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Automated Microfluidic System for Dynamic Stimulation and Tracking of Single Cells

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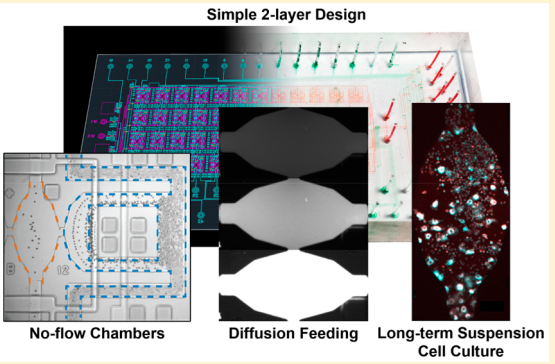
Supporting Information

ABSTRACT: Dynamic environments determine cell fate decisions and function. Understanding the relationship between extrinsic signals on cellular responses and cell fate requires the ability to dynamically change environmental inputs in vitro, while continuously observing individual cells over extended periods of time. This is challenging for nonadherent cells, such as hematopoietic stem and progenitor cells, because media flow displaces and disturbs such cells, preventing culture and tracking of single cells. Here, we present a programmable microfluidic system designed for the long-term culture and time-lapse imaging of nonadherent cells in dynamically changing cell culture conditions without losing track of individual cells. The dynamic, valve-controlled design permits targeted seeding of cells in up to 48 independently controlled culture chambers, each providing sufficient space for long-term cell colony expansion. Diffusion-based media exchange occurs rapidly and minimizes displacement of cells and eliminates shear stress. The chip was successfully tested with long-term culture and tracking of primary hematopoietic stem and progenitor cells, and murine embryonic stem cells. This system will have important applications to analyze dynamic signaling inputs controlling fate choices.



Quantitative continuous time-lapse imaging of live cells can be used to learn about the status and cell fate decisions of individual cells[1−6] and is routinely performed on environmentally controlled microscope setups.[2,7,8] A challenge in the field is the application of dynamic changes to the culture environment (e.g., transient or oscillating cytokine stimulation) while maintaining cell identity, especially when studying nonadherent cells during long-term differentiation experiments.[1,2] Microfluidic devices—especially large-scale-integrated-microfluidics (LSIM)[9]—permit users to automate cell culture and eliminate manual steps to alter cell culture conditions.[10−13] LSIM permits direct intervention with the chemical composition of the culture media in a programmable and automated fashion without interruption of time-lapse imaging.[14−17]

To date, most of such microfluidic systems rely on miniaturized flow chambers[18,19] in which media exchange occurs through fluid flow over the cells. This is not suitable for nonadherent cells, which would be readily displaced during each media change, rendering culture and continuous time-lapse imaging of these cells in most LSIM devices impossible.[20] Few systems have been recently reported that attempt to overcome this challenge, for instance, by using flow traps or microwells.[21,22] In one approach, which is based on a complex five-layer polydimethylsiloxane (PDMS) chip assembly, cells are seeded into hundreds of individual chambers and can be subsequently overflown by different culture media.[23] Other three-dimensional (3D) culture devices, which capture cell colonies in an array of gels during culture, have been implemented.[24] In addition, two-dimensional (2D) devices have been recently described that contain independent cell culture chambers and flow channels, connected by only small openings and provide a diffusive exchange of media.[25−27] In these cases, however, only a limited number of conditions can be tested per chip and the passive design of the devices may render fractions of the chambers unusable. Furthermore, individual wells often are not independently controllable for media exchange and seeding occurs randomly.

Here, we present a new microfluidic device that can be produced in a more robust manner while supporting controlled cell seeding, long-term cell expansion, and complex automated culture protocols. The cell culture device can control 48 miniaturized 2D cell culture chambers, independently and in a combinatorial fashion. Media exchange or chemical stimulation occurs in the absence of direct flow by means of diffusion and

