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Methods in Studying Red Blood Cells in Sickle Cell Disease

Kostyantyn Partola
University of Connecticut - Storrs, kostyantyn.partola@uconn.edu

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Methods in Studying Red Blood Cells in Sickle Cell Disease

Kostyantyn Rhys Partola, PhD
University of Connecticut, 2019

Sickle cell disease (SCD) is a genetically inherited blood disorder characterized by a single point mutation that produces hemoglobin S within red blood cells (RBCs). When such RBCs are deoxygenated hemoglobin S polymerizes and causes the cells to take on a sickle shape and become more viscous and stiffer than normal RBCs. The hallmark of SCD is painful vaso-occlusive crises in which the flow of blood to parts of the body is obstructed. Today, millions of people suffer from SCD around the world and life expectancy of individuals with SCD is in the 40’s. We developed several techniques to facilitate and expedite experimental investigation of SCD and in general blood biomechanics.

Vaso-occlusion is the result of interactions between blood cells and the endothelium. Atomic force microscopy (AFM) has been employed in single-molecule force spectroscopy (SMFM) and single-cell force spectroscopy (SCFS) assays to study these interactions. In order to process results from these assays quickly, we developed MATLAB-based custom software that expedited manual processing efforts from 1 to 2 hours to 10 to 15 minutes per experiment.

We explored microfluidic principles in order to further increase the throughput of the number of cells we can study. We have designed an experimental setup that implements
aspiration based assembly for microchannels which enables the use of most functionalization techniques, and a pressure controller that allows instant and precise changes in the microchannel flow. Utilizing this setup, we have quantified SS-RBC adhesion to the integrin αvβ3, a specific adhesion protein expressed on the endothelium which has been implicated in vaso-occlusion, and measured the shear modulus and viscosity of the SS-RBC membrane.

We have designed a portable, swift, easy-to-use, small sample, and sterilization-free rheometer capable of measuring the viscosity of Non-Newtonian liquids. This rheometer can quickly measure the viscosity of body fluids such as whole blood and blood plasma, which has been hypothesized to be of value in the assessment of SCD.

We explored expediting the development and review of simulations and three-dimensional data. To this end, we have developed a virtual reality environment for interactive multiphysics simulation and data visualization utilizing modern head-mounted displays.
Methods in Studying Red Blood Cells in Sickle Cell Disease

Kostyantyn Rhys Partola, PhD

B.S., University of Connecticut, 2013

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Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut

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2019
Doctor of Philosophy Dissertation

Methods in Studying Red Blood Cells in Sickle Cell Disease

Presented by
Kostyantyn Rhys Partola, B.A.,

Major Advisor: George Lykotrafitis
Associate Advisor: Biree Andemariam
Associate Advisor: Savas Tasoglu
Associate Advisor: Thanh Nguyen
Associate Advisor: Ying Li

University of Connecticut
2019
Acknowledgement

I feel that the entirety of my journey through graduate school has been a humbling experience. I have gained a profound appreciation for how little I know of all that we know, and how little we know of all that we intend to. It seems that every skill and quanta of knowledge that I’ve developed throughout my time creating this work has raised a median of ten further questions to answer and ten further paths to explore. I look forward to facing all of my future challenges with the same attitude of relentless persistence that I cultivated when I was forced to tackle problems that looked impossible at first glance. Of course, this work is not something that I could have accomplished alone and I want to thank everyone who has worked to make my success on this journey a possibility.

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Chapter 1: Introduction
Sickle cell disease (SCD) is a genetically inherited blood disorder characterized by a single point mutation. This mutation results in the replacement of the adult hemoglobin in red blood cells by hemoglobin S (HbS). When red blood cells are deoxygenated HbS polymerizes and causes the cells to take on a sickle shape and a rigid structure. As a result, the hallmark of SCD is painful vaso-occlusive crises in which blood flow to parts of the body is obstructed [1]. Vaso-occlusion is the result of interactions between blood cells and the endothelium [2-4]. Today, millions of people suffer from sickle cell disease around the world and life expectancy of individuals with sickle cell disease is in the 40’s [5, 6]. There is currently no drug available for patients of sickle cell disease that targets the pathophysiology of the disease [7].

It has been shown that the adhesive behavior and viscoelastic response of sickle cell red blood cells (SS-RBCs) are important factors in vaso-occlusion [8]. SS-RBC adhesion to specific proteins, such as human integrin $\alpha_v\beta_3$ and laminin, expressed on the endothelium has been implicated as part of the mechanism that triggers vaso-occlusive crises [2, 9-12]. During a vaso-occlusive crisis adherent SS-RBCs in the vasculature obstruct other SS-RBCs due to their viscoelastic and mechanical properties [13, 14].

A number of experimental tools have been used to quantify SS-RBC specific adhesion to proteins expressed on the endothelium. To this end, atomic force microscopy (AFM) has been employed in single-molecule force spectroscopy (SMFM) and single-cell force spectroscopy (SCFS) assays [10, 15-17]. Briefly, an atomic force microscope is capable of measuring a wide range forces (5 pN to 106 pN) applied on a cantilever probe by recording the deflection of the cantilever [18, 19]. Through the use of a controlled vertical
displacement applied to the probe by piezoelectric actuators the technique can produce force-displacement signals during both the approach and the retraction of the probe toward a cell. When the cantilever probe is functionalized with a specific ligand, the bond strength of the ligand and its receptor can be measured on the retraction force-displacement curve [17]. Significant data processing and additional experimentation was necessary of our publication using single-cell force spectroscopy studying the BCAM/Lu and laminin specific adhesion [16]. In particular, we reprocessed the entirety of the SMFS experimental data to ensure that each adhesion measurement represented specific binding with an RBC. We distinguished these sites from erroneous data points by identifying an effective spring constant of the adhesion complex and ensuring that we processed data in which this constant was less that 10 pn/nm. We measured this spring constant as the slope of the retraction curve after the cantilever retracted past the cell surface and before adhesion complex was broken. We also performed experiments to evaluate cell stiffness which was calculated by probing red blood cells (RBCs) with an AFM cantilever and curve fitting a theoretical model derived from the Hertz model to the force-displacement response curves.

In order to process these results quickly, we developed MATLAB-based custom software that expedited manual processing efforts from 1 to 2 hours per experiment to 10 to 15 minutes per experiment. The MATLAB-based data processing tools developed as part of this project were later published as FRAME, the Force Review Automation Environment [22].
We sought to further increase the throughput of the number of cells we could reasonably study and explored microfluidic principles to accomplish this. Typical microfluidic assays, which aim at quantifying mechanical properties of sickle cell red blood cells (SS-RBCs), suffer from a number of drawbacks in functionalization and flow control. Specifically, physical adsorption functionalization techniques produce inconsistent functional surfaces, and common volumetric flow pumps cannot be used to adjust the flow inside microchannels with minimal delay. We have designed an experimental setup that alleviates these complications by implementing aspiration for microchannel assembly that enables the use of most functionalization techniques and a pressure controller that allows instant and precise changes in the microchannel flow. Utilizing this setup, we have quantified SS-RBC adhesion to the integrin αvβ3, a specific adhesion protein expressed on the endothelium, as well as measured the shear modulus and viscosity of the SS-RBC plasma membrane.

We explored expediting the development and review of simulations and three dimensional data in sickle cell disease. To this end, we employed virtual reality. The recent commercial success of virtual reality based on head-mounted display (HMD) technology has the potential to fundamentally enhance the way we create, review, and interact with simulations and three-dimensional data. Representation of such data and simulations in virtual reality grants users the ability to explore them intuitively and personally. To this end, we have developed a virtual reality environment for interactive multiphysics simulation and data visualization. This environment integrates the visual fidelity, coding standards, and affordability of modern HMDs with (1) manual and immersive three-dimensional particle steering during simulation run-time and with (2) undistorted
volumetric rendering of static volumetric maps such as those produced by confocal microscopy. This method of visualization provides a deeper understanding of such data structures than previously explored methods, such as three dimensional anaglyph stereoscopy.

We encountered the hypothesis that leg ulcerations and vaso-occlusive episodes in sickle cell disease were related to whole blood viscosity. Unfortunately, current blood viscosity measurement techniques are inadequate for large scale measurements. In particular, commercially available rheometers are typically cumbersome, slow, necessitate constant cleaning, and are priced in the tens of thousands of dollars at the most affordable. Such devices are particularly inconvenient for clinical laboratory and point-of-care use because of the large amount of fluid needed and because of sterilization requirements before and after use.

Engineering, whether mechanical or not, is defined first and foremost by its key constituent: synthesis. It is in this spirit of synthesis that we designed a portable, swift, easy-to-use, small sample, sterilization-free, and cost-effective rheometer capable of measuring the viscosity of both Newtonian and Non-Newtonian liquids. This rheometer can quickly measure the viscosity of body fluids such as whole blood, blood plasma and urine which have been found to be important in cardiovascular health assessment and cognitive function evaluation as well as an important marker in certain diseases, such as Alzheimer’s disease, dementia, myeloma, rheumatoid arthritis, and Waldenström’s Macroglobulinemia as well as explore the aforementioned hypothesis in sickle cell disease. This device will also quantify the viscosity of liquids such as inks, bioprinting
inks, and oils, where viscosity is crucial during formulation, quality assurance, and, in the case of bioprinting inks, in regular use. An assembly based on this technique could produce a device that is fast (1-5 minutes), portable, easy to use, requires only a drop of the liquid (no more than 50 microliters), and does not need regular cleaning and sterilization thanks to the use of a disposable microchannel cartridge.
Chapter 2: FRAME (Force Review Automation Environment):

MATLAB-based AFM data processor
2.1 Introduction

An atomic force microscope (AFM) is capable of measuring a wide range forces (5 pN to 106 pN) applied on a cantilever probe by recording the deflection of the cantilever [27, 62]. Controlled vertical displacement is applied to the probe by piezoelectric actuators in order to produce force-displacement curves. Atomic force microscopy has several applications. In particular, resultant force-displacement curves have been used to measure the elastic modulus of biological samples [63] and the unbinding force between specific receptor-ligand bonds [27]. Manually processing these data requires researchers to separately review each measurement. This is very time consuming and susceptible to user error or bias. Few free software applications for automatic processing of AFM force measurements have been developed, and only two such programs have seen some use [64, 65]. OpenFovea processes unbinding measurements by curve-fitting either the Worm Like Chain (WLC) or the Freely Jointed Chain (FJC) models in order to identify unbinding events, while Hooke uses a fuzzy logic algorithm to identify unbinding events based on conformity with specific, pre-defined, characteristics. However, in AFM experiments that probe cellular surfaces directly, and in single cell force spectroscopy (SCFS) experiments, both aforementioned methods cannot be implemented since cellular surface deformation and multiple simultaneous ligand-receptor bonds alter the force-displacement curve shape [16, 53]. As a result, most AFM data are still manually processed, and no clear standard has emerged in the automated processing of both Young’s moduli and ligand-receptor unbinding measurements.

We have created an open-source software application based on MATLAB, the force review automation environment or FRAME, to greatly increase the speed at which AFM data are
Figure 2-1. Comparison of data processed manually and using FRAME. (a) FRAME displays the contact fit (dashed black line) used to compute a sample’s Young’s modulus, the contact point (black circle) and the end of fit point (green circle) based on the force-displacement curve (red and blue line plots). (b) Box-whisker plots showing the results of Young’s modulus measurements on Human Embryonic Kidney (HEK293T) cells as processed from the same data set manually and using FRAME. The two results do not pass the test of significant difference (nonparametric Mann-Whitney test, p>0.8). (c) Histograms showing the results of Young’s modulus
processed. FRAME is intuitive and easy to operate, even for the novice MATLAB user. It features a number of algorithms to streamline automated processing and a graphic user interface (GUI) to expedite manual processing and review of the data. Even if FRAME is only used in manual mode it can greatly accelerate data processing, especially for large sets of data. The results can be displayed in FRAME with MATLAB plotting tools, as well as exported as a Microsoft Excel file, for further statistical analysis. FRAME, the User Manual, and a demonstration video can be found online at the Cellular Mechanics Laboratory webpage (http://www.engr.uconn.edu/~gelyko/frame.html). FRAME is shared both as a Windows executable and as MATLAB code under the open-source BSD license.

2.2 Methods

FRAME can be used to compute Young's moduli and ligand-receptor unbinding forces. In particular, the Hertz contact model for spherical and pyramid shape cantilever tip has been implemented [66, 67]. Manual processing of data produced in a contact experiment requires the user to identify, on the force-displacement curve, the point where the cantilever tip comes in contact with the sample's surface. FRAME uses a least-squares minimization for the curve-fit of the Hertz contact model to identify both the Young's
modulus of the sample and the contact point on the curve. Before any force-displacement curve is fit with a contact model, it is first “flattened,” a process in which the curve’s force response before contact with the sample is defined to have an average force value of zero. In order to curve-fit the extension portion of force-displacement curves to the contact model and locate the contact point, FRAME effectively uses a least-squares minimization of the following expression: 

$$F(z) = g(z - z_1) \times [H(z - z_1) - H(z - z_2)]$$.

