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A uniform-shear rate microfluidic bioreactor for real-time study of proplatelet formation and rapidly-released platelets

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Abstract

Platelet transfusions, with profound clinical importance in blood clotting and wound healing, are entirely derived from human volunteer donors. Hospitals rely on a steady supply of donations, but these methods are limited by a 5-day shelf life, the potential risk of contamination, and differences in donor/recipient histocompatibility. These challenges invite the opportunity to generate platelets *ex vivo*. Although much progress has been made in generating large numbers of culture-derived megakaryocytes (Mks, the precursor cells to platelets), stimulating a high percentage of Mks to undergo platelet release remains a major challenge. Recent studies have demonstrated the utility of shear forces to enhance platelet release from cultured Mks. In this study, we performed a computational fluid dynamics (CFD) analysis of several published platelet microbioreactor systems, and used the results to develop a new 7- μ m slit bioreactor – with well-defined flow patterns and uniform shear profiles. This uniform-shear-rate bioreactor (USRB-7 μ m) permits real-time visualization of the proplatelet (proPLT) formation process and the rapid-release of individual platelet-like-particles (PLPs), which has been observed *in vivo*, but not previously reported for platelet bioreactors. We showed that modulating shear forces and flow patterns had an immediate and significant impact on PLP generation. Surprisingly, using a single flow instead of dual flows led to an unexpected 6-fold increase in PLP production. By identifying particularly effective operating conditions within a physiologically relevant environment, this USRB-7 μ m will be a useful tool for the study and analysis of proPLT/PLP formation that will further understanding of how to increase *ex vivo* platelet release.

Keywords

CFD; platelets; megakaryocytes; cell therapies; microfluidic bioreactor

Introduction

More than 2 million platelet units are transfused each year in the US to treat patients with thrombocytopenia (low platelet counts) or defective platelets.^{1,2} Platelets are small (2–3 μm) anucleate discoids responsible for thrombosis and hemostasis.³ Platelet units are collected from volunteer donors via apheresis or from the buffy coats of 4–8 whole blood donations. Hospitals depend on a steady supply of platelet donors. Disruptions of this supply together with a 5-day platelet shelf-life can result in critical shortages.⁴ In addition, because platelets require room temperature storage to maintain activity, there is risk of bacterial contamination prior to transfusion.⁵ The use of a current Good Manufacturing Practices (cGMP) process for platelet production from megakaryocytes (Mks) would allow for better control and characterization of transfusion units, and could transition the supply from fluctuating donors to a steady in vitro process. Finally, culture-derived platelet production could reduce the risk of immunogenic reactions by avoiding the need to provide platelets from multiple donors.⁴

Platelet formation starts from Mks, which undergo extensive cytoskeletal rearrangements to create proplatelets (proPLTs), the precursors to platelets. In the bone marrow, intravital microscopy studies in mice show that Mks directionally extend proPLTs into the blood sinuses, where shear forces (1.3–4.1 dynes/cm²) elongate and fragment proPLTs into preplatelets (prePLTs).⁶ High shear stress in the lung capillary bed shears proPLTs and prePLTs into individual platelets, and can also process Mks directly into platelets.^{7–10} The importance of shear forces has led many groups, including our own, to investigate the use of bioreactors to study and enhance platelet-like-particle (PLP) release from mature Mks (Fig. 1).¹¹ Estimates of platelet production in vivo are >1000 per Mk⁸; in comparison, in vitro studies typically report <100 – and in many cases <10 – PLPs per Mk.^{11,12}

A major challenge in the development of platelet bioreactors is that much remains unknown about the ex vivo initiation and regulation of proPLT formation, as well as how to maximize PLP release. Parallel-plate flow reactors (PPFRs) are the simplest bioreactors that have been used to study proPLT/PLP formation from adhered Mks under high (1800 s⁻¹)¹³ and low (400 s⁻¹) shear rates.¹⁴ However, it is difficult to carry out long-term analysis of individual Mks due to transient adhesions as Mks roll over the PPFR surface. Blin et al. improved on open-channel PPFRs by introducing an array of vWF (Von Willebrand factor)-coated columns in bioreactors.¹⁵ The anchoring of Mks to columns, at a shear rate of 5000 s⁻¹, allowed longer Mk retention for analysis and study of the proPLT formation step. Complex niche bioreactors occupy the other end of the bioreactor spectrum. For example, a 3D silk-based porous microtube surrounded by a silk sponge reproduced the structure of a blood sinus and the bone marrow niche.^{16,17} Using a shear rate of 60 s⁻¹, the system reproduced PLP production in a physiologically relevant environment, but real-time visualization was challenging. Therefore, insight into the factors that regulate proPLT formation and PLP release could be limited since immediate changes to the proPLT formation process cannot be analyzed. Similar limitations in real-time visualizations are present for the Avanzi et al. porous membrane system through which Mks extended proPLTs into a lower chamber with shear rates of 30 – 70 s⁻¹.¹⁸

In contrast, slit bioreactors, which use small features to create $< 10\text{-}\mu\text{m}$ openings that mimic gaps or fenestrations in endothelial cells lining sinuses in the bone marrow, offer the advantage of in situ study and analysis of proPLT and PLP formation that is difficult in the other types of bioreactors. Nakagawa et al.¹⁹ used a $4\text{-}\mu\text{m}$ slit bioreactor with unspecified shear rates and Thon et al.²⁰ introduced a $2\text{-}\mu\text{m}$ slit bioreactor with a shear rate of 500 s^{-1} . Although the PDMS-based fabrication of these slit bioreactors facilitate the opportunity to visualize the proPLT formation process in real-time, the flow patterns and shear rates within current systems have not been fully characterized. Developing an understanding of the bioreactor flow environment is important since non-uniformity in the flow patterns would lead to Mks experiencing different shear rates depending on slit location.

