Microfluidic device capable of medium recirculation for non-adherent cell culture

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(Received 17 June 2013; accepted 4 February 2014; published online 25 February 2014)

We present a microfluidic device designed for maintenance and culture of non-adherent mammalian cells, which enables both recirculation and refreshing of medium, as well as easy harvesting of cells from the device. We demonstrate fabrication of a novel microfluidic device utilizing Braille perfusion for peristaltic fluid flow to enable switching between recirculation and refresh flow modes. Utilizing fluid flow simulations and the human promyelocytic leukemia cell line, HL-60, non-adherent cells, we demonstrate the utility of this RECIR-REFRESH device. With computer simulations, we profiled fluid flow and concentration gradients of autocrine factors and found that the geometry of the cell culture well plays a key role in cell entrapping and retaining autocrine and soluble factors. We subjected HL-60 cells, in the device, to a treatment regimen of 1.25% dimethylsulfoxide, every other day, to provoke differentiation and measured subsequent expression of CD11b on day 2 and day 4 and tumor necrosis factor- α (TNF- α) on day 4. Our findings display perfusion sensitive CD11b expression, but not TNF- α build-up, by day 4 of culture, with a 1:1 ratio of recirculation to refresh flow yielding the greatest increase in CD11b levels. RECIR-REFRESH facilitates programmable levels of cell differentiation in a HL-60 non-adherent cell population and can be expanded to other types of non-adherent cells such as hematopoietic stem cells. © 2014 AIP Publishing LLC.

[<http://dx.doi.org/10.1063/1.4865855>]

I. INTRODUCTION

Microfluidic technology has evolved over recent years to engender an assortment of advanced microfluidic systems, including those capable of non-adherent cells culture.¹ Within some microfluidic devices, cells are entrapped in hydrogels that permit efficient nutrient

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transport and whole cell and extracellular matrix contact.² Some of the non-adherent microfluidic systems permit the biological analysis of cells in suspension, through use of various techniques including electrokinetics,³ lasers (including optical tweezing),^{4,5} acoustics, and magnetic fields.^{5,6} Other microfluidic systems can be used doubly to culture either adherent or non-adherent cells by exploiting confinement methods, such as chemical surface patterning,⁶ geometric arrays,^{6,7} and hydrodynamics.^{5,6} In some non-adherent hydrodynamic microfluidic systems, cells are segregated from mainstream flow and confined to a region positioned below, inside, or abutting the main channel.^{5,6,8,9} Such systems incorporate geometric traps,⁷⁻⁹ which may be combined with numerous flow modes to direct cells into traps.¹⁰⁻¹²

A useful feature absent from the aforementioned microfluidic systems is the ability to recirculate medium over confined non-adherent cells. Here, we detail a microfluidic device (coined RECIR-REFRESH) that uses a hydrodynamic trap, specifically a cell culture well, to confine cells, while enabling automated flow modes for cell seeding, medium recirculation, and medium refresh sequences using Braille actuation pumping.^{13,14} Braille actuation pumping gains primacy as a facile handheld platform for biological applications, including alignment of endothelial cells with flow,¹⁵ stretching cells to generate mechanotransduction,¹⁶ quantifying embryo metabolism,¹⁷ measuring real time oxygen dissolved content and its effect on cells,¹⁸⁻²⁰ and flow cytometry.²¹ Use of Braille actuated perfusion allows us the unique opportunity to not only program numerous flow schemes on this platform but to also gain a better understanding of how such schemes affect cells. We expand the utility of the system by presenting it as a standalone means to apply numerous flow scheme permutations to perturb biological behavior of non-adherent cells. In our preliminary experiments with mouse primary hematopoietic stem cells (HSCs), we demonstrated significant expansion of these cells in the RECIR-REFRESH device, without the presence of any extracellular matrix or feeder layers (Supplementary Figure S8).²²

To demonstrate the utility of this device for another non-adherent cell type, we characterized the effects of varying flow schemes on the differentiation of the HL-60 cells, a common non-adherent cell line model for acute myelocytic leukemia.²³ Previous studies have demonstrated that the HL-60 cells exhibit bi-potentiality and are therefore capable of differentiating into granulocytes, monocytes, and macrophages.²⁴ Dimethylsulfoxide (DMSO), trans retinoic acid, 1,25-dihydroxyvitamin D3, and 12-O-tetradecanoyl phorbol-13-acetate, among other agents are commonly sought as cancer therapeutics due to their ability to invoke differentiation of leukemia like cells into benign mature cells.²⁵ In this report, we showcase the differentiation of HL-60 cells with DMSO over 4 days of culture and observe the changes in their differentiation ability based on two fluid flow modes: medium recirculation or refresh. We perform cell-based assays for autocrine buildup (tumor necrosis factor-alpha, TNF- α) and differentiation ability (CD11b expression) on the chip.

