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Short communication

Separation of model mixtures of epsilon-globin positive fetal nucleated red blood cells and anucleate erythrocytes using a microfluidic device

Daniel Lee [a], Ponnusamy Sukumar [b], Aniza Mahyuddin [b], Mahesh Choolani [b,*], Guolin Xu [a,**]

[a] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669, Singapore
[b] Diagnostic Biomarker Discovery Laboratory, Department of Obstetrics and Gynecology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074, Singapore

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ABSTRACT

Microfluidic devices are capable of separating microparticles and cells. We developed and tested the efficiency of silicon cross-flow microfilters for the separation of primitive fetal nucleated red blood cells (FNRBCs) and adult anucleate red blood cell (AARBCs) from model mixtures. Stepwise improvements over three generations of device design resulted in an increasing trend in the recovery of FNRBCs. We obtained a recovery of FNRBCs ($74.0 \pm 6.3\%$, $p < 0.05$, $n = 5$) using the third generation device, with a depletion of $46.5 \pm 3.2\%$ AARBCs from the cell mixture. The purity of FNRBCs in the enriched fraction was enhanced by a factor of 1.7-fold.

1. Introduction

Microfluidic devices show considerable promise for the separation of cells, with potential applications in biology and medicine [1,2]. Separation of the blood components have focused primarily on obtaining plasma [3] or white blood cells [4,5]. Cell size, shape and deformability were considered in the design of microfluidic devices for blood cell separation: pillars [4], cross-flow filtration [5], hydrodynamic filtration [6], pinched flow fractionation [7] and lateral displacement [8] have all been examined.

Isolation of rare cells from blood such as circulating tumour cells (CTCs) [9] and fetal cells in maternal blood [10] has potential importance in disease diagnosis and monitoring. To date, these applications have been limited by being time and resource intensive, with limited efficacy. Recently, microfluidic devices have been explored for the isolation of cancer cells from model mixtures and patient blood [11].

Non-invasive prenatal diagnosis using fetal cells enriched from maternal blood would eliminate the risk of miscarriage associated with invasive testing such as amniocentesis and chorion villus sampling [10]. There is some evidence of the value of microdevices in sorting fetal cells. Mohamed et al. [12] sorted cord blood fetal nucleated red blood cells (FNRBCs) from WBCs in a microfluidic device with varying size channels. Huang et al. [13] demonstrated NRBC enrichment from maternal blood using a microfluidic device. Epsilon-globin positive(e+) FNRBCs are the ideal fetal cell target for non-invasive prenatal diagnosis. Separation of e+FNRBCs from abundant adult anucleate red blood cells (AARBCs) in maternal blood would enhance yield, as most e+FNRBCs are lost into the RBC pellet during density gradient centrifugation, the first step in enrichment protocols [10,14,15].

To date, there is no method that can satisfactorily separate e+FNRBCs from AARBCs. We hypothesized that a microfluidic device could be developed that would separate the two target cell types, e+FNRBCs and AARBCs based upon their physical properties such as size and deformability. We have designed, fabricated and tested the efficiency of a silicon-based cross-flow microfilter device for the separation of e+FNRBCs (~Ø15 μm) from model mixtures containing AARBCs (~Ø6.5 μm), and assessed recovery and purity of sorted samples. Flow rates were optimized to handle larger sample volumes in a shorter time period, as rare e+FNRBCs in maternal blood may require larger starting volumes for clinical diagnosis. Stepwise improvements over three generations of device design resulted in an increasing e+FNRBCs recovery. The microfilter device presented here recovered 74.0% e+FNRBCs and depleted 46.5% AARBCs from model mixtures.

* Corresponding author.
Tel.: +65 6772 4279; fax: +65 6779 4753.
** Corresponding author at: Institute of Bioengineering and Nanotechnology, Medical Device, 31 Biopolis Way, The Nanos, #04-01, Singapore 138669, Singapore. Tel.: +65 6824 7000; fax: +65 6478 9085.
E-mail addresses: obgmac@nus.edu.sg (M. Choolani), gxu@ibn.a-star.edu.sg, glxu@ibn.a-star.edu.sg (G. Xu).

