Journal of Visualized Experiments

A uniform shear assay for human platelet and cell surface receptors via cone-plate viscometry

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59704R1
Full Title:	A uniform shear assay for human platelet and cell surface receptors via cone-plate viscometry
Keywords:	Shear; Mechanosensation; Mechanoreceptors; platelet; Viscometry; biophysics; flow cytometry; Hemostasis
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Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Atlanta, Georgia, USA

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January 16th, 2019

To the Editors of *Journal of Visualized Experiments*,

Enclosed is a manuscript entitled "A uniform shear assay for human platelet and cell surface receptors via cone-plate viscometry", which I have been invited to submit to *JoVE* for publication.

Mechanosensation via cell-surface mechanoreceptors is an important mechanism by which cells can interact with and interpret their physical environment. Some common assays, while efficacious in testing the effects of many biochemical variables on receptor binding and activation, are ill equipped to explore the effects of mechanical force. Many cells that experience shear stress are circulating cells. As such, assays using flow chambers or microfluidics (sometimes paired with imaging) are often used to apply laminar flow. These assays permit specific control over shear time and rate. These assays are often used to interrogate interactions in which either the ligand or receptor is anchored/coating a surface. However, another paradigm in which cells may experience shear is in solution, not tethered to a surface. In this situation the effect of shear may not even be mediated via a specific ligand-receptor pair.

Our manuscript describes the method for a "uniform shear assay" whereby shear force is applied to cells in solution on a cone-plate viscometer. This assay allows for application of shear force to cell suspensions or biological fluids such as blood or plasma with control over specific shear levels and length of shear exposure. The shearing step of this method can be utilized upstream of many different biochemical or imaging readouts. Described herein, we have most recently paired the shearing step with high throughput detection methods like standard and imaging flow cytometry to detect the crosslinking of platelets by human-disease relevant autoantibodies and to reveal specific biophysical requirements for the activation of the platelet mechanoreceptor GPIb-IX by its ligands in solution.

Thank you for the invitation and for your consideration. I look forward to hearing from you soon

Sincerely,

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TITLE:

A Uniform Shear Assay for Human Platelet and Cell Surface Receptors via Cone-Plate Viscometry

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KEYWORDS:

Shear, mechanosensation, mechanoreceptors, platelet, viscometry, biophysics, flow cytometry, hemostasis

SUMMARY:

We describe an in-solution method to apply uniform shear to platelet surface receptors using cone-plate viscometry. This method may also be used more broadly to apply shear to other cell types and cell-fragments and need not target a specific ligand-receptor pair.

ABSTRACT:

Many biological cells/tissues sense the mechanical properties of their local environments via mechanoreceptors, proteins that can respond to forces like pressure or mechanical perturbations. Mechanoreceptors detect their stimuli and transmit signals via a great diversity of mechanisms. Some of the most common roles for mechanoreceptors are in neuronal responses, like touch and pain, or hair cells which function in balance and hearing. Mechanosensation is also important for cell types which are regularly exposed to shear stress such as endothelial cells, which line blood vessels, or blood cells which experience shear in normal circulation. Viscometers are devices that detect the viscosity of fluids. Rotational viscometers may also be used to apply a known shear force to fluids. The ability of these instruments to introduce uniform shear to fluids has been exploited to study many biological fluids including blood and plasma. Viscometry may also be used to apply shear to the cells in a solution, and to test the effects of shear on specific ligand-receptor pairs. Here, we utilize cone-plate viscometry to test the effects of endogenous levels of shear stress on platelets treated with antibodies against the platelet mechanosensory receptor complex GPIb-IX.

INTRODUCTION:

Mechanoreceptors are proteins that respond to mechanical stimuli, such as pressure or

mechanical perturbation/deformation. For some mechanoreceptors, sensing these mechanical perturbations is explicit to the function of the cell types in which they are expressed. Take, for example, the stretch receptors in baroreceptor neurons; these mechanosensitive ion channels regulate blood pressure by sensing vascular "stretch"[1,2]. In the inner ear, ion channels on hair cells detect mechanical deformations caused by sound waves[3], and cutaneous low threshold mechanoreceptors (LTMRs) facilitate the transmission of tactile information[4]. In other cases, mechanoreceptors provide important information to the cell for the establishment of adhesion or growth. Cells can sense the rigidity of their local environment, and may rely on contractile forces via the actin cytoskeleton and integrins to dictate growth or spreading[5,6].

When studying receptor-ligand interactions in cell or tissue-based models, common assays exist which can quickly and accurately report the effects of altering temperature, pH, ligand concentration, tonicity, membrane potential, and many other parameters which can vary in vivo. However, these same assays may fall short when it comes to detecting the contribution of mechanical force to receptor activation. Whether cells are sensing their microenvironment, detecting sound waves, or responding to stretch, one thing the aforementioned mechanoreceptors have in common is that they are participating in interactions where the ligand, receptor, or both, are anchored to a surface. Assays developed to test the effects of mechanical forces on receptor interactions often reflect this paradigm. Microfluidics and flow chambers are used to study the effects of shear flow on cells and receptors[7,8]. These types of experiments have the advantage of allowing fine-tuning of shear rates via established flow speeds. Other techniques employ fluorescent molecular probes to detect forces applied by cells on ligand-rich surfaces, yielding an accurate readout of the magnitude and orientations of forces involved in the interaction[9,10].

