Active Micropump-Mixer for Rapid Antiplatelet Drug Screening in Whole Blood

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Supporting Information

ABSTRACT: There is a need for scalable automated lab-on-chip systems incorporating precise hemodynamic control that can be applied to high-content screening of new more efficacious antiplatelet therapies. This paper reports on the development and characterization of a novel active micropump-mixer microfluidic to address this need. Using a novel reciprocating elastomeric micropump design, we take advantage of the flexible structural and actuation properties of this framework to manage the hemodynamics for on-chip platelet thrombus assay on type 1 fibrillar collagen, using whole blood. By characterizing and harnessing the complex three-dimensional hemodynamics of the micropump operation in conjunction with a microvalve controlled reagent injection system we demonstrate that this prototype can act as a real-time assay of antiplatelet drug pharmacokinetics. In a proof-of-concept preclinical application, we utilize this system to investigate the way in which rapid dosing of human whole blood with isofrom selective inhibitors of phosphatidylinositol 3-kinase dose dependently modulate platelet thrombus dynamics. This modular system exhibits utility as an automated multiplexable assay system with applications to high-content chemical library screening of new antiplatelet therapies.

Dual antiplatelet therapies combining aspirin and purinergic receptor antagonists, such as clopidogrel, are the standard of care for prevention of platelet activation in thrombosis.1 Currently approved drugs exhibit a limited therapeutic window due to dose dependent bleeding driving a continued need for the identification and development of newer drug molecules.2,3 The International Society on Thrombosis and Hemostasis has highlighted the need for new more reliable blood-flow based analytical tools that have utility both with respect to better management of patients on current antiplatelet therapies (APT) and in the pursuit of newer more effective APT.4,5 There is, therefore, a strong driver for effective high-throughput drug screening lab-on-chip technologies that model the complex hemodynamic environments pertinent to platelet based pathologies.

Shear based assays have been extensively utilized to explore and define the mechanically sensitive signaling (mechano-transduction) and adhesion mechanisms underlying platelet function.6−12 These experimental assays have historically harnessed microcapillary, parallel-plate, and cone-and-plate approaches.6−13 In addition, a number of microfluidic devices have been described for the assessment of the hemodynamic activation processes underpinning hemostasis and thrombosis; with some applied in proof-of-concept clinical applications.14−27 However, few if any of these microfluidic systems have been translated to high-content APT screening.

Screening for new APT, particularly in the case of large chemical/drug libraries, demands robust and rapid assay methods to identify, refine, and test possible drug candidates. To facilitate reliable high-content hemodynamic-based screening there is a key requirement for scalable lab-on-chip systems that are capable of handling large numbers of independent blood samples in combination with automated drug and reagent delivery. The ideal high-content APT screening system...
should ideally include: (i) a mechanism for discrete hemodynamic control facilitating rapid changes in applied shear rates; (ii) integrated sample and reagent switching control to allow for the rapid introduction of multiple blood samples and/or agonist/antagonist dilutions series; (iii) efficient reagent-to-blood mixing, facilitating homogeneous drug dosing; (iv) the capacity for flow recirculation to control incubation times and shear rate exposure; and (v) the potential for on-demand platelet sampling, allowing for ancillary off-chip platelet assays. Complex integrated microfluidic lab-on-chip systems have the potential to meet all these requirements; however, the development of such platforms is in its infancy, and significant research and development is required to realize such systems in the preclinical and clinical hematology settings.

A key bottleneck to the advancement of complex blood microfluidic systems is the ability to precisely and rapidly manipulate small blood volumes (≤200 μL), while minimizing the impact on blood composition and function. This requirement for both materials (biocompatibility) and operational (mechanical) hemocompatibility is particularly critical where platelet based assays are to be employed, due to the sensitivity of these blood cells to changes in shear rate (γ) and shear stress (τ).\(^9\,29\,32\) Flow-based platelet thrombosis assays typically require the perfusion of blood samples or platelet isolates over a range of wall shear rates (γ\(_w\)) = 300 s\(^{-1}\) to ≥10,000 s\(^{-1}\) while minimizing the impact on platelet function prior to delivery to downstream thrombogenic substrates such as type 1 collagen, fibrinogen, or von Willebrand factor.\(^9\,29\,33\)

To achieve the required flow velocities most flow-based assay systems have typically utilized external syringe drivers, peristaltic pumps, or pressure/gravity driven pumping systems to achieve controlled flow rates (Q) and flow rates (Q\(_w\)).\(^8\,34\) These external systems, while effective, are not ideal for high-content screening due to restrictions on the ability to control relatively small blood volumes, due to relatively large fluidic dead volumes in interconnecting tubing and limited capacity for rapid time-varying fluidic control.

