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On-Chip Open Microfluidic Devices for Chemotaxis Studies

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Abstract

Microfluidic devices can provide unique control over both the chemoattractant gradient and the migration environment of the cells. Our work incorporates laser-machined micro and nanofluidic channels into bulk fused silica and cover slip-sized silica wafers. We have designed “open” chemotaxis devices that produce passive chemoattractant gradients without an external micropipette system. Since the migration area is unobstructed, cells can be easily loaded and strategically placed into the devices with a standard micropipette. The reusable monolithic glass devices have integral ports that can generate multiple gradients in a single experiment. We also used cover slip microfluidics for chemotaxis assays. Passive gradients elicited from these cover slips could be readily adapted for high throughput chemotaxis assays. We have also demonstrated for the first time that cells can be recruited into cover slip ports eliciting passive chemoattractant gradients. This proves, in principle, that intravital cover slip configurations could deliver controlled amounts of drugs, chemicals, or pathogens as well as recruit cells for proteomic or histological analysis in living animals while under microscopic observation. Intravital cover slip fluidics will create a new paradigm for *in vivo* observation of biological processes.

Keywords

microfluidics; chemotaxis; laser etching; microscope

Introduction

Chemotaxis, the ability of cells to sense a chemical gradient and respond by migrating directionally toward or away from the source, is critical to cell development, wound healing, and a plethora of illnesses including cancer and autoimmune diseases (Johnson et al., 2004; Condeelis et al., 2005; Dorsam & Gutkind, 2007; De Paepe et al., 2009; Hansson, 2009; Wu et al., 2009; Lazennec & Richmond, 2010). The study of chemotaxis typically requires the experimenter to provide a gradient to the cells on a microscopic platform so various

characteristics of the cell can be observed and quantified. Since cells have a remarkable ability to detect small changes in receptor occupancy (~5% difference) across the length of the cell, it is critical that gradients remain stable and quantifiable (Lauffenburger et al., 1983; Postma & van Haastert, 2009). Classically, chemical gradients have been created using a micropipette assay (Bunning, 1989). A micropipette with a one micron-sized opening at the tip is used in conjunction with a micromanipulator and a pump to create a diffusive radial gradient within a one-well chamber on an inverted microscope (Bunning, 1989; Parent et al., 1998). Cells detect this passive diffusion gradient and migrate across the glass surface of the one-well chamber toward the micropipette source. Other devices have been created for measuring chemotaxis using diffusive gradients and include the Boyden chamber, Zigmond chamber, and the Dunn chamber, with the latter two being compatible with microscopic observation (Boyden, 1962; Zigmond, 1977; Zicha et al., 1997). While these devices are useful for determining qualitative differences over short time scales, these platforms also produce linear gradients that change over time, lack stability, and are difficult to manipulate.

The adaptation of microfluidics in biology has spurred many groups to make use of devices containing microchannels to create stable gradients (Andersson & van den Berg, 2003; El-Ali et al., 2006; Young & Beebe, 2010). Micro-fluidic gradient-generating devices such as hydrogel, polydimethylsiloxane (PDMS) devices (Kim et al., 2010; Raja et al., 2010), and serpentine channel devices (White-sides, 2006; Melin & Quake, 2007; Keenan & Folch, 2008; Young & Beebe, 2010) have given researchers added tools to understand gradient sensing mechanisms in more detail and in three dimensions (Knight et al., 2000; Cukierman et al., 2001; Hegerfeldt et al., 2002; Wolf et al., 2003). Although the small size and three-dimensional (3D) environment of these microfluidic devices are advantageous, the active mixing and constant fluid flow can introduce air bubbles, introduce shear and drag forces, and cause variations in the gradient if there are obstructions in the device (Walker et al., 2005; Keenan & Folch, 2008). Additionally, placement of cells within these devices is difficult because the systems are typically closed. Cell loading in the closed devices is usually performed by pumping cells through a microfluidic port.

Recently, “open” microfluidic devices (OMDs) have come on the scene and eliminate many of the problems associated with traditional closed microfluidic devices described above. These “open” platforms can provide spatial and temporal control over a reproducible chemical environment for chemotaxis and have been created to have multiple analysis capabilities, including the ability to stain cells and analyze protein and gene expression (Keenan et al., 2010; Lovchik et al., 2010; Dimov et al., 2011; Li et al., 2011). This type of OMD apparatus typically has no ceiling above the cell port and thus provides easy access for cell loading using a standard pipette and can also allow for the setting up of chemical gradients by a micropipette and micromanipulator system (Jowhar et al., 2010). For *Dictyostelium discoideum* amoebae, cell loading is accomplished in the absence of a matrix and several minutes before performing chemotaxis assays. With this type of OMD, it is trivial to lay down a matrix. All excess matrix and air bubbles can be easily flushed out with a pipette prior to cell loading. As this assay suggests, PDMS devices can be quite useful but are generally difficult to reuse and are optically inferior to glass, which limits their function

for high- and super-resolution microscopy. Furthermore, it is difficult to create 3D channels using PDMS and virtually impossible to make channels with features smaller than a few microns.

