Published in final edited form as:

Neurosci Lett. 2019 June 11; 703: 58–67. doi:10.1016/j.neulet.2019.03.025.

Morphometric and Computational Assessments to Evaluate Neuron Survival and Maturation within Compartmentalized Microfluidic Devices: The Influence of Design Variation on Diffusion-Driven Nutrient Transport

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Abstract

Burgeoning use of segregated microfluidic platforms that parse somas and neurites into discrete compartments is fueling unique examinations of neuronal structure and physiology in a manner impossible to achieve with non-compartmentalized systems. However, even though this line of axon-soma polarizing microfluidic devices stems from the same general design of a Campenot chamber set-up, slight deviations in device geometry appear to induce vastly different nutrient transport profiles that influence neuron survival and maturation. Here we examine the uptake of nerve growth factor (NGF) by a pheochromocytoma PC12 cell line cultured using two Campenot-like device designs, a “Standard” layout, representative of a commercial device, and a custom “Notch” layout, predicted to encourage more efficient nutrient transfer that gives rise to sustained neuron viability and extensive neurite elaboration. Exploiting *in vitro* culture schemes coupled with computational analyses, we identify the influence of device design geometry on the interplay between neuronal survival and maturation, gauged from morphometric assessments and the spatiotemporal distribution of NGF. Computer simulations of NGF transport within the devices revealed that the microfluidic neuron culture system is highly sensitive to change, where nutrient transport is intricately linked to device geometry and cell plating density, and premature depletion of nutrients is observed if specific design criteria are not met. This study underscores the

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Data Statement:

Research data will be made available upon request.

importance of validating specific device geometries for a particular neuro-based assessment, while showcasing computational modeling as a powerful tool to achieve this goal.

Keywords

Rat pheochromocytoma PC12 cell line; microfluidic devices; nerve growth factor (NGF); cell viability; nutrient depletion; mass transport

1. Introduction

Researchers have put forth considerable effort to develop *in vitro* culturing platforms capable of recapitulating the elaborate architecture of the nervous system. Microfluidic devices with semblance to Teflon Campenot chambers, parse soma and neurites into separate compartments linked by a series of grooves that serve as a passageway for neurites. These unique segregated culture schemes have been broadly adapted as *in vitro* models for axonal injury [1], axonal transport [2], and neurite-glia interactions [3–5]. However, slight deviations in device geometry among these Campenot-like microfluidic devices induce vastly different nutrient transport profiles that influence cell viability and neurite extension, and there is limited work offering a detailed rationale for these observations.

The geometry of a commercially-available Campenot-like device [6] has been reported to present unique challenges that could influence the behavior and viability of cells cultured within them: (1) difficulty with positioning a high density of neurons near the neurite-permissive microchannel openings [7–9] and (2) inherent limitations associated with nutrient and waste transfer within a diffusion-dependent fluid volume [7,10,11]. To circumvent these issues, alterations to device design or handling techniques have been made to supply better control over the neuron density and neurite elaboration within the device [4,6,8]. Attempts have included the application of centrifugal forces to coerce cells to align immediately adjacent to groove entrances [12], upon or following seeding [4]. Implementing centrifugal-force-induced cell seeding techniques can be cumbersome due to the required specialized instrumentation and experimental set-up. One group developed a “co-centric” device design displaying a central open well and outer ring compartment that were adjoined by radially oriented micro-grooves. Radial pressure generated from loading cells into the center well positioned cells close ($< 5\mu\text{m}$) to the groove entrance [6,8]. Supplanting the lengthy cell culture channel region, characteristic of the “Standard” device, with an open well cell culture region gives neurons proximate access to a pool of nutrient medium.

To further examine aforementioned reports of culturing challenges associated with the “Standard” device, we have designed a microfluidic device that also includes an open well cell culture region. Unlike other “open-well” designs, we have incorporated a small notch to contain cells, and thus, coined the name of this device to be “Notch.” We predict that this “Notch” design will offer more efficient nutrient transfer, sustained cell viability, and extensive neurite extension over the preexistent “Standard” device, by reducing the distance between the cell compartment and source of nutrient-rich medium. A comparison of feature dimensions for these two devices, “Standard” and “Notch” is shown in Figure 1A.