In this expression, $z$ is the vertical displacement, $F(z)$ is the force detected by the cantilever, $H$ is the Heaviside function, $g(z)$ represents the contact model, $z_1$ is the contact point and a design variable, and $z_2$ is the end the curve-fit range which can be either defined by the user or be a design variable. The initial conditions for the minimization are determined from the flattening process (see supplementary information). Other contact models can be easily implemented by the user through a simple guided modification of the MATLAB script. The step by step instructions for using custom contact models can be found in the user manual. The quality of the curve-fit, based on the root-mean-squared of the curve-fit residuals, can also be employed to ignore low quality curves from data sets. FRAME also provides users with the tools to manually identify the contact point and the curve-fit range on individual curves while retaining automated processing functionality on all others.

In addition, FRAME provides users with a set of algorithms to detect and quantify ligand-receptor unbinding events based on a number of user provided parameters. Potential unbinding events are selected by the algorithms based on force thresholds provided by the user. The unbinding event detection algorithms verify that high amplitude noise is not misinterpreted as an unbinding event by confirming that the abrupt curve shift at the location of a possible unbinding event is permanent. FRAME can fit the curve before an
unbinding event to the WLC model and produce the persistence and contour lengths associated with this event. Because the software treats each unbinding event on an individual basis and because users may alter many relevant parameters for unbinding event detection, these algorithms are applicable to any force range. FRAME is equipped with several functions and a user friendly GUI that allow users to very quickly review preliminary results and modify parameters based on a few representative curves per set of data in order to assure consistent performance. Users also have access to tools that allow them to manually select unbinding events on individual curves while retaining automated processing functionality on all others.

2.3 Results

We compared results derived from manual and automatic processing of data collected in receptor-ligand unbinding and in elasticity experiments.

2.3.1 Young’s Modulus Measurements

Measurements of the Young’s modulus of four Human Embryonic Kidney (HEK293T) cells were automatically and manually processed (see Supplementary information). The parameters for FRAME automated processing were chosen with regard to representative curves and the set of data was automatically processed using these parameters. We did not observe a statistically significant difference between manually processed and FRAME processed results (see Figure 2-1b). Based on these findings it is confirmed that the proposed automated method generates results consistent with manually processed results.
Figure 2-2. The FRAME graphical user interface (GUI) during typical use for unbinding event detection. All options, parameters, tools, and plots of force-displacement curves are available on one window.

2.3.2 Unbinding Events Measurements

Unbinding-force measurements were taken between the ICAM4 receptor expressed on the surface of a sickle human red blood cell (SS-RBCs) and a probe functionalized with the integrin αvβ3 and manually processed (see Supplementary information). The parameters for FRAME automated unbinding event detection were set with regards to representative curves. The curves were automatically processed using these parameters. We did not observe a statistically significant difference between manually processed and automatically processed results (see Figure 2-1e)
2.4 Discussion

FRAME offers a directly applicable array of modifiable tools to AFM users for expediting data processing. The software processes AFM data extremely quickly compared to the time required for manual processing. 1024 curves are processed in less than one minute for unbinding events and in less than five minutes for Young's modulus measurements in a work station with an Intel® Core™ 2 Duo CPU at 3.00 GHz and 8.00 GB of installed memory (RAM). FRAME also provides convenient manual processing tools that are integrated with automated processing. The GUI is intuitive and easy to use, requiring little to no user training. FRAME can automatically import force-displacement curves from AFMs by Asylum Research (.ibw files). Users of other brands of AFMs can import force-displacement measurements formatted as delimited text files into FRAME following instructions in the User’s Manual.

2.5 Supplementary Information

2.5.1 Cell Culture and Preparation

Human Embryonic Kidney (HEK293T) cells were plated on poly-L-lysine coated glass bottom petri dishes in solution containing Dulbecco’s Modified Eagle media, 10% fetal bovine serum, and 1% Penicillin-Streptomycin.

Heparin-anticoagulated venous blood was drawn from patients with sickle cell disease with the approval of the Institutional Review Boards of the UCONN Health Center and UCONN-Storrs. The whole blood, containing sickle cell human red blood cells (SS-RBCs), was centrifuged at 500 g for 10 minutes at 4°C to isolate the cells and discard the
buffy coat and plasma. The SS-RBCs were washed three times with Alsever’s solution and stored in Alsever’s solution. SS-RBCs were immobilized by incubation for 10 minutes at 37°C on a poly-L-lysine coated glass bottom petri dish. Excess and unattached SS-RBCs were washed away by rinsing.

Supplementary Figure 2-S1. The effects of varying the slope ratio $b$ in the FRAME stiffness algorithm on two hundred curves. (a) The detected contact point on individual curves does no change more than 60nm from the average measurement as the slope ratio $b$ is varied. (b) The corresponding Young’s Moduli are not significantly different from one another as the slope ratio $b$ is varied (nonparametric Kruskal-Wallis test, p>0.8).

2.5.2 Probe Functionalization
Silicon nitride cantilever probes (Bruker Probes, Camarillo, CA) were silanized with 2% v/v 3-aminopropyltriethoxysilane in acetone (10 minutes). Following silanization, the
probes were rinsed with deionized water and immersed in glutaraldehyde (0.5%, 30 minutes). The probes were then rinsed with deionized water and incubated in αvβ3 solution (100 μg/mL, 30 minutes). The probes were rinsed again and incubated in BSA (100 μg/mL, 5 minutes) in order to block any remaining aldehyde groups. Probes were stored at 4°C in phosphate buffered saline (PBS) and used within 48 hours.

2.5.3 AFM methods

For atomic force microscope (AFM) procedures in stiffness measurements of HEK293T cell please refer to the relevant sections of [68].

For AFM procedures in unbinding event measurements of SS-RBCs please refer to the relevant sections of [69].

2.5.4 Stiffness Curve Fitting Methods

Before curve-fitting is performed, each force-displacement curve is “flattened,” a process in which the non-contact portion of the curve is set to have an average force response of zero based on a linear regression in that portion of the curve. FRAME flattens the curve in two steps. In the first step, it roughly guesses the contact point based on the location of the relative decline in slope from the end of the approach curve (left side of the red curve shown in Figure 2-1a). The search algorithm determines, starting from the leftmost end, the point where the local slope of the approach curve is $b = 50\%$ lower than the slope at the leftmost end of the approach curve. We note that a value of $b$ between 10% and 90% produces consistent final results (see Supplementary Figure 2-S1). FRAME then applies a linear regression to the data from the start (rightmost end) of the approach
curve to this estimated point, and the curve is flattened based on the results of this linear regression. In the second step, the resultant curve from the first step is used to obtain bounds and initial conditions that are inserted in a least-squares-based curve-fit algorithm to produce the final flattened curve as explained next.

Specifically, FRAME minimizes the following equation:

\[
F(z) = f_1(z) + f_2(z) + f_3(z)
\]

\[
f_1(z) = k_1 z + k_2
\]

\[
f_2(z) = g(z - z_1) \ast [H(z - z_1) - H(z - z_2)]
\]

\[
f_3(z) = [k_4(z - z_2) + g(z_2 - z_1)] \ast [H(z - z_2)],
\]

where \( z \) is the vertical displacement, \( F(z) \) is force, \( H \) is the Heaviside function, \( g(z) \) represents the contact model (Hertz rigid pyramid and Hertz spherical models are included in FRAME) model, and \( k_1, k_2, k_4, z_1, \) and \( z_2 \) are the design variables. In addition, there is another design variable, \( k_3 \) which is part of the contact model. Function \( f_1 \) is determined by the linear behavior of the force-displacement curve before contact. Function \( f_2 \) defines the contact model and its active domain. Finally function \( f_3 \) is determined by the curve behavior after the contact model range (beyond \( z_2 \)). The contact model must be used in the flattening process because it affects the determination of the non-contact domain where the curve must be flat. The contact point is defined as \( z_1 \), while
$z_2$ defines the end of contact model domain. Users have the option of setting $z_2$ to an exact value relative to $z_1$ or to set it as a design variable with defined bounds. The exact expression of the function $g(z)$ depends on the geometry of the specific probe’s tip. In particular, for a rigid pyramid tip $g(z) = k_3 z^2$, where $k_3 = \frac{3 E \tan(\theta)}{4 (1-v^2)}$, $E$ is the sample’s Young’s modulus, $\theta$ is the angle between the sample and the side of the pyramid, and $v$ is the sample’s Poisson’s ratio. For a rigid spherical tip $g(z) = k_3 z^{3/2}$, where $k_3 = \frac{4 E}{3 (1-v^2)} R^{0.5}$, $E$ is the sample’s Young’s modulus, $v$ is the sample’s Poisson’s ratio, and $R$ is the radius of the spherical tip. The contact point is defined as $z_1$, while $z_2$ defines the end of contact model domain. Users have the option of setting $z_2$ to an exact value relative to $z_1$ or to set it as a design variable. To finalize flattening the function $f_1(z)$ is subtracted from the curve. To obtain the contact point and the stiffness of the sample after flattening, the minimization described above is repeated. We note that since flattening has already been performed the value of the new $k_1$ is approximately zero.
Authorship Contributions

This work was the product of a collaboration between Jamie L. Maciaszek, Jing Zhang, Biree Andemariam, George Lykotrafitis, and me, Kostyantyn Partola. Dr. Maciaszek designed experiments, performed experiments and data analyses, and wrote the manuscript. I performed experiments, edited the manuscript, and analyzed data. In particular, I processed and analyzed all data pertaining to SMFS experiments and produced the associated statistical evaluations. Jing Zhang performed experiments and data analyses. Dr. Andemariam designed experiments and edited the manuscript. Dr. Lykotrafitis designed experiments, performed data analyses, and edited the manuscript.

3.1 Introduction

Although red blood cells (RBCs) are considered to be relatively inactive due to their lack of a nucleus and organelles, they express surface adhesion receptors [17]. Many of these receptors mediate cell-cell interactions, or adhesion, on healthy and pathological RBCs. Knowledge of cytoadhesion is necessary to understand normal physiology, disease pathophysiology, and cellular-level effects of pharmacotherapies. Atomic force microscopy (AFM) [18] has introduced a wide range of approaches for measurements of biological samples under physiological conditions [15, 19-27]. Here, we present single-cell force spectroscopy (SCFS) as a tool [28-31] for the quantitative study of RBC adhesion while maintaining surface receptors in their native state. The technique is performed by measuring the adhesion between a single RBC attached to an AFM cantilever and a surface coated with purified proteins.
AFM studies of RBC adhesion have been utilized to provide quantitative evidence of changes in surface receptor distribution and values for unbinding forces with specific ligands [15, 26]. A technique known as single-molecule force spectroscopy (SMFS) measures the unbinding force, or detachment force, between a ligand on the AFM tip and its corresponding receptor on the cell membrane with piconewton sensitivity [15, 22, 25, 27, 32-34]. SMFS operates by collecting a set of force-distance curves of the approach-retract cycle between the functionalized tip and the cell surface. Specifically, the retraction curve gives the detachment force. This technique allows for (i) quantifying the unbinding force of specific receptor-ligand bonds, (ii) mapping the distribution and (iii) calculating the density of active receptors on cellular surfaces with high resolution [35-39]. While SMFS is a powerful technique to study RBC adhesion at the single-molecule level, it cannot provide the overall adhesion of a cell to a substrate or to another cell. Further, it is unknown how SMFS measurements compare to whole cell measurements.

To overcome these limitations, SCFS assays were developed [28-30, 40-42]. In SCFS, a RBC attached to an AFM cantilever is positioned above a functionalized substrate or another living cell. SCFS records force-distance curves of an approach/retract cycle between the RBC probe and the functionalized substrate or cell. The RBC is lowered onto the substrate/cell until a preset contact force is reached, and then held stationary for a defined time. Subsequently, the cell is withdrawn at a constant speed and bonds between the cell and substrate/cell break until separation. The detachment force is characterized as the force required to fully disengage the RBC from the functionalized substrate. While this in vitro assay can provide important details regarding RBC binding to laminin, it should
be noted that \textit{in vivo} RBCs may attach to a deformable layer of endothelial cells or interact with other glycoproteins.

Here, we established the technique of SCFS as a method to probe RBC adhesion, specifically with subendothelial laminin proteins. We found a correlation between SMFS measurements and SCFS measurements. We also showed that SCFS can detect significant changes in the adhesive response of RBCs to modulation of the cyclic adenosine monophosphate (cAMP) pathway. Based on these measurements, we identified variability in the RBC adhesion force to laminin amongst the human subjects, suggesting that RBCs maintain diverse intracellular levels of tonic protein kinase A (PKA).

3.2 Methods

3.2.1 Human subjects, blood samples, and blood preparation

Healthy volunteers at least 18 years old were eligible to participate in this study if they did not have a blood disorder or hemoglobinopathy, confirmed by screening blood samples via complete blood count and hemoglobin electrophoresis. This study was approved by the Institutional Review Boards of the UCONN Health Center and UCONN-Storrs. Experiments were performed using fresh-drawn, heparin-anticoagulated venous blood. Blood was centrifuged at 500$g$ for 10min at 4$^\circ$C to isolate the RBCs. The buffy coat and plasma were aspirated and discarded. RBCs were then washed 3x with Alsever’s solution.
3.2.2 Reagents

Alsever's solution, laminin, glutaraldehyde, biotinamido-caproyl-labeled bovine serum albumin (biotin-BSA), streptavidin, biotinylated-concanavalin A (ConA), 3-aminopropyltriethoxysilane (APTES), forskolin (*Coleus forskohlii*), KT-5720, epinephrine, BSA, were purchased from Sigma Aldrich (St. Louis, MO). BCAM protein was obtained from Novus Biologicals (Littleton, CO).