In addition to the mechanical constraints researchers encounter while performing migration assays, most labs are limited in their ability to access micromanipulation equipment for gradient generation as is commonly performed in many chemotaxis labs, including our own. To alleviate this problem, we created open passive gradient generators in a bulk-fused SiO₂ (silica) chip that could be used alone or assembled in tandem with PDMS or other fused silica migration devices. Fused silica has excellent optical properties, will not autofluorescence at visible wavelengths, and can be machined by a femtosecond laser. Recent advances in laser etching technologies make this technology possible (Grill et al., 2003; Ke et al., 2005; Kim et al., 2009). Channels and holes smaller than 200 nm have been demonstrated (White et al., 2008). Unlike PDMS, glass is also very rigid. This means that the channels will not have capacitance. Increases or decreases in pressure will result in precise and rapid changes in fluid flow or gradient manipulation. An on-chip OMD device for chemotaxis assays can be mass produced, is easy to use, and can provide stable gradients for highly quantitative experimentation. This article describes three reusable on-chip OMDs that elicit passive chemoattractant gradients. Each on-chip platform has unique features for defined experimentation. The first device was created in bulk fused silica, was bonded to a cover slip, and was used on an inverted microscope. The other two OMD platforms have gradient generators incorporated into fused silica where the thickness of the device itself is on the order of a microscope cover slip (100–200 μ m). Thus, gradients can be elicited from the cover slip upon which the cells are crawling. One of these cover slip-sized devices was useful for an inverted microscope while the other could be used on both an upright and inverted microscope. The latter two designs are proof of concept devices for chemotaxis platforms that can be used for drug discovery and intravital imaging. In all cases, *D. discoideum* cells were imaged using bright-field and fluorescence microscopy and exhibited robust chemotaxis toward cyclic adenosine monophosphate (cAMP) elicited from a glass port forming the controlled chemical gradient. Moreover, migrating cells were able to enter the gradient generating ports in the cover slip-sized on-chip OMDs.

Methods

Media and Buffers

HL-5 was purchased from Formedium (Hunstanton, Norfolk, UK). HL-5 media consist of 22 g of HL-5 powder, 10 g of dextrose, and 1 L of double distilled H₂O. Developmental buffer (DB) consists of 5 mM Na₂HPO₄, 5 mM KH₂PO₄, 1 mM CaCl₂, and 2 mM MgCl₂.

Strains Used

D. discoideum wild-type AX2 strain expressing RBD-GFP or LimE-RFP were used for cAMP chemotaxis (Muller-Taubenberger, 2006; Sasaki & Firtel, 2009). The plasmid pDM RBD-GFP was provided by Arjan Kortholt and Peter Van Haastert. pDM RBD-GFP confers G418 resistance.

cAMP Preparation

10 mM stock of cAMP (Sigma, St. Louis, MO, USA) solution was made in double distilled H₂O. For cAMP development a 2.5 μ M working solution was made in DB buffer. For cAMP chemotaxis, a 10 μ M working solution was made in DB buffer.

cAMP Development

50 mL of 2×10^6 cells per mL were centrifuged at 1,200 rpm and subsequently washed three times with DB buffer. The pellet was then resuspended in 5 mL of DB buffer resulting in 2×10^7 cells per mL. The 5 mL resuspension was transferred to a 150 mL flask and was shaken at 110 rpm for 1 h. After the initial 1 h shake, the cells were then pulsed with approximately 100 μ L of the 2.5 μ M cAMP every 6 min for 5 h. After the 6 h development, the cells were treated with 5 mM caffeine and shaken for 30 min to basalate the cells.

Gradient Characterization

The gradient measurements were carried out using 10 μ M fluorescein isothiocyanate (FITC) loaded into a syringe attached to a Harvard Apparatus syringe pump. To create the gradient, a small amount of positive pressure (50 μ L/h flow rate) was added to the syringe containing the FITC. Images were taken of the device with the FITC gradient at time 0 min and up to 60 min at 5 min intervals. The FITC fluorescence profile was measured using Image J's Plot profile feature (U.S. National Institutes of Health, Bethesda, MD) and the intensity values were plotted as a function of distance using Microsoft Excel.

