Published in final edited form as:

J Neurosci Methods. 2014 January 15; 221: . doi:10.1016/j.jneumeth.2013.09.018.

A Microchip for Quantitative Analysis of CNS Axon Growth under Localized Biomolecular Treatments

Jaewon Park^a, Sunja Kim^b, Su Inn Park^c, Yoonsuck Choe^c, Jianrong Li^{b,*}, and Arum Han^{a,d,*}

^aDepartment of Electrical and Computer Engineering, Texas A&M University, College Station, TX 77843, USA

^bDepartment of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843, USA

^cDepartment of Computer Science and Engineering, Texas A&M University, College Station, TX 77843, USA

^dDepartment of Biomedical Engineering, Texas A&M University, College Station, TX 77843, USA

Abstract

Growth capability of neurons is an essential factor in axon regeneration. To better understand how microenvironments influence axon growth, methods that allow spatial control of cellular microenvironments and easy quantification of axon growth are critically needed. Here, we present a microchip capable of physically guiding the growth directions of axons while providing physical and fluidic isolation from neuronal somata/dendrites that enables localized biomolecular treatments and linear axon growth. The microchip allows axons to grow in straight lines inside the axon compartments even after the isolation; therefore, significantly facilitating the axon length quantification process. We further developed an image processing algorithm that automatically quantifies axon growth. The effect of localized extracellular matrix components and brain-derived neurotropic factor treatments on axon growth was investigated. Results show that biomolecules may have substantially different effects on axon growth depending on where they act. For example, while chondroitin sulfate proteoglycan causes axon retraction when added to the axons, it promotes axon growth when applied to the somata. The newly developed microchip overcomes limitations of conventional axon growth research methods that lack localized control of biomolecular environments and are often performed at a significantly lower cell density for only a short period of time due to difficulty in monitoring of axonal growth. This microchip may serve as a powerful tool for investigating factors that promote axon growth and regeneration.

Keywords

Microfluidic culture platform; axon growth rate; compartmentalized culture; localized biomolecular treatment; quantitative axon length analysis; automated measurement

© 2013 Elsevier B.V. All rights reserved.

*Co-corresponding authors: Arum Han, arum.han@ece.tamu.edu 309C WERC, Texas A&M University, College Station, TX 77843-3128, USA (Tel: +1 979-845-9686, Fax: +1 979-845-6259) Jianrong Li, jrli@cvm.tamu.edu, Tel: +1 979-862-7155.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Unlike peripheral nervous system (PNS), adult mammalian central nervous system (CNS) axons normally do not regenerate after injury, which often results in permanent functional deficits. Extracellular inhibitory factors such as myelin-associated molecules dominantly attribute to the suppression of CNS axon regeneration (Fitch and Silver, 2008; Qiu *et al.*, 2000). However, neutralization of these known axon regeneration inhibitory factors allowed only limited axon regeneration *in vivo*, while manipulation of axon growth pathways has been shown to promote regeneration (Harel and Strittmatter, 2006; Park *et al.*, 2008; Yiu and He, 2006). Thus, both the extracellular inhibitory factors and the intrinsic growth capability of the neuron play a critical role in axon regeneration.

In order to better understand the growth or regeneration mechanisms of CNS axons and to find biomolecules that enhance axon growth or regeneration, methods that enable precise control of the biochemical environment and easy quantification of axon growth are critically needed. However, conventional culture plate-based *in vitro* neuron culture methods are significantly limited in conducting such studies. First, in *in vivo* situations, axons are often far away from the cell bodies and may encounter very different microenvironments. However, in most conventional culture methods, it is almost impossible to have different biochemical environments for neuronal soma and axon respectively, making it difficult to investigate the localized effect of a particular biomolecule on axonal growth under more *in vivo* like environment. Campenot chamber is probably the only conventional method with the capability to provide different biochemical environment for somata and axons (Campenot, 1977). The chamber utilizes a Teflon® divider assembled on a thin layer of silicone grease for isolating axons from neuronal somata or dendrites and has been widely used for studying axon-glia interaction and axonal biology of dorsal root ganglion (Ishibashi *et al.*, 2006; Ng *et al.*, 2007). However, the Campenot chamber involves complicated manual preparation steps, and has tendency to leak due to imperfect grease seal. Second, tracking and quantitatively analyzing the extent of axon growth in response to these factors over long culture period is challenging. Most *in vitro* neuron cultures are optimized at certain areal cell density (typically 250-1500 cells/mm²) for optimum paracrine support (Brewer *et al.*, 1993; Hartikka and Hefti, 1988; Ito *et al.*, 2010; Robert *et al.*, 2012). However, conventional axon growth studies are often performed at a significantly lower cell density (3-20 cells/mm²) and for only a short period of time after cell seeding, typically less than 5 days (Goldberg *et al.*, 2002a; Goldberg *et al.*, 2002b; Winzeler *et al.*, 2011). This is mainly due to difficulties in tracking and quantitatively analyzing the length of randomly grown axons that form complex networks when cultured at regular cell densities. Other analyses such as comparing the amount of proteins associated with axonal growth (*e.g.*, growth associated protein-43) have been used, but typically require time-consuming and labor-intensive sample preparation steps (Benowitz *et al.*, 2002; Goldberg *et al.*, 2004). Moreover, quantification of specific proteins associated with axon growth may not directly reflect the actual axon length. Therefore, an *in vitro* neuron culture platform that provides physically and biochemically controlled microenvironments, coupled with a capability to easily quantify axonal growth, all at commonly used *in vitro* cell densities, could lead to important advances in understanding and finding biochemical factors or pharmaceuticals that enhance the growth capability of CNS axons.