We applied computational fluid dynamics (CFD) to assess the flow environment of published slit bioreactors and used those results to develop an improved slit bioreactor with a well-characterized flow environment and uniform shear profiles across all the $7\text{-}\mu\text{m}$ slits. This uniform-shear-rate bioreactor (USRB- $7\mu\text{m}$) allows for the real-time visualization of proPLT/PLP formation. Furthermore, the environment within the USRB- $7\mu\text{m}$ promotes the rapid release of individual PLPs from Mks, which has been reported in vivo in mice²¹, but not reported in the aforementioned slit bioreactors that have used mouse-derived or human-derived Mks.^{19,20} We present how different real-time environmental changes (e.g., flow rates) impact Mk behavior within the USRB- $7\mu\text{m}$ and how CFD was further utilized to understand the associated changes in the flow environment. Importantly, the USRB- $7\mu\text{m}$, coupled with CFD, serves as a characterization tool to identify environmental conditions that have a positive impact on proPLT/PLP formation.

Materials and Methods

See Supplemental Section 1: Additional Methods for information on primary hematopoietic stem and progenitor cell (HSPC) culture for Mk differentiation. Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (St. Louis, MO).

CFD Modeling

Flow simulations were conducted using ANSYS v16.1 (ANSYS, Inc., Canonsburg, PA). 3D models of the slit bioreactors (Table S1) were created using Autodesk Inventor Professional software 2015 (San Rafael, CA) and the files imported into ANSYS Design Modeler. A computational grid (mesh) was individually optimized for each system (Fig. S1). Boundary conditions were no-slip at the walls, constant inlet velocity, and default gauge pressure of 0 Pa at the outlet. Additional details are included in Supplemental Section 2: Computational Fluid Dynamics (CFD) Modeling.

Bioreactor Fabrication

Briefly, a chrome mask (Front Range Photomask, Palmer Lake, CO) of the USRB- $7\mu\text{m}$ was used to create a master silicon wafer (WRS Materials, San Jose, CA) on which polydimethyl siloxane (PDMS) solution (Slygard 184 Kit; Electron Microscopy Sciences, Hatfield, PA) was poured to cast a mold. Additional details are included in Supplemental Section 1.

Bioreactor Perfusion with Mks

The USRB-7 μ m was positioned on a Lumascope microscope v500 (Etaluma Inc., Carlsbad, CA) placed inside an incubator (Thermo Scientific, Waltham, MA) maintained at 37°C and 5% CO₂. Separate syringe pumps (NE-300, New Era Pump Systems Inc., Farmingdale, NY) were used for each flow channel. A 5-mL glass syringe (81520, Hamilton Company, Reno, NV) was used for the outer channels and a 2.5-mL glass syringe (81420, Hamilton) was used for the center channel. Media (78% IMDM (Gibco, Carlsbad, CA), 20% BIT 9500 Serum Substitute (STEMCELL, Vancouver, BC, Canada), 1% Glutamax (Gibco), 1 μ g/mL low-density lipoproteins (Calbiochem, Whitehouse Station, NJ), 100 U/mL Pen/Strep) without cytokines was perfused throughout the bioreactor for 30 min at 6.5 μ L/min prior to Mk introduction. On day 10, 11, or 12 of Mk culture, Mks at density of 50,000/mL were stained for 15 min with 1 μ M Calcein AM at 37°C. After the 30-min media perfusion, 25,000 Mks (a sufficient number to observe the system dynamics and how often they might repeat and under what conditions, without clogging the slits) were microinjected into the tubing upstream from the reactor. No Mks were present within the syringes. A video was recorded of each bioreactor for 1–2 hrs.

Video Analysis

Videos (6 frames-per-second) were recorded for each experimental run using the Lumascope v500, equipped with High Sensitivity Monochrome CMOS Sensor camera, using a 20x or 40x objective. Each video was analyzed for every 5-min time interval for the duration of an experiment. One half of the bioreactor (10 slits) was analyzed at one time throughout 5-min time intervals for the entire video recorded. This process was repeated on the other half of the reactor. The data from each half of the reactor was then combined for each 5-min time interval. During each interval, only proPLTs and PLPs that originated from trapped Mks the slits were counted. Additionally, for some videos, pre-released particles flowing into and out of the slits were counted separately. Mks can give rise to particles without shear and these could be present in the suspension that was microinjected into the system. To increase accuracy, the videos were played at a slower speed during times of high PLP release activity. The 5-min interval was selected because it allowed us to effectively analyze and understand the dynamics of the process. Pre-staining Mks with Calcein AM allowed the Mks trapped in the reactor, as well as proPLTs/PLPs, to be clearly observed.

Statistical Analysis

JMP Pro 11 (SAS Institute Inc., Cary, NC) was used to generate histograms, distributions, and standard errors of the video analysis data for released proPLTs/PLPs.

Results

CFD Modeling of Shear Rates within Slit Bioreactors

We used CFD to evaluate the flow and shear conditions within several slit bioreactors (Table S1). CFD analysis of the Nakagawa bioreactor (Fig. S2), which uses 4- μ m slits,¹⁹ predicts slit shear rate that ranges from 400 s⁻¹ near the inlet to 30,000 s⁻¹ near the outlet (Fig. 2A). The calculated shear rate on proPLT extensions in the lower chamber ranges from 200 s⁻¹

near the inlet up to 6000 s^{-1} near the outlet (Fig. 2A). Additionally, simulations predict a net flow from the lower chamber into the upper chamber near the bioreactor inlets (Fig. S2B). Based on this CFD analysis, the flow and shear environment varies significantly across the bioreactor (Fig. 2A, Fig. S2B, Fig. S3).

