Journal of Visualized Experiments

Characterizing Single-Molecule Conformational Changes Under Shear Flow with Fluorescence Microscopy

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60784R1
Full Title:	Characterizing Single-Molecule Conformational Changes Under Shear Flow with Fluorescence Microscopy
Section/Category:	JoVE Bioengineering
Keywords:	single-molecule detection; Fluorescence microscopy; biopolymer; DNA; Von Willebrand Factor; Flow; Mechanobiology
Corresponding Author:	X. Frank Zhang Lehigh University Bethlehem, PA UNITED STATES
Corresponding Author's Institution:	Lehigh University
Corresponding Author E-Mail:	xiz310@lehigh.edu
Order of Authors:	X. Frank Zhang
	Avani V. Pisapati
	Yi Wang
	Megan E. Blauch
	Nathan J. Wittenberg
	Xuanhong Cheng
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bethlehem, PA, USA

TITLE:

Characterizing Single-Molecule Conformational Changes Under Shear Flow with Fluorescence Microscopy

AUTHORS AND AFFILIATIONS:

Avani V. Pisapati[1], Yi Wang[2#], Megan E. Blauch[3#], Nathan J. Wittenberg[3], Xuanhong Cheng[1,2], X. Frank Zhang[1,4]

[1]Department of Bioengineering, Lehigh University, Bethlehem, PA, USA

[2]Department of Materials Science and Engineering, Lehigh University, Bethlehem, PA, USA

[3]Department of Chemistry, Lehigh University, Bethlehem, PA, USA

[4]Department of Mechanical Engineering and Mechanics, Lehigh University, Bethlehem, PA, USA

[#]These authors contribute equally to the article.

Corresponding authors:

X. Frank Zhang

xiz310@lehigh.edu

Xuanhong Cheng

xuc207@lehigh.edu

Email addresses of other authors:

Avani Pisapati (avp210@lehigh.edu)

Yi Wang (yiw716@lehigh.edu)

Megan Blauch (meb416@lehigh.edu)

Nathan Wittenberg (njw@lehigh.edu)

KEYWORDS:

von Willebrand factor, lambda DNA, microfluidics, shear flow, TIRF, confocal, fluorescence microscopy

SUMMARY:

We present a protocol for immobilizing single macromolecules in microfluidic devices and quantifying changes in their conformations under shear flow. This protocol is useful for characterizing the biomechanical and functional properties of biomolecules such as proteins and DNA in a flow environment.

ABSTRACT:

Single-molecule behavior under mechanical perturbation has been characterized widely to understand many biological processes. However, methods such as atomic force microscopy have limited temporal resolution, while Förster resonance energy transfer (FRET) only allow conformations to be inferred. Fluorescence microscopy, on the other hand, allows real-time in situ visualization of single molecules in various flow conditions. Our protocol describes the steps to capture conformational changes of single biomolecules under different shear flow environments using fluorescence microscopy. The shear flow is created inside microfluidic channels and controlled by a syringe pump. As demonstrations of the method, von Willebrand factor (VWF) and lambda DNA are labeled with biotin and fluorophore and then immobilized on the channel surface. Their conformations are continuously monitored under variable shear

flow using total internal reflection (TIRF) and confocal fluorescence microscopy. The reversible unraveling dynamics of VWF are useful for understanding how its function is regulated in human blood, while the conformation of lambda DNA offers insights into the biophysics of macromolecules. The protocol can also be widely applied to study the behavior of polymers, especially biopolymers, in varying flow conditions and to investigate the rheology of complex fluids.

INTRODUCTION:

Mechanisms of how biomolecules respond to environmental stimuli have been studied widely. In a flow environment in particular, shear and elongational forces regulate the conformational changes and potentially the function of biomolecules. Typical examples include shear-induced unraveling of lambda DNA and von Willebrand factor (VWF). Lambda DNA has been used as a tool to understand conformational dynamics of individual, flexible polymer chains and the rheology of polymer solutions[1-4]. VWF is a natural flow sensor that aggregates platelets at wound sites of blood vessels with abnormal shear rates and flow patterns. Unraveling of VWF is essential in activating the binding of platelets to the A1 domain and collagen binding to the A3 domain. In addition, high shear-induced A2 domain unfolding allows the cleavage of VWF, which regulates its molecular weight distribution in circulation[5,6]. Thus, direct visualization of how these molecules behave under flow can greatly enhance our fundamental understanding of their biomechanics and function, which in turn can enable novel diagnostic and therapeutic applications.

Typical methodologies to characterize single-molecule conformations include optical/magnetic tweezers, atomic force microscopy (AFM) and single-molecule Förster resonance energy transfer (FRET)[7]. Single-molecule force spectroscopy is a powerful tool to investigate the force and motion associated with the conformational changes of biomolecules. However, it lacks the ability to map overall molecular conformations[8]. AFM is capable of imaging with high spatial resolution but is limited in temporal resolution[9,10]. In addition, contact between the tip and the sample may confound the response induced by flow. Other methods like FRET and nanopore analytics determine single-molecule protein folding and unfolding states based on the detection of intramolecular distance and excluded volumes. However, these methods are still in their infancy and limited in their direct observation of single-molecule conformations[11-14].

On the other hand, directly observing macromolecules with high temporal and spatial resolution under fluorescence microscopy has improved our understanding of single-molecule dynamics in many biological processes[15,16]. For example, Fu et al. recently achieved simultaneous visualization of VWF elongation and platelet receptor binding for the first time. In their work, VWF molecules were immobilized on the surface of a microfluidic channel through biotin-streptavidin interactions and imaged under total internal reflection fluorescence (TIRF) microscopy at varying shear flow environments[17]. Applying a similar method as Fu's, we here demonstrate that conformations of VWF and lambda DNA can be directly observed under both TIRF and confocal fluorescence microscopy. As shown in **Figure 1**, microfluidic devices are used to create and control shear flow, and biomolecules are immobilized on the channel surface. Upon the application of varying shear rates, conformations of the same molecule are recorded to measure the extensional length, also

shown in **Figure 1**. The method could be widely applied to explore other polymer behaviors under complex flow environments for both rheological and biological studies.

PROTOCOL:

1. Preparing VWF

1.1. Reconstitute human plasma VWF to prepare it for the labeling reactions. Add 100 µL of deionized (DI) water to 100 µg of lyophilized VWF to create a 1 mg/mL VWF stock solution.

1.2. Dialyze VWF stock solution in order to remove excess glycine, thereby increasing the biotin and fluorophore labeling efficiency.

1.2.1. Transfer 50 µL of VWF stock solution into a 0.1 mL dialysis unit with a 10,000 molecular weight cut-off and seal with a cap. Store the remaining stock solution at -20 °C. VWF stock will be stable for up to 1 year at -20 °C.

1.2.2. Run dialysis in 500 mL of 1x sterile phosphate-buffered saline (PBS) (0.01 M disodium phosphate, 0.0018 monopotassium phosphate, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4 at 25 °C) for 1 h at 4 °C with slow stirring. Repeat dialysis for an additional hour using 500 mL of fresh PBS.

1.3. Start biotin labeling reaction. Prepare a 2 mM solution of NHS-PEG_4-biotin by dissolving the solid in DI water immediately before the reaction. Allowing NHS-PEG_4-biotin to remain in water for extended time will cause the NHS-ester group to hydrolyze, thereby decreasing labeling efficiency.

1.3.1. Add 2.5 µL of 2 mM NHS-PEG_4-biotin to the dialysis unit containing the VWF stock solution. This will result in a 20-fold molar excess of biotin compared to VWF monomers. Primary amines of VWF will react with the NHS-ester groups, thereby covalently binding to PEG_4-biotin groups via amide linkages.