Here, we present a microchip that is capable of isolating axons from neuronal somata or dendrites for quick and easy quantitative axonal growth analysis. The microchip, similar to the Campenot chamber, utilizes height difference of microstructures to isolate axons from neuronal somata and dendrites, yet provides perfect seal against the substrate and can be mass fabricated in much reduced time. In addition, the microchip physically guides the isolated axons to grow in straight lines for easy length quantification that could not be done

by the conventional Campenot chamber or other compartmentalized neuron culture platforms (Majumdar *et al.*, 2011; Park *et al.*, 2009; Taylor *et al.*, 2005). Together with an image processing method that can automatically measure and quantify the length of axons through pattern recognition algorithm, we investigated the effects of various extracellular matrix (ECM) components including Matrigel™, laminin, fibronectin, collagen, and chondroitin sulfate proteoglycan (CSPG) as well as brain derived neurotrophic factor (BDNF), and show that their effect on axon growth could be significantly different depending on which part of the neurons are exposed to such factors.

2. Materials and methods

2.1. Quantitative axon growth analysis microchip design

The microchip is composed of three poly(dimethylsiloxane) (PDMS) components; a top compartment layer, a bottom axon-guiding microgroove layer, and a cylindrical ridge structure (Figure 1A). The top compartment layer has one soma compartment in the center and six surrounding axon compartments that are 800 μm apart from the soma compartment. All compartments are designed to be a single well-type open configuration where each compartment also serves as a reservoir that can hold 400-800 μl of culture medium. The open compartment configuration not only provides better cell culture environments by facilitating CO_2 exchange but also allows easier management of the areal cell density. In addition, this open configuration allows physical axotomy to be performed on the isolated axons without any special equipment (such as laser setup) by simply transecting the isolated axonal layer inside the axon compartment to study axon regeneration or cellular interactions upon axonal damage (Supplementary Figure S1). The bottom layer has a radial array of 3 μm deep, 20 μm wide, and 10000 μm long axon-guiding microgrooves. Unlike previously reported compartmentalized neuron culture microdevices where two compartments are connected by microchannels (Park *et al.*, 2012; Taylor *et al.*, 2005), the microchip we developed here utilizes microgrooves patterned on the bottom layer to connect the central soma compartment to the surrounding axon compartments. Each of the six satellite axon compartments is connected to the central soma compartment by approximately 70 axon-guiding microgrooves. The shallow depth (3 μm) of the microgrooves confines neuronal somata to the soma compartment, while allowing axons to grow into the neighboring axon compartments. The unique feature of this microchip is that the microgrooves not only enable isolation of neuronal somata and dendrites from axons but also continue to physically guide axons to grow in straight lines even within the axon compartment (Figure 1B). The ridge structure was added to the center of the soma compartment to minimize the required number of cells by reducing the cell culture surface area and to increase the axon isolation efficiency.

2.2. Microchip fabrication

The compartment layer is replicated from a PDMS master that has been cast molded from a poly(methyl methacrylate) (PMMA) master mold, fabricated by a bench-top CNC milling machine (MDX 40, Roland, Irvine, CA). The microgroove layer was replicated from a SU-8™ (Microchem, Inc., Newton, MA) patterned Si master mold fabricated through a photolithography process. Both the PDMS and the Si master molds were vapor coated with (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (United Chemical Technologies, Inc., Bristol, PA) and rinsed with isopropyl alcohol (IPA) prior to the replication to facilitate PDMS release from the master mold. The center ridge structure was cut out from a 2 mm thick PDMS slab using a round punch (diameter: 10 mm). The three components were exposed to oxygen plasma for 120 seconds prior to assembly to improve bonding. Assembled microchips were immersed in deionized water followed by an autoclave process

for sterilization. The overall microchip fabrication process is illustrated in Supplementary Figure S2.

2.3. Tissue dissociation and cell preparation

Primary CNS neurons were prepared from forebrains of embryonic day 16 Sprague-Dawley rats as described in the previous publication (Koito and Li, 2009). Briefly, forebrains free of meninges were dissected in ice-cold dissection buffer ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's Balanced Salt Solution containing 10 mM HEPES), dissociated with L-cysteine activated papain (10 units/ml) for 5 minutes at 37°C, and resuspended in dissection medium containing trypsin inhibitor (10 mg/ml) for 2-3 minutes. Following two more washes with the trypsin inhibitor solution, the tissue was resuspended in a plating medium (NBB27 + glutamate: neurobasal medium containing 2% B27, 1 mM Glutamine, 25 μM glutamic acid, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin) and triturated with a fire-polished glass Pasteur pipette. The cells were then passed through a 70 μm cell sieves and live cells were counted using a hemocytometer and trypan blue exclusion assay. The viability of isolated cells was constantly greater than 90-95%. $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution, neurobasal medium, B27, penicillin, streptomycin and goat serum were from Invitrogen (Carlsbad, CA). Poly-D-lysine, papain, trypsin inhibitor, glutamine, and glutamic acid were from Sigma Aldrich (St. Louis, MO).

