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Study of Chemotaxis and Cell–Cell Interactions in Cancer with Microfluidic Devices

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Abstract

Microfluidic devices have very broad applications in biological assays from simple chemotaxis assays to much more complicated 3D bioreactors. In this chapter, we describe the design and methods for performing chemotaxis assays using simple microfluidic chemotaxis chambers. With these devices, using real-time video microscopy we can examine the chemotactic responses of neutrophil-like cells under conditions of varying gradient steepness or flow rate and then utilize software programs to calculate the speed and angles of cell migration as gradient steepness and flow are varied. Considering the shearing force generated on the cells by the constant flow that is required to produce and maintain a stable gradient, the trajectories of the cell migration will reflect the net result of both shear force generated by flow and the chemotactic force resulting from the chemokine gradient. Moreover, the effects of mutations in chemokine receptors or the presence of inhibitors of intracellular signals required for gradient sensing can be evaluated in real time. We also describe a method to monitor intracellular signals required for cells to alter cell polarity in response to an abrupt switch in gradient direction. Lastly, we demonstrate an *in vitro* method for studying the interactions of human cancer cells with human endothelial cells, fibroblasts, and leukocytes, as well as environmental chemokines and cytokines, using 3D microbioreactors that mimic the *in vivo* microenvironment.

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1. INTRODUCTION

Microfluidic devices can be designed to control the flow of liquid inside cell-sized channels and to thereby enable a variety of biological studies. The dimensions of the channels in microfluidic devices are typically 10s–100s of microns, and hence with appropriate fluid controls and sensors, can support the manipulation and analysis of very small volumes. Fabrication of these microdevices requires the use of techniques adapted from semiconductor microfabrication and plastic molding, such as photolithography or micromachining to create molds, and replica casting or embossing or glass etching to create the actual devices. Many of the devices are ideally suited to high-resolution microscopic imaging of chemotaxis.

Chemotaxis is a directional cell movement during which cells sense a chemical gradient in a chemokine or chemoattractant and move toward the chemical source. Many types of cells use chemotaxis to actively move to specific locations. The inflammatory process provides an excellent example of chemotaxis, wherein immune cells respond to a gradient of chemokines or chemoattractants, and move up the gradient to reach the site of infection. Once the immune cells “sense” the gradient, they extravasate from vascular vessels and move toward the infection site within the adjacent tissue to destroy bacteria, remove dead cells, and heal the wound area. To set up an *in vitro* chemotaxis assay requires generation of a reliable chemokine/ chemoattractant gradient. Traditional *in vitro* chemotaxis assays use a passive diffusion mechanism to generate the gradients, such as a modified Boyden chamber (Boyden, 1962) or agarose- or collagen-based assays (Haddox, Knowles, Sommers, & Pfister, 1994; Haddox, Pfister, & Sommers, 1991; John & Sieber, 1976; Nelson, Quie, & Simmons, 1975), and other techniques like Zigmond or Dunn chambers (Zicha, Dunn, & Brown, 1991; Zigmond, 1977). With the Boyden chamber or modified Boyden chamber, transwells covered with polycarbonate filters with tiny pores (from 3 to 10 μm in diameter) are used to separate two different concentrations of chemokine. The assay relies on diffusion between the two chambers to generate a gradient across the membrane. The Zigmond and Dunn chambers generate the gradient through a very small bridge area between two chemokine reservoirs. Assays based upon agarose or collagen rely on chemokine diffusion through the agarose or collagen gel and require cells to crawl through or under the agarose or collagen up the gradient of chemotactic factors.

All of these traditional chemotaxis assays have common disadvantages. (1) They can generate only linear gradients and cannot provide either a variety of gradient shapes or rapid alterations of gradient direction or gradient profiles, all of which occur in the tissues *in vivo*. (2) Changing the type or concentration of chemokines within the gradient region is not feasible during experiments. After the gradient has been established, any change to the chemokine requires a significant amount of time before the new gradient can be established and this change most likely will disturb cell migration. (3) The steepness and range of the gradient with these assays are determined by both the difference in the concentration of the chemokines in the two solutions on either side of the barrier and the thickness of the barrier that separates them, and this steepness cannot be altered without replacing one of the two solutions, with concomitant hydrodynamic forces. For all of these reasons, it is desirable to develop a new chemotaxis system that can create and maintain a stable concentration

gradient and also allow manipulation of the slope and direction of the gradient to better understand the factors involved in “gradient sensing.” Microfluidic devices are ideal tools for creating and controlling chemokine gradients. Microfabrication methods enable creation of arbitrary microchannel designs through which cells can chemotax. Microchannel dimensions can be created small enough that diffusion occurs in minutes to seconds, thus reducing the waiting time for a gradient to become established (Li Jeon et al., 2002; Lin, Nguyen et al., 2004; Lin, Saadi et al., 2004).

