

METHODOLOGICAL ARTICLE

A sensitive and adaptable method to measure platelet-fibrin clot contraction kinetics

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Funding information

National Heart, Lung, and Blood Institute, Grant/Award Number: HL138179, HL150818 and HL56652; U.S. Department of Veterans Affairs, Grant/Award Number: I01BX003877

Handling Editor: Prof. Yotis Senis

Abstract

Background: Platelet-fibrin clot contraction is critical for wound closure and maintenance of vessel patency, yet a molecular understanding of the process has lagged because of a lack of flexible quantitative assay systems capable of assaying multiple samples simultaneously.

Objectives: We devised a sensitive and inexpensive method to assess clot contraction kinetics under multiple conditions.

Methods: Clot contraction was measured using time-lapse digital photography, automated image processing with customized software, and detailed kinetic analysis using available commercial programs.

Results: Our system was responsive to alterations in platelet counts and calcium, fibrinogen, and thrombin concentrations, and our analysis detected and defined three phases of platelet-fibrin clot formation: initiation, contraction, and stabilization. Lag time, average contraction velocity, contraction extent, and area under the curve were readily calculated from the data. Using pharmacological agents (blebbistatin and eptifibatide), we confirmed the importance of myosin IIA and the interactions of integrin $\alpha_{IIb}\beta_3$ -fibrinogen/fibrin in clot contraction. As further proof of our system's utility, we showed how 2-deoxyglucose affects contraction, demonstrating the importance of platelet bioenergetics, specifically glycolysis.

Conclusions: Our system is an adaptable platform for assessing the effects of multiple conditions and interventions on clot contraction kinetics in a regular laboratory setting, using readily available materials. The automated image processing software we developed will be made freely available for noncommercial uses. This assay system can be used to directly compare and define the effects of different treatments or genetic manipulations on platelet function and should provide a robust tool for future hemostasis/thrombosis research and therapeutic development.

KEYWORDS

clot contraction, metabolism, platelet function test, platelet-rich plasma, washed platelets

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Essentials

- Our method measures clot contraction kinetics using automated digital image processing.
- Our method confirmed requirements for integrins, myosin IIA, and glycolysis in clot contraction.
- Our method uses readily available resources and can be easily adopted in a laboratory setting.
- Our method is adaptable for analyzing multiple samples.

1 | INTRODUCTION

Platelets are small, anucleate, cell fragments that are important for maintaining vascular homeostasis.¹⁻³ Vascular disruption activates platelets, leading to $\alpha_{IIb}\beta_3$ integrin activation and fibrinogen-binding, linking the extracellular fibrin/fibrinogen network to the platelet actomyosin cytoskeleton.⁴ Clot contraction is an active squeezing of the clot, reducing its volume and consolidating its mass to form a semiporous thrombus for preventing blood loss.^{3,5} Platelet-driven clot contraction is important for promoting clot stability and for maintaining blood vessel patency.^{6,7} Contractile forces generated by the nonmuscle myosin, myosin IIA, are transmitted through the actin cytoskeleton to the fibrin meshwork, resulting in the expulsion of fluid, decrease of clot size, and increase of clot density.^{8,9} Microscopy studies are consistent with this view showing that during clot formation, platelets send out filopodia with longitudinal thin actin filaments that contact and “pull” on the fibrin fibers.^{3,8} Despite these insights, a refined understanding of platelets in clot contraction at the molecular and cellular level is lacking.

Several assays for monitoring clot contraction have been developed. Some use single images of clots formed in whole blood, platelet-rich plasma, or washed platelets (with exogenous fibrinogen).^{10,11} Others determine the amount of fluid extruded at an endpoint.^{7,12,13} These analyses can yield little kinetic information. Alternative methods periodically take “snapshots” during the contraction process.^{14,15} The images are then quantified using software, such as ImageJ, to approximate contraction rates.^{14,16} However, these methods use only a limited number of images and lack temporal resolution, thus misestimating lag time and contraction rates and being insensitive to subtle alterations in kinetics. Because there is clinical evidence that clot contraction is important in several pathological conditions (e.g., stroke),¹⁷ better understanding of contraction is needed to more precisely define the cellular and molecular mediators that influence the process.

The Thrombodynamics Analyzer solves many of the past problems and is valuable for evaluating clinical samples, especially whole blood.^{3,6,17} However, it compares only two samples simultaneously and has a costly setup. We report an easily implemented, cost-effective method for measuring clot contraction. Our method can use any digital camera with an intervalometer and measure multiple samples simultaneously. Importantly, we developed a custom software that automates the processing of the clot images and their quantification. Like the Thrombodynamics Analyzer, our system detected three phases of the clot contraction process, which was dependent on platelet number, calcium, thrombin, fibrinogen, myosin IIA, and fibrin-integrin $\alpha_{IIb}\beta_3$ interaction. We applied our system to probe the importance of platelet glycolysis to clot contraction.

2 | METHODS

2.1 | Imaging setup configuration

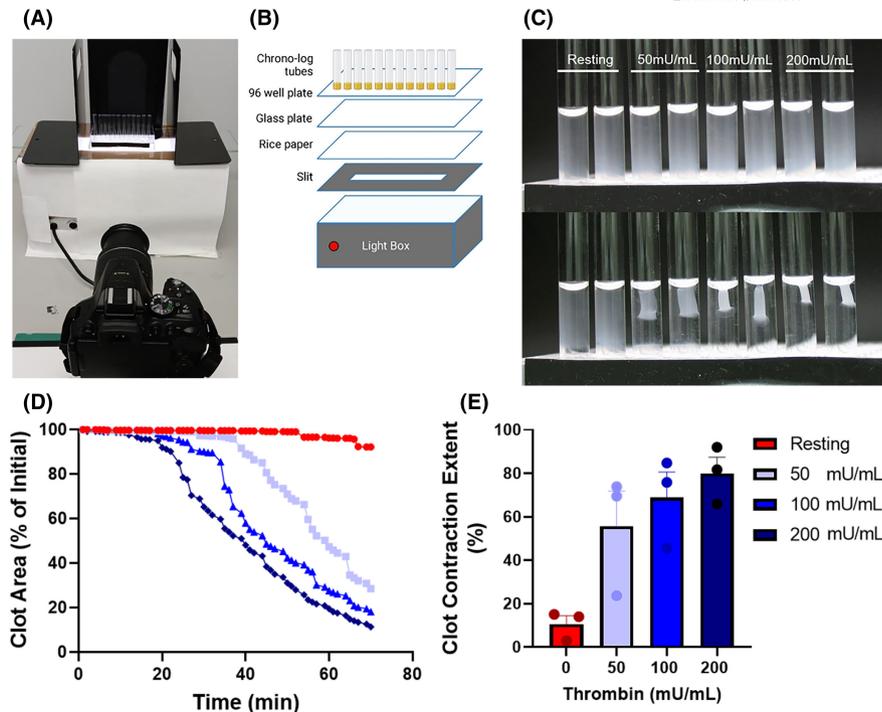
Generating quality images is essential to our method. Because platelet-fibrin clots are pale, it was important to optimize the lighting and camera system to maximize the images' contrast. To minimize interfering ambient light, the setup was housed in a dark room; however, a dark box or dark-curtained booth sufficed. Optimal sample illumination was from below. As shown in Figure 1A,B, a lightbox (Hall productions, HPE0612, 12×6 inches) was placed under a cardboard box with a rectangular opening (10×2 inches) cut into its top side. The opening was covered with thin rice paper to further diffuse the light. One or two clear, 96-well plates (Thermo Scientific AB0796) were placed on a glass plate over the opening to serve as tube holders for 12 or 24 samples, respectively. The back rows of the plate were covered with black paper/tape to suppress stray light. The front of the plate was covered with black tape to provide a sharp contrast at the bottom of the tubes. A black background was placed behind the plates.

