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Microfluidic generation of orthogonal chemical gradients to study cellular decision making during 3D migration

Joshua M. Campbell

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by

Joshua M. Campbell

Undergraduate honors thesis under the direction of

Dr. Adam T. Melvin

Cain Department of Chemical Engineering

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Louisiana State University
& Agricultural and Mechanical College
Baton Rouge, Louisiana

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Chapter 1: Investigation into the migratory behavior of cancer cells can be enhanced by the development of more physiologically-relevant systems

It is estimated that in the United States there were over 600,000 deaths related to cancer and over 1.7 million new cases of cancer diagnosed in 2018, with more than 80% of these deaths occurring after metastasis[3]. Additionally, over 250,000 women will be diagnosed and over 40,000 women will die of breast cancer in the United States during 2018[3]. Due to these overwhelming numbers, and cancer being the second leading cause of death in the United States, there has been a great deal of interest among researchers to better understand all aspects cancer so that these numbers may decrease in the future. Cancer metastasis begins with a cancer cell undergoing epithelial to mesenchymal transition (EMT) and migrating from a tumor to the vasculature through the process of chemotaxis[4]. In order to better understand metastasis, researchers must first better understand chemotaxis and how cancer cells are directed to migrate from a tumor to blood vessels.

Triple negative breast cancer cell lines, such as MDA-MB-231 cells, are negative for the estrogen receptor, progesterone receptor, and for HER2. As such, they are harder to treat than most other types of cancer using traditional therapies and are highly metastatic, making the mortality rate of patients with triple negative strains of breast cancer relatively high[5]. MDA-MB-231 cells have been shown to migrate towards single chemical gradients of the chemokines stromal cell-derived factor-1α (SDF-1α) and epidermal growth factor (EGF) within a microfluidic device[1]. Both SDF-1α and EGF are well-known proteins normally found within the body, and they both function as signaling molecules, directing cells to certain location within the body. These chemokines are also present within the tumor microenvironment resulting in competing gradients which can bias the migration of cancer cells[6]. There have been no studies, however, examining the chemotactic response of MDA-MB-231 cells to multiple chemical gradients which is more representative of *in vivo* conditions. The goal of this work is to fabricate a microfluidic device capable of making multiple gradients to which these cells can chemotax in order to gain a better understanding of how breast cancer cells respond to competing external cues.

Microfluidics started to become popular around 20 years ago with polydimethylsiloxane (PDMS) being adapted as the optimal material for making microfluidic devices[7]. PDMS is a flexible, gas-permeable, and transparent silicon-based polymer that is easy to work with and easy to imprint small channels into. Previously, microfluidic devices required more complex machinery

and tools, often being printed on rigid silicon chips or glass; however, PDMS allowed for rapid fabrication of microfluidic chips, taking less than 24 hours to make a working device[7]. PDMS replication, the process of undergoing the polymerization reaction resulting in solid microfluidic replicas, coupled with photolithography, made it easy to run experiments using microfluidic devices. Techniques of photolithography included the precise layering of photoresist on a silicon wafer prior to exposing parts of the photoresist to UV by covering the wafer with a mask. This technique allowed for the creation of molds that had channels as small as 20 μm wide[7]. Some of the early microfluidic devices included gradient generators, droplet generators, and cell culturing devices. One of the first gradient-generating devices worked by slow mixing and splitting of channels containing chemicals and buffer, resulting in a precise concentration gradient downstream (Figure 1.1)[2].The device worked by taking advantage of the laminar flow caused by the small network of channels, so the mass transfer through the device was caused primary by diffusion instead of convection. Other microfluidic gradient generators included porous hydrogels for molecules to diffuse through. One such example was a three-channel microfluidic device made of agarose, a polysaccharide chain extracted from algae, which forms a permeable membrane through which chemicals can diffuse[1]. Additionally, many other devices take advantage of flow in different ways in order to produce concentration gradients[8-11]. Eventually microfluidic devices were used to measure the chemotactic response of cells towards concentration gradients. Another device that is capable of measuring chemotaxis is the Boyden chamber, or Transwell assay[12, 13]. In this type of experiment, a well is divided into two sections separated by a membrane in the middle which

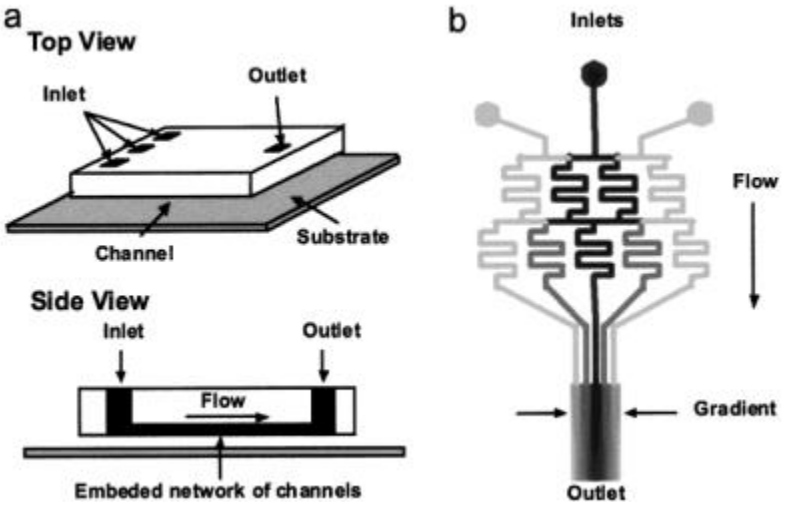

Figure 1.1. Microfluidic gradient generator. (a) Schematic of gradient-generating microfluidic device. The device is made of PDMS plasma bonded to glass and contains ports for fluid flow. (b) Device geometry included serpentine channels that promote mixing of buffer and chemical. A concentration gradient is formed downstream of the mixing channels. Figure reproduced from Jeon et al[2].

allows for diffusion of a chemical from the bottom of the well to the top. Cells are then placed in the top part of the chamber, and after a certain amount of time, cells that have burrowed through the membrane separating the two chambers are counted. This type of study, however, has disadvantages as it is an endpoint study in which cells are observed only at the beginning and end of the experiment. Though more difficult to set up, microfluidic devices allow for single cell, transient analysis of migration over the duration of a chemotactic experiment.

Many research groups have employed microfluidic devices to study the chemotactic behavior of cells to single chemical gradients[14]. Haessler et al. created a three-channel microfluidic device made out of agarose to make single chemical gradients to study the 3D chemotactic response of murine dendritic cells[10]. Kim et al. utilized a similar device to show the directed migration of MDA-MB-231 cells to single gradients of SDF-1α or EGF[1]. Park et al. used a modified version of the device using PDMS plasma bonded to glass in order to study mesenchymal stem cell migration to a single gradient of SDF-1α[9]. Frick et al. recently utilized a PDMS on glass microfluidic device to show dendritic cell migration to a single gradient of CCL19[11]. While these are a few examples of microfluidic devices used to create singular chemical gradients in order to study cell chemotaxis, there are many other similar devices reported in the literature.

The device created by Kim et al[1] is of special importance to this thesis project because their device formed the foundation for the method by which the gradient generators developed as the goal of this thesis operate. In the device from Kim et al, the chemoattractant flowed through the top channel of a three-channel arrangement while buffer flowed through the bottom channel to create a 'flow-free' chemical gradient (Figure 1.2). Cells were loaded in the middle channel of the device and diffusion through the porous agarose hydrogel allowed for a chemical gradient to form. The cancer cells within the device were subsequently tracked in order to measure the migratory response of the cells to these gradients, and the group showed that MDA-MB-231 cells will migrate up a single gradient of only SDF-1α or a single gradient of SDF-1α and EGF combined, while the presence of an EGF gradient alone resulted only in an increase in cell motility without a specific direction[1]. The first goal of this thesis aims to develop a similar three-channel microfluidic device presented by Kim et al while improving on the design to allow for broader applicability[1].

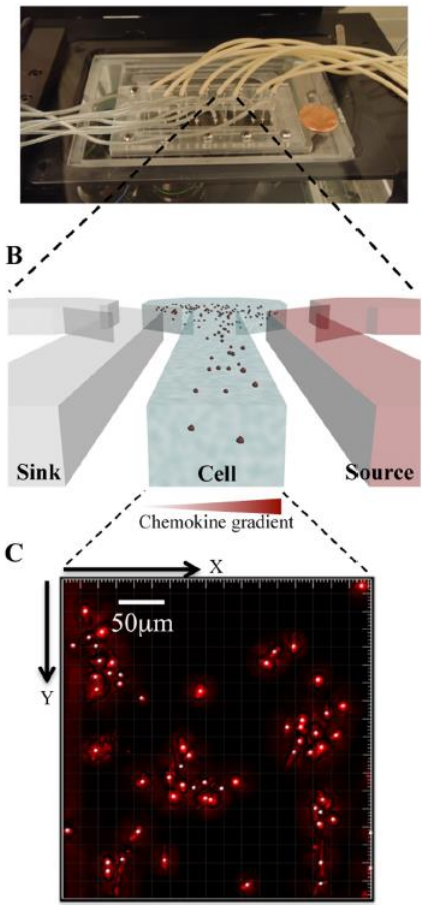

Figure 1.2. Three-channel device design and implementation. (A) Experimental setup of microfluidic device showing agarose between Plexiglas manifold and glass slide. Tubing provides flow of chemokines and buffer to device. (B) Individual device geometry contains three parallel microchannels with the outer channels comprised of a chemokine-containing source and a buffer-containing sink. The middle channel houses the cells in a 3D extracellular matrix and is the location of the concentration gradient. (C) Cells present within the middle channel for a single experiment. Figure reproduced from Kim et al[1]

In these previous microfluidic devices, single gradients of a chemical were formed, and cell migration was monitored in order to track their movement in relationship to the gradient within the device. After a migratory experiment takes place, the cell migratory data is analyzed to determine whether or not the cells moved preferentially up the chemical gradient. In order to quantify cell movement during the experiment, there are a few common parameters acceptable in literature. These include measuring migratory speed, distance travelled, directional persistence, and/or the chemotactic index[15]. Migratory speed is the speed at which individual cells are moving for the duration of an experiment. The migratory speed in the presence of the chemical gradient can be compared to a control experiment without the chemical in order to test if there is a statistical difference between the two. This parameter is calculated by tracking single cells in order to determine the total distance that the cells travel during an experiment and dividing by the total time of the experiment. Usually migratory speed would be analyzed on a single cell basis and not as part of a bulk analysis. Distance travelled can refer to either the total distance that a cell travels during an experiment or the displacement of the cell in the direction of the chemical gradient. Again, these values would then be compared to a control experiment without a chemical gradient present in order to determine if cells have a biased response to the chemical gradient being tested. Directional persistence is a cell's ability to travel in the same direction over time, meaning if a cell were to travel in only one direction for the entire duration of an experiment, that cell would be considered as having strong directional persistence. This

parameter typically involves monitoring cells and tracking their directional angle relative to some set angle. The last migratory parameter is chemotactic index, and this parameter is typically the most indicative of whether or not a cell type is migrating preferentially to a chemical gradient. The chemotactic index is calculated by dividing a cell's displacement in the direction of the gradient by the total distance travelled by the cell during the experiment. As such, a cell that travels only in the direction of the gradient would have a chemotactic index of one.

While there are numerous reports in the literature document the chemotactic response of cells migrating in response single chemical gradients, there are relatively few studies, and microfluidic devices, examining how cells respond to multiple chemical gradients that are orthogonal to one another. Only two such microfluidic devices have been reported in the literature, each capable of producing multiple chemical gradients and utilizing a similar geometry in order to do so – a diamond or square shaped cell channel surrounded by four flow channels to induce gradient formation[16, 17]. Fan et al developed a device using PDMS plasma bonded to glass with collagen in order to produce multiple gradients to study both epithelial (MCF-10A) and cancer (MDA-MB-231) cells[16]. Uzel et al. developed a cross-shaped device capable of producing orthogonal chemical gradients in order to show a rotational change in cancer cell migration as well as stem cell differentiation into motor neurons similar to the neural tube during development[17]. The device developed in this thesis is similar to these, but the device here will contain a central diamond shaped channel in PDMS with [17]a bottom agarose layer in order to gain advantages of multiple previous devices.