Thon et al. analyzed their $2\text{-}\mu\text{m}$ slit reactor using CFD.²⁰ We also provide a CFD analysis of their system (Fig. S4). Consistent with Thon et al., our simulations showed that the shear rate in the slits increases from the inlet towards the outlet of the bioreactor. The calculated shear rate in the open slits ranges from 5000 s^{-1} at the inlet to 7500 s^{-1} at the outlet (Fig. 2B). Our calculated shear rate below the slits, along the lower channel wall, ranges from 250 s^{-1} near the inlet to 500 s^{-1} at the outlet (Fig. 2B), similar to that reported by Thon et al.²⁰ This reactor provides a more uniform shear profile compared to that of Nakagawa et al. However, there is still an increase in slit-shear rates towards the bioreactor outlet (Fig. 2B, Fig. S5).

CFD-Driven Design and Assessment of a Uniform-Shear-Rate Bioreactor

A thorough CFD analysis was conducted on potential new slit bioreactor designs to avoid the CFD-predicted non-uniform flow and shear profiles of current slit bioreactors. In our optimized bioreactor system, Mkcs enter a center channel where a V-shaped array of twenty $7\text{-}\mu\text{m}$ slits separates the Mkcs from outside flows converging at 90° (Fig. 3, Fig. S6, S7). The slit dimension was chosen to retain mature Mkcs – usually $>20\text{ }\mu\text{m}$ – and to prevent large pressure drops and flow stagnation. The height of the bioreactor was chosen to be $40\text{ }\mu\text{m}$, similar to blood sinusoid dimensions.²² To experimentally visualize and confirm flow patterns in the system, $1\text{-}\mu\text{m}$ fluorescent beads were used to map the streamlines of the cell-free system, and showed good agreement with the CFD streamlines (Fig. 4). Uniform shear profiles across and downstream of the slits were confirmed through CFD simulations of the cell-free system. For center channel and combined outer channel flow rates of $1.5\text{ }\mu\text{L}/\text{min}$ each, the $7\text{-}\mu\text{m}$ slits have a maximum calculated shear rate of 5000 s^{-1} , except for a maximum shear rate of 2800 s^{-1} for the two slits at the end of the V where the flow in the outer channels converges (Fig. 5A, B and Fig. S8). ProPLTs extending through the slits would experience a shear rate range from $100\text{--}200\text{ s}^{-1}$ (past the slits in the open channel) (Fig. 5A, B). Increasing the combined outer channel flow rate to $5\text{ }\mu\text{L}/\text{min}$ did not affect the shear rate through the slits (maximum remained 5000 s^{-1}), but the shear rate that would be experienced by proPLTs increased to $250\text{--}350\text{ s}^{-1}$ (Fig. 5C, D). Therefore, the USRB- $7\text{-}\mu\text{m}$ allows Mkcs trapped at the slits and extending proPLTs to experience similar shear rates regardless of location within the bioreactor.

Understanding Shear Forces in the Presence of Cell Blockages

Cell-blockage scenarios for the slits were simulated using CFD, with a center channel flow rate of $1.5\text{ }\mu\text{L}/\text{min}$ and a combined outer channel flow rate of $5\text{ }\mu\text{L}/\text{min}$. First, $20\text{-}\mu\text{m}$ sized spheres were modeled just upstream of slits (i.e., partial blockage). The simulations predict that these cells would experience a shear rate of $1500\text{--}3000\text{ s}^{-1}$ (Fig. 6). Second, the system was modeled with only 2 open slits by completely blocking the remaining 18 slits. ProPLTs extending past the slits are expected to experience a shear rate of $100\text{--}900\text{ s}^{-1}$ (Fig. 7A). The shear rate is the highest (900 s^{-1}) near the 2 open slits where the velocity is the highest (Fig.

7A, B). The lowest shear rate (100 s^{-1}) occurs upstream of the open slits where it appears that the flow from the open slit is re-directing the outside flow away from the slits (Fig. 7A, B). Finally, similarity between the simulation streamlines and experimental streamlines was confirmed using cells and $1\text{-}\mu\text{m}$ fluorescent beads (Fig. 7B vs 7C). Thus, CFD can help understand the flow profile, as well as estimate the shear rates, in the USRB- $7\mu\text{m}$ when Mks are trapped at the slits.

Uniform-Shear-Rate Bioreactor Promotes proPLT and Rapid PLP Generation from Mks

After design and fabrication of the USRB- $7\mu\text{m}$ (Fig. 3), and validation of the flow patterns (Fig. 4, Fig. 7C), the capability of the system to promote proPLT formation from mobilized peripheral blood (mPB)-derived Mks was assessed. Experiments showed that, depending on the size of the trapped Mks, 1–3 cells can occupy a slit. Importantly, the shear environment within the USRB- $7\mu\text{m}$ could stimulate proPLT formation from trapped Mks (Fig. 8). Mks were stained with Calcein to allow clearer visualization of the proPLT formation process (Fig. 8A). Trapped Mks within the slits extruded their bodies and elongated into the characteristic proPLTs with beads-on-a-string morphology (Fig. 8B, Supplemental Video 1). Interestingly, the USRB- $7\mu\text{m}$ microenvironment also promoted trapped Mks to release individual PLPs directly from their bodies. Some Mks, immediately after slit capture, rapidly released dozens of PLPs within seconds (Fig. 9, Supplemental Video 2).