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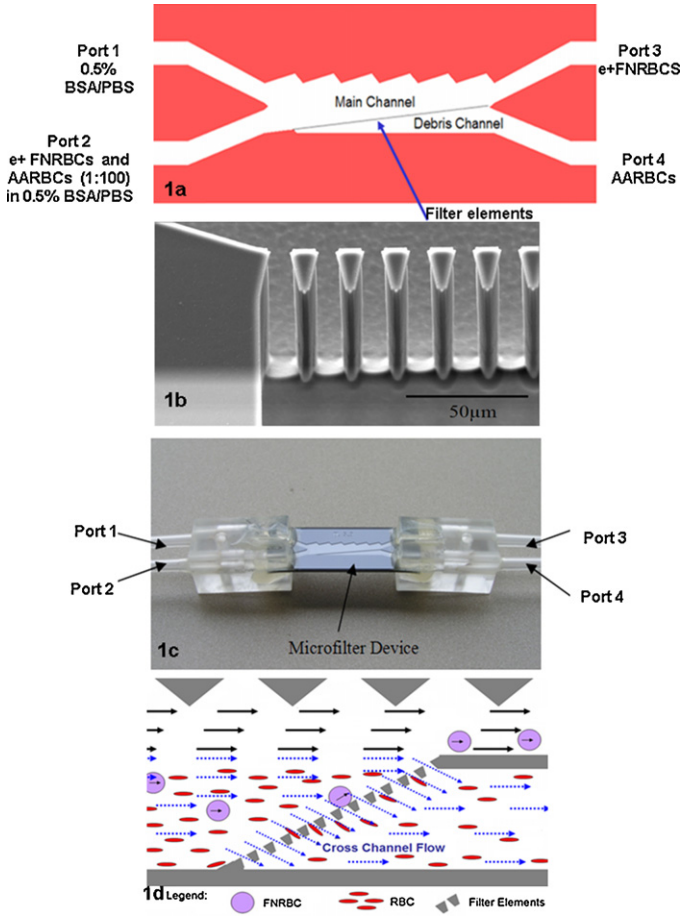

Fig. 1. Microdevice working principle: (a) schematic of microdevice: Ports 1 and 2 are inlets for PBS and model mixture sample(s), respectively. Ports 3 and 4 are collection ports for e+FNRBCs and AARBCs, respectively; a microfilter array in the main channel creates a debris channel as shown; (b) ESEM image of microfilter showing the filter elements; (c) photomicrograph of the microfilter device; (d) dashed arrows indicate flow of 1× PBS containing cells mixture from Port 2. Solid arrows indicate flow of 1× PBS/0.5% BSA buffer from Port 1, flowing alongside the cell mixture from Port 2. The flow from Port 1 pushes the cell mixture to encounter the filter array, whereby the smaller AARBCs can pass through the filter gap and get collected at Port 4; whereas larger FNRBCs cannot pass through the filter gaps and are diverted to Port 3.

While the current enrichment is encouraging, further improvement will be required for the device to be useful for fetal non-invasive prenatal diagnosis.

2. Experimental

2.1. Device structure and principle

The microfluidic filter device used to separate e+FNRBCs from model mixtures containing AARBCs was based on size and known deformability of AARBCs. A main conduit channel with an inclined silicon filter array in its path creates a second debris channel. The device has two inlets (Ports 1, 2; P1, P2) and two outlets (Ports 3, 4; P3, P4). P1 and P2 are for flow of buffer and sample mixture, respectively. P3 and P4 are for e+FNRBC collection and AARBC collection, respectively (Fig. 1a and c).

1× Phosphate-buffered saline (PBS) buffer/0.5% BSA was injected through P1 and flowed alongside the sample mixture injected through P2. The flow from P1 pushed the flow of sample mixture to encounter the filter array. Filter gaps (Fig. 1b) were designed to allow deformable AARBCs to pass through under a hydrodynamic pressure between the main channel and debris channel across the filter and collect at P4.

Larger, less deformable e+FNRBCs get diverted to the cell collection P3 (Fig. 1d). Device characteristics of the three devices designed are described in Table 1. Buffer and sample flow rates were manipulated to obtain maximum e+FNRBC recovery.