A two-fold approach was taken for this thesis to accomplish the goal of developing a microfluidic device capable of generating multiple concentration gradients to study how breast cancer cells respond to competing extracellular cues. First, a three-channel microfluidic device was designed that was inspired by previous geometries using a new method of PDMS on agarose. Once similar results with respect to gradient development and cell motility were obtained using the modified three-channel device, a new device would be designed with the capability of forming multiple concentration gradients orthogonal to one another As such, the second chapter of this thesis contains work related to the development of the three-channel device, and the third chapter discusses the design and fabrication of a workable microfluidic device for orthogonal gradient generation. Concentration gradients present within both of these devices were confirmed using fluorescent microscopy and modeled by numerical simulations. Each of the devices was also

optimized to allow for the 3D migration of breast cancer cells. As such, much of the work contained in this thesis focuses on troubleshooting the problems encountered while developing this new technology, culminating in a working orthogonal device ready to be used for cell migration experiments.

Chapter 2: Developing an alternative to common three-channel gradient generators to study cellular chemotaxis

2.1 Introduction

The first task to accomplish for this thesis was to fabricate an alternative version of the three-channel microfluidic device capable of delivering a single flow-free chemical gradient. Because the orthogonal device, which is the ultimate goal for this thesis, combines both a new device setup and a new geometry, it was first necessary to test the device setup to ensure workability and performance. The three-channel design was chosen because previous studies used similar methods to create concentration gradients as the PDMS on agarose method proposed in this thesis[1]. This device geometry was also "flow-free", which allowed for a more accurate recreation of the tumor microenvironment. The results obtained from the three-channel device could also be able to replicate previous findings and provide a comparison between the response of cells migrating in response to single concentration gradients and competing, orthogonal concentration gradients. With these goals in mind, a three-channel microfluidic device was fabricated using a PDMS on agarose approach, and the concentration gradients present within the device were characterized by fluorescent microscopy and computer simulations prior to optimizing the device for experimentation with cells.

2.2 Materials and Methods

2.2.1 Design of the three-channel microfluidic device

A library of three-channel device geometries were designed and fabricated on silicon masters using normal photolithography techniques. The microfluidic device setup is composed of two layers: a top PDMS slab with the three fluid channels imprinted into it from a silicon wafer and a bottom layer composed of agarose to allow diffusion of chemokines to occur (Figure 2.1). The two layer device is encased in two Plexiglas pieces on the top and bottom which are screwed together to keep the PDMS and agarose from separating and allowing fluid to leak out of the channels within the device, ruining the chemical gradient. The device is similar to the device

developed by Kim et al (Figure 1.2) in that the chemical gradient forms within the flow-free central channel where the cells are housed within a 3D collagen matrix, allowing for cell movement[1]. Top and bottom flow channels are used as a source channel that has some concentration of chemoattractant (top) and an opposite sink channel that contains only buffer without any chemoattractant (bottom). With this setup, either a single gradient can be formed or parallel gradients of two different chemoattractants in opposite directions may exist by having the source channel of one chemoattractant be the sink channel for the other chemoattractant and vice versa. The specific geometry used in this chapter was chosen based on its potential to form stable chemical gradients. The chosen dimensions were 600 µm wide fluidic channels that were spaced 200 µm apart (Figure 2.1 A). The total contact area for the three channels was 10 mm and the height of the fluidic channels was 150 µm.

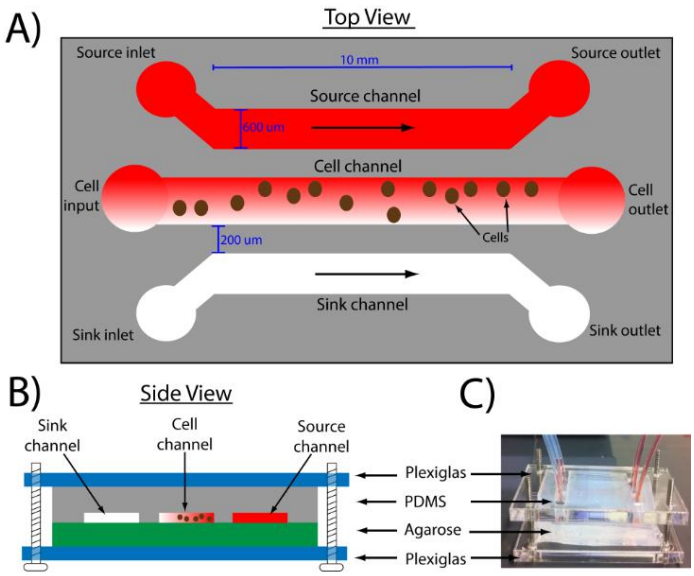

Figure 2.1. Three channel design and fabrication. (A) Three channel device schematic showing chosen device dimensions and overall device geometry. The top channel acts as a source for chemoattractant while the bottom channel provides a sink, therefore allowing diffusion to take place from the top of the device to the bottom. The center channel houses cells in a collagen matrix where the concentration gradient is present. (B) Three channel device setup with completed device. Each layer of the device is labelled with the blue representing the Plexiglas pieces, the gray representing the PDMS with imprinted channels, and the green representing the agarose layer through which diffusion takes place. All layers are held in place by tightening screws through the Plexiglas pieces. (C) Tubing can also be seen attached to the PDMS layer and going through the top Plexiglas.

2.2.2 Wafer fabrication

To make the silicon master, a modified photolithography protocol was followed as previously described (Figure 2.2)[18]. The geometry of the device was designed with AutoCAD software prior to fabrication. A two-step soft-lithography was used to fabricate the 150 µm tall

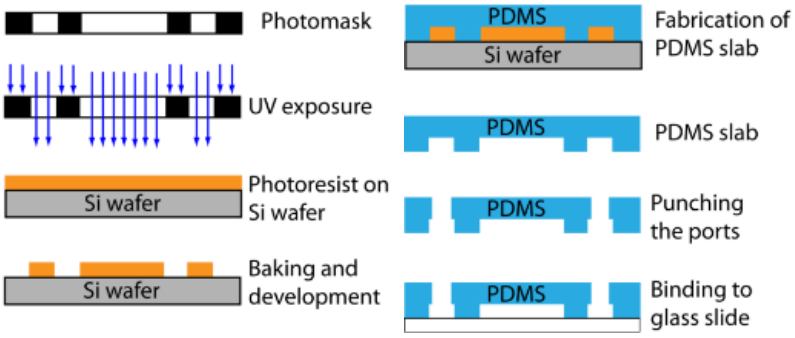

Figure 2.2. Process of constructing a silicon master and a PDMS device using standard techniques of photolithography and PDMS replication.

fluidic channels onto the silicon master. First, a 75 µm layer of a negative photoresist polymer, SU-8 2050 (Microchem) was deposited on a clean 3" silicon wafer and baked at 65 °C for 10 min followed by a second bake at 95 °C for 20 min. Next, a second 75 µm layer of SU-8 2050 was deposited onto the wafer to make a 150 µm thick layer of SU-8. After cooling down, the wafer was exposed to UV light with 1.2 mW/cm^2 power intensity for 80 seconds using a transparency mask (CAD/Art) to create the fluidic channels. The wafer was baked again at 65 °C for 15 min and at 95 °C for 30 min, post UV exposure. The silicon wafer was developed with an SU-8 developer solution (Microchem) to remove the un-crosslinked SU-8 to produce the microfluidic patterns. The wafer was hard baked at 120 °C for 60 min to increase wafer durability. The following day, the wafer was placed in a desiccation chamber for an hour under vacuum with a petri dish containing a few drops of a trichloro(1H,1H,2H,2H-perfluorooctyl)silane in order to coat the wafer. After silanization, the wafer was ready to be used in order to make PDMS devices (Figure 2.3 A).

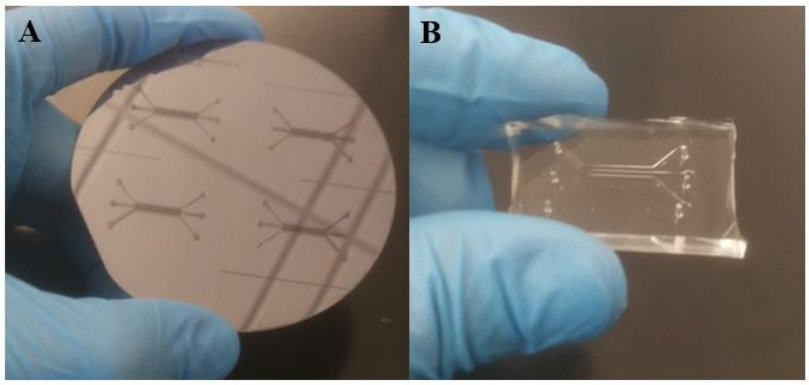

Figure 2.3. Three-channel wafer and PDMS device. (A) Three-channel device silicon wafer for a 600 µm channel width, 200 µm spacing between channels and 150 µm tall channels. (B) PDMS cut out of three-channel device with holes punched out for tubing ports.

2.2.3 Assembly of three-channel microfluidic device

To construct the working PDMS on agarose device, a 5:1 ratio of PDMS base to curing agent was mixed thoroughly until homogenous. The mixture was degassed in a desiccation

chamber for approximately 45 minutes or until all the bubbles were removed. The PDMS mixture was next poured over the silicon master (see details on wafer fabrication in section 2.2.2) within a petri dish and placed on a hot plate at 65°C overnight. After the PDMS cured and the wafer was removed from the hot plate, the PDMS was peeled off the wafer, and the individual three-channel devices were cut out with holes punched for tubing ports using a 23 gauge blunted needle (Figure 2.3 B). Tubing was then fixed into the ports using uncured PDMS with the top Plexiglas piece in place. Three percent agarose (w/v) was made using deionized water and autoclaving the mixture for the agarose powder to enter into solution. All device components including the PDMS with tubing and top Plexiglas layer, bottom Plexiglas layer, and screws and nuts were autoclaved to ensure sterility. All the pieces were placed in a biosafety cabinet to assemble the complete device under sterile conditions. An ~1 mm thick slab of agarose was poured into a 60 mm dish and cut out in an ~1 in. x 1.5 in. rectangle to fit the bottom of the device. The agarose layer was placed on top of the bottom Plexiglas housing before laying the PDMS layer and top Plexiglas piece on top of the agarose. The top Plexiglas piece contains holes for threading the tubing through. The two Plexiglas pieces were secured together using screws and nuts, which were subsequently tightened to prevent leaking of the device (Figure 2.1 C).

2.2.4 Fabrication of a custom-built temperature control box for a light microscope

Typical cell migration experiments are performed at 37°C for 12-24 h. An advantage of the microfluidic approach is the ability to directly visualize cell migration using light microscopy; however, prior to experimentation it was necessary to build a temperature control chamber for the lab microscope. The box was designed in AutoCAD, and then the sides were cut out of quarter-inch thick Plexiglas using a laser printer within the chemical engineering machine shop. The sides of the box were assembled using epoxy so that they would stay together. The box was designed so that when put together, two sides would be placed around the lab microscope enclosing the stage where the microfluidic device will be positioned during experimentation. A removable top allowed for the placement of the device on the microscope after preheating the chamber to 37°C. Holes were cut out of the side of the box to allow for a heating element to be connected and to allow for microtubing to pass through to connect the device to the syringe pumps. A sliding door was also added in order to manipulate the stage and focus of the microscope after the device was set up

(Figure 2.4). To heat the box to 37°C, a hairdryer was connected to tubing that was connected to a hole in the box, and the hairdryer was plugged into a potentiostat to control the amount of power supplying the hairdryer, which in turn controls the amount of heat produced by the hairdryer. The potentiostat was varied in its output, and a thermocouple connected to a temperature gauge was used to monitor the temperature until it reached the desired 37°C, at which point the potentiostat output would be set. A bowl of water was placed in the box to create a humidified environment.