3.2.3 Surface coating with laminin

Glass petri dishes (Ted Pella, Redding, CA) were divided using a hydrophobic PAP pen (Sigma Aldrich, St. Louis, MO) enabling us to separate RBCs from the laminin surface (Figure 3-1a). Half of the glass surface was silanized with 2% APTES for 10min, rinsed with deionized (DI) water, treated with 0.5% glutaraldehyde for 30min, rinsed, incubated in laminin solution for 30min, rinsed, and incubated in BSA (100μg/mL) for 5min.

3.2.4 Cantilever preparation

Tipless silicon nitride AFM cantilevers (NanoWorld, Neuchâtel, Switzerland) were soaked in acetone for 5min, UV irradiated for 15min, incubated overnight in 0.5mg/ml biotin-BSA at 37°C, rinsed and treated with 1% glutaraldehyde for 30s, rinsed, incubated in 0.5mg/ml streptavidin for 10min, rinsed, and incubated in 0.5mg/ml biotinylated Con A for 10min at 25°C.

Pyramid-tipped silicon nitride cantilevers (Bruker Probes, Camarillo, CA) for single-molecule experiments were functionalized with BCAM protein as follows. Cantilevers were silanized with 2% APTES for 10min, rinsed with DI water, immersed in 0.5%
glutaraldehyde for 30min, rinsed, incubated in BCAM solution (100μg/mL) for 30min, rinsed, and incubated in BSA (100μg/mL) for 5min.

### 3.2.5 Cell capture and experimental setup

Cells were allowed to bind weakly to the non-functionalized region of the glass substrate via 10min incubation at 37°C followed by rinsing with Alsever's solution, thus allowing for cantilever engagement with a chosen RBC. Single cells were captured by positioning the ConA-functionalized cantilever above the cell center (Figure 3-1a) and gently lowering onto the cell for ~30s with a contact force of 1nN near the end of the probe (Figure 3-1c) to prevent the cantilever surface from interacting with the laminin substrate. The cell was lifted from the surface and allowed to establish firm adhesion to the cantilever before
relocation to the laminin-functionalized region of the dish.

**Figure 3-1** Measurement of red blood cell adhesion to subendothelial matrix laminin using single-cell force spectroscopy. (a) The apex of a tipless ConA-functionalized AFM cantilever is positioned above a RBC. It is then gently pushed (~1 nN) for 30s onto the cell. The cantilever-bound RBC is separated from the substrate and allowed to establish firm adhesion to the cantilever for 1 min. The cantilever is then slowly moved to the functionalized segment of the petri dish to perform adhesion measurements. (b) Representative force-distance curves obtained from experiments for an RBC probing a laminin substrate. The measured detachment forces are 0.64nN in the first curve which shows a single rupture and 1.06nN in the second curve which shows multiple rupture events. (c) Optical microscopy image showing a human RBC attached to the apex of a ConA-functionalized cantilever.
3.2.6 AFM setup

Experiments were conducted using the MFP-3D-BIO AFM (Asylum Research, Santa Barbara, CA) mounted on an inverted optical microscope (Zeiss Axiovert A1). For SCFS, the nominal spring constant of the employed cantilever was 80mN/m. For SMFS, the nominal spring constant of the employed cantilever was 30mN/m. The effective spring constant was obtained via a thermal noise-based method and used in all calculations [43]. All experiments were performed in Alsever’s solution at 37°C, and biochemical modulators were added 30min prior to experiments. For each assay, 5 RBCs were tested from each subject’s blood. Values reporting “N=” denote the number of subjects per condition and “n=” denote the total number of tested cells.

Each SMFS measuring session involved probing a 1µm x 1µm region with a lateral resolution of ~31.2nm. For substrate measurements, 3 areas of a functionalized substrate were chosen at random and probed. For RBC measurements, one 1µm x 1µm region was recorded per RBC. Experiments were performed at a retraction speed of 800nm/s. Only measurements with a single break between laminin on the cantilever tip and BCAM/Lu on the laminin substrate or RBC surface were processed.

Each SCFS measuring session involved testing a single RBC probe attached to a previously calibrated cantilever for 100 approach/retraction cycles on one section of a laminin-functionalized glass substrate. As monitored by optical microscopy, the captured cells rarely detached from the cantilever during pulling. Tests were performed with a trigger force of 500pN with a contact time of 0.1s (unless noted), and the cantilever was retracted at 800nm/s until the RBC completely detached from the laminin substrate.
Force-distance curves were used to determine the detachment force of the RBC from the substrate. A detachment force is defined as the maximum downward force associated with the force-distance curve [28]. The median effective spring constant for the cantilever-RBC-laminin adhesion complex was ~4.6pN/nm. We note that during the course of testing each specific cell the median effective spring constant did not change, meaning that the mechanical properties of the RBC membrane did not change during this experiment. Representative curves are shown in Figure 3-1b. Further, while we could have also computed the work of detachment [31], we preferred not to do so in order to properly compare the SCFS measurements with SMFS measurements.

For both assays, we found that at 800nm/s hydrodynamic forces do not significantly affect detachment measurements (Supplementary Material). We considered only measurements for which the effective spring constant of the adhesion complex was less than 10pN/nm with a median value of ~4.6pN/nm.

3.2.7 Statistical methods

Values for detachment forces are reported using box-and-whisker plots and frequency distributions. In the frequency distributions reported for SCFS experiments, each bin contains the number of detachment forces with values within the bin’s range divided by the total number of approach/retraction cycles. For SMFS, frequency (%) is defined as the total number of all non-zero force points divided by the total number of sampled sites for each experimental condition.
3.3 Results And Discussion

3.3.1 Surface density of laminin for functionalized substrates

To obtain the experimental parameters used in our SCFS assay, we first determined the appropriate solution concentration of laminin to be used for substrate functionalization. SMFS experiments were performed using a BCAM-functionalized probe against substrates prepared with various solution concentrations of laminin (1µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml). For each substrate, 1024 measurements were recorded in three different 1µmx1µm areas yielding a total of 3072 measurements for each laminin solution concentration.

The detachment force of BCAM with laminin was nearly constant across all solution concentrations (Figure 3-2b). Interestingly, this detachment force was larger than what we previously measured for the inverse interaction between BCAM on the RBC and laminin on the AFM probe using SMFS [15, 26]. We conjecture that more BCAM proteins attached to the AFM tip make contact with multiple laminin proteins on the substrate since we recorded multiple distinct rupture forces. A representative curve is shown in Figure 3-1b. BCAM proteins are approximately 6nm [44, 45], whereas laminin is a cruciform structure with arms of lengths ranging between 40nm and 80nm [46]. Upon functionalizing a substrate with BCAM and measuring the detachment force using a laminin-functionalized probe, we confirmed that the median unbinding force is 41.4pN, in agreement with previous measurements (Figure 3-2a).
Figure 3-2 Determination of laminin solution concentration to be employed in SCFS assay. (a) Frequency distributions obtained from SMFS experiments of measured detachment forces between (i) a substrate coated with laminin (50 µg/ml) and a BCAM/Lu probe, and (ii) a substrate coated with BCAM (50 µg/ml) and a laminin probe. (b) Box-and-whisker plots of the unbinding forces measured at each tested laminin solution concentration shows that the median values are nearly consistent (0 µg/ml: 66.1pN; 1 µg/ml: 182.2pN; 5 µg/ml: 225.0pN; 10 µg/ml: 185.3pN; 25 µg/ml: 144.8pN; 50 µg/ml: 138.9pN; Mann-Whitney test for 25 µg/ml vs. 50 µg/ml). (c) Plots of the substrate density measured at each tested laminin concentration shows that the density appears to reach a plateau at 25 µg/ml (median values at 0 µg/ml: 1.63%; 1 µg/ml: 10.4%; 5 µg/ml: 4.3%; 10 µg/ml: 10.0%; 25 µg/ml: 7.8%; 50 µg/ml: 10.9%; Kruskal-Wallis one-way test for 10 µg/ml, 25 µg/ml, and 50 µg/ml). The median values and the respective ranges are shown. (d) Frequency distributions obtained from SCFS experiments of detachment forces between a RBC probe and laminin substrate (50 µg/ml) measured in the (i) absence and (ii) presence of bath laminin. The significant reduction in the number of curves showing detachment forces, from 66% to 12% (p<0.0001) demonstrates the specificity of the measurements. (e) Box-and-whisker plots of detachment forces for human RBC probes (N=2, n=10) at each laminin solution concentration. The median values of the detachment forces were found to increase with higher laminin concentrations (0 µg/ml: 130.3pN; 1 µg/ml: 113.9pN; 5 µg/ml: 215.8pN; 10 µg/ml: 243.1pN; 25 µg/ml: 154.9pN; 50 µg/ml: 559.9pN; 100 µg/ml: 678.0pN), appearing to plateau at the 50 µg/ml concentration. (f) Plot of RBC binding frequency shows an increase in binding as laminin substrate concentration increases (median values at 0 µg/ml: 16.5%; 1 µg/ml: 34.2%; 5 µg/ml: 43.0%; 10 µg/ml: 96.0%; 25 µg/ml: 46.6%; 50 µg/ml: 39.4%; 100 µg/ml: 72.4%). The median values and the respective ranges are shown.
For laminin-functionalized substrates, the percentage of sampled sites where a detachment force was detected, reflecting the density of laminin on the substrate, increased with higher laminin concentrations (Figure 3-2c; hyperbolic fit). Variability reflects different substrate and probe preparations. To determine background noise, measurements were recorded on a substrate where the laminin-functionalization step was omitted. Interactions were detected in 4.1±0.65% of sampled sites.

We next determined the effect of different laminin solution concentrations on the adherence of RBC probes to laminin substrates. Background noise was quantified by using a RBC to probe a substrate where the laminin-functionalization step was omitted. Small forces with a median of ~130pN were detected in 16.5% of curves. The interaction specificity was demonstrated by performing experiments in 100μg/mL bath laminin, thus blocking BCAM/Lu receptors. By recording approach/retraction cycles between RBC probes and a substrate functionalized with 50μg/ml laminin, the number of curves showing detachment forces reduced from 66% to 12% (p<0.0001, Figure 3-2d) while the median detachment force was ~112pN, similar to background noise. Further, detachment forces recorded in bath laminin decreased five-fold, from a median value of 559pN to 106pN (p<0.0001) reflecting bath laminin bound to many BCAM/Lu receptors which significantly reduced the propensity for binding of the RBC probe to the laminin substrate.

RBC probes were used to record forces against each laminin substrate. Detachment forces were found to increase with higher laminin solution concentrations (p<0.0001), appearing to stabilize around the 50μg/ml concentration (Figure 3-2e). For this concentration, we obtained force curves characterized by median detachment forces up
to 0.56nN. In contrast, lower concentrations resulted in median detachment forces of less than 0.24nN, reflecting the need for a denser substrate laminin concentration to maximize BCAM/Lu receptor binding. These detachment force values are comparable to those observed for RBCs in other experiments [40]. Moreover, the frequency of RBC binding to the laminin substrate also directly increased with laminin concentrations (Figure 3-2f; p<0.0001).

From the experiments employed to determine the appropriate solution concentration of laminin for substrate functionalization, we found that detachment forces appear to plateau at the 50µg/ml laminin concentration (Figure 3-2e), whereas the surface density detected via SMFS appears to plateau at ~10µg/ml (Figure 3-2c). Tethered polymer layers are comprised of polymer molecules attached to a surface, with a density high enough to cause the chains to stretch away from the surface [47, 48]. By maximizing the brush height, the free-energy cost is minimized, thus minimizing inter-chain repulsion. This may affect the binding of tested cells to proteins attached to a surface. The surface distribution
of a tethered polymer layer can be characterized by the distance between attachment points, $D$. As $D$ approaches the radius of gyration, $R_g$, of the polymer chain, the molecules interact and the polymer layer structure passes from the single-chain (mushroom) regime to the brush regime. In the 'mushroom' regime the reduced tethered density, $\Sigma = \sigma \pi R_g^2$, where $\sigma = 1/D^2$, is $\Sigma < 1$, the 'mushroom-to-brush' transition regime occurs for $1 < \Sigma < 5$, and the transition to 'brush' regime occurs at $\Sigma = 5$. The value $\Sigma = 1$ corresponds to $D = D_2 = \sqrt{\pi R_g}$ [49]. For the case of laminin, where $R_g \cong 80 \text{ nm}$ [50, 51], the 'mushroom' regime appears for $D_1 > 140 \text{ nm}$ and the transition to 'brush' regime occurs at $D = D_2 = D_1/\sqrt{5} \cong 63 \text{ nm}$. Because the lateral step size was $\sim 31.2 \text{nm}$ in SMFS experiments, after the first transition point is reached, it is difficult to distinguish between different surface densities. Based on Figure 3-2c, we conjecture that the first transition point, from 'mushroom' to 'mushroom-to-brush', was reached at the 10μg/ml laminin concentration. This is reinforced by the fact that the binding frequency for SCFS experiments plateau at the same laminin concentration, meaning that at this concentration, most of the substrate is covered with laminin chains in the 'mushroom' form. We found however, that the detachment force reached a plateau at $\sim 50 \mu g/ml$ solution concentration, meaning that the binding between all active RBC adhesion receptors and laminin molecules reaches equilibrium. We conjecture that this is the second transition point, from 'mushroom-to-brush' to 'brush' regime, and determined the corresponding concentration of 50μg/ml to be most suitable for SCFS assays of human RBCs.
3.3.2 Dependence of BCAM/Lu-mediated RBC adhesion on contact time

To investigate the time dependence of cell adhesion, we varied the contact time of RBCs with 50µg/ml laminin substrates between 0.1s and 10s and determined the detachment forces. For the 0.1s contact time, the median force required for RBC detachment from laminin substrates was ~0.63nN. As the contact time increased to 1s, the median detachment force increased to ~0.95nN. For contact times greater than 1s and up to 10s, the median detachment force did not significantly change (Kruskal-Wallis test). We opted to use the 0.1s contact time for experimental convenience because at higher time scales the RBC had a greater probability of detachment from the probe prior to the completion of 100 cycles resulting in experimental failure.

