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Combined Microfluidics / Protein Patterning Platform for Pharmacological Interrogation of Axon Pathfinding

Peng Shi^a, Stephane Nedelec^b, Hynek Wichterle^b, and Lance Kam^a

^aDepartment of Biomedical Engineering, Columbia University, New York, NY, USA

^bDepartments of Pathology, Neurology and Neuroscience, Center for Motor Neuron Biology and Disease, Columbia University Medical Center, New York, NY, USA

Abstract

This report combines microfabricated, multi-compartment chambers and protein-micropatterned surfaces into a new platform for the study of localized, sub-cellular signaling on axon guidance. We focus on the specific question of local crosstalk between N-cadherin and fibroblast growth factor receptor (FGFR) signaling. Motor neurons differentiated from embryonic stem cells extend axons from one compartment through a microchannel barrier into a second compartment containing patterns of N-cadherin against a background of laminin. N-cadherin was effective in both guiding and accelerating motor axon outgrowth. Selective inhibition of FGF receptor (FGFR) function in the axon but not the cell body reduced the rate of axon outgrowth while not affecting guidance along N-cadherin, indicating a differential role of crosstalk between these pathways in helping axons navigate the complex extracellular environment. This approach is readily applicable to other systems of guidance cues.

Introduction

Proper function of the nervous system relies on the formation of precise connections between nerve cells that span complex substrates and long distances. Axon guidance is a key process in establishing this architecture, in which cells must appropriately integrate and respond to multiple signals presented in the extracellular environment ¹. These cues come from a wide range of signaling systems, including “canonical” guidance molecules (*e.g.* Netrins, Slits, Semaphorins, and Ephrins), morphogens (*e.g.* Hedgehog, BMP, and Wnt families), growth factors (*e.g.* NGF, BDNF, HGF, and FGFs), and cell adhesion proteins (*e.g.* N-cadherin, NCAM, L1-CAM). Extensive cross-talk between these pathways has been demonstrated, introducing additional layer of complexity to the mechanisms controlling axonal navigation. Moreover, guidance molecules that are often associated with extracellular matrix components are received and interpreted by nerve cells in a highly localized manner within the growth cones found at the tips of growing axons. Axon guidance is thus an intricate process occurring on a subcellular scale, requiring highly refined experimental techniques to study and manipulate.

Contemporary microfabrication methods have much promise for enhancing the tools that are used to study how localized signaling drives cell function. For example, Campenot chambers, which consist of millimeter-scale barriers that isolate axons and cell bodies into separate compartments, have been widely used to investigate local signaling by restricting the activity of pharmacological agents to distinct parts of the neuron ². This allows localized study of receptors on the cell surface and limits the effects of detrimental or toxic drugs to the rest of

the cell. Microfabricated versions of the Campenot chamber have been more recently developed that provide higher reproducibility and better compatibility with a broad range of nerve cells by eliminating the need for a sealant compound between the chamber and substrate through which neurons must extend their axons³⁻⁶. The classic stripe assay method, in which matrices are patterned with two different guidance molecules to study axonal substrate preference, has similarly been updated by microfabrication. Specifically, the use of microchannel and microcontact printing methods allow patterning of finer features as well as the combination and alignment of more than two molecular cues^{7, 8}.

In this report, we combine the benefits of the microfabricated chamber with those of the microcontact printing to study local signaling in motor axons guided along multicomponent protein patterns⁸. Our chamber system is modified from previously described systems to accommodate aggregate cultures, such as explants or embryoid bodies containing embryonic stem cell-derived neurons, both being more suitable for the study of axon guidance than dissociated cells. As a first demonstration of this system, we investigate guidance of embryonic stem cell-derived motor neuron axons by N-cadherin, a well-known type I cadherin that mediates homophilic, calcium-dependent cell-cell interactions⁹ and plays multiple roles in the vertebrate nervous system development, including stimulation of axonal extension¹⁰⁻¹⁴. These actions are mediated in large part through classic cadherin/catenins interactions. Crosstalk of N-cadherin with FGF receptor (FGFR) and downstream PLC γ /DAG lipase/CAM kinase/Ca2⁺ pathway has been posed as an additional mechanism^{15, 16}, global inhibition of FGFR reduces neurite outgrowth on N-cadherin, with no effect on axon outgrowth on other proteins such as laminin^{17, 18}. However, the precise mechanism of this interaction remains unclear. In this study we utilize the new microfabricated system to examine localization of N-cadherin / FGFR interaction in axon guidance.