Pneumatically actuated reciprocating diaphragm micropumps and associated microvalve-gate systems offer significant potential as a solution for discrete on-chip automated blood/platelet handling due to their flexibility, scalability, facility for on-chip integration and automation, and the capacity for multiplexing in complex microfluidic circuits.\(^35\,\,37\)\(^–\,40\) Reciprocating diaphragm micropumps consist of a series of pump-chambers, in which a thin planar membrane surface or surfaces is inset into the microfluidic wall or walls. The pumping chamber is generally in the form of a deformable elastomeric diaphragm.\(^37\,\,40\) Pneumatic actuation/inflation of the diaphragm creates a peristaltic pressure wave that induces unidirectional or bidirectional fluid displacement, contingent on upstream fluidic resistance, valve format, and actuation mode.\(^37\,\,40\) The pressure capacitance, duty cycle (actuation frequency), degree of diaphragm inflation, and actuation regime determine overall flow rates and displaced volumes.\(^40\)

We describe the development, fabrication, and characterization of a microfluidic thrombosis assay that utilizes a pneumatically actuated reciprocating micropump-mixer as part of a proof-of-concept design that satisfies the requirements for a rapid screening system for candidate APT molecules.

### EXPERIMENTAL SECTION

**Device Description.** The overall dual-layered design is shown (Figure 1). Hemodynamic control and active mixing were achieved via an integrated reciprocating elastomeric micropump-mixer consisting of a pump chamber within the primary fluidic channel (bottom layer) housing three 2.4 mm\(^2\)/35–40 μm thick diaphragms (V1, V2, and V3). These diaphragms separate the primary fluidic channel (bottom layer) and vertically superimposed pneumatic actuation chambers (top layer; Figure 1a). Three pneumatic actuation channels provide independent positive and negative pressure control of the three-way pump cycle and are connected to a custom built pneumatic valve driver/control unit.\(^41\) The valve driver was coupled to the microfluidic housed within a custom 37 °C incubator via 0.75 mm inner diameter tygon tubing (Cole Parmer) with a length of 1.1 m. The custom pneumatic driver utilized microsolenoid valves to switch between two pneumatic reservoirs held at either negative or positive pressures, acting to open or close the on-chip valves. The micropump actuation regime, controlled through a custom Python script, is depicted in Figure 1b, with peristalsis achieved...
using a 120° cycle. In brief, each valve or valve set was actuated with a phase offset of 120°, where ⊥ represents a closed valve, or application of positive pressure (25–100 kPa), and ⤲ represents an open valve, or application of negative pressure (−20 kPa): ⊥⊥⊥, ⊥⊥⊥, ⊥⊥⊥, ⊥⊥⊥, ⊥⊥⊥ (Figure 1c). The integrated micropump-mixer thrombosis assay/drug screening prototype is depicted in Figure 1c. Reagent/drug delivery was achieved by incorporation of laterally placed dual straight-gate-valved injection ports positioned laterally 4 mm downstream of the blood sample reservoir (Figure 1c). Straight gate valve design and operation was as per Szendzik et al. (2018). CAD files and Python scripts are available on request. The thrombosis assay segment consisted of a downstream 500(μ) × 50(μ) × 14750(μ) μm (10:1 aspect ratio) straight microchannel coated with 100 μg/mL type 1 fibrillar collagen (Helena Laboratories).

**Device Fabrication.** Multilayer microfluidic device fabrication was carried out as per the Supporting Information.

**Blood Collection and Platelet Handling.** Ethics approval was obtained from the Alfred Hospital and Monash University Standing Committees on Ethics in Research involving Humans. All experiments were performed in compliance with relevant laws and guidelines and followed Monash Universities policies regarding the handling of human samples. Whole blood samples were obtained from consenting subjects via venesection using a 12G butterfly needle set, at a slow withdraw rate, into 50 mL syringes containing either 2% w/v citrate or 3.2% ACD. Samples were gently inverted to mix anticoagulant, transferred to 50 mL Falcon tubes, and allowed to rest for 10 min at 37 °C prior to use. Full blood examination, including platelet and erythrocyte counts, were conducted using a Cell-Dyn Emerald hematology analyzer (Abbott Diagnostics). Washed human platelets were isolated from human whole blood as previously described.