In addition to mechanosensation occurring where one or both partners are anchored to a surface, shear stress may affect proteins and cells in solution. This is often observed in blood cells/proteins which are constantly in the circulation, and may manifest via activation of mechanoreceptors that are normally surface-anchored[11], or through exposure of target sequences which would be occluded under static conditions[12]. However, relatively fewer techniques assay the effects of shear force on particles in solution. Some in-solution approaches introduce shear via vortexing cells in fluid suspension with varying speeds and durations, although these approaches may not allow a very precise determination of the shear stress generated. Rotational viscometers measure viscosity by applying a specific shear force to fluids. Herein we describe an applied method for determining the effect of specific laminar shear rates on cells or cell fragments in solution.

One of the most highly expressed proteins on the platelet surface is the glycoprotein (GP) Ib-IX complex. GPIb-IX is the primary receptor for the plasma protein von Willebrand Factor (VWF). Together, this receptor-ligand pair has long been recognized as the foundation of the platelet response to shear stress[13]. In the event of vascular damage, VWF binds to exposed collagen in the sub-endothelial matrix[14], thus recruiting platelets to the site of injury via the VWF-GPIb-IX interaction. VWF engagement to its binding site in the GPIbα subunit if GPIb-IX under physiological shear stress induces unfolding of a membrane-proximal mechanosensory domain

(MSD) which in turn activates GPIb-IX[15]. In a recent study, we have shown that antibodies against GPIbα, like those generated in many immune thrombocytopenia (ITP) patients, are also capable of inducing platelet signaling via MSD unfolding under shear stress[11]. However, unlike VWF, which facilitates shear-induced GPIb-IX activation by immobilizing the complex under normal circulation, the bivalent antibodies are able to crosslink platelets via GPIb-IX and unfold the MSD in circulation. In this way, a mechanoreceptor which is normally activated by surface immobilization under shear can be activated in solution. In the present report, we will demonstrate how a viscometer-based uniform shear assay was leveraged to detect the effects of specific levels of shear stress on receptor activation in solution.

PROTOCOL:

All methods using donor-derived human platelets described herein were approved by the Institutional Review Board of Emory University/Children's Healthcare of Atlanta.

1. Blood Draw and Platelet Isolation

1.1) Draw human blood from consenting healthy adult donors via venipuncture on the day of the experiment into 3.8% trisodium citrate. One 4.5 mL tube of blood is sufficient to yield enough platelet rich plasma (PRP) for 20–25 conditions in donors whose platelet counts are close to 250 x 10^3 per µL.

NOTE: Avoid drawing blood via narrow gauge needles (smaller than 21 G).

1.2) Prepare PRP via centrifugation at 22 °C and 140 x *g* for 12 min with a long brake. This will result in two distinct layers, with red blood cells at the bottom and the light colored PRP at the top.

1.3) Isolate the top, cloudy, yellow layer of PRP via careful pipetting through a pipette tip cut at a 45° angle and obtain the platelet count via a complete blood count (CBC).

1.4) If necessary, wash platelets in PIPES-buffered saline (150 mM NaCl, 20 mM PIPES) in the presence of prostaglandin E1 (PGE1) and resuspend in Tyrode's buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.35) with 5 mM glucose, otherwise, proceed to step 1.5. The following steps describe the washing in brief.

1.4.1) Adjust PRP volume to 10 mL with PIPES-buffered saline and add 0.6 µM PGE1.

1.4.2) Centrifuge for 8 min at 1,900 x *g*, then discard supernatant and let the platelet pellet sit in 400 µL of Tyrode's and glucose solution for 5 min.

1.4.3) Gently resuspend the platelet pellet and keep it undisturbed for 30 min.

1.5) Adjust the platelet count to ~250 x 10^3 platelets per µL with pooled human platelet-poor plasma (PPP) and maintain the suspension at 22 °C undisturbed or under gentle rotation.

2. Ligand and uniform shear treatment

NOTE: All steps in section 2 that require pipetting should be done slowly, so as not to introduce any shear.

2.1) Add the desired antibody or ligand to the PRP or washed platelets and mix gently by pipetting up and down or stirring with a pipette tip. Leave it undisturbed at room temperature for 5–10 min. Add an equivalent volume of PPP or Tyrode's buffer to a negative control.

2.2) Turn on the cone-plate viscometer, set the plate temperature to 22 °C and allow time to let the plate reach this temperature.

2.3) Pipette the treated PRP or washed platelets onto the temperature-controlled cone-plate viscometer directly at the center of the plate. Ensure that all of the sample is deposited between the cone and plate at the point of contact, and not on the outside of the cone's rim.

2.4) Shear at an appropriate rate and duration.

2.4.1) Calculate shear as indicated by the viscometer manual, or as previously shown[15,16].

2.4.2) Determine shear rate from viscosity and desired shear stress via Newton's law of viscosity; $\tau = \mu\frac{du}{dy}$; plasma viscosity is 1.5–1.6 centipoise (cP)[17]. For example, a normal shear range for human circulation is 5–30 dyn/cm^2 and shear should be applied on the single digit minute time scale.