Our previous computational fluid modeling suggests that medium recirculation can bolster cell signaling^{13,14} through the concentration of autocrine factors.¹³ Also, it was shown that medium recirculation can lead to nutrient depletion concomitant with enhancement of autocrine factors. The degree of nutrient depletion versus the autocrine factor enhancement can be controlled by setting the fraction of the flow which is redirected from the outlet of the cell containing region back to the inlet of the channel.¹³ Here, we have characterized the convective-diffusive phenomena of the chemical factors in the cell culture well during different flow conditions using both experimental methods and computer-aided simulations.

II. EXPERIMENTAL PROCEDURES

A. Materials

1. Cell culture

The following reagents were required for cell culture: Iscove's Modified Dulbecco's Medium (ATCC 30-2005), antibacterial-antimycotic (Invitrogen 15240-062), fetal bovine serum (Invitrogen 10438-026), 0.25% trypsin-EDTA (Invitrogen 25200-072), dimethylsulfoxide, and DMSO (ATCC, 4-X).

2. Immunocytochemistry

The following reagents were needed for immunocytochemistry: Anti-TNF- α (rabbit polyclonal, Assay Biotech C10265), CD11b/Mac-1-PE (Mouse IgG1, BD Pharmingen 555388), secondary antibody goat-anti-rabbit AlexaFluor488 (Invitrogen A11008), Hoechst 33342 (Invitrogen H1399), bovine serum albumin, BSA (Invitrogen A1470), and phosphate buffered saline, PBS (Invitrogen 10010-023).

3. Device fabrication

The following materials were purchased for device fabrication: Polydimethylsiloxane (PDMS) elastomer and curing agent (Sylgard 184, Dow Corning), epoxy resin and hardener (CYTEC, FR-1080), Photoresist SU-8 2075 (MicroChem), hypodermic needles, 25 G $\times$ 5/8 in. (BD, 305122), sealing tape (Nunc 250050), microscope slides, 50 $\times$ 75 mm (Corning 2947-75X50), rectangular cover glasses, 24 $\times$ 40 mm (Fisher, 12-545-D) and 48 $\times$ 65 mm (Fisher, 3335), round cover glass, 5 mm diameter (Warner Instruments, CS-5R 64-0700), biopsy punch, 4 mm and 5 mm diameter (Miltex, 33-38; 33-34), biopsy punch w/plunger, 0.75 mm, 1.5 mm and 2 mm diameter (Harris Unicore, 15075; 15075; 15076), scalpel (Fisher, 08-927-5C), razor scraper (Titan 12037), and (Tridecafluoro-1,1,2,2-Tetrahydrooctyl)-1-Trichlorosilane (United Chemical Technologies, Bristol, PA, T2492).

B. Methods

1. Microfluidic chip design

The device comprises of a medium reservoir, with port that allows access to a funnel inlet and outlet channel openings, and it is positioned atop a PDMS base with an underlying channel network containing an embedded cell culture well. The design of the microchip is illustrated in Figure 1, which provides a backside view of the device, including all film backing and device components existing beneath the microchannel network, and a cross section view, near the reservoir region and of the cell culture well. The width and height of the microfluidic channel network are 300 μ m and 30 μ m, respectively.

Fluid actuation was mediated through Braille display platform,¹⁴ a custom-made device containing two Braille cells (Braille cell is a module for one programmable Braille character and it has eight individual pins) (SC9, KGS, Saitama, Japan). The Braille cells were controlled with a computer via Universal Serial Bus (USB). The microfluidic bioreactor chip interfaces with the pin actuator module when the microchip is clamped so that its channels align over the pins, which can push upward, closing the channel. The pin movements for valving (raised pin) and pumping (moving pin) were controlled with a custom computer program written in C#.

The microfluidic chip rests on a metal fingerplate that exposes braille pins from the underlying Braille cell, while the power supply and circuit board are encased in a detached box. Channel regions above the braille pins have a bell-shape cross sectional profile that allows them to conform to the shape of the Braille pins.²⁶

Medium flow status and patterns (seeding, recirculation, and refresh) as well as the location of Braille pins are all detailed in Figure 2. There are three flow schemes: (1) refresh, in which fresh medium from a reservoir is continually passed over cells, (2) recirculation, wherein a circulating loop of medium is passed over cells in the cell culture well, and (3) recirculation-refresh, in which there is an alternation (1:1 used here) of both flow patterns. During the seeding mode, cells are steered from the seeding funnel to the cell culture well. Pins along this path are depressed to prevent trauma to cells, and therefore, there is pseudo passive flow in this mode. The recirculation setting guides medium from the cell culture well through a continuous channel loop that covers the two inner rows of pins. The refresh mode directs medium from a pool contained in the medium reservoir through the outer channel network, starting from the inlet, passing through the cell culture well, and ending at the outlet. The reader is directed to

FIG. 1. Microfluidic device design overview. The front view of the actual microfluidic chip is shown in (a). The positions of individual components are detailed through schematics of the backside (b) and cross section (c) views of the chip. A superimposed cross-section of the device is created from cross-sections taken through both the medium reservoir (a') and cell culture well (b'), at positions shown in (a).

