the chamber is projected onto the TV monitor.