Separation of platelets by size in a microfluidic device based on controlled incremental filtration†

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The significant biological and functional differences between small and large platelets suggested by recent studies could have profound implications for transfusion medicine. However, investigating the relationship between platelet size and function is challenging because separating platelets by size without affecting their properties is difficult. A standard approach is centrifugation, but it inevitably leads to premature activation and aggregation of separated platelets. This paper describes the development and validation of a microfluidic device based on controlled incremental filtration (CIF) for separating platelets by size without the cell damage and usability limitations associated with centrifugation. Platelet samples derived from whole blood were used to evaluate the performance of the CIF device separation on design parameters and flow rate, and to compare the properties of PLT fractions generated by the CIF device with those produced using a centrifugation protocol in a split-sample study. This was accomplished by quantifying the platelet size distribution, mean platelet volume (MPV), platelet-large cell ratio (P-LCR) and platelet activation before and after processing for all input and output samples. The ‘large platelet’ fractions produced by the CIF device and the centrifugation protocol were essentially equivalent (no significant difference in MPV and P-LCR). Platelets in the ‘small platelet’ fraction produced by the CIF device were significantly smaller than those produced by centrifugation (lower MPV and P-LCR). This was because the CIF ‘small platelet’ fraction was contaminated by much fewer large platelets (~2-times lower recovery of >12 fL platelets) and retained the smallest platelets that were discarded by the centrifugation protocol. There was no significant difference in platelet activation between the two methods. However, centrifugation required a substantial amount of additional anticoagulant to prevent platelet aggregation during pelleting. Unlike centrifugation, the CIF device offered continuous, flow-through, single-step processing that did not cause platelet aggregation. Such a capability has the potential to accelerate the basic studies of the relationship between platelet size and function, and ultimately improve transfusion practice, particularly in the pediatric setting, where the need for low-volume, high-quality platelet transfusions is most urgent.

Introduction

Platelets (PLTs) are the smallest blood cells, shaped as 1 μm-thick oblate spheroids (discoids) ranging from 2 to 7 μm in diameter.1,2 They primarily contribute to hemostasis and play significant roles in wound healing,3,4 the innate immune response,5,6 and inflammation.7 Generated in the bone marrow as fragments of the cytoplasm of megakaryocytes, PLTs vary greatly in size, with cell volume ranging from single to tens of femtoliters (fL).8,9 The ‘mean platelet volume’ (MPV) for healthy adults typically falls within the range of 7.2–11.7 fL.10 PLTs with cell volumes larger than 12 fL are commonly considered ‘large’, and their prevalence is quantified via the so-called ‘platelet-large cell ratio’ (P-LCR), which is a standard parameter measured by automated hematology analyzers. P-LCR represents the percentage of the total PLT population that has a cell volume greater than 12 fL. A P-LCR value of 15–35% is considered normal.8,11 Although there is no standard definition of ‘small’ PLTs, cells with a volume smaller than the MPV of the PLT size distribution (but larger than the ~0.5 fL volume of the largest PLT-derived microparticles12) are typically considered ‘small’.13

Recent studies have suggested that there are potentially significant biological differences (beyond the cell size) that exist between large and small PLTs, and these differences lead to distinct functional roles for the two PLT fractions,
with large PLTs mediating hemostasis and small PLTs having primarily immunomodulating and pro-inflammatory effects.13-15 This notion is supported by clinical observations that thrombocytopenic patients with higher MPV (i.e., a larger fraction of large PLTs) have fewer bleeding problems than those with lower MPV,16 while patients experiencing chronic inflammation have lower MPV (i.e., a larger fraction of small PLTs).17 Furthermore, using transfusion thresholds based on PLT mass (PLT count × MPV) to account for the size of transfused PLTs, rather than relying solely on PLT count, has been shown to significantly reduce the number of transfusions without increasing the risk of bleeding.18,19

Conclusive studies regarding the relationship between PLT size and function have proven elusive, partly due to the difficulty in separating PLTs by size without affecting their properties. Currently, centrifugation stands as the sole practical method available for size-based PLT separation.13,14 However, centrifugation subjects PLTs to significant mechanical forces during pelleting, leading to cell damage, premature activation, release of granule contents, and refractoriness.20-24 PLTs pelleted through centrifugation inevitably form macroscopic aggregates, which, if transfused, can potentially embolize into the microcirculation, leading to ischemia.25,26 To minimize this risk in clinical settings, PLTs are allowed to ‘rest’ for up to 60 minutes, often followed by an additional 15–60 minutes on a PLT agitator, to facilitate the dissociation of these aggregates.20,21 In research settings, a substantial amount of additional anticoagulant (e.g., ACD-A) is introduced to prevent PLT activation and aggregation, although this may potentially interfere with subsequent analyses.14