Evaluating PLP-Release Kinetics under Different Flow Conditions

We analyzed the kinetics of the USRB- $7\mu\text{m}$ to identify conditions that change Mk behavior by counting the number of Calcein-stained PLPs that originate from Mks trapped at the slits per 5-min time interval across individual experimental runs (Fig. 10). The number of PLPs-on-a string that were observed on proPLTs were also counted as released PLPs (Fig. S9) and we estimated that ~30% of PLPs released were from proPLTs. We observed that, when an incoming Mk blocked the flow of an open slit, proPLT/PLP formation and the number of slits making PLPs greatly increased within the bioreactor (Fig. 10A – green arrows, Supplemental Video 3). The blockage most likely increased the pressure drop across the slits, thus, trapped Mks were exposed to an immediate higher pressure and shear that increased their productivity. This observation led us to hypothesize that introducing a step-increase in the center channel flow rate may mimic the effects of cell-blockage. Indeed, similar responses after multiple step-increases in flow rate were observed during five separate experimental runs (Fig. 10B, Fig. S10A). The calculated CFD pressure drop across the slits increased continuously as more slits were occupied, especially when few slits remained open (Fig. S11A). Further, increasing the flow rate of the system with a constant number of open slits increased the calculated CFD pressure drop across the slits in a linear manner (Fig. S11B). The experimental observations and CFD analysis support our hypothesis that a pressure drop increase by a blockage event or flow rate change could increase Mk productivity, as both types of changes increased the immediate number of PLPs released by ~3-fold and the number of active slits by 30–50% (Fig. S12).

While trying to remove a small bubble from the outer channel during an experiment, we inadvertently stopped the flow of the outer channels. To our surprise, when the outer channel flow was completely stopped, the rate of proPLT and PLP release dramatically increased

(Fig. 11 vs. Fig. 10, Fig. S10B). The outer channel flow-rate is intended to impose shear forces on the extending proPLTs, so it is also a key parameter of the system. Yet, we discovered that turning off the outer channel flow rate dramatically changed the Mk behavior and greatly increased PLP release (Supplemental Video 4). Further, we could still observe an increase in productivity when an incoming Mk blocked the flow of an open slit (Fig. 11A – green arrows) or by introducing a step-increase in flow rate in the center channel (Fig. 11B), similar to that seen when the outside flow was maintained at 5 $\mu\text{L}/\text{min}$ (Fig. 10). Under this new operating condition, upon capture, some Mks continued to rapidly release dozens of PLPs within seconds (Supplemental Video 5).

We compared bioreactor runs using outer channel combined flow rates of 5 $\mu\text{L}/\text{min}$ (9 bioreactor runs across 3 different Mk cultures) and 0 $\mu\text{L}/\text{min}$ (4 bioreactor runs across 2 different Mk cultures) (Fig. 12). The average number of open slits was 2 for 5 $\mu\text{L}/\text{min}$ and 1 for 0 $\mu\text{L}/\text{min}$ combined outer channel flow rates (Fig. 12A). On average, 40% of the occupied slits were actively making proPLTs/PLPs under the 5 $\mu\text{L}/\text{min}$ outer flow condition, whereas 61% were active when operating at 0 $\mu\text{L}/\text{min}$ outer flow condition (Fig. 12B). The number of PLPs released per 5-min time interval had a mean of 55 and followed an exponential decay curve for the 5 $\mu\text{L}/\text{min}$ outer flow condition, while there was a 6-fold higher mean of 351 PLPs released with a log-normal distribution for 0 $\mu\text{L}/\text{min}$ (Fig. 12C). Thus, unexpectedly, an outer channel flow rate of 0 $\mu\text{L}/\text{min}$ greatly increased PLP production compared to 5 $\mu\text{L}/\text{min}$. An interesting observation from both environments is that the productivity increase from a blockage event (green arrow) could carry over into the next interval if the blockage occurred near the end of that interval (Fig. 10A – intervals 9 to 10, Fig. 10B – intervals 11 to 12, Fig. 11B – intervals 13 to 14, and Fig. S10B – intervals 5 to 6).

It is important to demonstrate that the PLPs produced exhibit functional activity. Due to the higher productivity, we analyzed the effluent of three bioreactors operated with an outer channel combined flow rate of 0 $\mu\text{L}/\text{min}$. Calcein⁺ PLPs were ~67% CD41⁺CD42b⁺ (Fig. 13A). Functional activity of CD41⁺CD42b⁺ PLPs was evaluated via expression of CD62P – a transmembrane glycoprotein that is translocated by granules to the surface of platelets after activation – both before (Fig. 13Bi) and after adding thrombin (Fig. 13Bii) to activate the PLPs. The average percentage of CD62P⁺ PLPs increased from ~20 to ~70% after thrombin addition (Fig. 13Biii). Confocal analysis of PLPs on fibrinogen revealed a characteristic tubulin ring in the absence of thrombin and highly spread PLPs in the presence of thrombin (Fig. 13C), which is similar to the behavior of fresh platelets.²³

The effluent most likely contained a combination of pre-released particles (present in the Mk suspension introduced into the system) and PLPs generated at the slits. We analyzed the videos using the counting strategy described earlier to determine the rate at which pre-released Calcein-stained particles entered and exited the slits. The mean rate was 125 per 5-min time interval (Fig. S13A), which is higher than the mean rate of PLP generation for a combined outer channel flow rate of 5 $\mu\text{L}/\text{min}$ (55), but less than half than the mean rate for a combined outer channel flow rate of 0 $\mu\text{L}/\text{min}$ (351). By counting flow-through and newly produced PLPs in the same experiment, we estimated that ~76% of Calcein-stained PLPs were generated by the slits in the reactors with no flow in the outer channels (Fig. S13B).

Therefore, the Calcein⁺ PLPs characterized in the effluent were largely generated by Mks trapped at the slits.

Finally, to verify our PLP counting process, we introduced expired blood platelets, stained with Calcein, into a cell-free bioreactor. During a 30-min perfusion, platelets were counted per 5-min time interval for outer channel combined flow rates of 5 $\mu\text{L}/\text{min}$ or 0 $\mu\text{L}/\text{min}$ (Fig. 14A). The number of platelets counted per time interval was about the same for either condition, as expected, since platelets would only enter via the center channel. Additionally, we provide images of expired platelets flowing through the USRB-7 μm (Fig. 14B) to compare them to the PLPs released from trapped Mks to further support our count strategy (Fig. 14C).