2.5) Lift the cone off of the plate slighlty (~2 mm) so that the sample remains in contact with both the plate and cone, and use a gel-loading or other long pipette tip to collect 5–10 µL from the center of the sample volume.

2.6) Incubate the sheared samples with the desired markers for 20 min at room temperature. For markers of phosphatidylserine, β-galactose, and P-selectin exposure use Lactadherin C2 domain (LactC2) at 0.08 µM[18], Erythrina cristagalli lectin (ECL) at 6.25 µg/mL, and anti-P-selectin antibody (20 µg/mL), respectively.

2.7) Fix samples in 2% paraformaldehyde for 20 min at RT prior to dilution or cold storage, and proceed to step 3.1 or store samples at 4 °C for no longer than 12 h.

3. Detection of surface markers and crosslinking via flow cytometry

3.1) Analyze the sample via flow cytometry, collecting at least 20,000 events for each condition.

3.2) Quantitate signal strength of the fluorescent markers using the height value for the intensity

of each fluorophore, or the geometric mean fluorescence intensity (MFI).

3.3) If aiming to detect platelet crosslinking following shear treatment, analyze the sample on an imaging-capable flow cytometer and quantitate crosslinking by area and aspect ratio parameters.

3.3.1) Plot a histogram of area and/or aspect ratio.

3.3.2) Use a negative control with bovine serum albumin (BSA) or vehicle to draw a gate excluding most fully circular events (this gate is usually drawn at an aspect ratio ~0.8[19,20]) and quantitate the percentage of events inside of this gate. Events with a lower aspect ratio are more likely to be crosslinked.

REPRESENTATIVE RESULTS:

Figure 1 outlines how the trigger model of GPIb-IX activation, initially introduced to explain shear-dependent receptor activation when anchored to the vessel wall, may also support activation of platelets crosslinked by a multivalent ligand. **Figure 2** shows readouts of human platelet activation treated by two antibodies targeting the N-terminal domain of GPIb-IX (6B4 and 11A8), and one control antibody (normal IgG) under sheared and static conditions. In **Figure 3**, platelets were treated with 6B4 and an antibody with an unbinding force too low to trigger GPIb-IX activation, AK2. **Figure 4** shows a time course. Markers of platelet activation were probed and detected at progressive time points during shear treatment with 11A8 or AK2. In **Figure 5a**, antibody crosslinking platelet-sized beads decorated with the GPIb-IX N-terminal domain was assayed via conventional flow cytometry. **Figure 5b** shows the 6B4-mediated crosslinking of platelets as imaged via imaging flow cytometry. In **Figure 6**, clinical samples of serum from patients with immune thrombocytopenia were used in place of the divalent monoclonal antibodies against GPIb-IX used in previous figures. Patient plasma-containing antibodies against GPIb-IX triggered shear-dependent responses, in contrast with plasma from ITP patients without antibodies targeting GPIb-IX.

FIGURE AND TABLE LEGENDS:

Figure 1: Platelet surface mechanoreceptor GPIb-IX can bind a soluble divalent ligand (such as an antibody). When two GPIb-IX complexes on opposing platelets bind to the same divalent ligand, crosslinking occurs. Under physiological shear, the crosslinked platelets can generate a pulling force to act on GPIb-IX and unfold the MSD therein. As a consequence of MSD unfolding, GPIb-IX is activated, and induces platelet signaling and downstream platelet clearance, as illustrated. Figure was adapted from Quach et al.[21]

Figure 2: Expression of markers of platelet activation in human PRP treated with control IgG or anti-GPIb-IX antibodies 11A8 and 6B4 under static or sheared (30 dyn/cm^2) conditions. In these representative results, platelet surface exposure of phosphatidylserine (PS), β-Galactose, and P-Selectin were probed by green fluorescent protein (GFP)-conjugated Lact-C2, FITC-conjugated Erythrina cristagalli lectin (ECL) and APC-conjugated anti-P-Selectin antibody, respectively. Fluorescence was detected via flow cytometry. Data are expressed as percentage of events with

fluorescence above negative control. ***p ≤ 0.001, ****p ≤ 0.0001; significance determined via student's *t*-test. Error bars represent standard deviation (SD).

Figure 3: Human PRP treated with two anti-GPIb-IX antibodies. Human PRP treated with two anti-GPIb-IX antibodies, one with high unbinding force (6B4) and one with low unbinding force (AK2) under static (0 dyn/cm^2), low shear (5 dyn/cm^2), and high shear (30 dyn/cm^2) conditions. Graphs show percentage of events positive for surface expression of β-Galactose, PS, and P-Selectin. *p ≤ 0.05, ***p ≤ 0.001. Significance was determined via a one-way ANOVA with Tukey test. Error bars represent SD. Figure has been adapted from Quach et al.[11]

Figure 4: Time course of shear exposure (20 dyn/cm^2) for human PRP treated with control IgG, 11A8, or AK2. Platelet surface exposure of PS and P-Selectin was detected via flow cytometry for samples sheared for 0, 0.5, 1, 3, and 5 min. ***p ≤ 0.001, ****p ≤ 0.0001. Significance was determined via a one-way ANOVA with Tukey test. Error bars represent SD.