For time-lapse photography, we used cameras with an intervalometer function to take a timed series of images. We have used a Canon Powershot Pro1 and a Nikon D5300 DSLR Camera with an 18–55 mm lens. The time intervals between two consecutive images were determined by the desired time resolution, minimally allowed intervals of the camera, maximally allowed numbers of total images, and the duration of the experiment. We used intervals of 1 min, which is the minimally allowed intervals of Canon Powershot Pro1 and allows for 100 and 999 min of recording for Canon Powershot Pro1 and Nikon D5300, respectively. For higher temporal resolution, intervals of 15 sec or less are possible for Nikon D5300. To obtain close-up images of the tubes while staying in focus, it is critical to either use the Macro function (Canon Powershot Pro1) or set the 18–55 mm lens to 55 mm (Nikon D5300). Many low-end digital cameras should prove adaptable for this application. For consistency, we recommend using the same working distance and other camera settings (e.g., speed [ISO 100], white balance, shutter speed, image pixels, and no flash) for all experiments.

2.2 | Preparation of reaction tubes

The reaction tubes are critical. They must be cleaned, inside and out, and have an acrylamide gel pad at the bottom to ensure that the clot contracts upward toward the solution meniscus.¹⁶ To clean, siliconized tubes (Chrono-Log P/N 312 cuvettes) were soaked in distilled water, followed by 95% ethanol, for 5 min each, and then

FIGURE 1 Assay configuration and examples of data. (A) Image of the imaging setup used. (B) Diagram of the samples and the under-sample lighting system. (C) Representative images at 0 (upper panel) and 70 min (lower panel) of clot formation in resting (R) and thrombin (50–200 mU/ml) stimulated mouse platelet-rich plasma (PRP). (D) Representative contraction curves from a thrombin titration of clot contraction of mouse PRP in (C). (E) Extent of clot contraction percent calculated as $(100 - \% \text{ area at } t_{\text{end}})$. Error bars indicate the SEM for $n = 3$. GraphPad Prism and Excel were used for all calculations



air-dried. The acrylamide solution for 12 tubes (one set of reactions) was: 2 ml of 10% polyacrylamide gel prepared by mixing 667 μ l of acrylamide (IBI InstaPAGE Acrylamide 40% Solution, 37.5:1), 500 μ l Protogel resolving buffer (National Diagnostics, EC-892 resolving gel), and 833 μ l distilled water. Polymerization was initiated with 9 μ l 10% ammonium persulfate and 2 μ l TEMED. Immediately, 100 μ l of the acrylamide solution was transferred to the bottom of each tube without touching the inner wall. The acrylamide solution was carefully topped with 10 μ l of distilled water and allowed to polymerize for ~2 h. The tubes were rinsed, twice, with HEPES-Tyrode buffer (pH 7.4), to remove unreacted polyacrylamide and solutes. As prepared, the tubes are single use.

2.3 | Platelet preparations

The animals were euthanized via CO₂ asphyxiation and blood was harvested by cardiac puncture. Platelet-rich plasma (PRP) or washed platelets were prepared from C57BL/6 mice.¹⁸ An anticoagulant master mix (100 μ l) was drawn into a 1-ml tuberculin slip tip syringe with a 26G needle so that the final concentrations in 1 ml of blood was 0.38% sodium citrate, 0.2 U/ml apyrase, and 10 ng/ml PGI₂. Blood was pooled, diluted 1:1 (v/v) with PBS or HEPES-Tyrode buffer (pH 6.5) containing 0.2 U/ml apyrase and 10 ng/ml PGI₂, and incubated for 5 min. PRP was harvested by centrifugation at 215g in a swinging-bucket rotor centrifuge (Beckman Coulter Avanti J-15R centrifuge with acceleration and deceleration set as 5 and 2, respectively) for 5 min.¹⁸ Platelets were harvested from PRP by centrifugation at 675g for 7–10 min. The pelleted platelets were gently resuspended in HEPES-Tyrode buffer (pH 6.5), supplemented with 1 mM EGTA, 0.2 U/ml apyrase, and 10 ng/ml PGI₂ using large

disposable transfer pipets. The platelets were again sedimented and resuspended in 1–2 ml HEPES-Tyrode buffer (pH 7.4), and their concentrations were measured using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Indianapolis, IN). The platelet concentrations were adjusted with HEPES-Tyrode's buffer (pH 7.4) and incubated at room temperature or 37°C for 30 min. Reactions containing platelets, CaCl₂, and human fibrinogen (Cat #F4883, Sigma), with or without inhibitors (final volume of 500 μ l) were gently mixed in Eppendorf tubes then initiated by adding thrombin (Chronolog, Haverton, PA). The mixture (450 μ l) was mixed and immediately transferred to a cleaned tube and the camera was activated to start the photo series.