2.4. Microchip cell culture

Dissected primary neurons diluted in 200 μl of plating medium (NBB27 with glutamate) were loaded into the soma compartment of microchip at an areal density of 500-2000 cells/ mm^2 . 150 μl of culture medium was added to each of the six axon compartments to prevent the substrate from drying. The microchip was then left inside a 37°C humidified 5% CO_2 incubator for 30 minutes to allow cells to settle down and attach to the bottom, followed by adding 600 μl of culture medium to the soma compartment and 200 μl of culture medium to each of the axon compartment. Cells were then cultured inside a 37°C humidified 5% CO_2 incubator without culture medium change for four days. At DIV 4, the culture medium was replaced with NBB27 without glutamate. Culture medium was exchanged with NBB27 without glutamate containing either ECM or BDNF at DIV 7 for localized treatment. The growth of axons was analyzed at DIV 11 without any additional culture medium exchange.

2.5. Localized biomolecular treatment

Fluidic isolation between the neuronal soma/dendrites and the axons was achieved by generating fluidic level difference between the soma and the axon compartments (Park *et al.*, 2012; Taylor *et al.*, 2005). During the localized soma treatment, small but sustained medium flow from the axon compartment (higher fluidic level) toward the soma compartments (lower fluidic level) prevents biochemicals applied to the soma compartments from flowing or diffusing into the axon compartments. In the case of localized axon treatment, the small flow from the soma compartment (higher fluidic level) toward the axon compartments (lower fluidic level) maintains fluidic isolation.

For the localized biomolecular treatments, selected ECMs (Matrigel™, laminin, fibronectin, collagen, and CSPG) and BDNF were added directly to either the soma or the axon compartment as an alternative method to coating at DIV 7, a time point when numerous axons have already grown across the sealed microgrooves and have been established inside the axon compartments (Supplementary Figure S3). This is because the coating of cell culture surfaces with ECMs often results in blockage of the sealed microgrooves and lead to failure in isolating axons. Matrigel™ was from BD Biosciences (San Jose, CA). Fibronectin (F1141) and collagen (C-3867) were from Sigma Aldrich (St. Louis, MO). Laminin

(23017-015) was from Invitrogen (Carlsbad, CA) and BDNF (GF029) and CSPG (CC117) were from Millipore (Billerica, MA).

2.6. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10-20 minutes, washed with PBS, and blocked with TBS-T (50 mM Tris-HCL, pH 7.4, 150 mM NaCl and 0.1 % Triton X-100) containing 5% goat serum. The fixed cells were incubated overnight at 4°C with antibodies against neurofilament-H (NF) at 1:1000 dilution (Chemicon, Temecula, CA) or microtubule-associated protein 2 (MAP-2) at 1:1000 dilution (Chemicon, Temecula, CA). After washing with TBS-T, secondary antibody conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (1:1000, Molecular Probes, Inc., Eugene, OR) was incubated with the cells for 1 hour at room temperature. Cell images were captured using a fluorescent microscope (Olympus IX71) equipped with a digital camera (Olympus DP70).

2.7. Axon growth analysis

Calcein-Am (1 μ M) was added to the axon compartment at DIV 11 for visualization of axons. Isolated axons were imaged with a fluorescent microscope (Olympus IX71) equipped with a digital camera (Olympus DP70). Effects of localized ECM treatments on axon growth presented in the paper were manually analyzed and effects of localized BDNF and Matrigel™ treatments on axon growth were analyzed by the developed algorithm.

2.7.1. Manual axon growth analysis—Lengths of isolated axons were measured with NIS-Element 2.30 (Nikon Instruments, Inc.) software from acquired images by measuring the length of the longest grown axon of each microgroove from the sealed microgroove outlet.

2.7.2. Automated axon growth analysis—An image processing and axon tracing algorithm has been developed to automate the entire axon growth quantification process as shown in Figure 2 (see Supplementary Information for details). (1) For simplicity in later processing, the original image is rotated to a standard orientation so that the microgrooves, hence the axons within the microgrooves, are aligned to the vertical axis. (2) The rotated image is convolved with a Difference-of-Gaussian (DoG) filter to remove luminance irregularities and to enhance the edges. (3-4) The baseline, defined as the line connecting the starting points of all axons where the axons exit from the sealed microgroove section (sealed microgroove outlets), is detected through the following steps: oriented Gabor filter (horizontal), thresholding, histogram analysis, and finally quadratic curve fitting. (5-6) Starting from the bottom part of the image, end points are detected through the following steps: thresholding, denoising, oriented Gabor filter (vertical), scanning, and candidate point selection. (7) Among detected end points, candidate axons for each microgroove are found by searching around each end point using long oriented lines. (8) The candidate axons from step (7) include spurious cases, including redundant axons anchored to the same end point and axons that are out of bound (those that can or do grow beyond the image boundary). These spurious candidate axons are eliminated and a final sanity check is done by comparing the location and orientation of the neighboring candidate axons. After running the axon tracing algorithm, the lengths of the axons in all images are calculated based on their start point and end point.