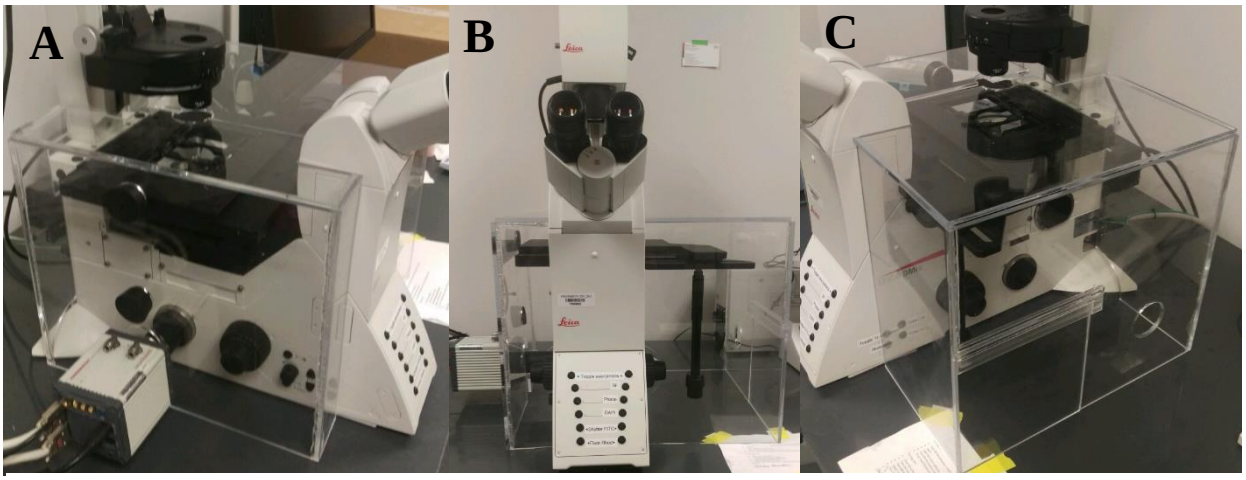

Figure 2.4. Constructed microscope temperature control box. (A) Left side of temperature control box, containing two holes for inserting tubing that are covered with rubber stoppers while not in use to prevent heat loss and a hole for the camera to fit through; (B) front view of temperature control box, the whole box is in two pieces that fit around the microscope; (C) right side of temperature control box, containing hole for tubing connected to heating unit, sliding door for stage control and focusing during an experiment, and removable top to access stage.

2.2.5 Fluorescent gradient characterization

Experimental characterization of the chemical gradient within the microfluidic device was performed using fluorescent microscopy. The gradient was visualized using and a green fluorescent molecule, fluorescein isothiocyanate (FITC), conjugated to 6 kDa dextran (Sigma). This 6 kDa version of FITC-dextran was used due to its similar size to SDF-1α and EGF, which are 8 kDa and 6 kDa respectively. The similarity in size of the molecules more closely mimics the diffusion to be expected by the chemoattractants while allowing the fluorescence to be detected using a fluorescent microscope. A fluorescent DMi8 inverted microscope (Leica microsystems) with a digital CMOS camera C11440 (Hamamatsu Photonics K.K.) and LAS X software 3.3.0 were used to image and analyze fluorescent signals. The following excitation/emission filters

(Chroma Tech. Corp) were used: fluorescein isothiocyanate - FITC (λ_{ex}: 440-520 nm and λ_{em}: 497-557 nm) and rhodamine (λ_{ex}: 536-556 nm and λ_{em}: 545-625 nm). The gradient data gathered from the fluorescence microscopy experiments would allow any possible future gradient experiments with chemotaxing cells to be directly linked to the magnitude and direction of the gradient at the specific time during the experiment and location of the cell within the central channel.

2.2.6 COMSOL simulations of chemical diffusion within the microfluidic device

COMSOL Multiphysics is a powerful simulation platform used to model fluid, heat, and mass transport. To use COMSOL, the device geometry was drawn and the materials used within the device were defined. The device dimensions for the channel width, height, length, and spacing were all specified within COMSOL along with the corresponding thickness and dimensions of the agarose slab (Figure 2.5). The porosity of 3% (w/v) agarose used within the simulation needed to be user-defined, as COMSOL does not contain agarose in its pre-defined library of materials, and the value for porosity was taken from Pluen et al who reported a porosity of 0.971 for 3% agarose[19]. The fluid component, water, was already defined within COMSOL. The Laminar Flow physics tab was used to simulate the flow of fluid through the microfluidic channels, which is valid since the flow is well within the laminar flow regime with a Reynold's number less than ten, and the Transport of Diluted Species physics tab was used to model the mass transport through the device. Both convective and diffusive mass transport were used in the simulation. The Laminar Flow tab assumes the no slip boundary condition in its calculations with incompressible flow. The fluid material was set to water, and a flowrate of 15 μL/min in the top and bottom channels was specified. For the Transport of Diluted Species tab, diffusion through a porous membrane was selected to simulate the diffusion through the agarose slab, and the diffusion coefficient for SDF-1α and EGF through 3% (w/v) agarose was

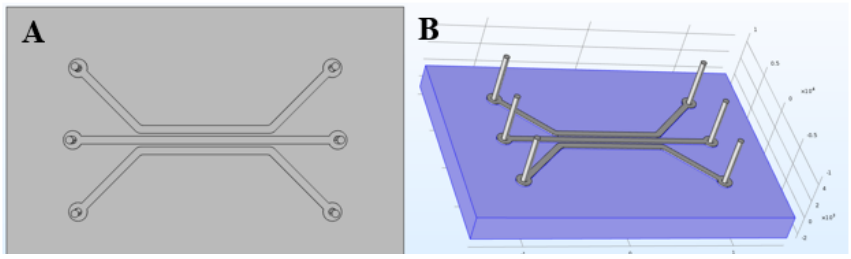

Figure 2.5. Three-channel device geometry as input into COMSOL. (A) Top view of three-channel device showing the channel spacing and overall device geometry, (B) 3D schematic of entire device setup as input into COMSOL with block highlighted in blue as agarose, channels on top of agarose, and tubing visualized above.

set to 110 $\mu m^2/s$ as reported by Kim et al[1]. Convective mass transport was also simulated by using the velocity results from the Laminar Flow tab calculations. The concentration of chemoattractant in the top source channel was specified as 40 nM. COMSOL uses the continuity equation as well as Fickian diffusion to simulate the mass transfer within the fluid and through the agarose. The simulation calculates the effective diffusion coefficient through pores based on the input porosity and the Millington and Quirk model.

2.2.7 Cell preparation and injection into the microfluidic device

For experiments with cells, all device assembly was done in a biosafety cabinet to prevent device contamination. MDA-MB-231 cells were cultured with DMEM (Corning) supplemented with 10% calf serum, MEM essential and nonessential amino acids (1x each), sodium pyruvate (2 mM), and insulin (10 μg/mL) in T-75 flasks and passed regularly when flasks reached approximately 80% confluency. The initial procedure for loading a device with cells comprised of autoclaving all device parts and assembling the complete device under a biosafety cabinet to prevent contamination. After constructing the device, cells would be prepped by suspending cells at approximately one million cells per milliliter in complete media followed by mixing with collagen type I (Corning) to achieve a final concentration of 2 mg/mL collagen. Mixing of cells and collagen was done on ice so that collagen did not polymerize prior to loading into the central channel of the microfluidic device. After gently mixing cell and collagen solution to ensure homogeneity and preventing bubbles within the sample, the mixture was loaded into a syringe and manually injected into the center channel of the microfluidic device. The tubing leading into the center channel was then capped on both sides using blunted and closed-off 23 gauge needles to prevent evaporation of liquid from the channel and to prevent contamination. Flow of culture media through the device would then be established at 15 μL/min in an incubator maintained at 37°C and 5% CO_2. Cells would be allowed to attach and spread in the collagen matrix within the device overnight prior to starting a migratory experiment. For a random migration experiment, the device was supplied with complete media in both the top and bottom channels. For a directed migration experiment, the device was supplied with media supplemented with 20% FBS or 60 nM EGF in the top channel and complete media in the bottom channel. Typical migratory experiments would last anywhere from 12 to 20 hours with images collected every 3 minutes. All experiments were performed on the Leica DMi8 microscope described above using phase contrast microscopy.

2.3 Results and Discussion

2.3.1 Experimental characterization of gradient formation within the microfluidic device using fluorescent microscopy

Gradient characterization experiments were performed by first assembling a three-channel microfluidic device. After the device was assembled and checked for the presence of any leaks,, it was placed under the fluorescent microscope. FITC-dextran (10 mM) was flowed through the top channel, acting as the source, and DI water was flowed through the bottom channel, acting as the sink. The development of the gradient was visualized using fluorescent microscopy by collecting images every 3 minutes for a total of eight hours (Figure 2.6). After the experiment ended, the images were collected and analyzed with the Leica software by taking a line scan across the width of the center channel (600 μm) to collect the fluorescent intensity values across the width the 'flow-free' channel. Because fluorescent intensity is proportional to the relative concentration of FITC-dextran, the observed gradient can be qualified by the intensity changing in the channel from source to sink. The intensity values were plotted against the position within the channel at regular time intervals and shown that a gradient does exist within the center channel (Figure 2.7), and the shape of the plot suggests that this gradient is linear, or constant, across the width of the channel.

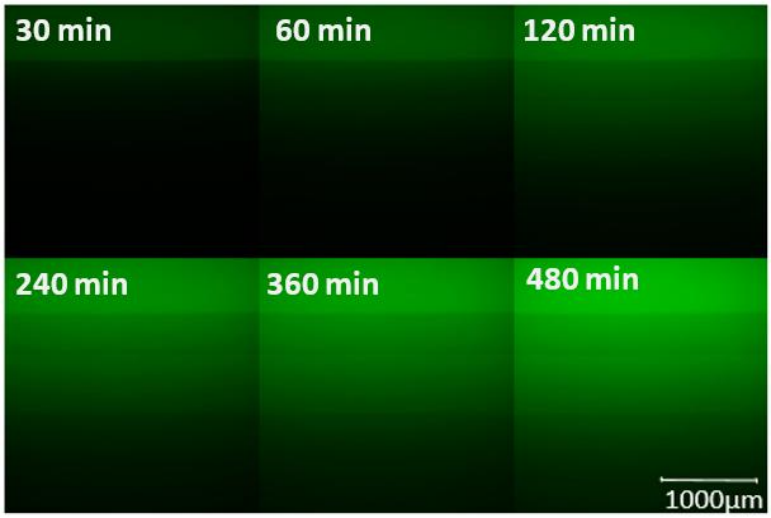

Figure 2.6. Montage of fluorescent images during gradient characterization in three-channel device. As seen in the pictures from a fluorescent experiment, a concentration gradient forms across the middle channel as FITC-dextran diffuses from the top source channel to the bottom sink channel. Scale bar is 1000 μm.

A complete characterization of the chemical gradient within the microfluidic device could not be done by fluorescent imaging alone. Due to the thickness of the agarose layer, it was observed that the FITC-dextran would accumulate within the agarose slab in the z-

direction. This made approximating the concentration across the center channel difficult because the intensity recorded by the camera was dependent not only on the concentration of fluorophore present within the sample, but also on the physical amount of fluorophore. This meant that the intensity was more closely estimated by the concentration times the volume instead of the concentration alone. This reasoning was verified with a few sample calibration tests in which the same concentration of FITC-dextran with different volumes was placed in wells within a 96-well plate under the microscope, and the intensity values recorded increased as the volume increased (data not shown). Interestingly, the presence of the agarose was also found to affect the observed intensity values. Tests were performed by adding layers of agarose at the bottom of the wells with varying thickness in addition to dilute amounts of FITC-dextran. It was observed that the presence of agarose within the well caused changes in the observed intensity of the FITC-dextran (data not shown). To obtain a quantitative value for the concentration profile within the device, it was decided to perform numerical simulations using COMSOL Multiphysics to simulate both fluid and mass transport. Thus, the simulations became a quantitative tool to characterize the gradient within the microfluidic device whereas the fluorescent microscopy experiments remained a qualitative test to verify experimentally that a concentration gradient was actually being generated within then center channel.

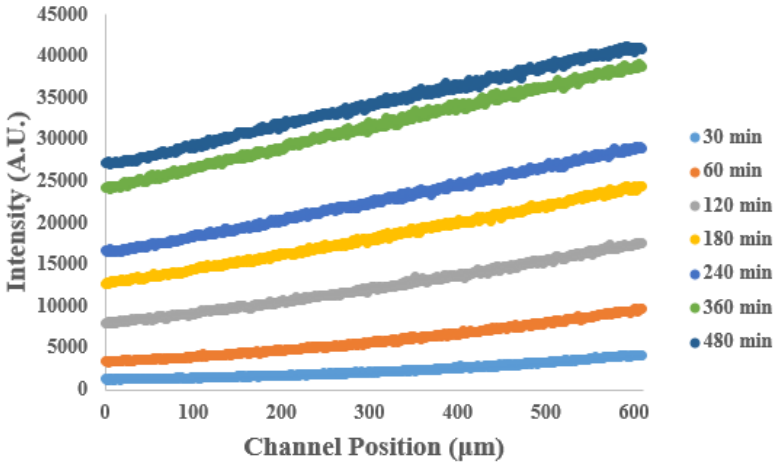

Figure 2.7. Plot of fluorescent intensity values through the center channel in the three-channel device. Intensity values were reported from the microscope software, and a channel position of 0 µm corresponds to the side nearest the sink channel whereas a channel position of 600 µm corresponds to the side nearest the source channel. A distinct linear gradient can be seen across the channel.