3.3.3 Increases in SCFS detachment forces correlates with increases in total forces recorded by SMFS on 1 µm² membrane regions

To determine the relationship between single-molecule and single-cell measurements, we performed both assays using n=5 cells from human subjects (N=3). SMFS experiments probed 1µmx1µm areas of the RBC surface with a lateral resolution of ~31.2nm, enabling us to measure single-rupture events. For the SCFS assay, RBCs probed a laminin-functionalized substrate (50µg/ml laminin solution concentration) with a contact time of 0.1s. For comparison of SMFS to SCFS, the resultant total force associated with each 1µmx1µm membrane area was calculated as the product of the frequency of detected events and the mean detachment force. For example, an area with a mean detachment force of 39.51pN detected in 2.83% of sampled sites yields a total force of 1.15nN. Resultant total forces ranged from 0.77nN to 3.92nN. In contrast,
detachment forces obtained between single RBCs with a laminin substrate were lower, ranging from 0.11nN to 1.8nN (Figure 3-4a). The mean detachment force for an entire RBC corresponds to the simultaneous formation of ~20 active bonds between BCAM/Lu proteins on the RBC surface and laminin on the substrate, calculated by dividing the average single-cell detachment force by the average single-molecule detachment force. The median detachment force of one BCAM/Lu receptor from laminin was found to be 40.62pN. We note that the SCFS technique reflects the overall RBC adhesion to the substrate, whereas SMFS measurements represent direct ligand-receptor interactions.

Since multiple cells were tested from each subject, detachment forces could be compared between subjects. The Pearson correlation test was used to determine the strength of relationship between SMFS and SCFS assays (Figure 3-4b). We found that SMFS resultant mean total force correlated strongly with the mean detachment force obtained via SCFS ($r=0.99$, $R^2=0.99$). Due to the fact that SCFS measurements reflect currently active bonds, as well as innate cellular features, they can be used as an innovative tool to study RBC adhesion. However, it is important to note that detachment of the cell from the substrate is a complicated process, and the detachment force obtained is a result not only of receptor-ligand binding strength and spatial distribution of receptors on the cell membrane but also of other factors such as membrane fluctuations, cellular elasticity, and cell shape [28, 52, 53]. RBC deformability plays a significant role in SCFS measurements, whereas SMFS measurements represent direct ligand-receptor interactions. Increases in RBC compliance may allow the cell to spread, leading to an increased number of ligand-receptor interactions and in effect, greater detachment forces measured via SCFS [42].
Figure 3-4. Correlation of single-cell measurements with traditional SMFS measurements. (a) Box-and-whisker plot of the detachment forces from SMFS and SCFS assays. Experiments were performed on 5 cells from each human sample (N=3). SCFS experiments used single RBCs to probe a laminin functionalized substrate (50µg/ml) with a contact time of 0.1s. SMFS experiments probed BCAM/Lu receptors on 1 µm x 1 µm areas of the RBC surface with a laminin-functionalized probe. The resultant total force for each area was calculated as the product of the frequency of detected events and the mean detachment force. (b) Correlation of the mean resultant total force on 1 µm x 1 µm areas of the RBC surface obtained via SMFS with the mean detachment force obtained via SCFS measurements (Pearson correlation test; r=0.99, R²=0.99). Each plotted point represents 5 tested cells from one healthy subject.

3.3.4 cAMP-dependent modulation of red blood cell adhesion to laminin via BCAM/Lu

To establish the sensitivity of the assay in the adhesion of BCAM/Lu to laminin, we investigated changes in the detachment force of single RBCs with laminin via pharmacologic modulation of the cAMP-signaling pathway. Previous experimental approaches utilizing flow adhesion assays have shown that the interaction between BCAM/Lu and laminin is mediated by cAMP-dependent PKA [54]. Stimulation of the β2-
adrenergic receptor (β2-AR) [55, 56] activates the G-protein coupled receptor Gαs, which stimulates adenylyl cyclase (AC) [57]. AC catalyzes the conversion of adenosine triphosphate (ATP) to cAMP, resulting in PKA activation and ensuing BCAM/Lu-laminin adhesion. In human RBCs, basal cAMP levels are controlled through the activity of phosphodiesterases [58, 59], which limit the signaling lifespan of cAMP and the distance it can diffuse from its production site [60].

RBCs were treated with forskolin (30μM, FSK), a strong AC activator. Compared to untreated RBCs, forskolin-treated RBCs exhibited significantly higher detachment forces (Figure 3-5a,b,e; p<0.0001, Mann-Whitney U test). To quantify the effect of direct PKA inhibition on the RBC detachment force from laminin, RBCs were treated with KT-5720 (1μM), a PKA inhibitor, which resulted in a significant decrease in detachment forces from baseline (Figure 3-5c; p<0.0001). Based on these results, we conjecture that RBCs maintain varying intracellular levels of tonic PKA [60, 61]. We speculate that this tonic PKA is modulated by cAMP levels and must be raised for the activation of BCAM/Lu receptors to occur, resulting in elevated adhesion to subendothelial laminin. The reduction
Figure 3-5. Single-cell force spectroscopy can clearly detect significant changes in RBC adhesion to laminin substrates (50µg/ml) following treatment with biochemical agents modulating the cAMP-dependent pathway. (a) Frequency distribution of the detachment forces associated with healthy RBCs at baseline (N=3, n=15) shows a
in the number of active BCAM/Lu receptors on the RBC surface following inhibition of PKA activity with KT-5720, reflected by the significant decrease in detachment forces, is suggestive of a tonic drive. Even in the absence of PKA inhibitors and activators, PKA exerts a sustained, steady-state activation of BCAM/Lu receptors. Finally, the physiologic implication of cAMP-stimulated adhesion was studied by treating RBCs with epinephrine (1 µM), a hormone which stimulates the β2-AR. This resulted in a significant increase in the detachment forces compared to baseline (Figure 3-5d; p<0.0001). These data demonstrate that the single-cell AFM assay can detect differences in the detachment of whole RBCs from a laminin substrate in response to pharmacologic modulation (Figure 3-5e).

### 3.4 Conclusion

SCFS was established as a method to quantify the strength and modulation of adhesion of human RBCs to functionalized substrates. Using SMFS and SCFS, we determined the
appropriate solution concentration of laminin to utilize for substrate functionalization via the density of protein adsorption to the substrate. From these tests, we determined the 50µg/ml solution concentration of laminin to be most effective. Then, we probed laminin substrates with RBCs using contact times ranging from 0.1s to 10s. We chose 0.1s because of the low potential for experimental failure prior to the completion of 100 approach/retract cycles and for comparison between single-molecule and single-cell measurements. We found a significant correlation between results from SMFS measurements probing BCAM/Lu with a laminin-functionalized probe and SCFS measurements probing a laminin substrate with an RBC probe. Finally, we established that SCFS can detect variations in the detachment force of RBCs to laminin based primarily on the number of active BCAM/Lu receptors, which was modulated via biochemicals affecting the cAMP-PKA pathway. This study shows important implications for AFM-based SCFS measurements in understanding and evaluating the pharmacologic response of adhesion receptors on RBCs.

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Chapter 4: Red Blood Cell Specific Adhesion and Mechanical Properties in Microfluidics
4.1 Introduction

Sickle cell disease (SCD) is a genetically inherited disorder characterized by painful vaso-occlusive crises in which blood flow to parts of the body is obstructed [1]. Vaso-occlusion is the result of interactions between blood cells and the endothelium [2-4]. It has been shown that the adhesive behavior and viscoelastic response of SS-RBCs are important factors in vaso-occlusion [8]. SS-RBC adhesion to specific proteins, such as human integrin $\alpha_v\beta_3$, expressed on the endothelium has been implicated as part of the mechanism that triggers vaso-occlusive crises [9, 10]. During a vaso-occlusive crisis, adherent SS-RBCs in the vasculature obstruct other SS-RBCs due to their viscoelastic and mechanical properties [13, 14].

A number of experimental tools have been used to quantify SS-RBC adhesion to proteins expressed on the endothelium. To this end, atomic force microscopy (AFM) has been employed in single-molecule force spectroscopy (SMFM) and single-cell force spectroscopy (SCFS) assays [10, 15, 16]. These assays offer insight into molecular level interactions, but only a few RBCs can be tested this way under each condition. In contrast, perfusion chambers and microfluidic devices have been utilized with the same aims [11, 70]. These setups provide the potential for concurrent high-throughput assays that are more clinically relevant than AFM-based techniques. However, current microfluidic assays face many design challenges in terms of functionalization and flow control. Specifically, physical adsorption of targeted proteins directly on the microchannel surfaces has become a staple of microfluidic assays due to the ease of application and because of the difficulty in utilizing other strategies when microchannels are assembled.
using oxidative sealing [71]. Unfortunately, this functionalization technique produces weak and unstable immobilization bonds between the proteins and the substrate. In addition, proteins do not bind specifically, orient themselves randomly, and are capable of forming as many bonds as they have binding sites [72]. This can lead to experimental protocols that are marred by background RBC adhesion to the substrate itself and adhesion to protein binding sites that are not of interest to the specific study. On the other hand, the use of volumetric flow pumps in microfluidic assays prevents instant changes in flow parameters, thus severely limiting options in assay design.

The mechanical properties of wildtype RBCs have been explored both empirically and in simulations [73-75]. As such, optical tweezers and micro-pipettes have also been employed to study SS-RBC viscoelastic properties [8, 76]. Also, the mechanical properties of SS-RBCs have been investigated using AFM [77]. Because these assays feature direct manipulation of individual SS-RBCs they are time consuming, have low throughput, and contain generalizations and potential errors arising from the type and shape of the device. Tangentially, a microfluidic assay enabled by a custom made pressure driven flow has been used to study viscoelastic properties of sickle cell trait RBCs [78].

In this work, we propose a microfluidic approach that addresses the complications found in adhesion assays by using a functionalization strategy in which a known protein binding site covalently bonds with the substrate in order to create a uniformly dense functionalized surface. In order to facilitate the functionalization, the microchannel is assembled through
aspiration, which keeps the functionalized areas inside of the microchannel untouched [79].

In concert with this design, a pressure controlled pump allows us to introduce a well-controlled flow inside the microchannel. The use of a pressure pump allows us to quantify SS-RBC adhesion to a functionalized substrate not only by the fraction of remaining adherent RBCs at the end of each experiment but also by their ability to withstand wall shear stress increases during experiments. This feature empowers us to quantify the mechanical properties of SS-RBCs by instantly modulating the flow while tracking the morphological changes in SS-RBCs that have adhered directly to a glass substrate.

4.2 Materials and Methods

4.2.1 Equipment

Precise and steady control of microfluidic flow was achieved with a pressure controller (OB1 Microfluidic Flow Control System; Elveflow, Paris, France). Both the inlet and outlet ports of the microfluidic device were independently controlled by the pressure controller in order to increase precision. The microchannel was observed and recorded digitally by an Orca-Flash 2.8 high speed camera (Hamamatsu Photonics K.K.; Hamamatsu City, Japan) attached to an Olympus IX73 inverted microscope (Olympus Corporation; Shinjuku, Tokyo, Japan). A diagram of the setup is shown in Figure 4-1A. In adhesion experiments a 20x magnification objective lens, numerical aperture (NA) of 0.45, was
used, while recovery experiments utilized a 40x magnification objective lens, NA of 0.6. The high speed camera captured 45 frames a second. A custom LabVIEW user interface
was used to control both devices digitally and record pressure readings and video footage simultaneously.

4.2.2 Microchannel fabrication

Polydimethylsiloxane (PDMS) microchannels were created using photolithography to give all features a height of approximately $32\,\mu m$. The master mold was created following standardized permanent epoxy negative photoresist processing guidelines provided by MicroChem (USA; Westborough, MA). To summarize briefly, the master mold was created by covering a 100 mm diameter silicon wafer with SU-8 2025 negative photoresist, spin coated to achieve the desired feature height, covered with the mask of the design, and exposed to ultraviolet (UV) light in order to crosslink the polymer. The relief structure was then developed using 1-Methoxy-2-propyl acetate (SU-8 developer) and covered with PDMS prepolymer, which was then cured to create the PDMS elastomer. The PDMS microchannels were cut from the wafer and peeled off from the master mold. The microchannel device featured a 15 mm long and 1 mm wide microchannel, connected at either end to 6 mm diameter antechamber wells. The size of the wells was necessary to promote structural stability of the microchannel.