In this chapter, we first demonstrate a method using a simple microfluidic device that allows the recording of cell migration in response to a chemokine gradient (Fig. 1) (Walker et al., 2005). The design of this device includes a Christmas tree-like mixer and splitter, in which the concentrated chemokine is injected through one input (IN_B) and the buffer (without chemokine), is input from the other (IN_A), both driven by a single-syringe pump with two syringes. Within the mixer/splitter, whenever two input flows with different concentrations merge, the resulting laminar flows mix by lateral diffusion in the serpentine section and the concentration of the mixed solutions becomes uniform with a concentration equal to the average of the two inputs and twice the flow rate. When a single flow is split into two flows, the concentration in each branch is equal to the concentration before the split but the flow rates are halved. The splitting and merging of the two input flows can produce three different streams, which are split and then merged into four, and, for the device shown in Fig. 1, these flows in turn create five flows. Through three tiers (or stages) of this splitting and mixing, five streams of chemokine fluids with different concentrations are generated in the order of increasing concentrations that span those of the two original flow streams from the two syringes. Then these five streams of chemokine fluids are merged again into the main channel, where a chemokine gradient is formed from low to high across the main channel all the way to the output end (OUT). Depending on the location in the main channel, the chemokine gradient shows a step-wise gradient at the entrance of the main channel and becomes a relatively smoother gradient with the help of lateral chemokine diffusion within the laminar flow of the channel (Fig. 1). The cells are seeded in the main channel through the cell input (IN_{cell}), and time-lapse video microscopy is taken in the mid-location of main channel.

The second microfluidic device used in chemotaxis studies shown in this chapter is a microfluidic switching system (Fig. 2) (Liu et al., 2008). This device allows one to change the direction of the gradient in less than 1 min and is a very useful tool to study how the cells respond to the directional change of the gradient. Similar to the previous microfluidic chemo-taxis chamber, the two pairs of input fluid streams are driven separately by two pumps and three tiers of mixer are used to generate gradients. Only one pump runs at a time: when pump I runs, it generates a low-to-high gradient across the main channel from the bottom, and when pump II starts running and pump I stops, a reversed gradient is generated.

Our third microfluidic device described in this chapter is a miniature tumor microenvironment system in which a cluster of tumor cells (i.e., a spheroid), fibroblasts, and endothelial cells are cocultured in a system that mimics the tumor microenvironment (Fig. 6) (Hockemeyer et al., 2014). This device provides an ideal tool to study these cell–cell interactions in a simulated tumor microenvironment.

2. METHODS

2.1 Chemotaxis in Microfluidic Chemotaxis Chamber

2.1.1 Making Microfluidic Chemotaxis Chamber—Microfluidic devices that consist of three tiers of “divide and mix” micro-channels that enter into the main microchannel (Fig. 1) were fabricated in a class-100 clean room (less than 100 particles per cubic foot of air). The splitting/mixing microchannels were designed to generate five streams of that span a range of concentrations of fluid as described above. A stable gradient of chemical thus forms across the streams in the main microchannel. In the models and experiments presented here, the main microchannel dimensions are 500 μm wide, 100 μm tall, and 1 cm long. Polydimethylsiloxane (PDMS) was chosen as the construction material because it allows devices to be fabricated rapidly and is optically transparent, facilitating observation of both the concentration gradient and the cells involved in chemotaxis. Microfluidic devices were made by molding microchannels in the PDMS (Sylgard 184, Dow Corning, Midland, MI) and then bonding the mold to glass coverslips using standard methods in soft lithography (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001).

Materials

Class 100 clean room

PDMS (Sylgard 184, Dow Corning, Midland, MI)

Glass coverslips, No. 1, 24 mm $\times$ 50 mm

SU-8-2100 (MicroChem Corp., Newton, MA)

Programmable hotplate (Barnstead/Thermolyne International, Dubuque, IA)

16-gauge blunt needle: outer diameter (OD) 1.65 mm; inner diameter (ID) 1.19 mm (Becton, Dickinson and Company, Franklin Lakes, NJ)

Plasma cleaner (Harrick Scientific Corporation, Ossining, NY)

Tygon tubing, OD: 1.52 mm; ID: 0.508 mm (Cole Parmer, Vernon Hills, IL)

Procedures

1. Curing agent and prepolymer are mixed together (1:10 wt/wt) and placed in a desiccator for 45 min. The mixture is then poured over a SU-8-2100 (MicroChem Corp., Newton, MA) master in a tissue culture dish which contains the positive relief of the microchannel design. The PDMS mold is cured for 2.5 h at 80 $^{\circ}\text{C}$ on a programmable hotplate.
2. Once the mold has cooled, it is peeled off the master, cut to size, and access holes are punched for tubing. A blunt 16-gauge needle with an OD of 1.65 mm and an ID of 1.19 mm is used as the punching tool.
3. Each PDMS mold is then treated with a plasma cleaner for 20 s and placed on a number 1 24 mm $\times$ 50 mm glass coverslip.

4. The bonded device is treated again with the plasma cleaner for 15 s to facilitate filling with liquid.
5. Tygon tubing with an OD of 1.52 mm and an ID of 0.508 mm, cut into four 30.5 cm lengths, is inserted into each access hole.
6. Sterilized DI water is injected into the device via the waste line until the device and the remaining three tubes are filled with water. Bubbles are removed by pinching the three lines closed and applying pressure to the waste line syringe. The increased fluid pressure within the device causes the air in trapped bubbles to diffuse through the PDMS.