2.4 | Image processing and analysis

Raw images were cropped using ImageJ macros (see software in <https://www.dropbox.com/sh/nce88konldxwzfa/AACn-ZbPO1Dep-wk0k76cErFa?dl=0>). Raw or cropped images (Figure 1C) were used to quantify clot areas or to generate movies of the whole clot contraction process (Supplemental software files [Data S1]). To analyze the images, we took a machine learning/neural network-based approach to generate a custom software, PROCTUBE, which automatically tracks the clots and quantifies their areas (Video S1). The quantified clot areas were compiled by the software into an Excel file for further analysis. The executable files for PROCTUBE (Windows 10 compatible) are provided at <https://www.dropbox.com/sh/nce88konldxwzfa/AACn-ZbPO1Dep-wk0k76cErFa?dl=0> PROCTUBE can be opened by double-clicking the executable file or by typing "proctube" or "proctube -m [modelfile]" in the command window. When the software starts, a window opens and you are directed to select

the folder with the saved pictures from your experiment. PROCTUBE outputs images with the recognized clots outlined in blue and areas of the clots quantified (in pixels), as well as compiles an Excel file containing all quantified clot areas for each image (Figure S1 and ReadMe documentation in <https://www.dropbox.com/sh/nce88konldxwzfa/AACn-ZbPO1Dep-wk0k76cErFa?dl=0>). These images and the table are saved to the same folder as the raw source images. Clot area, as a percent of the initial area (for PRP, Figure 1D), was calculated and plotted versus time using GraphPad Prism (8.4.3).

2.5 | Data analyses

Image data were analyzed (using GraphPad and Excel) to determine the lag time (initial time at which the point-to-point change in area exceeded 1%); area under the curve (AUC, calculated with GraphPad); clot contraction extent (relative clot size at 100 min) and average velocity (first derivative average, calculated with GraphPad Prism 8.4.3 for the time course; Figure 2A). As done by Tutwiler et al.,³ a piece-wise analysis was carried out by dividing the curve into three parts based on a smoothed first derivative plot (Figure 2A). The times, t_1 and t_2 , were determined from the local maxima and minima of the plots, respectively. The size of the smoothing window can be varied for improved resolution as needed. Using the scheme of Tutwiler et al.,³ the initial or initiation phase (t_0-t_1) was fit to an exponential curve equation; the second or contraction phase (t_1-t_2) was fit to a linear equation; and the third or stabilization phase (t_2-t_{end}) was fit to an exponential equation. The rates (k_1 , k_2 , and k_3) for each phase of the contraction curve were calculated using available software (Excel or GraphPad) and the following equations:

Phase 1: Initiation phase $t_0 < t < t_1$ $y = y_0 - (y_0 - y_1)(1 - e^{-k_1 t})$

Phase 2: Contraction phase $t_1 < t < t_2$ $y = y_1 - k_2 t$

Phase 3: Stabilization phase $t_2 < t < t_{end}$ $y = y_2 - (y_2 - y_3)(1 - e^{-k_3 t})$

Statistical significance was assessed using one-way analysis of variance followed by a multiple comparison test with the Bonferroni method (GraphPad Prism (8.4.3)).

2.6 | Study approval

All animal work was approved by the University of Kentucky Institutional Animal Care and Use Committee, protocol #2019-3384.

3 | RESULTS

3.1 | Assay optimization

Clot contraction is affected by the concentrations of platelets, calcium, thrombin, and fibrinogen. Initially, we tested different concentrations of washed mouse platelets, stimulated with thrombin (20 mU/ml) and fibrinogen (250 μ g/ml). Platelet concentrations

between 5 and 50 $\times 10^6$ /ml had a significant decrease in lag time as counts increased (Figure 2B,C). The average velocity and clot contraction extent increased and was the highest at 50 $\times 10^6$ platelets/ml (Figure 2D,E). AUC is inversely correlated with both average velocity and extent and thus is a less-sensitive metric of contraction (Figure 2F). Similar results were seen when each of the three phases: initiation (phase 1), contraction (phase 2), and stabilization (phase 3), were analyzed, though at the lowest platelet concentration used, none of the phases were readily detected from the data using the first derivative plot (Table S1). Given the robust contraction rate and clear delineation of all three phases, we chose 50 $\times 10^6$ platelets/ml for our standard assay conditions.

To address the calcium dependence, the concentration of exogenously supplied CaCl_2 was varied from 0 to 1 mM and clotting was initiated with 20 mU/ml thrombin in the presence of 250 μ g/ml fibrinogen (Figure 3A-E). EGTA was added to the platelet preparation washes but not to the final suspensions unless indicated. Although there were limited differences in clot contraction when calcium was included (between 0.1 and 1 mM), the average contraction velocity was only significantly reduced when EGTA was included. Phase 1 was most affected by increasing calcium concentrations and none of the three phases was detectible in the presence of EGTA (Table S1). These results paralleled previous data.^{3,12} Because calcium was required, but all tested concentrations worked similarly, we chose 0.5 mM as our standard concentration.

We next tested the importance of thrombin, which was varied from 5 to 100 mU/ml (Figure 1C-E for mouse PRP and Figure 4A-E under standard conditions for washed mouse platelets). As expected, with increased thrombin concentration, the extent of clot contraction remained unchanged but the lag time, which is indicative of clot initiation decreased. Phase 1, initiation, was most affected by increased thrombin, with its rate increasing ~5-fold over the concentration range tested (Table S1). Based on these data, we chose 20 mU/ml thrombin as our standard concentration for subsequent assays.

Cross-linking of fibrin and attachment of platelets to fibers are important steps in clot contraction.^{16,19} Thus, the ratio of fibrinogen to platelets is critical in determining the rate and extent of contraction.³ When clot contraction was initiated without added fibrinogen, the average velocity and extent were severely affected, suggesting the platelet pools of fibrinogen are insufficient (Figure 5). We tested fibrinogen levels varying from 62 to 1000 μ g/ml, which, although lower than physiological levels, provided a sensitive window for the assay. The extent of clot contraction and average velocity of contraction were highest at 125 and 250 μ g/ml. At the highest fibrinogen concentration tested (1000 μ g/ml), the fibrinogen/platelet ratio was too high for efficient contraction. When fibrinogen was omitted, none of the three phases were detectible (Table S1). For standard conditions, we chose 250 μ g/ml, which showed the best average velocity and extent of contraction.