This study describes the development and validation of a novel microfluidic device for the separation of PLTs by size, eliminating the need for centrifugation. The operating principle of the device was based on ‘controlled incremental filtration’ (CIF), a high-throughput microfluidic technology for separating cells based on size.27,28 When developing this device, we combined the latest CIF design innovations (previously validated in various leukocyte separation projects29-32) to yield an entirely new functionality, which has not been demonstrated by any other microfluidic technology, including our own previous work. We conducted extensive testing of the CIF device using samples of platelet-rich plasma (PRP) obtained from freshly-collected whole blood. This involved measuring PLT count and the distribution of PLTs by size for both input and output samples to assess separation efficiency and quantify the device’s performance in relation to CIF design parameters and processing flow rate. Finally, we compared the properties of PLT fractions generated by the CIF device with those produced using a centrifugation protocol in a split-sample study.

Materials and methods

Fabrication of microfluidic devices

Microfluidic devices were fabricated as previously described.28,30,31,33 Briefly, a master wafer was patterned using photolithography of SU-8 photoresist (MicroChem, Newton, MA, USA) spin-coated on a 4″ silicon wafer (University Wafer, South Boston, MA, USA). Polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning, Midland, MI, USA) casts were cured, cut, and peeled from the master wafer. Through-holes for inlet and outlets were made using biopsy punches (Acuderm Inc, Fort Lauderdale, FL, USA). Each CIF device was assembled by bonding a PDMS replica to a PDMS-coated flat substrate using a plasma oxidizer (PDC-001, Harrick Plasma, Ithaca, NY, USA). Each device was incubated with a 1% (w/v) solution of methoxy-poly(ethylene glycol)-silane (mPEG-Silane, MW 5000; Laysan Bio Inc, Arab, AL, USA) at 70 °C for 20 minutes and then flushed with isotonic buffer (9 mM Na2HPO4, 1.3 mM NaH2PO4, 140 mM NaCl, 5.5 mM glucose, 1% w/v bovine serum albumin, 290 mmol kg-1, pH 7.4) at room temperature. Polyurethane (PU) and polyethylene (PE) tubing was used to convey the PRP sample to the device inlet (PE, 0.034″ I.D. × 0.060″ O.D.; Scientific Commodities, Lake Havasu City, AZ, USA) and collect separated fractions from the two outlets (PU, 0.012″ I.D. × 0.025″ O.D.; Scientific Commodities). All assembled devices underwent visual inspection, and those exhibiting signs of fabrication defects, such as delamination due to weak bonding or the presence of missing and/or slanted posts, were deemed defective and subsequently discarded.

Preparation of blood samples

Samples of whole blood were collected from healthy volunteers (n = 9, including 7 male and 2 female) after obtaining informed consent under a study protocol approved by the University of Houston Institutional Review Board (Committee for the Protection of Human Subjects 1, protocol #16272-01). Blood was drawn by venipuncture (8.5 mL, Vacutainer, BD Biosciences, Franklin Lakes, NJ, USA), anticoagulated with acid citrate dextrose solution A (ACD-A) and used in experiments within 4 hours of collection. Whole blood was centrifuged at 250 × g for 15 minutes to produce platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifuging a separate sample of the freshly collected blood at 1600 × g for 20 minutes and then centrifuging the resulting supernatant at 4000 × g for an additional 5 minutes.

Separating PLTs by size using centrifugation

The separation of PLTs by size via centrifugation followed a protocol recently developed by Handtke et al.14 Briefly, 300 μL of additional ACD-A was added to 3 mL of the PRP sample to prevent PLT activation and aggregation during centrifugation. The sample was then centrifuged at 650 × g for 7 minutes (without a brake) to pellet PLTs (so called ‘large PLT’ fraction, or LPF). The resulting supernatant was transferred into a 15 mL tube and centrifuged again at 650 × g for 7 minutes (also without a brake) to pellet PLTs that remained suspended after the first spin (so called ‘small PLT’ fraction, or SPF). The supernatant produced by the second spin (including any PLT that remained suspended) was discarded to remove the extra ACD-A added earlier. Each
pellet was resuspended in 1.5 mL of PPP collected from the same donor. It took approximately 50–60 minutes to fully process the initial 3 mL PRP sample (about 20–25 minutes per each centrifugation/resuspension stage and additional 10 minutes for the required manual manipulations).