Results and Discussion

Chamber Design and Fabrication

The technical goal of this study is to build a system that allows neurons to interact with and choose between multiple biomolecular signals while providing pharmacologically isolated access to axons and cell bodies. As illustrated in Figure 1, our implementation consists of an elastomer (polydimethylsiloxane, PDMS) chamber with two open compartments, one for cell bodies and the other for axons, connected by a series of microscale channels that limit diffusion of chemicals between the compartments while allowing and directing axon growth between these structures. For this study, we chose an optimized set of microchannel dimensions of 500 μm in length, 10 μm in width, and 5 μm in height, with a center-to-center spacing of 25 μm , which supported good fluidic isolation between the two compartments while allowing robust axonal outgrowth. On the cell body side, we introduced into the microchannel barrier a 50 μm tall, 50 μm wide overhang (Figure 1B) to hold the explants in close proximity to the barrier. The microchannels and overhang were cast from a multi-height master fabricated out of photoresist using conventional techniques. The resultant structure was cut to create compartments, and then assembled onto planar glass substrates (modified with protein patterns as described below) to produce the chamber device. The cell body and axon chambers were chemically well-isolated from each other, as illustrated in Figure 2A. The axon chamber was loaded with 10 μM Cy5 fluorescent dye (MW 792 Da) in cell culture media while the cell body side contained media alone. The fluorescence image was taken after incubating the chamber at 37°C for 24 hours. Minimal fluorescence was detected in the cell body compartment, demonstrating chamber isolation. Molecular transport simulations, assuming a typical diffusion coefficient of 1 $\mu\text{m}^2/\text{msec}$ for small molecular agents and the dimensions stated above, indicate that the concentration in the cell body compartment would reach 1.7% of axon compartment concentration after 24 hours. This limited transport could be further compensated

by maintaining liquid level of cell body compartment slightly higher than that of axon compartment⁵. Simulations indicate that modest rate of media flow (10 $\mu\text{m/s}$) created by increasing the liquid depth in the cell body side by 250 μm effectively offsets solute transport; resulting in 0.053% increase in the cell body compartment concentration over 24 hours (Figure 2B & C).

Proteins in the axon chamber are patterned onto the bottom surface prior to assembly. Specifically, we use an indirect affinity approach^{7,8} to avoid drying and potential denaturation of the protein during chamber assembly. Microscale patterns of Protein A are microcontact printed onto the coverslip, which is then aligned with the microchannel barrier using a custom-built aligner (Figure 3A). A slight angle (typically 30 degrees) between the stripes and microchannels is adopted to better detect axon guidance. After assembly, fusion proteins containing the active region of the target protein appended with an Fc domain were captured from solution by the patterned Protein A (Figure 3B). Figure 3C illustrates the capture of Ncad-Fc, a fusion of the extracellular domain of N-cadherin with human Fc. The captured protein concentration was approximately 100 molecules/ μm^2 as reported previously⁸, and this capture is specific, as incubation of a Protein A-patterned surface (blocked with BSA) with fibronectin (which lacks an Fc domain) does not reveal similar patterning (Figure 3D). This indirect capture approach is highly flexible, robust, and, as will be described later in this report, extensible to include multiple proteins.

Axon Response in the Microfabricated System

Figure 4 illustrates the use of these chambers with motor neurons. HBG3 ES cells expressing a GFP transgene under the control of motor neuron specific promoter HB9 were differentiated as Embryoid Bodies (EBs) into spinal motor neurons¹⁹. Chambers were prepared by microcontact printing lines of Protein A and backcoating sequentially with polyornithine and laminin, providing a permissive reference substrate; the activity of surface-captured Ncad-Fc or other target protein is evaluated in comparison to this surface. EBs were then loaded into the cell body compartment and manipulated into position under the overhang using a glass pipette. Figure 4A illustrates the position of EBs against the barrier. Over the course of 48 hours, axons extended from these EBs progress through the microchannels and interact with the target proteins. As illustrated in Figure 4B, axons encountering lines of Ncad-Fc (25 μm width, spaced 75 μm apart) reoriented and followed these lines in contrast to the intervening regions of laminin that did not elicit turning response from motor axons. Axon guidance was N-cadherin specific, as axons on surfaces incubated with pre-immune Rabbit IgG antibody were not similarly guided (Figure 4C). To further demonstrate the specificity of N-cadherin guidance we patterned chambers with a repulsive cue, an Fc-tagged version of EphrinB2 (Eph-Fc). Axons on these surfaces avoided the lines of Eph-Fc, growing instead preferentially along the intervening laminin regions (Figure 4D). We thus demonstrated that the developed system can be utilized to study mechanisms underlying both attractive and repulsive axon guidance.