1.3.2. Place the dialysis unit inside a 1.5 mL microcentrifuge tube. Seal the dialysis unit with its corresponding cap. Secure the tube-dialysis assembly with Parafilm. Keep upright and leave at room temperature for 40 min.

1.4. Start fluorophore labeling reaction. Prepare a 2.8 mM solution of Alexa 488 tetrafluorophenyl-ester (TFP-ester) fluorescent dye ($excitation_{max}$ = 498 nm, $emission_{max}$ = 519 nm) by dissolving the fluorophore solid in DI water. Do this immediately before the reaction to prevent the TFP-ester group from hydrolyzing.

1.4.1. Add 2.9 µL of the 2.8 mM 488 fluorophore to the dialysis unit. This will result in a 34-fold molar excess of fluorophore compared to VWF monomers. Remaining primary amines of VWF will react with the TFP-ester groups, thereby covalently binding to fluorophores via amide linkages.

1.4.2. Add 2.0 µL of 1 M sodium bicarbonate (dissolved in DI water) to dialysis unit.

This adjusts the pH of the reaction closer to 8.0, which increases the efficiency of the TFP-ester and primary amine reaction.

1.4.3. Secure the dialysis unit in a microcentrifuge tube just as in step 1.3.2. Store in the dark to prevent photobleaching and leave at room temperature for 1 h and 30 min.

1.5. Place the dialysis unit in 900 mL of 1x sterile PBS and dialyze overnight at 4 °C. This will yield approximately 70 µL of labeled VWF at a concentration of 0.71 mg/mL or 2.84 µM (monomer concentration).

1.6. Transfer labeled VWF into a microcentrifuge tube. Cover the tube with aluminum foil and protect from light. Store at 4 °C. For long-term storage, add anti-microbial agent sodium azide to a final concentration of 0.02% (w/v).

NOTE: The protocol can be paused here.

2. Preparing Lambda DNA

2.1. Biotinylate linear lambda DNA by filling its cohesive end sites (cos sites) with biotin-14-dCTP nucleotides according to standard protocols, repeated here in step 2.1[18]. Fill in the remainder of the cos sites with dATP, dTTP and dGTP nucleotides.

2.1.1. Prepare 1 mM solutions of dATP, dTTP, dGTP and biotin-14-dCTP in a 10x reaction buffer (500 mM sodium chloride, 100 mM Tris hydrochloride, 100 mM magnesium chloride, 10 mM dithiothreitol, pH 7.9 at 25 °C).

2.1.2. Place 48 µL of 500 ng/µL lambda DNA in a PCR tube and heat for 5 min at 65 °C. The cos sites of circular lambda DNA will separate under heat, linearizing the molecule and making single-stranded overhangs ready for biotinylation. Immediately after, place on ice to prevent cos sites from re-annealing.

2.1.3. Add 5 µL of 1 mM dATP, dTTP and dGTP and 4 µL of 1 mM biotin-14-dCTP to the lambda DNA. Also add 2.5 µL of 5 U/µL Klenow Fragment (3'→5' exo-) to catalyze the DNA synthesis.

2.1.4. Incubate the reaction mixture for 1 h at 37 °C.

2.1.5. Add 1.2 µL of 0.5 M EDTA. Then heat the reaction mixture for 5 min at 70 °C. This will deactivate the Klenow Fragment and biotinylation reaction.

2.2. Remove excess nucleotides from lambda DNA using a spin column that can hold 10-70 µL and has a 6,000 molecular weight cut-off.

2.2.1. Place the column inside a 2 mL microcentrifuge tube. Centrifuge the column and tube at 1000 x *g* for 2 min. Dispose of flow-through that collects in the tube.

2.2.2. Replace the column buffer with a 1x solution of the same reaction buffer from

step 2.1.1. Do this by adding 500 µL of 1x buffer to the column. Centrifuge for 1 min at 1000 x *g*. Dispose of flow-through. Repeat these 2 more times so that a total of 1500 µL has been added to the column.

2.2.3. Place the column in a 1.5 mL microcentrifuge tube. Carefully add the solution from step 2.1.5 to the top layer of the column. Centrifuge for 4 min at 1000 x *g*.

2.2.4. Collect the flow-through (40-70 µL) from the microcentrifuge tube and place in a PCR tube. This contains the purified, biotinylated lambda DNA.

2.3. Label lambda DNA with fluorescent YOYO-1 dye ($excitation_{max}$ = 490 nm, $emission_{max}$ = 509 nm) according to standard protocols, repeated here in step 2.3.1[20].

2.3.1. Prepare a solution of YOYO-1 dye and lambda DNA with a dye to base pair molar ratio of 1:10. Assume no DNA was lost in the purification step to calculate the base pair concentration of lambda DNA. Full-length lambda DNA has 48,502 base pairs.

NOTE: For example, if 50 µL of solution was recovered from step 2.2.4, add 7.4 µL of 500 µM YOYO-1 dye.

2.3.2. Heat the solution for 2 h at 50 °C in the dark to complete the reaction.

2.3.3. Cover the tube with aluminum foil and protect from light. Store at 4 °C. The solution is now ready to be injected into microfluidic devices.

NOTE: The protocol can be paused here.

3. Creating microfluidic channel molds in silicon wafer

3.1. Use photolithography to create microfluidic channels with appropriate dimensions **(Figure 2)** on a master silicon wafer according to standard protocols[19].

4. Preparation of polydimethylsiloxane (PDMS) microfluidic device

4.1. Add 5 parts silicone elastomer base to 1 part curing agent (by mass) in a weigh boat. Stir the contents thoroughly for 1 min to create pre-cured PDMS solution.

4.2. Place the master silicon wafer in a plastic Petri dish. Pour the PDMS solution over the wafer to create a 5 mm layer. Cover the dish and leave in a desiccator under vacuum for 1 h to remove air bubbles.

4.3. Incubate covered Petri dish at 60 °C overnight to cure PDMS into a flexible solid. Curing will result in microfluidic channels molded into the PDMS at the PDMS-wafer interface.

4.4. Cut 20 x 10 mm rectangles into the PDMS, around each microfluidic channel, using a razor. Remove the rectangular PDMS blocks with tweezers.

4.5. Use a 25 G blunt end needle with sharpened edges to punch a hole 0.5 mm in diameter at one end of the channel, making sure the hole goes completely through the PDMS block (**Figure 2**). Use a thin needle to punch out PDMS from hole. Repeat this at the other end of the channel. This will create an inlet and outlet for flow through the channel.

4.6. Clean the surface of the PDMS block with vinyl cleanroom tape. Blow compressed nitrogen gas over a No. 1 ½, 22 x 50 mm coverslip to remove debris.

4.7. Place the PDMS block with the channel side up and the coverslip into the chamber of a plasma bonding machine. Start the treatment.

4.8. When treatment is complete, quickly place the PDMS block on a coverslip so that the channel is in contact with the slip. Apply pressure along the edges of the block. Place the coverslip-PDMS assembly on a hot plate at 115 °C for 15 min to reinforce the permanent bond.

4.9. Insert 10 cm-long, 0.25 mm inner diameter tubing into the outlet hole at the top of the PDMS block. This allows fluid to easily flow out of the channel. The device is now complete.

5. Treating surface of microfluidic device

5.1. Inject <10 µL of 10 µg/mL biotinylated bovine serum albumin (BSA-biotin) dissolved in sterile 1x PBS into the inlet of microfluidic device for VWF experiments. Inject <10 µL of 1 mg/mL BSA-biotin for lambda-DNA experiments. Withhold a few microliters of BSA-biotin in the pipette tip after injection and allow the tip to remain embedded in the inlet.

5.1.1. Always keep a droplet of DI water around the tip. This will prevent air bubbles from entering the channel. Apply this technique every time a new solution is injected into the channel.

5.1.2. Allow BSA-biotin to incubate in the device for 2 h. The BSA will nonspecifically bind to the coverslip surface (**Figure 3A**).