Figure 5: Quantitating crosslinking by area and aspect ratio parameters. (**a**) Platelet-sized (4 μm) beads were conjugated with the N-terminal domain of GPIbα and incubated with IgG (gray), AK2 (red), or 6B4 (blue) under sheared (20 dyn/cm^2) conditions. Shown here are contour plots of forward scatter (FSC-A) versus anti-mouse antibody fluorescence. (**b**) Histogram for aspect ratio of platelets in PRP incubated with 6B4 and fluorescently labeled anti-CD41 (a platelet marker) antibodies under shear. Representative images at various aspect ratios are shown. Figure has been adapted from Quach et al.[11]

Figure 6: Clinical samples of serum from patients with immune thrombocytopenia were used in place of the divalent monoclonal antibodies against GPIb-IX. (**a**) GPIb-IX binding capacity of plasma from patients with ITP, assayed via enzyme-linked immunosorbent assay (ELISA). (**b**) Washed human platelets reconstituted in ITP patient plasma under static and sheared (30 dyn/cm^2) conditions. Graphs show percentage of events positive for surface expression of P-Selectin or PS. ****p ≤ 0.0001. Significance was determined via one-way ANOVA with Tukey test. Error bars represent SD. Figure has been adapted from Quach et al.[11]

DISCUSSION:

The protocol described in this manuscript allows quick and versatile assessment of the effect of laminar shear on platelet and cell surface receptors. The specific representative results presented here underscore how the effects of multimeric or bivalent ligands can be affected by shear flow. In addition to this application, a uniform shear assay has broad applications in observing shear-dependent effects. In the absence of a known ligand-receptor pair, a uniform shear assay can also detect the effects of shear on factors such as cell shape and signaling, especially when paired with flow cytometric analyses. Alluded to in part by **Figure 5b**, a uniform shear assay lends itself to a wide variety of detection methods other than or in complement to standard flow cytometric analyses outlined in this protocol, including imaging, biochemical, and cell-based assays.

Figure 2 exemplifies the basic application of the assay, specifically to discriminate between a shear-dependent ligand-receptor interaction and a negative control. 6B4, an antibody which is

capable of crosslinking platelets via binding to GPIb-IX receptors and sustaining enough force to activate the receptor[11], activates platelets when exposed to shear (depicted in the model in **Figure 1**), unlike the control IgG which does not bind platelet GPIb-IX. This application simultaneously provides evidence that the effect is specific to the chosen receptor-ligand pair and is dependent on shear. The uniform shear assay can also distinguish between the effects of ligands which can and cannot sustain their bonds to a receptor under specific shear levels. In **Figure 3**, we see a lack of signaling readout from the anti-GPIb-IX antibody AK2, which binds its epitope with a particularly weak unbinding force[11]. This figure also demonstrates the effects of varying the level of shear stress applied by the cone-plate viscometer from a low to high shear.

Figure 4 demonstrates that this assay can be used to modulate the length of shear exposure and suggests a strategy for displaying this data. In **Figure 5**, two alternative flow-cytometry based readouts for the assay are used, both of which do not rely on fluorescent markers of receptor activation but instead may be used to describe crosslinking and size/shape change. **Figure 5b** shows how forward scatter, which increases proportionate to the size of an event, can distinguish between distinct populations of unlinked (singlet), doublet, triplet, or higher multiplet crosslinked events. As in **Figure 5b**, the assay can be applied to beads conjugated to a receptor of interest, which eliminates the possibility of other receptors participating in the crosslinking events seen via other methods. In **Figure 6**, plasma, a biological fluid containing potential ligands for our receptor of interest is used in place of purified monoclonal antibodies. This illustrates an application in which a specific known ligand or binding partner for a receptor of interest may not be necessary.

Although the assay is fairly accessible as written, there are a few key steps which should be performed with care. Foremost among these is to be careful to not introduce shear from external sources other than the actual shear treatment itself. When drawing blood, as mentioned in step 1.1 of this protocol, it is important to draw it through proper gauge needles. For human blood collection via venipuncture from consenting adult donors, this can be accomplished with a 21 G blood collection set (listed in the **Table of Materials**). Once blood is drawn and PRP is isolated, it can be stored at room temperature for up to 6 h on the bench or on a slow rotisserie.

If the centrifuge used to isolate PRP can brake at different speeds it is best to choose a slow brake so as to minimize disturbance of the PRP layer and application of unnecessary force. Once PRP is isolated or the cell type of interest is in fluid suspension, avoid pipetting the mixtures too quickly. Instead, aspirate and expel samples in a slow and steady fashion especially during step 2.5, where the sample is drawn through a particularly narrow pipette tip. For sample volumes which can accurately be pipetted via pipette tips of different sizes, it is advisable to use the largest among them. For especially sensitive or viscous samples, the very end of the pipette tip can be cut at an angle to widen the opening through which the sample is to be drawn.

Many assays which test the effect of shear on cellular mechanosensation rely on an interaction between an anchored ligand and its receptor. Microfluidics and flow chambers are one such example, where the interaction between an anchored ligand and a receptor, usually on a cell in solution, can be observed in real time[7,8]. Other techniques use microscopy to detect events

occurring at the interface between a cell layer and the underlying substrate or probe[9]. These techniques have been used to particularly great effect in studying blood cells in circulation. On the other hand, techniques which allow the interrogation of events that occur entirely in solution under flow are more limited. For generic application of force in solution, vortexing a sample may serve as a "quick and dirty" method. However, it is difficult to determine the precise force applied, and the shear is not certain to result from laminar flow. This is one of the advantages of the uniform shear assay. On the other hand, the uniform shear assay's primary drawback is a time gap between the application shear and observing the cells themselves. Shear is applied, then cells are stained and fixed, but imaging techniques cannot observe the cells during the shear process itself. One adaptation of this method would involve adding the cells directly to the desired markers and shearing with the markers present. This would allow the immediate detection of any transient effects.