2.1.2 Chemotaxis Assay for Differentiated HL-60 Cells—The HL-60 cell line is a promyelocytic cell line derived from human leukemia (Collins, Gallo, & Gallagher, 1977). The cells are promyelocytes, but can be induced *in vitro* to differentiate into different lineages of mature myeloid cells depending on the reagents used for induction (Collins, Ruscetti, Gallagher, & Gallo, 1978). If dimethyl sulfoxide at 1–1.5% is provided for HL-60 cell culture, the cells will differentiate into granulocyte-like cells, or neutrophils. Chemotaxis is one of the most important characteristics of neutrophils in inflammatory response. Differentiated HL-60 cells are widely used to study neutrophil chemotaxis, since they are readily available and easy to genetically modify. Although it has been reported that the differentiation of HL-60 may boost the expression of chemokine receptor, CXCR2, a major receptor to the inflammatory chemokine CXCL8 (Collins, 1987), the expression level is too low to drive an efficient chemotactic response to CXCL8 (Elvin, Kerr, McArdle, & Birnie, 1988). Therefore, HL-60 cells were stably transfected with a CXCR2 expression vector and the transduced cells exhibited a robust chemotaxis in response to a CXCL8 gradient (Sai, Walker, Wikswo, & Richmond, 2006). The response of these cells to a chemotactic gradient of CXCL8 can be visually observed with a microfluidic device, as described in Liu et al. (2008), Sai et al. (2006), and Walker et al. (2005).

Materials

Stable HL-60 cells expressing CXCR2

Modified Hank's balanced salt solution (HBSS): 150 mM sodium chloride, 4 mM potassium chloride, 1.2 mM magnesium chloride, 10 mg/ml glucose, and 20 mM HEPES, pH 7.2

Dimethyl sulfoxide (Endotoxin-low, Sigma-Aldrich)

RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco)

CO₂ incubator

Fibronectin (human) (BD Biosciences, San Jose, CA)

Bovine serum albumin (BSA) (Sigma-Aldrich)

CXCL8 or MIP-2 (Recombinant protein from a commercial source) Syringe, 1 ml (Becton, Dickinson)

Syringe pump, Harvard PHD 2000 (Harvard Apparatus, Holliston, MA)

Blunt end needles, 25 gauge, ID=0.3 mm, and OD=0.5 mm (Howard Electronic Instruments, Inc., El Dorado., KS)

Inverted microscope with CCD camera (Axiovert 200M, Zeiss, Germany)

Procedures

1. To prepare the differentiated HL-60 cells 2×10^5 , HL-60 cells are inoculated into 10 ml antibiotic-free RPMI medium supplemented with 10% FBS and 1.3% endotoxin-low DMSO is added. Cells are incubated at 37 °C, 5% CO₂ for 5–7 days. The morphology of cells will change during the process of differentiation from a perfectly round shape to a randomly irregular shape with multiple protrusions on the cell membrane.
2. To coat the microfluidic chemotaxis chamber, the microfluidic device is refilled with HBSS buffer and the device is viewed under an inverted microscope to verify that no air bubbles are trapped; if bubbles are present, they are removed by the method described in step 6 above (see Section 2.1.1). 100 µg/ml fibronectin solution is injected into the device through the cell injection line (Fig. 1) with inputs A and B blocked so that the fibronectin can coat the slide at room temperature for 1 h. The excess fibronectin solution is then washed out from the device by injecting 1 ml HBSS buffer through the waste line just prior to loading the cells.
3. The differentiated HL-60 cells are washed with serum-free RPMI-1640 medium and resuspended in serum-free RPMI-1640 medium at 2×10^6 cells/ml. The cells are injected into the microfluidic device and incubated for 20 min at 37 °C and 5% CO₂ to allow the cells to settle and attach to the microchannel floor. The cells should be uniformly distributed on the microchannel floor prior to exposing them to the chemokine.
4. The microfluidic device containing the cells is placed on the inverted microscope (Zeiss Axiovert 200M) with a controlled environmental chamber set at 37 °C and 5% CO₂. A 1 ml syringe is loaded with chemokine (CXCL8) in serum-free RPMI-1640 medium at a concentration of 50 ng/ml and another syringe is loaded with medium only as control, and then the syringes are connected to the syringe pump. The input lines A and B are connected to the chemokine syringe or the medium syringe, respectively. Pumping is applied at a high flow rate (50 µl/min) for 1.5 min with the cell loading line open and waste line closed to allow quick replacement of HBSS buffer in the input lines with chemokine or medium.
5. The pumping is paused, the waste line is opened while the cell loading line is closed, and then a slower pump rate of 1 µl/min is utilized for the remainder of experiment.
6. Time-lapse video recording is initiated once the slower pump rate is initiated.