**Micron-Resolution Particle Image Velocimetry.** μ-PIV was carried out at the midplane of the micropump and collagen assay channel to measure the velocity field and the resulting strain rate (γ). dH2O seeded with 2 μm red fluorescent polystyrene particles (ThermoFisher Scientific) was perfused through the microfluidic system. Imaging was performed on a motorized inverted (Ti2, Nikon) microscope equipped with a G-2A filter cube for epifluorescence imaging and a high-speed PCO.dimax HS4 camera (4 Megapixels, 12bit). Fluorescent particles were illuminated using a diode-pumped solid-state laser with a continuous wave output at 532 nm (Verdi Family, Coherent). Images in the micropump and collagen channel were captured at 4000 and 7000 Hz through a 4X/0.13 and 10X/0.3 objectives, respectively. The optical magnification for these cases were 2.76 and 1.1 μm/pixel, respectively. A total of 10000 images were captured. Images were calibrated using a micron-sized calibration grid and preprocessed by applying histogram equalization. PIV vector computation was performed using DAVIS (LaVision, GmbH) using a multipass algorithm with grid deformation; final interrogation size = 64 × 64 pixels. In order to minimize spurious vectors, a 5 × 5 spatial filter and a temporal filter over 10 ms was applied to the velocity fields in postprocessing. Due to the periodic nature of the pump, the velocity fields were phase-averaged over seven pump cycles to enhance the signal-to-noise ratio.

**Confocal Mixing Analysis.** Micropump mixing capacity was assessed as per Stroock et al. through injection of FITC-conjugated, PEG species of M = 350 Da, 2000 Da, and 20 kDa (NANOCS Pty Ltd.). PEG-FITC concentrations were 28.6 (350 Da), 5.0 (2000 Da), and 0.5 μM (20 kDa). In brief, the method involved conducting a z-stack of the average intensity of all images, the background fluorescence was subtracted. A line scan was performed across the width of the channel to assess fluorescence arising from the PEG-FITC species. Standard deviations in fluorescence across the channel were calculated in GraphPad Prism.

**Platelet Imaging.** Washed platelets (200 × 10⁹/L) were loaded with DiOC₆ (2 μg/mL) for 10 min at 37 °C. Dye-loaded platelets (final platelet count = 200 × 10⁹/L) were then reconstituted with isolated RBC prior to perfusion through the device. Platelet adhesion and activation were blocked with theophylline (10 mM) and epifibatide (Integrilin, 20 μg/mL). Reconstituted blood samples were run through the micropump at the described actuation pressures and duty cycle. Epifluorescence (488ex/510em) images were acquired at 20 fps (1280 × 1024) using an Andor Zyla sCMOS camera attached to inverted Nikon TiU microscope (Nikon Plan Fluor 5X/0.50 objective). Images were compiled as maximum z-projections off-line in ImageJ.

**Platelet Ca²⁺ Assay.** Ca²⁺ analysis was carried out as per Supporting Information.

**FACS Analyses.** FACS analysis was carried out as per Supporting Information.

**Hemolysis Assay.** Hemolysis assay was carried out as previously described.

**Platelet Thrombosis Assay.** The microfluidic device and 0.1 × 1 mm glass microcapillaries (VitroCom Pty Ltd.) were derivatized with purified type 1 collagen (100 μg/mL; Helena Laboratories), according to previously published methods. Collagen was infused 1.4 mm downstream of the micropump assembly and allowed to physiosorb for 24 h at 4 °C before removal of excess collagen solution and blocking of the microfluidic with 10% (w/v) BSA for 10 min at room temperature. Thrombus formation was monitored at 37 °C using a Nikon Air confocal and analyzed as per published methods.

**Statistical Analysis.** Statistical analysis was performed where indicated in GraphPad Prism using unpaired two-tailed t tests. Analysis of variance (ANOVA) was performed using either GraphPad Prism or SPSS statistics. All variables were coded as continuous. Continuous results were assumed to have a normal distribution (using a normal probability plot), linearity (using a residual plot), and constant variability (using a scatter plot).

## RESULTS AND DISCUSSION

**Micropump Mechanical Performance.** To characterize the blood perfusion performance of the microfluidic flow rates (Q) with human citrated whole blood were assessed as a function of actuation pressure, and frequency (Figure 2a). Micropump performance increased with actuation pressure, with maximal Q for whole blood achieved at the maximum achievable actuation pressure of 100 kPa (the upper safety limit of the pneumatic valve driver; Figure 2a), with univariate analysis demonstrating a statistically significant effect of actuation pressure on output Q (F(32884.0) = 2214.6; p < 0.0005; partial η² = 0.994). Examination of the relationship between actuation frequency at constant pressure demonstrated a nonlinear (bell-shaped) relationship such that the peak pump Q was achieved within a frequency range of 3–9 Hz (Figure 2a), with univariate analysis demonstrating a statistically significant effect of actuation frequency on output
surface area of 2 mm² (Figures 2a and S1). Calculated average performance scaled with a micropump valve diaphragm size, blood volume per valve stroke (Figure 2b). Micropump at higher actuation frequencies is due to a reduced displaced $Q$ increasing actuation frequency, suggesting that the drop in actuation, the overall extent of valve actuation decreased with microvalve closure.