Chemotaxis Assay Using the Glass Passive Gradient Devices

The device was connected to an inlet and outlet syringe on a Harvard Apparatus syringe pump using microfluidic tubing. Once the plumbing was connected, the device was equilibrated with DB buffer at a rate of 50 μ L/h. Once the DB buffer had equilibrated in the device and there were no air bubbles, the developed AX2:RBD-GFP or AX2:LimE-RFP expressing cells were loaded into the cell ports using a micropipette. The cells were allowed to settle on the bottom of the device for 10 min. After the cells were settled, the DB buffer syringe was replaced with a syringe containing 10 μ M cAMP (bulk silica gradient generating device) and 100 nM cAMP (silica coverslip device) and the flow rate was returned to 50 μ L/h.

Imaging

Images were acquired on a Marianis Workstation equipped with a Cool Snap charge-coupled device (CCD) camera and an Extended QE, high-speed cooled CCD camera (Cascade II 512). The microscope used was an inverted, wide-field epifluorescence microscope (Zeiss Axio Observer Z1; Carl Zeiss, Oberkochen, Germany). Images were acquired by SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA). All time-lapse images were taken every 15 s. Images were also collected on an upright Zeiss Axioskop 2 FS with an Achroplan 40 $\times$ 0.8 NA water immersion objective. All time-lapse images were taken every 15 s.

Visualization and Analysis of Chemotaxis

To visualize the migration of cells and record the specifics of chemotactic response, a program was written with Lab-VIEW™ software. Time-lapse image data from Slidebook were exported to avi movie format. The movie files were then loaded in the cell tracking program. The user can view the image data in a window, play the file, or examine individual frames. Tracking of cells uses the machine vision algorithms from LabVIEW™ Vision Development Package. The user selects the cell to track with a mouse, and the software records the x, y pixel coordinates of the centroid of intensity in the image for the fluorescent cell. The identified results of the search and the centroid are displayed in a window for visual verification of the software performance. As the user plays the movie, the software tracks the cell automatically and builds an array of x, y coordinates over the desired length of the track. The measured centroids are overlayed on the image data so that the tracking of the cell can be visually verified. The software has adjustments for the size and intensity of object identification for user flexibility.

Once the x, y path of the cell is known and the source of the chemoattractant is identified, the chemotactic response can be quantified. The measured responses are the velocity of travel, the direction of travel (chemotactic index), and the persistence. The velocity is simply the distance between x, y pairs of the cell path divided by the time between points. Velocity can be measured point-to-point or integrated over several time steps. Chemotactic index (CI) is the cosine of the angle formed by the travel vector and the vector from the cell to the source. The travel vector is determined by the x, y track of the cell. The travel vector can be taken at each frame increment or integrated over several frames.

$$CI(n) = \cos(\text{angle } SX_nX_{n+m}),$$

where CI is the chemotactic index of the cell at time n , S is the point source of the chemoattractant, X_n is cell's position at the start point of the travel vector, and X_{n+m} is the cell's position at the end point of the travel vector after the integration time increment m . It is useful to calculate the velocity and chemotactic index over short time scales corresponding to the movement of one cell length. For these time scales the chemotactic persistence, defined as the length of cell travel path divided by the change in distance to the source, is equal to the chemotactic index. The calculation of persistence is more useful over long time scales.

Device Design

The four-sided bulk silica gradient generating device is shown in Figure 1a. The device's microfluidic channels span over the bottom surface of a $10 \times 10 \times 0.5 \text{ mm}^3$ fused silica chip, which houses four chemical concentration gradient forming structures consisting of three ports each. Each one of the silica chip edges houses one of the gradient forming structures, in accordance with the device layout shown in Figures 1b and 1c. An enlarged view of one of these gradient forming structures is depicted in Figure 1d. The microfluidic channels are sealed from below by a $170 \mu\text{m}$ thick cover glass that is coated with a $20 \mu\text{m}$ thick layer of PDMS. The microfluidic channels stem from a central chemoattractant reservoir comprised

of a 500 μm thick fused silica chip and a reservoir in the acrylic manifold that is silicone-bonded to the top surface of the fused silica chip. Rubber o-rings seal the connections between the chemoattractant supply tubing and the acrylic manifold. This device is designed to operate on an inverted microscope. Once the device is mounted and the microscope objective is focused on the PDMS surface under a cell loading port, the device is flushed with buffer solution. Cells suspended in buffer solution are pipetted into the cell loading port and allowed to settle and attach to the PDMS surface. The chemoattractant reservoir is then loaded with chemoattractant solution. As the chemoattractant seeps through the gradient forming structure, the cells chemotax over the smooth PDMS surface. This ensures direct and clear imaging of the individual cell responses.