Separating PLTs by size using the CIF device
When processing with the CIF device, the PRP samples were used as-is, without dilution or any other modifications. The PRP was transferred into a 10 mL syringe and passed through the CIF device using a syringe pump (GenieTouch, Kent Scientific Corporation, Torrington, CT, USA) to separate the sample into two fractions: the middle channel output (CIFmid, retaining most of the large PLTs) and the side channel output (CIFside, containing primarily small PLTs). It took approximately 10 minutes for a CIF device operating at a flow rate of 30 μL min\(^{-1}\) to process a volume of sample sufficient for the downstream analyses (∼300 μL).

Measurements of PLT size distributions and percent recovery calculations
In each experiment, the input and output samples were analyzed using a hematology analyzer (XS-1000i, Sysmex America, Inc., Mundelein, IL, USA) to measure PLT concentration, MPV, and P-LCR. The PLT histograms recorded by the hematology analyzer were processed using ImageJ (NIH, Bethesda, MD, USA) and Excel (Microsoft Corporation, Redmond, WA, USA) to extract the detailed PLT size distribution data. For each sample, the PLT size histograms were quantized into 1 fl bins (e.g., a ‘2 fl PLTs’ bin combined all PLTs with cell volume of at least 2 fl but less than 3 fl). The total number of PLTs within each size bin was calculated by multiplying the corresponding PLT concentrations by the volume of the sample.

‘Recovery’ was defined as the percentage of processed PLTs of a specific size range that ended up in a particular sample. For example, ‘recovery in filtrate’ of the CIF device for 2 fl PLTs was calculated as [recovery of 2 fl PLTs in filtrate] = [number of 2 fl PLTs in filtrate]/[total number of 2 fl PLTs in filtrate and retentate] × 100%. Similarly, ‘>12 fl recovery’ was defined as the percentage of processed PLTs with volume larger than 12 fl that ended up in a particular sample. For example, ‘>12 fl recovery’ for filtrate of the CIF device was calculated as [>12 fl recovery in filtrate] = [number of >12 fl PLTs in filtrate]/[total number of >12 fl PLTs in filtrate and retentate] × 100%, and ‘>12 fl recovery’ for the ‘small PLT’ fraction of the centrifugation protocol was calculated as [>12 fl recovery in ‘small PLT’ fraction] = [number of >12 fl PLTs in ‘small PLT’ fraction]/[total number of >12 fl PLTs in all fractions produced by the centrifugation protocol] × 100%. Finally, ‘overall recovery’ was defined as the percentage of all processed PLTs that ended up in a particular sample. For example, ‘overall recovery’ for filtrate of the CIF device was calculated as [overall recovery in filtrate] = [number of PLTs in filtrate]/[total number of PLTs in filtrate and retentate] × 100%, and ‘overall recovery’ for the ‘small PLT’ fraction of the centrifugation protocol was calculated as [overall recovery in ‘small PLT’ fraction] = [number of PLTs in ‘small PLT’ fraction]/[total number of PLTs in all fractions produced by the centrifugation protocol] × 100%.

Flow cytometry
PLT samples from before and after separation were analyzed as collected (resting) and after additional activation (activated). Additional activation was accomplished by incubating PLTs with 70 μM TRAP-6 (Sigma-Aldrich, Saint Louis, MO, USA) for 15 minutes at 37 °C. Resting and activated samples were then incubated for 15 minutes at room temperature with mouse anti-human CD41a/fluorescein isothiocyanate (FITC; BD Biosciences, San Jose, CA, USA) and mouse anti-human CD62P/phycoerythrin antibodies (PE; BD Biosciences, San Jose, CA, USA) to label PLTs and P-selectin, respectively. Flow cytometry was performed using the Amnis ImagestreamX Mk II (Luminex Corporation, Austin, TX, USA), P-selectin mean fluorescence intensity (MFI) was measured on PLTs (CD41a-positive cells) and normalized to the value obtained for each donor’s own resting PRP.