2.8. Statistical Analysis

All data presented are mean $\pm$ standard deviation from at least three independent experiments for each condition. The differences among the experimental conditions were

analyzed by one-way analysis of variance (ANOVA) and Bonferroni post-test for multiple comparisons using Prism Graph Pad (GraphPad Software Inc., CA) and SPSS (IBM Corporation, NY), with $p < 0.05$ considered as statistically significant.

3. Results and discussions

3.1. Axon isolation and guidance

After loading and culturing CNS neurons from E16 rats for 11 days inside the soma compartment, isolation of axons was observed inside the axon compartment (Figure 3A). In addition, axons that crossed into the axon compartments continued to grow straight due to the physical guidance of the microgrooves (Figure 3B). The difference in growth morphology of isolated axons with and without these axon guiding microgrooves is evident (Figure 3B-inset). This axon-guiding feature is the key factor that facilitates easy quantitative and automated analysis of axon length even for high-density cell cultures by preventing axons from tangling with those from neighboring cells.

Having established the isolation and guidance capability of the developed microchip, we wanted to further increase the efficiency of axon isolation. We have previously demonstrated that minimizing the distance of neuronal somata from the microchannel inlet significantly improves the axon isolation efficiency (Park *et al.*, 2009). This is still true in this present design as can be seen through retrograde staining of the isolated axons, where most of the somata with isolated axons inside the axon compartments are located in the vicinity of the sealed microgroove inlets (Figure 3C). To further increase the isolation efficiency of the microchip, a cylindrical ridge structure was added to the center of the soma compartment so that the majority of neurons are loaded within 2.5 mm of the sealed microgroove inlets (Figure 3D). This ridge structure not only helped more cells to be located close to the sealed microgroove inlets but also reduced the number of neurons needed to achieve the same areal cell density by approximately 56% due to reduced effective surface area (177 mm² to 78.5mm²). After 11 days of culture at plating density of 1000 cells/mm², more than $97 \pm 2\%$ ($n > 3$, number of tested microchips) of the microgrooves were filled with axons when the ridge structure was present, compared to $59 \pm 15\%$ ($n > 3$, number of tested microchips) without the ridge structure (Figure 3E). To further characterize the relationship between the cell plating density and axon isolation efficiency, neurons were plated inside the soma compartment with the ridge structure at three different cell densities (500, 1000, and 2000 cells/mm²). The axon isolation efficiency was evaluated by the percentage of channels filled with axons. Cells plated at an areal density of 1000 cells/mm² showed significantly higher isolation efficiency compared to 500 cells/mm² (96.8% vs. 84.9%, $p = 0.040$), while cells plated at 2000 cells/mm² showed only a minute increase compared to 1000 cells/mm² with no statistical significance (98.0% vs. 96.8%, $p = 1.000$) (Figure 3F). This axon isolation efficiency was uniform for all six axon compartments (Supplementary Figure S4). Based on this result, 1000 cells/mm² was selected as the optimal neuron plating density for all subsequent experiments.

3.2. Fluidic isolation between multiple compartments

Since biomolecules applied to the axon compartments are fluidically confined, the satellite axon compartments are isolated from each other as well as from the soma compartment, thereby allowing six different localized biomolecular treatments to be performed simultaneously on the isolated axons in a single device. In order to demonstrate the fluidic isolation and the localized treatment capability, isolated axons inside the axon compartments were exposed to CSPG (5 µg/ml), a family of proteoglycan known to negatively regulate axon growth and cause retraction/degeneration of the established CNS axons (Wang *et al.*, 2008). After 4 days of treatment, most of the CSPG treated axons were degenerated, with

only a few very short axon segments remaining around the outlet area of the sealed microgrooves. In contrast, neurons in the soma compartment and axons inside the sealed microgrooves were not affected by CSPG applied to the axon compartment, suggesting that CSPG applied to the axon compartment was properly confined within the compartment (Figure 4A).

Next, the parallel localized biomolecular treatment capability was tested by applying six different concentrations of CSPG (0–25 $\mu\text{g/ml}$) to the isolated axons in each of the six axon compartments. After 4 days, we found that CSPG at concentrations lower than 250 ng/ml was not sufficient to cause degeneration of pre-established axons. However, at concentrations higher than 2.5 $\mu\text{g/ml}$, CSPG induced axonal retraction (Figure 4B). These results are consistent with previously reported effective dosage of CSPG (3 $\mu\text{g/ml}$) for inhibition of neurite outgrowth in conventional cell culture (Lingor *et al.*, 2007). Parallel localized treatment feature of the microchip opens up the possibility of further developing the current multi-compartment microchip into a higher-throughput screening platform by further segmenting the axon compartments. The microchip also has the potential to minimize the batch-to-batch or replicate-to-replicate variation since a single soma compartment is shared by multiple axon compartments that can be exposed to different localized axonal treatments within a single microchip.