2.2. Device fabrication

Silicon microfilter devices were fabricated at the Institute of Bioengineering and Nanotechnology, Singapore, using standard micromachining techniques. Device layout was prepared and a photo-mask created. Silicon dioxide (SiO_2) was deposited on deionised water (DI) cleansed silicon wafers using plasma enhanced chemical vapor deposition (PECVD, 5 min) and coated with hexa-methyl-di-silizane (HMDS) before photo-resist (PR) was spun-coated (2000 rpm, 30 s) and soft-baked (100 °C, 3 min). Filter design was realized on PR-coated silicon wafers by UV exposure (EVG 620). Wafers were soft-baked (110 °C, 3 min) developed in AZ300MIF-developer solution, DI-water rinsed, spun-dried and hard-baked.

Reactive ion etching (RIE) enabled removal of SiO_2 from regions on silicon wafers not protected by PR. Residual PR on silicon wafers were stripped off by rinsing with acetone and N-methyl-pyrrolidone (NMP)-soak/sonication (70 °C, 2 h). Deep reactive ion etching (DRIE, Alcatel AMS100 I-Speeder) on silicon wafer created microchannels and silicon pillars of microfilter devices.

SiO_2 mask on silicon wafers were stripped off using buffered oxide etchant (27 °C, 20 min), DI-water rinsed and spun-dried. Following this a 500 ± 25 μm thick Pyrex 7740 glass wafer (4″) was anodically bonded onto the silicon wafer (305 °C, 1000 V, 40 min, EVG 520 Anodic Bonder).

Silicon microfilter devices were singled-out from glass-bonded silicon wafer by dicing process (DISCO-DAD3350). Tubing adaptors were fabricated using a three-dimensional fast prototyping machine (Objet Eden-2600) and mounted onto both ends of the microfilter device using epoxy resin.

2.3. Preparing e+FNRBCs and AARBCs

Placental tissues were obtained from women undergoing elective first trimester surgical termination of pregnancy (fetal gestational age: 7–10 weeks) at the Department of Obstetrics and Gynecology, National University Hospital, Singapore. Institutional Review Board approved written informed consent was obtained in each case. e+FNRBCs were isolated from trophoblast tissue using our own protocol [16]; AARBCs were prepared from blood obtained from healthy volunteers.

2.4. Sorting e+FNRBCs and AARBCs from model mixtures

Model mixtures comprised 1:100 ratio of e+FNRBCs:AARBCs (Device 1: 10^5:10^7; Devices 2, 3: 10^4:10^6) suspended in 3.0 mL 0.5% BSA/PBS. At start, devices were primed with 0.5% BSA/PBS through both inlets using syringe pumps until the entire volume within the device was filled; care was taken to ensure that no air bubble remained within the device. 3.0 mL sample mixtures

Table 1
Separation element features for devices.

Parameters	Device number		
	Device 1	Device 2	Device 3
Microfilter elements gap size (μm)	7	5	4
Angle of microfilter elements array (°)	30	5	5
Outlet Port 3 channel width (μm)	200	1200	1200

Table 2
Separation flow rate and model mixtures containing e+FNRBCs and AARBCs using microdevices.

	Flow rates (mL min^{-1})	Port 3		Port 4	
		FNRBCs recovered mean ± SEM	AARBCs contamination mean ± SEM	FNRBCs lost mean ± SEM	AARBCs depleted mean ± SEM
Device 1	P1 = 0.1, P2 = 0.1	40.5 ± 12.6	56.1 ± 12.0	18.3 ± 4.5	44.2 ± 13.3
	P1 = 0.1, P2 = 0.2	31.5 ± 8.4	48.3 ± 11.4	23.1 ± 5.6	36.7 ± 10.5
	P1 = 0.1, P2 = 0.3	38.3 ± 11.3	42.1 ± 17.2	13.2 ± 8.4	19.5 ± 20.2
Device 2	P1, P2 = 0.1	39.4 ± 14.4	31.9 ± 5.0	32.0 ± 12.6	53.7 ± 14.5
	P1, P2 = 0.3	56.0 ± 9.7	37.4 ± 9.9	34.8 ± 11.9	41.0 ± 11.1
	P1, P2 = 0.45	61.0 ± 8.3	47.0 ± 2.7	25.4 ± 11.7	18.4 ± 8.1
Device 3	P1, P2 = 0.1	47.6 ± 4.8a	45.9 ± 4.4	26.0 ± 8.2	45.6 ± 7.6
	P1, P2 = 0.3	74.0 ± 6.3b	42.5 ± 9.4	18.4 ± 1.6b	46.5 ± 3.2
	P1, P2 = 0.45	67.2 ± 5.6b,c	46.0 ± 4.0	34.4 ± 6.5c	50.9 ± 6.5

The results are presented in percentages. Means with the different alphabets are significantly different ($p < 0.05$). SEM: standard error of mean.