The microfluidic device was attached, through aspiration, to a functionalized glass slide substrate (See Figure 4-1B) [79]. This method of assembly allows substrates to be functionalized before the device is assembled since the functionalization is not altered or destroyed during assembly, as in oxidative sealing. Truncated and polished 20 gauge needles were employed as the inlet and outlet ports of the microchannel, and were connected to 6 mm diameter antechamber wells of the microfluidic device. The aspiration
network was connected by a 2mm diameter tygon tube to a vacuum pump that supplied a negative pressure of 75 kPA. The microchannel was brought into contact with the functionalized glass slide substrate while submerged in phosphate buffered saline (PBS), in order to prevent the formation of an air bubble inside the microchannel.

4.2.3 Functionalization

Human integrin $\alpha_v\beta_3$ (EMD Millipore; Billerica, MA, USA) was covalently bound to an N-hydroxysuccinimide (NHS) functionalized glass slide (NHS surfaces; MicroSurfaces, Inc., Englewood, NJ, USA) in order to facilitate a uniform coating [72]. In order to bind the protein, it was deposited onto the slide at a concentration of 270 mg/mL in Triton-X 100 and incubated on the slide for 20 minutes at 37°C. The excess solution was then rinsed off the slide with PBS, the slide was coated with a proprietary buffer solution (MicroSurfaces, Inc., Englewood, NJ, USA) to remove unbound NHS groups and incubated for 30 minutes at room temperature to this end. The excess buffer solution was rinsed off the slide with PBS. The functionalized slide was then stored in PBS and immediately used.

4.2.4 Sample Preparation

This study was approved by the Institutional Review Boards of the UCONN Health Center and UCONN-Storrs. Volunteers with SCD, at least 18 years of age, were eligible to participate in this study. RBCs from consented volunteers were separated from freshly drawn, heparin-anticoagulated, venous blood by centrifuge, at a relative centrifugal force of 500g for 10 minutes at 4°C. Afterward, the buffy white coat and plasma were removed by aspiration, and the RBCs were washed three times with Alsever's solution, a saline
solution used to store blood [80]. RBCs were diluted down to a hematocrit of 0.5% for the adhesion assay and 1% for the viscoelasticity assay in Alsever's solution before being introduced into the microchannel. This hematocrit was selected in order to maximize the acquisition of useful images during video recording, such that there were as many non-overlapping RBCs in each frame as possible.

Epinephrine was added to the SS-RBC solution to produce a final concentration of 16.4 nM. The SS-RBCs solution with Epinephrine was incubated for 30 minutes before the experiment was conducted. Propranolol was added to the SS-RBC to produce a final concentration of 10 μM. The SS-RBC solution with propranolol was incubated for 30 minutes before the experiment was conducted.

4.2.5 Adhesion Assay

SS-RBCs from one concenting patient in Alsever’s Solution (Sigma-Aldrich, St. Louis, MO, USA) at a 0.5% hematocrit were introduced into the microchannel and allowed to adhere to the functionalized substrate for 10 minutes at a pressure drop that produced a 0.1 dynes/cm² wall shear stress. After this initial period, all RBCs in a 3 mm² area inside the microchannel were recorded. The pressure drop across the microchannel was then increased to produce 1, 5, 10, 20, and 30 dynes/cm² wall shear stress and the adherent RBCs were recorded in the same 3 mm² area 1 minute after each increase. After all wall shear stresses measurements were taken, the microchannel was discarded.
4.2.6 Viscoelasticity Assay

Untreated, baseline, SS-RBCs from one concenting patient, in Alsever's Solution at a 1% hematocrit were introduced into the microchannel and allowed to adhere to the glass substrate for 10 minutes with no pressure drop across the microchannel. The recoveries of adherent SS-RBCs due to the instant change in wall shear stress from \(1 \text{ dynes/cm}^2\) (physiological wall shear stress) to \(0 \text{ dynes/cm}^2\) were recorded.

4.2.7 Data Processing

A custom MATLAB script was used to detect and quantify adherent RBCs. A background image of each recorded video was created by averaging all individual pixel values over all frames. This filtering method obfuscates all moving objects and retains all stationary ones in the background image. The background was then cropped to the area of interest, devignetted, and sharpened using unsharp masking. The edges of all RBCs were quantified by the Canny edge detection algorithm. The convex hulls of all object edges were then labelled, and sorted based on size: objects smaller than a potential RBC (smaller than \(4 \mu m\) in diameter) were discarded while objects larger than a potential RBC (larger than \(12 \mu m\) in diameter) were broken up into multiple objects using the watershed algorithm. The final set of objects were again sorted by size such that objects too small (smaller than \(4 \mu m\) in diameter) or too large to be an RBC (larger than \(12 \mu m\) in diameter) were discarded. The remaining objects were counted and overlaid on the original image, so that the computed result could be double checked and adjusted manually to ensure its accuracy (see Supplementary Figure 4-S1).
In viscoelastic experiments, RBCs were identified on a frame by frame basis after the frames were cropped, sharpened, and devignetting. Each individual RBC was identified and tracked throughout a video. The tracking was automated using a custom MATLAB code, and the results were verified manually and corrected where necessary. RBC morphology was determined by fitting the identified shape of the RBC with an ellipse. Only adherent yet freely deformable and mobile RBCs were selected, manually, for processing (see Supplementary Figure 4-S2 for an example of SS-RBC morphology at different applied wall shear stresses).

Though these simple MATLAB codes have been highly customized for our use and are of little scientific interest in and of themselves, we have made them available online (http://www.engr.uconn.edu/~gelyko/additionalsoftware.html) as an example of the exact processing logic, algorithms, and working parameters we used.

4.3 Results and Discussion

4.3.1 Adhesion Assay

One patient’s SS-RBC’s affinity to adhere to a \( \alpha_v\beta_3 \) functionalized substrate was modulated by epinephrine, an agonist, which has been shown to increase SS-RBC adhesion to the endothelial protein [9]. Epinephrine produces this effect by upregulating cAMP in RBCs and stimulating protein kinase A, which increases SS-RBC adhesion [2]. Consistent with previous findings, the adherent population of SS-RBCs treated with epinephrine was not only greater but resisted higher wall shear stresses than SS-RBCs not treated with epinephrine (See Figure 4-2). Additionally, we predict that antagonists,
such as propranolol, may not only block the action of agonists, but actively reduce SS-RBC adhesion to a $\alpha_v\beta_3$ functionalized substrate. Consistent with this conjecture, we found that the adherent population of SS-RBCs treated with propranolol was lower and could not withstand wall shear stresses as high as that of untreated SS-RBCs (see Figure 4-2).

**Figure 4-2.** Adherent populations of untreated SS-RBCs alongside those treated with epinephrine and propranolol relative to their respective initial adherent populations to a substrate coated with human integrin $\alpha_v\beta_3$ at increasing wall shear stresses. When treated with epinephrine, SS-RBCs adhere in greater relative numbers at larger stress than when untreated. SS-RBCs treated with propranolol appear to adhere in fewer relative numbers than untreated SS-RBCs. Due to the large number of initial SS-RBCs ($n>300$ for each data point), the 95% confidence intervals of each data point, if displayed, would be obscured entirely by the symbols representing the measured values.

Each measurement is based on a minimum of 300 initially adherent RBCs spread uniformly over a 3 $mm^2$ area. Background adhesion, evaluated with a slide coated only with the background PEG layer, was found to be negligible below a shear stress of $5 \frac{dynes}{cm^2}$ and non-existent above (data not shown). Thus, we are quantifying, almost exclusively, SS-RBC adhesion to human integrin $\alpha_v\beta_3$. By incrementally increasing wall shear stress on the group of adherent SS-RBCs and recording each change, we are able to
meaningfully measure the strength of adhesion of SS-RBCs to the specific receptor $\alpha_v\beta_3$ with high throughput. Due to this uniformity and the large number of processed cells, we expect that the reported values are true for the entire sample and that this precision will remain consistent in future patient samples. As with AFM measurements, we expect to observe large variability between samples drawn from different patients and even samples drawn from the same patient on different dates. However, when we treated the patient sample with epinephrine and propranolol the modulation of the adhesion was in agreement with the expected trend that we have previously reported based on AFM. We expect that using the proposed method we will be able to measure the same modulation in future patient samples.

We note that the adhesion levels of individual RBCs from the same sample are different from cell to cell. This has been observed in whole RBC adhesion experiments where the adhesion force between a RBC and a functionalized substrate was measured via AFM [16] and in single molecule force spectroscopy experiments where the expression of active adhesion receptors was measured [15, 81]. Because of this, we expect that the same level of wall shear stress will not have the same effect on all RBCs in terms of detachment from the substrate. Nonetheless, we observed that higher wall shear stress results in a lower adherent fraction and after a sufficiently high value almost, depending on the RBC adhesion level, all RBCs are detached.

4.3.2 Continuous Flow and RBC Elongation

Based on the pressure pump’s internal pressure sensors, which record pressure drop experienced across the microchannel every 0.1 seconds, we have found that the
The response time of our microfluidic system is 0.1 seconds at the slowest. Observations of RBC morphology suggest that the system responds even faster and clearly indicate that continuous input pressure changes are experienced across the microchannel corresponding to continuous pressure changes. We have verified this by applying an oscillating pressure drop across the channel and simultaneously measuring the major and minor axes of an RBC as it undergoes recovery from a wall shear stress of \( 7 \text{ dynes/cm}^2 \) near the 2 second mark. The RBC recovery is curve-fit to the solution of the Kelvin-Voigt model in which no external forces are present. Box plots of the measured shear modulus and viscosity of adherent SS-RBCs \( (n = 21) \) as they recovered from 1 \( \text{ dyne/cm}^2 \) wall shear stress. The notches on the box-plots represent the 95% confidence intervals of the measured medians.
minor axes lengths of an adherent RBC and evaluating its elongation index as $EI = \frac{\text{major axis} - \text{minor axis}}{\text{major axis} + \text{minor axis}}$ (see Figure 4-3A).

### 4.3.3 SS-RBC Recovery

The viscoelastic properties of the SS-RBC membrane were quantified by measuring the shear modulus and viscosity using the Kelvin-Voigt model for a Neo-Hookean near incompressible material with linear viscous response:

$$\frac{T_s}{2\mu} = \frac{1}{4} (\lambda^2 - \lambda^{-2}) + t_c \frac{\partial \ln(\lambda)}{\partial t},$$

where $\mu$ is the membrane shear modulus, $t_c \equiv \frac{\eta}{\mu}$ is the characteristic time, $\eta$ is the membrane viscosity, $\lambda = \frac{\text{deformed major axis RBC length}}{\text{undeformed major axis RBC length}}$ is the extension ratio, and $T_s = \frac{T_1 - T_2}{2}$ is the membrane shear stress where $T_1 = \frac{\tau \cdot \text{deform RBC area}}{\text{deformed RBC minor length}}$ and $T_2 = 0$ are the in-plane principal membrane stresses, and $\tau$ is the wall shear stress applied on the RBC [75, 82]. The shear modulus was obtained via the expression $\mu = \frac{2T_s}{(\lambda^2 - \lambda^{-2})}$, where $\lambda$ is the extension ratio between two steady-state conditions. The viscosity was evaluated as $\eta = \mu * t_c$ where $t_c$ was found by curve fitting the relaxation data of the instantaneous extension ratio $\lambda(t)$ with the analytical solution $\lambda(t) = \sqrt{\Lambda + \exp(-t/t_c) \Lambda - \exp(-t/t_c)}$ of the governing equation for zero applied membrane forces (see Figure 4-3B), where $t$ is the time, $\Lambda = \frac{\lambda_m^2 + 1}{\lambda_m^2 - 1}$, and $\lambda_m$ is the extension ratio at the instance when the applied membrane forces are set to zero.
The viscosities and shear moduli of SS-RBCs were measured using this method as shown in Figure 4-3C and 4-3D. The measured shear moduli are of the same order of magnitude, though nominally higher, as those reported by [82]. This is consistent with our expectations, since the aforementioned work investigated wild type RBCs and previous efforts from our lab have measured higher Young’s Moduli in SS-RBCs with respect to their wild type RBC counterparts [83]. The time constant, $t_c$, was found to be on the same order of magnitude as the original findings by [75], signifying that the reported viscosity measurements are in agreement with both works. The width of the 95% confidence of the shear modulus measurements ($\sim 2 \frac{\mu N}{m}$) is a product of the large number of processed RBCs, and we expect future processed samples to continue to produce measurements of comparable precision.