2.3.2 COMSOL simulations to quantify the concentration profile within the microfluidic device

Time-dependent diffusion studies were simulated across the center channel of the device for up to 24 hours with data points collected every 15 minutes. Concentration data were taken for a line scan across the width of the center channel and plotted using Origin (Figure 2.8). Heat maps showing the mass transfer across the entire device were also generated to compare with the fluorescence images taken from the microscope (Figure 2.9). The results from COMSOL correlate with the results obtained from the fluorescent microscopy experiments in terms of gradient shape which provides confidence in the experimental values and confirms the presence of a chemical gradient within the microfluidic device.

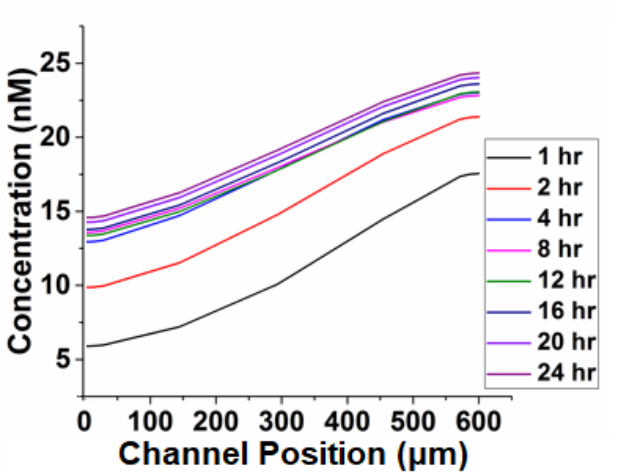

Figure 2.8. Concentration of chemoattractant in the center channel of the three-channel device as generated by COMSOL. Concentration data were taken by drawing a line across the middle of the center channel from the bottom of the channel to the top. 0 μm corresponds to the wall of the center channel closest to the sink, and 600 μm corresponds to the wall of the center channel closest to the source.

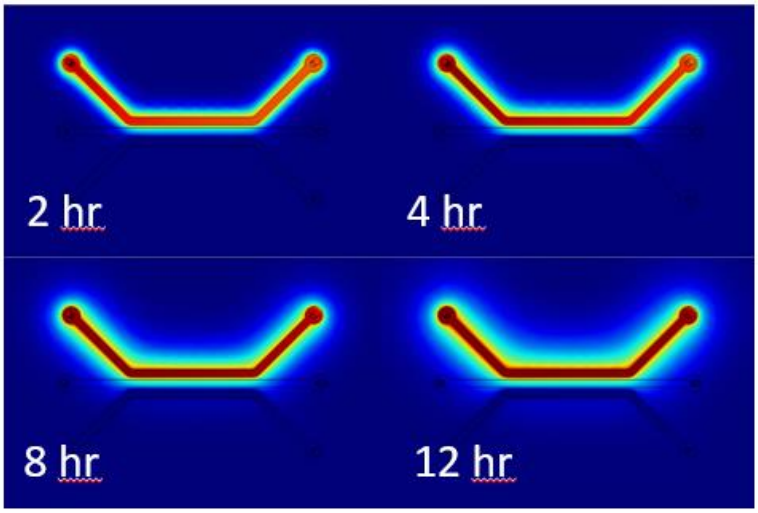

Figure 2.9. Heat map of concentration in three-channel device as predicted from COMSOL simulations. Red represents the highest concentration coming from the source channel – 40 nM, and blue represents a concentration of 0 nM. COMSOL shows a concentration gradient form within the center channel as chemoattractant diffuses from the source channel to the sink channel over time.

2.3.3 Optimization of three-channel microfluidic device for cell migration

Initially, some complications were encountered while running migration experiments using the microfluidic device. These problems included the center channel drying out, cells rupturing and/or lacking polarized morphology within the migration channel, and collagen detaching from channel walls (Figure 2.10). To prevent the device from drying out, the experimental protocol was

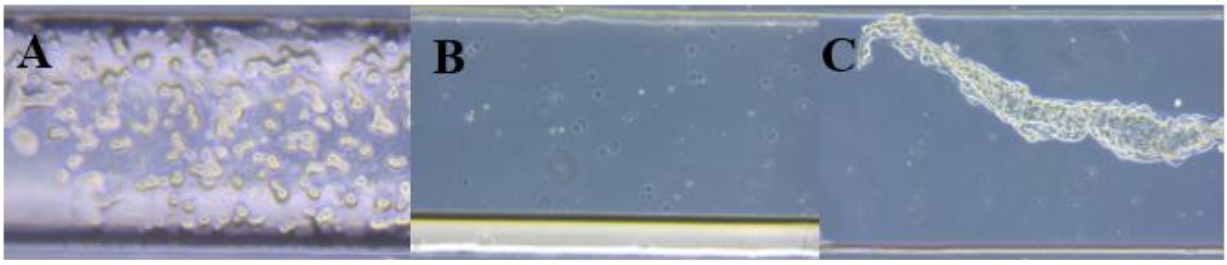

Figure 2.10. Complications encountered while optimizing three-channel device for experiments with cells. (A) Center channel of device drying out during an experiment, (B) cells within center channel dark and without a polarized morphology after 24 hours within device, (C) collagen detaching from PDMS walls and folding in on itself.

modified (1) to flow media through the outer channels for at least an hour after assembling the device, prior to cell injection, and (2) to immerse the device in media during this priming step to saturate the agarose layer with liquid. The device was also wrapped in Parafilm to retain moisture within the device while in the incubator. The collagen used is acidic, so in order to maintain cell viability and observe a mesenchymal morphology, 1M sodium hydroxide was added to the cell/collagen suspension to adjust the pH to slightly basic conditions. Using a basic pH had the dual effect of increasing cell viability and normal phenotype as well as causing the density of the collagen matrix to increase, as increasing pH has been shown to increase the polymerization of collagen as well[20]. The collagen detachment was alleviated by allowing for the collagen to solidify at room temperature for at least 45 minutes prior to moving

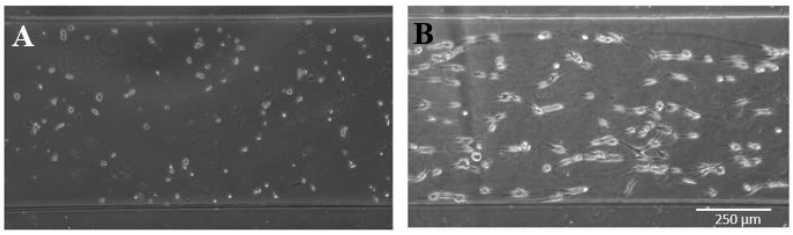

Figure 2.11. MDA-MB-231 cells within the center channel of a three-channel device after implementing protocol changes. (A) Cells within the three-channel device directly after injecting them into the center channel, (B) the same set of cells within the three-channel device 24 hours later directly before beginning a migratory experiment. Cells are seen attached to the collagen matrix, and many have adopted a mesenchymal morphology.

the device to the incubator and re-establishing flow through the device. Once these changes to the protocol were implemented, the device became useable for migratory experiments with cells (Figure 2.11).

Initial migration experiments were performed without the presence of a chemical gradient to observe their random motility at room temperature. However, these early experiments did not result in the movement of the cells. Upon reviewing the literature, , it was noticed that many groups run their experiments in a temperature and humidity controlled environment similar to that of an incubator in which the cells are normally kept or similar to that of the normal human body. It was decided, therefore, that experiments should be run at 37°C in a humidified chamber instead of at ambient temperature of around 22°C. In order to maintain a constant temperature around the microscope where experiments are performed, a temperature control box was made to place around the microscope while experiments were being run (Figure 2.4).

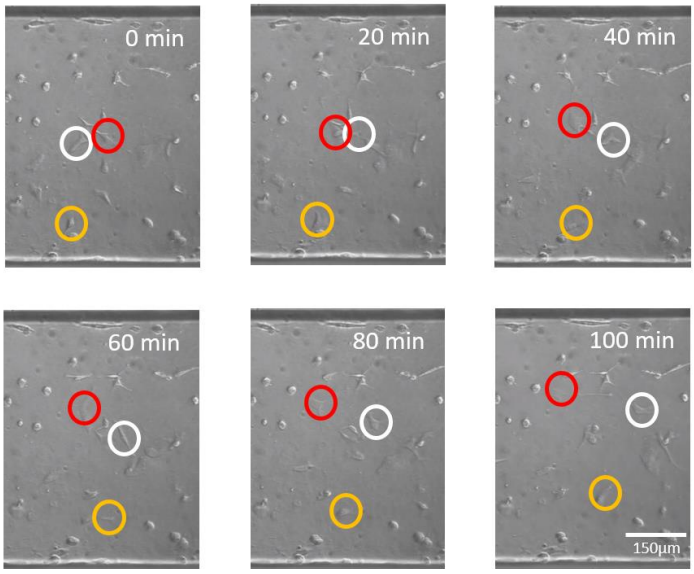

Figure 2.12. Tracking single cells through the center channel of a three-channel device during a random migratory experiment. Manually tracking the movement of three cells (indicated by the three colored circles) over time during a random migratory experiment. For this experiment, pictures were taken every two minutes. Time steps shown are normalized with time 0 min occurring ~2 h into the actual experiment.

2.3.5 Single cell tracking of migrating cells within the three-channel device

Once the setup for experiments was optimized, random migratory experiments were conducted by flowing full cell media through the top and bottom channels of the device in order to observe the normal movement of MDA-MB-231 cells seeded in collagen within the center channel. These cells were observed for periods between 16 and 20 hours. During experiments, cells were oftentime observed to undergo epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET) as the cells switch between highly migratory and stationary phenotypes (Figure 2.12).

To provide some information on the movement of the cells, single migrating cells were tracked using the software Tracking Tool™ PRO v2.1 to generate migratory plots to visualize the movement of cells over time (Figure 2.13). Cells that moved out of the visual field of the microscope, or cells that underwent cell division during the experiment, were excluded from the analysis and not tracked. Cells within the device showed normal movement consistent with previously published results[1]. Migratory plots such as in Figure 2.13 are used in order to show which cells had an overall displacement in the direction of the gradient or opposite the gradient. In Figure 2.13, though the specific experiment was under full media conditions, the tracks shown in blue represent cells that had a net displacement in the positive y-direction (moved towards the top channel), and tracks in red show cells that had a net displacement in the negative y-direction (moved towards the bottom channel) over the duration of the experiment.

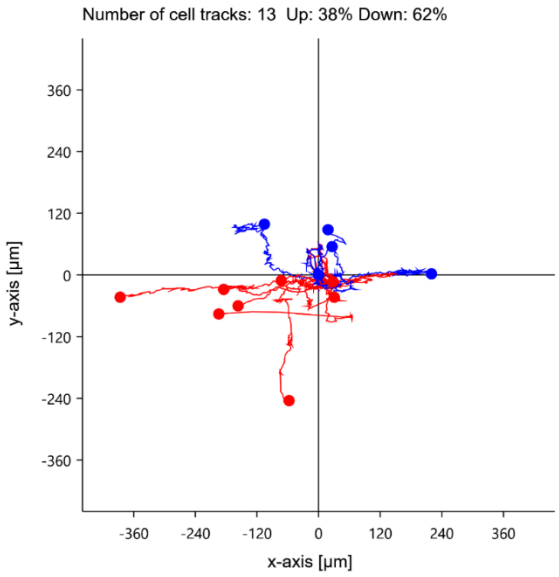

Figure 2.13. Example migratory plot from tracking single cells during a random migration experiment with full media in the top and bottom channels of the three-channel device.

Chemotaxis studies were also run in order to measure the migration response of MDA-MB-231 to a concentration gradient of EGF. A solution of 40 nM EGF was flowed through the top channel of the three-channel device for an 8 h period before switching to 1x PBS through the top channel for the next 8 h (total experiment time 16 h). A solution of 1x PBS was flowed through the bottom sink channel for the entire experiment. The cells were tracked using tracking software and were observed to initially not migrate towards the source channel during the first 8 hours, but during the last half of the experiment with 1x PBS flowing through both flow channels, the cells began to show directed migration towards up the gradient of EGF (Figure 2.14). These results suggest that the cells exhibited a memory response towards the gradient that formed during the

first eight hours of the experiment by migrating up that gradient during the second half of the experiment when the gradient was disappearing.