(Fig. 1a) and equal volume 0.5% BSA/PBS were injected simultaneously through respective ports using syringe pumps. Syringe pumps were used to maintain flow rates through inlets (Table 2). Clogging and passing capacity of devices were qualitatively inspected using an optical microscope. At end, devices were flushed using 6 mL 0.5% BSA/PBS through each inlet. Devices 2 and 3 were stepwise, iterative improvements from their antecedent version.

Cells collected at P3, 4 were centrifuged (3000 rpm/10 min). Pellets were suspended in 100–200 μL of 0.5% BSA/PBS, and P3 FNRBC percentage recovery and loss at P4 calculated by haemocytometer. Similarly, P4 AARBC depletion and contamination at P3 determined. Recovered cells were cytospun and Wright's stained [16] for morphology, and immunostained for e-globin for verification of integrity and identity [16] (Fig. 2c and d).

2.5. Statistical analysis

Data were analyzed using SPSS v13 software. One-way ANOVA with Bonferroni post hoc test or non-parametric Kruskal–Wallis tests were carried out to compare the results for statistical significance. Two groups were compared either by *T*-test or Mann–Whitney *U*-test.

3. Results and discussion

Prior to designing and fabrication of the microdevice 1, we measured the diameter(s) of e+FNRBCs and AARBCs ($n = 300$ each): mean ± SD e+FNRBC was 15.0 ± 2.1 μm and AARBC was 6.5 ± 1.1 μm.

3.1. Cross-flow silicon filter

Silicon was chosen for fabrication of our cross-flow filter as it is rigid, can maintain a defined pore size, and is readily manufactured using lithography-based fabrication techniques. Silicon-based microfilters have been used previously for WBC separation from blood [4,17]. Comparison of four silicon-based microfilter designs: cross-flow, pillar, weir and membrane filters suggested that the cross-flow design was superior for whole blood cell separation because of its ability to handle larger blood volumes and nucleated cell trapping [5], and have less pore-clogging that can extend filter life [3].

3.2. Model mixtures for efficiency studies

Rare cell detection is probably the most challenging of all cell sorting exercises. Novel devices and protocols are best evaluated in model mixtures where the recovery can be accurately determined. In contrast, in *in vivo* models, it is impossible to accurately determine enrichment efficiencies as the true number of cells present in any sample of maternal blood is not known with certainty. Model mixtures are prepared by mixing cells in higher proportions than their physiological concentration. This is necessary to determine device efficiencies, as very few target cells present would make the estimation of device performance inaccurate. Most investigators recognize the importance of such model mixtures: tumour cells were mixed with same number of normal cells (10^4 each) to test the capture efficiency of an antibody based microdevice [18] and equal numbers of nucleated erythroleukemic K562 cells and AARBCs were mixed when studying a MEMS-DEP device [19].

Model mixtures have been used for studying enrichment protocols for non-invasive prenatal diagnosis. *In vivo* assessments are limited by the lack of information on the actual number of target fetal cells circulating in maternal blood, and the estimated low numbers of target cells per millilitre of maternal blood make the assessment of enrichment efficiency of the device itself inaccurate [16]. Limited access to the appropriate target fetal cell type (first trimester fetal erythroblasts) limits the effectiveness of some model mixtures. As surrogates, various authors have used cord blood in adult blood [14,20] male fetal liver cells in adult female mononuclear (MN) cells [21] and cultured cord blood NRBCs in adult MN cells [22]. We and Voullaire et al. [15,16,23] have used the target first trimester FNRBCs for our model mixture experiments to generate data more representative of non-invasive prenatal diagnosis.