CFD Analysis of Changes to the Outer Channel Flow Rate

CFD was used to evaluate what environmental factors could explain the differences in Mk behavior at 5 $\mu\text{L}/\text{min}$ vs. 0 $\mu\text{L}/\text{min}$ flow rates in the outer channels, while keeping the center channel flow rate constant at 1.5 $\mu\text{L}/\text{min}$. Wall shear rate, pressure, velocity, strain rate, and the structure of the flow patterns were the primary factors of interest. Simulations with the outer flow rate of 0 $\mu\text{L}/\text{min}$ did not show changes to the wall shear rates within the slits of the bioreactor (Fig. 15 vs. Fig. 5). Next, we focused on CFD-predicted pressure and velocity profiles across the slits at the center height of the bioreactor, $z = 20 \mu\text{m}$ (Fig. S14A). The average CFD pressure drop across the slits was similar for the two outer channel flow rate conditions, but at 5 $\mu\text{L}/\text{min}$ the variability between slits was greater (Fig. 16A, Fig. S14B). The outside flow likely imparts some back pressure in the center channel flow, evident by the higher relative pressures shown in Fig. 16A. Stopping the outside flow potentially reduced the pressure downstream of the slits and may allow Mks to release more PLPs. The velocity profile across the slits is also more variable when the combined outside channel flow rate is 5 $\mu\text{L}/\text{min}$ vs. 0 $\mu\text{L}/\text{min}$, but the average velocity profiles were similar (Fig. 16B, Fig. S14C, D).

Next, we examined the strain rate (rate of deformation) within the bioreactor slits. Strain rates represent extensional flow that is created due to a velocity gradient in the direction of flow. The CFD outputs of our bioreactor showed an increase in velocity along the slits, due to the hyperbolic-like-converging region (Fig. 16B). In CFD, the strain rate can be easily extracted from the velocity gradient tensor output as dV_x/dx . Plotting the average strain rate across the slits did not show any large differences for combined outer channel flow rates of 5 $\mu\text{L}/\text{min}$ or 0 $\mu\text{L}/\text{min}$ (Fig. 16C). The maximum strain rates predicted are 336 s^{-1} for 5 $\mu\text{L}/\text{min}$ and 346 s^{-1} for 0 $\mu\text{L}/\text{min}$ outside combined flow rate. Based on this analysis, though there were no differences in the strain rates, we can observe extensional flow conditions within our slits.

Finally, we assessed the structure of the flow patterns using the CFD streamlines, as well as 1- μm fluorescent beads to map the experimental streamlines. There is strong agreement between the predicted and experimental streamlines under the two different outside flow conditions (Fig. 17). The flow patterns are very different at the two flow rates. For the 5 $\mu\text{L}/\text{min}$ combined outer channel flow rate, the streamlines are compressed towards the center of the reactor along the posts (Fig. 17A, B). On the other hand, with no outer channel flow,

the streamlines are not compressed, but rather expand downstream of the slits (Fig. 17C, D). Furthermore, overlaying images from Mk experiments with the CFD streamlines demonstrates how the flow structure influences the behavior of the Mks (Fig. 18). This is further supported by the observation of switching the flow from 5 $\mu\text{L}/\text{min}$ to 0 $\mu\text{L}/\text{min}$ shown in Supplemental Video 4. Also, the velocity vectors past the slits show that the flows through individual slits seem to interact with each other when the outside flow is at 5 $\mu\text{L}/\text{min}$, whereas a more isolated slit environment is generated with no outside flow (Fig. 19A vs. Fig. 19B).

Discussion

CFD modeling has been used to analyze numerous biotechnology processes²⁴, as well as microfluidic-based systems.²⁵ Using CFD to guide platelet bioreactor design, analyze forces on Mks, and examine bioreactor performance could greatly advance the design of platelet bioreactors – for which CFD has been minimally applied to-date. In this study, we used CFD to evaluate published slit bioreactors and develop a USRB-7 μm design with improved flow and shear uniformity. The 4- μm slit bioreactor introduced by Nakagawa et al. had no specified shear rates.¹⁹ Therefore, we proceeded to evaluate the Nakagawa et al. system with CFD. Shear rates on proPLTs (along the length of the bioreactor) were within the sinusoids range (200 s^{-1}) and above physiological rates (6000 s^{-1}), depending on the location. However, the shear rate within the 4- μm slits where Mks would be trapped had a much higher range of 400 – 30,000 s^{-1} . The substantial non-uniformity of shear rates along the bioreactor length might make it difficult to study proPLT/PLP formation real-time since Mks at different regions of the bioreactor would experience substantially different microenvironments.

The 2- μm slit bioreactor developed by Thon et al. had a narrower range of shear rates than Nakagawa et al.²⁰ The authors used CFD to analyze their system and reported a shear rate of $\sim 500 \text{ s}^{-1}$. Our CFD outputs showed that, along the length of the bioreactor, proPLTs would experience shear rates from 250–500 s^{-1} , confirming the author's findings, and that the slit shear rates ranged from 5000–7500 s^{-1} . Additionally, Thon et al. provided real-time visualization of the proPLT formation process. However, the shear rates within the slits showed a steady increase along the bioreactor so that Mks trapped at various slits would be exposed to different shear rates.