Thus far we have applied this assay to detect the shear-dependent in-solution effect of antibodies targeting the GPIb-IX complex. While the antibodies provide a convenient, simple, divalent binding partner, it is logical to assume that crosslinking of mechanoreceptors on opposing cells in circulation can be accomplished via myriad multivalent ligands with sufficiently high unbinding forces. This technique may be useful in the future in detecting the specific effects of such ligands. Additionally, the uniform shear assay may be used in conjunction with imaging techniques not described herein such as standard fluorescence microscopy. Ultimately, the uniform shear assay provides a reasonably cheap, quantitative, and specific method for applying laminar shear to cells and cell fragments in solution, and can be used upstream of many detection methods.

ACKNOWLEDGMENTS:

Work pertinent to this study was supported in part by National Institutes of Health (NIH) National Heart, Lung, and Blood Institute grants HL082808, HL123984 (R.L.), and F31HL134241 (M.E.Q.). Funding also provided by NIH National Institute of General Medical Sciences grant T32GM008367 (M.E.Q.); and pilot grant funds from Children's Healthcare of Atlanta and Emory University Pediatric Flow Cytometry Core. The authors would like to thank Dr. Hans Deckmyn for sharing the 6B4 antibody, and the Emory Children's Pediatric Research Center Flow Cytometry Core for technical support.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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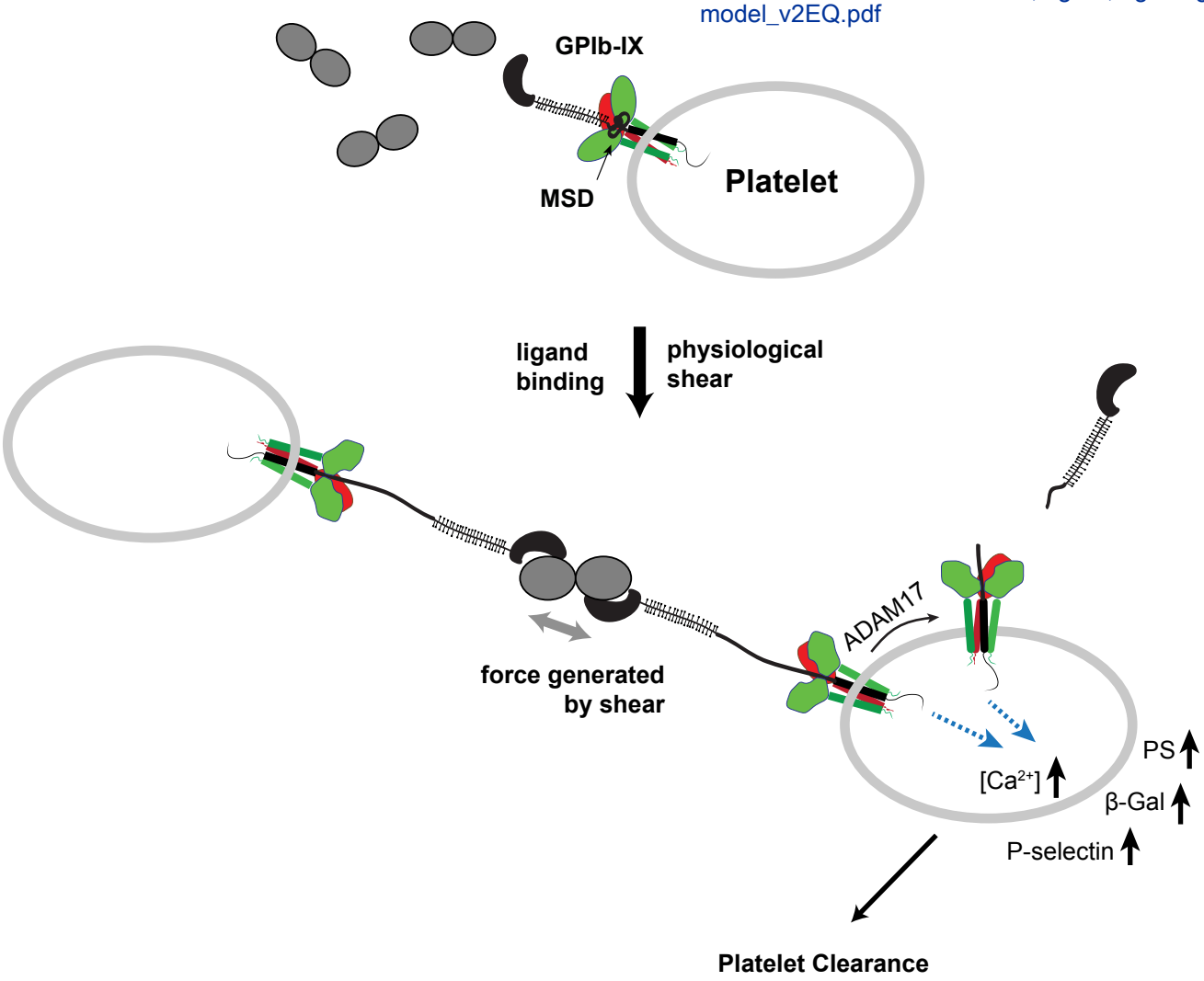