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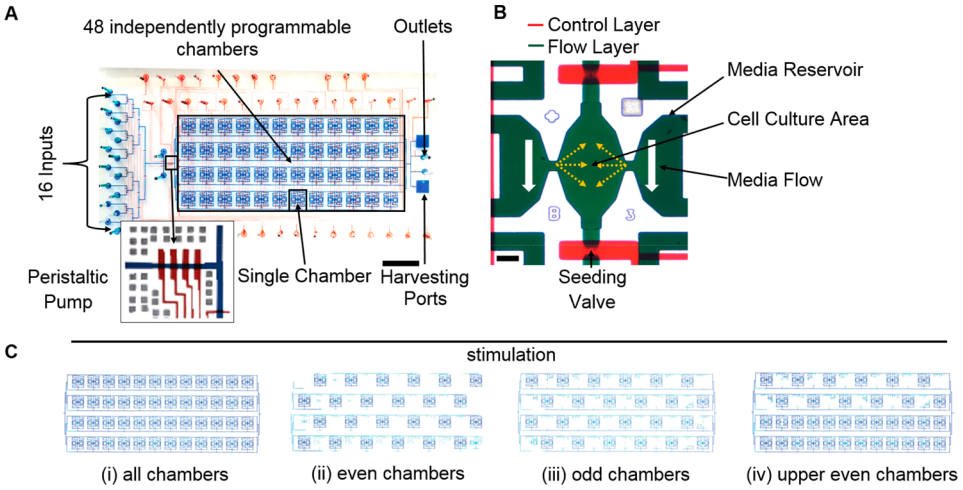

Figure 1. Design and operation of suspension cell culture chip. (A) Photograph of the two layer PDMS device filled with food dye. Red lines represent control lines operating the valves, and blue lines represent flow lines for media exchange and cell loading. The flow regime is from left to right. Sixteen (16) independent inputs are located on the left, while 2 outlets are on the right. A four-valve on-chip peristaltic pump allows for accurate fluid manipulation. The core of the device consists of 48 independent cell culture chambers. Scale bar = 5 mm. (B) Picture of a single cell culture unit. The cell culture area is placed in the middle. For cell seeding and surface functionalization, two inlet channels are placed for fast fluid exchange (on top/bottom). Two media supply channels are placed adjacent the chamber to maintain the steady-state concentration. Valves allow a flow-switching regime, to avoid cross-chamber flow. Diffusion occurs between media reservoirs and the cell culture area in order to establish equilibrium (yellow dashed arrows). Media flow is limited to areas within the media reservoirs (white arrows). Scale bar = 100 μm (C) In total, 48 diffusion chambers for suspension cell culture can be independently addressed. A representative sample sequence of different stimulation regimes is shown.

thus without interruption of information about cell identity. This system allows simultaneous dynamic stimulation and microscopy tracking of individual weakly adherent or non-adherent cells and is suited for culture conditions where shear flow across cells should be avoided. We provide proof-of-concept data for the use of fluorescent protein fusion reporters, allowing in situ readouts of individual cell states such as differentiation status and signaling dynamics in murine hematopoietic progenitor cells and murine embryonic stem cells.

MATERIALS AND METHODS

Chip Design and Fabrication. The two-layer PDMS chip was designed using AutoCAD (Autodesk, Inc., San Rafael, CA, USA). Photolithographic masks were printed with 40 kdpi resolution (Fine Line Imaging, Minneapolis, MN, USA) and mold structures were generated on 4-in. silicon wafers, as previously described.[15] The channel network had a height of 25 μm for both control and flow layers. All features were generated using SU-8 3025 (Microchem, Westborough, MA, USA), while we deposited and reflowed AZ-50XT (Merck KGaA, Darmstadt, Germany) at valve positions to produce parabolic surfaces. For molding, 66 g of RTV 615 PDMS (10:1; polymer: catalyst) (Momentive Performance Materials GmbH, Leverkusen, Germany) were cast on the wafer and the polymer was cured at 80 °C for 45 min. O_2 plasma treatment, alignment to the control layer, and bonding was conducted after inlet holes were punched into the cured flow layer. After 2 h of thermal bonding at 80 °C, we punched control layer inlets and plasma-treated the assembly to bond the device to a coverslip. Each fully assembled chip was then cured for an additional 12 h at 80 °C.