3.3. Automated axon growth analysis

Results of the image processing and axon tracing algorithm from key intermediate steps are shown in Figure 5A–C (see Supplementary Figure S5 for detailed results). Figure 5D shows the final result of the image processing overlaid on top of the original image for comparison. The developed algorithm successfully traced the isolated axons and quantified the length in significantly reduced time. Fidelity was validated by comparing the average length of axons measured by the automated method against the manual tracing method and the automatically quantified axon lengths were less than 1% different from the manual measurement results (Figure 5E, Supplementary Figure S6).

3.4. Effects of localized ECM treatment on axon growth

To enhance the survival and growth of neurons *in vitro*, culture substrates such as glass coverslips or multi-well culture plates are often coated with extracellular matrices (ECMs) (Baron-Van Evercooren *et al.*, 1982; Gingras *et al.*, 2008; Serra *et al.*, 2007; Taylor *et al.*, 2007; Vyas *et al.*, 2010). However, the direct effect of ECMs on axon growth has not been fully characterized. We have chosen four ECMs (MatrigelTM, laminin, fibronectin, and collagen at protein concentration of 50 $\mu\text{g/ml}$) that are most widely used as substrate coating for cell cultures to investigate their effect on axon growth. CSPG (5 $\mu\text{g/ml}$) was used as a negative control for axon growth. First, the direct effect of ECMs on isolated axons was investigated by adding ECMs only to the axon compartment. Average lengths of the isolated axons inside the axon compartments were analyzed 4 days later (Supplementary Figure S7A). All tested ECMs were found to promote the isolated axons to grow approximately 35–50% longer than that of the control, with MatrigelTM being the most effective and collagen the least effective (MatrigelTM – 53.4%, $p < 0.001$; collagen – 36.1%, $p < 0.001$) (Figure 6A). The effects of laminin and fibronectin were comparable to that of MatrigelTM (laminin – 48.5%, $p < 0.001$; fibronectin – 53.2%, $p < 0.001$) and no statistical significance was observed among these three treatments (MatrigelTM vs. laminin, $p = 0.910$; MatrigelTM vs. fibronectin, $p = 1.000$; laminin vs. fibronectin, $p = 0.992$). CSPG, on the other hand, caused all pre-existing axons to degenerate as expected with no trace of axons left inside the axon compartment.

Next, to investigate how local exposure of neuronal somata to ECMs influences axon growth, ECMs were applied to the soma compartment at the same protein concentration as described above (Supplementary Figure S7B). Matrigel™, laminin, and collagen treatments to the neuronal somata were found to be more effective than localized axon treatment and promoted axon growth (Matrigel™ – 74.2%, $p < 0.001$; laminin – 67.2%, $p < 0.001$; collagen – 80.3%, $p < 0.001$) (Figure 6B). In contrast, although fibronectin promoted axon growth as effective as Matrigel™ or laminin when added directly to the isolated axons, it had only minimal effect when added to the neuronal somata (soma vs. axon: 11% vs. 53% with $p = 0.016$ and $p < 0.001$, respectively). Interestingly, CSPG had contrasting effects on axon growth depending on where it acts on. Whereas local CSPG treatment of isolated axons resulted in robust axon retraction and degeneration (Figure 4, Figure 6A), localized neuronal somata exposure to CSPG actually promoted axon growth (27% increase compared to control, $p < 0.001$, Figure 6B). This was unexpected as CSPGs are mainly known for their axon growth inhibitory activities. However, in most previous *in vitro* studies, CSPGs were used either as substratum or bath-applied to cultured neurons. CSPGs are a complex family of proteoglycans that consist of a core protein and covalently attached chondroitin sulfate glycosaminoglycan side chains, and are up-regulated in the CNS in response to injury (Laabs *et al.*, 2005). They are the predominant extracellular matrix molecules with axon growth inhibitory activities in glial scars that impede CNS axon regeneration (Rolls *et al.*, 2009; Silver and Miller, 2004). However, several studies have suggested neuron growth-promoting features of CSPGs (Rolls and Schwartz, 2006). Our finding is also consistent with a recent study that demonstrated enhanced local protein synthesis of RhoA within distal axons underlies CSPG-induced inhibition of axon growth (Walker *et al.*, 2012). It should be noted that the CSPG used in our study contains a mixture of large extracellular chondroitin sulfate proteoglycans from embryonic chicken brain and includes neurocan, phosphacan, versican, and aggrecan. At present, it is not known whether the repelling activity of CSPG on distal axons and the growth-promoting activity on neuronal somata is due to distinct CSPG components or to the sugar side chains since modifications of the polysaccharide side chains modulates biological activities of CSPGs. Nevertheless, the contrasting effect of CSPGs on CNS neurons and distal axons revealed in this study underscores the need of spatial dissection of molecular mechanisms of biofactors that impact axon growth.

