Probing DNA-DNA Interactions with a Combination of Quadruple-Trap Optical Tweezers and Microfluidics


2.1 Abstract

DNA metabolism and DNA compaction in vivo involve frequent interactions of remote DNA segments, mediated by proteins. In order to gain insight into such interactions, quadruple-trap optical tweezers have been developed. This technique provides an unprecedented degree of control through the ability to independently manipulate two DNA molecules in three dimensions. In this way, discrete regions of different DNA molecules can be brought into contact with one another, with a well-defined spatial configuration. At the same time, the tension and extension of the DNA molecules can be monitored. Furthermore, combining quadruple-trap optical tweezers with microfluidics makes fast buffer exchange possible, which is important for in situ generation of the dual DNA-protein constructs needed for these kinds of experiments. In this way, processes such as protein-mediated inter-DNA bridging can be studied with unprecedented control. This chapter provides a step-by-step description of how to perform a dual DNA manipulation experiment using combined quadruple-trap optical tweezers and microfluidics.

2.2 Keywords

Quadruple-trap optical tweezers, microfluidics, force detection, single molecule, protein-mediated inter-DNA bridging
2.3 Introduction

Over the past two decades, optical tweezers have been used extensively to elucidate the mechanics of individual DNA molecules and their interactions with proteins \[8, 31, 32, 33\]. It has, however, remained a challenge to perform well-defined investigations of biological processes involving multiple DNA domains, such as protein-mediated DNA-DNA bridging. While such processes can, to some extent, be probed on an individual double-stranded DNA (dsDNA) molecule using dual-trap optical tweezers, the exact spatial configuration of the interacting DNA segments cannot be resolved or controlled.

![Figure 2.1: Quadruple-trap optical trapping can be used for dual DNA experiments. (A) Schematic of the setup. A single 1064 nm laser is used for generation of all four traps, and each trap is movable by rotation of a steerable mirror. The flow cell is placed on a movable stage between the objective and the condenser of the microscope. Within this flow cell, four laminar buffer flows are present, allowing fast buffer exchange and easy tethering of DNA-protein complexes to beads. Steps (i)-(iv) illustrate the typical experimental workflow in the flow cell: in the bead-containing channel (i) beads are held in the optical traps and then moved to channel (ii), which contains DNA. Here, individual DNA molecules are tethered between bead pairs. Next, the DNA-bead assemblies are moved to the buffer channel (iii) where mechanical probing ensures that only 1 DNA molecule is tethered between each bead pair and the DNA is brought into the desired configuration (wrapped/crossed). Finally, the DNA-bead assemblies are moved to the protein channel (iv) where the DNA construct is exposed to the protein of interest. Adapted from \[30\]. (B) Image of the fluorescence signal emitted by 2 48.5-kb λ dsDNA molecules in half-wrapped configuration (see text) stained 2-5 nM with Sytox Orange, showing the capability of the instrument to independently manipulate two DNA molecules in three dimensions.

To overcome these limitations, quadruple-trap optical tweezers have been introduced \[34\], which allow independent manipulation of two individual DNA molecules in three dimensions. The optical setup used for these experiments (Figure 2.1(A)) has been described previously \[30, 34, 35\]. Concomitant fluorescence imaging can be used to confirm the presence of two DNA molecules and their configurations (Figure 2.1(B)). In brief, the setup consists of four optical traps that are capable of trapping four micrometer-sized polystyrene beads. The four trapped beads are arranged in two pairs, after which each pair is coupled to the ends of a DNA molecule in a dumbbell configuration, typically using...
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biotin-streptavidin linkages. The other important functionality needed for dual DNA experiments is a laminar flow cell that facilitates fast exchange between multiple buffers solutions, containing beads, DNA, protein and buffer only [33] (Figure 2.2). Fast exchange allows for efficient and effective assembly of two DNA dumbbells and then cycling through the subsequent steps of the biochemical/biophysical process of interest. Typically, we use a microfluidic flow cell with 6 laminar flow channels [34].

Three previous studies [30, 34, 35] have applied this technique to successfully unravel interactions between two DNA molecules. In the first case [34], one DNA molecule was held taut between two trapped beads, while another DNA molecule was wrapped around the first molecule. This second DNA construct was then slid along the first DNA molecule and used as a mechanical probe to detect roadblocks, such as proteins bound to the other DNA molecule. The mechanical force required for removal of these roadblocks could then be measured directly. In the second study [35], bridging between two DNA molecules by the bacterial nucleoid-associated H-NS protein was studied using dual DNA manipulation. Here, quadruple-trap optical tweezers enabled full control over the distinct molecular configurations in which either a shearing force is exerted over multiple H-NS bridges, or an unzipping force is exerted over a single H-NS bridge, by which the nature of DNA bridging by H-NS could be unraveled. In the

Figure 2.2: Schematic representation of the flow system. Beads, DNA, protein and buffer solutions are stored in individual syringes. By using pressurized air, a constant flow can be created through the flow cell, creating multiple laminar flows as shown in Figure 2.1. Valves are used to switch the flow on or off in the individual channels. Adapted from [36].