2.2 Gradient Switching in Microfluidic Device

One of the advantages of microfluidic devices is that they are capable of providing a diverse range of gradients. The controlled and reproducible generation of a spatiotemporally complicated chemoattractant gradient is very important in the study of the mechanisms of cellular chemotaxis. For example, microfluidic devices are excellent for studying how cells respond to two opposing chemokine gradients, how cells change their morphology when the direction of gradient switches, and how the movement of cells alters as the steepness of the gradient changes. In the methods listed below, we describe a modified microfluidic chemotaxis device that allows switching of the direction of gradient in seconds (Fig. 2).

2.2.1 Making a Gradient Switching Microfluidic Device

Materials

Class 100 clean room

PDMS (Sylgard 184 Dow Corning, Midland, MI)

Glass coverslips, No. 1, 50 mm

SU-8-2050 (MicroChem Corp., Newton, MA)

Programmable hotplate (Barnstead/Thermolyne International, Dubuque, IA)

16-gauge blunt needle, OD: 1.6 mm; ID: 1.2 mm (Becton, Dickinson, Franklin Lakes, NJ)

Plasma cleaner (Harrick Scientific Corporation, Ossining, NY)

Tygon tubing, ID: 0.02 in.; OD: 0.05 in. (Saint-Gobain Performance Plastics Corporation)

Procedures

1. A 100 μm thick layer of photoresistant SU8-2050 is exposed to UV light through a chrome mask to generate negative master patterns on a silicon substrate.
2. PDMS mixed with a curing agent at a ratio of 10:1 is cast onto the master to replicate the master patterns and cured for 2.5 h at 80 °C.
3. The cured PDMS is peeled from the master, and with a 16-gauge blunt needle, holes are punched for connecting the Tygon tubing, which serve as the inputs and outputs.
4. The surfaces of the channel side of the cured PDMS and a clean glass cover slide (No. 1) are treated with plasma for 20 s and bonded together to form an irreversible seal.
5. Approximately, 30 cm long Tygon tubing is inserted into the input holes to connect the device to a pair of two syringe pumps.

2.2.2 Chemotaxis Assay for Differentiated HL-60 Cells with Gradient Switch

Materials

HL-60 cells stably expressing CXCR2

Modified HBSS: 150 mM sodium chloride, 4 mM potassium chloride, 1.2 mM magnesium chloride, 10 mg/ml glucose, and 20 mM HEPES, pH 7.2

Dimethyl sulfoxide (Endotoxin-low, Sigma-Aldrich)

RPMI-1640 medium supplemented with 10% FBS (Gibco)

CO₂ incubator

Fibronectin (human) (BD Biosciences, San Jose, CA)

BSA, Sigma-Aldrich

CXCL8 or MIP-2 (Commercially available)

Syringe (1 ml, Becton-Dickinson)

Syringe pump, Harvard PHD 2000 (Harvard Apparatus, Holliston, MA) Blunt end needles (25 gauge, ID=0.3 mm, and OD=0.5 mm) (Howard Electronic Instrument, Inc., El Dorado., KS)

Inverted microscope (Axiovert 200M, Zeiss, Germany), with a charge-coupled device (CCD) camera (Hamamatsu, Japan)

MetaMorph software (Molecular Devices, Inc., Sunnyvale, CA)

Procedures

1. The main channel of the device is coated with human fibronectin at a 100 µg/ml concentration for 1 h at room temperature and rinse with HBSS buffer to remove excess fibronectin in the channels before loading cells.
2. HL-60 cells are differentiated by culturing 2×10^5 CXCR2-expressing HL-60 cells/ml in antibiotic-free medium that contains 1.3% DMSO for a week.
3. The differentiated CXCR2-HL-60 cells are washed and resuspended with serum-free RPMI-1604 medium at a concentration of 4×10^6 cells/ml. The prepared cells are injected into the device through the loading channel and seed the cells for 5 min at 37 °C and 5% CO₂.
4. The device with cells is set on the stage of an inverted microscope with a temperature and CO₂ controlled chamber. The four tubing inputs are connected to syringes filled with either CXCL8 solution or buffer alone. The solutions are injected into the device driven by two syringe pumps, as indicated in Fig. 2A. The key feature is the use of two pumps and four syringes, such that one pump drives one gradient and the second pump drives the reverse gradient.
5. Initially, both pumps are run at a high flow rate (50 µl/min) to quickly fill the four segments of tubing (about 1 min). A single pump is then run at low flow rate (0.5 µl/min) to maintain a chemokine gradient that is high on the side of the

channel of the top (Fig. 2B). At the same time, time-lapse video recording is initiated. After running the pump for 20 min, stop the first pump and run the second to generate a gradient with the high concentration on the opposite side of the channel (Fig. 2C), and continue time-lapse video recording for an additional 20 min.