Supplementary Figure S1 to view the detailed pumping sequence of pins for each flow pattern.²²

2. Microfluidic chip preparation

The negative mold for microfluidic channel features, containing cross-sections with both straight and bell-shaped sidewalls (to conform to braille pins), was fabricated as previously described.^{14,26} Briefly, a $30\text{ }\mu\text{m}$ layer of SU-8 2075 photoresist was spun onto a cover glass ($75\text{ mm} \times 50\text{ mm}$), dried on a $95\text{ }^{\circ}\text{C}$ hot plate, sandwiched with aligned top and bottom photo-plotted film masks (20 000 dpi, CAD/Art Services) that defined the channel segments having rectangular sidewalls and smooth sidewalls, respectively. The film-SU-8-cover glass-film layers were further sandwiched between two glass slides. The topside was exposed to collimated 365 nm UV and the backside diffused 365 nm UV. The exposure doses for topside and backside exposure were 8 mW/cm^2 and 1 mW/cm^2 . The exposed SU-8-coated cover glass was post-exposure-baked and developed according to manufacture instructions. Chemical vapor deposition of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane was used to facilitate de-molding. The cover glass with SU-8 channel features was reinforced by attaching to a microscope slide with double-sided tape, and then the microscope slide to the base of a square Petri-dish.

PDMS prepolymer was prepared from a mixture containing 10 parts elastomer and 1 part hardening agent and cured under the indicated conditions. The FR-1080 epoxy mixture, used to create replica molds, was comprised of resin and hardener in a ratio (wt./wt.) of 100:83, and stored at $4\text{ }^{\circ}\text{C}$ until all the mixture was degassed (usually occurs in 24 h).

FIG. 2. Medium flow schemes. (a) Schematic of channel network positioned over braille pins, with relative location of funnel, outlet, and cell culture well indicated. (b) Illustration of seeding, recirculation, and refresh flow modes achieved with indicated pin positions. The movement of medium can be restricted based on pin configurations.

Small and thin accessory PDMS components were prepared prior to bulk device assembly: PDMS membrane, doughnut shape well spacers, reservoirs, and reservoir top covers. PDMS films (200 μm thickness) were prepared by curing (120 $^{\circ}\text{C}$, 2 h) PDMS prepolymer mixture spin coated onto a silanized glass slide. Doughnut shapes (5 mm outer diameter and 750 μm inner diameter) were perforated from the PDMS film. Epoxy negative molds of a reservoir with a funnel were replicated from a master fabricated with conventional machining using two-hold casting of PDMS and epoxy. Top covers comprised 2 cm $\times$ 3.5 cm $\times$ 3 mm (l $\times$ w $\times$ h) PDMS slabs with centered holes or access ports (4 mm diameter). Channel feature layers and reservoir layers were fabricated by casting of PDMS against epoxy negative molds. For expedited fabrication, several epoxy negative channel mold copies of the original SU-8 negative channel master mold were fabricated by a reverse replica molding technique.

Serial alignment was used to join all individual PDMS components, and the detailed fabrication sequence is illustrated in Supplementary Figure S2.²² Briefly, PDMS prepolymer was used to attach a medium reservoir to the face of a 2 mm-thick PDMS base (cured 2 h at 60 $^{\circ}\text{C}$) opposite the face containing channel features. Holes were pierced in the reservoir at the funnel tip (1.5 mm diameter) and outlet (2 mm diameter), and through the opposing channel side of the PDMS construct. A hole (0.75 mm diameter) was also perforated in the designated well region of the PDMS membrane, where it would add a height of 200 μm to the well. Plasma oxidation was used to seal the PDMS film against channel network. Construction of the well was completed by using corona to sequentially attach a spacer, providing an additional 200 μm of wall height, and a round cover glass (5 mm diameter), serving as a base, center aligned below the well opening in the PDMS membrane. The PDMS chip was heated at 120 $^{\circ}\text{C}$ for 1 min to facilitate bonding and allowed to cool for 1 min. Distilled water was introduced into the channel outlet until the entire channel network was primed. Corona was used to affix a top cover to the ledges of the medium reservoirs, and the device was allowed to stand at room temperature for 30 min to enable complete bonding. The reservoir was filled with water and then the finished chip was sterilized with 256 nm UV for 30 min. Sterilized devices were stored at 4 $^{\circ}\text{C}$ to minimize evaporation.

