Response to Editorial and Reviewer Comments

for

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

by

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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.