We conducted experiments in which platelet-rich plasma (PRP) was reconstituted with isolated autologous red blood cells at fixed Hct of 0, 20, 40, and 60% and perfused at 100 kPa/6 Hz. Figure 2c demonstrates that Hct within the physiological (20–40%) to supraphysiological range (60%) has no significant effect on micropump performance compared to PRP alone, suggesting that the system is insensitive to donor to donor variability in Hct. In addition, the mechanical effects of micropump operation on red blood cell handling were negligible, with minimal levels of hemolysis observed across the actuation range tested (Figure S2). This data suggests that, due to the mechanical displacement effects of the micropump valves, red blood cells are effectively partitioned and shunted in the direction of overall flow. It should be noted that blood is non-Newtonian, such that fluid viscosity is not constant, but varies as a function of flow velocity. It is therefore possible that the increased bulk fluid viscosity due to increased Hct may be counterbalanced by transient changes in cell margination during each pump cycle.

Taken together this mechanical analysis demonstrates that the micropump design can act as a relatively efficient driver of whole blood flow and can deliver platelets to the downstream collagen assay segment at pathophysiologically relevant $\gamma_w$. Fluid Dynamics Assessment of Micropump Operation. To investigate the fluid dynamics of micropump operation, we conducted $\mu$-PIV experiments under select actuation conditions of 100 kPa/6 Hz and 50 kPa/3 Hz. Snapshots of the instantaneous streamwise shear rate field ($\gamma$) and instantaneous velocity vectors ($\nu$) at six valve positions in one cycle of the micropump are shown in Figure 3a,b (Figure S3; Videos S1 and S2). $\mu$-PIV demonstrated that complex flow behaviors were apparent, such as the presence of counter-rotating vortices (steps 1, 2, and 5) and stagnation points (steps 2 and 5), suggesting that a high degree of blood component mixing may be achievable. Discrete nodes of elevated transient $\gamma$ were observed at the interface between V2 and V3 during steps 2 and 4, with transient increases primarily occurring during the filling phase (step 6), centered under and between V1 and V2, with peak transient $\gamma$ observed at the midpoint of V2 (Figures 3a,b and S3a,b; Videos S1 and S2).

Comparison of micropump operation at 100 kPa/6 Hz and 50 kPa/3 Hz demonstrated predominantly similar flow patterns per cycle with marginally differing peak $\gamma$ transients (Figures 3a,b and S3a,b; Videos S1 and S2), with the 50 kPa/3 Hz actuation regime producing marginally smaller flow accelerations and lower instantaneous $\gamma$ for a given $Q$ (Figures 3a,b and S3a,b; Videos S1 and S2). Micropump operation resulted in a pulsatile instantaneous flow rate waveform within the downstream collagen assay segment of the device, with pulse frequency mapping to micropump actuation frequency (Figures 3a,b and S3a,b).

Taken together, this analysis suggests that, due to the generation of vortical flow structures, the micropump may act as a highly efficient blood mixer. It should be noted that due to...

Q ($F(4, 434.88) = 333.9; p < 0.0005$; partial $\eta^2 = 0.971$). Peak micropump performance with whole blood was limited to a maximum $Q = 58.2 \mu$L/min at 100 kPa/6 Hz. A significant but minor interaction effect of frequency and pressure was observed ($F(12, 135.047) = 103.7, p < 0.0005$; partial $\eta^2 = 0.969$).

To examine the effect of increasing actuation frequency on microvalve closure efficiency, we conducted line-scanning confocal microscopy experiments in which valve operation was recorded in a single cross-sectional $xz$ plane as a function of time. This analysis demonstrated that, apart from 100 kPa actuation, the overall extent of valve actuation decreased with increasing actuation frequency, suggesting that the drop off in $Q$ at higher actuation frequencies is due to a reduced displaced blood volume per valve stroke (Figure 2b). Micropump performance scaled with a micropump valve diaphragm size, with optimal micropump performance achieved at a diaphragm surface area of 2 mm² (Figures 2a and S1). Calculated average $\gamma_w$ values within the downstream collagen assay segment as a function of micropump actuation mode were 362 s⁻¹ at 25 kPa/1 Hz, 1100 s⁻¹ at 50 kPa/3 Hz, 1454 s⁻¹ at 50 kPa/15 Hz, and 4461 s⁻¹ at 100 kPa/6 Hz, well within the optimal range for in vitro thrombosis models.