5.2. Remove the pipette tip. Inject <10 µL of casein blocking solution into the channel and allow it to incubate for 30 min. The casein will block any free sites, reducing nonspecific binding of biomolecules to the surface (**Figure 3B**).

5.3. Remove the tip and inject <10 µL of 10 µg/mL streptavidin dissolved in sterile 1x PBS into the channel for VWF experiments. Use 100 µg/mL streptavidin for lambda DNA experiments. Incubate for 10 min. The streptavidin will bind to the biotin groups of the BSA-biotin (**Figure 3C**).

5.4. Remove the tip and inject <10 µL of 1x detergent solution (0.05% Tween 20 in PBS) into the channel to wash away excess streptavidin.

5.5. Remove the tip and inject <10 µL of either 28.4 nM VWF diluted in casein solution or lambda DNA from step 2.3.3. Incubate VWF for 3 min. Incubate lambda DNA for 45 min

(**Figure 3D**).

5.6. Remove the tip and inject <10 µL of 5 mM free biotin diluted in casein solution. Free biotin will block excess streptavidin binding sites on channel surface (**Figure 3E**).

6. Visualizing VWF and Lambda DNA under fluorescence microscopy

6.1. Prepare 1 mL of casein blocking solution with 2.2 mM protocatechuic acid and 37 nM protocatechuate-3,4-dioxygenase (to minimize photobleaching). Load into a syringe and secure in a syringe pump. Take 30 cm long, 0.25 mm inner diameter tubing and attach one end to the syringe needle. Flow in the solution to remove air bubbles. Attach the other end of the tube to the inlet of the microfluidic device.

6.2. Select the highest magnification objective (i.e., 60-100X) of a total internal reflection (TIRF) or confocal fluorescence microscope. Add a drop of immersion oil on its objective if needed. Place the microfluidic device on the microscope stage so that the coverslip is flush with the objective.

6.3. Start brightfield microscopy. Adjust focus so that any features, like debris and bubbles, are visible. Then adjust the stage in the X and Y direction until the edge of the microfluidic channel is visible and bisects the frame.

6.4. Switch to the 488 channel (FITC). Adjust Z-level and TIRF angle as needed until individual green, globular molecules can be distinguished. These are either VWF or lambda DNA molecules.

6.5. Adjust exposure time and laser intensity to visualize fluorescent molecules without photobleaching them too quickly. Adjust contrast to also visualize molecules more clearly.

6.6. Start flow from the syringe pump so that casein blocking solution flows into the channel and out of the outlet. Stop and start flow to observe the changes in the conformation of molecules. When applying flow, use rates between 5,000 and 30,000 µL/h. Repeat this throughout various areas of the microfluidic device. Continue this process to locate molecules that can extend and relax upon multiple cycles of stopping and starting flow.

6.7. Note how long it takes for molecules to reach maximum extension and completely relax into globules. Record videos of the continuous behavior of molecules under shear flow, selecting the best exposure time, exposure frequency and video duration that will capture the full range of extensional behavior and minimize photobleaching.

6.8. Save the videos as .AVI files with a scalebar.

NOTE: The protocol can be paused here.

7. Image analysis of conformational changes

7.1. Calculate the wall shear rate ($\dot{\gamma}$) applied to macromolecules using the flow rate (Q) and

the height (h) and width (w) of the rectangular microfluidic channel. Use the following equation to do so:

$$\dot{\gamma} = \frac{6Q}{wh^2}$$

7.2. Determine the length of any biomolecule under various shear rates using a customized MATLAB code (see **Supplementary Files**). Create a folder titled **videos analysis** which includes the following MATLAB codes: **main.m**, **save_each_frame.m**, **get_length.m** and **get_length.fig**. Create a subfolder within **videos analysis** titled **videos** and add .AVI files to be analyzed into it.

7.3. Open **main.m** using MATLAB 2019a and run the code. Type in the name of the video file to be analyzed in the command window under **Please input the data file to analyze:**.

7.4. In the opened graphical user interface (GUI), set threshold (text in the box on the top right of the window) to **20** and click on the **Set threshold button** to confirm.

7.5. Use data cursor in the top tool bar of the window to choose one pixel anywhere on the scale bar. Click **Start point** in the **Scale bar** section on the right of the window. The (x,y) position of the chosen pixel will appear on the right of the button. Click on the **Pixel size (μm)** button. The pixel size needs to be measured only once in each video.

7.6. Choose any pixel on the molecule of interest. Click on **Start point** in the **VWF** section. After the position of the chosen pixel appears on the text box on the right, click on **Left end**, **Right end** and **String length** to get the molecular length in the image.

NOTE: This step can be used to analyze the conformational changes of any biomolecule, despite the code having a specific section named **VWF**.

7.7. Double-check the left and right end of the molecule. Zoom in and use the data cursor to check the pixel position of interest. Manually choose the pixel as an end and recalculate the length (in pixels) when necessary.

7.8. Record the pixel size in μm and string length in pixel number into an Excel sheet and calculate the string length in μm.

7.9. Repeat the steps above for every image. Use **Last**, **Next** button in the bottom right corner of the GUI to switch among images in the same video file. Click on **Close** to close the GUI window.

REPRESENTATIVE RESULTS:

Observing the dynamic behavior of biomolecules such as VWF and lambda DNA is highly dependent on optimizing their binding to the device surface. Incubating surface treatments for the recommended times in the microfluidic device is crucial to obtaining binding with a few anchorage points, so that molecules can freely extend and relax upon changing flow. If the proteins or DNA are bound too strongly with multiple linkages, they will either extend to

limited lengths or not extend at all. This occurs particularly with VWF when it remains without flow on the device surface for more than 3 min prior to free biotin blocking. The longer VWF remains on the surface stagnant, the more VWF biotin groups bind to the surface streptavidin groups and the less flexibility the molecule has to unravel. If molecules are bound too weakly, on the other hand, they will detach upon flow and disappear from view. This can occur if VWF or lambda DNA is incubated for too short of periods, causing too few biotin-streptavidin interactions to form. Molecules can also break free when extremely high shear rates (>200,000 s^{-1}) are applied, weakening the biotin-streptavidin interactions.

An ideal molecule binds to such an extent that it can unravel and relax upon multiple cycles of stopping and starting flow. The flexibility of a molecule to change conformation like this is often demonstrated by its ability to extend to increasing lengths as higher shear rates are applied within a range of increasing flow. Images of VWF obtained with TIRF microscopy demonstrate this relationship in **Video 1**. The extension versus shear rate curve of this same VWF molecule in **Figure 4** precisely captures the shear-induced behavior of a VWF molecule and is useful for characterizing the biomechanical properties of the protein. Images of lambda DNA obtained with confocal fluorescence microscopy similarly show increased extension upon higher shear rates and gradual relaxation over 2 min, as is captured in **Video 2** and **Video 3**. The recoiling characteristics of lambda DNA after stopped flow is also graphically represented in **Figure 5**.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematics of single-molecule flow experiment in microfluidic channel under fluorescence microscopy. The channel surface is coated with BSA-biotin and blocked with casein. Streptavidin is bonded with biotin on the channel surface and also biotinylated VWF/lambda DNA to immobilize single molecules on the surface. As shear rate increases from **A** to **C**, the molecule is stretched from a folded state to an elongated state along the flow direction from the left to the right.

Figure 2: Microfluidic channel dimensions. The shape and structure of the PDMS microfluidic device are shown together with the channel dimensions. The channel is 50 µm in height and ranges from 0.1 to 1.0 mm in width. The narrowing region in the middle of the channel is 0.7 mm in length. The inlet and the outlet are 0.5144 mm (25 G) in diameter. Flow direction is from the left to right.

