Characterization of a membrane-based gradient generator for use in cell-signaling studies†

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Received 5th October 2005, Accepted 16th January 2006
First published as an Advance Article on the web 1st February 2006
DOI: 10.1039/b514133h

This paper describes a method to create stable chemical gradients without requiring fluid flow. The absence of fluid flow makes this device amenable to cell signaling applications where soluble factors can impact cell behavior. This device consists of a membrane-covered source region and a large volume sink region connected by a microfluidic channel. The high fluidic resistance of the membrane limits fluid flow caused by pressure differences in the system, but allows diffusive transport of a chemical species through the membrane and into the channel. The large volume sink region at the end of the microfluidic channel helps to maintain spatial and temporal stability of the gradient. The chemical gradient in a 0.5 mm region near the sink region experiences a maximum of 10 percent change between the 6 and 24 h data points. We present the theory, design, and characterization of this device and provide an example of neutrophil chemotaxis as proof of concept for future quantitative cell-signaling applications.

Introduction

Chemical gradients play an important role in mediating biological activity in vivo. Insight into the interplay between a chemical gradient treatment and the corresponding cellular response may help to determine the cues that are responsible for regulating specific cellular activities. Understanding the importance of these chemical cues may help researchers develop controlled microenvironments where the desired cellular response is produced by combining the effects of exogenous controlled gradient treatments with ongoing endogenous cell–cell signaling.

Prior to the development of laminar flow based gradient generators, it was difficult to accurately develop and predict the chemical microenvironment that cells were exposed to. Laminar flow based systems create chemical gradients by taking advantage of diffusional mixing across the interface of adjacent flowing streams. With these devices, it is possible to treat a cell population with a controlled chemical gradient and observe the biochemical and morphological responses in vitro.3–5

The continuously flowing streams of fluid provide precise control over the stability, gradient profile, concentration range and slope of a chemical gradient. The stimulus of interest can be changed “on the fly” to create a sequential chemical gradient treatment scheme. Flow based devices have successfully been used to study neutrophil chemotaxis and neuronal differentiation in vitro.6–8 While these devices are robust and provide excellent control over the chemical gradient characteristics, the continuously flowing streams that are necessary to maintain chemical gradients make these devices unsuitable for addressing biological questions where soluble factors are important in regulating cell behavior.

One way that cells respond to chemical cues in their environment is by secreting signaling factors that either affect the secreting cell itself (autocrine), or affect other types of cells (paracrine).9,10 In flow based systems, autocrine/paracrine factors cannot accumulate because flowing fluid streams immediately carry away secreted factors. In situations where cell–cell communication (via soluble factors) plays a critical role in regulating biochemical activity, the removal or accumulation of secreted factors may lead to distinctly different cellular behavior.11,12

In this paper we discuss the design, modeling and characterization of a microfluidic gradient generator that does not require flowing fluid streams to develop a stable chemical gradient. The chemical gradient is maintained by using a source/sink construct that includes a high fluidic resistance membrane. Diffusive transport is allowed through the membrane while the membrane fluidic resistance minimizes convective flows. We also present an example of neutrophil chemotaxis in response to a gradient developed in the system.

Neutrophils are extremely sensitive to chemical gradients, and they are used here as an illustrative example for future studies. The characterization of this system is an essential step that lays the foundation for quantitative use of this device to determine when cell–cell communication (via soluble factors) is an important consideration.

Experimental

The device is created in (poly)dimethylsiloxane (PDMS) using soft lithography and rapid prototyping.13 The device is fabricated from three layers of PDMS; the bottom channel

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© Electronic Supplementary Information (ESI) available: Video of neutrophil chemotaxis in response to a gradient of chemoattractant developed in the system. See DOI: 10.1039/b514133h
layer, the middle sealing layer, and the top fluid reservoir layer (see Fig. 1). Aligned access ports are punched in the layers using sharpened coring tools (2 mm and 3 mm diameter ports) and an elliptical sink region is cored in the bottom channel layer (6 mm width × 8 mm length). The access port between the bottom and middle PDMS layers (source region) is covered with a 0.2 μm pore diameter polyester membrane (Osmonics T02CP04700). The access port between the middle and top PDMS layers (cell addition port) is covered with a 10 μm pore diameter polyester membrane (Sterilitech PET10025100). The PDMS layers are then aligned and permanently bonded using oxygen plasma treatment (sandwiching and sealing the membranes in between the layers of PDMS).