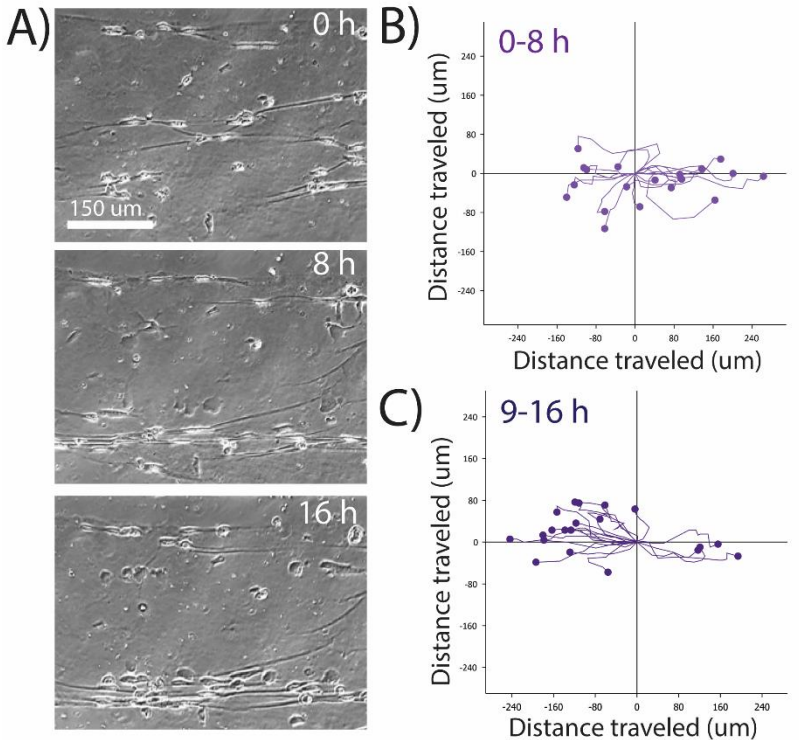

Figure 2.14. Cellular chemotaxis response to an EGF concentration gradient. (A) Pictures of MDA-MB-231 cells migrating through collagen matrix within the center channel of a three-channel device in response to a concentration gradient of EGF. (B) Cell migration tracks during the first 8 h while flowing 40 nM EGF at 15 μL/min in the top source channel. Cells initially move primarily in the x direction, parallel to the gradient formed in the device. (C) Cell migration tracks during the last 8 h while 1x PBS was flowed through both top and bottom channel of the device. Cells show a positive chemotactic response where the gradient was previously generated.

2.4 Conclusions

A three-channel microfluidic gradient generator comprised of PDMS on agarose was developed for tracking single cell migration in response to single concentration gradients. Further experiments could be run in the three-channel device to track the directed movement of certain cells to different chemoattractants; however, because single cue migration has been well studied in the literature, it was chosen to begin developing the orthogonal device which is more similar to *in vivo* conditions and has rarely been observed in the literature. The three-channel device, however, set the basis for microfluidic gradient generators for this project, as we obtained similar chemical gradient results to those found in literature along with similar cell movement within a collagen matrix. Furthermore, the PDMS on agarose design was novel and allows for some advantages over similar agarose only or PDMS plasma-bonded to glass devices. Some of these advantages include taking the device apart after experimentation to stain or further experiment with specific cells, superior gradient control over long periods of time, and a flow free environment where cells are migrating.

Chapter 3: Design and optimization of a two-layer microfluidic device capable of producing stable orthogonal chemical gradients to observe cellular chemotaxis

3.1 Introduction

Different cells within the human body such as cancer cells, fibroblasts, and neutrophils have been shown to respond to concentration gradients of different types of chemicals and proteins[1, 9-11]. Because cells will respond to single gradients of many different chemoattractants, it is necessary to study the decision making of these cells in the presence of many different chemical gradients simultaneously to better understand cellular migration and chemotaxis and thus better understand the cellular processes that are guided by chemotaxis such as the immune response and cancer metastasis. There are limited available technologies that are capable of producing multiple competing chemical gradients[16, 17, 21] and only some of these devices have been used to study cell chemotaxis[17]. As such, the focus of this chapter is to address this limitation to design, characterize, and utilize a microfluidic gradient generator that is able to produce stable chemical gradients orthogonal to one another to study single cell migration and decision making. Similar to Chapter 2, the device geometry was created in AutoCAD and the chemical gradients produced in this new design were simulated using COMSOL. Once a device geometry was shown to produce stable linear gradients over time, photomasks were ordered and a silicon master was fabricated using the chosen device geometry. The chemical gradients produced in the device were then verified using fluorescence, and a working and functional device was developed.

3.2 Materials and Methods

3.2.1 Design of the microfluidic orthogonal gradient device

AutoCAD was used to draw the geometry for the orthogonal gradient device to determine channel spacing and visualize overall device workability (Figure 3.1). The orthogonal gradient

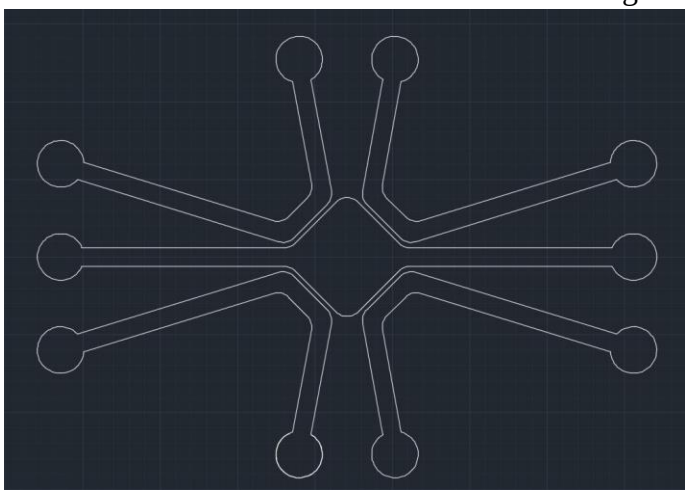

Figure 3.1. AutoCAD drawing of proposed orthogonal device geometry. Spacing between inner diamond and outer flow channels is 150 μm, and diamond is a square with 3 mm side length. Scale bar is 2.5 mm

device was designed to produce chemical gradients orthogonal, or perpendicular, to one another across a channel containing a 3D matrix of cells. With this goal in mind, the device has a central cell chamber in the shape of a diamond with flow channels surrounding each of the four sides of the middle channel. With this design, it was theoretically possible to produce concentration gradients of two different chemicals orthogonal to one another by using the top two channels as sources for the first chemical and using the left two channels as sources for the second chemical. The top left channel would have a combination of both of the chemicals (Figure 3.2).

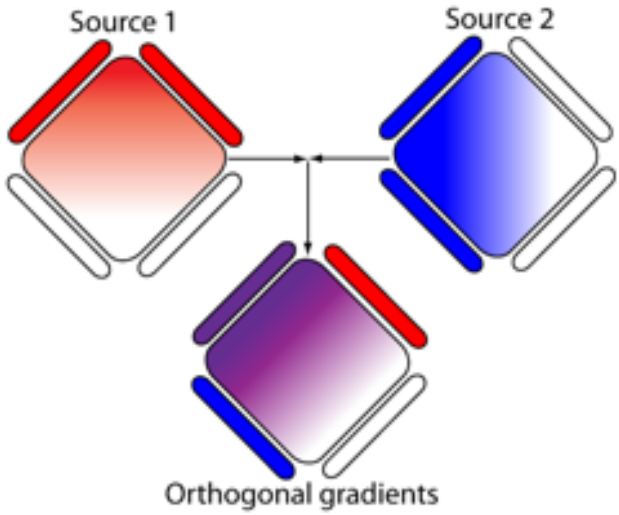

Figure 3.2. Schematic showing the formation of the orthogonal gradients within the device. As shown, one gradient forms from the top down, and one gradient forms from the left to the right.

The chosen orthogonal geometry has a central diamond that is a square with a 3 mm side length and rounded corners. The channels surrounding the middle channel are 600 µm wide, and there is a 150 µm space between the outer flow channels and the middle cell culture diamond (Figure 3.3). A few other device geometries were drawn and tested, including a 5 mm side length diamond, a 1.5 mm side length diamond, and each diamond size with 150 µm, 600 µm, and 1200 µm spaces separating the outer channels from the center channel. The 3 mm side length with 150 µm side channel spacing was chosen due to the steepness of the chemical gradients formed within the center channel as well as the flow profile through the device. All geometries, however, were designed such that the ports

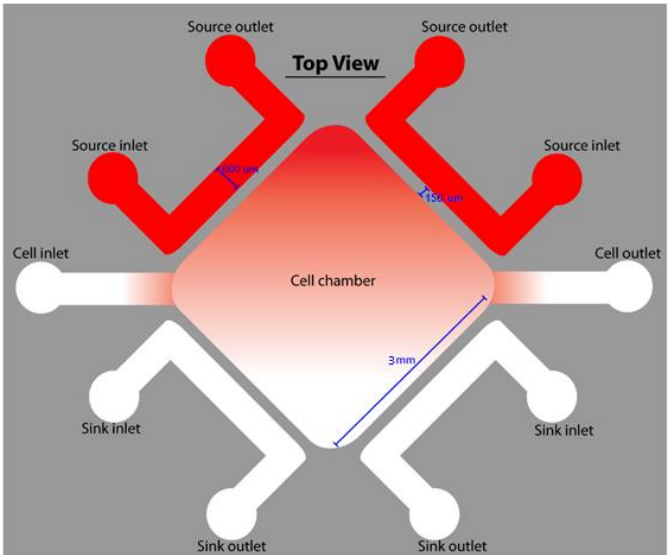

Figure 3.3. Orthogonal device with chosen geometry and spacing. The cell chamber is a diamond with side length 3mm. Each of the flow channels has a width of 600 µm, and the space between the flow channels and center channel is 150 µm.

for each of the channels of the different spacings lined up to the same distance relative to the middle of the center channel. They were designed this way so that only one set of top Plexiglas pieces could be constructed to fit all possible geometries.

3.2.2 Fabrication and assembly of the microfluidic orthogonal gradient device

To assemble the device, a similar protocol was used as was described in section 2.2.3. PDMS was mixed in a 5:1 ratio of base to curing agent before being degassed and pouring over the new orthogonal wafer to polymerize on a hot plate at 65°C overnight. The PDMS was then cut out and ports were punched using a 23 gauge blunted needle (Figure 3.4) prior to connecting tubing to the ports and sealing with freshly mixed PDMS. All parts of the device are autoclaved prior to assembly, and agarose is prepared at 3% (w/v) using deionized water. The entire device was assembled as described in section 2.2.3with the PDMS layer on top of the agarose layer and the entire device enclosed by two Plexiglas pieces which were held together by six screws instead of four. Flow could then be established into the device through the four outer channels. For experiments in which two sets of channels contained the same fluid (e.g., both top channels containing the same concentration of fluorescent molecule and the both bottom channels containing buffer without fluorescent molecule) the channels containing the same fluid were connected together by the tubing from those ports using a blunted 23 gauge needle that was open on both ends. Using the connector, the experiment would be able to run with only one syringe pump, as each pump is capable of holding up to only two syringes at one time.

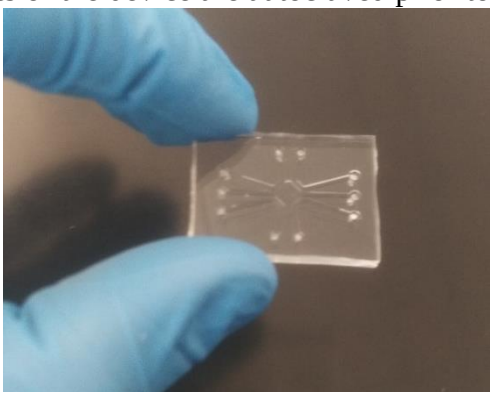

Figure 3.4. PDMS replica of the orthogonal gradient device with holes punched at the inlets and outlets of all of the channels.