Figure 3: Surface treatment steps for single-molecule immobilization. All steps occur at room temperature. (**A**). BSA-biotin is coated on the surface for 2 h. (**B**). Casein is injected into the channel for 30 min to block the surface. (**C**). Streptavidin is incubated in the channel for 10 min to bind with BSA-biotin. (**D**). After washing away excess molecules in the former steps, fluorophore and biotin labeled VWF/lambda DNA is injected into the channel and immobilized through bonding with streptavidin. (**E**). Free biotin is flowed in, blocking extra streptavidin binding sites to minimize its interference with the molecule during conformational changes.

Figure 4: Extensional behavior of VWF under shear flow. The molecule reversibly unravels at 7 different shear rates: 0 s^{-1}, 33,333 s^{-1}, 66,667 s^{-1}, 100,000 s^{-1}, 133,333 s^{-1}, 166,667 s^{-1} and 200,000 s^{-1}. Length of the stretched molecule increases from 0.52 µm at zero shear rate to 3.44 µm at 200,000 s^{-1} shear rate.

Figure 5: Relaxation behavior of lambda DNA after shear flow stops. Flow with 33,000 s^{-1} and 66,667 s^{-1} shear rates are applied from 0 to 30 s to the same molecule. Relaxation is recorded from 30 s to 150 s. At 66,667 s^{-1} shear rate, the DNA molecule elongates to 15.00 µm and relaxes back to 5.83 µm after the flow has been stopped for 2 min. At 33,333 s^{-1} shear rate, the molecule extends only to 8.75 µm and is 3.33 µm in length after 2 min of relaxation.

Video 1: Reversible unraveling of VWF under increasing shear rates using total internal reflection fluorescence (TIRF) microscopy. The molecule in the middle of the view reversibly unravels to different length at shear rates 33,333 s^{-1}, 66,667 s^{-1}, 100,000 s^{-1} and 133,333 s^{-1}. A syringe pump is used to control the flow rate from which shear rates are calculated. Flow direction is from the left to right. Images are taken with 15 s intervals to allow complete relaxation and extension processes.

Video 2: Relaxation of lambda DNA after 33,333 s^{-1} shear rate. Images are taken under confocal fluorescence microscopy. Lambda DNA are stretched under 33,333 s^{-1} shear flow and relaxed back to a folded state after the flow is stopped at 30 s. Duration of the relaxation is 2 min. Flow direction is from the left to right. Images are taken with 30 s intervals in between.

Video 3: Relaxation of lambda DNA after 66,667 s^{-1} shear rate. Settings are identical to the ones in **Video 2** except for the initial shear rate.

Supplementary Files. MATLAB codes.

DISCUSSION:

To obtain high quality data of single-molecule conformational changes using fluorescence microscopy as described in this method, it is critical to incubate the molecule for the appropriate amount of time, minimize its nonspecific interactions with the surface and establish microscope settings that reduce photobleaching. The ability of the molecule to freely change conformation is related to the number of biotin-streptavidin interactions formed between the molecule and the surface. As mentioned previously, this must be controlled by incubating the molecule without flow for the appropriate amount of time. Additionally, protein or DNA may nonspecifically bind to the coverslip if the coverslip is not blocked effectively. Without the recommended blocking solution, molecules can attach to the glass nonspecifically and be unresponsive to any flow rate applied. Applying the casein block during early surface treatment and maintaining its presence during flow is essential for reducing these nonspecific interactions. Finally, capturing the continuous, dynamic behavior of a single molecule requires frequent fluorophore excitement during image capture. This can cause rapid photobleaching if laser intensity, exposure time and exposure frequency are too high. It is therefore necessary to adjust these settings in tandem and strategize how to reduce their values without compromising the time or image resolution of the data.

If extension and relaxation of the molecule are not observed, additional steps should be followed. Incubate the molecule in the device for longer and shorter times than what is advised in the protocol. For each time that is tested, vary BSA-biotin and streptavidin concentrations by factors of 10. These tests may be necessary to optimize the number of biotin-streptavidin anchorage points formed between the molecule and surface. For example,

if the biotin labeling density is very high, due to deviations from the recommended concentrations or reagents in the labeling protocol, shorter molecular incubation time and lower BSA-biotin and streptavidin concentrations may be needed. To further improve the success of the experiment, scan the entire microfluidic device for molecules that reversibly unravel. The surface may not be treated uniformly with streptavidin or casein block, causing molecules in certain areas to have greater unraveling responses than others.

This method is limited by a lack of information about the size and tethering points of the molecule, the difficulty in producing 0 s^{-1} shear rate and the optical resolution of fluorescence microscopes. Previous work has shown a large variation in the unraveling behavior of VWF, potentially explained by the wide distribution in the number and location of biotin-streptavidin tether points and the molecular weight of each VWF molecule[18]. At the moment, the method we present cannot define tether points and molecular size. However, Brownian dynamics simulations of a coarse-grained VWF model published by Wang et al. incorporate these variables and can be run alongside experimental findings to explain such variation[18]. Furthermore, flow does not stop instantaneously when the syringe pump is stopped, confounding the observation of recoiling dynamics. This is due to the deformation and slight dilation of the PDMS channel during the intended flow period. When the pump is stopped, fluid continues to flow until the PDMS is fully relaxed. An improved system should use more rigid PDMS or microchannels fabricated in hard plastic materials, allowing fluid to reach a 0 s^{-1} shear rate more quickly. Finally, one can only resolve molecules whose size is on the same order of magnitude as the optical resolution of the fluorescence microscope, which may be no smaller than a few hundred nanometers. Thus, there is a minimum size requirement for the molecules that can be directly observed with this method.

The current protocol concerns mainly quantification of conformational changes of protein and DNA molecules under physiological flow. However, the method can also be used to visualize real-time interactions between biological molecules and further characterize protein and DNA function. For instance, Fu et al. have shown that tethered VWF can activate under high shear flow and further capture the platelet adhesion molecule GPIb□ under varying flow conditions[17]. This binding event is preserved even when VWF is bound to the surface by biotin-streptavidin linkages, demonstrating the effectiveness of this protocol to study physiologically relevant functions and mechanics[17]. Similar mechanistic insights could be obtained while studying the interactions between unraveled DNA and regulatory proteins in flow environments[21,22]. Additionally, our method pertains mostly to observing conformational changes in macromolecules. Nevertheless, one could adapt it for the purpose of studying smaller molecules that are large enough to be resolved under fluorescence microscopy. For example, by noncovalently or covalently attaching a small molecule to a much larger, immobilized lambda DNA, one could increase the shear-sensitivity of the smaller molecule and more easily observe its behavior. In conclusion, other single-molecule characterization methods, such as AFM or optical tweezers, provide high-resolution data on the structural and functional properties of macromolecules; however, these alternative methods cannot observe the dynamic, conformational changes of proteins and DNA that take place in a physiological flow environment, as is presented in this protocol.

ACKNOWLEDGMENTS:

This work was supported in part by a National Science Foundation grant DMS-1463234, a National Institutes of Health grant HL082808 and Lehigh University internal funding.

DISCLOSURES:

The authors declare no competing interests.