The open passive gradient port generators in a SiO_2 cover slip are shown in Figure 2a. The device houses five chemical concentration gradient forming ports that stem from a single microfluidic channel, which passes along the bottom surface of a $10 \times 10 \times 0.1 \text{ mm}^3$ fused silica chip. The gradient forming holes traverse the 100 μm thick fused silica chip from the chemoattractant channel to the cell recruitment site, as indicated in the device layout shown in Figures 2b and 2c. The microfluidic channels are sealed from below with a PDMS coated, 100 μm thick cover glass, which draws directly from the chemoattractant supply via the acrylic manifold. The manifold is silicone-bonded to the top surface of the fused silica chip. Rubber o-rings seal the connections between the chemoattractant supply tubing and the acrylic manifold.

This device is designed to operate on an inverted microscope. Once the device is mounted and the microscope objective is focused on the cell recruitment site, the device is flushed with buffer solution. Cells suspended in buffer solution are pipetted into the cell loading port and allowed to settle on the top surface of the fused silica chip. The microfluidic channel is then loaded with chemoattractant solution. As the chemoattractant seeps through the gradient forming holes, the cells chemotax up the concentration gradient and can be imaged from below.

Open Passive gradient port generators in SiO_2 cover slip are shown in Figure 3a. The device houses a single cell recruitment site with three 15 μm diameter chemoattractant delivery ports that stem from a single microfluidic channel embedded inside a $10 \times 10 \times 0.2 \text{ mm}^3$ fused silica chip, as shown in Figure 3. The microfluidic channel, located 60 μm below the surface of the fused silica chip, draws directly from the chemoattractant supply via the acrylic manifold that is silicone-bonded to the opposite surface of the fused silica chip, as indicated in the device layout shown in Figures 3a and 3b. Rubber o-rings seal the connections between the chemoattractant supply tubing and the acrylic manifold.

This device can be operated on both an inverted and an upright microscope. In the inverted microscope configuration (Fig. 3b), cells are loaded with the device upright, allowed to settle, and then the apparatus is inverted and placed into a bath with a Mylar film bottom suspended over the microscope objective. The individual cells responding to the concentration gradient are imaged through the Mylar film. In the upright microscope configuration (Figs. 3a, 3d), cells suspended in buffer solution are pipetted onto the cell-seeding surface near the cell recruitment site. The buffer solution was used for water

immersion using an upright Zeiss Axioskop 2 FS with an Achroplan 40× 0.8 NA water immersion objective. In both cases, the device is flushed with buffer solution and loaded with chemoattractant solution prior to use.

Device Fabrication

The devices were fabricated and assembled in an ISO 1000 class clean room, in accordance with the procedure outlined in detail in Costa et al. (2011). The microfluidic channels and the chemical concentration gradient forming structures were patterned on ultraviolet grade fused silica using the femtosecond laser micromachining system (White et al., 2008; Zalloum et al., 2010). The three delivery ports and the embedded microfluidic channel of the glass intravital/perfusion device were machined using water cavitation assisted, femtosecond laser micromachining (Ke et al., 2005), in accordance with the schematic shown in Supplementary Figure 1.

Results

Open Passive Gradient Generators in Bulk Silica Chip

We have developed an “open” chemotaxis device that allows quantitative measurement of the migration rates of cells in multiple gradients within a single device. With an “open” system, the experimenter can pipette cells into a cell loading port that is accessible to the outside environment (Jowhar et al., 2010). In these OMDs, the cell loading port remains open throughout the experiment, amenable to buffer addition, additional treatments, or perfusion. The benchmarks for this device included developing a platform that would allow simple cell loading, dynamic control of gradients, high-resolution fluorescence microscopy, visualization of multiple gradient generating ports simultaneously, and reusability. Fused silica material was chosen as a substrate because of its toughness, excellent optical properties, low background fluorescence, and machinability by a femto-second laser (Supplementary Fig. 1). We constructed an on-chip gradient generating device capable of producing multiple cAMP chemoattractant gradients (Fig. 1a). The chemoattractant entered the device via a chemoattractant supply inlet, traveled through the 2 mm thick acrylic manifold and down the center of the device where chemicals entered the machined 500 μm thick silica chip (Fig. 1b). These gradient producing ports and channels in the silica chip were plasma bonded to a 170 μm PDMS-coated cover slip (Figs. 1c, 1d). While we made a number of successful iterations of the device with varying numbers of ports (data not shown), we report here a four-sided device, with three ports each, which was used in our initial studies. The ports depicted in Figure 1d were 3.0 μm wide. These ports can be varied in size from a few hundred nanometers to several microns for the production of varying gradients in separate cell loading areas (Fig. 1a).