3.3. Separation of e+FNRBCs and AARBCs from model mixtures using microdevices

FNRBC recovery and AARBC contamination at P3, and AARBC depletion and FNRBC loss in P4, are presented in Table 2, for all three devices. In our experiments, cells recovered after a single pass through our novel cross-flow filters were expressed in relation to their initial numbers in model mixtures to represent the absolute recovery of e+FNRBCs at P3, and depletion of AARBCs at P4. Isolation of WBCs from whole or diluted blood [5,6] is relevant in several clinical circumstances, but less challenging than rare cell detection. With rare cells, cell recovery is crucial; modest purity is acceptable. Our recovery of 74.0% of target cells (e+FNRBCS) in P3 suggests that the design and flow rates of fluids through the main channel directed most of the target cells appropriately, comparable to other cross-flow filters [5].

In Device 1, three flow rates were compared. No significant difference was observed for e+FNRBC recovery or AARBC depletion between the three flow rates (Table 2). Most AARBCs contaminated P3 at all three flow rates. In Device 2, filter-gap size and array angle were reduced, and P3 channel width was increased 6-fold (Table 1). Three identical flow rates in both inlets (0.1, 0.2, 0.3 mL/min) were

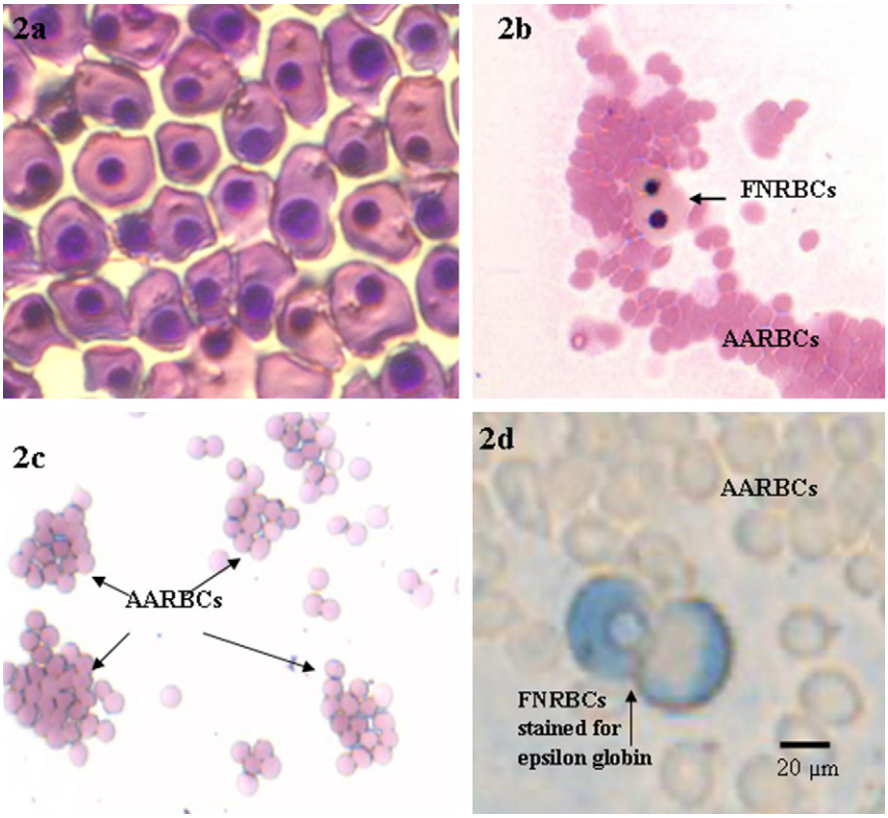

Fig. 2. Cells used for model mixture experiment: (a) Wright's stained e+FNRBCs prepared from placental villi; (b) model mixture of e+FNRBCs and AARBCs before sorting using microdevice (Wright's stained); (c) AARBCs collected at Port 4 (Wright's stained) showing intact morphology; (d) e+FNRBCs collected at Port 3 and immunostained for cytoplasmic epsilon-globin using anti-epsilon-globin antibody showing the intact morphology of the cells after staining, unstained AARBCs are seen at the background.

tested; the results, though not statistically significant, showed an encouraging increasing trend of e+FNRBC recovery (39.4–61.0%), but AARBC contamination also showed a similar trend (Table 2).