Figure 2

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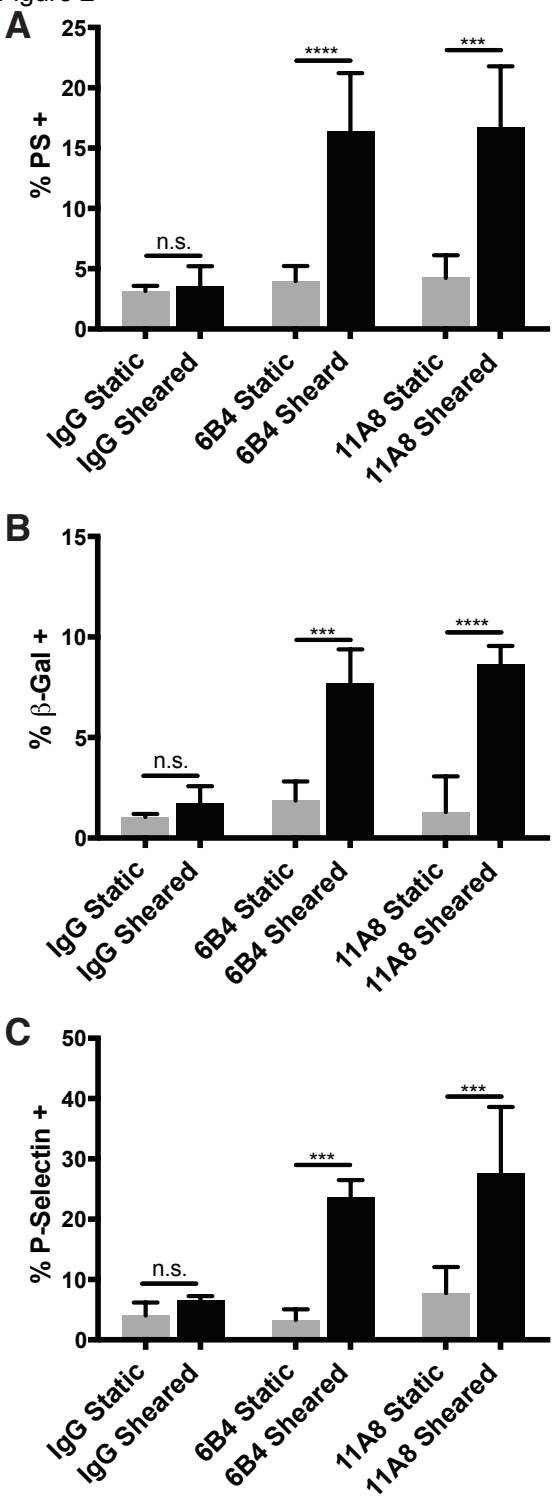