4.3.4 Conclusion

The implemented experimental setup presents an opportunity to quickly and precisely quantify the mechanical and pharmacological properties of SS-RBCs in many different assays. This is enabled by the combination of equipment, specifically the high-speed camera and the precise and instantly modifiable pressure flow, the surface functionalization protocol, and custom image processing software. Minor tweaks to the assay parameters, such as the concentration of endothelial protein during functionalization, may also be appropriate to maximize the sensitivity of the system and elucidate SS-RBC properties. The current setup can be easily modified to enable experiments under controlled oxygen levels.
These experimental methods may potentially also be conducted in similar assays on wildtype RBCs or in efforts to study other related diseases, such as malaria or diabetes, where either RBC adhesion or mechanical behavior plays a major role in pathology. The same advantages and improvements presented in this report may prove to be applicable is such studies. Thus, this potential is an avenue for future work in this area.

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4.4 Supplementary Information

**Supplementary Figure 4-S1.** The intermediate outputs of the custom video data processing MATLAB script used to detect and quantify adherent RBCs. **A.** A typical frame of a recorded video. **B.** The background of the video. Note that only adherent RBCs can remain in the image. **C.** The devignetted, cropped background. **D.** Output of the Canny edge detection algorithm on the processed background. **E.** The convex hull regions of all edges. **F.** Independent regions are identified, labelled, approximated by an ellipse, processed based on size (rejected or split), and finally the approximated ellipses are overlaid over the devignetted and cropped video background. The user adjusts program count by accounting for missed or lumped cells as well as false-positive identification of objects other than SS-RBCs.
**Supplementary Figure 4-S2.** The approximated morphology of a SS-RBC, denoted cell 2, that has been deformed at **A** $7 \text{ dynes/cm}^2$ wall shear stress, **B** $5 \text{ dynes/cm}^2$ wall shear stress, and **C** $0 \text{ dynes/cm}^2$ wall shear stress.
Chapter 5: Assembly for Measuring the Viscosity of Fluids Using Microchannels
5.1 Introduction

The viscosity of nearly all liquid products, whether consumer, industrial, or medical in nature, must be ascertained during both formulation and quality assurance. The viscosity of body fluids, such as whole blood and blood plasma has been shown to be a powerful tool in cardiovascular health evaluation and in monitoring several diseases. For instance, non-medical rheology measurements are important in both the development and the quality assurance of inks[113], paints[114], petroleum[115], cosmetic products[116, 117], oils[118], coatings[119], adhesives[120], consumer care products[121], extrusion polymers[122], foods and beverages[116, 117, 123], and pharmaceutical formulations[117, 124].

Both viscometers and rheometers suffer from numerous drawbacks. Most notably, such devices require a significant amount of sample liquid, typically a full vial. This requirement often makes such instruments unfeasible in applications where the sample is expensive, such as in bioprinting inks. Rheometers are also typically heavy and require peripheral equipment to operate. This immobility prevents technicians from bringing the device to the sample. Constant cleaning is necessary between the measurement of every sample. The measurement process itself can be very slow. One sample can take half an hour or more to process. Such a long processing time may exclude samples which undergo a change, such as adhesives, inks, and epoxies. Many affordable models also necessitate the need for a trained technician with an engineering background and a familiarity with the theoretical expressions exploited by their device. Without double checking the measurement data, these instruments may produce results that are 10 or 100 times off
from reality. The price of trained personnel combined with the long measurement times can costs companies significant sums. Instruments which fix most of these issues are orders of magnitude more expensive than their counterparts.

Due to the technological challenges involved, body fluid viscosity measurements such as whole blood viscosity (WBV) and blood plasma viscosity have not been a prevalent medical measurement despite evidence of their value in cardiovascular risk management and in evaluating cognitive ability in Alzheimer’s Disease. High WBV measurements have been strongly correlated with cardiovascular events, such as stroke and ischemic heart disease. A 1996 study measured the WBV of more than 1,500 people and followed up with them 5 years later in order to identify any cardiovascular events. The investigators found WBV to be a powerful long term predictor of cardiovascular events, stronger even than conventional risk factors such as LDL cholesterol, smoking, or diastolic blood pressure [125]. A recently published study that followed more than 3,000 subjects over the course of approximately 17 years found similar results for both WBV and plasma viscosity [126]. A number of other studies have reported comparable findings [127, 128]. An affordable point-of-care rheometer may also be of particular useful in Alzheimer’s Disease evaluation. WBV has been found to be correlated with cognitive ability and can be used as a biomarker in monitoring the disease [129, 130]. In Waldenström’s Macroglobulinemia (WM), which is a type of cancer that causes hyperviscosity syndrome (HVS), a condition in which the viscosity of blood is too high, oncologists actively need to monitor their patients’ blood plasma viscosity [131]. Other disease which produce the risk of HBS include multiple myeloma [132], paraproteinemia [133], and polycythemia [134]. Blood viscosity measurements may also be useful in monitoring risk of leg ulceration and
possibly vaso-occlusive episodes in sickle cell disease [135]. We further hypothesize that a small sample body fluid rheometer may also be of consequence in dialysis, diabetes monitoring, as well as in sports medicine.

5.2 Final Product

We are have designed a small-sample microfluidics-based rheometer that will provide viscosity results over a continuous range of shear rates in at most 5 minutes. Our device will not require peripheral equipment to operate and will also be portable. The device itself consists of two parts: the core device that conducts and records the measurements and a refillable microchannel cartridge that is the only component which comes in contact with any liquid sample. This is particularly useful for certain liquids, such as inks, adhesives, epoxies, and especially in body fluids because the device will not need regular cleaning or sterilization between uses. Furthermore, the cartridges will be tagged with an RFID chip that will provide important information to the core device in order to improve the fidelity of the measurement and ensure that only our cartridges can be used with the core device. Thanks to the nature of microchannels, the rheometer will only require a small volume of the sample liquid for a viscosity measurement. We anticipate that 500 \( \mu L \) of a sample will be necessary at most per measurement. For WBV measurements, medical practitioners will only need to administer a finger prick to their patients in order to acquire enough blood for a complete measurement and a full blood draw procedure will not be necessary. In bioprinting inks the use of this device will produce significant savings by reducing the waste of expensive sample. The device will also be able to measure plasma
viscosity, an already established laboratory measurement, as well as any low viscosity body fluids and industrial liquids.

To perform the measurement, our rheometer will continuously record the position of a liquid sample as it traverses a microchannel. This information can be used in combination with the microchannel dimensions to determine the sample viscosity at a continuous range of shear rates. The shear rate range can be controlled by the microchannel dimensions. The sample will traverse the microchannel from end to end by means of capillary pressure, which must be quantified during each measurement. If the measurement of capillary pressure is not reliable enough for our customers or does not provide them with sufficient measurement options, samples will be made to traverse the microchannel by means of a controlled negative pressure. This addition, however, will still require a measurement of capillary pressure because it is non-negligible. The measurement of liquid viscosity based on the detection of the position of the interface and its speed in a microchannel was established by Srivastava et al [1]. The biggest challenge for the automation of this method is the detection of the sample location. The answer to this challenge has typically been the digital image processing of captured video. However, this technique is computationally demanding

**Figure 5-1** A mock-up of the final device with an inserted cartridge.
because the traveling interface of the sample must be established through digital image processing of every video frame. This method requires the use of a computer powerful enough to quickly process the necessary video processing algorithms. Our device meets this challenge in tracking and processing the sample through the microchannel by exploiting the increase of the absorption and scattering of light that illuminates the channel as the sample flow progresses through it. Essentially, a bright collimated light is emitted above the microchannel and is recorded by a photodiode below it. As the sample enters and traverses the microchannel less and less light can reach the photodiode due to the shadow the sample casts. The signal from the photodiode can processed to find the exact positions of the sample column along the microchannel with respect to time and then establish the flow speed. A mockup of what the final device may look like can be seen in Figure 5-1. A diagram displaying the physical principles used by the sensor is presented in Figure 5-2.

5.3 Technical Discussion

We can quantify liquid viscosity by recording the position and velocity of a liquid column as it is introduced to and traverses a microchannel open on both sides to atmospheric pressure. We measure the position of the liquid column through a single photodiode receptor, essentially a 1-pixel camera (Figure 5-2). In order to track and record the process, we first record how much collimated light passes through an empty microchannel. As a liquid enters and begins to traverse the microchannel it obscures the light source from the photodiode and we continuously record the drop in the voltage across the photodiode. Once the liquid has traveled the entire length of the microchannel
the voltage levels out and the cartridge can be removed from the device. The recorded voltage signal can then be converted directly to liquid column length. To do so we first normalize the signal by the final and initial voltage levels and then we multiply the length of the microchannel cartridge by 1 minus the signal. The resultant signal is the position of the liquid column over time and its numerical derivative with respect to time produces the average liquid velocity (or volumetric flow rate if multiplied by the cross-sectional area of the microchannel). We have validated the exactness and repeatability of this measurement technique in several experiments (one example in Figure 5-3). The quantification of viscosity itself is discussed in detail in Power Law Index section.

![Diagram of rhoemeter design and physical principle](image)

**Figure 5-2** Rhoemeter design and physical principle: the position of the liquid column is identified by the shadow it casts on a photodiode.

This technique has a number of critical advantages. First of all, the simplicity of the core devices means that the final product can be made durable, reliable, and portable while
still being affordable. Furthermore, because the signal is normalized during the signal processing, there is no need to calibrate the photodiode sensor or the light source. Even if the liquid is partially transparent, if some significant amount of light is blocked the device will function correctly. There is also no need to account for temperature variance effecting the power of the light source so long as it is not happening in the middle of the measurement. It also means that the microchannel itself does not need to have any micro-electro-mechanical system components in order to conduct the measurement. Therefore, the final microchannel cartridge can be extremely affordable to mass produce. The main drawback of this technique is the inability to detect liquids that are too transparent. To accommodate such liquids, we are currently developing a version of the device with dark-field microscopy principles which we have already validated will allow a single photodiode to detect transparent liquid traversal in a laboratory setting.

5.3.1 Capillary Pressure inside the Microchannel Cartridge

The pressure applied on the liquid column is one of several factors necessary to properly evaluate the viscosity of the travelling liquid, though it is the only one that cannot be evaluated before the measurement. The full viscosity measurement is very sensitive to changes in this pressure and this pressure must thus be accurately quantified during
every single viscosity measurement. We have chosen to use capillary pressure as the
driving force of the measurement because it greatly reduces system complexity and
removes the need to install, calibrate, and validate a pressure pump. Furthermore,
capillary pressure quantification would be necessary even in the presence of a pressure
pump since a non-negligible capillary effect would still be present in low viscosity liquids.
The Young-Laplace equation describes mathematically the pressure experienced by the
liquid ($\Delta P$) in terms of the liquid surface tension ($\sigma$), radius of curvature of the liquid
surface inside the microchannel ($R_1$) and the radius of curvature of curvature of the droplet
outside the microchannel ($R_2$) as

$$\Delta P = \sigma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)$$

This expression [136] may simplify for certain liquids and particularly large droplets to
$\Delta P = \sigma / R_1$ in the case that $R_2 \gg R_1$. The capillary pressure can be further expressed in
terms of the contact angle $\theta$, the microchannel height $d$, and the microchannel width $w$
as $P_{\text{capillary}} = 2\sigma \cos(\theta) \left( \frac{1}{d} + \frac{1}{w} \right)$ [137]. Since $d \ll w$ in a typical microchannel, this
expression can be simplified to

$$\Delta P = \frac{2\sigma \cos(\theta)}{d}$$

However, the surface and contact angle are specific to every liquid sample. It is not only
impractical to measure both of these values before conducting a viscosity measurement
but introduces further potential sources of error into the final capillary pressure
measurement. Instead, capillary pressure must be measured during the experiment and, because in some measurements $R_2$ might still play an effect and change during the measurement, it must be measured and recorded continuously throughout the experiment. We note here that based on preliminary analysis other forces, such as the hydrostatic force of the liquid droplet, are negligible.

Capillary Pressure itself can be measured through several techniques. For instance, the sample droplet can be exposed to a secondary microchannel of the same dimensions which is closed on the far end. As the sample enters and begins to traverse the microchannel, it will begin to compress the air inside. Eventually, the pressure of the compressed air will equal the capillary pressure and will stop the sample from moving forward. This air pressure, which equals the capillary pressure ($P_{\text{capillary}}$) can be measured with a pressure sensor or by relating the new volume the air encompasses ($V_{\text{new}}$) in the microchannel to the original microchannel volume ($V_{\text{org}}$) with relation to the atmospheric pressure ($P_{\text{atm}}$) such that $P_{\text{atm}}V_{\text{org}} = (P_{\text{atm}} + \Delta P)(V_{\text{new}})$ that is

$$\Delta P = P_{\text{atm}} \frac{V_{\text{org}}}{V_{\text{new}}} - P_{\text{atm}}$$

Alternatively, the main channel can be blocked off with a valve, after the air has pressurized and a value is reached, the valve can be opened to continue the experiment.
5.3.2 The Power Law Index

The principle of quantifying fluid viscosity based on microchannel traversal was described by Srivastava et al. in 2005 for Newtonian liquids [138] and 2006 for Non-Newtonian liquids [139]. For non-Newtonian liquids, the aforementioned method used a Hagen-Poiseuille flow equation modified to conform to a Power Law Fluid. Power Law Fluids are idealized models used to describe real liquids. The expressions the authors developed are

\[
\frac{1}{L(t)} = C v(t)^n
\]

\[
\eta(t) = \frac{d^2}{S} \frac{1}{\left(\frac{2}{3} + \frac{1}{3n}\right)} \frac{\Delta P}{v(t)L(t)}
\]

Where \( n \) is the power law index which describes the change in viscosity with respect to shear rate, \( L(t) \) is the liquid column length, \( v(t) \) is the liquid column mean velocity (which is directly proportional with volumetric flow rate), \( \eta(t) \) is the viscosity, \( d \) is the thickness of the microchannel, \( S \) is a constant factor characterizing the geometry, \( C \) is a constant dependent on the power law index, and \( \Delta P \) is the pressure applied on the microchannel. As expected, this expression simplifies to the Hagen-Poiseuille flow equation when \( n = 1 \), which is a Newtonian liquid described by the power law. If the power law index \( n \) is constant, it is possible to curve-fit the first expression in order to quantify \( n \).
We note here that a constant value of $n$ does not describe many liquids correctly. Applying the aforementioned expression directly and conforming to the assumption that $n$ is constant can produce erroneous viscosity results. Based on preliminary data we have found the value of the power law index depends on shear rate. Future work may develop an algorithm to identify the values of $n$ of the liquid continuously throughout the measurement.