The device is filled by placing a small gauge syringe needle into the sink region and using a pipette tip to inject fluid into the system through the large pore membrane. The needle acts as a pressure release and helps to fill the device without requiring separate fluid inlet ports (open ports can result in unwanted fluid motion because of unstable air/liquid interfaces). Once the device is filled, the needle is removed and the PDMS seals around the puncture hole.

Channel dimensions are 3 mm long, 1 mm wide and 0.025 mm tall (volume = 0.075 μL). The fluorophore Alexa 488 (Molecular Probes) is used to visualize the development of the gradient. Fluorescent images are taken using an inverted microscope (Nikon IX70) and captured using Metamorph (Molecular Devices). ImageJ (version 1.33i) is used for image analysis.

Primary human neutrophils for the chemotaxis demonstration were obtained as described in ref. 14.

**Results**

As a representative device we tested a system containing a 3 mm long, 1 mm wide, 0.025 mm tall channel (channel volume = 0.075 μL). The sink volume is 100 μL and the source volume is 10 μL. The device was filled with deionized water and 8 μL of 25 μM Alexa 488 was added to the source region. The probe diffuses through the membrane and into the channel creating a concentration gradient from the source region to the sink region. To visualize the development of the gradient, the system was imaged at different time points and the fluorescence intensity of Alexa 488 was measured. Using a calibration curve, the pixel intensity was converted to probe concentration (data not shown).

Upon introduction of the fluorophore into the source of the test device, approximately 6 hours is required for the gradient to stabilize (gradient development period). Fig. 2 shows the concentration profile in a 1.5 mm region of the channel at 6, 7, and 24 hours. The concentration ranges from 1.25 μM to 0.125 μM over the 1.5 mm distance (corresponding to a slope of 0.75 μM mm⁻¹). In a 0.5 mm subsection of the measured region (close to the entrance of the sink region) the concentration shifts by a maximum of 10% between 6–24 hours.

A mathematical model is used to guide device design, determine the length of the gradient development period, and predict the concentration profile of the pseudo steady state gradient. In the test device, the experimental data correlates well with the data from the predictive model (Fig. 2).

**Theory**

Volumetric flow rate is proportional to the pressure gradient along the fluid path and inversely proportional to the fluidic resistance (Q = ΔP/R). Theoretically, it is possible to limit flow rate by ensuring that the fluid levels at the inlet and outlet are equal (ΔP = 0). This approach is problematic for microfluidic systems because it is difficult to precisely match fluid levels. Surface tension effects can also cause observable fluid flow. In order to limit fluid flow resulting from small pressure differences, we incorporate a high fluidic resistance porous membrane into the system. The porous membrane helps limit fluid flow due to any pressure imbalances by increasing the fluidic resistance of the system (i.e. as R increases, Q decreases). For small molecules, the resistance of the membrane does not affect diffusive transport into the system.

The chemical species enters the channel by diffusing through the membrane in the source region and creates a concentration gradient along the length of the channel. A source/sink concept is used to create a pseudo steady state in the system where the concentration at a point does not vary dramatically with time.

![Fig. 1](image1.png) Schematic (a) side and (b) top representations of the gradient device. The chemical stimulus of interest is added over the membrane in the source region. The molecules diffuse through the membrane and into the channel creating a chemical gradient along the axial (x) direction of the channel. The large volume of the sink region (>100 μL) helps to maintain the stability of the gradient. Cells are added to the system by placing them over the membrane in the cell addition port.

![Fig. 2](image2.png) Plot showing the gradient profile along the axial direction of the channel at 6, 7, and 24 hours along with the model data. The concentration changes a maximum of 10% in a 0.5 mm region near the sink entrance.
Ideal source/sink setups maintain constant concentrations in the source and sink (i.e. infinite sources and sinks). Typically this is achieved by using flowing fluid streams to maintain the desired concentrations at the source and sink. The same idea is achieved without using fluid flow by using source and sink reservoirs with volumes that are much larger than the channel volume. The large volume sink reservoir at the end of the channel helps to maintain the chemical gradient by not allowing the chemical species to accumulate in the channel. Without the large volume reservoir, the chemical species accumulates in the channel (dc/dx = 0) and the gradient does not reach a pseudo steady state value.