Chip Control and Setup. All chip experiments used microfluidic control manifolds based on the solenoid valve controlled system made available at https://sites.google.com/site/rafaelsmicrofluidicspage/home.[15] The valve closing pressure varied between 1.7 bar and 2.5 bar, while flow layer liquids were pressurized to 0.5 bar. A custom graphic user interface (GUI) for experimental scripting and control (available in the Supporting Information) was created using Matlab (Matlab, Inc.).

Environmental Control for Cell Culture. Environmentally controlled microscopes were used for chip operation. Microscopes were installed in custom built aluminum-slot frame boxes lined with black, foamed-insulation boards and maintained at 37 °C using a feedback-controlled heating unit (Life Imaging Services). Within a smaller stage-top enclosure (Life Imaging Services) humidity (>98%) and gas composition (5% CO_2, 5% O_2, 90% N_2, 30 L/h) for cell culture was maintained using roughly 100 mL of water absorbed by sodium polyacrylate (Sigma−Aldrich Corp.) to provide a reliable source of humidity for a 3-day-long experiment. A data logger (Sensirion AG, Zurich, Switzerland) monitored the relative humidity.

Microscopy and Image Data Analysis. Experiments were conducted on a Nikon Ti-eclipse inverted microscope that was equipped with an Orca Flash IV camera (Hamamatsu Photonics K.K.), using a 10× objective (NA 0.45) (Nikon Instruments Europe B.V.). Custom scripts were written for Youscope (www.youscope.org) Fiji and Matlab (Matlab, Inc.) to perform image acquisition and data processing procedures. Single-cell tracking procedures were performed using tTt.[28]

Diffusion-Based Cell Culture. We accomplished exchange of the media in the cell culture area by first flushing media reservoirs (Figure 1B) adjacent to each cell culture chamber, using unimpeded flow from the media input or modulated flow produced by the peristaltic pump (Figure 1A). To this end, we sequentially refilled channels to the left and the right of the cell culture chamber with fresh medium and let diffusion occur into the cell culture chambers in 30 min intervals.

Chip Validation. To ensure that, during media exchanges, no direct flow may enter the cell culture area, we experimentally confirmed the absence of flow in the culture chambers using polystyrene beads with a diameter of 10 μm (Spherotech, Inc.).

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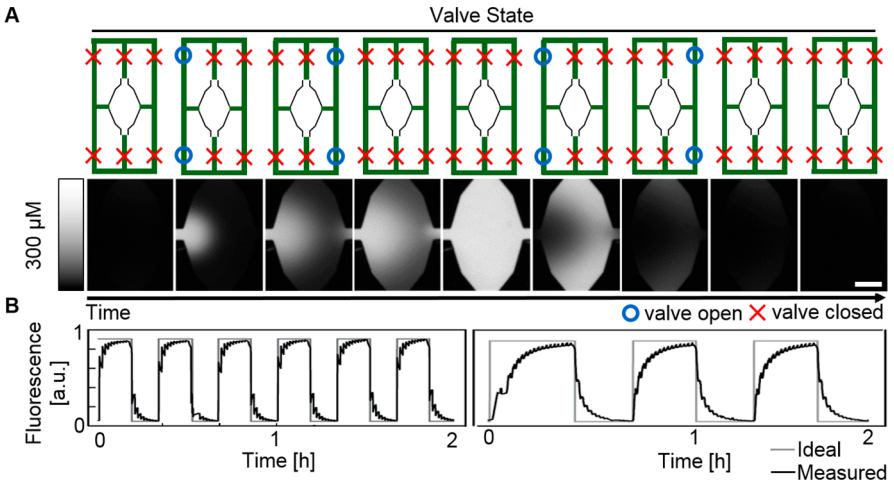

Figure 2. The suspension cell culture chip permits multiple stimulation and withdrawal patterns to be applied simultaneously across 48 chambers. (A) Oscillation pattern of Rhodamine−Dextran application and withdrawal. The top portion depicts the sequence of valve actuation to provide rapid media exchange by applying the flow switching strategy. Valves are operated to periodically alternate between opening left and right media supply channels. This prevents direct flow through the cell culture area. The seeding valves remain closed during culture. The bottom portion shows a typical sequence of a full cycle of flow switching to supply 1 of the 48 cell culture areas. Tiles are 15 min apart. Scale bar = 100 μm. (B) Rapidly oscillating rhodamine−dextran (40 kDa) patterns are created through sequential stimulation steps: (left) 20 min oscillations and (right) 40 min oscillations.