We used 50 $\times 10^6$ platelets/ml, 0.5 mM CaCl_2 , 20 mU/ml thrombin, and 250 μ g/ml fibrinogen at room temperature as our standard reaction conditions for washed mouse platelets. This gave a

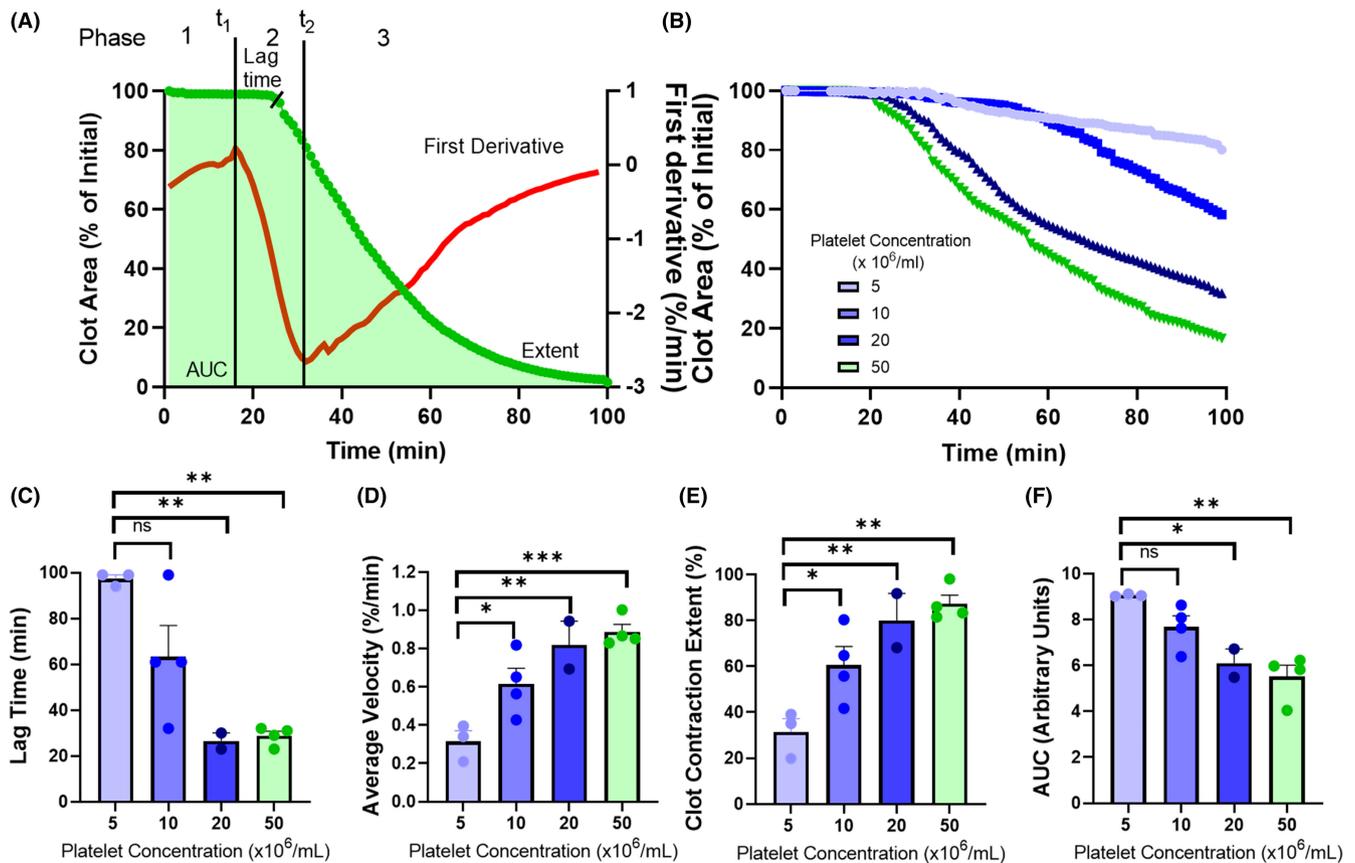


FIGURE 2 Analysis of clot contraction: platelet dependence. (A) A representative contraction curve with green line showing clot area (extent of clot contraction percent calculated as $100 - \% \text{ area at } t_{\text{end}}$) and red line showing the calculated first derivative smoothed with 10 neighbors on each side. The three phases of the curve were identified by the maxima (t_1) and minima (t_2) of the first derivative curve. Lag time was calculated as the time point at which point-to-point area measurements decreased by more than 1%. Extent was the contraction at 100 min (t_{end}). Area under the curve (AUC) was calculated using GraphPad Prism. (B) A representative dataset recovered from a mouse platelet titration stimulated with thrombin (20 mU/ml, 0.5 mM CaCl_2 , and 250 $\mu\text{g/ml}$ fibrinogen). The lag time (min; C) average velocity (%/min; D) extent of contraction ($100 - \% \text{ area at } t_{\text{end}}$; %; E) and AUC (F) were calculated and plotted versus platelet concentration. Error bars show SEM for $n = 2-4$; not significant: ns; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$ when compared with 5×10^6 platelets/ml. Statistical significance was calculated as described in the Methods section using GraphPad Prism

predictable lag time, average velocity, extent, and AUC, all of which could be compared across treatment groups. All three phases of the contraction process (initiation, contraction, and stabilization) were detectable. There was a reduction in the area over time, in resting/unstimulated controls, which likely represents some platelet activation during their preparation. We recommend optimizing these parameters: platelet number, calcium, fibrinogen, and agonist when using our system to tailor it to the experimental questions being asked and to assure that all three phases are detected and can be compared.

Our standard conditions differed from Tutwiler et al.,³ where whole blood was analyzed. We used lower concentrations of platelets, fibrinogen, and thrombin and lower incubation temperatures with longer time courses. Our intent was to focus on intrinsic properties of platelets; thus, we optimized conditions for washed mouse platelets. Using our system, contraction was measurable in PRP (Figure 1D and E) and whole blood from both human and mouse (data not shown). Because red blood cells (RBCs) dose-dependently inhibit contraction and thus are a variable in the

process,³ we chose to optimize our reactions in their absence. RBCs can be added to address specific experimental questions. In contrast to Tutwiler et al.,³ our system detected all three contraction phases in absence of RBCs, suggesting that the phases are intrinsic to platelet function.

3.2 | Troubleshooting

If a clot falls off the meniscus, it should not be analyzed. This occurred randomly, but thorough cleaning of the tubes lessened its chance. The camera position must be consistent to assure image uniformity and should be optimized using both the camera and the image processing software. We suggest having a dedicated place for experiments. Place the equipment in a dark room or box that can be isolated from external light sources. Stray light is a problem because it causes streaks on the tube surfaces, which may be misread by the software.