Statistical analysis
The statistical significance of the differences in MPV and P-LCR between the samples was evaluated using the two-sided paired t-test. Normalized CD62P MFI were compared to resting PRP levels using two-way repeated measures ANOVA with Dunnett’s multiple comparisons test. A value of p < 0.05 was considered statistically significant.

Results
Design and operation of the microfluidic device
A typical CIF device operates by siphoning a relatively small (and precisely calculated\(^{27,28}\)) fraction, \(f_{gap}(t)\), of the flow in the middle channel, \(Q_m(t)\), into two side channels through a series of identical filtration gaps on either side, \(Q_{gap}(t) = f_{gap}(t)Q_m(t)\) (Fig. 1). In such a design, the width of the middle channel and the magnitude of \(f_{gap}(t)\) determine the width of the fluid lamina, \(w\), that is diverted into the side channel through each gap \(i\), and thus the size cut-off for the particles that are sufficiently small to be pulled into the side channel by \(Q_{gap}(t)\) (Fig. 1B).\(^{27,34}\) Because the fluid is siphoned off continuously along the length of the device, particles that are larger than the size cut-off become progressively concentrated in the middle channel (retentate, CIFmid), whereas smaller particles distribute between the retentate and the side channel output (filtrate, CIFside) at approximately the same concentration (Fig. 1A).

When designing a CIF device, the initial values for the width of the middle channel and the filtration fraction \((f_{\ast})\) are selected based on empirical data suggesting that these values are likely to produce a size cut-off suitable for the desired
application. Then, the values of $f_{\text{gap}}(i)$ are iteratively adjusted with each subsequent gap $i$ by incrementally increasing the fluidic resistance of the middle channel and/or decreasing the fluidic resistance of the side channels to maintain the desired size cut-off throughout the device. Because the typical values of $f_{\text{gap}}$ are very small (on the order of $10^{-4}$ for most applications involving blood cells), the fluidic resistance of the side channels must be initially high. Instead of making the side channels correspondingly narrow (and therefore difficult to fabricate), we replaced them with a series of relatively wide but long ‘meandering’ segments of equivalent fluidic resistance (Fig. 2A, inset). The fluidic resistance of these segments is then progressively reduced by reducing their length (while keeping the width constant) to pull incrementally more fluid into the side channels (Fig. 2B). The same is accomplished in the linear section of the device (Fig. 2A) by progressively increasing the width of the side channels and decreasing the width of the middle channel (Fig. 2B).

In addition to the separation channels, each CIF device included a single inlet with an integrated filter for catching large aggregates and debris that may be present in the initial PRP sample and two distinct outlets, one for collecting the middle channel output and another one for collecting the combined output of the two side channels (design layout: Fig. 2A; assembled device: Fig. 3A). For a given $f_{\text{gap}}$, the total number of filtration gaps in a design (and therefore the overall length of the device) determines how much filtrate is extracted from the sample by the device. In this study, we limited the volume of extracted filtrate to ~50% to avoid concentrating PLTs in the retentate excessively and maintain an appropriate level of pH in the sample. The overall footprint of the CIF device was ~3.2 mm × 75.8 mm, and the void volume was 14 μL, not including the tubing connections (Fig. 3A).

Driven through the CIF device by a syringe pump (at 30 μL min$^{-1}$), PRP samples were separated into two fractions: CIF$_{\text{mid}}$ (retentate from the middle channel), and CIF$_{\text{side}}$ (filtrate from the two side channels) (Fig. 3A). Analysis of the PLT size distribution histograms produced by the hematology analyzer confirmed that large (>12 fL) PLTs were almost exclusively confined to the CIF$_{\text{mid}}$ fraction (Fig. 3B). For example, in a CIF device ($f_{\text{gap}} = 1.04 \times 10^{-4}$) designed to separate the initial PRP sample approximately in half, the CIF$_{\text{mid}}$ fraction contained 49.9 ± 1.6% of the sample volume, 87.3 ± 3.8% of all PLTs and 96.9 ± 1.5% of large (>12 fL) PLTs. The CIF$_{\text{side}}$ fraction of the same device received 50.1 ± 1.6% of the sample volume, 12.7 ± 3.8% of all PLTs and 3.1 ± 1.5% of large PLTs. As a result of separation, P-LCR of the CIF$_{\text{mid}}$ fraction increased to 18.2 ± 2.6%, and of the CIF$_{\text{side}}$ fraction decreased to 3.8 ± 1.3%, from the initial value of 16.2 ± 2.3% measured for the input PRP samples.