3.5. Effects of localized BDNF treatment on axon growth

Next, we investigated whether axonal growth could be further enhanced by a combination of an ECM with a growth factor. BDNF, which is known to promote the survival, growth and differentiation of neurons (Acheson *et al.*, 1995; Huang and Reichardt, 2001), was mixed with Matrigel™ to examine if it further enhances axon growth. Among the four ECMs tested, Matrigel™ was chosen because it is one of the most commonly used ECM for neuron cultures and because it had similar degree of axon growth promotion effect regardless of whether it was applied locally to somata or axons. Matrigel™ (50 µg/ml), BDNF (1 ng/ml), and their combination was added to either the soma compartment or the axon compartment at DIV 7, and axonal growth was analyzed at DIV 11. When added to axons only, Matrigel™ and BDNF promoted axon growth by approximately 34.1% ($p < 0.001$) and 33.7% ($p < 0.001$) respectively and in combination, by approximately 63% ($p < 0.001$) (Figure 6C). Significant synergistic effect of combined Matrigel™ and BDNF treatment was not observed, however additive effect was evident. Interestingly, BDNF, which was as effective as Matrigel™ when locally added to the isolated axons, had only a small effect on axon growth when added only to the neuronal somata (9.4% increase, $p < 0.001$) (Figure 6D). Combination of Matrigel™ with BDNF was not statistically different from that of Matrigel™ treated alone ($p = 1.000$). In summary, our results show that some of the previously known biomolecules have contrasting effect on axon growth depending on which part of a neuron is exposed to the biomolecules. The results also demonstrate the need for an

in vitro culture platform where localized biomolecular environments can be simply manipulated and axon growth can be readily quantified to further understand the mechanism of axonal growth, as well as to find potential factors or therapeutics that can promote axon regeneration.

4. Conclusions

We have demonstrated a microchip capable of isolating and guiding axons from neuronal somata as well as dendrites for easy manipulation of cell culture microenvironments for investigating effects of localized biomolecular treatment of different parts of neurons (in this case somata and axons) on axon growth. The microchip resembles the isolation scheme of the Campenot chamber but provides perfect fluidic seal throughout the culture period and could be mass fabricated in much reduced time without complicated preparation steps. More importantly, the device contains physical axon-guiding features that could not be achieved with the Campenot chamber or other current neuron microdevices. Axon guiding feature along with the newly developed image processing algorithm enabled automated quantitative analysis of the axonal growth. This is the first step towards utilizing a microchip to better understand the mechanism of CNS axon growth. Our microchip overcomes limitations of conventional methods where localized control of biochemical environment and monitoring of axonal growth over a longer period of time are restricted. The multi-compartment design can be further developed into a high-throughput screening platform. Using this microchip, we found a contrasting effect of CSPGs on axon growth depending on spatial activation of CSPG signaling pathways in distal axons or cell bodies. While the mechanism by which CSPGs act on cell body to promote axon growth remains to be identified, our results are consistent with recent studies that suggest neuron growth-promoting features of CSPG. Above all, our observation of an opposing effect of CSPGs on axon and somata underscores the importance of spatial considerations when biological activities of a molecule are investigated.

We believe that the microchip described here has the potential to be used not just as a tool for studying axon growth but also as a tool for a much broader neuroscience-related research such as studying axonal transport under tightly controlled biochemical environments or screening of drugs that affects the soma and the axon differently for finding combinations that are optimal in promoting axon growth. We also believe that this capability, combined with the simple axotomy capability of the microchip as shown through preliminary studies, will allow studying axonal regeneration under the influence of different biochemicals. The axon guiding feature of the microchip along with the automated quantitative analysis tool will provide a significant technical framework for studying axon growth and regeneration *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health / National Institute of Mental Health grant #1R21MH085267 and by the National Institutes of Health / National Institute of Neurological Disorders and Stroke grant #NS060017.