Figure 3

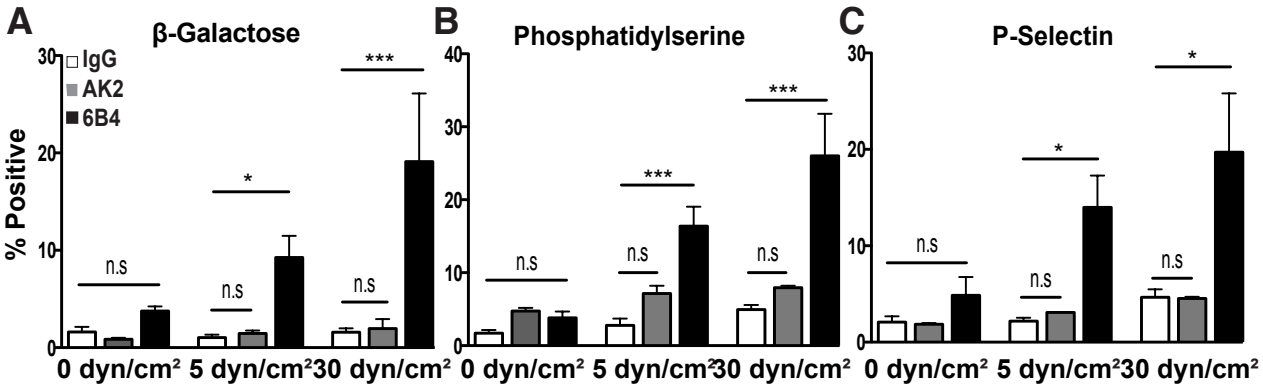


Figure 4

Click here to access/download;Figure;Fig 4 Time Course_v2EQ.pdf

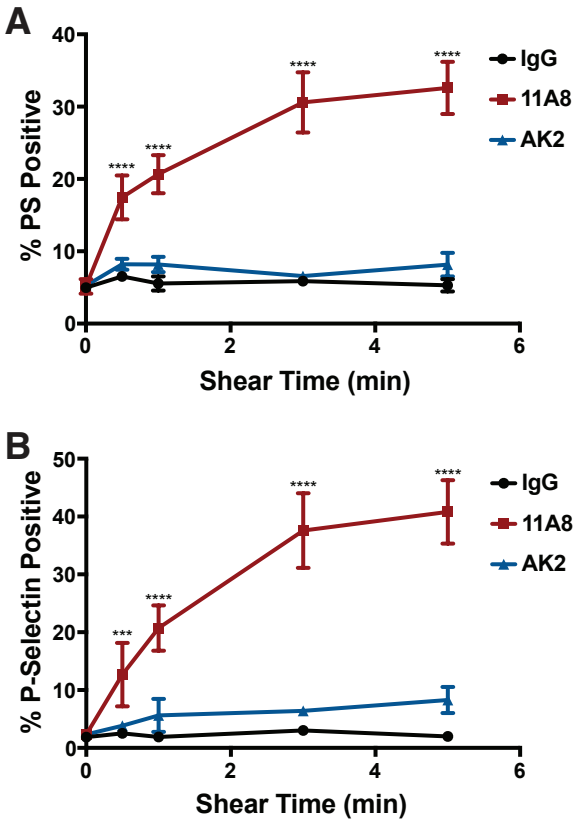


Figure 5

Click here to access/download;Figure;Fig 5 PBAIT Beads and Imaging Flow _V2.pdf

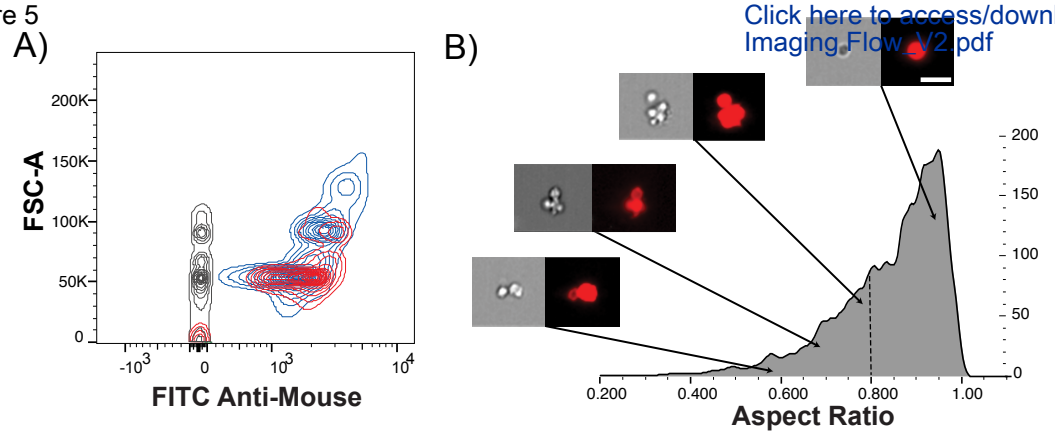
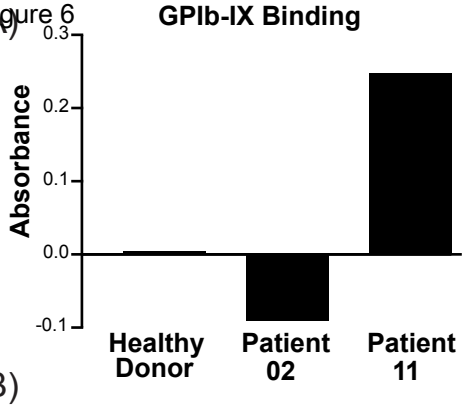


Figure 6

Click here to access/download;Figure;Fig 6 Patient Samples_v2.pdf

A)



B)

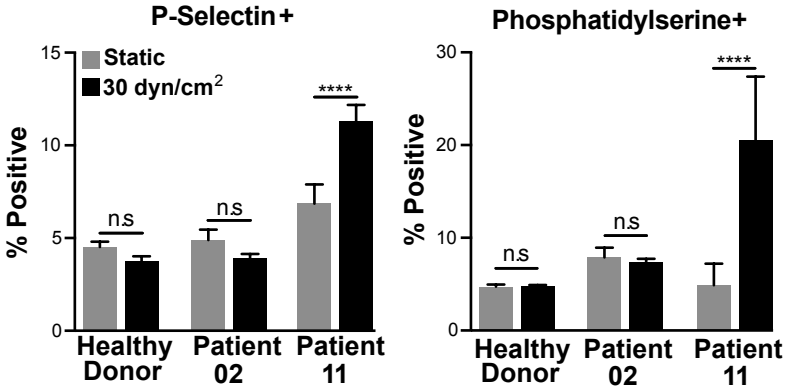


Table of Materials

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Name of Material/ Equipment	Company
APC anti-human CD62P (P-Selectin)	BioLegend
Brookfield Cap 2000+ Viscometer	Brookfield
FITC-conjugated Erythrina cristagalli lectin (ECL)	Vector Labs
Pooled Normal Human Plasma	Precision Biologic
Vacutainer Light Blue Blood Collection Tube (Sodium Citrate)	BD
Vacutainer Blood Collection Set, 21G x ¾" Needle	BD

Catalog Number
304910
-
FL-1141
CCN-10
369714
367287

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Author(s): M. Edward Quach, Anum K Syed, Renhao Li

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Date:	1/16/19

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Figures 6,5,3, and 1 have been adapted in whole or in part from two previous publications in Blood. All figure legends now reflect proper attribution as requested. See below for a link to Blood's republication policies. Specifically, under the subheading "Authors reusing their own material":

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Email addresses for each author are included in the editorial manager for this manuscript, and the corresponding author's email is included in the text of this manuscript.