2.2.2.1 Cell Tracking and Data Analysis

1. Time-lapse video data are generated by taking pictures every 20 s using a CCD camera. MetaMorph software is used to track and analyze cell movement. The HL-60 cells that migrate more than 20 μm in the initial gradient and keep moving after the gradient switching are defined as exhibiting a directionally biased migration response to the chemokine gradient and are included in the quantification of the chemotaxis, while the cells that remain within a 20 μm radius of their original positions are excluded from the analysis, as are cells that stopped or detached from the substrate.
2. As shown in Fig. 3, the first 5 min following initiation of flow (the cell accommodation interval) was excluded from data extraction because during this time the cells respond to the chemokine gradient, become polarized, and begin to move toward the gradient. Thus, counting the events during this time frame would adversely affect the precise analysis of cell movement. The initial response interval was defined as the time between the beginning of cell movement and the time the gradient direction was shifted (15 min). The gradient was then switched, a process which required approximately 1 min. The 5-min interval that began with the reversal of two pumps was termed the “prompt response interval.” During this period of time, the response of cells to the directional change of the gradient was taken into account. The later response interval was the next 10 min, which was continued until the end of the experiment. Chemotactic indexes (CI) before and after switching were calculated as mean and standard error, and the cell response time and average moving angles were also determined for comparison (Fig. 4).
3. The CI is defined as the displacement along the direction of the gradient divided by the total migration distance and is used to quantify the cell motility toward the chemokine gradient. The results were evaluated by the Student's *t*-test and single factor analysis of variance (ANOVA). The number (*N*) of cells is indicated in Fig. 5.
4. Conclusions: In summary, we present a simple microfluidic system for studying cell migration in a time-dependent gradient environment and describe the response of PI3K-inhibited neutrophil-like HL-60 cells to such a gradient condition. The inhibited HL-60 cells showed normal chemotaxis but reduced cell motility, as indicated by slower response time to the gradient switch, less polarization, and a reduced number of cells that turned upon reversal of the CXCL8 gradient.

2.3 Cell–Cell Interaction in Tumor Microenvironment

Microfluidic devices can support cell–cell signaling analyses with more analytical depth than can other coculture methods. For example, cancer is not a cellular or genetic disease of one cell type, but a complex cascade of interactions among several different cell types, proteins, cytokines, and circumstances in a microenvironment. The process of cancer progression and metastasis is complicated, encompassing recruitment of fibroblasts as well as leukocytes to the developing tumor or the premetastatic niche. Numerous pathways are activated simultaneously across several different types of cells. In order to study these interactions, microfluidics offers what other current models cannot: a method to mimic and observe an *in vivo*-like microenvironment, while still maintaining control over the system as a whole. Herein, we describe a microfluidic device that is capable of creating such a microenvironment, complete with spheroids (aggregates of cancer cells that behave more like an *in vivo* tumor than cells on 2D surfaces), fibroblasts, and endothelial cells that form a blood vessel-type structure.

2.3.1 Creation of the Microfluidic Device—The microfluidic device (see Fig. 6) was designed in AutoCAD and fabricated on a planar silica chip using a computer-controlled, femtosecond laser machining station. The chip was then etched in 10 M KOH at 80 °C for 1 h to form channels within the silica. Fabrication and assembly were conducted in an ISO 1000 stage clean room (Hockemeyer et al., 2014). Once the device is built, it can be reused several times for repeat experiments. To sterilize before initiating an experiment, 70% ethanol is flown through the device, followed by flowing 1× PBS supplemented with 400 µg/ml penicillin, 400 µg/ml streptomycin, and 1 µg/ml amphotericin B with a rate of 20 µl/min.

Materials for spheroid formation

MDA MB 231-GFP⁺ cells (MDA MB 231 cells from ATCC were transduced with a lentiviral GFP vector and selected by antibiotic resistance and expression of GFP by flow cytometry)

Trypsin/EDTA (Sigma)

DMEM (10% FBS plus 2 mM L-glutamine) (Gibco) with 20% methylcellulose (Sigma)

96-well sterile non-tissue-culture-treated U-bottom plate

Procedures for spheroid formation

1. MDA MB 231-GFP cells are detached from a culture flask using Trypsin/EDTA.
2. Live cells are collected by centrifugation and resuspended in DMEM (10% FBS, L-glutamine) and 20% methylcellulose solution to a density of 1000 cells/ml.
3. One milliliter of this cell suspension is pipetted into each well of the 96-well U-bottom plate.
4. The culture is placed in the water-jacketed incubator (37 °C) for a period of 2–3 days, until a small spheroid forms in each well.

Materials for endothelial cell loading

HMVECad cells (Life Technologies)

Cell Tracker Blue (CTB) (Invitrogen)

MCD-131 medium supplemental with microvascular growth supplement (Life Technologies)

Trypsin (0.05%)/EDTA (0.53 mM) in HBSS, without calcium, magnesium (Corning)

25 mM HEPES-containing medium

100 µl gas-tight syringe

Blunt 23-gauge dispensing needle

Procedures for endothelial cell loading

1. Endothelial cells are stained with 8 µM CTB in serum-free MCDB-131 for 1 h at 37 °C.
2. CTB is removed and cells are incubated in normal media for 30 min.
3. Cells are detached using Trypsin (0.05%)/EDTA (0.53 mM) in HBSS solution.
4. Cells are centrifuged at 800 g for 5 min.
5. Cells are resuspended in 25 mM HEPES-containing MCDB-131 media at density of 5×10^6 cells/ml.
6. 500 µl HEPES containing MCDB-131 medium is allowed to flow through the channel.
7. 100 µl HEPES containing MCDB-131 medium is added to the semicircular chamber.
8. Endothelial cells are loaded into the device's channel using the gas-tight syringe and a 23-gauge needle.
9. The device is positioned on an angle for 3 h so cells will seed on interface between the channel and semicircular well.