However, in the cases that the power law exponent $n$ is, in fact, constant, the expression

$$\frac{1}{L(t)} = C \nu(t)^n$$

is an ordinary differential equation which may be solved as

$$L(t) = \left[ (t + t_0) \left( \frac{n + 1}{n} \right) \left( \frac{1}{C} \right)^n \right]^{\frac{1}{n+1}}$$

Where $t_0$ is a constant that is $t_0 = 0$ if the measurement begins at $t = 0$. This expression can be curve-fit to the data of the liquid column length with respect to time to find the value of $n$.

### 5.3.3 Shear-Rate Range

The Shear-Rate applied to the liquid is dependent on how far into the channel it has pervaded. The wall shear rate can be evaluated as

$$\dot{\gamma}_w = \frac{6Q(t)}{wd^2} \left[ \frac{2}{3} + \frac{1}{3n} \right]$$
Where \( Q(t) \) is the volumetric flow rate and the other parameters are as described in the Power Law Index section [139]. We note that the Hagen-Poiseuille flow equation is known to fail in particularly short pipes due to the molecular limitations in liquids that are not accounted for in the equation. In our case this is analogous to a sample first entering the microchannel.

![Image of device components](image)

**Figure 5-4** A Early prototype featuring core device components B. Microfluidic cartridges comprised of glass and specialized hydrophilic microfluidic polyester films. C. Preliminary viscosity data of black ink (top) and shoe shine (bottom).

### 5.4 Results

We have created a preliminary stationary prototype using premanufactured components in order to demonstrate the feasibility of the sensor. This device (Figure 5-4) has demonstrated the feasibility of tracking liquid traversal through a microchannel. The
prototype features the vital working components that will comprise the final core device. Using this $1,500 prototype, we have collected preliminary viscosity measurements of a black ink and a shoe shine and corroborated the measurements using a $70,000 industrial rheometer (AR G2 Magnetic Bearing Rheometer by TA Instruments). These preliminary measurements were done by estimating capillary pressure, manually managing the curve-fit of the data to find the power law index of the liquid sample, and manually eliminating the potentially erroneous shear rate range of the measurement. This prototype was created in order to minimize the time required to collect meaningful result using the physical principles we wish to exploit. A number of parameters still need to be accounted for with greater fidelity using additional sensors, most notably the exact value of the capillary pressure must be recorded with greater precision. Future work may also include the application of dark field microscopy principles to the set up in order to accurately track transparent liquids.

Our microfluidic cartridges are comprised of two constantly hydrophilic microchannels. The cartridge is comprised of a glass slide, a central polyester layer which has been cut to the dimensions of a desired microchannel, and a transparent polyester layer of which has been laser cut to provide inlet and outlet ports for the device and give a hydrophilic coating. None of the three materials are air permeable, which is an important factor in several potential techniques of determining the capillary pressure experienced by the liquid. Both of the polyester films already have FDA approval and a number of medical conversion companies use them to mass produce microfluidic devices.
We have developed a functional circuit board outfitted with a photodiode, a pressure sensor, and external input and output to communicate with a personal commuter (Figure 5-5). The circuit board records the amount of light collected by the photodiode and the pressure experienced by the pressure sensor and streams the information through a USB connection at up to 10,000 Hz. The streamed data is processed by MATLAB and LabView codes to convert the results into viscosity measurements. This circuit board may be integrated into a second portable version of the prototype in Future Work. The second prototype will act as the main tool in the development of solutions for the associated technological challenges of quantifying the capillary pressure, the sample’s power law index, and the working shear rate range.

**Figure 5-5** Printed circuit board for our next prototype design and experimental setup.

**Figure 5-6** Viscosity measurements for the same sample (black ink) are consistent, varying approximately 20%. This results assumes a consistent capillary pressure, a consistent manual selection of an appropriate constant factor characterizing the microchannel, and assumes that the power law exponent is constant. The appropriate shear rate range was also manually selected and results at either extreme were discard.
Conflict of Interest Statement

We, Prof. George Lyktorafitis and Kostyantyn Partola, have filed a US and PCT patent application through the University of Connecticut (who own the patent application) based on this technology. We have also have founded a company, Simvize LLC (formerly Eir Medical Devices LLC), in order to commercialize it.
Chapter 6: Simulation and Visualization in Virtual Reality
Author Contributions

This work was the product of a collaboration between Vi Q. Ha, Kyle Blummer, Daniel Balla, George Lykotrafitis, and me, Kostyantyn Partola. I wrote this manuscript which was edited by Prof. Lykotrafitis. Dr. Ha and I worked on this project together and contributed equally to final product. While Dr. Ha and I have contributed to all parts of the final product, Dr. Ha’s efforts focused on the original IMPETUS code itself in order to facilitate its integration with virtual reality and to enable runtime interactivity in IMPETUS. My work focused on visualization of simulations, the user experience, interactivity, and the interface between simulations and the Unreal Engine 4. I also designed and implemented the code and method for the visualization of three-dimensional data. Kyle Blummer and Daniel Balla provided investigatory support into methods and shaped early version of the code. Prof. Lykotrafitis administered and supervised the project and edited this manuscript.

6.1 Introduction

Intuition, conjecture, and experimentation are essential driving forces behind innovation and scientific discovery. These principles may be intrinsically augmented thanks to the appearance of immersive virtual reality in the public eye. The success of this tool invariably forces us to inquire into the fundamental nature of how we perceive and interact with the world around us. Within this framework, interactive simulations in a virtual reality (VR) environment border on becoming virtual experiments conducted by the researcher. Interactive simulation has already been used in surgical simulations to train medical professionals in place of cadavers or animals [84, 85], to assist and save cost in parts
design [86], and even in flood threat analysis [87]. Furthermore, the development of any one simulation benefits directly from a design philosophy that integrates constant visualization and interactivity [88]. The use of contemporary virtual reality enables swift and intuitive detection of errors and conflicts between simulation parameters and reality.

Virtual reality in general has enjoyed an enthusiastic academic and commercial community of developers for a number of decades. Numerous attempts at immersion in a virtual world have been explored with varying degrees of success in that time. The early 1990’s saw a rise in public interest in VR with the developments of a number of VR systems such as the CAVE system [89], a cubic room of screens meant to immerse multiple users, though the system suffered from its cumbersome size and high price-tag, PowerWalls and its derivatives [90], arrays of screens or very large screens such as the ImmersaDesk and Infinity Wall, and commercial attempts at releasing low resolution head-mounted displays (HMDs). These tools were studied for their effectiveness in various applications, such as the delivery of exposure therapy, though the associated technological challenges proved too cumbersome for the time [91].

Despite public focus leaving VR, development and enhancement of the technology continued steadily. Over the last five years, HMDs have breached public discourse on a massive scale once again thanks to technological advancements that provide unprecedented visual fidelity packaged with affordability. These advancements include high-refresh rate, high resolution, and low-persistence displays, fast and accurate motion tracking, and personal computers capable of continuously rendering graphically impressive virtual worlds. As a result of this high visual fidelity, the best modern HMDs
(e.g. HTC Vive, Oculus Rift) provide users with a superior sense of presence, a self-reported quality of “being there” [92]. Modern HMDs, which became available for application development in 2013 and to the public commercially in 2016, have experienced relative commercial success in entertainment and, concurrently, a number of VR-centric studies have reported success in a varied assortment of clinical applications [91, 93-97]. Modern HMDs provide the academic community at large the opportunity to utilize VR in applications that were previously unattainable. The standardized interface code that is now employed commercially ensures that VR applications will remain compatible with upcoming HMDs.

One simulation technique that can considerably benefit from integration with modern HMDs is molecular dynamics (MD). Virtual reality provides an immersive experience that can bestow a deep intuitive understanding of the molecular interactions and their effect on the emergent system. Without the use of modern HMDs such an intuitive understanding of MD simulations is challenging to acquire even with the aid of contemporary visualization tools, such as VMD [98]. Interactivity with simulations during run-time is certainly possible in programs such as VMD but the associated screen-based interfaces, traditional input controllers (e.g. mouse and keyboard), and classical VR systems often complicate three-dimensional interactions and exploration to the point of impracticality. Integration of MD with modern HMDs will allow researchers to intuitively perturb and interact with their simulations in VR by applying external forces, steering components, and by causing shape changes.
Visualization of volumetric images, as those produced by techniques such as confocal microscopy, super-resolution microscopy [99], magnetic resonance imaging, and cryo-electron microscopy [100] has long suffered due to the underlying complication of having to display innately three-dimensional images on incompatible two-dimensional monitors. Even three dimensional anaglyph stereoscopy is used in order to ease the difficult interpretation of volumetric data [101, 102]. Similar to its application in MD in this regard, virtual reality offers a natural and compatible way to reconstruct and explore volumetric images and simulations by invoking them in a three-dimensional environment. A number of academic and commercial packages, such as UCSF ChimeraX [103] and VMD, currently provide shape rendering in both classic and contemporary VR systems, and integration with classic VR systems has been available since their inception. These tools use algorithms that attempt to display a solid shape meant to represent the scanned object, commonly referred to as an “iso-surface.” However, such algorithms may significantly distort particular volumetric maps, especially those that display gradients, subtle effects, or “soft boundaries.” Sculptor [104], a notable open-source visualization package, provides users with a direct volume rendering technique in order to render gradients or “soft boundaries” [105] and even integrates with classic VR systems. However, the software is not currently directly compatible with modern HMDs.

The recent technological breakthroughs in HMD technology in the last five years have paved the way for us to undertake these challenges to integrate the visual fidelity, coding standards, and affordability of modern HMDs with (1) manual and immersive three-dimensional particle steering during simulation run-time and with (2) undistorted volumetric rendering of static volumetric maps such as those produced by confocal...
microscopy. We have developed an interactive multiphysics simulation and visualization environment in virtual reality called Interactive MultiPhysics EnvironmenT for Unified Simulation in Virtual Reality (IMPETUS-VR). Using this tool, researchers will be able to experience and manually interact with their simulations and steer individual components during runtime. IMPETUS-VR offers researchers interactive VR integrated particle-based simulations that interact with and run in parallel with gradient continuum field simulations and long-range force network simulations. Additionally, they will be able to literally walk through the data and explore any static volumetric image (defined by cross-sectional volumetric maps) in the very same three-dimensions inherent in their image while maintaining the integrity of the image. The fundamental dimensional agreement between the simulation or image space and the visualization space grants ineffable understanding of the simulation or image details and allows the researcher to exert immediate control over their work. The dimensional agreement also allows researchers to not only instantly select any desired perspective, including inside the simulation itself, but to observe and interact with previously unobservable blind spots that could not be accessed through two-dimensional representations.

6.2 Design and Implementation

Simulations in IMPETUS-VR are powered by the Interactive MultiPhysics EnvironmenT for Unified Simulation (IMPETUS) [106] which is a powerful C++ based, object oriented simulation engine. IMPETUS is capable of simultaneous and interdependent evaluation of continuum models, long-range particle networks, and short-range particle interactions. This code is available independently, with associated tutorials, online. All IMPETUS
functionality has been fully implemented in IMPETUS-VR. Thus, users can create and visualize simulations using the IMPETUS libraries, or even import molecular dynamics configurational files, such as .cfg, in order to view them in virtual reality. They can also employ the code structure that we have provided with IMPETUS-VR, which is based on Unreal Engine 4, as a foundation for implementing their preferred MD simulation code. The demonstration video of IMPETUS-VR (Supplementary Video 1) illustrates the general functional principles of the software and some of its practical applications.