A key variable associated with blood handling is the effect of donor to donor variability in red blood cell density (hematocrit) that may affect overall blood viscosity and therefore micropump performance. To determine the impact of hematocrit (Hct), we conducted experiments in which platelet-rich plasma (PRP) was reconstituted with isolated autologous red blood cells at fixed Hct of 0, 20, 40, and 60% and perfused at 100 kPa/6 Hz. Figure 2c demonstrates that Hct within the physiological (20–40%) to supraphysiological range (60%) has no significant effect on micropump performance compared to PRP alone, suggesting that the system is insensitive to donor to donor variability in Hct. In addition, the mechanical effects of micropump operation on red blood cell handling were negligible, with minimal levels of hemolysis observed across the actuation range tested (Figure S2). This data suggests that, due to the mechanical displacement effects of the micropump valves, red blood cells are effectively partitioned and shunted in the direction of overall flow. It should be noted that blood is non-Newtonian, such that fluid viscosity is not constant, but varies as a function of flow velocity. It is therefore possible that the increased bulk fluid viscosity due to increased Hct may be counterbalanced by transient changes in cell margination during each pump cycle.

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the limitations of using aqueous media rather than blood, μ-PIV analysis may represent a simplification of overall hemodynamics, where partitioning of red blood cells and cell–cell interactions may further modify mixing dynamics. **Micropump Reagent–Blood Mixing.** Given the complex hydrodynamic effects revealed by μ-PIV, we posited that our device would act as an effective active drug molecule-to-blood mixer. To investigate this, we conducted a series of experiments in which FITC conjugated polyethylene glycol (PEG) species with M of 350 Da, 2000 Da, and 20 kDa (modeling the M range of small molecule inhibitors to high M heparin) were injected continuously into the perfused blood sample via lateral injection ports (Figure 1c) with micropump actuation at 50 kPa/3 Hz. Scanning confocal imaging of cross-sectional FITC fluorescence was conducted at the following micropump locations, with mixing time ($t_{\text{mix}}$) calculated based on flow velocities measured by μ-PIV relative to the drug injection ports: [1] Drug-port ($t_{\text{mix}} = 0$ s, $d = 0$ mm); [2] cross-section prepump ($t_{\text{mix}} = 0.74$ s, $d = 2.1$ mm); [3] cross-section postpump ($t_{\text{mix}} = 8.8$ s, $d = 7.8$ mm); [4] cross-section at the collagen assay channel ($t_{\text{mix}} = 10.9$ s, $d = 23.6$ mm). Ext. pump—external syringe driver control; Micropump—micropump driven control; Premixed—premixed control samples perfused through the test manifold. (d) Proportional axial distribution of DiOC$_6$-labeled platelets in human whole blood as a function of micropump actuation step. Gray-shaded blocks indicate the relative position of V1, V2, and V3. (e) Proportional lateral distribution of DiOC$_6$-labeled platelets in human whole blood as a function of micropump actuation step. Arrows indicate valve upstroke or downstroke phase. Representative of $n = 3$ independent experiments.

**Figure 3.** μ-PIV assessment of micropump performance. (a) μ-PIV contour maps showing planar phase-averaged strain rate magnitude ($\gamma$) and velocity vectors for micropump operation at 50 kPa/3 Hz (Video S2). Note that panels approximate steps 1–6 of micropump operation. Dotted marques denote opening microvalves; solid marques denote fully closed microvalves. Overall flow direction is bottom-to-top. (b) μ-PIV analysis of instantaneous flow rates at cross-section within the downstream collagen assay channel at 50 kPa/3 Hz, respectively. Representative of $n = 2$ independent experiments for each case.