Materials for extracellular matrix (ECM) preparation

HEPES buffer solution (NaOH, HEPES, and 10× PBS in water)—pH 7.2

Matrigel (BD Biosciences)

Rat tail collagen type 1 (BD Biosciences)

Procedures for preparation of ECM

1. The ECM is prepared on ice; once the Matrigel is added, subsequent steps/protocols are completed swiftly to ensure the ECM does not start to polymerize before the fibroblasts/spheroids are added.

2. A desired amount of ECM is prepared, using a 1:1 ratio of collagen and Matrigel, with a final concentration of 1.5 mg/ml collagen and 10% Matrigel. The collagen is added to an Eppendorf tube first, then buffer with the HEPES solution. The Matrigel is added last and then the cells are loaded into this ECM suspension.

Materials for fibroblast loading

Fibroblasts (NAFs and/or CAFs,)—normal mammary-associated fibro-blasts (NAFs) and breast cancer-associated fibroblasts (CAFs) were the generous gifts of Drs. Harold L. Moses and Simon Hayward (Vanderbilt) MCDB-131 medium supplemented with 10% FBS, 1× insulin amp in, 1× nonessential amino acids, 1.4 mM L-glutamine (Gibco), 13% amniomax basal medium, and 2.1% amniomax C100 supplement (Gibco)

Cell Tracker Red (Life Technologies)

TrypLE (Life Technologies)

ECM solution

Procedure fibroblast loading

1. Fibroblasts are incubated in 7 μ M Cell Tracker Red for 1 h at 37 °C.
2. Fibroblasts are detached using TrypLE.
3. Detached fibroblasts are collected by centrifugation at 800g for 2 min.
4. Cells are resuspended and counted.
5. Cells are diluted to a concentration of 2.5×10^5 cells/ml and added to the ECM solution.
6. The fibroblast/ECM solution is set aside until it is ready for spheroid integration.

Materials for tumor spheroid loading

1.5 ml centrifuge tube

Cut pipette tips

Experimental medium (DMEM, 10% FBS, LG, and 25 mM HEPES)

Fibroblast loaded, ECM solution

Spheroids

Procedures for tumor spheroid loading

1. Spheroids to the 1.5 ml centrifuge tube carefully, using the cut pipette tips so as not to disrupt their integrity.
2. Spheroids are washed twice with experimental medium and placed on ice.

3. Supernatant is aspirated from spheroids and aliquots are placed into the premade fibroblast/ECM suspension to achieve a final concentration of 50–60 spheroids in 200 μ l of the ECM.
4. 7.5 μ l of this fibroblast/spheroid/ECM solution is loaded into the semicircular chamber.
5. The device is incubated at 37 °C and 5% CO₂ for 1.5 h to allow polymerization of the ECM, inverting the orientation intermittently to ensure an even cell density spread within the chamber.
6. After 1.5 h, 100 μ l experimental medium is added to the top of the semicircular chamber. Then, depending on the type of experiment, one may add 100 μ l of CXCL12 ligand diluted to 10 ng/ml, or 100 μ l vehicle solution to the top of the semicircular chamber as well. This part of the protocol can be altered to incorporate a variety of chemokines/ stimuli, as it is not limited solely to CXCL12. Alternatively, inhibitors of chemokines/chemokine receptor interactions may be added.
7. Flow can be established by connecting tubing to the inlet and outlet of the channel and to two syringe pumps. Depending on the rates of the pumps, one can establish either interstitial or regular perfusion flow through the channel.
8. The device is placed on a microscope stage (Zeiss Axiovert 200M) equipped with an incubated stage set at 37 °C and CO₂ chamber set at 5% CO₂ and imaged at 15 min intervals over 20 h under a 10 $\times$ objective lens in bright field and for fluorescence at excitations of 488 nm and 546 nm to visualize GFP-expressing cancer cells and Cell Tracker Red-labeled fibroblasts, respectively.
9. The time-lapse imaging data are quantitated using MetaMorph software (Figs. 7 and 8).

3. LIMITATIONS

Despite the many advantages of microfluidic devices as applied in chemotaxis assays, some problems have appeared. First, a constant flow rate is required to maintain a stable chemokine gradient, and the shear force consequently generated within a very small volume places some stress on the cells. Thus, it is very important to carefully regulate the flow rate. To resolve this problem, some devices have been designed to generate a gradient without using an external pump (Gao, Sun, Lin, Webb, & Li, 2012; Xu et al., 2012). These devices use either gravity-based passive pumping to generate and maintain the gradient (Gao et al., 2012), a low flow-rate osmotic pump (Xu et al., 2012), pure diffusion within very small grooves, or even a flow-free design (Abhyankar, Lokuta, Huttenlocher, & Beebe, 2006; Kim & Kim, 2010; Zhu et al., 2012).