Local crosstalk between FGFR and N-cadherin signaling

The remainder of this report focuses on the main strength of our system, the ability to localize pharmacological interventions in order to identify the mechanisms of axon growth and guidance on micropatterned surfaces. We first quantify the guidance along N-cadherin stripes illustrated in Figure 2B by measuring the angle formed between the microchannels and axons. Growth parallel to the microchannels and perpendicular to the barrier edge was chosen as a zero degree reference. Figure 5A shows a histogram of the angle formed by individually identifiable axons on surfaces incubated with either Ncad-Fc or an inert antibody, collected from three separate experiments, measured 48h hours after EB plating. For these studies, the Protein A lines were patterned at an angle of 30 degrees, as notated over this graph, and the increased population of axons extending along this direction illustrates guidance by Ncad-Fc.

As a further comparison, Figure 5B reports the fraction of axons extending along the Protein A lines (within a ± 10 degree window). The fraction of axons within this window is approximately four-fold higher on Ncad-Fc lines than on lines coated with the control antibody of rabbit IgG ($P < 0.001$ by ANOVA, $n = 3$, 179, and 184 axons drawn from three separate experiments).

N-cadherin activity on axon outgrowth has been previously shown to depend upon FGFR signaling, but the localization of this crosstalk is not determined. To first test whether axon's guidance by Ncad-Fc is dependent on FGFR signaling, we measured the fraction of axons aligned along the Ncad-Fc lines (within the same window) in the presence of the FGFR inhibitor, PD173074. This inhibitor selectively inhibits FGFR tyrosine kinase activity, blocking autophosphorylation of FGFR1²⁰, and also prevents signaling through FGFR2-5. When supplemented at 100 nM (a concentration previously demonstrated to disrupt FGFR signaling¹⁷) into either the axon or cell body compartment, PD173074 does not affect the selective growth of axons along Ncad-Fc lines as illustrated by the last two columns of Figure 5B ($P = 0.747$ by ANOVA, $n = 3$, 129, and 168 axons drawn from three independent experiments for inhibitor loaded into the axon and cell body compartment, respectively). Thus, guidance selectivity of axons by N-cadherin is not dependent on FGFR signaling.

We next examined the rate of axon outgrowth on Ncad-Fc as a function of FGFR inhibition. Figure 6 compares outgrowth rate averaged over a 12-hour period, starting at 36 hours after seeding of EBs. When added to the cell body compartment, PD173074 did not decrease the rate of axon outgrowth on Ncad-Fc, compared to that with no inhibitor ($P = 0.63$, $n = 86$ and 63 axons for uninhibited and cell-body inhibited samples, drawn from three independent experiments). In contrast, FGFR inhibitor added to the axon compartment significantly reduced the rate of Ncad-Fc stimulated axon outgrowth by 40%, from $43.3 \pm 15.9 \mu\text{m/h}$ to $24.1 \pm 10.9 \mu\text{m/h}$. ($P < 0.001$, mean $\pm$ s.d, $n = 94$ axons drawn from three experiments for axon-inhibition). Importantly, axon outgrowth rate on laminin (LN) coated stripes was not influenced by the presence of FGFR inhibitor in either compartment and stayed at a basal level in each condition. These results demonstrate that stimulation of axons on N-cadherin by FGF is localized to within the growth cones and axon.