REFERENCES:

1. Shaqfeh, E.S. The dynamics of single-molecule DNA in flow. *Journal of Non-Newtonian Fluid Mechanics*. **130** (1), 1-28 (2005).
2. Smith, D.E., Babcock, H.P., Chu, S. Single-polymer dynamics in steady shear flow. *Science*. **283** (5408), 1724-1727 (1999).
3. LeDuc, P., Haber, C., Bao, G., Writz, D. Dynamics of individual flexible polymers in a shear flow. *Nature*. **399** (6736), 564-566 (1999).
4. Huber, B., Harasim, M., Wunderlich, B., Kröger, M., Bausch, A.R. Microscopic Origin of the Non-Newtonian Viscosity of Semiflexible Polymer Solutions in the Semidilute Regime. *ACS Macro Letters*. **3** (2), 136-140 (2014).
5. Springer, T.A. Biology and physics of von Willebrand factor concatamers. *Journal of Thrombosis and Haemostasis*. **9** (0 1), 130-143 (2011).
6. Springer, T.A. von Willebrand factor, Jedi knight of the bloodstream. *Blood*. **124** (9), 1412-1425 (2014).
7. Merchant, K.A., Best, R.B., Louis, J.M., Gopich, I.V., Eaton, W.A. Characterizing the unfolded states of proteins using single-molecule FRET spectroscopy and molecular simulations. *Proceedings of the National Academy of Sciences of the United States of America*. **104** (5), 1528-1533 (2007).
8. Neuman, K.C., Nagy, A. Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy. *Nature Methods*. **5** (6), 491-505 (2008).
9. Siedlecki, C.A. et al. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*. **88** (8), 2939-2950 (1996).
10. Roiter, Y., Minko, S. AFM single molecule experiments at the solid-liquid interface: In situ conformation of adsorbed flexible polyelectrolyte chains. *Journal of the American Chemical Society*. **127** (45), 15688-15689 (2005).
11. Aznauryan, M. et al. Comprehensive structural and dynamical view of an unfolded protein from the combination of single-molecule FRET, NMR, and SAXS. *Proceedings of the National Academy of Sciences of the United States of America*. **113** (37), E5389-E5398 (2016).
12. Schuler, B., Eaton, W.A. Protein folding studied by single-molecule FRET. *Current Opinion in Structural Biology*. **18** (1), 16-26 (2008).
13. Howorka, S., Siwy, Z. Nanopore analytics: sensing of single molecules. *Chemical Society Reviews*. **38** (8), 2360-2384 (2008).
14. Talaga, D.S., Li, J. Single-molecule protein unfolding in solid state nanopores. *Journal of the American Chemical Society*. **131** (26), 9287-9297 (2009).
15. Moerner, W.E., Fromm, D.P. Methods of single-molecule fluorescence spectroscopy and microscopy. *Review of Scientific Instruments*. **74** (8), 3597-3619 (2003).
16. Xia, T., Li, N., Fang, X.H. Single-Molecule Fluorescence Imaging in Living Cells. *Annual Review of Physical Chemistry*. **64**, 459-480 (2013).
17. Fu, H. et al. Flow-induced elongation of von Willebrand factor precedes tension-dependent activation. *Nature Communications*. **8** (324), 1-12 (2017).

562 18. Smith, S.B., Cui, Y., Bustamante, C. Overstretching B-DNA: The Elastic Response
563 of Individual Double-Stranded and Single-Stranded DNA Molecules. *Science.* **271** (5250), 795-
564 799 (1996).
565 19. Wang, Y. et al. Shear-Induced Extensional Response Behaviors of Tethered von
566 Willebrand Factor. *Biophysical Journal*. **116** (11), 29092-2102 (2019).
567 20. Carlsson, C., Johnsson, M., Åkerman, B. Double bands in DNA gel electrophoresis
568 caused by bis-intercalating dyes. *Nucleic Acids Research.* **23** (13), 2413-2420 (1995).
569 21. Collins, B.E., Ye, L.F., Duzdevich, D., Greene, E.C. DNA curtains: Novel tools for
570 imaging protein–nucleic acid interactions at the single-molecule level. *Methods in Cell*
571 *Biology*. **123**, 217-234 (2014).
572 22. Green, E.C., Wind, S., Fazio, T., Gorman, J., Visnapuu, L. DNA Curtains for High-
573 Throughput Single-Molecule Optical Imaging. *Methods in Enzymology.* **472**, 293-315 (2010).
574