A mass balance of the system shows that the gradient inside the channel does not change as long as the chemical flux entering the channel equals the chemical flux leaving the channel (dc/dt = 0 when dc/dx = dc/dx_{out}). Once these fluxes match (at the end of the gradient development period), the system enters a pseudo steady state where the gradient does not dramatically change with time. The finite period of time before the fluxes match is a function of the input concentration, molecular diffusion coefficient, and the channel length. A simple numerical model is used to predict the duration of the gradient development period.

**Model**

A 1D simulation of the diffusion equation (eqn (1)) is used to model the behavior of the system. The gradient in the z direction can be neglected if the z dimension is much smaller than the axial dimension (H/L ≪ 1). The y dependence is neglected based on experimental observations. Fig. 3 shows that the pixel intensity along the transverse direction is constant (i.e. zero gradient). The solution to the diffusion equation (with appropriate boundary conditions) provides information about the duration of the gradient development period and the pseudo steady state concentration profile.

The equation, initial and boundary conditions, respectively, are listed below (eqns (2)–(5)). The molecular diffusion coefficient for the species is denoted by $D$, $C_0$ is the initial concentration input into the source region, and $C_\infty$ is the sink concentration. Eqn (3) describes the initial condition of the system. Eqn (4) describes the rate at which the chemical species enters the channel. The factor $K$ is the partition coefficient of the membrane and is a function of pore diameter (void volume) and $A_m$ is the surface area of the membrane. The boundary condition at the entrance of the sink region (eqn (5)) states that the rate at which the chemical species leaves the channel is proportional to the difference between the concentrations in the channel and the sink region scaled by the product of the molecular diffusivity and cross-sectional area of the channel ($A_c$).

$\frac{dc}{ct} = D \nabla^2 c$  \hspace{1cm} (1)

$\frac{dc}{ct} = D \frac{c^2}{c^2}$  \hspace{1cm} (2)

$c(x,0) = 0$  \hspace{1cm} (3)

$\frac{dc(0,t)}{cx} = KA_m(c_0 - c)$  \hspace{1cm} (4)

$\frac{dc(L,t)}{cx} = -DA_c(c - c_\infty)$  \hspace{1cm} (5)

Eqn (2) is solved using the Matlab solver PDEPE. The model is useful as a predictive tool and is used to guide design modifications.

**Chemotaxis demonstration**

Neutrophil chemotaxis in response to a gradient of formyl-Met-Leu-Phe (fMLP) is demonstrated using this system. The neutrophils are added to the system by adding a small volume of cells over the membrane in the cell addition region (where membrane pore size > cell size) at the edge of the sink region shown in Fig. 1. The neutrophils settle through the membrane and into the sink region. The addition of a small volume of cells (<1 μL) to the large volume of the sink reservoir (>100 μL) ensures that the system is not disturbed during the addition process.

In the absence of a chemotactic gradient, the neutrophils neither polarize nor migrate into the channel. They remain within the footprint of the cell addition port (as shown in Fig. 4). In the presence of a gradient, the neutrophils migrate out of the sink region and begin chemotaxis along the fMLP gradient developed in the channel (see migration video in supplementary information†). Fig. 5 shows still images captured from a video of neutrophil chemotaxis in this system. Migrating cells do not take a linear path along the gradient but rather move in an ambulatory fashion with net motion in the direction of the increasing gradient.

The mathematical model and observed cell behavior can be used to correlate the chemical gradient and the cellular response. This simple demonstration opens up the door for future applications where endogenous cell–cell signaling is coupled with exogenous chemical treatments to determine how cell–cell signaling affects cellular behavior.
Discussion

Flow based gradient generation systems are gaining recognition in biological applications because they precisely control chemical gradient characteristics. However, the continuous fluid flow required to maintain the chemical gradient removes secreted soluble factors that are often important for cell–cell communication. Flow based systems also require specialized equipment (syringe pumps) to maintain fluid flow. For long experiments, the continuous flow means that large volumes of expensive reagents are used (e.g. 1 μL min⁻¹ flow rate corresponds to 1.1 mL of reagent used in a 24 hour period).¹⁴ The static generation device provides a simple method to create stable linear gradients using small stimulant volumes. The system does not require external equipment, and can easily be incorporated into existing biological studies. The device is easy to fabricate and the design can be modified to produce the desired gradient characteristics.