Beads were flushed into the device after treatment with 0.1% Triton-X detergent, to prevent bead aggregation. Diffusion rate and dynamic input calibration was conducted using a 40 kDa Rhodamine-conjugated Dextran (300 μM), which is comparable in size to low-molecular-weight proteins, such as cytokines. A single pulse of rhodamine-dextran was introduced into PBS-filled media reservoirs and the fluorescence signal was monitored.

Reagents, Surface Functionalization, and Cell Seeding. To set up chips for biological experiments, first, all excess air was forced out of the device by prefilling it with PBS and applying pressure via the liquid supply lines while leaving the outlet valves of the device closed. To ensure comparable culture conditions to normal cell culture vessels, biological functionalization of the flow channels was performed for 3 h or overnight with fibronectin for hematopoietic progenitors or E-Cadherin for murine embryonic stem cells, both at a concentration of 150 ng/mL (Merck KgaA, Germany). This step was followed by extensive washing with PBS and then cell culture media. Cells were seeded into the desired chambers by connecting a pressurized microcentrifuge tube to the first inlet via a 25-cm-long segment of PEEK tubing with an interior diameter of 65 μm. Using the peristaltic pump, deposition of a precise number of cells in individual chambers was possible.

Primary Murine Bone Marrow Cell Isolation. For experiments with primary murine cells, male C57BL/6J mice 12−14 weeks old, expressing enhanced green fluorescent protein (GFP) from the Lysozyme M gene locus (LysM-GFP),[29] were used in accordance with the ordinance provided by Canton Basel-Stadt and approved by the veterinary office of Canton Basel-Stadt, Switzerland (Approval No. 2655). Granulocyte and macrophage progenitor cells (GMPs) were defined as lin-/Sca1-/c-Kit+/CD34+/CD16/32+, as described previously.[7] Bones were dissected, pulverized in a 4 °C mortar, and strained through a 40 μm nylon mesh (Becton Dickinson AG, Switzerland). Following staining with anti-CD16/32 (BD2.4G2; BD Pharmingen, Switzerland) conjugated to AlexaFluor 700 (Fc-block), a lineage (lin) depletion step using biotinylated antibodies for CD3e (145-2C11), CD11b (M1/70), CD19 (1D3), CD41 (MWReg30), B220 (RA3-6B2), Gr-1 (TER-119), TER-119 (RA3−6B2), and Streptavidin-conjugated beads Roti-MagBeads (Carl Roth AG, Switzerland) was performed on an EasySep magnet (Stem Cell Technologies, USA). Cells were stained with fluorescently conjugated antibodies for Sca-1, c-Kit, and CD34 for 30−60 min and sorted on a FACS Aria (BD Biosciences). Cell culture media were composed of Stem-Span SFEM (Stem Cell Technologies), 1% penicillin/streptomycin, 10% FCS, with 100 ng/mL SCF, 10 ng/mL IL3, 10 ng/mL IL6, or with 20 ng/mL M-CSF (all cytokines from Peprotech). Anti-F4/80 antibody (clone BM8) (eBioscience) of a concentration of 0.2 mg/mL was added at a dilution of 1:10 000[30] to all media to additionally report full differentiation toward macrophages.

Embryonic Stem Cell Culture. Murine embryonic stem cells (line R1) expressing the ERK kinase translocation reporter (ERKKTR)[31] were used as a source of adherent cells. Approximately 10 cells were seeded in each chamber of the microfluidic device. The cells were cultured for 2 days under self-renewing conditions (DMEM (Gibco) containing 10% FCS11 (PAA) and 1000 U/mL leukemia inhibitory factor (Lif) (CellGS)). During this time medium in the chambers was replaced every 30 min. Prior to stimulation experiments, cells were starved overnight for 16 h in 0.5% FCS containing DMEM without Lif with the same feeding pattern mentioned above. To create dynamically changing media conditions with periodically increasing and decreasing levels of a cell extrinsic signaling molecule over the course of several hours, the chip was programmed to switch periodically between a ligand containing recombinant mouse FGF1 (10 ng/mL, R&D Systems) in starvation medium for 5 s, followed by a 32 min incubation prior to dilution of the FGF1 ligand by applying a wash for 2 s with starvation medium, followed by a 15 min incubation. Images were taken every 3 min to monitor ERK activity. For experiments comparing our chip to traditional cell culture devices, cells were cultured in channel slides (μ-slide VI, Ibidi, Inc.).