Figure 1

Click here to access/download;Figure;Figure 1.png

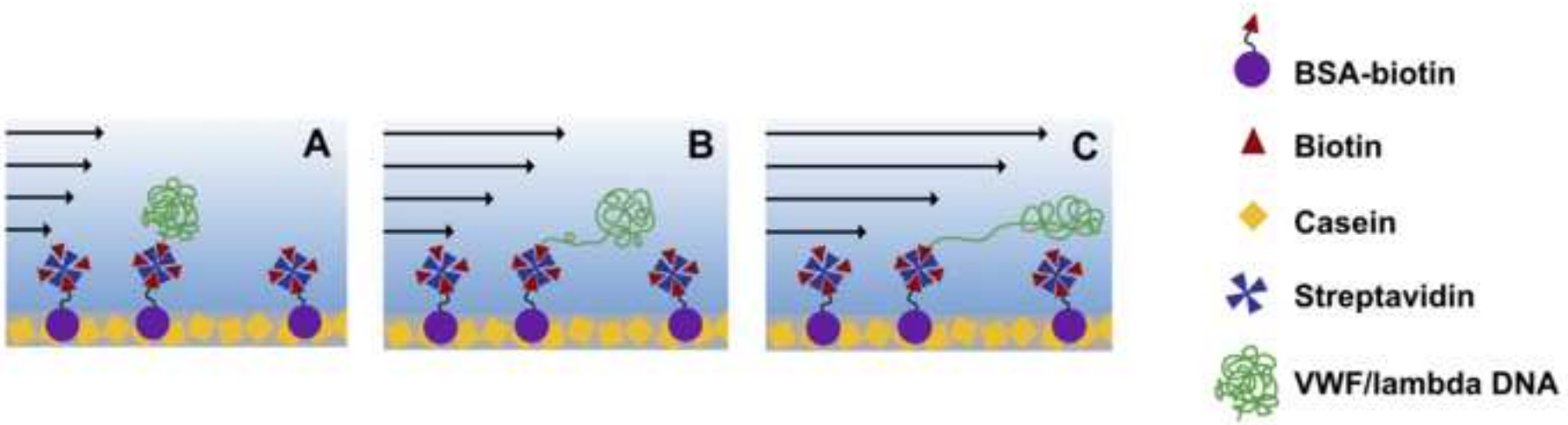


Figure 2

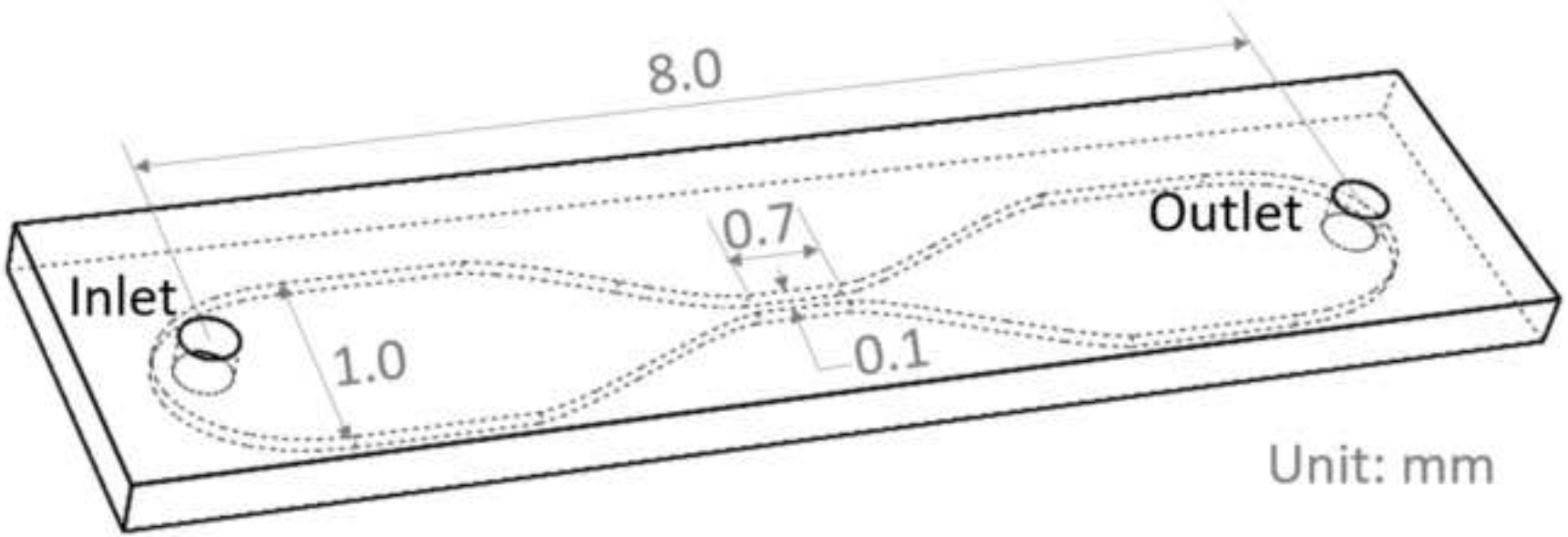


Figure 3

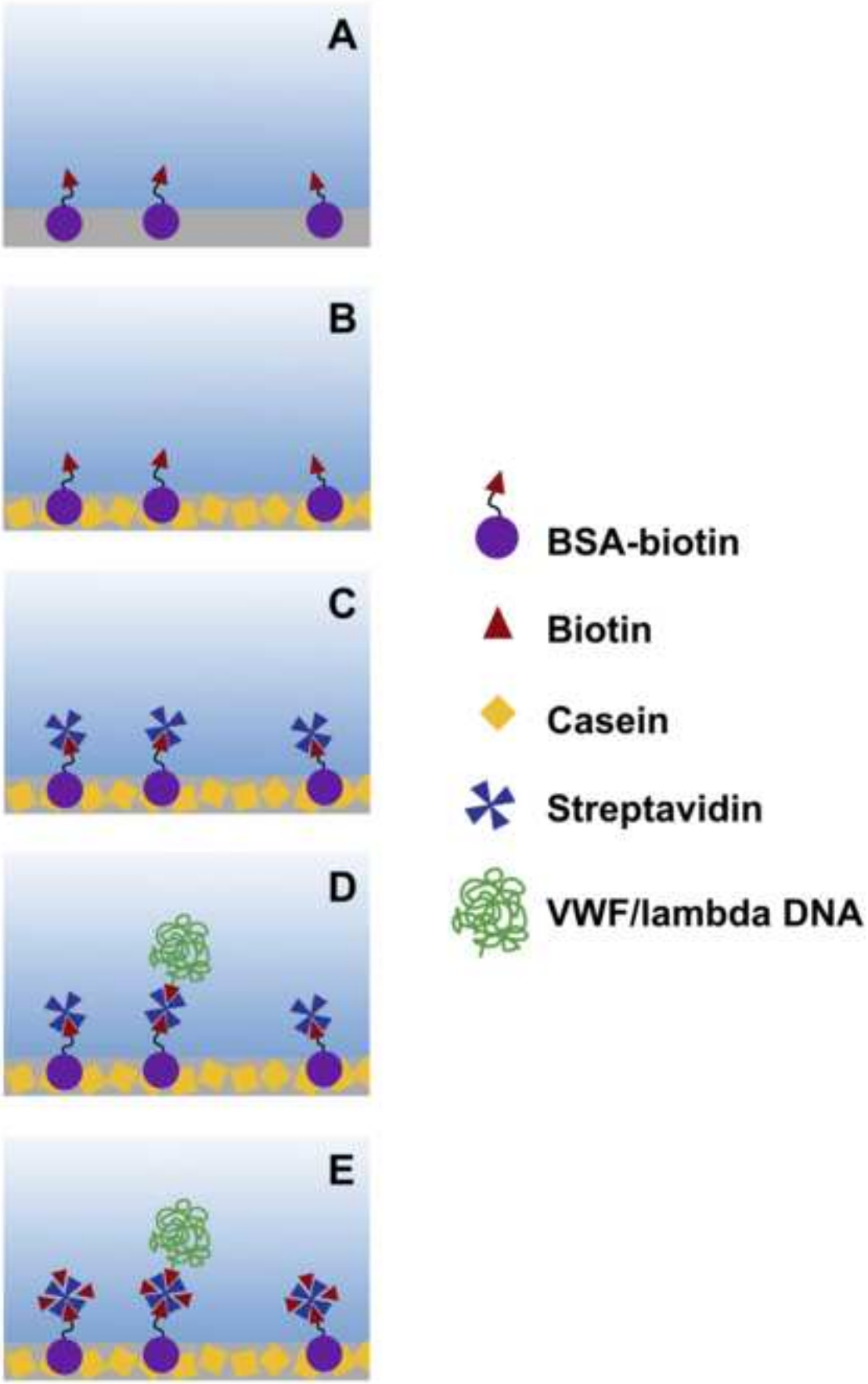


Figure 4

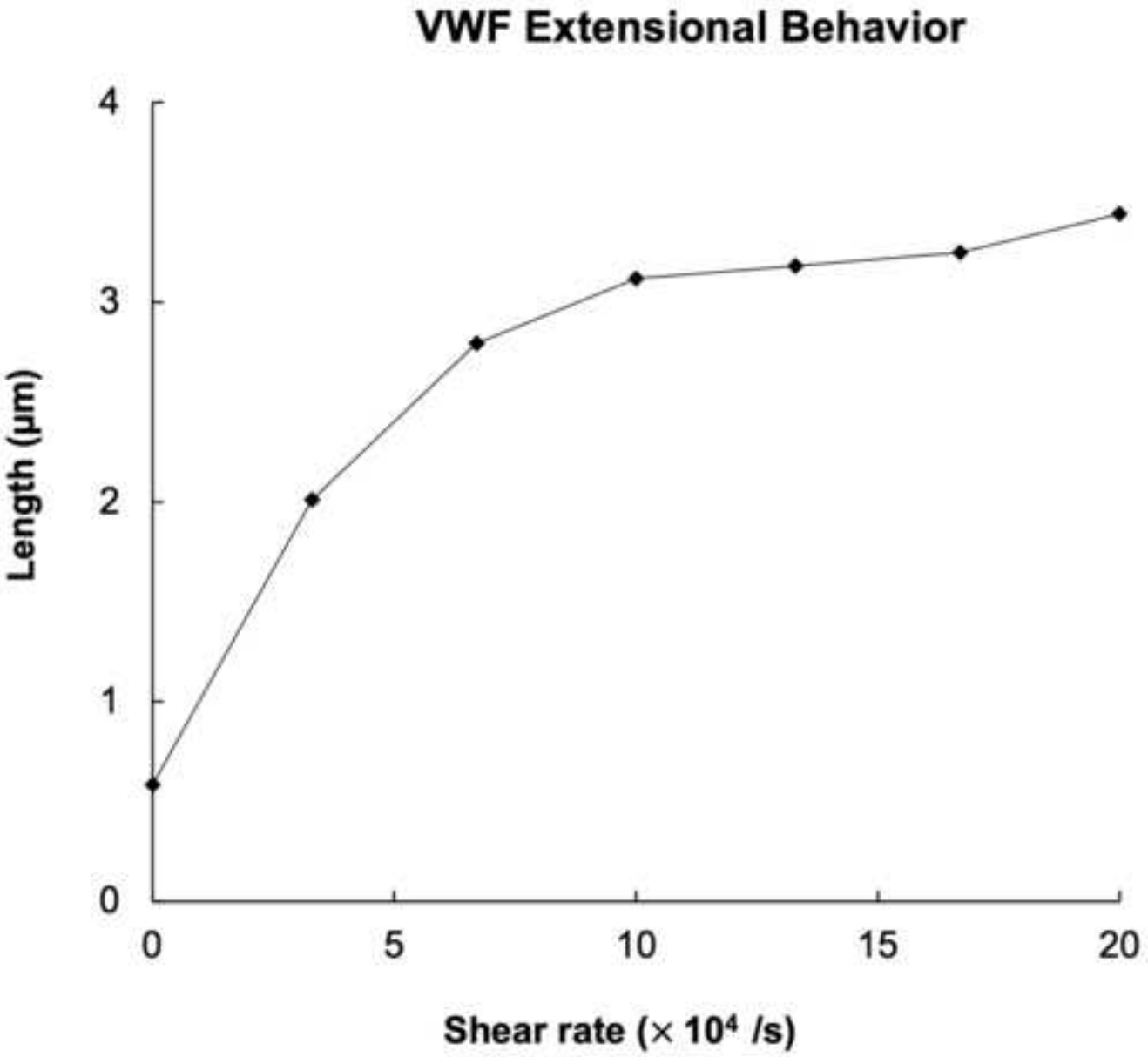
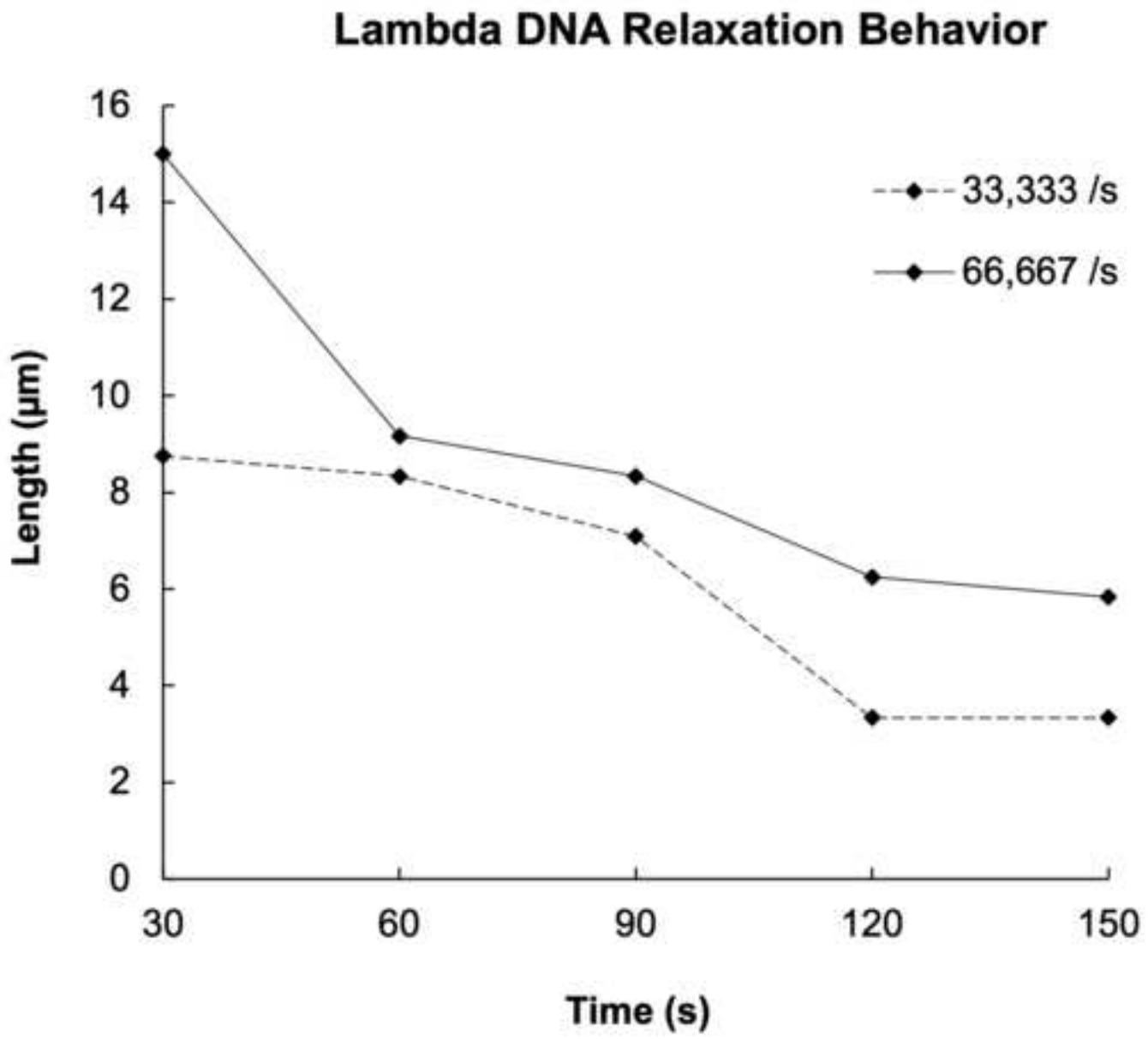


Figure 5



Video 1

Click here to access/download

Video or Animated Figure

Video1.mov

Video 2

Click here to access/download

Video or Animated Figure

Video2.mov

Video 3

Click here to access/download

Video or Animated Figure

Video3.mov

Name of Material/Equipment	Company	Catalog Number
Alexa Fluor 488 Labeling Kit	Invitrogen	A30006
Bio-Spin P-6 Gel Columns	Bio-Rad	7326221
Biotin	Sigma-Aldrich	B4501
Biotin-14-dCTP	AAT Bioquest	17019
BSA-Biotin	Sigma-Aldrich	A8549
Coverslips	VWR	48393-195
dNTP Set	Invitrogen	10297018
Float Buoys for Mini Dialysis Device	Thermo Scientific	69588
Klenow Fragment (3'→5' exo-)	New England BioLabs	M0212S
Lambda DNA	New England BioLabs	N3011S
Mini Dialysis Device	Thermo Scientific	69570
NEBuffer 4	New England BioLabs	B7004S
NHS-PEG4-Biotin	Thermo Scientific	21330
Protocatechuate 3,4-Dioxygenase	Sigma-Aldrich	P8279
Protocatechuic acid	Santa Cruz Biotechnology	sc-205818
Silicone Elastomer Kit for PDMS Fabrication	The Dow Chemical Company	4019862
Streptavidin	Sigma-Aldrich	85878
The Blocking Solution	CANDOR Bioscience	110 050