**Figure 4.** Micropump mixing efficiency. (a–c) Confocal image analysis showing pixel × pixel fluorescence standard deviation ($\sigma$) of PEG-350-FITC, PEG-2000-FITC, and PEG-20,000-FITC, respectively, in a cross-sectional flow at 50 kPa/3 Hz or external syringe pump perfusion ($Q = 13.5$ $\mu$L/min); [1] cross-section at drug injection port ($t_{\text{mix}} = 0$ s, $d = 0$ mm); [2] cross-section prepump ($t_{\text{mix}} = 0.74$ s, $d = 2.1$ mm); [3] cross-section postpump ($t_{\text{mix}} = 8.8$ s, $d = 7.8$ mm); [4] cross-section at the collagen assay channel ($t_{\text{mix}} = 10.9$ s, $d = 23.6$ mm). Ext. pump—external syringe driver control; Micropump—micropump driven control; Premixed—premixed control samples perfused through the test manifold. (d) Proportional axial distribution of DiOC$_6$-labeled platelets in human whole blood as a function of micropump actuation step. Gray-shaded blocks indicate the relative position of V1, V2, and V3. (e) Proportional lateral distribution of DiOC$_6$-labeled platelets in human whole blood as a function of micropump actuation step. Arrows indicate valve upstroke or downstroke phase. Representative of $n = 3$ independent experiments.
Prepump ($t_{\text{mix}} = 0.74$ s, $d = 2.1$ mm); 
Postpump ($t_{\text{mix}} = 8.8$ s, $d = 7.8$ mm); 
Collagen-assay channel ($t_{\text{mix}} = 10.9$ s, $d = 23.6$ mm) (Figure 4a–c). Quantitation of the extent of mixing as a function of PEG-FITC fluorescence standard deviation ($\sigma_{\text{PEG-FITC}}$) at cross-section, according to the method of Stroock et al. (2002), demonstrated that at 50 kPa/3 Hz significant mixing was achieved immediately downstream of V3 ($t_{\text{mix}} = 5.09$ s), with complete homogeneity achieved (relative to premixed controls) 15.9 mm downstream of V3 (at the collagen assay channel, $t_{\text{mix}} = 10.9$ s), across the $M$ range tested (Figure 4a–c). In comparison, experiments in which blood flow was controlled by an external syringe driver (in the absence of micropump actuation) demonstrated that diffusion limited mixing was negligible across the microfluidic circuit (Figure 4a–c).

To investigate the way in which micropump hemodynamics and mixing impacts blood platelet distribution in whole blood, we conducted epifluorescence imaging of DiOC$_6$-labeled platelets at micropump positions V1, V2, and V3, under dynamic micropump operation. Examination of the axial distribution of platelets at centerline during pump cycling demonstrated a relative increase in platelet distribution centered under the microvalves during valve upstroke, due to downstream valve closure and the negative pressure of valve opening (Figure 4d,e). A relative increase in platelet density was observed immediately downstream of V3 upon concomitant closure of V2 and V3 (step 4), leading to positive displacement of the platelet population in the flow direction (Figure 4d,e). Observation of platelet lateral distribution bisecting V1, V2, and V3 demonstrated distinct behaviors, dependent on the phase of micropump actuation, with redistribution of platelets to the valve margins during downstroke and centralization of the platelet population during valve upstroke (Figure 4e).

Taken together, this data demonstrates that the micropump can act as both hemodynamic controller and as a highly effective and rapid reagent to blood mixer, without the requirement for additional passive mixing components, such as circuitous mixing structures, that would increase blood contact times with the device and potentially lead to increased levels of platelet activation.

**Effect of micropump operation on blood platelet activation and function.** A key consideration for an effective micropump-mixer is the ability to control blood hemodynamics while minimizing direct effects on blood platelet function (mechanical hemocompatibility) prior to downstream assay. Our $\mu$-PIV analysis and platelet distribution experiments suggested that blood platelets may experience localized transient $\gamma$ gradients during passage through the micropump-mixer valve structures. To directly examine the effect of micropump-mixer operation on platelet activation, we monitored real-time platelet $[\text{Ca}^{2+}]$, as a function of position within the valve manifold. Detailed analysis correlating platelet $[\text{Ca}^{2+}]$, with lateral position (across valves) and axial position...
within the micropump demonstrated that $[\text{Ca}^{2+}]_c$, mapped to the observed platelet redistribution effects and hemodynamics identified at the individual micropump valves (Figure S5a,b), suggesting that overall blood mixing and associated hemodynamics may transiently affect platelet function. Analysis of platelet $[\text{Ca}^{2+}]_c$ at 100 kPa/6 Hz in the direction of blood flow, demonstrated that mean $[\text{Ca}^{2+}]_c$ (averaged over the complete pump cycle) increased above a baseline of $\sim$50 nM, approaching $\sim$200 nM at V3 (Figure 5c). At 50 kPa/3 Hz $[\text{Ca}^{2+}]_c$ approached $\sim$130 nM (Figure 5c). Blockade of platelet amplification loops (ALB) reduced overall $[\text{Ca}^{2+}]_c$ to $\sim$150 and $\sim$100 nM for 100 kPa/6 Hz and 50 kPa/3 Hz, respectively, suggesting that the canonical platelet activation loops have a partial effect on platelet activation in the device (Figure S4). Significantly, however, while some increase in mean platelet $[\text{Ca}^{2+}]_c$ was observed during passage through the micropump-mixer this increase was rapidly down-regulated immediately postpump, approaching baseline levels (Figures 4c and S4c), demonstrating that micropump dynamics had only a transient effect on overall platelet activation.