This device was capable of producing multiple linear gradients similar to the single gradient made using a micro-pipette in an OMD previously published by our group (Fig. 1) (Jowhar et al., 2010). *D. discoideum* cells expressing the Ras Binding Domain of Raf1 and fused to the green fluorescent protein (RBD-GFP) were loaded into the open cell ports and exposed to gradients of the chemoattractant cAMP (Fig. 1e) (Sasaki et al., 2004). This device was designed with a unique 100 μm recessed area so that cells would not settle directly on the

gradient generating ports (Figs. 1c, 1d). Each 3 mm cell loading port was exposed to three different sources of cAMP (from three 3.0 μm gradient generating ports) under the control of a syringe or Harvard apparatus pump (data not shown and Fig. 1d). The syringe pump was used to apply a constant positive pressure to the loaded syringe, thus preventing fluctuations in the gradient from potential backflow created by negative pressure. Cells rapidly migrated over the surface of the PDMS and toward all the gradient generating ports (Supplementary Movie 1).

This allowed us to perform three replicate experiments at the same time. These gradients were stable for at least 60 min (Figs. 4a, 4b), and the cells were capable of migrating over a distance of several hundred microns during the experiments (Fig. 1e; Supplementary Movie 1). Cell tracking was difficult because large numbers of cells converged as they neared the gradient generating ports. A number of solitary cells that could be followed continuously were tracked using the Cell Tracker program written in Lab-VIEWTM software (Fig. 5a). Cells migrated with high chemotactic efficiency toward the microfluidic gradient generating port (see Fig. 5b for chemotactic index and cell persistence).

Open Passive Gradient Port Generators in Thin Silica Cover Slip (I)

The four-sided OMD in bulk silica is a useful platform for performing general chemotaxis studies. However, a gradient generating device within a thin silica cover slip provides better bright-field optics and also would allow us to machine vertical surface ports that provide passive chemoattractant gradient sources to a large field of cells. We reasoned that machining ports in an imaging cover slip could provide an avenue for multiple chemoattractant gradients for cells to crawl toward and would provide flexibility for added microfluidic configurations (Fig. 2a). The device would also be more simple because the gradient is generated from the cover slip and not an additional silica component. This OMD consisted of an acrylic manifold, an upper-machined 100 μm silica cover slip, a 20 μm thick layer of PDMS, and a bottom 100 μm thick silica cover slip (Fig. 2b). Chemoattractants were pumped in through the acrylic manifold and then down into microfluidic channels machined horizontally in the cover slip and connected to vertically machined channels at the center of the device (Figs. 2c, 2d). The large horizontal channel was loaded with chemoattractant and relieved of any air bubbles (Fig. 2b). Cells were readily loaded by micropipetting into the cell loading area where they were allowed to settle and adhere to the silica cover slip. The channels were primed with buffer by a syringe; we observed that the cells randomly migrated on the surface. Once the cells adhered, cAMP was pumped in to form a chemoattractant gradient. To perform this function, a Harvard apparatus pump was used to create positive pressure on the chemoattractant loaded syringe. This resulted in a stable, relatively linear gradient over time (Figs. 6a, 6b). *D. discoideum* cells expressing RBD-GFP rapidly chemotaxed toward the cAMP gradient elicited from the ports and, in many cases, continued on and entered the port. Five cells are marked with white asterisks at the beginning of the movie (time 0 min), and two more cells are recruited into the ports from distances outside of the initial field of view during the 20 min movie (Fig. 2e; Supplementary Movie 2). The cell paths were tracked (see Fig. 7a) and their chemotactic efficiency is summarized in Figure 7b.

Open Passive Gradient Port Generators in Thin Silica Cover Slip (II)

As proof of principle, we next developed an OMD where the manifold was on the side opposite of the gradient generating ports as a prototype for the construction of a similar cover slip gradient generator as a window for intravital imaging (see the Discussion section). As in the previous cover slip device, channels were etched through a cover slip sized ($22 \times 22 \text{ mm} \times 170 \mu\text{m}$ thick) square piece of silica adhered to an acrylic manifold for long-term support (Figs. 3a, 3b). These channels extended 4 mm from the manifold port through the cover slip and are connected to the surface in the viewing area by three $15 \mu\text{m}$ sized ports (Fig. 3c). The manifold was connected to a chemoattractant supply tube fed by a syringe. We first demonstrated the viability of the device on an upright microscope using a $40\times$ water immersion lens. The cell buffer was used for water immersion. *D. discoideum* cells expressing the actin marker LimE-RFP rapidly migrated toward and into the cAMP gradient cover slip ports (Fig. 3e; Supplementary Movie 3).