As an alternative to primary neurons, we use the rat pheochromocytoma PC12 cell line, a common model system for neuronal development and function [13,14], to assess neuron viability and maturation for different device designs. The PC12 cell model has been extended to microfluidic devices in the examination of biological stimulation [15,16], cytotoxicity [14], neuro-electrode interfaces [17], and topographical influence on neuron architecture [18]. The sole dependency of differentiated PC12 cells on nutritive NGF for survival and process formation, makes them an excellent system for characterizing the distribution of nutrients and wastes, as well as the consequent impact on cell health within a compartmentalized device.

We hypothesized that NGF-dependent, neuron-like PC12 cells, confined within a chamber, abutting microchannel barriers, and maintained in stationary medium, will be more apt to survive and exhibit extensive neurite formation when cultured in the “Notch” device versus the “Standard” device. Neurite-bearing PC12 cells were loaded into “Standard” or “Notch” devices pre-coated with a mixture of collagen and poly-DL-ornithine (PDLO) and cultivated for several days. We employed a combination of morphometric analyses and finite element modeling to identify the influence of device design geometry on the interplay between neuronal survival and maturation and the spatiotemporal distribution of NGF. This manuscript serves as a research note that aims to provide the neuroscience community with a better understanding of how alterations in device geometry influences the likelihood of successfully culturing neurons in Campenot-like microfluidic devices.

2. Materials and Methods

2.1 Medium Formulations

Complete culture medium was composed of RPMI medium (Invitrogen A10491–01) supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal bovine serum, and 1% Antibiotic-Antimycotic (Invitrogen 15240–062).

Incomplete culture medium was composed of RPMI medium supplemented with 1% heat inactivated horse serum and 1% Antibiotic-Antimycotic.

Differentiation medium consisted of incomplete medium enriched with 100 ng/mL NGF (Millipore NC010).

2.2 Maintenance of PC12 Cells

A 50:50 mixture of 0.08 mg/mL collagen gel (0.08 mg/mL, diluted in 0.02 N acetic acid, rat tail collagen, type 1, BD 354236) and poly-D,L-ornithine (PDLO) solution (0.5 mg/mL, dissolved in borate buffer, pH ~8.5, Sigma P8638), referred to as collagen-PDLO, was used as a surface coating. A 10-cm diameter petri-dish was covered with desired coating solution (5 mL collagen or 2.5 mL collagen-PDLO mix), which was allowed to dry out in the cell culture hood overnight. The dish was rinsed thrice with sterile distilled water and exposed to UV for 30 min prior to cell culture.

The PC12 cell line was originally derived from a pheochromocytoma of the adrenal medulla of a rat in 1976 [19]. PC12 cells (kindly donated by Dr. Christin Carter-Sue,

University of Michigan, Department of Molecular & Integrative Physiology) with a cluster like morphology, were maintained on collagen-coated dish in 10 mL of complete culture medium. Half of the complete medium was replaced with fresh medium every 3 days. Upon reaching confluency, all but 5 mL of conditioned medium was removed from the dish, and the cells were physically detached by forcefully triturating the medium repeatedly over dish surface. Cell clusters were then dissociated by passing the medium twenty times through a 21-gauge needle. Cells were pelleted by centrifugation (200 x g) for 5 minutes and re-suspended in complete medium. To create a single cell solution for cell counting, cells were dissociated by sequentially passing the solution through a 21-gauge and then a 23-gauge needle.

2.3 PC12 Differentiation into Neuron-like Cells

To induce neuronal differentiation, PC12 cells from one frozen vial were thawed, diluted in differentiation medium, pelleted via centrifugation, resuspended in differentiation medium, and dissociated by passing cells twenty times through a 21-gauge needle. The cell suspension was added to 10 mL of differentiation medium on a 10-cm diameter dish coated with the collagen-PDLO mixture. Half of the medium was replaced with fresh differentiation medium every two days. At day 7, the monolayer of neuron-like cells was detached from the dish using the aforementioned physical method. Cells were pelleted by centrifugation at ~125 x g (reduced speed to separate neurite debris from cells [19]) for 5 minutes and re-suspended in differentiation medium.