As expected, we observed a significant accumulation of PLTs retained in the middle channel near the filtration gaps (Fig. 3C) (we have previously observed a similar behavior for red blood cells, particularly at low hematocrits). In a CIF design, the flow laminae closest to the filtration gaps are continually diverted away from the middle channel (Fig. 1B).
PLT accumulation became increasingly more pronounced along the length of the device (compare Fig. 3C(i) and (iii)).

Shaped roughly as oblate spheroids, PLTs of all sizes have about the same thickness (minor/polar axis, 2c) of ~1 μm, and range in diameter (major/equatorial axis, 2a) from about 2.0 μm for the smallest 2 fL PLTs to more than 4.8 μm for the largest (>12 fL) PLTs. For particles with a radius smaller than the width of the extracted flow lamina (a < w), we would expect their distribution between the filtrate and retentate to approximate that of the fluid volume (for example, see the distribution of 1 μm spherical beads between retentate and filtrate of the CIF device in Fig. S1A of ESI†). In our experiments, however, recovery of the smallest 2 fL PLTs (a ≈ 1 μm) in the filtrate was consistently lower than the percent of extracted fluid volume (Fig. 4). We reasoned that this discrepancy was likely due to crowding of the flow laminae near the filtration gaps by retained PLTs (Fig. 3C), which impeded the free outflow of the smallest PLTs into the side channels. For a given f gap, increasing the flow rate to increase the frequency of cell-cell interactions would lessen the accumulation of retained PLTs near the filtration gaps (see Fig. S2, ESI†) and thus reduce the effect of crowding on the recovery of smallest PLTs. Indeed, the difference between the percent of fluid volume and the percent of 2 fL PLTs recovered in the filtrate (filtrate recovery difference) declined significantly from 23.2 ± 4.6% to 9.8 ± 3.0% at 30 μL min⁻¹ (Fig. 4A).

In a CIF device, we would expect particles with radii exceeding the width of the extracted flow lamina (a > w) to remain in the middle channel (Fig. 1B and 3C). However, when the flow laminae near the filtration gaps become crowded, these larger particles may be pushed into the side channels due to the interactions with other particles (as opposed to being pulled by the extracted flow lamina, Q gap, as shown in Fig. 1B for smaller particles). Therefore, more interactions between the cells at the higher flow rate also increased the number of large PLTs escaping into the side channels. Indeed, recovery of 12 fL PLTs (2a = 4.8 μm) in the filtrate increased from 4.9 ± 0.9% at 30 μL min⁻¹ to 13.3 ± 2.9% at 90 μL min⁻¹, decreasing the filtrate recovery difference from 45.6 ± 3.7% to 36.7 ± 3.4% at 30 and 90 μL min⁻¹, respectively (Fig. 4A). As the particle concentration increases, the interactions between particles also increase and, therefore, the recovery of large particles in the filtrate should also increase. Indeed, increasing the concentration of 4.7 μm spherical beads from ~100 beads per μL to ~1700 beads per μL increased their recovery in the filtrate from 1.8% to 6.8%, respectively (Fig. S1B, ESI†).

Conversely, reducing PLT concentration in the input sample through serial dilution (and thus decreasing the interactions between the cells) increased the filtrate recovery difference for PLTs of all sizes and this effect was more pronounced for smaller PLTs (Fig. S3, ESI†).

Finally, we reasoned that increasing f gap to widen the extracted flow lamina would expand the size range of PLTs being pulled by Q gap into the side channels (see Fig. 1B),...
thereby increasing the filtrate recovery of smaller PLTs. At the same time, removing more PLTs from the crowded region near the filtration gaps in the middle channel would reduce the likelihood of larger PLTs being pushed into the side channels due to interactions with other cells, thus decreasing the filtrate recovery of larger PLTs. Indeed, as the gap of the CIF design increased (from $1.04 \times 10^{-4}$ to $1.28 \times 10^{-4}$), the filtrate recovery difference for smaller PLTs decreased and for larger PLTs increased (Fig. 4B).