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third study [30], quadruple-trap optical tweezers were used in a similar way to study the DNA-organizing activity of two Archaeal proteins, Alba1 and Alba2. A combination of dual optical trapping and quadruple optical trapping was used to show that the Alba proteins can switch between DNA stiffening and bridging modes in a concentration-dependent manner. In all three studies, quadruple optical trapping was essential to control and probe the molecular architectures in order to uncover information that is otherwise not directly available. In addition, the integration of microfluidics enabled the fast generation of the protein-DNA complexes of interest. Together, these studies highlight that the combination of quadruple-trap optical tweezers and microfluidics provides a powerful means to study biological processes involving multiple DNA molecules. In addition, it is possible to further improve this methodology by combining quadruple optical trapping with fluorescence microscopy, as previously has been shown for dual optical tweezers [31, 23], in order to concurrently acquire spatially resolved dynamic information on the molecular architecture of the DNA-protein complex (Figure 2.1(B)). This improvement can further enhance the capabilities to unravel protein dynamics on DNA at the single-molecule level.

Figure 2.3: Schematic representation of different configurations for quadruple-trap experiments. (A) Crossed configuration (i) of the DNA molecules as suitable for friction experiments. Possible observations are the build-up of tension if there is sufficient local friction between the molecules (ii) or a lack of tension build-up if the DNA can slide over the other molecule (iii). (B) Half-wrapped configuration (i) suitable for probing protein-mediated inter-DNA bridging. Possible observations are linked DNA molecules linked together by bridges (ii), or, a lack of bridging between the DNA molecules (iii). (C) Fully-wrapped configuration (i) suitable for scanning probe experiments [35]. Possible observations are that the wrap is immobile causing tension build up (ii), or a mobile wrap that lacks tension build up (iii).

In this chapter, we provide a step-by-step guide on how to operate a quadruple-trap optical tweezers system to investigate the (protein-mediated) interactions between two DNA molecules. The optical system is shown schematically in Figure 2.1(A). Although quadruple-trap optical tweezers allow the user to perform experiments at any desired DNA-DNA configuration, here we will focus on three particular configurations (Figure 2.3): crossed, wrapped, and half-wrapped configurations. These three configurations can be used to address a range of biological questions involving multiple DNA molecules or domains. The first DNA-DNA configuration of interest is crossed DNA (Figure 2.3(A), top panel). This configuration is especially suitable for studying the friction be-
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tween two DNA molecules and how this friction is affected by proteins. When the upper DNA molecule (between beads 3 and 4) is lowered to push down on the lower DNA molecule, the force between the two molecules increases. Having established a contact between the two DNA molecules, two beads are then moved simultaneously such that one DNA molecule is slid over the other DNA molecule. If the local interactions are sufficiently strong (due to a bound protein, for instance), the upper DNA can stick to the lower DNA molecule and tension will build up (Figure 2.3(A), bottom left panel). If the molecule can slide without hindrance, no buildup of tension will occur (Figure 2.3(A), bottom right panel). The two molecules are shown in Figure 2.3(A) with a perpendicular orientation, but in principle any desired angle between the DNA molecules can be achieved by moving bead 3 to the left or right.

The second DNA-DNA configuration that can be used is the half-wrapped configuration (Figure 2.3(B), top panel). This is particularly suitable for studying protein-mediated inter-DNA bridging, as it ensures a single point where the two DNA molecules touch and provides a potential bridging site. In a typical experiment, the DNA molecules are brought into a half-wrapped configuration, and then incubated in the protein-containing channel of the flow cell. Once they are brought back to the (protein-free) buffer channel, the DNA molecules are unwrapped. If bridging has occurred successfully, the DNA molecules will be held together by the bridge (Figure 2.3(B), bottom left panel). If no bridge has formed, unwrapping results in two separate DNA molecules (Figure 2.3(B), bottom right panel). When a bridge is detected, the mechanical properties of this bridge can be probed by applying a shearing and the unzipping forces (Figure 2.4). In a shearing geometry (Figure 2.4(A)) a uniform force is applied along all DNA-protein bridges. In a typical experiment, a bead tethered to one DNA molecule is moved parallel to the bridged region, away from the bridge, while a force is measured on the bead that tethers to the other DNA molecule on the opposite side of the bridged region. The results of such an experiment on Alba1-mediated bridges (Figure 2.4(C)) show clear rupture events corresponding to the rupture of protein bridges. The unzipping force, on the other hand, represents the force required to open up or unzip the bridged region from one side (Figure 2.4(B)). This can be measured by moving one of the beads to which one DNA molecule is tethered perpendicular to the bridge, while measuring the force on the bead tethered to the other DNA molecule, but on the same side of the bridged region. As such, the bridged region can be unzipped one protein at a time. An example of the results of such an experiment (Figure 2.4(D)) on Alba1-mediated bridges shows a step-wise release of DNA stretches due to the unzipping of the bridges.