3.2.3 Fabrication of the silicon master for the orthogonal gradient device

The next step in the design process was to fabricate a silicon master based on the geometry of the orthogonal device. The fabrication process used to make the wafer was the same as described in section 2.2.2 with the difference of using a new photomask that had the desired orthogonal

device design (Figure 3.5). To change the height of the channels (further discussed in 3.3.3.1), the wafer fabrication had to be altered so that instead of two layers each of 75 μm of SU-8 2050, four layers each of 75 μm of SU-8 2050 were added to the silicon master. These additional layers also required more baking steps between layers, and each subsequent bake between spin coats had to increase in duration so that the most recent layers were properly solidified prior to adding the next coat. The thicker layer of SU-8 also posed the challenge of increasing the UV exposure time so that complete layer underneath geometry was properly reacted before developing. Using this modified protocol, a new wafer was made that had the same geometry as before but with a taller channel height.

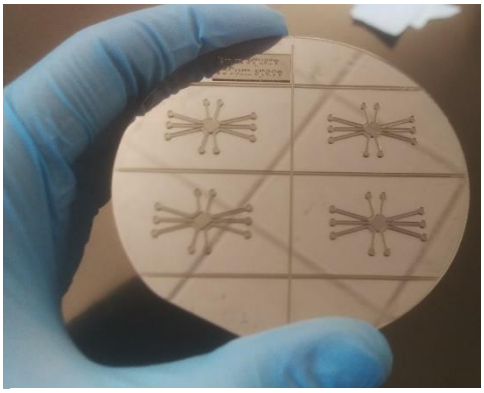

Figure 3.5. Silicon wafer with orthogonal design. Chosen geometry was a 3 mm diamond side length and a 150 μm spacing between outer channels and central diamond.

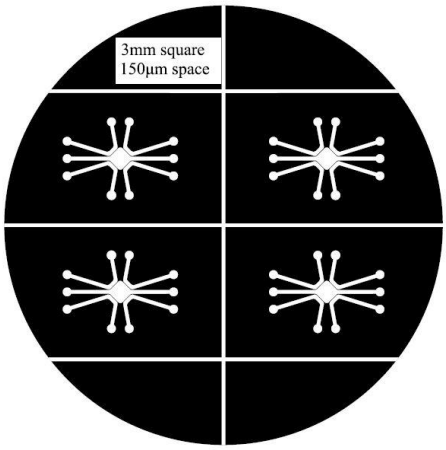

Figure 3.6. Example orthogonal wafer design built in AutoCAD. White regions in the drawing represent clear spots on the actual photomask where UV is allowed to pass through, initiating the photo-catalyzed reaction to take place in the layers of SU-8 2050 underneath.

Additionally, there are alternate configurations that would lead to different combinations of gradients forming within the central channel of the device. This device, therefore, is more robust than the original three-channel device in that it improves on the number of gradients capable of forming within the device simultaneously since not only can the orthogonal device produce a chemical gradient of a single chemoattractant from top to bottom but also can produce combinations of gradients in order to better replicate conditions that cells would be exposed to within the human body while they are undergoing cellular processes. The AutoCAD file would eventually be used to design the masks that were ordered for silicon wafer fabrication (Figure 3.6).

3.2.4 Fabrication of the Plexiglas housing for the orthogonal gradient device

Because the new orthogonal device has more ports where tubing is placed than the three-channel device, and because the ports are in different locations than the three-channel, it was necessary to design and fabricate a new set of top and bottom Plexiglas pieces specific for the orthogonal device. In order to make the new Plexiglas pieces, the new design was drawn using AutoCAD (Figure 3.7).

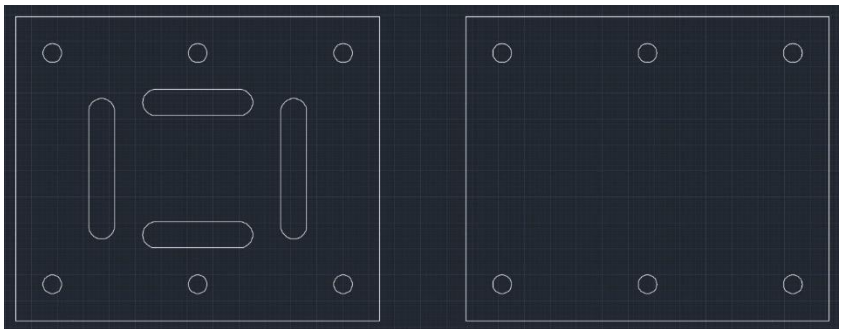

Figure 3.7. Orthogonal top and bottom Plexiglas pieces as designed using AutoCAD. The piece on the left is the top piece with holes for tubing. These pieces have six holes around the edges for screws to go through in order to tighten the device.

The new top piece design has four separate holes through which tubing goes through from the PDMS ports. Both the bottom and the top layers were designed with six holes around the outside for screws to go through. The original Plexiglas pieces for the three-channel device had four holes for screws to be placed; however, the orthogonal pieces were designed using six holes because the original pieces with four would sometimes cause the middle of Plexiglas to bend, causing the device to leak in the middle due to lack of pressure. This flaw was fixed by the addition of screws being placed in the middle of the pieces along with the preexisting screws in the corners.

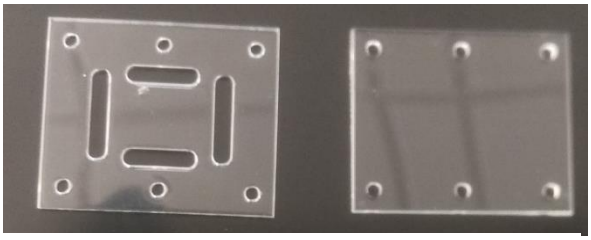

Figure 3.8. Completed orthogonal Plexiglas pieces after being cut out using a laser printer.

After designing the new pieces in AutoCAD, they were then cut out of 1/16 inch thick Plexiglas using the same laser printer that cut out the sides of the temperature control box (Figure 3.8). The top and bottom pieces were then used in making the full PDMS-on-agarose orthogonal device.

3.2.5 COMSOL simulations of the orthogonal gradient device

After coming up with the initial design for the orthogonal device, the geometry was imported into COMSOL to model the fluid and mass transport occurring within the device (Figure 3.9). Because the orthogonal gradient device utilizes agarose for mass transport in the same way

the three-channel device had, the same properties of agarose were input into COMSOL[19]. Similar to the three-channel COMSOL simulation, fluid transport was simulated using the Laminar Flow settings, and the mass transport was simulated using the Transport of Diluted Species settings. The flowrate through each of the four flow channels was set to 15 μL/min, and fluid flows in from the outer ports, close to the ports for the center channel, to the ports on the top and bottom of the device. Diffusive properties were copied from the three-channel simulation, and again, both diffusion and convection were modelled, with the convection being calculated using the flow data from the Laminar Flow study.

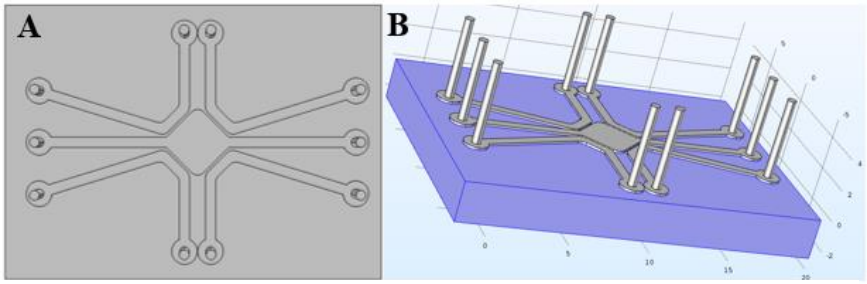

Figure 3.9. Orthogonal device geometry as input into COMSOL. (A) Top view of orthogonal device showing the channel spacing and overall device geometry, (B) 3D schematic of entire device setup as input into COMSOL with block highlighted in blue as agarose, channels on top of agarose, and tubing visualized above.

3.2.6 Observation of chemical gradients in the orthogonal device using fluorescent microscopy

Once the initial device assembly protocol was set and all the separate pieces for the device were designed and fabricated, the gradient within the device was experimentally observed using fluorescent microscopy similar to as described in section 2.2.5 As with the three-channel device, a 10 mM solution of 6 kDa FITC-dextran was used to visualize the gradient formation within the center diamond of the orthogonal device. Two syringes containing FITC-dextran or PBS were connected to outer flow channels of the orthogonal gradient device by inserting the needles into the tubing on the left side of the device relative to the image on the microscope. The syringe with FITC-dextran was attached to the upper left channel, and the two upper channels were joined together using a metal connector between the tubing of the two channels. Similarly, the syringe containing only 1x PBS was connected to the bottom channels. Flow was established using a syringe pump to control the flowrate at 15 μL/min, and the experiment was allowed to run for 22 hours with pictures taken every 3 minutes using the Leica DMi8 microscope described in section 2.2.5

3.2.7 Cell preparation and seeding within the orthogonal gradient device

The initial protocol for preparing cells to inject into the orthogonal device was the same as the procedure described in section 2.2.7. Cells would be passaged when reaching a confluency of approximately 80% and would be subsequently mixed with collagen to achieve a concentration of 2 mg/mL and diluted with sodium hydroxide to bring the mixture to slightly basic pH. This mixture would then be injected into the center channel of the orthogonal device which would be placed in an incubator overnight while full media flowed through the outer channels at 15 μL/min.

3.3 Results and Discussion

3.3.1 COMSOL simulations determine optimal device geometry for desired gradient formation

Several different time dependent studies were simulated using COMSOL to approximate the formation of the chemical gradients within the center cell culture channel of the orthogonal device for three different orientations of chemicals within the device (Figure 3.10). Based on the

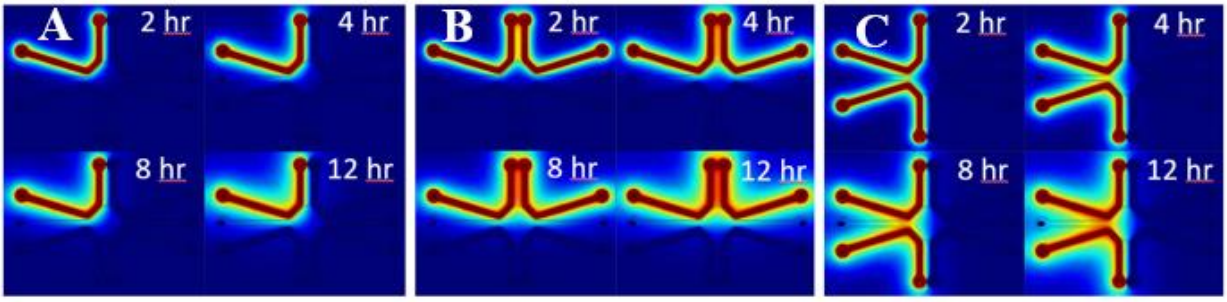

Figure 3.10. Formation of chemical gradients within COMSOL simulation for orthogonal device. (A) Gradient development for chemoattractant flowing in the top left channel, (B) gradient formation from top to bottom by flowing chemoattractant in the top two channels, (C) gradient formation from the left side of the device from flowing chemoattractant in the left two channels. Combining (B) and (C) would produce orthogonal gradient generation.

COMSOL simulations, the geometries used were capable of producing stable, linear chemical gradients over time within the central channel of the device (Figure 3.11). Specifically, these simulations indicate that when the device is set up to produce a gradient from the top or left sides of the device, the gradient produced is approximately linear after eight hours of flow through the outer channels. Additionally, the simulations demonstrate that the gradients that are formed reach steady state after ~16 hours from initiating flow through the device. Because COMSOL predicted

the capability of the proposed geometries to produce multiple gradient orthogonal to one another, these designs were then used to fabricate the actual microfluidic device.