Vinyl Cleanroom Tape	Fisher Scientific	19-120-3217
von Willebrand Factor, Human Plasma	Millipore Sigma	681300
YOYO-1 Dye	AAT Bioquest	17580
0.25 mm Inner Diameter Tubing	Cole-Parmer	EW-06419-00
25 Gauge Needle	Thomas Scientific	JG2505X

Comments/Description
Use as free biotin in Step 5.6
No. 1 ½, 22 x 50 mm
Use for 10X reaction buffer in Step 2.1.1 and 1X reaction buffer in Step 2.2.2
10K MWCO, 0.1 mL volume
Use as casein blocking solution throughout protocol

Response to Editorial and Reviewer Comments

for

Characterizing Single-Molecule Conformational Changes Under Shear Flow with Fluorescence Microscopy

by

Avani V. Pisapati, Yi Wang, Megan E. Blauch, Nathan J. Wittenberg, Xuanhong Cheng and X. Frank Zhang

In this letter, editorial and reviewer comments are written in italicized font and span the full page width between margins. Our responses are written below each comment or concern and have indented margins. Texts highlighted in yellow in this document indicate revisions made to the manuscript, "60784_R0.docx". Changes in "60784_R0.docx" itself are made in red. The line numbers associated with each of the highlighted revisions indicate the location of these changes in that document. All the references cited in the letter are the same as in the manuscript.

We thank the editors and reviewers for their time and responses.