The final DNA configuration of interest is the fully-wrapped configuration (Figure 2.3(C), top panel), which is suitable for scanning-probe experiments [34]. In this case the DNA molecules are fully wrapped around each other and one DNA molecule is used as a scanning probe by sliding along the other DNA molecule. If a roadblock is encountered, tension builds up and the sliding is blocked (Figure 2.3(C), bottom left panel) until the roadblock dissociates from the DNA and the tension is relieved. If no roadblock is encountered, the
Figure 2.4: Mechanical probing of bridges can be performed by applying either a shearing force (A) or an unzipping force (B). (A) To measure the shearing force, bead 2 is moved with constant velocity in the direction of the DNA (indicated with v). This exerts a uniform force along all DNA-protein bridges, which can be measured on bead 1 (indicated with F). (B) The unzipping force is the force required in the direction perpendicular to the DNA to unzip the bridges. In this case, bead 4 is moved with constant velocity (indicated with v) perpendicular to the DNA and the distance L and the force on bead 1 (indicated with F) are monitored. (C) Measurement of shearing force generated uniformly over Alba1 bridges. The force is measured on bead 1 and rises and relaxes as protein-mediated bridges ruptured. (D) Unzipping experiment where a force up to 50 pN is built up over the first bridge. As bead 4 is moved, the length L increases in discrete steps. Adapted from (6).

2.4 Materials

2.4.1 Experimental setup

1. The quadruple-trap optical tweezers setup (Figure 2.1(A)) is based on the following design. A trapping laser (a YLR-20-LP laser with a power of 20 W and a wavelength of 1070 nm, available from IPG laser GmbH) is used to generate four continuous optical traps, while the x- and y-positions of each trap within the focal plane are controlled using steerable mirrors (1 piezo mirror available from MadCityLabs (MCL) and 3 stepper-motor mirrors from the Agilis series available from Newport). Force can be detected in two of the four traps: back-focal-plane interferometry of the trapping laser is used to detect the force of one trap that has a polarization orthog-
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onal to the other three traps (trap 1), while interferometry of a spectrally distinct detection laser (with a power of 140 mW and a wavelength of 980 nm, available from Power Technology Inc.), co-aligned with one of the other traps, is used to detect the force on a second trap (trap 4). Two of the traps (traps 2 and 3) can also be moved in the z-direction, perpendicular to the focal plane, by misaligning a telescope along its optical axis (see Note 1). Each of the four traps can be switched on and off by independent shutters.

2. Microfluidic flow cell containing up to 6 inlet channels (available from LU-MICKS B.V.) (see Note 2). The flow cell is cleaned with bleach before use (see Section 2.5.1) and, depending on the proteins used for the experiments, is passivated (see Sections 2.5.2 to 2.5.4) to minimize interaction of the proteins interacting with the surfaces of the microfluidic system (see Note 3).

3. A flow system (schematically shown in Figure 2.2, available from LU-MICKS B.V.) based on pressurized air is used to regulate the buffer flow from individual syringes containing buffer solutions. Valves are used to switch the individual flows on and off. Tubing connecting the different components is made of Teflon or silica. The flow cell is mounted on a movable stage: this enables rapid (1-10 s) buffer exchange by moving the optical traps from one buffer flow to the other (Figure 2.1).

4. Custom-written LabVIEW software is used to:
   - create a template of an imaged bead (imaged via an LED with a wide-field camera) in order determine the bead positions and distances through a template-matching algorithm.
   - perform force calibration using the power spectrum of the measured bead displacements (in our case corresponding to the beads in trap 1 and 4).
   - perform real-time data acquisition of the bead displacements in the optical traps at a frequency of 50 kHz (force data) and 150 Hz (distance data).
   - control the x, y, and z-positions of the traps interfacing with a joystick.