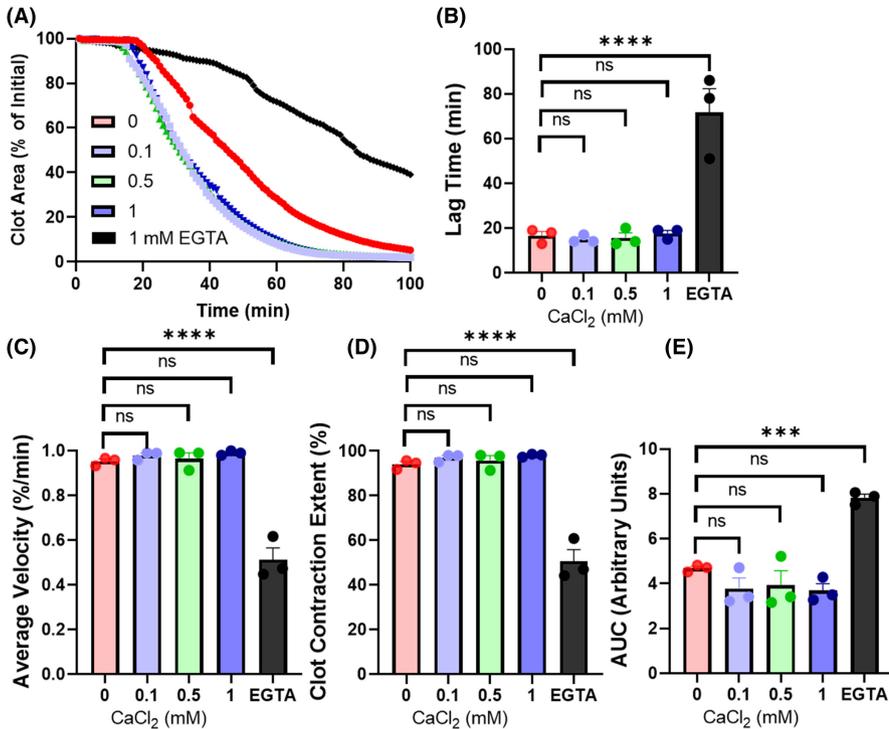


FIGURE 3 Analysis of clot contraction: calcium dependence. Shown are representative contraction curves (A), lag time (min; B), average velocity (%/min; C), the extent of clot contraction ($100 - \% \text{ area at } t_{\text{end}}$; %; D), and AUC (E) as determined with constant numbers of washed platelets ($50 \times 10^6/\text{ml}$ platelets) and the indicated concentrations of CaCl_2 or 1 mM EGTA. The concentrations of thrombin (20 mU/ml) and fibrinogen (250 $\mu\text{g}/\text{ml}$) were held constant. Error bars show SEM for $n = 3$; not significant: ns; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$ when compared with no calcium. Statistical significance was calculated as described in the Methods section using GraphPad Prism

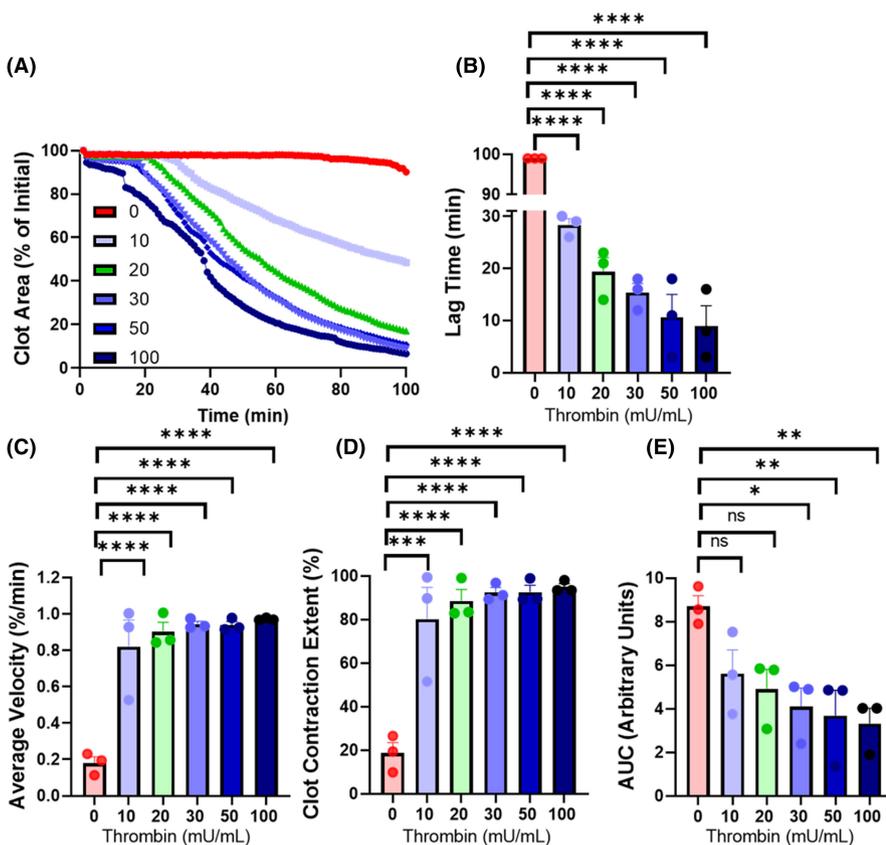


FIGURE 4 Analysis of clot contraction: thrombin dependence. Shown are representative contraction curves (A), lag time (min; B), average velocity (%/min; C), the extent of clot contraction ($100 - \% \text{ area at } t_{\text{end}}$; %; D), and AUC (E) as determined with constant numbers of washed platelets ($50 \times 10^6/\text{ml}$ mouse platelets) and the indicated concentrations of thrombin. The concentrations of CaCl_2 (0.5 mM), and fibrinogen (250 $\mu\text{g}/\text{ml}$) were held constant. Error bars show SEM for $n = 3$; not significant: ns; $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$ when compared with no thrombin. Statistical significance was calculated as described in the Methods section using GraphPad Prism

3.3 | Myosin IIA and fibrin/fibrinogen-integrin $\alpha_{\text{IIB}}\beta_3$ interactions are important for clot contraction

Myosin IIA and fibrin/fibrinogen-integrin $\alpha_{\text{IIB}}\beta_3$ interactions are important to clot contraction.^{20–22} Addition of a selective myosin IIA inhibitor, blebbistatin (50–200 μM)^{3,22}, resulted in a

dose-dependent increase in lag time and decreases in the average velocity and extent of clot contraction (Figure 6A–D). Consistently, there was an effect on phase 2, contraction, with an ~3-fold reduction in rate (Table S2). These results confirmed the importance of platelet myosin IIA-based force generation to clot contraction and indicated that the rate of contraction phase (phase 2) was