As a barrier against medium evaporation through the thin PDMS film, a layer of multiwell plate adhesive sealing tape, with a circular opening to fit around the well, was added to the bottom of the PDMS film. The extra 150 μm of thickness from this film does not hinder proper valving of Braille pins.^{15,19} Assembled devices and cover glasses (24 mm $\times$ 40 mm) were UV sterilized for 60 min. Following cell loading, a cover glass was sealed against the top cover to guard against contamination. Troubleshooting techniques for device construction and handling are provided in Supplementary Table S1.²²

3. Braille pumping

To generate the desired sequences of cell seeding and media recirculation and refreshing patterns shown in Figure 1(c), we used custom software (Supplementary Figure S3)²² in C# to control and interface with multiple Braille hardware (and thus multiple experiments and parameters) from a single laptop simultaneously. Our interface allowed us to select among various ratios of recirculation and refresh (1:1 alternation of recirculation to refresh time, 1:5, 5:1, 25:1, 50:1 as well as pure recirculation and pure refresh) and control the timing between pin movements in peristaltic Braille pumping and, therefore, control the length of each recirculation or refresh phase. *Note:* For our experiments, we used the 25:1 recirculation:refresh ratio as a pure recirculation mode to prevent medium from drying out in the microchannel network.

Owing to the inherent design of the Braille platform, the microchip does not warm up due to power dissipation. Most of power dissipation arises from the stand-by power of the High-voltage DC-DC converter on the controller circuit board. Any heat generated does not diffuse to the Braille display, which is comprised of pins aligned with a metal fingerplates that the microfluidic devices rests on.²⁷

4. HL-60 cell culture

a. Maintenance. HL-60 cells were maintained in Iscove's Modified Dulbecco's medium, supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic/antimycin, in an atmosphere of 95% air and 5% CO₂ at 37 °C. Two thirds of the medium was replaced with fresh medium every other day. To minimize unintended cellular differentiation during cultivation, cells were maintained at a concentration in the range of 1×10^5 cells/ml to 5×10^5 cells/ml.²⁸

b. Cell seeding and differentiation on chip. A 50 μl suspension of HL-60 cells (4×10^5 cells/ml) was added to the funnel of the microfluidic device. The device was clamped to the Braille platform, and the set-up was positioned on the "L" shaped microscope platform so that cells could be manually counted during live imaging. Braille program with pumping frequency of 100 ms/step was used to direct cells (250–300 manually counted) to the cell culture well, in a forward flow direction (inlet to outlet). Following seeding, medium flow was maintained at a Braille pumping frequency of 500 ms/step with the desired flow scheme, in a reverse flow direction (facilitates clearance of excess cells from microchannel), at 37 °C and 5% CO₂. Cells were then exposed to an initial 1.25% DMSO treatment on day 0 and a subsequent repeat treatment on days 2 and 4, to account for solvent degradation or absorption.

5. Immunohistochemistry

HL-60 cells were fixed at indicated time points and stained with antibodies specific for cluster of differentiation molecule 11b (CD11b), cell surface protein associated with macrophages and monocytes, or TNF- α , a pleiotropic inflammatory cytokine. All staining procedures were performed at room temperature using a 100 ms/step pumping frequency on the Braille platform. Cells were fixed by pumping ice-cold methanol (−20 °C) through the channels for 15 min. Methanol was displaced with a rinsing solution (PBS and 1% BSA) for 15 min. Blocking buffer (10% Normal Goat Serum + 1% BSA) was added to the channel and pumped overnight (not exceeding 15 h). Blocking buffer was displaced with the rinsing solution. To stain for CD11b, anti-CD11b conjugated to R-PE (R-Phycoerythrin, 1:100) was pumped through the

channels for 1 h and washed with PBS overnight. To stain for TNF- α , primary antibody, anti-TNF- α (1:50) was pumped through the devices for 1 h, followed with secondary antibody, goat-anti-Rabbit 568 (1:100) for an additional hour. The staining solutions were washed away with PBS overnight.

The fluorescent images were captured with SimplePCI software interfaced with a Nikon TE-300 microscope, outfitted with a CoolSnap HQ2 camera (Photometrics, Tucson, AZ). The images were analyzed with ImageJ software using custom javascript code to measure the average fluorescence intensity of each cell. Background removal and automated thresholding was applied to the original raw image to create a cell contour mask, which was then overlaid on the original image to exclude regions that did not contain cells, without regard to undesired fluorescence values from background artifacts.

6. Flow characterization

a. Device fluid flow velocity quantification. The fluid flow velocity was measured in the microchannels of the microfluidic device by using 6 μm fluorescent (emission 490–535 nm range) microsphere beads, which were pumped through the device at Braille pumping frequencies of 500, 1000, 1500, 2000 ms/step. A portion of the channel leading to the cell culture well was imaged in sequences acquired at 15 frames/s to determine the velocity of the microspheres that are representative of the fluid velocity for the aforementioned Braille pumping frequencies. A time-lapse series of approximately 10 min was collected for each flow rate. At each flow rate, the Braille pumping was equilibrated for 30–45 min before acquisition of data.