2.4.2 Buffer solutions

2.4.2.1 Buffer solutions for bleach cleaning protocol (see Section 2.5.1)
   - MilliQ water
   - Bleach solution: 0.7 M NaClO (see Note 4)
   - Sodium thiosulfate solution: 10 mM Na₂S₂O₃ (see Note 5)
2.4.2.2 Buffer solutions for passivation of the flow cell using casein (see Section 2.5.2)

- Blocking Reagent (available from Roche)
- Phosphate buffered Saline (PBS): 10 mM phosphate, 150 mM sodium chloride, pH 7.3-7.5 (available in tablet form from Invitrogen) (see Note 6).
- Casein storage buffer: PBS buffer with 10 mM Na\textsubscript{3} and 1 mM EDTA

2.4.2.3 Buffer solutions for passivation of the flow cell using a lipid bilayer (see Section 2.5.3)

- POPC stock solution: 2.5% solution of 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC, available from Avanti Polar Lipids) in methanol
- POPC buffer: 20 mM Tris (Tris(hydroxymethyl)aminomethane) pH 7.5 and 100 mM NaCl
- PBS

2.4.2.4 Buffer solutions for passivation of the flow cell using BSA with Pluronic\textsuperscript{®} F127 (BASF) (see Section 2.5.4)

- PBS
- BSA stock solution: 0.1% w/v BSA (available from Sigma-Aldrich) in PBS
- Pluronic stock solution: 0.5% w/v Pluronic\textsuperscript{®} F127 (available from Sigma-Aldrich) in PBS
- milliQ water

2.4.2.5 Buffer solutions for trapping experiments (see Sections 2.5.5 to 2.5.8)

- PBS
- Solution containing beads: Typically, streptavidin-coated (see Note 7) polystyrene beads with a diameter in the range of 1 to 5 µm (available from Spherotech) (see Note 8) are diluted in PBS solution (see Notes 9 and 10).
- DNA solution: A DNA construct is prepared with multiple biotinylated nucleotides on each end of the molecule and diluted to the tens of pico-molar range in PBS (see Note 9). Different designs of DNA constructs are possible, depending on the particular experiment (see Notes 11 to 14) [37].
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- Protein solution: The purified proteins of interest are diluted to the desired concentration in an appropriate buffer (see Notes 15 and 16).

2.5 Methods

2.5.1 Bleach cleaning protocol

1. Rinse all syringes with milliQ water: fill the syringes with milliQ and subsequently remove the milliQ to leave only 1 mL in each syringe. With the residual water, flush at least 0.5 mL per channel.

2. Remove any residual water and add 0.7 mL of bleach solution to each syringe. Flush this through all channels slowly for 30-60 minutes until the syringes are nearly empty.

3. Remove any residual solution from the channels and rinse all syringes with milliQ water: fill the syringes with milliQ and subsequently remove the milliQ to leave only 1 mL in each syringe. With the residual water, flush at least 0.5 mL per channel.

4. Remove any residual solution from the syringes and add 1.5 mL of sodium thiosulfate solution. and flush through all channels over a period of 5-10 minutes.

5. Remove any residual solution from the channels and rinse all syringes with milliQ water: fill the syringes with milliQ and subsequently remove the milliQ to leave only 1 mL in each syringe. With the residual water, flush at least 0.5 mL per channel.

2.5.2 Passivation of the flow cell using casein

1. Prepare a casein solution by dissolving the Blocking Reagent at 1% w/v in casein storage buffer. To sufficiently dissolve the casein, initially mix gently and then sonicate for 30-60 minutes (see Note 17) followed by 5 minutes of centrifugation at 16 000 x g (see Note 18). After centrifugation, keep only the supernatant and discard the precipitate in the pellet. Store the casein solution at room temperature (see Note 19).

2. Dilute the casein solution 5 x in PBS.

3. Flush through all channels of the flow cell that are to be passivated (see Note 20), switch off the flow (by closing the valve) and leave to stand for at least 30 minutes.

4. Flush all channels of the flow cell with 0.5 mL of the buffer to be used for the experiments.
2.5.3 Passivation of the flow cell using a lipid bilayer

1. Take 40 \( \mu \text{L} \) of POPC stock solution, put it in a 1.5 mL Eppendorf tube and evaporate the methanol in a strong flow of pressurized air (see Note 21).

2. Add 250 \( \mu \text{L} \) of POPC buffer to the dried POPC and flick strongly in order to re-suspend the lipids (see Note 22).

3. Sonicate the tube for at least 30 minutes until the solution is clear again (see Note 23).

4. Flush the flow cell with PBS buffer.

5. Make sure there are no bubbles in the flow cell.

6. Introduce 80 \( \mu \text{L} \) of the POPC solution into the flow cell through one of the channels, and incubate for 10 minutes.

7. Repeat step 6 two times.

8. Flush all channels of the flow cell with 0.5 mL of the buffer to be used for the experiments.

2.5.4 Passivation of the flow cell using BSA with Pluronic®

1. Flush all channels of the flow cell with 0.5 mL PBS.

2. Remove any residual solution from all syringes.

3. Flush 0.5 mL of the BSA solution through each channel of the flow cell. Then apply a gentle flow for 30 minutes (see Note 24).