FIGURE 5 Analysis of clot contraction: fibrinogen dependence. Shown are representative contraction curves (A), lag time (min; B), average velocity (%/min; C), the extent of clot contraction ($100 - \% \text{ area at } t_{\text{end}}$; %; D), and AUC (E) as determined with constant numbers of washed platelets ($50 \times 10^6/\text{ml}$ mouse platelets) and the indicated concentrations of fibrinogen ($\mu\text{g}/\text{ml}$). The concentrations of CaCl_2 (0.5 mM), and thrombin (20 mU/ml) were held constant. Error bars show SEM for $n = 3-5$; not significant: ns; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$ when compared with no fibrinogen. Statistical significance was calculated as described in the Methods section using GraphPad Prism

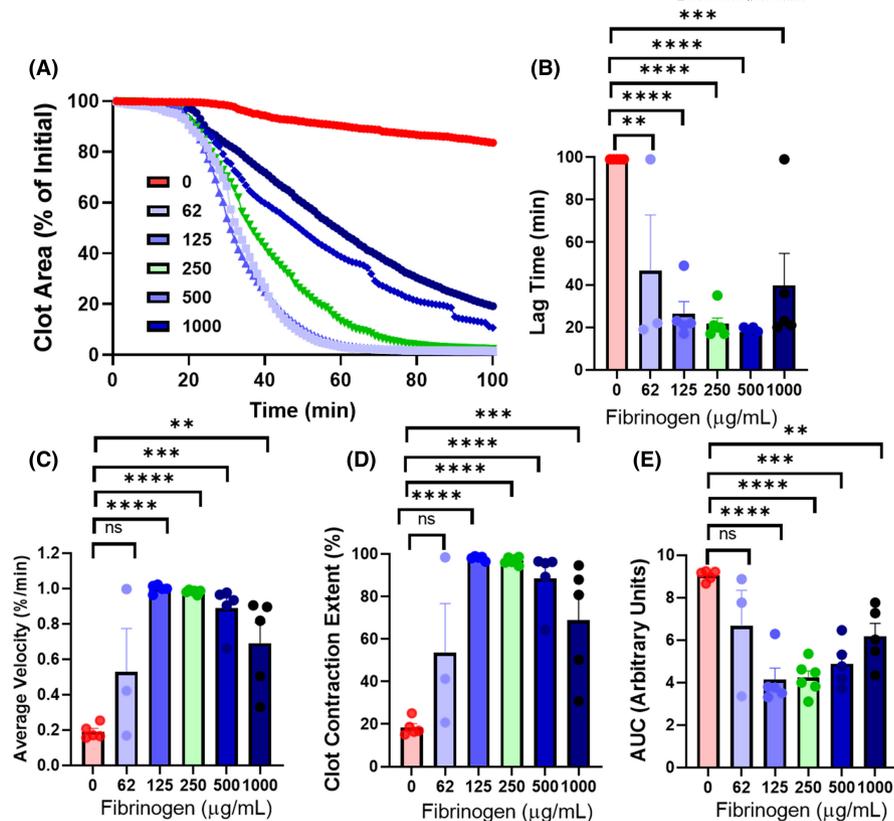
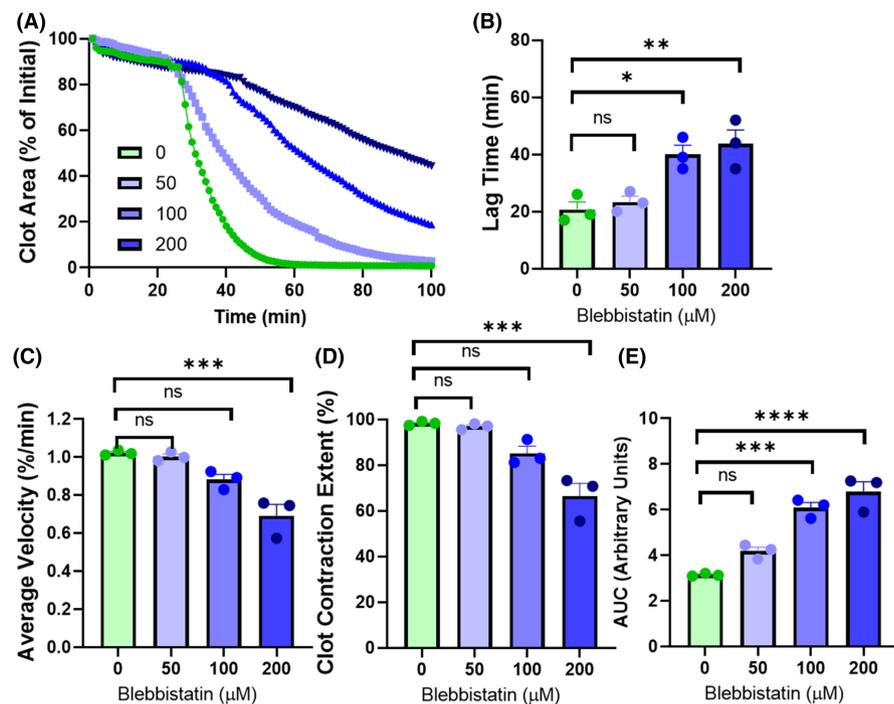


FIGURE 6 Role of myosin IIA in clot contraction. (A) Contraction in the presence of the indicated concentrations of blebbistatin (μM), under standard conditions (20 mU/ml thrombin, 250 $\mu\text{g}/\text{ml}$ fibrinogen, 0.5 mM CaCl_2 , and $50 \times 10^6/\text{ml}$ washed mouse platelets). The lag time (min; B), average velocity of contraction (%/min; C), extent of contraction ($100 - \% \text{ area at } t_{\text{end}}$; %; D), and AUC (E) were calculated and plotted. Error bars show SEM for $n = 3$; not significant: ns; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$ when compared with no blebbistatin. Statistical significance was calculated as described in the Methods section using GraphPad Prism



sensitive to defects in platelet myosin IIA. Our data parallel that in Tutwiler et al.³

Integrin $\alpha_{\text{IIb}}\beta_3$ is expressed at high levels on platelets and the fibrin/fibrinogen- $\alpha_{\text{IIb}}\beta_3$ interaction is crucial for the development of clot tension.²³⁻²⁶ To demonstrate its role in clot contraction, we tested the effects of eptifibatid, a competitive inhibitor of fibrin/fibrinogen binding to $\alpha_{\text{IIb}}\beta_3$.²⁷ Addition of 50 to 150 μM caused a dose-dependent

decrease in contraction extent (Figure 7A,D) and average velocity of contraction (Figure 7C). In this dosage range, there was an ~4-fold increase in lag time (Figure 7B). At the highest dose (150 μM), none of the three phases were detectible using the first derivative plot. Effects on phase 1 were dose-dependent at the lower inhibitor concentrations (Table S2). These results confirm the importance of fibrin/fibrinogen- $\alpha_{\text{IIb}}\beta_3$ interactions to clot contraction and parallel those in.³