A Nikon TE 2000 microscope interfaced with a Hamamatsu CCD camera with an L-shaped platform, outfitted for the Braille display, was used for real time device analysis. Time-lapse images of microchannel regions were collected with Metamorph software and analyzed using the Manual Tracker plugin in ImageJ. For image processing in ImageJ, only microspheres located at the center of the channel were selected for analysis. A circular ROI and a centering correction using the local maxima were used to isolate each microsphere so that its displacement could be tracked through consecutive frames.

b. Simulation. To better predict the change in flow pattern and autocrine factor concentration within and near the cell culture well during the different modes of device operation, a 3-D finite element analysis software (COMSOL Multiphysics with the Incompressible Navier-Stokes module and the convection and diffusion module) was used to analyze the fluid dynamics and diffusive/convective behaviors of the chemical factors in the media. A cross-sectional view close-up of the cell culture well, depicting channel height and spacer and membrane thickness, which supply additional well height, is provided in Figure 3(a). Figure 3(b) shows the meshed geometry of the analysis model based on the cell culture well size of 750 μm -diameter and 400 μm -height. The 90° turns of the microchannels at the inlet and outlet sides of cell culture well are included in the analysis model geometry to analyze the effect of turning flow near the entrance and exit of the cell culture well, especial for larger well designs. The extended lengths (3000 μm) of the microchannels beyond the 90° turns are placed to account for any possible entrance effect at the model flow boundaries. The ratio between entrance length and local channel width or height for fully developed laminar flow is proportional to Reynolds Number. The low Reynolds number nature of this microfluidic device (ranging from 0.005 to 0.05) indicates that the entrance length would be significantly less than the local channel width (300 μm), thus justifying the analysis model geometry. In simulation, the applied velocity flow boundary condition at the inlet is the uniform velocity based on the observed average speeds of the microspheres under different pumping conditions. The outlet boundary condition is based on the assumption that the fluid flow exits freely out of the analysis area into a non-pressurized region such as an open reservoir.

III. RESULTS AND DISCUSSION

There are several demonstrations in literature that explore mechanisms by which flow can alter biological profiles of cells,^{29–34} including shear induced cell aggregation of suspended

FIG. 3. Model of cell culture medium velocity profile near and in the cell culture well. (a) Close-up cross-section view of cell culture well region components, t = thickness, h = height. (b) Experimental measurement of average velocity of fluorescent microspheres (x axis, pumping frequency (s/step), y axis, velocity ($\mu\text{m/s}$)). (c) Overall model geometry with mesh (axis unit = mm). Cell culture well dimension: diameter = $750 \mu\text{m}$, height = $400 \mu\text{m}$. (d) Medium flow speeds and directions at various cross-sections of the microchannel and cell culture well (color scale units = m/s). Braille pump frequency = 500 ms/step (or 2 step/s); average inflow velocity = $210 \mu\text{m/s}$ based on microsphere flow observation. The arrows indicate the flow directions, not the speed magnitude. The local speeds are shown by color scales at selected cross-sections.

blood cells.^{31,32} However, there is a lack of the ability to assess how various flow regimes can perturb biological characteristics of cells confined while suspended. Taking into consideration that the body contains dynamic fluid-cell interactions, including regional specific velocities,³⁵ fluid-cell studies would benefit from an efficient system to program varied flow patterns that yield a unique presentation of soluble factors to non-adherent cells. A presentation of convective-diffusive phenomena of the chemical factors in the cell culture well of the RECIR-REFRESH device, along with an example of device utility using HL-60 cells, follows.

A. Model characterization of medium flow velocity and soluble factor concentration

First, we investigated the velocity distribution around and in the cell culture well. By tracking displacement of fluorescent microspheres along the microchannel, we obtained an average velocity of the fluid flow in the channel (Figure 3(c)). Figure 3(c) shows the result of the device fluid flow velocity quantification described in Sec. II B 6 a, which details quantification of the