To examine the downstream effect of micropump operation and assess the impact of the observed transient platelet $[\text{Ca}^{2+}]_c$, increase on global platelet activation we monitored the canonical platelet activation markers of, integrin $\alpha_{IIb}\beta_3$ activation (Pac1 binding), and $\alpha$-granule secretion (P-selectin expression) as a function of micropump actuation pressure and frequency following passage through the thrombus assay segment of the microfluidic and compared this with the "gold standard" microcapillary-based flow assay (Figure 4d,e). Micropump operation at 25 kPa/1 Hz, 50 kPa/3 Hz, 50 kPa/15 Hz, and 100 kPa/6 Hz had no significant effect on platelet integrin $\alpha_{IIb}\beta_3$ activation in bulk flow relative to the microcapillary benchmark (Figure 4d,e). Examination of $\alpha$-granule secretion in the form of P-selectin expression correlated with integrin activation, with no significant increase above that of the microcapillary benchmark controls (Figure 4e,f). Taken together, these data demonstrate that micropump-mixer operation has minimal effects on downstream platelet activation (at the collagen assay channel) when compared to the well-accepted microcapillary based flow assay and validate its utility as a hemocompatible blood handling system across a broad range of output shear rates.

**Application to Platelet Thrombus Dynamics Assay.**
Given the demonstrated effectiveness of the microfluidic as an active mixing and hemocompatible blood perfusion system, we next examined its effectiveness in driving platelet thrombosis at type 1 fibrillar collagen, a standard assay for exploring APT efficacy in vitro. Our $\mu$-PIV analysis revealed that the micropump-mixer generates a pulsatile Q within the downstream collagen assay segment (dependent on actuation frequency) that could affect platelet thrombus dynamics (Figures 3b and S3b). To investigate the impact of micropump pulsatile flow on platelet thrombosis we compared the micropump-mixer prototype operating at 25 kPa/1 Hz, 50 kPa/3 Hz, 50 kPa/15 Hz, and 100 kPa/6 Hz with a well published 1 mm(w) $\times$ 0.1 mm(h) glass microcapillary (VitroCom) based flow assay that utilized an external syringe driver (Harris PHD) delivering nonpulsatile flow at equivalent $\gamma_a$ to the micropump system. Figure 6a,b (Video S3) shows that there was no significant difference between micropump-mixer and microcapillary platelet thrombus assays across the $\gamma_a$ and actuation frequency range tested. An overall reduction in maximal thrombus volume was observed for both microcapillary and micropump systems at $\gamma_a = 1454$–4461 s$^{-1}$ that was a result of increased levels of observable embolization at the surface of type 1 collagen. A significant increase ($p < 0.0001$) in the extent of thrombus formation over time was observed with micropump actuation at 50 kPa/15 Hz compared to the microcapillary system (Figure 6b). This trend mapped to a nonsignificant trend observed for bulk-flow platelet activation markers Pac1 and P-sel (Figure 5d,e), suggesting that, at the highest actuation frequency of 15 Hz, the micropump-mixer may have a nominal impact on platelet aggregation dynamics.

Taken together, this data demonstrates that the micropump-mixer can effectively drive platelet thrombus formation across a relatively wide $\gamma_a$ range with equivalency to the well accepted microcapillary based assay. While the functional effects of time-varying (pulsatile) flow on platelet function is less well described than that of constant laminar flow, several studies suggest that shear rate transients, flow acceleration, and flow deceleration can modulate platelet signaling dynamics and therefore platelet activation and aggregation. While outside the scope of the present study, the ability of the micropump-mixer to control instantaneous flow pulsatility lends itself to further exploration of the effects of time varying hemodynamics on platelet function, primary hemostasis, and thrombosis, and may also open a window onto how time-varying flow impacts APT pharmacokinetics and efficacy.

**Proof-of-Concept Application to APT Screening.** To demonstrate the potential of the microfluidic as an effective and rapid APT screening platform we conducted experiments in which we assayed a series of isofom selective small molecule inhibitors of platelet phosphatidylinositol 3-kinase (PI 3-K): AS 2524224 (M = 305.28 Da, PI 3-Ky selective); TGX221 (M = 364.44 Da, PI 3-K$\beta$ selective); LY294002 (M = 10836

**Figure 6.** Micropump driven thrombus assay. Thrombus volume $v$ at the surface of Type 1 collagen for microcapillary $v$ micropump-mixer whole blood flow; $n = 2$ independent experiments.