3.4 | Glucose metabolism is important for clot contraction

Clot contraction is an actomyosin-dependent process that depends on glucose uptake and ATP hydrolysis. Glucose uptake and metabolism are important for platelet function.²⁸ Earlier reports, using conventional, qualitative approaches, showed that both glycolysis and oxidative phosphorylation contribute to the energy needs of platelets.²⁹ Our system is ideal for investigating how metabolism affects contraction. With washed platelets, reaction conditions can be tightly controlled, allowing an investigator to manipulate metabolites and energy sources. As an example, we used the phosphoglucosomerase inhibitor, 2-deoxyglucose (2DG).³⁰⁻³² Contraction time courses with varying 2DG concentrations showed significant inhibitory effects on lag time, average velocity, and extent of contraction (Figure 8). At the highest concentration (25mM) none of the three phases were detectable, using the first derivative plot. Interestingly, Phase 3 seemed most affected, even at the lower concentrations (rate reduced by ~5-fold with 10mM 2DG; Table S2). These experiments were performed in HEPES-Tyrode's buffer containing 5mM glucose. Omitting glucose reduced the contraction (data not shown). These results show that clot contraction relies on glycolysis and our method can be effectively used to study the effects of metabolic inhibitors.

4 | DISCUSSION

Clot contraction is important for hemostasis, promoting wound closure, and for vessel patency, reducing the size of potentially occlusive thrombi.^{3,33} However, the mechanistic understanding of clot contraction is limited, in part, because of limitations in the tools

available to quantitatively define it. Here, we report a simple yet sensitive method to assay clot contraction kinetics using a low-cost setup and a robust clot recognition and quantification software. Our system showed the expected dependence on platelet number, calcium, thrombin, fibrinogen, myosin IIA activity, and fibrinogen/fibrin-integrin interaction and our data parallel that reported by Tutwiler et al.³ Here, we showed how our assay system could be applied to investigate the dependency of clot contraction on platelet bioenergetics. Our system is ideal for such experiments since lower complexity samples such as washed platelets are required to control energy producing substrates. Plasma or whole blood would confound such studies. Our system is a cost-effective digital version of an old concept,^{7,15,16} and our contribution is the configuration details and software that allows clot contraction kinetics to be readily measured simultaneously for multiple samples in a regular laboratory setting. We hope that use of this method will facilitate the molecular dissection of the mechanisms and modulators of clot contraction, thus defining how dysfunction in clot contraction affects bleeding diatheses or occlusive thrombosis.

Although simple, our method requires careful implementation with the following being most important: (1) use and cleanliness of the Chrono-Log aggregometer tubes, (2) use of an acrylamide gel pad in the bottom of the tubes, (3) bottom illumination, (4) selection of a digital camera with an intervalometer and close-up features, (5) camera positions and setup, and (6) elimination of stray light. Gentle and thorough mixing after adding thrombin is necessary for uniform clot formation. Because samples are loaded sequentially, one should consider the time delay caused by sample loading into each tube when comparing lag times. The user can modify the start time post hoc to take into account sequential loading before the data is analyzed. Our system was highly reproducible. Comparing data generated over ~3years, using standard conditions (5×10^7 platelets/

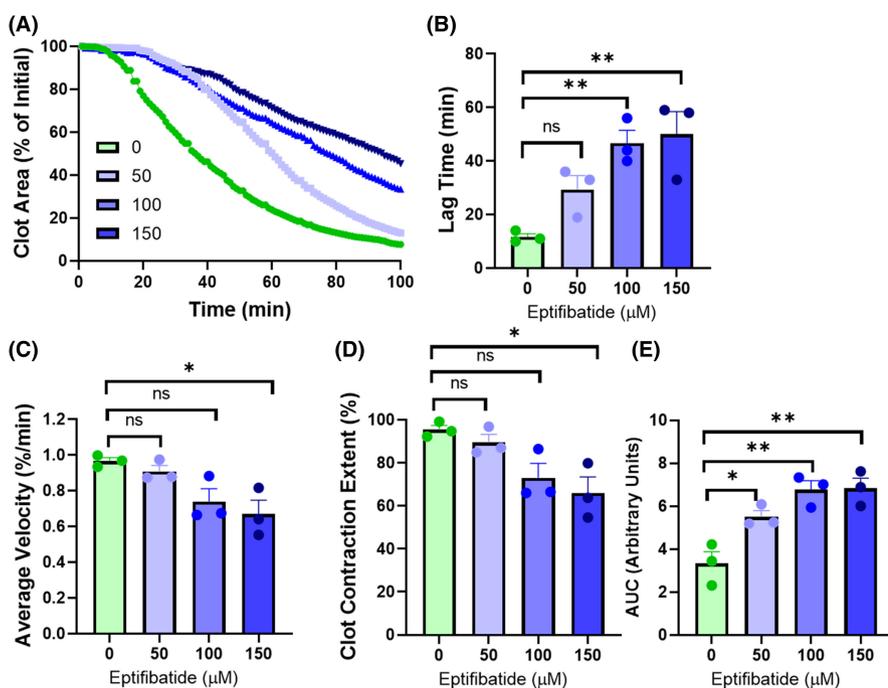
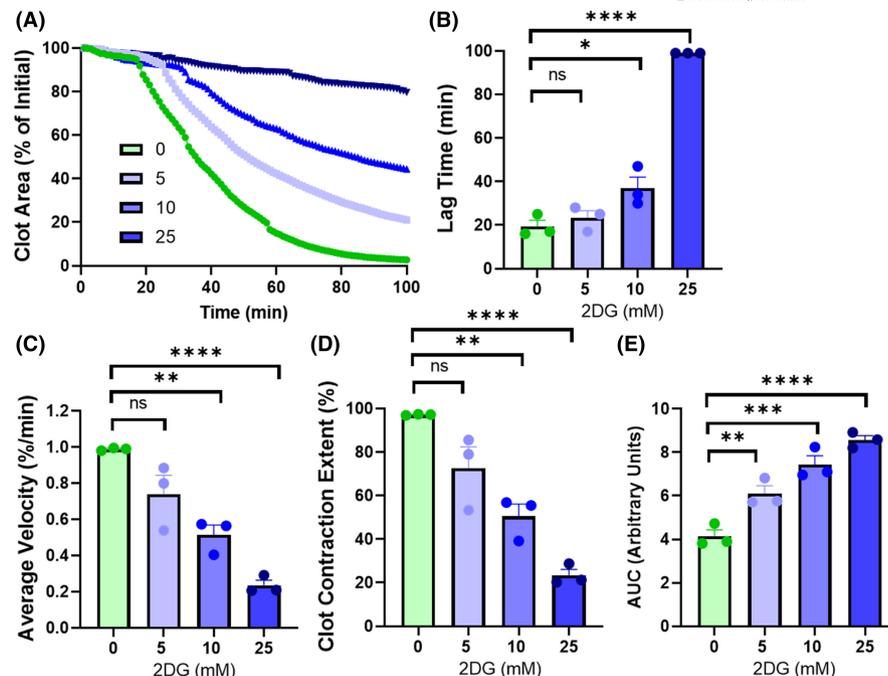