CFD analysis of the Nakagawa et al. and Thon et al. systems suggests the shear rate across slits increased along the bioreactor from the inlet to the outlet. This can be attributed to the design of the systems in which two parallel-like flows are separated by slits and where the top flow (pushing on the Mks) is re-directed into the lower channel at the end of the bioreactor length (Table S1). To avoid generating this increase of shear rates across the slits, our new design instead converged the two outer flows at a 90° V-shaped region. This arrangement allowed the center channel flow to push whole-Mk bodies into the 7- μm slits with similar maximum shear rates of 5000 s^{-1} . The outer flow converges at the slits and exerts nearly uniform shear rates (250–350 s^{-1}) on extending proPLTs. Mks exiting the bone marrow sinusoids can be trapped in the vascular bed of the lung where high shear forces are exerted on the whole-cell body and on proPLTs^{7,9}, thus, we also aimed to utilize high shear

forces on Mk bodies and physiological shear on the proPLTs. We extended our CFD analysis to include cell-blockages within the slits and confirmed the flow patterns experimentally to understand behavior of an occupied bioreactor and to estimate anticipated shear rates on proPLTs ($100\text{--}900\text{ s}^{-1}$).

Compared to Thon et al., our USRB-7 μm has a similar capture area of 20 slits vs. 15 slits, but a higher slit occupancy (90% vs 66%). More importantly, all the slits can be observed during an experimental run and we noted that on average 40–60% of occupied slits were actively making proPLTs/PLPs. In contrast, the length of the Thon et al. and Nakagawa et al. bioreactors makes it difficult to analyze proPLT/PLP formation from all the slits at the same time. In addition to supporting proPLT production, the USRB-7 μm also promoted rapid release of many individual PLPs, which has not been reported for other published bioreactor systems. This observation is physiologically relevant since Mks have also been observed to make platelets *in vivo* (in mouse) via a rapid fragmentation process that releases platelets without the propPLT formation step.^{21,26–28} Though we do not fully understand what factors influence proPLT vs. rapid-PLP release, we can observe that the rate of PLP production appears to be faster when PLPs are rapidly released (Supplemental Video 1 vs. Video 2). We hypothesize that rapid PLP generation within the USRB-7 μm is largely influenced by the unique slit geometry in which cells are pushed through a hyperbolic-like-converging region. As the area is reduced in this region, whole Mks bodies are squeezed and elongated through the 7- μm gap where the shear rate and strain rate are the highest.

We show that the flow microenvironment can greatly affect the behavior of Mks in real-time. Within the system, we observed that Mk capture at an open slit increased the release of PLPs across the other Mk-blocked slits. Slit-blockage events could be influenced by the size of the Mks being trapped and are not easily controlled, thus, our observations highlight the importance of understanding how the inherent dynamics of a bioreactor can impact the Mk response. A step-change in the center channel flow rate (while keeping the outside channel flow constant) transiently increased the rate of PLP releases, similarly to the slit-blockage events. The increase in immediate Mk productivity could be attributed to an increase in pressure drop across the slits, as presented by CFD analysis. While we can observe a temporary 3-fold increase in productivity after cell-blockages or flow rate changes in the USRB-7 μm , we recognize that the rates aren't sustained for the remainder of an experimental run largely due to the dynamic behavior of cell capture and slit openings. Nagakawa et al. did not study the effect of changing the flow conditions within their system.¹⁹ Thon et al. found that the average proPLT extension rate did not change at different flow rates (same flow for both channels, $12.5\text{--}100\text{ }\mu\text{L/hr}$),²⁰ but did not extend their CFD analysis to other flow regimes. It would be interesting to examine the Thon et al. system at higher flow rates or slit-blockage events to see if the number of active slits increased, even if the proPLT extension rate remained unchanged. Within the USRB-7 μm , we observed that step-changes to the center channel flow rate or a cell-blockage event led to ~30% and ~50% increases in active slits, respectively.

We anticipated that the presence of an outside channel flow would aid in shearing off proPLTs (increasing PLPs release rate), mimicking physiological blood flow *in vivo*. Thus, we were surprised that turning off the outside channel flow rate increased the average

number of PLPs released by almost 6-fold. Using CFD simulations, we probed the predicted environment to try and understand the variables responsible for these unforeseen results. Average velocity profiles, strain rates, and wall shear rates through the slits remained overall unchanged with or without flow in the outer channel. CFD predictions showed a slight increase in back-pressure by the outer flow on the center channel, which could inhibit Mks from releasing more PLPs. However, a significant change in the flow structure was observed, as confirmed by good agreement between the CFD streamlines and experimental streamlines. The CFD simulations showed that the slits appear to operate independently from each other with no outside flow (Fig. 19), and within our experimental runs, we observed Mk behavior that supported the simulations in which proPLTs and PLPs are not contracted towards the center of the bioreactor (Fig. 18B vs. A). Thus, we believe that the flow structure had the most significant impact on Mk behavior. Yet, the microenvironment that is being generated is certainly complex and the use of higher magnification, particle velocity imaging (PVI), and pressure transducers in the system could further refine our observations in future evaluations.

Analysis of the bioreactor effluent showed CD41⁺CD42b⁺ PLP populations that exhibited activation following thrombin addition. We acknowledge that some particles could have been pre-released before processing the Mks. Currently, we cannot discern which of the Calcein⁺ particles in the effluent were pre-released vs. generated in the USRB-7 μ m. However, our video analysis indicates that ~76% of Calcein-stained particles were generated at the slits in reactors with no outside flow. In recent work with the USRB-7 μ m, we are able to decrease the introduction of pre-released particles by ~50% by adding a low-spin step prior to introducing Mks into the system. We are optimizing this protocol and plan to permanently implement this step in future studies.

ProPLTs are subjected to a shear environment typical of the bone marrow sinusoids in many of the current published bioreactor systems (Fig. 1A). Additionally, Mk bodies can be directly exposed to high shear environments that approach and exceed estimated values within the lung (Fig. 1B). Single flow environments that transport Mks into regions with high shear forces (operating similar to the lung capillary bed) as well as extensional forces are sufficient for PLP generation, as demonstrated in this study and in Blin et al.,¹⁵ and challenge the need of using two flows to mimic the bone marrow niche. Though Nakagawa et al.¹⁹ and Thon et al.²⁰ used two flows, these systems also contain high shear regions and could potentially benefit from utilizing a single flow for Mk elongation and fragmentation at the slits.