average fluid speed in the microchannel network using different Braille pumping rates. This result was used as the basic input boundary condition for the simulations. The average velocity (Braille fluid actuation capability) at the pumping rate of 1000 ms/step (1 Hz) was found to be $80 \pm 14 \mu\text{m/s}$, and this value was interpreted as average flow velocity in the microchannel. The flow velocity at pumping rate of 500 ms/step was chosen as the inflow boundary condition of the model simulation in order to be consistent with the pumping condition used for cell culture experiments. Figure 3(d) shows the computed velocity distribution, from COMSOL model analysis, in and around the cell culture well (diameter = $750 \mu\text{m}$ and height = $400 \mu\text{m}$) using the Braille pumping frequency of 500 ms/step that resulted in an average fluid inlet velocity $210 \pm 112 \mu\text{m/s}$. The highest standard deviation of $112 \mu\text{m/s}$, resulting from the 500 ms/step pumping rate, may be due to errors in identifying the true position of the microsphere. As expected from continuity equation, we found that fluid flow velocities are dramatically reduced upon entrance to the cell culture well, due to the large cross-section area of this region in comparison to the inlet rectangular channel. The media flow velocity, within the confines of cell culture well, is one to two orders of magnitude below the average fluid speed in the surrounding microchannel network for a majority of the well volume. Supplementary Figures S4A and S4B²² further express this great reduction in flow speed once the fluid enters the cell culture well. This implies that cells that enter the well would tend to reside in the well, as the fluidic drag force pushing the cell toward the well exit dramatically reduces in the well, given that drag force, at the low Reynolds Number creeping flow, varies proportionally with velocity. This is an important and advantageous characteristic of this novel device. The reduction in flow speed and the geometry of the cell culture well effectively enable cell-trapping in the well region for the non-adherent cell types. Simulation results revealed that with increasing dimensions of the cell culture well, an increasingly larger region of extremely low speed exist within the cell culture well, which enhances cells trapping. The same flow simulations also indicated that the depth of the cell culture well should not be overly shallow. A cell culture well with sufficient depth will compel the flow stream just entering the cell culture well to have a downward direction, as seen in Figure 3(d), which in turn drags the suspended cells entering the well downward, while still at relatively high entrance speed, toward the bottom of cell culture well and into the extreme low speed, thus low drag force region, thereby enhancing cell-trapping.

Second, we assessed how refresh flow modes would change the levels of these autocrines and soluble factors in the cell culture well. We used 40 kDa FITC-dextran molecules as a model for autocrine and soluble factors for our simulation, and to simplify analysis, we disregarded uptake of the autocrine/soluble factors by the cells. Figures 4(a) and 4(b) illustrates the change of FITC-dextran concentration in the cell culture well ($750 \mu\text{m}$ diameter and $400 \mu\text{m}$ height) over time during the refresh flow operation mode. The diffusivity of FITC-dextran is estimated to be $4.6 \times 10^{-11} \text{ m}^2/\text{s}$, based on molecular weight of 40 kDa and average inflow velocity of $210 \pm 112 \mu\text{m/s}$. The PDMS channel walls are assumed to be adequately blocked by the serum contained within the cell culture medium, and therefore, any amount of FITC-dextran that may absorb into the PDMS walls is considered to be negligible. As time progresses, the concentration of FITC-dextran in the cell culture well decreases in a disproportionate manner throughout the cell culture well. Simulations reveal that factor dilution time varies height-wise along the center of the well. At the bottom center of the cell culture well (height = $0 \mu\text{m}$), it took over 900 s for the FITC-dextran to be diluted from the original concentration of $0.625 \mu\text{M}$ to nearly $0 \mu\text{M}$. As height, starting from the bottom center of the well, increases to midway up the well (height = $200 \mu\text{m}$), clearance time is roughly 300 s. Due to the downward curvature of the flow streamline in the well, there is a slower flow speed region near the base and top of the well (shown by the darker blue regions in Figure 3(d)). This flow speed contributes to a slower washout rate and higher concentration of soluble factor (Figure 4(a)) at the base of the well. For non-adherent cells that have settled to the bottom of the well, it is essential to know what concentration of soluble factors could be achieved as a function of time, position in the well, and operating modes. The concentration and dilution of the autocrine/soluble factors in the culture well are a function of properties of factors under study, pumping speed, and the size of the

FIG. 4. Model of soluble factor concentration in vicinity of the cell culture well. (a) Cross sectional view of the change of FITC-dextran concentration in the cell culture well over time during refresh mode operation. Starting concentration is $25 \mu\text{g/ml} = 0.625 \mu\text{M}$. Unit of concentration color scale = mole/m³ (standard concentration unit for COMSOL software). $1 \text{ mole/m}^3 = 1000 \mu\text{M}$. (b) Change of FITC-dextran concentration at the center of the cell culture well with different heights (0, 100, 200, 300, and $400 \mu\text{m}$) from the bottom over time during refresh mode operation. The $0 \mu\text{m}$ height is at the bottom of the cell culture well, and the $400 \mu\text{m}$ height is flush with both the top of the well and the bottom of the inlet and outlet microchannels. Data curves for the heights of $200 \mu\text{m}$ and $300 \mu\text{m}$ nearly overlap. At these heights, the flow streamline has the highest speed (lighter region Fig. 3(d)), and the washout rate is the quickest (Fig. 4(a)). Due to the downward curvature of the flow streamline in the well, there is a slower flow speed region near the base and top of the well (darker region Fig. 3(d)), resulting in a washout rate that is slower (higher concentration of soluble factor) at the height of $400 \mu\text{m}$ than the heights of $200 \mu\text{m}$ and $300 \mu\text{m}$, for most of the simulation period.

culture well. The correct time-scale of chemical transport rate is essential in determining the time period of device operation modes during the non-adherent cell culturing experiments so that the cells can be exposed to the desired chemical environments for the preferred amount of the time. In this case, to completely washout the existing FITC-dextran concentration in the culture well using refresh-mode, it would take more than 15 min (900 s). Also, despite the chosen flow mode, due to distribution of medium volume throughout the device (Supplementary Figure S5),²² the medium in the reservoir is always considered “fresh,” since factors returning to the reservoir have a negligible impact on total concentration.