4. Remove any residual solution from all syringes.

5. Flush 0.5 mL of the Pluronic solution through each channel of the flow cell. Then, apply a very slow flow for 30 minutes.

6. Remove residual solution from the syringes.

7. Fill all syringes with milliQ water. Remove any residual solution, leaving only 0.5 mL in each syringe. Flush this through the flow cell.

8. Flush all channels of the flow cell with 0.5 mL of the buffer to be used for the experiments.
2.5.5 Starting up experiments

1. Make sure all shutters (for the four traps and the detection laser) are blocking the laser beams.

2. Flush 0.5 mL buffer through each channel of the flow cell (see Note 25).

3. Place immersion water / oil on the objective and condenser of the microscope as required (see Note 26). Using the back-focal plane camera, ensure that there are no air bubbles in the oil / water.

4. Adjust the objective height in order to focus in the middle of the flow cell (see Note 27).

5. Open the shutters of all four traps and the detection laser.

6. Insert beads solution into channel (i), DNA solution into channel (ii) and protein solution into channel (iv) (and if required, also in channels (v) and (vi) if using a 6-channel flow cell) (Figure 2.1(A)). The remaining channels (usually channel (iii)) are filled with a buffer solution in which the experiment is to be performed (see Notes 4 and 14).

7. Open the valves (Figure 2.2) and apply pressure to generate fluid flows in the channels with the beads, proteins and buffer.

8. Move the traps to the channel with the beads, catch four beads (See Note 28) and then move to the buffer channel.

9. Switch off all flow and calibrate the traps by fitting the power spectrum of the Brownian motion of the beads in trap 1 and 4 (see Notes 29 and 30).

10. Set the horizontal distance between bead pairs to at least 20 µm (see Note 31).

11. Reset the forces in both traps to 0 pN.

12. Check that the flow that can be generated in each channel is equal (see Note 32).

13. Move the traps to the channel with the DNA, open the flow and catch two DNA molecules. To catch the first DNA molecule, slowly approach bead 2 to a bead-to-bead distance of approximately 5 µm and then increase the bead-to-bead distance again to the initial position. As soon as a force is detected on the corresponding bead (bead 1 or 4) upon bead separation (see Note 33), set the distance to 16 µm and repeat this procedure for bead 3. Now, DNA has been tethered between both bead pairs.

14. Move the DNA-bead assemblies to the buffer channel and switch off all flows.
15. Test whether there is a single DNA molecule tethered between each bead-pair. To do so, record for each bead pair the force-extension curves of the DNA molecule by setting the bead-to-bead distance 5 µm and then slowly extending the DNA molecule while recording the force. The force-extension curve should look similar to force-extension curves previously described [8], with a steep force increase at the DNA contour length (16 µm for λ DNA) and an overstretching plateau at 65 pN (see Note 34).

![Diagram of bead positions](image)

**Figure 2.5**: Schematic (to scale) showing optimal bead positions during DNA catching for experiments in (A) crossed and (B) wrapped configuration. The force-detection beads (bead 1 and bead 4) should ideally not be moved after force calibration is done because the force calibration and force offsets may vary when these are moved. Therefore, these beads are directly positioned in their final locations. Furthermore, the bead-to-bead distance initially should be large enough to prevent unwanted tethering of DNA to the beads. Therefore, a distance above the contour length of the DNA molecule is advised. For λ DNA, for instance, a bead-to-bead distance around 20 µm is typically used. For half-wrapped and fully-wrapped DNA configurations the initial bead positions are identical. Indicated distances are measured from the bead edge to the other bead edge (so they are independent of bead diameter).

### 2.5.6 Performing a quadruple-trap dual DNA experiment in crossed configuration

1. Catch four beads as explained in Section 2.5.5 steps 5-7.

2. Position the beads at the desired positions in the flow cell as shown in Figure 2.5(A) (see Note 35).

3. Calibrate and reset the forces as described in Section 2.5.5, steps 8 and 10.

4. Switch the flow on and move to the DNA channel.

5. Catch two single DNA molecules as described in Section 2.5.5, steps 12-14.

6. Configure the DNA as shown in Figure 2.6(A) by moving traps 3 and 4 upwards (or downwards) in the z-direction, moving bead 3 to a crossed configuration and lowering beads 3 and 4 back to the original position (see Notes 36 and 37).
7. If a friction study in the presence of protein is to be performed (see Note 38), move the DNA construct to the channel with protein. If desired, the construct can be moved back to the buffer channel after a given incubation time.

8. Slide one DNA molecule along the other in the direction indicated in Figure 2.3(A). Use the force on bead 1 as readout for the friction.