Comparison of PLT fractions produced by the microfluidic device and centrifugation

Generally, the speed at which particles sediment during centrifugation depends on particle size (larger particles sediment faster).37 Because of this dependance, centrifugation has been the go-to method for separating PLTs by size.12,14 However, even the relatively gentle spin (650 × g for 7 min) required by the centrifugation protocol to produce the so called ‘large PLT’ fraction (LPF)14 invariably results in the formation of macro-scale PLT aggregates (clumps) (Fig. 5A). To alleviate this problem, the centrifugation protocol requires adding a significant amount of extra anticoagulant (100 μL of ACD-A per 1 mL of PRP),14 which prevents centrifugation-induced PLT aggregation almost entirely (Fig. 5B). Removing this extra ACD-A, however, requires centrifuging PLTs in the ‘small PLT’ fraction (SPF) a second time to pellet the cells and discard the supernatant. Consequently, any cells that remain suspended in the supernatant after the second spin – including the smallest of the PLTs originally contained in the PRP sample – also get discarded (Fig. 5B). In contrast, the CIF device separates PLTs into two fractions in a single step without needing any additional ACD-A (or dilution) because unlike centrifugation, microfluidic processing does not pellet PLTs and therefore does not produce PLT clumps (Fig. 5C).

We used paired PRP samples (from $n = 6$ donors) to compare the size characteristics of PLT fractions produced by the centrifugation protocol and the CIF device ($f^*_{gap} = 1.04 \times 10^{-4}$) operating at a flow rate of 30 μL min$^{-1}$ (Table 1). Both MPV and P-LCR of the PRP samples were within the normal range for healthy human adults.10,11 Although PLT recovery was significantly higher for CIFmid, the CIFmid fraction was essentially equivalent to LPF produced by the centrifugation protocol in terms of PLT size characteristics (no significant difference in MPV and P-LCR). However, PLTs comprising CIFside fraction were significantly smaller (lower MPV and
P-LCR) than those comprising SPF. This was because SPF was significantly more contaminated by the largest PLTs (~2-fold higher recovery of >12 fL PLTs) than CIFside. In terms of PLT size, CIFside fraction was more similar to the population of PLTs discarded by the centrifugation protocol (no significant difference in MPV and P-LCR). The CIFside fraction had a significantly higher overall PLT recovery, but the level of contamination with the largest PLTs was similarly low for both the CIFside and the discarded PLT fraction (no significant difference in >12 fL PLT recovery).

To better understand the effect of processing on PLT properties we measured activation for all PRP samples and of separated fractions at rest (Fig. 6A, top) and after incubation with thrombin receptor agonist peptide-6 (TRAP-6) (Fig. 6A, bottom) using imaging flow cytometry. When expressed as percent of all PLTs (CD41a+ cells) that were positive for CD62P, the baseline PLT activation in the PRP samples before processing was 9.1 ± 5.1% (range: 1.7–15.5%). Processing...
increased PLT activation, on average, by 4.4 ± 5.3 percentage points for centrifugation and by 4.9 ± 3.7 percentage points for the CIF device; however, there were no significant differences between the separation methods and different PLT fractions in paired analysis. We found a significant increase in PLT activation after the additional stimulation with TRAP-6 for the initial PRP as well as for the LPF and the CIFmid fractions. However, PLT activation in the SPF and the CIFside fractions was lower and not significantly different from the resting PRP control (Fig. 6B).

Discussion

This study demonstrates the feasibility of replacing centrifugation with a microfluidic device based on ‘controlled incremental filtration’ (CIF) for separating PLTs by size. In a paired comparison, the CIF device conferred several important advantages over the state-of-the-art two-stage centrifugation protocol recently published by Handtke et al.\textsuperscript{14} When performed on unmodified PRP samples, the centrifugation step of the protocol causes activation and massive aggregation of pelleted PLTs. Unlike centrifugation, the CIF device does not pellet PLTs and therefore could process PRP samples without the addition of extra anticoagulant (ACD-A) which was required by the centrifugation protocol. Because this extra anticoagulant needs to be removed to enable functional assays downstream, the ‘small PLT’ fraction (SPF) is pelleted again, and the supernatant is discarded. However, a significant fraction of the smallest PLTs is lost with the discarded supernatant. Additionally, PLTs comprising SPF are subjected to centrifugation twice while those within LPF only once, which may bias a comparison. In contrast, the CIF device separates the PRP sample into exactly two PLT fractions — CIFmid and CIFside — with both experiencing the same conditions while passing through the device. In terms of PLT size, CIFmid fraction was equivalent to LPF, while CIFside fraction was significantly less contaminated with large (>12 fl) PLTs than SPF and included the smallest PLTs that were discarded by the centrifugation protocol.