Conclusion

It is increasingly recognized that signaling within neurons must be considered on a localized, subcellular to nanoscale basis. Most prominently, Campenot chambers have been used to demonstrate that growth cone extension, collapse, and turning are modulated locally within this structure and often involve local protein synthesis²¹⁻²³. In this report, an improved, microfabricated Campenot chamber is combined with multicomponent, micropatterned surfaces to investigate the role of localized signaling in growth cone guidance of motor axon outgrowth. We demonstrate that different aspects of axon guidance by N-cadherin occur through different local pathways; specifically, enhanced axon growth rate, but not guidance, uses local crosstalk between N-cadherin and FGFR. Inhibition of FGFR in the cell body did not reduce growth rate, further supporting a model that this crosstalk is local; diffusion of signals downstream of FGFR signaling or FGFR/N-cadherin interaction from the cell body to axon does not contribute to accelerated axon outgrowth. While our report demonstrates the advantages of combining micropatterned surfaces and microfluidic chambers to study axon guidance, the system is sufficiently versatile to facilitate more complex studies of growth cone guidance behaviors in response to three or more signals or to gradients of signals in the axon chamber^{8, 24}. We anticipate that extensions such as this will provide important insights into molecular mechanism controlling growth cone responses to sequences of molecular cues more closely mimicking the complex substrates encountered by growing axons *in vivo*.

Experimental

Device Fabrication

Microfluidic chambers are fabricated using soft lithography technique²⁵ using a two-layer fabrication process⁴. The device consists of two open compartments bridged by multiple microchannels (5 μm depth, 10 μm width, and 500 μm length). A silicon wafer is spin-coated with a first layer of photoresist SU 8-2005 (5 μm), and exposed through a mask forming the layout of the microchannels. After development, a second layer of SU8-2050 (50 μm) is spin-coated and exposed through a mask containing patterns of both chambers, resulting in a master with positive relief patterns of the microfluidic device. A pre-polymer mixture of Sylgard 184 (Dow Corning) is cast against the master and cured to obtain a negative replica-molded piece. Two culture compartments are then cut into the elastomer, presenting the final chamber structure. The chambers are plasma treated for 2 minutes before assembling with a patterned coverslip to promote fluidic sealing of the final device

Protein Patterning and Substrate Preparation

Before patterning proteins, coverslips (25-mm, FisherSCI) were cleaned in Linbro 7 $\times$ detergent (ICN, mixed 1:4 with deionized water) at 80 $^{\circ}\text{C}$ for 45 minutes, rinsed extensively with deionized water, and baked at 450 $^{\circ}\text{C}$ for 6 hours. Protein A is patterned at specific location of each coverslip through microcontact printing. Briefly, PDMS stamps containing designed geometries were coated with 20 $\mu\text{g}/\text{ml}$ Protein A in PBS for 1 hour. For visualization purpose, 1/6 of the Protein A was prelabeled with Cy5 (GE Healthcare). The stamp was washed with DI water and dried by blowing nitrogen gas and placed in contact with the substrate for 10 seconds. The coverslip was further plasma treated for 2 minutes with the stamped region covered by a piece of plain PDMS.

The prepared chamber piece was assembled with the coverslips under our 6-axis aligner, to ensure that the exit of the microchannels is close to the protein A patterns. The entire inner surface of the chamber was blocked with 4% BSA for 2 hours. The axon compartment were then exposed to either Ncad-Fc (5 $\mu\text{g}/\text{ml}$, R&D Systems) or Ephrin-Fc (10 $\mu\text{g}/\text{ml}$) in PBS + 4% bovine serum albumin (BSA, Sigma) for 45 minutes at room temperature, and finally rinsed with PBS. And then, both compartments were sequentially incubated with polyornithine (Sigma) overnight (10 $\mu\text{g}/\text{ml}$ for cell body chamber, 2 $\mu\text{g}/\text{ml}$ for axon chamber) and laminin (10 $\mu\text{g}/\text{ml}$, Invitrogen) for 1 hour before loading EBs.

Cell Culture

Embryoid Body preparations of rostral cervical MNs were produced by addition of Retinoic acid and Hedgehog agonist to embryonic stem cell lines as previously described^{19, 26}. Briefly, HB9::GFP transgenic mouse-derived (HBG3) ES cells were grown on mouse embryonic fibroblasts in ES cell medium. ES cell colonies were dissociated after 2 days and cultured in DFK5 media. Media was replaced at 2 days and supplemented with all trans retinoic acid (1 μM , Sigma) and hedgehog agonist SAG (500 nM, Calbiochem). Medium was replaced on day 5 of differentiation and Embryoid Bodies (EBs) containing MNs were harvested on Day 6-7 for use in the axon guidance studies.