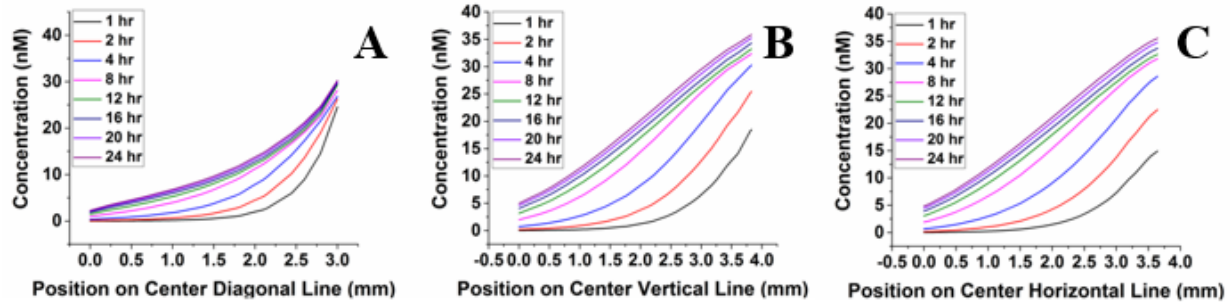

Figure 3.11. Concentration plots from data simulated through COMSOL. (A) Concentration plot for gradient formed with chemoattractant in the top left channel of the orthogonal device, (B) concentration plot for gradient formed with chemoattractant in the top two channels of orthogonal device, (C) concentration plot for gradient formed with chemoattractant in left two channels of orthogonal device. (B) and (C) form linear concentration gradients after approximately 8 hours and reach a pseudo steady state after 16 hours.

3.3.2 Observed chemical gradients in the orthogonal gradient device using fluorescent microscopy confirm COMSOL simulations

Similar to as described in Chapter 2, fluorescent microscopy experiments were performed to visualize the chemical gradient within the microfluidic device using 10 mM FITC-dextran. Images were collected during an 8 h time course that show a gradient developing within the device as a function of time with the overall shape of the gradient matching the COMSOL simulations (Figure 3.12). Because the similarities between the experimental and numerical approaches, it was decided to use the values obtained from the COMSOL simulations to determine the actual

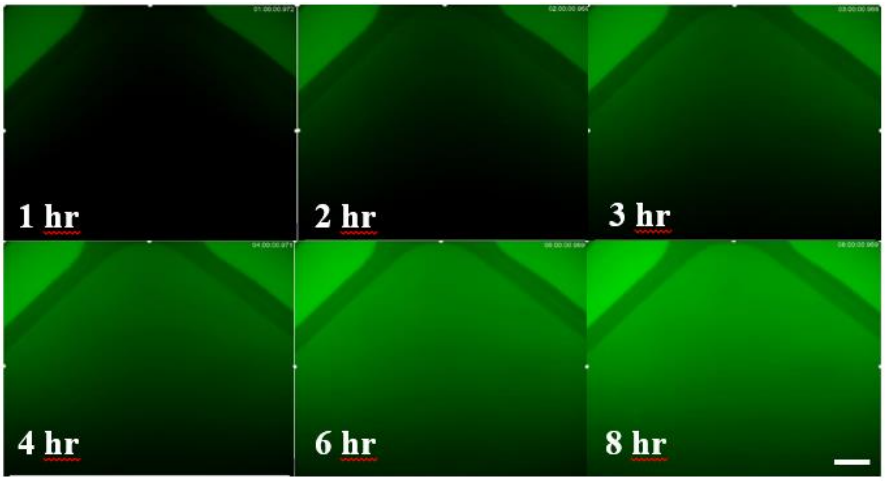

Figure 3.12. Fluorescent gradient development in the orthogonal device. 10 mM FITC-dextran was flowed through the top two channels of the orthogonal device at 15 µL/min. Scale bare is 300 µm.

concentration present within the device at a certain time point during an experiment. Now that the device has been fully assembled and the gradient present within the device has been characterized, the next step was to optimize the device for use with cells.

3.3.3 Optimization of orthogonal gradient device assembly for proper cell migration experiments

After designing and characterizing the orthogonal gradient device, the setup protocol had to be modified in order to run cell migration experiments within the device. Early on, there were several issues when transitioning to use the device for measuring the chemotactic responses in cells. These issues included (i) cells dying within the device by being compressed between PDMS and agarose layers, (ii) bubble formation within the center channel of the device during assembly and cell injection, and (iii) collagen detachment from the side walls of the center channel. A solution was found to each of these technical challenges and successful experiments have been run demonstrating cell migration within the orthogonal device. Briefly, to prevent cells from being compressed within the center channel, the channel height was doubled from 150 μm to 300 μm (see section 3.2.3 above). To keep bubbles from forming within the corners of the center diamond, the entire device was flooded with media prior to assembly so that the center channel was free of air before being assembled. To keep the collagen attached to the channel walls within the center culture channel, the same modification to the protocol of the three-channel device was employed – to allow the collagen to polymerize for longer prior to flowing media through the outer channels. Once these changes were made to the device protocol, cells within the device remain viable for the duration of an experiment (~18 h), and ideal conditions within the center channel were maintained for cells to migrate through the device.

3.3.3.1 Increasing channel height prevented channel compression

The first few times that cells were injected into the orthogonal device, there was a recurring problem of the cells in the very middle of the diamond turning a dark color under phase contrast

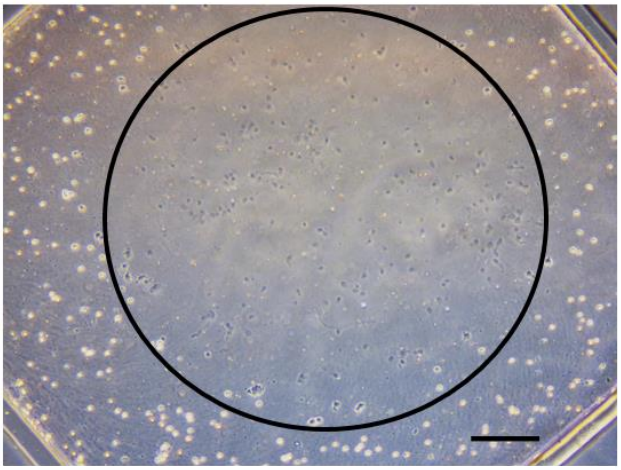

Figure 3.13. Cell death caused by agarose pushing on the PDMS layer. Inside the circled region, the cells can be seen as a darker color than those on the outside of the circle, suggesting cell death and additional pressure from the sagging PDMS. Scale bar is 400 μm.

microscopy suggesting that the cells were dying (Figure 3.13). It was determined that these cells were being compressed by the PDMS and agarose because the PDMS was most likely sagging in the middle of the diamond and coming in contact with the agarose due to the surface area to height ratio of the large center channel. This contact between the PDMS and agarose was not an issue in the three-channel device because the distance between walls was much shorter in the three-channel device than in the orthogonal gradient device which prevented channel sagging from occuring. In order to fix this issue, the height of the fluidic channels in the orthogonal gradient device was doubled from 150 μm to 300 μm. Alternate modifications could have been made to the device in order to prevent the sagging such as redesigning the center channel to include pillars in the middle of the diamond; however, this approach was not taken, as increasing the channel height was quicker and did not have the potential to impact the cell migration or the gradient formation within the device. After testing the new device with the taller channels, the sagging was prevented, and the cells in the center of the device remained viable for the duration of an experiment (Figure 3.14).

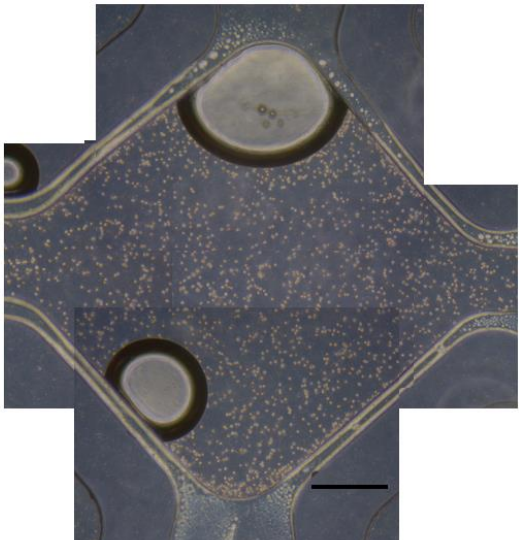

Figure 3.14. Orthogonal device with cells after modifying device to have 300 μm tall channels. This picture was taken prior to solving the bubble formation problem. Pictures must be stitched together because the entire device cannot be viewed under the microscope at once. Scale bar is 600 μm.

3.3.5.2 Perfusion of fluidic channels with media prior to cell seeding prevented bubble formation

The next problem that needed to be solved was the issue of bubbles forming in the corners of the

diamond within the center channel either during device assembly or during cell injection. Due to surface tension and the flow pattern in the center channel, it was challenging to remove a trapped air bubble from a corner of the center diamond once it became stuck there (Figure 3.14). Once a bubble formed in the corner, the surface tension effects between the liquid in the channel and the air bubble prevented any additional liquid injected into the channel from pushing the bubble out of the device as the liquid-air interface could not be broken at such small volumes. Additionally, the shape of the surrounding corner acts to lock the bubble into place, pushing against the flow of liquid through the device. The laminar flow of the liquid also prevented the liquid from dislodging the bubble as the small volume caused the liquid to flow in a straight path through the channel and around the bubble, preventing potential eddy currents caused by turbulent flow which could have moved the bubble out of the corner.

These bubbles were found to enter the device in a couple of different ways: by being present within the device during assembly due to air becoming trapped in the PDMS channel as the PDMS and agarose layers were placed together or by being introduced into the channel while injecting cells in to the device and becoming stuck in the corner. The main problem encountered by the presence of bubbles within the center channel was that collagen could not enter the part of the diamond that was occupied by the air bubble which prevented cells from migrating to this region of the device. This lack of collagen may also have had an effect on the gradient development through the device. In order to prevent bubbles from forming in the device, a few aspects of the assembly protocol were changed to remove all air from the device prior to device assembly. The tubing leading into the center channel was extended and filled with liquid media prior to assembling the device. Additionally, the entire device was assembled upside down, and the PDMS channels were flooded with media before combining the PDMS and agarose layers together. By flooding the device with media, no air would be able to become trapped in the channel when bringing the PDMS

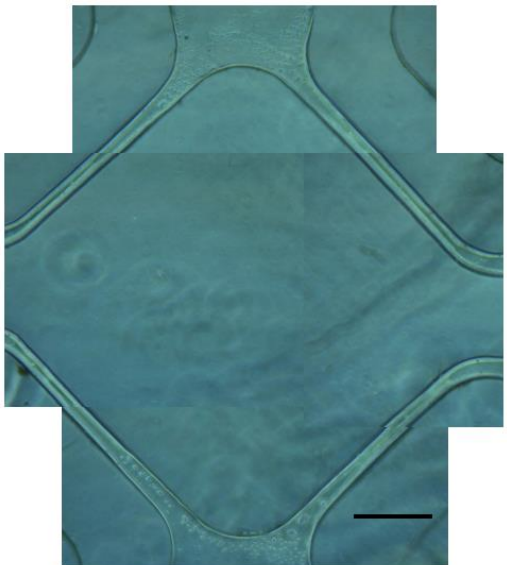

Figure 3.15. Orthogonal device without bubbles in center channel after device assembly. Bubble formation in the orthogonal device was prevented by flooding the channel with media prior to assembling the entire device. Scale bar is 600 μm.

and agarose together. By making these changes, the device was capable of being assembled without having air bubbles coalesce in the corners of the center diamond (Figure 3.15). An alternative to these changes could be to modify the device geometry so that there are vertical channels extending from the top and bottom corners of the device to allow for either back pressure to suction air bubbles from the device or to provide separate channels for injection in order to remove bubbles. With this new shape the central channel would resemble a cross shape with a diamond in the middle, and this new design could have some additional advantages for studying cell migration as these new channels would have different gradient formation in them than what is capable with the current device design.