Our data suggest that both centrifugation and the passage through the CIF device had a minimal effect on PLT activation. Centrifugation, however, was performed in the presence of a substantial amount of extra anticoagulant, and hence the increase in PLT activation due to centrifugation was much smaller than what is typically observed for unmodified PRP samples.\textsuperscript{33} Interestingly, the CIF device caused a similarly small increase in PLT activation as centrifugation, but without needing any extra anticoagulant. PLTs comprising the LPF and CIFmid fractions remained responsive to stimulus (TRAP-6) after processing and therefore the relatively low activation at rest was not a result of refractoriness.\textsuperscript{22,23} However, activation of PLTs comprising SPF and CIFside fractions in response to the additional stimulation was not different from the control (resting PRP), suggesting a lesser effectiveness of small PLTs. These findings are in agreement with the significant functional differences between large and small PLTs suggested by recent studies.\textsuperscript{13-15} The baseline values and variation in PLT activation among subjects were somewhat higher than reported in our previous work,\textsuperscript{28,33} likely because of the differences in the collection and processing of the initial whole blood samples, and the use of ‘samples of convenience’ in this proof-of-concept study (e.g., subjects were not monitored for medical conditions or use of medications that may affect PLT activation).

Several other microfluidic cell separation methods have been previously applied for separating PLTs. The inertial focusing device by Di Carlo \textit{et al.} enriched PLTs 100-fold in diluted whole blood (2% hematocrit) at a flow rate of 0.9 mL min\textsuperscript{−1}.\textsuperscript{38} The filtration method by Dickson \textit{et al.} removed residual red blood cells from units of apheresis-derived PLTs at a flow rate of 0.1 mL min\textsuperscript{−1}.\textsuperscript{39} A device based on deterministic lateral displacement (DLD) by Li \textit{et al.} purified PLTs at a rate of 1000 cells per second from whole blood diluted 50-fold.\textsuperscript{40} A device based on the hydrodynamic lift

### Table 1. Paired comparison of PLT fractions produced by centrifugation and the CIF device ($f_{gap} = 1.04 \times 10^{-4}$, 30 μL min\textsuperscript{−1}). Values are shown as mean ± standard deviation (minimum – maximum)

<table>
<thead>
<tr>
<th></th>
<th>Centrifugation protocol</th>
<th>CIF device</th>
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<tbody>
<tr>
<td></td>
<td>LPF</td>
<td>SPF</td>
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<tr>
<td></td>
<td>CIFmid</td>
<td>CIFside</td>
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<tr>
<td><strong>Concentration, ×10^3 μL\textsuperscript{−1}</strong></td>
<td>462 ± 81</td>
<td>638 ± 146</td>
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<tr>
<td>(352-548)</td>
<td>(473-833)\textsuperscript{a}</td>
<td>(63-145)</td>
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<tr>
<td><strong>MPV, fl</strong></td>
<td>9.1 ± 0.4</td>
<td>9.7 ± 0.3</td>
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<tr>
<td>(8.6-9.6)</td>
<td>(9.2-10.0)\textsuperscript{a}</td>
<td>(6.9-7.6)\textsuperscript{b}</td>
</tr>
<tr>
<td><strong>P-LCR, %</strong></td>
<td>19.1 ± 3.1</td>
<td>22.6 ± 2.4</td>
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<tr>
<td>(15.3-22.8)</td>
<td>(19.1-25.3)\textsuperscript{a}</td>
<td>(3.4-7.5)\textsuperscript{b}</td>
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<tr>
<td><strong>Overall recovery, %</strong></td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td></td>
<td>(75.5-85.4)\textsuperscript{a}</td>
<td>(91.9-16.5)</td>
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<tr>
<td><strong>&gt;12 fl. recovery, %</strong></td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td></td>
<td>(95.1 ± 1.3)</td>
<td>(95.1 ± 1.3)</td>
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<tr>
<td>(92.9-96.5)\textsuperscript{a}</td>
<td>(1.8-6.3)\textsuperscript{b}</td>
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<tr>
<td><strong>Activation, %</strong></td>
<td>9.1 ± 5.1</td>
<td>13.0 ± 5.1</td>
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<tr>
<td>(1.7-15.5)</td>
<td>(4.6-20.7)\textsuperscript{c}</td>
<td>(2.7-33.1)\textsuperscript{d}</td>
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</table>