In other experiments, we allowed *D. discoideum* cells to adhere to the silica surface. The OMD was then inverted and placed into a Mylar film imaging chamber created to hold the buffer and to provide a chemical sink for the chemoattractant. Live cell imaging was performed using a high NA $40\times$ oil immersion objective lens (Figs. 3b, 3f). Migrating cells were imaged on an inverted microscope (Fig. 3f; Supplementary Fig. 2).

Cells expressing RBD-GFP responded to the chemo-attractant gradient of cAMP and migrated toward and into the silica surface ports.

Discussion

The laser machined, on chip OMDs provide the flexibility to develop a variety of chemotaxis platforms for cell migration studies. We have described three unique devices here. On-chip OMDs and fused silica microfluidics in general have a number of distinctive features not found with PDMS devices. They are durable, easy to unclog and fill, and reusable. With glass, it is also possible to etch channels and ports down to a few hundred nanometers and microchannels can be easily created in three dimensions. Glass has the added benefit of being rigid, so that changes in flow can be regulated quickly and accurately. Microfluidic systems are considered uniquely suited for achieving a level of precision and gradient control that is necessary for quantitative studies of cell behavior. However, typical active mixing gradient generators require constant fluid flow and often fail to form reproducible gradients. Small changes in the device structure, cell debris, and air bubbles are common causes for these variations. Several groups have tried to bypass these problems by using microfabricated channels that respond to passive gradients (Jeon et al., 2002; Taylor et al., 2005; Abhyankar et al., 2006; Englert et al., 2009; Butler et al., 2010; Keenan et al., 2010; Lo et al., 2010; Lovchik et al., 2010; Dimov et al., 2011; Li et al., 2011) However, closed system microfluidic devices require a complicated cell loading process and are difficult to keep in working condition. To avoid these problems, we created a unique open system for migration studies.

Our previously published PDMS-based OMDs rely on microfluidic and micropipette technologies, but provided the experimenter with a platform that offered easy cell loading,

3D channels for migration, and a carefully defined passive gradient (Jowhar et al., 2010). This earlier type of platform is useful for laboratories that have access to a micromanipulator and a micropipette system. The on-chip OMD devices described here are capable of creating one or more passive gradients without the need for these expensive and large micromanipulators that commonly do not fit into microscope environmental and temperature control chambers. In their simplest form, the bulk glass devices described in Figure 1 can be easily bonded into a standard one well-chambered cover slip slide and will work on any inverted microscope for standard prokaryotic or eukaryotic chemotaxis analyses. Because these devices sit in an open system, they are typically submerged in several milliliters of buffer and provide a large sink for the chemoattractants so that the gradient can be maintained for several hours. We were able to observe hundreds of *Dictyostelium* cells migrating rapidly to multiple ports eliciting stable passive gradients. We found that cells detected and responded to a stable gradient in the bulk device for these long time periods. Cells were tracked and their velocity, chemotactic index, and persistence reported while migrating toward the cAMP source. These on-chip OMDs can be readily coupled to 3D microfluidic channels to perform quantitative experiments similar to those described using our PDMS-based OMD device (Jowhar et al., 2010). Furthermore, on-chip OMDs permit multiple experiments to be performed on the same chip. The basic designs described here are extremely easy to use and could be useful in a laboratory-teaching environment in both high school and university levels.

Machining microfluidic channels into the thinner cover slip-sized silica as described in Figures 2 and 3 will enable migration assays on any microscope. We demonstrated the utility of these OMDs by setting up passive gradients and visualizing cells chemotaxing toward and into the gradient generating ports. In the first cover slip device in Figure 2, the cells were imaged using an inverted microscope. A PDMS or machined glass chip with microfluidics could be mounted on top of a cover slip gradient producing port (and inside a larger cell loading area) for a variety of cell migration studies. Multiple ports could be integrated into the cover slip so that one could perform numerous experiments at the same time. The first cover slip design demonstrated that we could image cells through a machined cover slip device and showed that a stable radial gradient could emanate and recruit cells directionally toward the five source openings. The devices provided a chemoattractant gradient that could be sensed by cells. This was evident from the tracking and quantitative analysis of the migrating cells.

With the first cover slip device, the manifold was on the same side as the cell loading port. Cell observation was performed through the machined cover slip and a thin layer of PDMS. To obtain higher resolution images of cells, we created a second cover slip device where the input manifold was on the side opposite the gradient producing ports. Using the newer device, higher quality images of migrating cells were first obtained on an upright microscope with water immersion (Fig. 3e). We then used the same device for imaging on an inverted microscope, but coupled it to a specialized one-well chamber constructed with a 20 μm thick Mylar film bottom. This allowed us to perform high numerical aperture oil immersion microscopy while maintaining the cells in a large reservoir of buffer and preserving the chemoattractant gradient (Fig. 3f).