Imaging and Quantification

The cultures were observed by fluorescent and bright-field microscopy using a 10X objective on an inverted Olympus IX71 microscope. During imaging, the temperature and CO_2 level were maintained at 37 $^{\circ}\text{C}$ and 5% respectively using a live-cell imaging chamber (Pathology Devices, MD). To quantify the axons' growth speed, images were taken at 12 hour interval to

identify the extended length of each axon during that period, and to derive the averaged hourly growth rate.

Molecular Transport Simulation

Simulation of diffusive transport in the channels was done by finite element methods, using the COMSOL software package (COMSOL Multiphysics v3.2, Burlington, MA). The simulations presented here use the convection-diffusion model and assume a diffusion coefficient of $1 \mu\text{m}^2/\text{ms}$ and media viscosity at 37°C of $\mu=6.92 \times 10^{-4} \text{ Pa}\cdot\text{s}$. The initial concentration of chemical in the axon compartment was $10 \mu\text{M}$, and set to zero in the other compartment. For calculations of flow versus pressure head difference, the resistance of the rectangle microscale channels, which measured $500 \mu\text{m}$ in length (L), $10 \mu\text{m}$ in width (w), and $5 \mu\text{m}$ in height (h), was estimated as $R \approx 12\mu L/(h^3 w(1-0.63h/w))^{27}$.

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Figure 1. System illustration

(A) Graphic representation of our chamber system for combining cell body / axon isolation and outgrowth selectivity. (B) The compartments are isolated by a microchannel barrier, containing a linear array of 10 μm wide, 5 μm high channels spaced 25 μm apart. An overhang in the region next to the barrier simplified loading of aggregate samples.

Figure 2. Chemical isolation between compartments

(A) Fluidic isolation of Cy5 dye to the axonal compartment after 24 hours incubation at 37 °C, scale bar: 200 μm. (B) Simulated distribution of a diffusing chemical under no-flow or pressure-driven flow conditions. Constant flows of 0, 5, and 10 μm/s. (C) Concentration profiles of diffusion under flow conditions, derived from the simulations shown in Panel B.

Figure 3. Patterning of guidance proteins

(A) A combined internal reflection microscopy and fluorescence image showing alignment of microchannel walls (dark regions) with lines of protein A (red). (B) Fc-containing proteins are captured onto the patterned Protein A. (C,D) Demonstration of indirect capture using Ncad-Fc (panel C) onto protein A. Fluorescently-labeled fibronectin was not selectively captured onto these lines (panel D). Scale bar: 200 μm .

Figure 4. Axon guidance in response to attractive and repulsive cues

(A) EBs containing motor neurons expressing GFP (green) and extending axons maintain close proximity to the microchannels. (B) Guidance of motor axons by Ncad-Fc captured onto the Protein A lines (red). (C) No guidance was observed on patterned surfaces incubated with rabbit IgG. (D) EphrinB2-Fc, captured onto the Protein A, repelled motor axons. Axon outgrowth was limited to the intervening regions of laminin. Scale bar: 200 μ m

Figure 5. Axon guidance on N-cadherin

(A) Histogram of axon extension angle by neurons on surfaces incubated with Ncad-Fc or rabbit IgG. (B) Fraction of axons extending along the protein A lines. Addition of FGFR inhibitor, PD173074, into either the axon or cell body compartment does not affect guidance on N-cadherin. Data are mean $\pm$ s.d. from three independent experiments representing a total of 179, 184, 129 and 168 axons for each of the four conditions, respectively. * $P < 0.001$ compared to Ncad-Fc in the absence of inhibitor, by ANOVA.

Figure 6. Local crosstalk between FGFR and N-cadherin signaling

FGFR inhibitor impairs the ability of Ncad-Fc to stimulate axon outgrowth when applied locally to the axonal compartment, but not the body compartment. Similar effects of FGFR were not observed in axons growing on laminin region. Data are mean $\pm$ s.d. from three independent experiments, representing a total of 86, 63, 94, 51, 50, and 53 axons for each of the conditions (left-to-right). * $P < 0.001$ by ANOVA, comparisons only between axons on the same protein substrate.