FIGURE 7 Role of $\alpha_{IIb}\beta_3$ integrin in clot contraction. (A) Contraction in the presence of the indicated concentrations of eptifibatid (μM), under standard conditions (20mU/ml thrombin, 250 $\mu\text{g}/\text{ml}$ fibrinogen, 0.5mM CaCl_2 , and $50 \times 10^6/\text{ml}$ washed mouse platelets). The lag time (min; B), average velocity of contraction (%/min; C), extent of contraction ($100 - \% \text{ area at } t_{\text{end}}$; %; D); and AUC (E) were calculated and plotted. Error bars show SEM for $n = 3$; not significant: ns; * $p \leq 0.05$; ** $p \leq 0.01$ when compared with no eptifibatid. Statistical significance was calculated as described in the Methods section using GraphPad Prism

FIGURE 8 Role of glucose metabolism in clot contraction. (A) Contraction in the presence of the indicated concentrations of 2-deoxyglucose (2DG; mM) under standard conditions (20 mU/ml thrombin, 250 μ g/ml fibrinogen, 0.5 mM CaCl_2 , and 50×10^6 /ml platelets). The lag time (min; B), average velocity of contraction (%/min; C); extent of contraction (100 - % area at t_{end} ; %) (D) and AUC (E) were calculated and plotted. Error bars show SEM for $n = 3$; not significant: ns; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$ when compared with no 2DG. Statistical significance was calculated as described in the Methods section using GraphPad Prism



ml, 0.5 mM CaCl_2 , 250 μ g/ml fibrinogen, and 20 mU/ml thrombin; >24 distinct platelet preparations), the percent error (calculated as $[(\text{SEM}/\text{mean}) \times 100]$) was <6% for extent of contraction, AUC, average velocity, and lag time. The system is readily adaptable to both human and rodent washed platelets and PRP (Figure 1D); however, conditions should be optimized when switching species. Finally, in our studies, we used room temperature ($\sim 25^\circ\text{C}$); however, the assays can be performed at 37°C , as needed. Temperature substantially lower than 25°C inhibited clot contraction (data not shown) and should be avoided.

An interesting deviation from Tutwiler et al.³ is the effect of increasing hematocrit. They reported that RBCs inhibited contraction. In their absence, three phases were not detected.³ Using our conditions, RBCs were not required to detect all three phases, suggesting that the phases are intrinsic to platelets. Because the concentrations of platelets, thrombin, and fibrin(ogen) used in our system are lower than in Tutwiler et al.,³ it is plausible that, at the concentrations of platelets (2×10^8 /ml) they used, the early stages of the contraction are too rapid for delineation of the first phase. By adding the RBCs, they dilute the chances of interactions effectively slowing the process. Our reductionist system only partially reflects the events occurring in whole blood; however, it enables researchers to manipulate specific aspects of the reaction to define mechanistic details.

Tutwiler et al. stated in 2016 that "Blood clot contraction remains the least studied stage of blood clotting."³ Although some requirements for contraction have a clear logic (e.g., thrombin, fibrinogen, calcium) and genetic diseases affecting the cytoskeleton (Wiskot-Aldrich and May-Heglin Syndromes) or integrins (e.g., Glanzmann thrombasthenia) have clear mechanisms, some contraction dysfunctions are not understood.³⁴ In stroke patients, there is a poststroke contraction defect.¹⁷ One explanation given was metabolic exhaustion of the platelets, which is consistent with our studies showing the

importance of glucose metabolism to normal clot contraction.^{28,32}

We hope that our system, with its multisample capacity, robust software, and low cost, will give investigators a valuable tool to define these aspects of platelet function. Our system's adaptability to test multiple conditions with the same preparation of platelets is invaluable to quantitatively define the relative effects of platelet pathways (i.e., signaling, secretion, or bioenergetics) or different therapeutics. It will also aid the of screening transgenic mouse platelets for functional defects. Thus, it can address mechanistic questions in a basic research laboratory setting. Though our assay measures the bulk properties of a population of platelets, the data generated can augment the recent innovative biophysical methods performed on single platelets.^{4,35} However, our system does need further development for clinical studies where standardization is critical.^{17,36,37}

Overall, the described system and software have been used for >3 years and have been both robust and reproducible with an error of less than 6%. The system is a more facile, less instrument-intensive way to scan multiple treatment paradigms. In short, we envision our assay system as a "workhorse" tool for basic research laboratories to be used to better understand the molecular features of how platelets mediate clot contraction.

AUTHOR CONTRIBUTIONS

K.S.P. carried out all of the experiments and wrote the manuscript. J.A. assisted with some of the experiments. Q.J.W. designed the setup and wrote the ImageJ macros for image cropping and movie-making. Y.L. and X.H. wrote the PROCTUBE software. Q.J.W. and S.W.W. directed the research and edited the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. Jeremy P. Wood and the members of the Whiteheart Laboratory for their careful perusal of this manuscript

and their patience. The authors also wish to thank Dr. John W. Weisel and his group for their help and specifically thank Drs. Valerie Tutwiler and Fazoil I. Ataullakhanov for demonstrating the HemaCore Thrombodynamics Analyzer System. The work was supported by grants from the NIH, NHLBI (HL56652, HL138179, and HL150818), and a Department of Veterans Affairs Merit Award to S.W.W. Q.J.W. was supported by pilot grants from the NIH, NIGMS COBRE (P30GM127211 and P20GM121327), NIDDK (P30 DK020579), NCR/NACTS (UL1TR001998), and an AHA Grant-in-Aid.

RELATIONSHIP DISCLOSURE

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SUPPORTING INFORMATION

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How to cite this article: Prakhya KS, Luo Y, Adkins J, Hu X, Wang QJ, Whiteheart SW. A sensitive and adaptable method to measure platelet-fibrin clot contraction kinetics. *Res Pract Thromb Haemost.* 2022;6:e12755. doi: [10.1002/rth2.12755](https://doi.org/10.1002/rth2.12755)