3) Please define all abbreviations (PGE, etc.) before use.

The requested changes have been made.

4) Please abbreviate liters to L (L, mL, µL) to avoid confusion.

The requested changes have been made.

5) Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Thank you for catching this error. The protocol has been revised accordingly.

6) Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

The authors appreciate the provided editorial guidance on this matter. We have updated the protocol at several steps, reflected in the edited manuscript. Some specific changes per editorial suggestions:

1.1: What volume of blood is drawn?

The volume of blood drawn is dependent on the amount of PRP that individuals performing this protocol would require. This, in turn, is dependent on how many conditions they wish to include in their iteration of the protocol. Step 1.1 has been updated with some guidance on how to determine the amount of blood required.

1.3: How is the top layer isolated?

Some detail on the pipetting of the top layer has been added.

1.4: How to wash platelets in saline? Please provide the composition of Tyrodes buffer. If it is purchased, please cite the Table of Materials.

A washing scheme has been added, along with the composition of Tyrodes buffer and PIPES buffered saline, both of which can be made in-house.

1.5: How is the PPP obtained? It is not mentioned in previous steps.

Source of the PPP has been added to the materials table

2.1: Please specify the antibody or ligand added.

In this case the antibody or ligand added is the variable in the assay. This technique assesses the effect of shear on ligand-receptor pairs in solution. If the ligand is specified, then the technique is no longer generalizable to anything but the receptor-ligand pair our group has decided to investigate.

2.4: Please provide some guidance on an appropriate rate and duration.

Some rate and duration suggestions which are appropriate for human platelets have been added to this step.

2.6: Please provide examples on the markers that can be used.

Unless there is some confusion regarding this comment, this step already includes three suggested markers, their targets, and suggested concentrations.

2.7: At what temperature and for how long are the samples fixed?

These parameters have been updated.

3.3 Please describe how to quantitate crosslinking by area and aspect ratio parameters.

A brief description of the flow cytometry analysis has been added to this step

7) JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3‑6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have removed some of the discussion relating specifically to the findings from the assay as we have applied it in the past. We include a discussion of some critical steps and which steps to take special care during.

We have also added a few suggestions for what type of questions this technique could answer in the future, in addition to the discussion at the end of the first paragraph of the discussion. We have also added a paragraph regarding the significance with regard to other techniques and likewise, the limitations of the technique.

8) Figures 2‑4: Please add uppercase letters as labels to different panels.

The requested change has been made in our figure files.

9) Figures 2‑4, Figure 6: Please define error bars in the figure legend.

Thank you to the editor for noticing. Error bars have been defined in the legend.

10) Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

The table has been updated and alphabetized

11) References: Please do not abbreviate journal titles.

All journal titles in references have been expanded from their original abbreviations.
Note: The endnote style file on JoVE's website automatically formats citations with journal titles abbreviated

Reviewer 1 Comments

The authors thank reviewer 1 for his/her reading of the manuscript and characterization of our work as valuable and of interest to a broad audience.

Reviewer 2 Comments

The authors thank reviewer 2 for his/her reading of the manuscript and useful insights to improve the clarity of our methodology. Please see below for our specific responses to reviewer 2's comments.

1) Better description of methodology would improve ability to apply. Most of these are around the process of PRP isolation.

> *Step 1.1 Please define the gauge of needle recommended in this step. It is in the discussion, but not the cookbook.*
>
> We have added some clarifying guidance regarding the gauge of needle required for this step.
>
> *Step 1.3 Please provide better definition of the "top" layer Presumably, the cloudy suspension above the RBC pellet*
>
> Thank you for this advice. Both step 1.3 and 1.2 have been edited to make it clearer which layer is to be isolated
>
> *Step 1.4 Define the centrifugation conditions to pellet platelets*
>
> We have added some brief instruction on how to wash platelets to this step.
>
> *Step 1.5 How does one prepare platelet poor plasma (PPP)?*
>
> PPP can be prepared by centrifuging PRP at 1900 for 8-10 minutes, until platelets pellet out, which can be confirmed via hematocrit. However, it is also fairly cost effective to purchase pooled human PPP. We have therefore added pooled human PPP to the materials list for this manuscript.

2) How long can platelets sit before analyzing?

Once fixed, platelets can be analyzed right away or else they can be stored overnight at 4 degrees. This is indicated in step 2.7. Prior to fixation platelets can be kept at room temperature for 6 hours on a rotisserie. A

note about this has also been added to the discussion. The authors thank reviewer 2 for underscoring this important factor in working with platelets.

3) Step 2.6 Why 20 minutes? Can this be shorter/longer?

20 minutes is just a suggestion. In our hands 20 minutes usually is long enough for robust staining, and short enough for the negative control to remain dim. Of course, this period can be shortened or lengthened based on which markers the practitioner is using.

4) Check spelling of "brake" versus "brake" in the discussion.

The authors thank reviewer 2 for catching our mistake in usage even as (s)he fell victim to an adjacent error.