References

- Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM. A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature*. 1995; 374:450–3. [PubMed: 7700353]
- Baron-Van Evercooren A, Kleinman HK, Ohno S, Marangos P, Schwartz JP, Dubois-Dalcq ME. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *Journal of Neuroscience Research*. 1982; 8:179–93. [PubMed: 7154111]
- Benowitz LI, Goldberg DE, Irwin N. Inosine stimulates axon growth in vitro and in the adult CNS. *Prog. Brain Res*. 2002; 137:389–99. [PubMed: 12440381]
- Brewer G, Torricelli J, Evege E, Price P. Optimized survival of hippocampal neurons in B27 B27-supplemented neurobasal™, a new serum-free medium combination. *Journal of neuroscience research*. 1993; 35:567–76. [PubMed: 8377226]
- Campenot RB. Local Control of Neurite Development by Nerve Growth Factor. *PNAS*. 1977; 74:4516–9. [PubMed: 270699]
- Fitch M, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Experimental Neurology*. 2008; 209:294–301. [PubMed: 17617407]
- Gingras M, Beaulieu M-M, Gagnon V, Durham HD, Berthod F. In Vitro Study of Axonal Migration and Myelination of Motor Neurons in a Three-Dimensional Tissue-Engineered Model. *Glia*. 2008; 56:354–64. [PubMed: 18098124]
- Goldberg JL, Espinosa JS, Xu Y, Davidson N, Kovacs GT, Barres BA. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. *Neuron*. 2002a; 33:689–702. [PubMed: 11879647]
- Goldberg JL, Klassen MP, Hua Y, Barres BA. Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. *Science*. 2002b; 296:1860–4. [PubMed: 12052959]
- Goldberg JL, Vargas ME, Wang JT, Mandemakers W, Oster SF, Sretavan DW, Barres BA. An oligodendrocyte lineage-specific semaphorin, Sema5A, inhibits axon growth by retinal ganglion cells. *Journal of Neuroscience*. 2004; 24:4989–99. [PubMed: 15163691]
- Harel N, Strittmatter S. Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury? *Nature Reviews Neuroscience*. 2006; 7:603–16.
- Hartikka J, Hefti F. Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth, and expression of transmitter-specific enzymes. *The Journal of neuroscience*. 1988; 8:2967–85. [PubMed: 2842468]
- Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. *Annual review of neuroscience*. 2001; 24:677.
- Ishibashi T, Dakin KA, Stevens B, Lee PR, Kozlov SV, Stewart CL, Fields RD. Astrocytes Promote Myelination in Response to Electrical Impulses. *Neuron*. 2006; 49:823–32. [PubMed: 16543131]
- Ito D, Tamate H, Nagayama M, Uchida T, Kudoh S, Gohara K. Minimum neuron density for synchronized bursts in a rat cortical culture on multi-electrode arrays. *Neuroscience*. 2010; 171:50–61. [PubMed: 20800660]
- Koito H, Li J. Preparation of rat brain aggregate cultures for neuron and glia development studies. *Journal of Visualized Experiments*. 2009; 31:1304. [PubMed: 19794393]
- Laabs T, Carulli D, Geller HM, Fawcett JW. Chondroitin sulfate proteoglycans in neural development and regeneration. *Current opinion in neurobiology*. 2005; 15:116–20. [PubMed: 15721753]
- Lingor P, Teusch N, Schwarz K, Mueller R, Mack H, Bähr M, Mueller BK. Inhibition of Rho kinase (ROCK) increases neurite outgrowth on chondroitin sulphate proteoglycan in vitro and axonal regeneration in the adult optic nerve in vivo. *Journal of Neurochemistry*. 2007; 103:181–9. [PubMed: 17608642]
- Majumdar D, Gao Y, Li D, Webb DJ. Co-culture of neurons and glia in a novel microfluidic platform. *Journal of Neuroscience Methods*. 2011; 196:38–44. [PubMed: 21185867]
- Ng BK, Chen L, Mandemakers W, Cosgaya JM, Chan JR. Anterograde Transport and Secretion of Brain-Derived Neurotrophic Factor along Sensory Axons Promote Schwann Cell Myelination. *The Journal of Neuroscience*. 2007; 27:7597–603. [PubMed: 17626221]

- Park J, Koito H, Li J, Han A. Microfluidic compartmentalized co-culture platform for CNS axon myelination research. *Biomedical Microdevices*. 2009; 11:1145–53. [PubMed: 19554452]
- Park J, Koito H, Li J, Han A. Multi-compartment neuron-glia co-culture platform for localized CNS axon-glia interaction study. *Lab on a Chip*. 2012; 12:3296–304. [PubMed: 22828584]
- Park K, Liu K, Hu Y, Smith P, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Sci. STKE*. 2008; 322:963–6.
- Qiu J, Cai D, Filbin M. Glial inhibition of nerve regeneration in the mature mammalian CNS. *Glia*. 2000; 29:166–74. [PubMed: 10625335]
- Robert F, Cloix J-F, Hevor T. Ultrastructural characterization of rat neurons in primary culture. *Neuroscience*. 2012; 200:248–60. [PubMed: 22079571]
- Rolls, A.; Schwartz, M. Chondroitin sulfate proteoglycan and its degradation products in CNS repair. 2006.
- Rolls A, Shechter R, Schwartz M. The bright side of the glial scar in CNS repair. *Nature Reviews Neuroscience*. 2009; 10:235–41.
- Serra M, Leite SB, Brito C, Costa J, Carrondo MJT, Alves PM. Novel Culture Strategy for Human Stem Cell Proliferation and Neuronal Differentiation. *Journal of Neuroscience Research*. 2007; 85:3557–66. [PubMed: 17868148]
- Silver J, Miller JH. Regeneration beyond the glial scar. *Nature Reviews Neuroscience*. 2004; 5:146–56.
- Taylor A, Gifondorwa D, Newbern J, Robinson M, Strupe J, Prevette D, Oppenheim R, Milligan C. Astrocyte and Muscle-derived Secreted factors differentially regulate motoneuron survival. *Journal of neuroscience*. 2007; 27:634–44. [PubMed: 17234595]
- Taylor AM, Blurton-Jones M, Rhee SW, Cribbs DH, Cotman CW, Jeon NL. A Microfluidic Culture Platforms for CNS Axonal Injury, Regeneration and Transport. *Nature Methods*. 2005; 2:599–605. [PubMed: 16094385]
- Vyas A, Li Z, Aspalter M, Feiner J, Hoke A, Zhou C, O'Daly A, Abdullah M, Rohde C, Brushart TM. An in vitro model of adult mammalian nerve repair. *Experimental Neurology*. 2010; 223:112–8. [PubMed: 19464291]
- Walker BA, Ji S-J, Jaffrey SR. Intra-axonal translation of rhoa promotes axon growth inhibition by CSPG. *The Journal of Neuroscience*. 2012; 32:14442–7a. [PubMed: 23055514]
- Wang H, Katagiri Y, McCann TE, Unsworth E, Goldsmith P, Yu ZX, Tan F, Santiago L, Mills EM, Wang Y. Chondroitin-4-sulfation negatively regulates axonal guidance and growth. *Journal of cell science*. 2008; 121:3083–91. [PubMed: 18768934]
- Winzeler AM, Mandemakers WJ, Sun MZ, Stafford M, Phillips CT, Barres BA. The lipid sulfatide is a novel myelin-associated inhibitor of CNS axon outgrowth. *The Journal of Neuroscience*. 2011; 31:6481–92. [PubMed: 21525289]
- Yiu G, He Z. Glial inhibition of CNS axon regeneration. *Nature Reviews Neuroscience*. 2006; 7:617–27.