Third, we determined how changing the cell culture well geometry (diameter) affects autocrine/soluble factors in the cell culture well, while operating the device under the refresh flow mode. Supplementary Figure S6²² shows the simulation comparison of the wash-out of FITC-dextran from cell culture wells with various diameters. We disregard the low diameter to depth ratio ($<2:1$, $500 \mu\text{m}$ diameter with $400 \mu\text{m}$ depth) case where the flow streamlines are suppressed toward the higher portion of the cell culture well, thus causing a stagnant region on the lower part of cell culture well that inhibits convective factor transport. The simulation data reveal that increasing cell culture well diameter would demand longer times to wash out soluble factors. This trend indicates that if a rapid response in autocrine/soluble factor concentration is desired in the cell culture well during a specific device operating mode, the size of the cell culture well should be limited.

The size of cell culture well impacts the time needed to stabilize the concentration level of various factors when switching between different flow operating modes that change the inlet/outlet conditions of cell culture well. The aforementioned slowing of the liquid speed in the cell culture well, especially at the bottom of well where most the cells would settle, indicates that diffusion plays a key role in transport of autocrine and soluble factors. This phenomenon of more significant diffusion transport compared to convective transport near the bottom of the culture well is further illustrated in Supplementary Figures S4C, S4D, and S4E.²² The size of the cell culture well directly impacts the concentration gradient, which serves as the driving

force of diffusion transport of autocrine and soluble factors. In consideration of the above observations, the chosen dimensions for the cell culture well (respective diameter and depth of 750 μm and 400 μm) are considered to achieve a good balance between tempered flow and adequate concentration of autocrine/soluble factors.

B. Using RECIR-REFRESH to differentiate HL-60 cells

Differentiation has been modeled as a genetic network composed of several stimuli guided biological trajectories that converge or diverge to specific attractors, or steady state conditions for a particular cell fate. Given that cellular differentiation is a multiplex process comprised of a meshwork of signaling pathways, determining the conditions for HL-60 differentiation would benefit from more advanced analytical techniques.^{36,37} We utilized RECIR-REFRESH to observe the changes in the differentiation ability of HL-60 cells that were exposed to unique recirculation or refresh flow modes. Cells were exposed to an initial 1.25% DMSO treatment on day 0 and a fresh treatment, to account for solvent degradation or absorption, subsequently on days 2 and 4. We quantified HL-60 cellular differentiation sensitivity by monitoring the expression of two biological factors, CD11b and TNF- α .

The cell surface differentiation antigen CD11b has been well established as a marker of general differentiation of the HL-60 cells,^{25,38–40} although some studies suggest CD11b is specific to the macrophage differentiation pathway.³⁸ The expression of CD11b antigen is commonly used as a hallmark to quantify the level of leukemia cell differentiation. We have assessed levels of CD11b expression by measuring total fluorescence from CD11b specific fluorophore conjugated antibodies. The levels of CD11b expression doubled for the refresh and 1:1 recirculation-refresh flow mode and increased by half for recirculation flow mode. On day 4, the 1:1 recirculation-refresh flow scheme yields levels of CD11b expression that are about a fourth greater than that from the refresh mode, and 1:1 recirculation-refresh displayed the greatest increase in CD11b expression. Our data do suggest that CD11b expression in HL-60 cells is sensitive to different medium flow schemes (Figure 5). Comparing these results to those from simulations (see Supplementary Figure S7),²² it becomes clear that during the 1:1 recirculation-refresh flow mode, the wash out of the autocrine factor is balanced by the incoming nutrient stream, creating an optimal condition for HL-60 differentiation. In fact, for this reason, we chose to operate the device under the 1:1 recirculation-refresh flow mode for expansion of HSCs over a 12 day time period, to optimize the balance between the secreted factors produced by the hematopoietic cells and the fresh nutrients from the serum free defined medium (Supplementary Figure S8).²²