The cover slip devices described here provided evidence that small ports etched in glass can set up a passive chemo-attractant gradient and can recruit cells into the ports. We are now developing microfluidic windows for use in intravital imaging in mice. Having the ability to image through glass cover slips that have incorporated microfluidics will be a powerful tool for intravital imaging for biomedical and clinical studies (Hak et al., 2010; Weigert et al., 2010). Drug dosing, for example, is a critical issue for the efficacy of many cancer therapies (Hochberg & Tamhane, 1987). Furthermore, having the capacity to add controlled amounts of drugs or reagents to a localized area that is under observation in a living vertebrate will be extremely useful for the study of many processes such as the firing of neurons in the brain, the development of organs in the abdomen, or the recruitment of neutrophils to a site of infection. Our system may allow the careful titration of vital dyes to optimize contrast in various tissues during imaging. While we can make submicron ports for setting up gradients, we can also make larger ports within a cover slip for the addition of various labeled cell types in order to watch their infiltration into an organ or tissue. Such a system will also allow the addition of controlled amounts of pathogens to visualize a site of infection and the immune response. In fact, gradients of chemoattractants could be setup on the inner side of the cover slip for the recruitment of specific cell types into the glass coverslip for further histochemical or proteomic analysis. These devices are extremely flexible and could support the addition of multiple ports or micro-fluidic channels for multiple simultaneous treatments. Providing drugs from two ports would produce a smooth gradient of the ratio of one drug to another, so that nonlinear double-dosage dependent effects could be studied.

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Figure 1.

Bulk SiO₂ passive chemoattractant gradient generator for recruiting migrating cells. **a:** Photograph from above of the entire device containing four cell-loading ports used in independent chemotaxis assays. **b:** Schematic of two cross sections of the open multiport gradient generator. This device was made in bulk silica and for use on an inverted microscope. Section OA is made along the direction of a gradient forming structure. Sections OB and OB' are made along the direction of the chemoattractant supply tube channel located in the acrylic manifold. **c:** Schematic of the bottom surface of the femtosecond laser machined fused silica chip. The device's microfluidic channels span over the bottom surface of a 10 × 10 × 0.5 mm³ fused silica chip. The microfluidic channels are sealed from below by a 170 μm thick cover glass that is coated with a 20 μm thick layer of PDMS. Dimensions are in μm. The manifold is silicone-bonded to the top surface of the fused silica chip. Rubber o-rings seal the connections between the chemoattractant supply tubing and the acrylic manifold. **d:** Optical microscope image of a chemical concentration gradient forming structure machined by a femtosecond laser on the bottom surface of a fused silica chip. Inset is a magnified image of upper gradient generating port. The scale bar is 60 μm. **e:** Image prior to (0 min) and after continuous

exposure to a chemoattractant gradient (80 min). Cells rapidly migrated toward the cAMP source (see Supplementary Movie 1). All units are in microns unless otherwise specified. Scale bar is 40 μm .

Figure 2.

Cover slip gradient generating device for chemotaxis assays and cell recruitment. **a:** Photograph from the side of the cell recruitment device. Like the open multiport chemoattractant device, this open device is designed for use on an inverted microscope. **b:** Schematic of the longitudinal cross section of the device. Unlike the device in Figure 1, cells crawl directly on the silica cover slip. **c:** Schematic of the bottom surface of the femtosecond laser machined fused silica cover slip. Dimensions are in μ m. **d:** View of the cell recruitment site loaded with FITC dye showing a line of five chemical concentration gradient forming 15 μ m diameter holes. **e:** Fluorescent images at time 0 and after 20 min of exposure to a cAMP gradient. Cells migrate directionally on the silica toward and in some instances into the ports. Five cells are marked with white asterisks at time 0 and two more cells are recruited into the ports from distances outside of the initial field of view during the 20 min time-lapse video (see Supplementary Movie 2). All units are in microns unless otherwise specified. Scale bar is 30 μ m.

Figure 3.