Figure 1.

(A) Schematic illustrations of the top and bottom PDMS layers composing the developed quantitative axon growth analysis microchip. (B) Illustration showing axon isolation and guidance via the array of shallow microgrooves patterned on the bottom substrate.

Figure 2.

A schematic illustration showing image processing and axon tracing algorithm steps for automated axon growth quantification process.

Figure 3.

(A) Sealed microgrooves ($3 \times 20 \times 800 \mu\text{m}^3$) successfully confined neuronal somata in the soma compartment and prevented dendrites from crossing into the axon compartment. No dendrites could be observed inside the axon compartment at DIV 11 (axon: NF – green, dendrites: MAP2 – red). White dotted line indicates inlets and outlets of the sealed microgrooves. (B) Microgrooves formed on the bottom substrate physically guided axons (stained with Calcein-AM) to grow in straight lines once axons crossed into the axon compartment. Inset shows axons inside a compartmentalized microdevice having similar configuration but without the axon-guiding microgrooves, showing tangling of axons that make quantitative and automatic growth analysis challenging. Scale bar: 50 μ m. (C)

Retrograde staining of isolated axons by Calcein-AM loaded into the axon compartment shows that most of the somata with axons extending into the axon compartment are located in the vicinity of the inlet area of the sealed microgrooves. (D) Illustration and DAPI stained neurons showing the distribution of neurons inside the soma compartment. Well-type open compartment configuration and the ridge structure enabled most of the sealed microgrooves to have multiple neurons at inlets. (E) Isolated and guided axons inside the axon compartment without (left) and with (right) the cylindrical ridge structure. White dotted lines indicate the boundary between the sealed microgroove and the compartment. (F) Axon isolation efficiency at different neuron plating densities measured at DIV 11 (mean $\pm$ SD). More than $96.8 \pm 1.9\%$ of the sealed microgrooves were filled with axons when neurons were plated at initial density of 1000 cells/mm² (* $p < 0.05$ compared to 500 cells/mm²).

Figure 4.

(A) Degenerated axons inside the axon compartment after 4 days of localized CSPG treatment (5 $\mu\text{g/ml}$). (B) The fluidic isolation feature and the multi-compartment configuration enabled six different concentrations of localized CSPG treatments to be performed on a single microchip for screening effective CSPG dosage (2.5 $\mu\text{g/ml}$) that caused axon degeneration. The images are representative results from more than three independent experiments.

Figure 5.

(A-D) Results from key intermediate steps of the automated axon growth quantification process. (A) Original image. (B) The original image is rotated so that the microgrooves and the axons are vertically oriented. The boundary between the sealed microgrooves and the axon compartment is located and shown as the red curve (dubbed the “baseline”). (C) End points of the axons are found by sweeping the image from the bottom toward the top. Identified end points are marked with yellow circles. (D) Final result of the axon detection algorithm is shown (yellow lines indicate successfully traced axons). (E) Comparison of the measured axon length by automated (314.3 ± 85.41 pixels) and manual measurements (315.1 ± 84.01 pixels) (mean $\pm$ SD, n : number of analyzed axon compartments).

Figure 6.

The effect of localized biomolecular treatments on axon growth. The results shown are obtained from 3-6 independent experiments (mean $\pm$ SD, *n*: number of analyzed axons). (A) The effect of ECMs and CSPG on axon growth when added only to the axon compartments. (B) The effect of ECMs and CSPG on axon growth when added only to the soma compartment. (C) The effect of MatrigelTM with BDNF on axon growth when added only to the axon compartments. (D) The effect of MatrigelTM with BDNF on axon growth when added only to the soma compartments. * $p < 0.05$, *** $p < 0.001$ to control.