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RESULTS AND DISCUSSION

Design and Operation. By combining actuated valves, implemented for adherent cell culture,[14,15,32,33] and 2D lateral-diffusion-based culture for suspension cells,[25−27,34] we are able to create independently controllable culture conditions across 48 chambers on a single chip (see Figures 1A−C and Video S1). Our two-layer design simplifies production and operation in contrast to previously proposed 3D designs.[23,24,35]

Liquids and cells can be added by connecting a pressurized vessel to 1 of 16 inputs. Liquid flow from the reservoir and through the device is driven by a pressure difference or using an on-chip peristaltic pump. Waste products or samples of cell suspensions are removed on the right side of the device via the outlets or harvesting ports (see Figure 1A).[36]

The core of the chip contains 48 independently addressable 1000 μm × 350 μm and 25 μm tall chambers for cell culture (see Figures 1B and 1C, and Video S1). Two media reservoirs connect to the culture chambers using 50-μm-wide openings for diffusion-based feeding.

This device is especially suited to long-term culture experiments where large numbers of cells will result from clonal expansion and certain delays in establishing a homogeneous gradient for chemical compounds across the entire chamber appears tolerable against the duration of the biological process under observation.

Flow Switching Allows Diffusion-Based Cell Feeding and Stimulation. Medium flow in suspension cultures results in rapid dislocation or removal of cells. In our device, reagents diffuse between cell culture chambers and media reservoirs through a thin connection port via Fick's Law (see Figure 1B and Video S2), allowing us to modify the contents of the cell culture chamber without dislocating individual cells by replacing the contents of the media reservoirs via flow.

To prevent flow created by small differences within the supply channels during simultaneous flushing, we applied an alternating flow-switching strategy and replaced media reservoirs sequentially (see Figure 2A). We first filled the left reservoir with fresh medium or stimulant, while the valves for the right side remained closed. We then mirrored this process to fill the right reservoir. Volume replacement by flow is fast, compared to diffusion, which allowed us to refill channels sequentially and expand our experiments with discrete conditions for each chamber.[36−38]

Characterizing Diffusion-Based Media Exchange Using Dextran. To assess diffusion-based material exchange in our device, we simulated a periodic compound input by flowing media conditioned with rhodamine-conjugated dextran (40 kDa) through media reservoirs (see Figure 2B and Video S3). Oscillations with periods of 20 min (Figure 2B and Video S4) or 40 min (Figure 2B and Video S5), using sequential flow switching in 1 min intervals, reached steady state within 10 min.

During this time, gradients of compound concentrations form within the cell culture area (Video S2). The duration of gradients can be reduced by longer reservoir channel washing times and repeated filling of the reservoir channels (Video S3). We chose wash times and intervals resulting in gradients lasting a maximum of 20 min.

In order to observe the consequences of transiently occurring gradients on cell states in a rapidly responding system, we stimulated murine embryonic stem cells (line R1) expressing the ERK kinase translocation reporter (ERKKTR)[31] with 1000 U/mL FGF1 containing media. 93% of cells directly adjacent to the media reservoirs and 80% of cells furthest from media reservoirs responded within the first 3 min. All cells responded within 10 min of stimulation (see Figures S2A and S2B, and Video S9). This lag time must be considered when designing experiments with biological processes occurring at rapid timescales.

Absence of Flow during Media Exchange. We assessed the movement of nonadherent polystyrene beads within the cell culture chambers during rapid flow within media reservoirs. Bead translocation within the cell culture chambers was <2 μm after 15 s of media reservoir flushing (see Figures 3A−C and Video S6). Accordingly, murine GMPs loaded into the chip and subjected to oscillations between rhodamine−dextran-containing culture media and rhodamine−dextran-negative media showed a comparable lack of movement (see Video S10).