The diverse Mk responses to different real-time environmental changes within the USRB-7 μ m support its use as a characterization tool to study ex vivo PLP formation. Further work will focus on utilizing the USRB-7 μ m to study aspects and characteristics of the bone marrow niche that could be incorporated, such as ECM protein coatings, and to leverage CFD to identify more effective PLP-generating microenvironments. Additionally, preliminary results show the capability of the USRB-7 μ m to process Mks derived from umbilical cord-blood (CB) HSPCs with proPLT and PLP behavior similar to that presented in this study (data not shown). Thon et al. observed that, even using 2- μ m slits, Mks were able to deform, pass through the slits, and enter the lower channel.²⁰ We observe similar

behavior of Mks passing through our 7- μ m slits. Therefore, a secondary system, such as the spinning-membrane separator we described previously²⁹, must be used to separate PLPs from Mks, which could then be recycled back to the bioreactor. Overall, future USRB-7 μ m studies will increase understanding of proPLT and PLP formation in a uniform-flow environment and can reveal important variables and operating parameters through which ex vivo platelet production can be increased.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Shear rate range that proPLTs experience within published bioreactor systems and this work. (B) Maximum shear rates that Mk bodies would experience within cell-free published bioreactor systems. The maps show the wide spectrum of shear rates that have been studied in bioreactors, as well as in vivo values for bone marrow sinusoids²² and the lung³⁰.

Figure 2. CFD analysis of the published slit bioreactors

(A) Shear profile of Nakagawa et al. system.¹⁹ Inlet flow rates = 16.7 $\mu\text{L}/\text{min}$. (B) Shear rates in the Thon et al. system.²⁰ Inlet flow rate for each channel was set to 6.25 $\mu\text{L}/\text{h}$ (total combined inlet flow rate 12.5 $\mu\text{L}/\text{h}$). White arrows indicate the flow direction. Insets of both systems are shown for details around the slit channels that Mks occupy (insets rotated to show all the channel walls).

Figure 3. Design concept of the uniform-shear-rate bioreactor and experimental set up
 (A) The newly designed system uses an array of 7-μm slits to capture Mks. Outside flows converge at the slits to apply shear forces on extending proPLTs. (B) Two syringe pumps are used for the bioreactor operation, allowing for independent flow rate changes to the center and outer channels. The bioreactor is positioned over a microscope equipped with real-time imaging in brightfield and green fluorescence. The entire system is placed inside an incubator at 37°C, 20% O₂, and 5% CO₂. (C) Cell-free fabricated bioreactor. Scale bar = 50 μm.

Figure 4. Qualitative validation of the bioreactor flow profiles

Comparison of streamline plots from the (A) CFD simulations and (B) experimental flow visualizations using 1- μ m fluorescent beads. The flow rate is 1 μ L/min in the center channel and in the combined outer channels. White arrows indicate the flow direction. Scale bar = 50 μ m.

Figure 5. Shear rate analysis of cell-free bioreactor introduced in this study

Shear rates in the entire slit region (A) and close-up view of individual slits (B) for $1.5 \mu\text{L}/\text{min}$ flow rates in the center and combined outer channels. Shear rates through the entire region (C) and details for individual slits (D) for a flow rate of $1.5 \mu\text{L}/\text{min}$ in the center channel with $5 \mu\text{L}/\text{min}$ in the combined outer channels. White arrows indicate the general flow direction. Estimated shear rates on proPLTs (dashed lines) are within $100 \mu\text{m}$ from the slits.

Figure 6. Shear analysis using 20-μm spheres near the slits (2-μm gap size upstream of posts)
 Center of spheres are placed at the center of the bioreactor height ($z = 20 \mu\text{m}$). Shear rates through the entire region (A) and details for individual slits (B) for a flow rate of $1.5 \mu\text{L}/\text{min}$ in the center channel with $5 \mu\text{L}/\text{min}$ in the combined outer channels. White arrows indicate the flow direction. Estimated shear rates on cells designated by (*). Estimated shear rates on proPLTs (dashed lines) are within $100 \mu\text{m}$ from the slits.

Figure 7. Modeling cell blockage of bioreactor slits

(A) Shear rates after blocking all but 2 of the slits. (B) Velocity streamlines of a system with 2 open slits. (C) Visualization of the system dynamics with fluorescent beads and Calcein-stained cells. Simulation and experimental flow rates were $1.5 \mu\text{L}/\text{min}$ in the center channel with a combined flow of $5 \mu\text{L}/\text{min}$ in the outer channels. White arrows indicate the flow direction. Estimated shear rates on proPLTs are within $100 \mu\text{m}$ from the slits. Scale bar = $50 \mu\text{m}$.

Figure 8. Shear-driven proPLT formation in the bioreactor

(A) Green fluorescence observation of Calcein-labeled Mks with proPLT formation. (B) Brightfield time-lapse images of cells trapped in a slit and exposed to shear. The center channel flow elongates Mks through the slits. Flow in the outer channels applies shear on the extensions further elongating them leading to fragmentation after several minutes. Black arrows indicate proPLTs. Scale bars = 50 μm . Flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with 5 $\mu\text{L}/\text{min}$ in the combined outer channels. Blue arrows show direction of flow.

Figure 9. Shear-driven rapid PLP release in the bioreactor

Time-lapse images of trapped Mks in a slit, rapidly releasing many individual PLPs in seconds. Time units: h:min:s. Orange arrows point to individual PLPs. Scale bar = 35 μm . Blue arrows show direction of flow. Flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with 5 $\mu\text{L}/\text{min}$ in the combined outer channels.