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Figure 7. Micropump-mixer APT screening. (a) Microcapillary based drug dosing under flow at \( \gamma_w = 1100 \text{ s}^{-1} \) showing thrombus volume vs time at the surface of Type 1 collagen (all drugs premixed 10 min prior to sample perfusion). (b) Micropump-mixer drug dosing at 50 kPa/3 Hz (\( \gamma_w = 1100 \text{ s}^{-1} \)) showing thrombus volume vs time at the surface of Type 1 collagen (all reagents were continuously injected into the sample stream with a mixing time at homogeneity of \( \sim 10.9 \text{ s} \)). Control-citrated whole blood; DMSO, whole blood + 0.025% w/v dimethyl sulfoxide; Wortmannin, 100 nM; LY294002, 25 \( \mu \text{M} \); TGX221, 0.5 \( \mu \text{M} \); AS 2524224, 2 \( \mu \text{M} \). (c) Representative confocal reconstructions of micropump-mixer whole blood flow experiments (\( t = 4 \text{ min} \)). (d–g) Dose response curves showing % inhibition of thrombus growth following injection of Wortmannin (final \([\text{Wortmannin}]\) after micropump mixing–incubation \( t = 10.9 \text{ s} \)); LY294002 (final \([\text{LY294002}]\) after micropump mixing–incubation, \( t = 10.9 \text{ s} \)); TGX221 (final \([\text{TGX221}]\) after micropump mixing–incubation, \( t = 10.9 \text{ s} \)); AS 2524224 (final \([\text{AS 2524224}]\) after micropump mixing–incubation, \( t = 10.9 \text{ s} \)) into the prototype microfluidic vs microcapillary blood flow experiments (Wortmannin mixed and incubated for 10 min prior to perfusion); \( n = 3 \) independent experiments.
micropump-mixer to perform as a reliable and rapid preclinical assay system. Direct head to head comparison of this system with an established laminar microcapillary flow assay (well represented in the preclinical platelet literature) demonstrated equivalency, if not superiority, of the micropump-mixer system. Significantly, by integrating drug/reagent delivery on-chip and harnessing the rapid active mixing attributes of the micropump-mixer design, we were able to achieve controlled drug–blood incubation times ~55-fold shorter than that of the standard assay. Taken together, these data demonstrate that this component microfluidic system offers significant flexibility with respect to on-chip automation of blood handling and provides the capacity for high density multiplexed blood circuits for high-content APT screening. In addition, due to the relatively small dead volumes, this prototype system may have significant utility in the assay of murine blood samples, where total sample volumes are limiting. Future developments are focused on the incorporation of an addressable fluidic-logic based control system, facilitating true automated multiplexable high-content lab-on-chip drug screening functionality.

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02486.

Supplementary Figures S1, S2, S3, and S4 (PDF)
Supplemental Video S1: µ-PIV of dynamic micropump operation at 100 kPa/6Hz. µ-PIV analysis showing instantaneous velocity vectors and velocity magnitude ($V_{mag}$ mm/s) of the micropump-mixer operating at 100 kPa/6 Hz. Dotted line and graph (top) show average output flow rate (µL/min) immediately downstream of V3. Video at 0.0075X normal speed (AVI)
Supplemental Video S2: µ-PIV of dynamic micropump operation at 50 kPa/3Hz. µ-PIV analysis showing instantaneous velocity vectors and velocity magnitude ($V_{mag}$ mm/s) of the micropump-mixer operating at 50 kPa/3 Hz. Dotted line and graph (top) show average output flow rate (µL/min) immediately downstream of V3. Video at 0.0075X normal speed (AVI)
Supplemental Video S3: 3D platelet thrombus growth on type 1 collagen in micropump-mixer device. Reconstructed line scanning confocal images of DiOC6 labeled platelet thrombus growth dynamics driven by the micropump-mixer operating at 1 Hz/25 kPa, 3 Hz/50 kPa, 6 Hz/100 kPa, and 15 Hz/50 kPa, respectively. Each frame represents a 30 s time point scan (AVI)

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†These authors contributed equally to this work (C.S. and R.J.B.). The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. C.S. designed, fabricated, and ran mechanical and biological testing of the microfluidic device and cowrote the manuscript. R.J.B. designed and carried out biological experiments and cowrote the manuscript. F.A. and C.d.S. developed and ran the µPIV experiments and system. M.M. carried out the biological experiments. I.M. and A.O. ccodeveloped the fluid dynamics experiments. H.H.N. and R.J.H. contributed to the biological experimental design. A.M. contributed to the microfluidic device development and fabrication methods. W.S.N. developed the experimental design, supervised the study, and wrote the manuscript.

Notes
The authors declare no competing financial interest.

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