Editorial Comments:

The manuscript has been modified and the updated manuscript, ***60784_R0.docx****, is attached and located in your Editorial Manager account.* ***Please use the updated version to make your revisions.***

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
2. *Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."*
3. *Please define all abbreviations before use.*
4. *Please sort the items in alphabetical order according to the name of material/equipment.*
5. *Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.*

Response: We thank the editors for their modifications and comments. We have proofread the manuscript to correct for all spelling and grammar issues and have defined all abbreviations. Such corrections are marked in red in the document "60784_R0.docx". All submitted figures are original and do not require copyright permission for their publication. The "Table of Materials.xlsx" has also been corrected so that the materials/equipment are now in alphabetical order. Finally, all figures have been converted to .png with 300 dpi for your convenience.

Reviewer #1 Summary:

In this manuscript, Pisapati et al., reported about their protocol, which describes the steps to capture conformational changes of single biomolecules under different shear flow environments using fluorescence microscopy. After reading this manuscript, I am confident that this is worthy of publication in JOVE as soon as possible. In addition, this protocol can also be widely applied to study the behavior of polymers, especially biopolymers, in varying flow conditions and to investigate the rheology of complex fluids. Really enjoyed reading this protocol.

Response: We thank the reviewer for reading the manuscript and for their positive comments.

Reviewer #2 Summary:

This manuscript describes the use of microfluidic channels and high resolution fluorescence microscopy to study the role of fluid shear on extentional (unravelling) response of macromolecules. The particular macromolecule under consideration is von Willebrand Factor (vWF) that plays a critical role in clotting function in the body. Unravelling of vWF under high shear stress has been implicated as a major cause of bleeding events due to the fact that unravelled vWF being cleaved by ADAMTS13. This therefore is a critical study that can highlight the range of shear stresses under which high MW vWF multimers can be cleaved into more inefficient smaller fragments.

The described protocols for device fabrication, device functionalization, microfluidic flow control, labeling and fluorescence microscopy are extensive and clearly laid out and will enable easy adoption by others. This approach can be used to study other shear sensitive molecules.

Response: We thank the reviewer for reading the manuscript and for their positive comments.

Reviewer #2 Minor Concerns:

This may be limited to larger macromolecules. Also, care may need to be taken to ensure that the anchoring groups do not impact function of the bound molecules.

Response: We thank the reviewer for raising these two critical points. It is true that our current protocol presents a method for observing larger macromolecules. Conformational changes in smaller molecules are more difficult to observe due to their lower sensitivity to shear and inability to be resolved by fluorescence microscopes. We have now addressed this point in the discussion of limitations. However, it is possible to modify our method by binding smaller molecules to these same larger macromolecules tethered to the surface, thereby increasing the shear-sensitivity of any small molecules that can be resolved by fluorescence microscopy. Therefore, we have made this additional point in the discussion of future applications. Finally, Fu et al. have demonstrated the ability of VWF to bind to the platelet receptor GPIbα while VWF was bound by biotin-streptavidin linkages to a microfluidic surface. This binding event helps initiate platelet aggregation and is critical to VWF's function in the blood. This therefore supports that the anchoring groups are not likely to affect the function of bound molecules. We appreciate that this issue was raised and have now added further clarification in our discussion about it.

Action taken:

Previous text: (Lines 474-499)

This method is limited by a lack of information about the size and tethering points of the molecule as well as the difficulty to produce 0 s^{-1} shear rate in the channel. Previous application of this method has illustrated a large variation in the shear-induced unraveling behavior of VWF[18]. Possible explanations for this are that extensional responses are influenced by the number and location of biotin-streptavidin interactions as well as the molecular weight of VWF, all of which vary from molecule to molecule. At the moment, the method we present cannot define tether points and molecular size. However, Brownian dynamics simulations of a coarse-grained VWF model published by Wang et al. incorporate these

variables and can be run alongside experimental findings to explain variation[18]. Furthermore, flow does not stop instantaneously when the syringe pump is stopped, confounding the observation of recoiling dynamics. This is due to deformation and slight dilation of the PDMS channel during the intended flow period. When the pump is stopped, fluid continues to flow until PDMS is fully relaxed. An improved system should use more rigid PDMS or microchannels fabricated in hard plastic materials, allowing fluid to reach a 0 s^{-1} shear rate more quickly.

The current protocol concerns mainly quantification of conformational changes of protein and DNA molecules under physiological flow. The method can also be used to visualize real-time interactions between biological molecules and further characterize protein and DNA function. For instance, Fu et al. has shown that tethered VWF can activate under high shear flow and further capture the platelet adhesion molecule GPIbα under varying flow conditions[17]. Similar mechanistic insights could be obtained while studying the interactions between unraveled DNA and regulatory proteins in flow environments[21,22]. Other single-molecule characterization methods, such as AFM or optical tweezers, provide high-resolution data on the structural and functional properties of macromolecules. However, these alternative methods cannot observe the dynamic, conformational changes of proteins and DNA that take place in a physiological flow environment, as is presented in this protocol.

Revised text: (Lines 478-514)

This method is limited by a lack of information about the size and tethering points of the molecule, the difficulty in producing 0 s^{-1} shear rate and the optical resolution of fluorescence microscopes. Previous work has shown a large variation in the unraveling behavior of VWF, potentially explained by the wide distribution in the number and location of biotin-streptavidin tether points and the molecular weight of each VWF molecule[18]. At the moment, the method we present cannot define tether points and molecular size. However, Brownian dynamics simulations of a coarse-grained VWF model published by Wang et al. incorporate these variables and can be run alongside experimental findings to explain such variation[18]. Furthermore, flow does not stop instantaneously when the syringe pump is stopped, confounding the observation of recoiling dynamics. This is due to the deformation and slight dilation of the PDMS channel during the intended flow period. When the pump is stopped, fluid continues to flow until the PDMS is fully relaxed. An improved system should use more rigid PDMS or microchannels fabricated in hard plastic materials, allowing fluid to reach a 0 s^{-1} shear rate more quickly. Finally, one can only resolve molecules whose size is on the same order of magnitude as the optical resolution of the fluorescence microscope, which may be no smaller than a few hundred nanometers. Thus, there is a minimum size requirement for the molecules that can be directly observed with this method.

The current protocol concerns mainly quantification of conformational changes of protein and DNA molecules under physiological flow. However, the method can also be used to visualize real-time interactions between biological molecules and further characterize protein and DNA function. For instance, Fu et al. have shown that tethered VWF can activate under high shear flow and further capture the platelet adhesion molecule GPIbα under varying flow conditions[17]. This binding event is preserved even when VWF is bound to the surface by biotin-streptavidin linkages, demonstrating the effectiveness of this protocol to study physiologically relevant functions and mechanics[17]. Similar mechanistic insights could be obtained while studying the interactions between unraveled DNA and regulatory proteins in

flow environments[21,22]. Additionally, our method pertains mostly to observing conformational changes in macromolecules. Nevertheless, one could adapt it for the purpose of studying smaller molecules that are large enough to be resolved under fluorescence microscopy. For example, by noncovalently or covalently attaching a small molecule to a much larger, immobilized lambda DNA, one could increase the shear-sensitivity of the smaller molecule and more easily observe its behavior. In conclusion, other single-molecule characterization methods, such as AFM or optical tweezers, provide high-resolution data on the structural and functional properties of macromolecules; however, these alternative methods cannot observe the dynamic, conformational changes of proteins and DNA that take place in a physiological flow environment, as is presented in this protocol.

Supplemental MATLAB code

Click here to access/download

Supplemental Coding Files

main.m

Supplemental MATLAB code

Click here to access/download

Supplemental Coding Files

get_length.fig

Supplemental MATLAB code

Click here to access/download

Supplemental Coding Files

get_length.m

Supplemental MATLAB code

Click here to access/download
Supplemental Coding Files
save_each_frame.m