3.3.5.3 Enhancing collagen polymerization time within the microfluidic device decreased channel wall detachment

The final technical issue encountered with the orthogonal gradient device while introducing cells was an issue similar to that encountered with the three-channel device. Initially, when the collagen and cell suspension was injected into the center channel, the suspension would regularly spread through the entire center diamond. However, when the device was inspected the next day after ~12-18 h of incubation to allow the cells to attach to the collagen and become accustomed to the device environment, sometimes the collagen within the middle channel would become detached from the walls of the channel and fold in upon itself (Figure 3.16). The retraction of the collagen matrix was an issue for studying cell migration as the cells cannot move as easily within the non-uniform collagen layer. To fix this issue, the collagen was allowed to fully polymerize at room temperature by leaving the device under a biosafety cabinet for at least 45 minutes before reestablishing flow to the outer channels of the device. Having the collagen fully form into a cross-

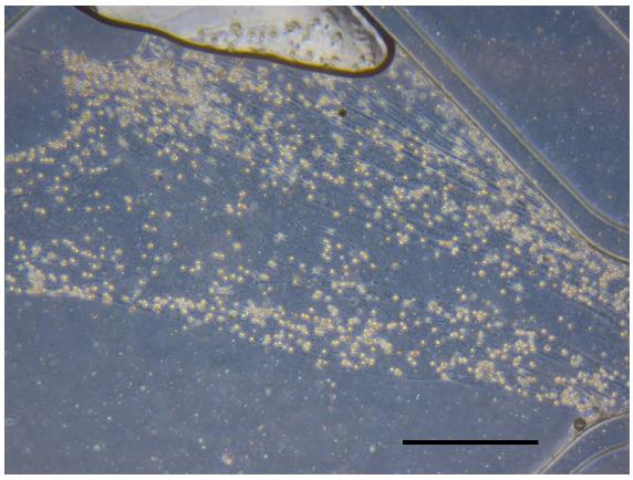

Figure 3.16. Orthogonal device showing collagen detachment from sides of center channel. Scale bar is 600 μm.

matrix mesh prior to flowing again through the device prevented the collagen from detaching from the walls of the channel (Figure 3.17). Upon reviewing the literature, there are many groups that have pretreated the inside of their microfluidic channels with BSA, poly-lysine, and/or fibronectin to encourage surface adhesion of the collagen to the channel walls[13, 16, 17, 22-24]. A method that does not use these treatments, however, would be preferred as it does not alter the chemistry within the device, which could affect the cell migration due to the introduction of new chemicals to the cellular environment.

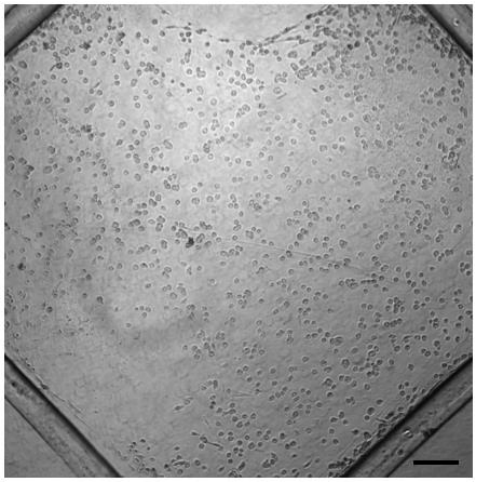

Figure 3.17. Orthogonal device with cells prior to random migratory experiment. Cells within device were viable, and the collagen remained attached to the side walls. Scale bar is 200 μm.

After making these changes to the orthogonal device setup, random migratory experiments were run using MDA-MB-231 cells in the presence of full media, and similar responses were observed to those in the three-channel device (Figure 3.18). It was decided to perform random migration experiments first to ensure that the cells could spread and migrate similar to what was observed in the three-channel device. Several cells were found to migrate in a similar manner in the orthogonal gradient device to those in the three-channel device. The result is a working microfluidic platform capable of generating orthogonal chemical gradients and directly visualizing 3D cell migration.

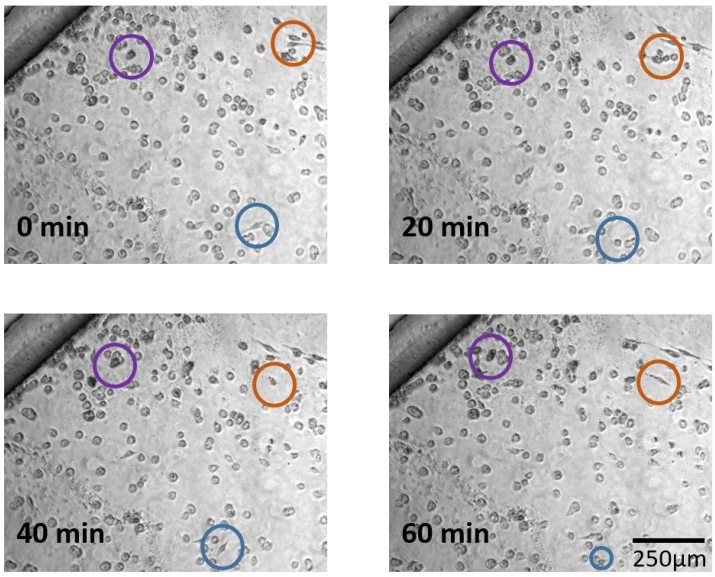

Figure 3.18. Cell migration of MDA-MB-231 cells through the orthogonal device in the presence of full media. Times for the pictures are normalized with time 0 min corresponding to approximately one hour.

3.4 Conclusions

Now that the orthogonal device has been optimized, the next step moving

forward with this project will be to expose cells to competing chemical gradients to measure cellular decision making. For MDA-MB-231 cells, one combination of chemoattractants to test would be EGF and SDF-1α. Though the chemotactic response of these to single gradients of each of these chemoattractants has been previously studied in this cell line, decision making in these and other cell lines is more complex than moving towards only one chemical gradient. Within the body, there are often multiple competing gradients of proteins or other cell excretions present for which migratory cells such as aggressive cancers could potentially move towards. As such, it is necessary to obtain a more complete understanding of how these types of cells decide how to migrate in order to potentially prevent these cells from spreading and causing further harm to patients. The device that was created through this project has been well characterized and provides an easily configurable environment to test cellular chemotaxis.

Furthermore, the orthogonal device could be used for testing other cellular processes such as the response of certain cells to single and combinatory gradients of specific drugs. One such example would be to test the effectiveness of certain antibiotics to kill bacteria that have formed biofilms. With the advent of antibiotic-resistant strains of bacteria, it is essential to identify treatment approaches for patients infected with bacteria that are not affected with traditional therapies. One proposed solution is to use combinations of already identified and approved antibiotics to increase the efficacy of the drugs[21]. The orthogonal device made through this project is capable of testing such drugs for enhance combinatory effects on bacteria, and this device has some advantages over devices that have already been seen in literature. The developed orthogonal device is able to be taken apart after running an experiment for harvesting of specific cells to run subsequent test or assays such as polymerase chain reaction to look for certain genes or to stain for certain biomarkers or for viability. Similar microfluidic devices that are plasma bonded to glass are unable to be removed after cells are exposed to concentration gradients, making it impossible to subject these cells to future experimentation. Moving forward, the orthogonal device will next be tested to show its capability to measure cellular responses of interest within the device.

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References

1. Kim, B.; Hannanta-Anan, P.; Chau, M.; Kim, Y.; Swartz, M.; Wu, M., Cooperative Roles of SDF-1 alpha and EGF Gradients on Tumor Cell Migration Revealed by a Robust 3D Microfluidic Model. *Plos One* **2013,** *8* (7).
2. Jeon, N.; Dertinger, S.; Chiu, D.; Choi, I.; Stroock, A.; Whitesides, G., Generation of solution and surface gradients using microfluidic systems. *Langmuir* **2000,** *16* (22), 8311-8316.
3. Siegel, R.; Miller, K.; Jemal, A., Cancer Statistics, 2018. *Ca-a Cancer Journal For Clinicians* **2018,** *68* (1), 7-30.
4. Wirtz, D.; Konstantopoulos, K.; Searson, P., The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nature Reviews Cancer* **2011,** *11* (7), 512-522.
5. Aysola, K.; Desai, A.; Welch, C.; Xu, J.; Qin, Y.; Reddy, V.; Matthews, R.; Owens, C.; Okoli, J.; Beech, D.; Piyathilake, C.; Reddy, S.; Rao, V., Triple Negative Breast Cancer - An Overview. Hereditary Genetics: 2014.
6. Gilkes, D.; Semenza, G., Role of hypoxia-inducible factors in breast cancer metastasis. *Future Oncology* **2013,** *9* (11), 1623-1636.
7. Duffy, D.; McDonald, J.; Schueller, O.; Whitesides, G., Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Analytical Chemistry* **1998,** *70* (23), 4974-4984.
8. Sia, S.; Whitesides, G., Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis* **2003,** *24* (21), 3563-3576.
9. Park, S.; Jang, H.; Kim, B.; Hwang, C.; Jeong, G.; Park, Y., Directional migration of mesenchymal stem cells under an SDF-1 alpha gradient on a microfluidic device. *Plos One* **2017,** *12* (9).
10. Haessler, U.; Kalinin, Y.; Swartz, M.; Wu, M., An agarose-based microfluidic platform with a gradient buffer for 3D chemotaxis studies. *Biomedical Microdevices* **2009,** *11* (4), 827-835.
11. Frick, C.; Dettinger, P.; Renkawitz, J.; Jauch, A.; Berger, C.; Recher, M.; Schroeder, T.; Mehling, M., Nano-scale microfluidics to study 3D chemotaxis at the single cell level. *Plos One* **2018,** *13* (6).

12. Kao, W.; Lin, C.; Lee, L.; Lee, P.; Hung, C.; Lin, Y.; Chen, S.; Ke, F.; Hwang, J.; Lee, M., Investigation of MMP-2 and -9 in a highly invasive A431 tumor cell sub-line selected from a Boyden chamber assay. *Anticancer Research* **2008,** *28* (4B), 2109-2120.

13. Zhang, C.; Barrios, M.; Alani, R.; Cabodi, M.; Wong, J., A microfluidic Transwell to study chemotaxis. *Experimental Cell Research* **2016,** *342* (2), 159-165.

14. Toh, A.; Wang, Z.; Yang, C.; Nguyen, N., Engineering microfluidic concentration gradient generators for biological applications. *Microfluidics and Nanofluidics* **2014,** *16* (1-2), 1-18.

15. Irimia, D.; Geba, D.; Toner, M., Universal microfluidic gradient generator. *Analytical Chemistry* **2006,** *78* (10), 3472-3477.

16. Fan, Q.; Liu, R.; Jiao, Y.; Tian, C.; Farrell, J.; Diao, W.; Wang, X.; Zhang, F.; Yuan, W.; Han, H.; Chen, J.; Yang, Y.; Zhang, X.; Ye, F.; Li, M.; Ouyang, Z.; Liu, L., A novel 3-D bio-microfluidic system mimicking in vivo heterogeneous tumour microstructures reveals complex tumour-stroma interactions. *Lab on a Chip* **2017,** *17* (16), 2852-2860.

17. Uzel, S.; Amadi, O.; Pearl, T.; Lee, R.; So, P.; Kamm, R., Simultaneous or Sequential Orthogonal Gradient Formation in a 3D Cell Culture Microfluidic Platform. *Small* **2016,** *12* (5), 612-622.

18. Landwehr, G.; Kristof, A.; Rahman, S.; Pettigrew, J.; Coates, R.; Balhoff, J.; Triantafillu, U.; Kim, Y.; Melvin, A., Biophysical analysis of fluid shear stress induced cellular deformation in a microfluidic device. *Biomicrofluidics* **2018,** *12* (5).

19. Pluen, A.; Netti, P.; Jain, R.; Berk, D., Diffusion of macromolecules in agarose gels: Comparison of linear and globular configurations. *Biophysical Journal* **1999,** *77* (1), 542-552.

20. Li, Y.; Asadi, A.; Monroe, M.; Douglas, E., pH effects on collagen fibrillogenesis in vitro: Electrostatic interactions and phosphate binding. *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* **2009,** *29* (5), 1643-1649.

21. Kim, S.; Masum, F.; Kim, J.-K.; Chung, H. J.; Jeon, J. S., On-chip phenotypic investigation of combinatory antibiotic effects by generating orthogonal concentration gradients. Lab on a Chip, 2019.

22. Wu, X.; Wu, J.; Li, H.; Legler, D.; Marshall, A.; Lin, F., Analysis of CCR7 mediated T cell transfectant migration using a microfluidic gradient generator. *Journal of Immunological Methods* **2015,** *419*, 9-17.

23. Yang, K.; Wu, J.; Peretz-Soroka, H.; Zhu, L.; Li, Z.; Sang, Y.; Hipolito, J.; Zhang, M.; Santos, S.; Hillier, C.; de Faria, R.; Liu, Y.; Lin, F., M-kit: A cell migration assay based on microfluidic device and smartphone. *Biosensors & Bioelectronics* **2018,** *99*, 259-267.

24. Yang, K.; Wu, J.; Xu, G.; Xie, D.; Peretz-Soroka, H.; Santos, S.; Alexander, M.; Zhu, L.; Zhang, M.; Liu, Y.; Lin, F., A dual-docking microfluidic cell migration assay (D-2-Chip) for testing neutrophil chemotaxis and the memory effect. *Integrative Biology* **2017,** *9* (4), 303-312.