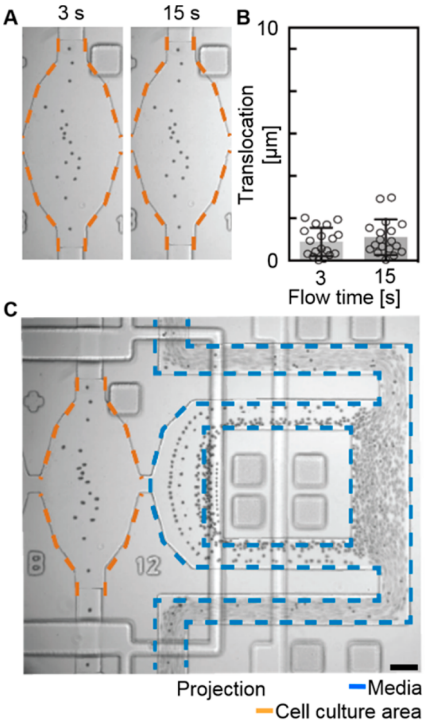

Figure 3. Flow-switching principle permits change in the chemical media composition by diffusion in the absence of direct fluid flow. (A) 10 μm polystyrene beads simulate suspension cells within each chamber. Beads were loaded into each chamber before being subjected to up to 15 s of flow within the right media reservoir: (left) bead locations after 3 s of continuous flow and (right) bead locations after 15 s of continuous flow. (B) The mean displacement of each bead in the cell culture area during 15 s of flow is less than 2 μm. (C) The minimum projection of recorded bead locations over 15 s of flow shows rapid bead movement in media diffusion channels and very minor bead movement inside the cell culture chamber. Scale bar = 100 μm.

Long-Term Culture of Murine Hematopoietic Stem and Progenitor Cells. We seeded a channel slide (μ-slide VI, Ibidi) and each chamber of our microfluidic device with 4−90 LysM-GFP GMPs per chamber and performed continuous culture over 3 days in stem-span expansion medium (SFEM) (StemCell Technologies) supplemented with 1% penicillin/streptomycin, 10% FCS, 100 ng/mL SCF, 10 ng/mL IL3, and 10 ng/mL IL6 with diffusion feeding every 30 min. Estimates of cell cycle length and proliferation within our device over the course of 3 days are comparable to standard cell culture devices, such as the Ibidi VI channel slides (see Figures S1A and S1B in the Supporting Information).

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Differentiation of Murine Hematopoietic Stem and Progenitor Cells. We observed HSPC differentiation under controlled conditions by seeding our device with 4−10 LysM-GFP GMPs per chamber and culturing each chamber in either SFEM supplemented with 1% penicillin/streptomycin, 10% FCS, 100 ng/mL SCF, 10 ng/mL IL3, and 10 ng/mL IL6 or 20 ng/mL M-CSF with media reservoir replenishment every 30 min. We monitored cell growth and LysM-GFP expression continuously by time-lapse imaging while tracking individual cells during growth and media exchange (see Figure 4D and Video S7). In the presence of constant M-CSF, 77% of colonies upregulated LysM-GFP within the 96 h observation period, while only 54% did so in the absence of M-CSF (p-value = 0.001636, Two-sample Kolmogorov−Smirnov test, Figure 4A and Video S7). Accelerated LysM-GFP induction in GMPs exposed to constant M-CSF stimulation is consistent with previous results under nonmicrofluidic culture conditions[29] and indicates that our device maintained different culture conditions simultaneously during time-lapse imaging.