![Figure 2.6: Schematics (to scale) showing how (A) crossed and (B) wrapped DNA configurations are generated. (A) To obtain a crossed DNA configuration with the DNA stretched to its contour length (16 µm for λ DNA) the following steps are taken: (i) Both DNA molecules are stretched to their contour lengths and (ii) the molecule between traps 3 and 4 is moved upwards (in the z-direction) to allow (iii) bead 3 to pass over the other DNA molecule. Finally, (iv) the DNA molecule between traps 3 and 4 is lowered again to its original height. (B) To achieve a half-wrapped DNA configuration with DNA stretched to its contour length (16 µm for λ DNA), take the following steps: (i) Position the beads in a square with 10 µm length of each side. (ii) move beads 3 and 4 upwards (in the z-direction) and (iii) move bead 2 such that it passes over the grey DNA molecule. (iv) Then lower beads 3 and 4 until they are well below the focal plane, and (v) move bead 2 back to its original position. Finally, (vi) move beads 3 and 4 back to their original z-positions. (C) To achieve a fully wrapped DNA configuration, (i) start with the half-wrapped configuration as described in (B). (ii) Move beads 3 and 4 upwards (in the z-direction) to allow (iii) bead 2 to pass below the blue DNA molecule. Then (iv) lower beads 3 and 4 (in the z-direction) until all four beads are in the focal plane. Then move (v) bead 3 and (vi) bead 4 to their final positions.

2.5.7 Performing a quadruple-trap dual DNA experiment in half-wrapped configuration

1. Catch four beads as explained in Section 2.5.5 steps 5-7.

2. Position the beads at the desired positions in the flow cell as shown in Figure 2.5(B) (see Note 35).

3. Calibrate the beads and catch DNA as described in Section 2.5.6, steps 3-5.
4. Configure the DNA as shown in Figure 2.6(B) (see Notes 39 and 40) by moving trap 3 and 4 downwards in the z-direction and moving bead 2 over the other DNA molecule. Next, move beads 3 and 4 upwards in the z-direction until they are well above the other 2 beads. Then move bead 2 back to its original position and consequently move beads 3 and 4 upwards to their original z-position (see Notes 37, 41 and 42).

5. Move the DNA constructs to the protein channel. If desired, the constructs can be moved back to the buffer channel after a given incubation time.

6. Detect possible bridging by moving bead 3 slightly to the right. If a force increase is detected on bead 1 and bead 4, a bridge has been formed. If a force change is only detected on bead 4, no bridge is present.

7. If a bridge has formed, measure the shearing and/or unzipping forces by moving bead 2 and/or bead 4 in the directions indicated in Figure 2.4 (See Notes 43 and 44).

2.5.8 Performing a quadruple-trap dual DNA experiment in fully-wrapped configuration

1. Repeat steps 1-6 as described in Section 2.5.7 to obtain a half-wrapped DNA configuration.

2. Wrap the DNA further to a fully wrapped configuration as shown in Figure 2.6(C) by moving bead 3 and 4 upwards (in the z-direction) and moving bead 2 below the other DNA molecule. Then lower beads 3 and 4 to their original positions (see Note 37) and position beads 3 and 4 to their final positions, 10 µm to the right of beads 1 and 4 as indicated in panel (vii) of Figure 2.6(C) (see Note 45).

3. To perform a scanning-probe experiment, move beads 2 and 4 as indicated in Figure 2.3(C). Use the force as readout; a sudden increase in force shows that a roadblock is encountered (Figure 2.3(C), bottom left panel). If no roadblock is detected, the wrap will slide along the DNA (Figure 2.3(C), bottom right panel).

2.6 Notes

1. In our particular implementation, trap 2 is controlled using a piezo mirror which supports absolute positioning of this trap. Traps 1, 3 and 4 are controlled using stepper-motor mirrors which do not support absolute positioning but have a very large range over which the traps can be moved.

2. Other (non-commercial) options could include a flow cell design consisting of a glass slide, a parafilm layer and a cover slip [34].
3. Many proteins have the tendency to bind to the glass flow cell or the tubing connecting this to the syringes. Equilibration of a protein solution with the microfluidic surfaces can take up to 24 hours [38], which means that protein concentration depletion or leakage of previously used proteins into solution can occur on a timescale greatly exceeding a typical experiment. To reduce this effect, surface passivation can be used. Typical passivation agents include casein, BSA or lipid bilayers (see Sections 2.5.2 to 2.5.4). Typically, trial-and-error is used to determine which surface passivation gives the best results for each protein of interest. Keep in mind that the lipid bilayer only works for glass and silica surfaces. If the tubing used is made of a plastic, this should then be passivated separately by casein or BSA-Pluronic passivation.