Cover slip gradient generating device for chemotaxis assays and cell recruitment designed for both upright and inverted microscopes. **a:** Schematic of the device cross section when used with an upright microscope. As is the case in Figure 2, cells in this open device migrate on the surface of the silica cover slip. **b:** Schematic of the same device when inverted and used with an inverted microscope. This device differs from the upright apparatus because it has an added culture chamber with a 20 μm thick Mylar film bottom that the apparatus rests on. **c:** Schematic of cell seeding surface of the silica device surface showing the three chemoattractant releasing ports. **d:** Photograph of the cover slip device on an upright microscope. Inset is magnified image of three 15 μm diameter gradient generating ports in the silica cover glass. Scale bar is 50 μm . **e:** Shown are frames of cells migrating toward and into the three gradient generating ports at the beginning and end of an 80 min time-lapse series. White arrows mark the gradient generating ports. The left picture shows the initial time point (0 min) just prior to increasing Harvard syringe pump flow 50 $\mu\text{L}/\text{h}$ and forming a cAMP gradient. The right picture represents the last time point of the 80 min movie (Supplementary Movie 3). During the course of the movie, eight cells actually migrated into the ports. Scale bar is 15 μm . **f:** Image of cells migrating toward the same three ports as above except these cells were imaged on an inverted Zeiss microscope

in phase contrast. Cells were loaded with the device upright, allowed to settle, and then the apparatus was inverted and placed into a bath with a Mylar film bottom. Cell migration is observed through the Mylar film. Left image represents the initial image (0 min) just prior to cAMP gradient addition, and the right image represents the 80 min time point from Supplementary Figure 2.

All units are in microns unless otherwise specified. Scale bar is 15 μm .

Figure 4.

Gradient profile of the four-sided SiO₂ passive gradient generating device. **a:** Image of the device and a FITC gradient with a flow rate of 50 $\mu\text{L/h}$ applied by a Harvard syringe pump. The white dotted line represents the line intensity measurements in panel B averaged over 60 min. The scale bar is 100 μm . **b:** To determine the difference in concentrations the cells might encounter along the device, we calculated the relative changes in fluorescence intensity that a 20 μm cell might encounter in the gradient. Graph of the fluorescence intensities of the dotted line in panel **a** plotted as a function of distance in microns. Cell 1 (green) in region 1 (50–70 μm) depicted by two yellow lines and cell 2 (green) in region 2 (170–190 μm) depicted by two blue lines represents the gradient that a 20 μm cell would encounter in this region of the device. The intensity change from the front to the rear of cell 1 in region 1 is 4%. Cell 2 in region 2 only displays a change in intensity of only 8%.

Figure 5.

Cell chemotaxis toward a point source of cAMP in the bulk glass device. **a:** Cell migration tracks of five independent cells (A–E) were analyzed from the same experiment. The cells migrate directionally toward the cAMP source labeled with an asterisk in the figure. The starting point for each cell is marked with an X. Scale bar is 10 μm . **b:** Quantitation of the chemotaxis parameters for cells A–E. In the table, columns V, C.I., and P represent velocity ($\mu\text{m}/\text{min}$), chemotactic index, and persistence, respectively. The last four cells (B–E) were measured at multiple sections of the traveled path to the source and are scored more than once. All cells exhibited strong chemotaxis indices and persistence in the passive chemoattractant gradient. Cell E likely becomes adapted to the high cAMP concentration at the source, wanders away until it de-adapts, and then again returns to the source. As a negative control cells were tested for their ability to chemotax to a buffer loaded syringe. The cells exhibited random motility and did not move directionally toward the buffer source (data not shown).

Figure 6.

Gradient profile of the passive gradient generating ports in the SiO_2 cover slip device. **a:** Image of the cell recruitment device and FITC gradient with a flow rate of $50 \mu\text{L/h}$ using the Harvard syringe pump. The image was extracted from a 60 min movie.

The white dotted line represents where the gradient profile was measured and averaged over 60 min. Scale bar is $60 \mu\text{m}$. **b:** Graphical representation of the FITC fluorescence gradient profile (dotted white line) as a function of distance. Cell 1 (green) in region 1 ($70\text{--}90 \mu\text{m}$) depicted by two yellow lines and cell 2 (green) in region 2 ($170\text{--}190 \mu\text{m}$) depicted by two blue lines represent the gradient that a $20 \mu\text{m}$ cell would encounter in this region of the device. Similar to the bulk silica device, changes in concentrations were calculated to be 3% for cell 1 and 6% for cell 2.

Figure 7.

Cells directionally migrating toward the gradient generating openings in the cell recruitment device. **a:** Tracks of seven individual cells (A–G) migrating toward the cAMP source openings (open circles). Cell G migrated into the port. The starting point for each cell is marked with an X. Scale bar is 20 μm . **b:** Quantitation of the migration parameters for cells A–G. In the table, columns V, C.I., and P represent velocity ($\mu\text{m}/\text{min}$), chemotactic index, and persistence, respectively. The multiple data points in the table for cells A–G represent different sections of the path as the cell traveled toward the cAMP source. The majority of the cells tracked exhibited good chemotaxis indices and persistence. A negative control was conducted measuring the ability of the cells to directionally migrate toward a buffer loaded syringe. The cells exhibited random migration and did not move directionally toward the buffer source and cells did not enter the ports (data not shown).