Only the filter-gap size was reduced in Device 3; all other device characteristics and flow rates were identical to Device 2 (Table 1). There was a significant increase in the recovery of spiked e+FNRBCs in model mixtures at a flow rate of 0.3 mL/min ($74.0 \pm 6.3\%$, $p < 0.05$). At this flow rate, e+FNRBCs loss into P4 was also minimized ($18.4 \pm 1.6\%$). The best recovery and lowest loss of e+FNRBCs was seen with medium flow rates (0.3 mL/min); at this flow rate $46.5 \pm 3.2\%$ of AARBCs were depleted.

These data were obtained on model mixtures of 1:100 e+FNRBCs:AARBCs. The rarity of fetal cells in maternal blood would require further improvements to these devices to sort AARBCs, and also to subsequently deplete white blood cells using an antibody-type depletion strategy.

3.3.1. Purity of enriched sample

The percentage purities of e+FNRBCs in cell mixtures before and after separation were calculated. The purity of e+FNRBCs in enriched cell-mixture collected at Port 3 using the Device 3 at a flow rate of 0.3 mL/min was found to be enhanced by a factor of 1.7-fold that of initial mixture.

3.4. Comparison of the efficiency of devices

The best e+FNRBC recovery of each device were compared. There was an increasing trend in e+FNRBC recovery at P3 from Devices 1 to 3, and a decreasing trend in contaminating AARBCs (Table 3). e+FNRBC recovery was significantly better in Device 3 ($74.0 \pm 6.3\%$) compared with Device 1 ($40.5 \pm 12.6\%$). Except for the filter-gap width, all device characteristics between Devices 2 and 3 were similar, but AARBC depletion was significantly greater in Device 3 (50.88 ± 6.50) compared with Device 2 (18.36 ± 8.07) at a flow rate of 0.45 mL/min ($p < 0.05$).

Stepwise, iterative improvements in device design allowed improved e+FNRBC separation from model mixtures without clogging of small pores (4 μm) common with weir and membrane filters [5]. Our 74.0% e+FNRBC recovery from model mixtures using Device 3 (0.3 mL/min flow rate) is the best separation of e+FNRBCs from AARBCs described to date.

3.5. Flow rate and sample volume

Most microdevices to-date use small volumes of whole or diluted blood (1–100 μL) to either enrich WBCs [4–6,24,25] or obtain plasma for analysis [3,8,26], with flow rates of a few μL/min. But in order to enrich rare cells from blood, larger volumes need to be processed. It is anticipated that in clinical applications, about 20 mL of maternal blood would need to be processed to isolate sufficient numbers of e+FNRBCs for non-invasive prenatal diagnosis [16]. Some microdevices used for FNRBC separation from cord

Table 3
Comparison of the best performance(s) of each device to recover the maximum e+FNRBCs from model mixtures.

	% recovery of e+FNRBCS in Port 3 Mean ± SEM	% contamination of AARBCs in Port 3 Mean ± SEM
Device 1	40.5 ± 12.6a	56.1 ± 12.0
Device 2	61.0 ± 8.3	47.0 ± 2.7
Device 3	74.0 ± 6.3a	42.5 ± 9.4

Means with same alphabet are significantly different ($p < 0.05$). SEM: standard error of mean.

blood were limited by slow flow rates of 0.35 mL/h [12], but others were more able to handle larger volumes of maternal blood samples [13]. Our device permitted an optimal flow rate of 0.3 mL/min suggesting that 20 mL of maternal blood could be processed in just over an hour.

3.6. e+FNRBC integrity after cell sorting

e+FNRBCs are nucleated, and unique in morphology (Fig. 2a and b). After cell sorting, FNRBCs were cytospun and immunostained for epsilon-globin (Fig. 2d). Epsilon-globin immunostaining of FNRBCs showed that target cell morphology and cytoplasmic content were intact, and not adversely affected by the hydrodynamic pressure of moderately high flow rates.

4. Conclusions

This proof of concept study suggests that size-based separation of the two cell types e+FNRBCs and AARBCs is possible using a cross-flow microfilter with appropriate hydrodynamic pressures in the system. Our best results suggest a 74.0% fetal cell recovery with a 46.5% red blood cell depletion. Future studies would test the device in *in vivo* experiments to isolate e+FNRBCs from maternal blood, but in fact the size-based microfiltration device design could be exploited for the separation of other large and less deformable cells that circulate in patient blood such as circulating tumour cells.

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