Another disadvantage of microfluidic devices is the limited number of samples that may be assayed at the same time due to the complexity of the assay. Additional modifications are needed to allow the testing multiple samples under multiple conditions. New designs with multiple repeating units, such as arrayed microdevices (Berthier, Surfus, Verbsky,

Huttenlocher, & Beebe, 2010), allow the testing of multiple samples or multiple conditions. These devices have the potential to be used to analyze a patient's neutrophil chemotaxis in response to multiple chemokine/ chemoattractant gradients and will be very useful in the diagnosis of a primary immunodeficiency disorders. This type of device also demonstrates the capability for increased throughput in microenvironmental studies designed for screening targeted therapies for specific human diseases. Moreover, 3D microbioreactors are currently being designed for high-throughput screening (Wen, Zhang, & Yang, 2010; Yang, Zhang, & Wen, 2008), and we hope to utilize a simplified microbioreactor for this purpose in the future.

4. PERSPECTIVES

4.1 Shearing Force and Calculation of Chemotactic Force

As described above, the shearing force generated by constant flow places some stress on the cells being analyzed. The value of this is that it can mimic the flow that leukocytes experience in circulation, and with a microfluidic device the impact of the flow force on chemotaxis can be calculated. A typical cell in the microfluidic chemotaxis device as described in Section 2.1 encounters two different forces from orthogonal directions: the shearing force applies externally along the direction of flow and the internal chemotactic force generated from the chemokine gradient. Under these two different forces applied at a 90° angle, the trajectory of the cell migration will be the result from the balance between these two forces (Fig. 9). If the cell moves along the 45° angle line between these two forces, it is predicted that the chemotactic force generated under this specific chemokine gradient will be equivalent to the shearing force by the flow (Fig. 9). Of course, the final calculation will need to evaluate the surface area of the cell that encounters the shearing force and the friction between the cell and the chamber surface (Sai et al., 2006).

4.2 Cell Polarity Change Versus Cell Turning During a Gradient Change of Direction

With the gradient switching device, we tested the ability of the dHL-60 cells to respond to a switch in the direction of the gradient in the presence or absence of inhibition of PI3K. In the presence of PI3K inhibitor, the dHL-60 cells exhibited a slower change in the direction of cell migration. Moreover, when the cells that were not treated with PI3K inhibitor changed the direction of migration in response to the switch in the direction of the gradient, the majority of the cells changed their polarity (head became tail and tail became head) and migrated in the opposite direction, and only a minor proportion of cells turned around without changing polarity. This change of polarity is probably the most efficient way for a cell to change the direction of cell migration (Liu et al., 2008).

4.3 Potential Future Uses of Microfluidic Devices: Analysis of Circulating Tumor Cells

A more promising application of microfluidic devices is for the purification of circulating tumor cells (CTCs) from the peripheral blood of cancer patients. The earliest steps of metastasis involve the shedding of malignant tumor cells from the primary tumor followed by intravasation of these tumor cells into the blood vasculature. Careful monitoring of the number of CTCs can provide very important index for the prognosis of cancer patients as well as an indicator for response to ongoing therapy. However, among millions of different

types of other cells in the bloodstream, it is not easy to identify the sparse tumor cells. With the help of microfluidic devices, the tumor cells can be concentrated by binding to an antibody that specifically detects CTCs. The antibody is precoated on the surface of micropoles in the micro-fluidic channels (Lim et al., 2012; Nagrath et al., 2007; Stott et al., 2010; Zheng, Yang, & Li, 2010; also see review in Qian, Zhang, & Chen, 2015), and as CTCs bind to the antibodies they are concentrated for identification and quantitation by flow cytometry.

4.4 Advantages of Using 3D Microbioreactors to Investigate Factors that Influence the Tumor Microenvironment

A major problem that preclinical translational studies in oncology deal with is the fact that mouse models utilizing human tumor cells do not allow testing the interaction of human tumor cells with human immune cells, endothelial cells, and other stromal cells. Microbioreactors allow one to test the effects of therapeutic drugs on the interaction of immune cells with the tumor and its stroma in a manner that can readily be imaged in real time and analyzed. Immunotherapeutic agents can be evaluated using these micro-bioreactors, where the activity of cytotoxic T cells can be monitored visually and biochemically. Moreover, organoid cultures of human tumors interacting with the patient's own immune cells, fibroblasts, mesenchymal stem cells, and endothelial cells can readily be examined and tumor metabolomics can be analyzed in the presence and absence of combinations of therapeutic agents. Once these microbioreactors can be scaled for moderate throughput, we will have a unique method for preclinical studies that utilize all human cells in a microenvironment that mimics the patient's tumor.

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Figure 1.

Gradient formation in microfluidic device monitored with FITC-dextran. In this device, a constant 1 $\mu\text{l/m}$ flow of 100 $\mu\text{g/ml}$ FITC-dextran (MW 10,000) in Hank's buffer from one syringe and a flow at the same rate of buffer alone was driven by a single-, dual-syringe pump. The fluorescent images were taken at different locations along the main channel. The intensity of the fluorescence was quantitated across the channel for each image and is shown in the plots. *Adapted from Walker et al. (2005).*

Figure 2.