Figure 10. PLP-release kinetics in the bioreactor

(A) Number of PLPs released per 5-min time interval during a bioreactor run with constant center channel flow rate (indicated above plot) and three significant cell blockages of open slits during that time interval denoted by green arrows. (B) Number of PLPs released per 5-min time interval during a bioreactor run with three center channel flow rate increases (dashed lines - indicated above plot) and three significant cell blockages of open slits denoted by green arrow. For (A) and (B), combined outer channels flow rate = $5 \mu\text{L}/\text{min}$. Color legend in (A) and (B) depicts the number of slits making PLPs during each 5-min time interval.

Figure 11. PLP release kinetics in the bioreactor with no outer channel flow

(A) Number of PLPs released per 5-min time interval during a bioreactor run with no center channel flow rate changes (indicated above plot) and four significant cell blockages of open slits denoted by green arrow. (B) Number of PLPs released per 5-min time interval during a bioreactor run with two center channel flow rate changes (dashed lines - indicated above plot) and three significant cell blockages of open slits denoted by green arrow. For (A) and (B), combined outer channels flow rate = 0 $\mu\text{L}/\text{min}$. Color legend in (A) and (B) depicts the number of slits making PLPs during each 5-min time interval.

Figure 12. Distributions of PLP release kinetics in the bioreactor

(A) Number of slits open per 5-min time interval for (i) 5 $\mu\text{L}/\text{min}$ and (ii) 0 $\mu\text{L}/\text{min}$ combined outside channel flow rate. (B) %Occupied slits that were making PLPs per 5-min time interval for (i) 5 $\mu\text{L}/\text{min}$ and (ii) 0 $\mu\text{L}/\text{min}$ outside flow rate. (C) PLPs released per 5-min time interval for (i) 5 $\mu\text{L}/\text{min}$ (exponential fit) and (ii) 0 $\mu\text{L}/\text{min}$ (log-normal fit) outside flow rate. Error bars indicate $\pm\text{SEM}$.

Figure 13. Characterization of recovered PLPs from effluent

(A) CD41 and CD42b expression of Calcein⁺ PLPs. (B) Representative plots of activation of recovered CD41⁺CD42b⁺ PLPs in the absence (i) or presence (ii) of thrombin and (iii) summary of %CD62P⁺ PLPs for 3 bioreactor experiments from two Mk cultures with different donors. (C) Recovered PLPs adhere to BSA and fibrinogen (FIB) with a characteristic tubulin ring, and spread extensively after activation with thrombin. (green – beta tubulin, red- actin, blue – DNA). Scale bar = 10 μm. Bioreactor conditions: 1.5 μL/min center channel and 0 μL/min outer combined flow rate.

Figure 14. Expired blood platelets in the bioreactor compared to PLPs released from Mks
 (A) Profile of expired platelets per 5-min time interval within a bioreactor (counted three times). Error bars indicate $\pm\text{SEM}$. (B) Images of Calcein-stained platelets flowing through bioreactor with either (i) $5 \mu\text{L}/\text{min}$ or (ii) $0 \mu\text{L}/\text{min}$ combined outer channel flow rate. (C) Images of Mks releasing PLPs from the slits with either (i) $5 \mu\text{L}/\text{min}$ and (ii) $0 \mu\text{L}/\text{min}$ combined outer channel flow rate. For (A-C), the center channel flow rate was maintained at $1.5 \mu\text{L}/\text{min}$. Blue arrows indicate the flow direction. Yellow arrows indicate platelets or PLPs.

Figure 15. Shear rate analysis with no outer channel flow

Shear rates through the entire region (A) and details for individual slits (B) for a flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with 0 $\mu\text{L}/\text{min}$ in the outer channels. White arrows indicate the flow direction. Estimated shear rates on proPLTs (dashed lines) are within 100 μm from the slits.

Figure 16. Average CFD outputs across the slits along the x-axis

(A) Average pressure, (B) velocity profile, (C) and strain rate across the slits for two combined outer channel flow rates. Blue = 5 μL/min, Red = 0 μL/min. The center channel flow rate was maintained at 1.5 μL/min. Dashed line on plots represent the 7-μm slit opening where velocity is the highest.

Figure 17. Streamline observations under different outside channel flow rates

(A) CFD streamlines and (B) experimental streamlines using 1- μm fluorescent beads and Calcein-stained cells for a flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with 5 $\mu\text{L}/\text{min}$ combined outer channel flow rate. (C) CFD streamlines and (D) experimental streamlines using 1- μm fluorescent beads and Calcein-stained cells for a flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with 0 $\mu\text{L}/\text{min}$ in the combined outer channel flow rate. White arrows indicate the flow direction. Scale bar = 50 μm .

Figure 18. CFD streamlines overlaid on images from Mk experiments

Center channel flow rate of 1.5 $\mu\text{L}/\text{min}$ with (A) 5 $\mu\text{L}/\text{min}$ and (B) 0 $\mu\text{L}/\text{min}$ combined outer channel flow rate. Blue arrows indicate direction of flow. Black arrows indicate proPLTs. Yellow arrows indicate PLPs. Scale bar = 50 μm .

**Bioreactor Run 5 $\mu\text{L}/\text{min}$ combined outer channel flow:
Constrained-regime flow**

**Bioreactor Run 0 $\mu\text{L}/\text{min}$ combined outer channel flow:
Free-regime flow**

Figure 19. Difference of flow structures depicted with velocity vectors

(A) Velocity vectors within slits for a flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with a 5 $\mu\text{L}/\text{min}$ combined outer channel flow rate. (B) Velocity vectors within slits for a flow rate of 1.5 $\mu\text{L}/\text{min}$ in the center channel with a 0 $\mu\text{L}/\text{min}$ combined outer channel flow rate. 20- μm spheres shown within slits simulate the effects of cells in the slits, while dashed lines represent direction of proPLTs/PLPs.