6.2.1 Code Structure

This environment was made possible by integrating a serialized version of the Interactive Multi-Physics Environment for Unified Simulations (IMPETUS) [106] with the Unreal Engine 4. We chose Unreal Engine 4 because it offers openly available source code. In IMPETUS-VR the Unreal Engine 4 software is responsible for the interactivity and the visualization of the simulation state: the software renders the state and handles user interactions and commands. In turn, IMPETUS processes the simulation in a separate thread, a technique known as multithreading, which functions on the principle of evaluating multiple processes concurrently. This means that as each visualization frame is rendered for display any spare computational power is allocated to the numerical computation of the simulation. The structure of the code can be seen in Figure 6-1. Throughout the course of simulation development in virtual reality, we have found that an inconsistent or jarring frame rate is difficult to observe at best and nauseating at worst. Multithreading empowers IMPETUS-VR to maintain an adequate visualization frame rate at the cost of potentially slowing down the computation time of particularly large simulations.
Figure 6-1. IMPETUS-VR connects the Modular Interactive Domains (MIDs) of IMPETUS to the Unreal Engine. These domains are currently the Particle Dynamics domain, Interactive Finite Difference domain and the Network domain. Unreal Engine 4 renders and presents the scene to the user through the HTC Vive. The Vive controllers allow users to directly interact with and influence the simulations.

6.2.2 Volumetric Visualization Algorithm

We implemented the visualization of volumetric three-dimensional microscopy images following a heuristic approach where every cross-section of the image is displayed as an individual image separated in the normal direction from adjacent cross-sections. Each slice is partially transparent such that the transparency of every pixel is linearly inversely
proportional to its brightness. The method is demonstrated in Figure 6-2. This implementation of volumetric representation produces little computational overhead without sacrificing the fidelity of the source image. Volume rendering algorithms that also

Figure 6-2. Algorithm for volumetric representation of a 3D confocal microscopy image via IMPETUS-VR. (A,B) The fluorescent channel of the z-stack of the images recorded through a confocal microscope (A) are combined to produce a full-color image for each slice (Bii). B(i-iii) are sampled slices in the z-stack generated as explained above. (C) These slices are then loaded in IMPETUS-VR in an ordered manner and made partially transparent on a pixel-by-pixel basis such that dark pixels are more transparent than bright pixels. (D) The individual images are then placed in a three-dimensional space on top of one another and presented to the viewer as a full volumetric image.
preserve image integrity, such as volume ray casting or three-dimensional texturing, are too computationally taxing, and thus preclude the use of modern HMDs. Conversely, algorithms, such as the shear warp algorithm [107] or splatting [108], which are sufficiently inexpensive to render in virtual reality degrade image quality and often misrepresent the acquired data. This is due to the algorithm’s core goal to represent the volumetric object as a solid shape with a well-defined boundary. The conversion from volumetric map to solid object may significantly distort the original cross-sectional volumetric maps especially when gradients, subtle effects, or “soft boundaries” are present in the map. These and similar algorithms are currently the most popular methods of volumetric visualization of the majority of modern VR applications.

6.2.3 Computer Specifications

IMPETUS-VR was tested with the HTC Vive on a custom built computer running Windows 10 (Intel® Core™ i7-5820K CPU @ 3.30GHz, Radeon RX480 graphics card, 16GB of RAM, and MSI X99A Raider motherboard) and visualized more than 15,000 individual low polygon count spheres or more than 65,000 individual cubes without a frame rate drop in virtual reality. The number of seamlessly rendered simulation components is a hardware limitation that will improve with the release of superior hardware.

6.3 Ethics Statement

The individual in this manuscript (Supplementary Video 1) has given written informed consent to publish these case details.
6.4 Results

In order to highlight the capabilities of IMPETUS-VR we have produced a number of fully interactive simulations that users may modify by using a personal virtual reality head mounted display and associated controllers. Specifically, we have developed a solvent-free model of a phospholipid bilayer (Figure 6-3A, Supplementary Vid. 2) [109], and a simulation of a spectrin network which corresponds to the cytoskeleton connected to the red blood cell membrane (Figure 6-3B) [110]. The specific models are discussed in detail in [109] and [110] respectively. These simulations demonstrate the ability to explore interactively the properties of emergent systems as they are perturbed and rearranged in virtual reality. Furthermore, using IMPETUS-VR, we modeled the interaction between cranial neural crest and cranial placode cells during collective cell migration (Supplementary Figure 6-2A, Supplementary Vid 3) to reproduce experimental results produced in Theveneau, E. et al. [111]. This simulation is an example of a continuum field whose gradient is visualized as green vectors with orientations and magnitudes that are explicitly apparent in virtual reality. This clarity is not practically achievable outside of the realm of VR. We have further produced an exemplary simulation of a network of particles that interact via long-range spring forces while at the same time are subjected to the Lennard-Jones potential and to the Nose-Hoover thermostat (Supplementary Figure 6-2B, Supplementary Vid 4). This simulation demonstrates the visualization of long-range forces as “tethers” within virtual reality. We note that these simulations are described in greater detail in their original non-interactive non-VR states in the IMPETUS publication [106]. Similar manual particle steering during simulation run-time applications have historically been available in classic VR systems, such as VMD. However, these tools
lack integration with modern HMDs and are generally meant as two-dimensional “on-screen” applications. As a result, their practical usability suffers in terms of affordability and three-dimensional freedom of movement, especially when manual steering and view selection requires the use of mouse and keyboard, which physically restrict user inputs to two dimensions.

Figure 6-3. Exemplary simulations provided with IMPETUS-VR. A. A simulation of a solvent-free model of a phospholipid bilayer where each blue shape represents a cluster of lipids, while the red and yellow shapes represent arbitrary membrane proteins. B. A spectrin network which corresponds to the cytoskeleton connected to the red blood cell membrane. The red particles represent actin junctions and the green particles represent spectrin chain repeats.

We note here that we have developed a number of user controls meant to improve the user experience. We implemented simple “floating menus” in order to allow users to navigate the software while using the HMD itself. These menus are inspired by similar menu options seen in modern VR entertainment applications. Our experience with the IMPETUS-VR environment has shown that floating menus, which make the use of keyboard and mouse unnecessary, greatly improve practical usability of the virtual environment as well as suggest enhancement of the sensation of presence (the self-reported quality of “being there”). Another feature developed with the user in mind are the
exploration controls which we designed to minimize the potential for “virtual reality sickness” (also known as “cyber sickness” or simply “motion sickness”) [112]. In IMPETUS-VR, users directly control the rotation and position of the visualized object itself, rather than their own virtual position. These controls are intended to promote a sense of control over the visualized object while maintaining the stability of the viewer in the virtual world.

To further showcase the abilities of IMPETUS-VR in terms of volumetric visualization, we have provided a number of volumetrically rendered confocal microscopy images. A 102 µm by 102 µm by 19 µm confocal microscopy image of a kidney mouse section can be seen in IMPETUS-VR (Figure 6-5A, Supplementary Vid 5). The three-dimensional structure of the mouse kidney section can be partially inferred in the figure itself, however it is self-evident when viewed in three dimensions. We have also included a confocal scan of ICAM4 expressed on the surface of a Sickle Red Blood Cell (Figure 6-4). We note that the integrity and clarity of gradients and subtle effects visible in the individual cross-sections of the volumetric maps remain observable in final volumetric render. However, this particular method prevents users from changing image channel representations (i.e. individual colors meant to identify different components of the volumetric map) in real-time due to the way each individual cross-section is represented. This is a fundamental limitation of the technique itself, and although a menu and tools can be added to allow users to adjust channel intensities in real-time the response would likely be too slow using modern personal computers to be considered practical. Another limitation comes from the fact that the render is not comprehensible when viewed exactly from the side, where the individual layers appear to have no thickness. We note that these limitations can be
overcome with the use of certain established direct volume rendering techniques, such as those used by Sculptor [105], though these and similar algorithms may be too computationally taxing for high resolution volumetric maps to be used in current HMDs.

The preceding figures and others are available as 360 panoramas and can be viewed online through your browser, cell phone, or personal virtual reality headset (http://www.engr.uconn.edu/~gelyko/impetusvr.html). We have also made the program code (under the GNU General Public License version 3) and associated libraries, the demonstration video, a user manual, and a tutorial video available online at the same web address.
**Figure 6-4** Volumetric representation of a three-dimensional confocal microscopy image of a sickle-cell red blood cell (SS-RBC). The green color represents ICAM4 receptors expressed on the surface of a red blood cell while the red color represents $\alpha_\nu\beta_3$ protein that is adherent to the SS-RBC surface. Data courtesy of Dr. Krithika Abiraman, reproduced here with her permission.
Figure 6-5. Volumetric representations of three-dimensional confocal microscopy images. **A.** A 102 \( \mu \text{m} \times 102 \mu \text{m} \times 19 \mu \text{m} \) volumetric image of a kidney mouse section. Here the blue color represents actin, the green color represents wheat germ agglutinin (a general stain for the surfaces of cells), and the red color represents the nuclei. **B.** A 290 \( \mu \text{m} \times 290 \mu \text{m} \times 20 \mu \text{m} \) scan of a hippocampus section where the red color represents cell nuclei stained with DAPI and the green color represents activated glial cells stained with GFAP.
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Supplementary Figure 6-S1. An IMPETUS-VR render of exemplary simulations. A. The interaction between cranial neural crests (represented by blue particles) and cranial placode cells (represented by red particles) during collective cell migration as it viewed by the user inside the head mounted display. The green arrows represent the three-dimensional gradient field of the chemoattractant produced by the placode cells. The nature of the gradient field is clearly apparent in virtual reality. B. A simulation
highlighting the representation of the network of long-range particle interactions displayed as red “tethers.”

Supplementary Videos 1 through 5 can be found online at

http://www.engr.uconn.edu/~gelyko/impetusvr.html
We have worked to expedite the acquisition of results, whether simulation or experimental, and to increase the throughput of measurements in studies which utilize atomic force microscopy, explore cell adhesion to specific proteins, in simulations which explore cell mechanics, and in rheological measurements. Overall we have explored several techniques to accomplish this, namely automated data processing, virtual reality, and microfluidics. These methods provide the possibility of accelerating the acquisition of sound experimental and simulation data.

Data analysis of force spectroscopy curves from atomic force microscopy has historically been a very time-consuming and tedious endeavor. Researchers can spend several hours processing the results from just several. To alleviate this massive time waste we developed FRAME. Our software offers a directly applicable array of modifiable tools to AFM users for expediting data processing. FRAME algorithms process AFM data extremely quickly compared to the time required for manual processing. 1024 curves are processed in less than one minute for unbinding events and in less than five minutes for Young’s modulus measurements in a typical work station. FRAME also provides convenient manual processing tools that are integrated with automated processing. FRAME can automatically import force-displacement curves from AFMs by Asylum Research (.ibw files). Users of other brands of AFMs can import force-displacement measurements formatted as delimited text files into FRAME following instructions in the User’s Manual.

Early versions of FRAME developed out of our exploration of Single Cell Force Spectroscopy where the need for increased processing speed was readily apparent. We
used the original functions and GUI-free environment to establish SCFS as a method in quantifying the adhesion of RBCs to functionalized substrates. This method allowed us to explore the modulation of RBC adhesion via physiological reagents, such as epinephrine, by affecting the cAMP-PKA pathway.

We needed to explore the adhesive and mechanical properties of a larger number of RBCs at one time because the data may be too variable for 5 cells to describe the properties of the population in a meaningful way. To meet this need, we produced a high-throughput microfluidics testing setup. This experimental setup presents an opportunity to quickly and precisely quantify the mechanical and pharmacological properties of SS-RBCs in many different assays. This is enabled by the combination of equipment, specifically the high-speed camera and the precise and instantly modifiable pressure flow, the surface functionalization protocol, and custom image processing software. Minor tweaks to the assay parameters, such as the concentration of endothelial protein during functionalization, may also be appropriate to maximize the sensitivity of the system and elucidate SS-RBC properties. The current setup can be easily modified to enable experiments under controlled oxygen levels.

The experimental methods above may potentially also be conducted in similar assays on wildtype RBCs or in efforts to study other related diseases, such as malaria or diabetes, where either RBC adhesion or mechanical behavior plays a major role in pathology. The same advantages and improvements presented in this report may prove to be applicable is such studies. Thus, this potential is an avenue for future work in this area.
Current solutions for measuring whole blood viscosity are not practical for the application: tests are slow, require a lot of blood and sterilization, and the associated equipment is complex and not portable. We have endeavored to enable simple and accessible whole blood viscosity measurements by designing and developing our own microfluidics based rheometer. Our assembly for measuring the viscosity of various liquids through the use of microchannels provides a promising improvement in currently available rheometers. Several key features, such as fast measurements, small sample size, and lack of sterilization may produce a device which enables regular whole blood viscosity measurements even at the point of care. This measurement is important in hyperviscosity syndrome, a condition which may arise in certain illnesses such as Waldenström’s Macroglobulinemia. Whole blood viscosity may also be important in monitoring overall cardiovascular health.

We have produced IMPETUS-VR to create truly interactive three-dimensional simulations and an intuitive three-dimensional data visualization environment. This tool greatly improves the fidelity, development, and understanding of IMPETUS simulations. Furthermore, it provides researchers a method of visualizing their three-dimensional data in three-dimensions, instead of distorting it into two-dimensions.
Appendix

Publications

Journal Publications and Patent Filings


J. MACIASZEK, K. PARTOLA, J. ZHANG, B. ANDEMARIAM (MD), and G. LYKOTRAFITIS, 'Single-cell force spectroscopy as a technique to quantify human red blood cell adhesion to subendothelial laminin', *Journal of Biomechanics, 47, Issue 16*, 3855-3861, 2014

Conference Proceedings

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