(A) Schematic of a microfabricated gradient device with a pair of dual-syringe pumps for gradient switching; (B) chemokine gradient generated by running pump I; (C) switching of the chemokine gradient is generated by running pump II. The white arrows in (B) and (C) indicate the flow direction in the main channel. *Originally published in* Liu, Sai, Richmond, and Wikswo (2008).

a: Cell accommodation interval
 b: Initial response interval
 c: Prompt response interval
 d: Later response interval

Figure 3.

Summary of intervals used to quantify HL-60 response to changing chemo-attractant gradients. (a) Cell accommodation interval for 5 min; (b) initial response interval toward a forward gradient for 15 min; (c) prompt response interval toward the switched/reversed gradient for 5 min, which includes 1 min of high flow during gradient reversal; (d) later response interval after the gradient reversed for 10 min. *Originally published in Liu et al. (2008).*

Figure 4.

Examples of displacement of the HL-60 cells and the change in their morphology in response to a switch in the direction of the gradient. Displacement is measured relative to the initial position of the cells before exposure to the gradient. The time of the onset of switching is indicated by the dashed vertical line, and the return to low velocity by the dotted line. (A) An HL-60 cell quickly changed direction; (B) a Wortmannin-inhibited HL-60 cell turned slowly; (C) a Wortmannin-inhibited HL-60 cell kept moving in the initial direction without turning. *Adapted from Liu et al. (2008).*

Figure 5.

Chemotaxis of HL-60 cells as measured by the chemotaxis index (CI) in control experiments without PI3K inhibitor as compared to that in the presence of PI3K inhibitor. Data are for the three intervals of the gradient switching shown in Fig. 4. The gradient direction is indicated either as forward or as reverse. *Originally published in Liu et al. (2008).*

Figure 6.

Isometric (A) and sectional (B) views of the device etched in a silica chip and sealed to a coverslip. A zoomed in orthogonal view of the semicircular well and channel (C) displays spatial orientation of cancer cell spheroids (green), fibroblasts (red), and micro-vascular endothelial cells (blue) during an experiment along with rates of inlet flow, outlet flow, and interstitial flow. Images of the fabricated device (D and E) give a size reference with scale bars 5 mm and 200 μm , respectively. (D) Assembled device glued to the acrylic manifold. Inlet and outlet port supply is sealed by red o-rings on the top and bottom of the diagram. (E) A view of the cell well, interstitial flow channel, and bridge between the two containing 6 μm flow channels every 30 μm . (F) A simulated vascular wall through endo-thelial cell monolayer is created by HMVECs labeled with Cell Tracker Blue cultured on channel side of porous membrane spatially mimic blood vessel wall. 20 $\times$ magnification of channel in brightfield shows some visibility of cells in monolayer. (G) 20 $\times$ magnification of DAPI channel visually confirms HMVECs are beginning to form a monolayer. *Originally published in* Hockemeyer et al. (2014).

Figure 7.

Spheroid sprouting with cultured spheroids alone, in the presence of cancer-associated fibroblasts, or in the presence of normal tissue-associated fibroblasts. Shortly after polymerization of the reconstituted basement membrane, the device was imaged over 12 h. Cancer cells are GFP-expressing and fibroblasts were labeled with Cell Tracker Red. (A) Cancer cell spheroids alone did not exhibit sprouting. (B) Cancer cells sprouted into the surrounding matrix when cultured with CAFs. (C) When cultured with NAFs, cancer cells moved around the spheroid or within small clusters, but very little migration occurred outside of local movement. *Originally published in Hockemeyer et al. (2014).*

Figure 8.

Spheroid sprouting in response to addition of CXCL12. After polymerization, medium supplemented with 10 ng/ml CXCL12 was added to the well and the device was imaged over 20 h. (A) When stimulated with CXCL12 without the presence of fibro-blasts, cancer cells sprouted extensively from the spheroid and were highly mobile within the surrounding matrix. (B) When stimulated with CXCL12 in the presence of normal fibroblasts, spheroid sprouting was abrogated and cancer cells remained localized *to the spheroid*. *Originally published in* Hockemeyer et al. (2014).

Figure 9.

Chemotactic migration of CXCR2-expressing differentiated HL-60 cells under different levels of CXCL8 gradient steepness. Different levels of CXCL8 gradient steepness were generated by constant flow of a certain concentration of CXCL8 in Hank's buffer and buffer alone at a rate of 1 μ l/min. The real-time video of cell movement was recorded and the data were analyzed using the MetaMorph program to trace movement of 8–12 cells for each experiment. The left panel shows the trajectories of the cells under each level of gradient steepness, and the right panel shows the mean trajectory of cell movement for each experiment. Since the cell movement was driven by two forces, flow and chemotactic force, the trajectory of cell movement represents the net result of these two forces. With the increase of the steepness of chemokine gradient, the angle of the trajectories was more in line with the direction of the gradient. *Originally published in Sai et al. (2006).*