4. Bleach will efficiently degrade biological materials with hydrophilic degraded products, facilitating cleaning of the syringes, tubing and flow cell.

5. Sodium thiosulfate effectively destroys any residual bleach.

6. For long-term storage of either PBS solution or protein buffer, it is advised to add 1 mM of EDTA and 10 mM NaN₃ to prevent bacterial growths in the buffer solutions.

7. In principle, other DNA-bead attachment schemes such as Anti-Digoxigenin-coated beads in combination with Digoxigenin-labelled DNA constructs are also possible.

8. When choosing the appropriate bead diameter, one important consideration is that smaller beads afford a higher force resolution after filtering [39]. On the other hand lower laser power is required to trap larger beads [20], reducing possible DNA damage caused by the trapping laser. In addition, larger beads ensure a spatial separation between the (diffraction-limited) focus of the trapping laser beam and the DNA-protein complex of interest.

9. In principle, any buffer with a pH of 7.0-8.0 and a monovalent salt (NaCl or KCl) at a concentration of 25-200 mM is suitable. Typically, a good buffer to use for the DNA and beads dilutions is PBS. At higher salt concentrations, the efficiency of tethering a DNA molecule is higher. The DNA concentration has to be adjusted accordingly.

10. The exact concentration of beads needs to be determined experimentally. As a rough guideline: for particles with a diameter of 2 µm a 1000 x dilution of a stock solution of 1% w/v of polystyrene is used (i.e. a concentration of 0.4 fM) and for particles with a diameter of 4.5 µm a 100 x dilution of a stock solution of 0.5% w/v is used (i.e. a concentration of 2 fM). In the rest of the methods and schematics a bead diameter of 4.5 µm is assumed.

11. The main disadvantage of using biotins on both ends of the DNA molecule to link it to the beads is that the orientation of the tethered DNA molecule
remains unknown. This problem is especially apparent when studying sequence-dependent DNA-protein interactions. Possible ways to overcome this issue would be to use different labels on each end of the DNA molecule: for instance, one end of the DNA could be attached to the bead via biotin-streptavidin linkages while the other end could be linked via Digoxigenin-Anti-Digoxigenin interactions.

12. If the biotin labels on each end of the DNA molecule are on the same strand, the other strand can be melted off in situ using force-induced melting [37], thus generating single-stranded DNA.

13. Most constructs we use are based on the 48.5-kb λ-phage DNA or the 8.5-kb pKYB-I DNA because these are commercially available and are sufficiently long to efficiently catch the molecules by flow stretching. In the rest of the methods and schematics it is assumed that a 48.5 kb λ-DNA construct is used.

14. Besides DNA constructs that are of full-length double- or single-stranded DNA, other constructs are also possible, such as molecules that are partially single- and partially double stranded or torsionally constrained [37].

15. The minimum volume of protein solution needed for a typical experiment is around 500 µL.

16. Often, the buffer in which the experiments are performed will be the same as in which the protein is diluted but in principle, a different buffer can be used.

17. The casein solution will appear opaque because it contains micelles.

18. Centrifugation is necessary to remove larger particles and aggregates. This cannot be done by filtering, because the blocking agent casein will efficiently clog the filter pores.

19. Casein can be stored for months at room temperature if stored in the presence of 1 mM of EDTA and 10 mM NaN₃. The quality is most easily checked by the smell. If it starts to smell like cheese, discard the solution.

20. Because air bubbles tend to stick to the casein on the surface of the flow cell, it is advantageous to passivate only the channels of the flow cell which are exposed to proteins.

21. It should take several minutes to evaporate the ethanol.

22. After resuspension, the mixture should be opaque.

23. Make sure to place the tube in the sonicator in a place where the water level is strongly agitated.

24. Make sure there are no bubbles in the flow cell during the passivation steps.
25. Use the appropriate buffer for each channel. For the beads channel, use the buffer that the beads are diluted in, for the DNA channel, use the buffer that the DNA is diluted in, etc.

26. In our system, we use a water-immersion objective and an oil-immersion condenser, so water is used for the objective-flow cell interface and oil for the condenser-flow cell interface.

27. This is most easily done by focusing first on the edge of the lower fluid-glass interface and then moving the objective up by 50 µm (the height of the fluid layer in our flow cell is 100 µm).

28. The four trapped beads should look very similar. If one or more beads do not appear homogeneous, there is most likely a piece of dirt from one of the buffer solutions stuck on the bead. If one or more look significantly bigger, there might be multiple beads in that particular trap. Also, some of the beads in the bead sample might be of a different size. In all of these cases, discard those beads and catch new beads, until four homogenous beads with similar diameters are in the traps.