The static gradient system requires small volumes of stimulus (e.g. 10 μL of chemical stimulus in source reservoir). The chemical stimulus is easily introduced into the system by placing 6–8 μL of sample over the membrane in the source region and allowing it to diffuse through the membrane and into the channel. The system parameters are easily changed to adjust the gradient development period (e.g. the model predicts a 1 mm wide, 0.025 mm tall, 1 mm long channel reaches pseudo steady state in 3 hours at room temperature). The relative placement of the source and sink regions determines the slope of the pseudo steady state gradient; sources and sinks placed further apart result in less steep slopes at pseudo steady state. The mathematical model is used as a predictive tool as well as a method to guide system design.

The normalized concentration \( \frac{c}{c_0} \) value in the pseudo steady state gradient is a function of the porosity (percent void volume) of the membrane used (10% void volume). Important design parameters when choosing a membrane for this application include the hydrophilicity (to ensure proper wetting), membrane thickness, protein binding capacity and pore size.

The pseudo steady state gradient found from experimental data is not completely constant over time because the concentration in the sink region is not maintained at exactly zero (because the sink is not perfectly mixed). From eqn 5 the rate of species leaving the channel is proportional to the difference in concentration between the channel and the sink. As the diffusing species leaves the channel, the concentration in the sink close to the channel becomes non-zero and the rate of the species leaving the channel changes slightly in time. However, comparison between the model and experimental data suggests that the approximation of zero sink concentration is valid because the small incoming chemical flux is diluted by the large fluid volume in the sink. One way to mimic an ideal sink would be to periodically flush out, replace or mix the reservoir volume.

The slope of a chemoattractant gradient is thought to influence the migration rate of cells in vivo.¹⁵ This concept can be quantitatively tested using the static gradient system by employing a multiple source construct where each source is placed a different distance from the sink (resulting in a different pseudo steady state slope). Cells are introduced into a central location and the cellular migration that occurs in response to different gradient slopes can be observed in parallel to determine a correlation between cell migration and gradient slope. Similarly, multiple chemoattractants can be investigated to determine preferential behavior in response to

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Fig. 4 (a) Cells are added into the system by placing a small volume of cells over the membrane in the cell addition port. The cells settle through the membrane and into the sink reservoir. The added cells settle straight through the membrane and remain contained within the footprint of the cell addition port. (b) Neutrophils in the sink region of an untreated system (i.e. no chemoattractant present) do not polarize or migrate into the channel entrance. The white line marks the footprint of the cell addition port.

Fig. 5 Images of chemotaxing neutrophils captured by time-lapse video microscopy show the positions of four different neutrophils as marked. Images (a)–(d) are in sequential order and show the position of the marked neutrophils at 10 minutes intervals (30 minutes total). The source region was filled with 100 nM fMLP. Scale bar = 100 μm. See online video†.
an array of chemoattractants. The mathematical model is a valuable tool in the design of such experiments.

Conclusions

Chemical gradients are important for many biological processes including embryonic development, gene patterning and the regulation of immune system responses.\textsuperscript{18–20} Researchers suspect that cancer metastasis is caused in part by a concentration gradient of chemical factors that are secreted by cancerous tissue.\textsuperscript{21,22}

The ability to treat cells with chemical gradients in an \textit{in vitro} environment (where secreted factors are allowed to accumulate) is valuable when cell–cell communication plays a vital role in regulating cell behavior.\textsuperscript{23} A platform that incorporates this capability may lead to a deeper understanding behind the signaling mechanisms that promote the formation of diseased or abnormal tissue.

The primary characterization of the static gradient system provides a foundation for future studies investigating the interaction between exogenous stimulation and endogenous cell–cell communication. Experiments using this device can help to determine when cell–cell communication (autocrine/paracrine signaling) is an important factor by comparing the cellular response observed in flowing and static gradient generation schemes. This device has potential applications in drug discovery, immunology, stem cell differentiation, and cancer biology.

Acknowledgements

VVA and DJB thank the MMB research group for helpful discussions and the NIH for funding.

References