When HL-60 cells are treated with TNF- α , a pleiotropic cytokine, their growth is suppressed and they are pushed towards monocytic pathway differentiation.^{41–44} In concert with DMSO, TNF- α demonstrates a synergic effect on HL-60 cell differentiation through the activation of ERK pathway.⁴⁴ However, TNF- α is also an autocrine produced by HL-60 cells, and these cells express high-affinity receptors for TNF- α .^{43,44} Endogenous TNF- α release from HL-60 cells can be induced by treatment with TNF- α , interferon-gamma (IFN), and lipopolysaccharides (LPS), either in combination or alone.⁴³ In this report, we chose to observe and quantify the TNF- α autocrine levels produced by HL-60 cells while undergoing DMSO induced differentiation in 3 different flow modes, without potentiating TNF- α release via any agents such as IFN, LPS, or TNF- α . We observed no statistically significant difference in the three distinct fluid flow modes when comparing the TNF- α fluorescence on day 4 from the DMSO induced differentiated HL-60 cells. This might be due to any of the following three reasons: (1) our ability to detect TNF- α might be limited with fluorescence imaging as compared to an ELISA assay, (2) given that HL-60 cells express high affinity receptors for TNF- α , the autocrine released TNF- α might have been taken up by the HL-60 cells immediately prior to imaging, or (3) without the presence of an inducing agent, HL-60 cells might produce low levels of endogenous TNF- α . Additionally, PDMS is known to uptake autocrine and soluble factors,⁴⁵ which poses a significant problem with use of PDMS devices for biological applications. However, in these experiments, we compare the experimental conditions against control

FIG. 5. HL-60 differentiation in the RECIR-REFRESH chip. (a) Representative fluorescent images of antigen CD11b and (b) antigen TNF- α . The relative fluorescence intensity is shown for antigens (c) CD11b and (d) TNF- α . For the control groups on day 0, cells were fixed immediately following seeding. For CD11b expression, symbol ($\ddagger$) signifies that the mean CD11b expression for the flow mode is significantly different from both the mean CD11b expression of the identical flow mode on day 2 and of the day 0 control. For TNF- α expression, ($\ddagger$) indicates that the flow mode mean is significantly different from the mean of the day 0 control. $p > 0.05$. Data from each group were taken from at least 3 separate devices ($n \geq 3$). Significant differences between group means were determined with one way ANOVA and Tukey's post hoc tests. Scale bar = 50 μm .

experiments, which were also conducted within the PDMS devices. If a fraction of the auto-crine factors are absorbed into the PDMS, the response observed from the cells as result of autocrine or soluble factor signaling could be skewed.

Interestingly, our data do reveal a similar trend on day 2 for CD11b expression, as on day 4 for TNF- α expression, with levels of expression increasing for the flow modes in respective order, refresh, 1:1 recirculation:refresh, and recirculation. This observation could evidence synergistic activity between CD11b and TNF- α . Other researches, making use of flow cytometry techniques, reported a total of 30% CD11b positive cells on day 2 as compared to less than 5% on day 0,^{44,46} using a DMSO inducer, CD11b expression increased further with a joint dose of DMSO and TNF- α .⁴⁶ Further experiments must be conducted to determine synergistic expression of CD11b and TNF- α in response to varied flow schemes.

IV. CONCLUSION

We have fabricated a Braille actuation pump-driven microfluidic device that enables recirculation and refresh sequences of medium perfusion, thereby facilitating programmable levels of cell differentiation in a non-adherent cell population. Imaging of the cells is a simple process, as the cell culture well contains a transparent base. Further, cells may be harvested in a single step by removing the cover slip forming the base of the cell culture well. We

demonstrated that HL-60 cells display a differential expression of CD11b with different flow schemes. The ability to achieve recirculation of medium in the device also ensures a sufficient concentration of TNF- α and other autocrine factors necessary for cell proliferation. The utility of this device can be expanded to determine the impact of fluid flow variation on autocrine regulation and/or differentiation of other non-adherent cells, aside from HL-60 cells. Our preliminary findings, detailed in the supplementary material (Supplementary Figure S8 and Experimental Procedures Section 3),²² indicate that RECIR-REFRESH can provide ideal cell culture conditions for promoting efficient expansion of fluorescence-activated cell sorting (FACS)-isolated murine HSCs and their subsequent harvesting from the device for downstream assays.²² This system can not only be utilized to program flows characteristic of native niches but also program cellular response, which has implications for guiding cell behavior towards a desired response (i.e., pure stem cell population). Modeling complex biological flow patterns and observing the resulting modulation of cell behavior demands new tools of this complexity, and this device fits the bill. We expect that in the future, this device can be used to explore the impact of fluid flow variation on autocrine regulation and/or differentiation of other non-adherent cells.

ACKNOWLEDGMENTS

We acknowledge the support of the Department of Biomedical Engineering and the University of Michigan (U-M) Undergraduate Research Opportunities Program (UROP). R.W., a UROP research scholar, is grateful for support from UROP Biomedical and Life Sciences Summer Fellowship Program. We are grateful to former UROP research scholars, Jane Xiao, Elisa Quiroz, and Kim Jun Young (visiting scholar) for their contributions towards device fabrication. We are thankful to David Lai for assistance with photolithography. We thank Brian Johnson and Professor Mark Burns (Department of Chemical Engineering, U-M) for use of their clean room facilities. This material is based upon work supported by the seed funding from the U-M Office of Research at the University of Michigan (G.M.), and by NIH Grant No. GM096040 (S.T.).

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