29. Here, the power spectrum of the Brownian motion of a trapped bead is used for force calibration. Recently, a method has been developed to calibrate the forces using momentum transfer [40]. In principle, this method could be implemented in the current system. This would be advantageous because it does not require a priori knowledge of the size and shape of the trapped particle. In addition, it would allow for force detection in 3 dimensions while the power spectrum motion method only allows for calibration of the forces in x, and y-directions.

30. Before starting the power spectrum calibration, make sure that both the trapping laser and the detection laser have been switched on sufficiently long to have stable power outputs.

31. This large distance prevents unintentional and uncontrolled tethering of DNA between the bead pairs.

32. If the force increases are not approximately equal for all channels, there is either a blockage in one of the channels or an air bubble in the tubing or the flow cell. By visual inspection of the flow cell, one can determine if or where such bubbles exist. If this is the case, they can be removed either by flushing the buffer solution briefly at a high pressure or by sucking the bubbles out from the exit channel with a syringe while all inlet channels are open. If no bubbles are visible but one of the channels is still not flowing as much as the others, there is most likely a blockage in one of the tubes. Here, it is advisable to disconnect the tubing, trim its end (where blockages are most likely to occur) and reconnect.

33. The rationale behind these approach and retraction cycles is that DNA will spontaneously attach to the beads and, with the buffer flow, extend in
the direction of the flow. By approaching and retracting the second bead, one can fish the other end of the flow-stretched DNA and form a stable tether.

34. If the force rises at a distance below the contour length of the DNA molecule and the overstretching plateau is at forces much higher than 65 pN, there are most likely multiple DNA molecules tethered, in which case the options are either to wait (at a relatively high tension) for the additional molecules to break or to discard the bead pair and start the catching procedure again.

35. These configurations are designed based on the following considerations: (1) only traps 3 and 4 can move in the z-direction; (2) the x, and y-positions of the force detection beads (traps 1 and 4) do not change during wrapping or crossing because that would require a new force calibration.

36. The distances indicated in Figure 2.6 are chosen such, that at the final configuration (Figure 2.6(A)(iv), Figure 2.6(B)(vi) and Figure 2.6(C)(vii)) the DNA molecules are at their contour length. In principle, these configurations can be achieved at other DNA extensions as well. One has to keep in mind that, at end-to-end lengths well below the DNA contour length, the DNA will be quite floppy and thus the exact molecular configuration might be less well defined. At much larger end-to-end lengths, there is the risk that during wrapping or crossing, when beads 3 and 4 are moved in the z-dimension, the absolute force on the DNA (including the z-component of the force) might be so large, that the DNA structure changes due to the overstretching transition. In this case, it can no longer be assumed that an interaction between two purely double-stranded DNA molecules is studied.

37. To determine whether the beads are in the same plane, look closely at their diffraction rings. These should be identical to those of the other traps (traps 1 and 2) when the z-positions are the same. Alternatively, one could use the template matching score provided by the bead tracking algorithm to determine the correct z-position of these beads.

38. In principle, the crossed configuration is also suitable for studying DNA-protein bridging. The reason why the half-wrapped configuration is suggested for bridging experiments, is that in the crossed configuration it is difficult to directly control whether the DNA molecules are in contact in the z-direction, as there is no bead tracking in this dimension. In a wrapped configuration, it is certain that the DNA molecules are in contact at the wrapping point. If the crossed configuration is to be used for a bridging assay, the easiest way to see whether the DNA is in contact is, after crossing, to keep moving bead 3 and 4 down until a force increase is detected on bead 1. Then the DNA molecules are definitely in contact.
39. These steps will generate a right-handed DNA wrap. If a left-handed wrap is desired, reverse the direction of motion of beads 3 and 4 in the z-dimension.

40. A possible improvement to the current system could be to automate the wrapping process. When DNA wraps consisting of multiple turns are desired, this can significantly speed up the experiments.

41. Another possible improvement of the system could be to implement both force detection [40] and bead tracking [41] in the z-direction. Currently, only force detection and bead tracking tracking in x-, and y-directions is possible.

42. After wrapping one DNA around the other, check whether the DNA molecules are successfully wrapped by slightly moving bead 2 to the right. A resulting force change should be detectable in both traps 1 and 4.

43. Keep in mind that moving beads 1 and 4 will change their respective force calibrations or force offsets.

44. In principle, the most accurate way of measuring both the shearing and unzipping forces is by moving bead 2 (because that trap supports absolute positioning) and detecting the force on bead 1 (that trap has a unique polarization for force detection giving the highest force resolution). In that case, the positions of bead 2 and 4 have to be exchanged (with respect to the positions shown in Figure 2.4(B)) after DNA catching.

45. For DNA wraps consisting of n turns, repeat these steps n times.