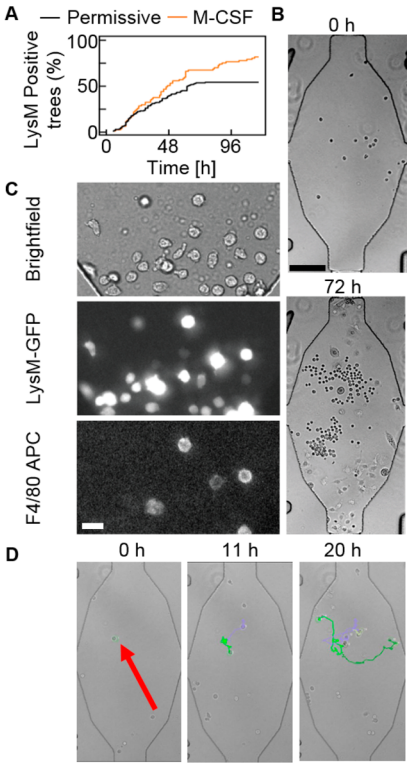

Figure 4. The suspension cell culture chip permits continuous and dynamic differentiation assays of blood progenitor cells. (A) Continuous culture of nonadherent granulocyte macrophage progenitor cells (GMPs). The cells were cultured on the chip for >96 h in different media with automated, diffusion-based media exchange occurring through the side channels every 30 min. LysM-GFP signal shows the impact of M-CSF containing medium (orange) over control medium (black) on cell differentiation. (B) Nonadherent GMPs can be cultured and monitored over 3 days. Top portion shows the initial cell density, and the bottom portion reflects the cell density after 72 h of culture. Scale bar = 100 μm (C) Colonies expressing LysM-GFP as a marker for differentiation into the macrophage lineage. The top portion shows bright-field images, the middle portion shows GFP channel images of cells with a range of LysM-GFP expression levels, and the bottom portion shows APC channel images of GMPs stained with an in-culture anti-F4/80-APC antibody as a marker for macrophage differentiation. Scale bar = 10 μm. (D) Tracking of individual nonadherent GMP cells in multiple generations during continuous feeding via diffusion. Red arrow at t = 0 h shows the initially tracked single cell; colored traces are tracks of individual cells. See Video S7 for a full recording of this experiment.

In-Culture Continuous Antibody Staining of Live Cells on Chip. Automated fluid replacement within our device permitted us to culture GMPs in media containing fluorescent antibodies targeting F4/80, a molecular reporter of commitment in macrophages (Figures 4A−D and Video S8). The resulting fluorescence signals were readily detectable on differentiated cells, indicating cell commitment to the macrophage lineage.

CONCLUSIONS

We present a robust, valve-based microfluidic chip design for culture of both adherent and suspension cells in rapidly changing chemical media conditions. Each of its 48 chambers can be precisely addressed individually to accurately deposit cells and exchange chemical compounds by diffusion. These cultures can then be observed in the device by continuous time-lapse imaging across several days. Notably, the small size of the diffusion ports limits the rate at which equilibrium can be reached in a given chamber to ∼10−20 min (Figure 2B). This observation must be considered when designing experiments with the relevant biological readouts in mind.

The device will be most beneficial for studying cell fate choices following dynamically altered media conditions occurring at long time-scales across several days. Compared to more-complex 3D devices, we improve and simplify production through a two-layer chip assembly. Our design presents a robust and user-oriented device for stem cell biologists. It offers a versatile experimental tool that can be easily implemented.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00312.

Figures showing that the suspension cell culture chip permits continuous culture assays of blood progenitor cells (Figure S1) and the diffusion-based stimulation of ERKKTR expressing murine ESCs with FGF1 (Figure S2) (PDF)

MATLAB files necessary to execute the graphic user interface written for control of our device (ZIP)

CAD designs for the device (ZIP)

Video showing the generation of a transient checkerboard pattern in the device using blue food dye (AVI)

Video showing rhodamine−dextran diffusion from diffusion channels into cell culture chamber (AVI)

Video showing rhodamine−dextran diffusive oscillations on a 30-min interval (AVI)

Video showing rhodamine−dextran diffusive oscillations on a 20-min interval (AVI)

Video showing rhodamine−dextran diffusive oscillations on a 40-min interval (AVI)

Video showing that 10-μm polystyrene beads, simulating non-adherent cells within a cell culture chamber, are minimally perturbed by flow through the diffusion channel (AVI)

Video showing GMPs cultured for 20 h in serum-free media supplemented with M-CSF (50 ng/mL) (AVI)

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Video showing in-culture staining of differentiated GMPs with Alexa Fluor 647 labelled anti-F4/80 and Alexa Fluor 488 labelled anti-CD11b antibodies (AVI)

Video showing diffusion-based stimulation of ERKKTR expressing murine ESCs with LIF conditioned media (AVI)

Video showing diffusion-based application of rhodamine−dextran to murine GMPs (AVI)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Author Contributions

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Notes

The authors declare no competing financial interest.

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