Statistically significant differences are denoted as: \textsuperscript{a} CIFmid vs. LPF. \textsuperscript{b} CIFside vs. SPF. \textsuperscript{c} CIFside vs. discarded PLTs.
principle implemented by Geislinger et al. recovered PLTs from diluted whole blood (hematocrit <1%) at a flow rate of 0.0125 mL min\(^{-1}\).\(^{1,41}\) Finally, a hydrophoresis device developed by Choi et al. processed whole blood diluted 10-fold to separate PLTs with ~40% recovery and ~80% purity at 0.02 mL min\(^{-1}\).\(^{1,42}\) Although useful for separating PLTs from other types of blood cells, none of these microfluidic cell separation methods have been applied specifically to separating small from large PLTs.

Separating PLTs based on size is not a trivial task because PLTs of all sizes have essentially the same thickness (~1 μm) and the differences in cell diameter between ‘large’ and ‘small’ PLTs are very small. For example, the difference in diameter between a 12 fl PLT and a 6 fl PLT is only about 1.4 μm (for comparison, the difference between a PLT and a leukocyte could be ~10 μm, which simplifies the size-based separation dramatically).\(^{28}\) PLT shape presents another significant challenge due to the characteristic tumbling motion oblate spheroids undergo when flowing near the channel walls.\(^{36}\) Notwithstanding these separation challenges, the CIF device resolution was sufficient to effectively extract a significant fraction of the smallest PLTs with the filtrate (CIF\(_{side}\)), while preserving virtually all (97–99%) of large PLTs in the retentate (CIF\(_{mid}\)). Additionally, the width of the extracted streamline (which is determined by the CIF design parameters) and the flow rate at which the sample was passing through the device provided two independent ways to adjust the size distribution of PLTs extracted with the filtrate which could be particularly useful when studying the biological differences between PLTs of different size.

Finally, removing or replacing the suspending medium from PLT units before transfusion is known to significantly reduce the risk of adverse reactions\(^{43–45}\) including transfusion-associated circulatory overload,\(^{46,47}\) allergic reactions,\(^{48,49}\) febrile non-hemolytic transfusion reactions\(^{50,51}\) and transfusion-related acute lung injury.\(^{52,53}\) In current clinical practice, the PLT unit volume is reduced via centrifugation by pelleting PLTs, removing ~50% of the original suspending medium, and resuspending the PLT pellet in the remaining volume. These volume-reduced PLTs are then allowed to ‘rest’ for up to 60 min (often followed by an additional 15–60 min on a PLT agitator) to dissociate the PLT aggregates formed by pelleting.\(^{20,21}\) Approximately 80% of the PLTs initially present in the unit are typically recovered by this centrifugation-based process.\(^{20,21}\) The developed CIF device was able to remove ~50% of the suspending medium in the CIF\(_{side}\) fraction, while recovering ~90% of all PLTs and ~98% of the largest (>12 fl) PLTs in the CIF\(_{mid}\) fraction, thus exceeding the separation performance typically reported for the centrifugation-based protocols employed clinically. However, further research is needed to increase the throughput of the device (e.g., by further reducing the fluidic resistance of individual CIF units and by multiplexing many CIF units in parallel)\(^{30–33}\). Nevertheless, the continuous, flow-through operation of the CIF device provides another major advantage over batch centrifugation, suggesting a possibility of scalable, on-demand processing that could potentially be done at a patient’s bedside. Such a capability would be particularly useful in the pediatric setting, where the demand for high quality, low-volume PLT transfusions is most urgent.\(^{44,45,54,55}\)
Author contributions

MTPD and SSS designed the study. SCG designed microfluidic devices. SSS fabricated the master wafer. MTPD assembled the microfluidic devices. MTPD, AM, KA, and FWL performed the experiments and collected the data. MTPD, FWL, SCG and SSS analyzed and interpreted the data. SSS supervised the project. MTPD and SSS wrote the manuscript. All authors critically reviewed and approved the manuscript.

Conflicts of interest

SSS and SCG are inventors of U.S. Patent #9789235 “Separation and concentration of particles” describing the ‘controlled incremental filtration’ technology, and are co-founders of Halcyon Biomedical, Incorporated, a company that would benefit from its commercialization. MTPD, AM, KA, and FWL declare no conflict of interest.

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References


34 D. W. Inglis and N. Herman, *Lab Chip*, 2013, 13, 1724–1731.