2.4 Device Fabrication

The preparation of “Standard” devices is described elsewhere [1]. Molds for the “Notch” device were prepared with a similar photolithography process, using dimensions included in Figure 1. The device molds were treated with trichlorosilane, a release agent that prevents material adhesion to the mold. Polydimethylsiloxane (10 parts elastomer and 1 part crosslinking agent) was cast against the molds, and holes were machined into the well regions [1].

Cover glasses were coated with PDMS. The features side of the devices and the PDMS-coated side of the cover glasses were exposed to oxygen plasma for 4 minutes, and then bonded together. “Standard” and “Notch” devices were respectively bonded to 30 × 40 mm and 22 × 22 mm sized cover glasses. The bonded devices were heated 10–15 min at 60°C to ensure bonding. The assembled devices were subjected to 4 minutes of oxygen plasma treatment to render their interior regions hydrophilic.

2.5 Device Culture

Within 30 minutes of the final plasma oxidation treatment, the assembled devices were transferred to Petri-dishes (60mm-dia) and the inner device chambers were filled with a collagen-PDLO mix (5 µL/chamber for “Notch” and 10 µL/chamber for “Standard” devices). Petri-dishes, housing devices, were wrapped in Parafilm to prevent coating evaporation and stored at room temperature in the cell culture hood overnight. The next day, the coating was aspirated and rinsed twice with sterile distilled water. Chambers and wells were filled with water, and the device was sterilized with UV light for 35–40 min.

Immediately following resuspension of primed PC12 cells in differentiation medium, the water was aspirated from the device openings, and a $2.25\text{--}2.5 \times 10^6$ cells/mL suspension was injected directly into a single enclosed chamber in each device ($1.5 \mu\text{L}$ /chamber for “Notch” and $3 \mu\text{L}$ /chamber for “Standard” devices). To encourage cell adhesion to the substrate, the devices were incubated at 37°C , for a duration (up to 5 min) brief enough to prevent evaporation of cell suspension. The opposite chamber and all wells were filled with differentiation medium ($40 \mu\text{L}$ /well for “Notch” and $65 \mu\text{L}$ /well for “Standard” devices). The device cultures were maintained at 37°C , differentiation medium was exchanged every 2 days, and experiments were terminated on the eighth day. Brightfield images were acquired every 2 days at 10x magnification in 3 and 8 discrete non-overlapping regions, parallel to the neurite-microchannel region, respectively for the “Notch” and “Standard” devices.

2.6 Immunohistochemistry

Unless otherwise stated, all steps were performed at room temperature. Cells were fixed with a 4% paraformaldehyde, PFA, solution (diluted in 1X PBS from 20% stock, EMS 15713) for 20 min. Traces of PFA were removed with a 1X PBS rinse (3x, 4 min/rinse). Permeabilization was achieved with a 30-min incubation in 0.2% Triton X-100 solution that was rinsed away with 1X PBS (3x, 4 min/rinse). Cells were incubated in a blocking buffer, consisting of 10% normal goat serum, NGS (Sigma G9023, in 1X PBS), at room temperature for 1 hr. Cells were incubated overnight at 4°C with anti-tubulin β -III antibody, clone TU-20, conjugated to Alexa Fluor 488 (Millipore CBL412X, 1:250 dilution). The next day, cells were rinsed (2x, 4 min/rinse) with 1X PBS, counterstained with DAPI ($1 \mu\text{g/mL}$, Molecular Probes, Invitrogen D1306), and rinsed with 1X PBS (3x, 4min/rinse). Fluorescent images were acquired at 10x magnification in 3 and 8 slightly overlapping regions, parallel to the neurite-microchannel region, respectively for the “Notch” and “Standard” devices.

2.7 Neuron Viability and Morphology Analysis

All morphometric measurements and counts were extracted from either brightfield or fluorescent images using the ImageJ software program (v 1.50i, National Institute of Health, Bethesda MA). For analysis, cell culture regions were subdivided into eight regions of interest (R1-R8, R1 and R8 closest to wells and R4 and R5 farthest from wells) for the “Standard” device (Figure 2Ai) and three regions of interest (R1-R3, all bordering well) for the “Notch” device (Figure 2Bi). Cell counts were manually obtained from brightfield images taken on day 2, 4, and 8 using an extension of ImageJ, Cell Counter (De Vos, 2001), and cell counts from day 2 were used in the Comsol multiphysics simulations. Grayscale, fluorescence images of labeled nuclei and process-bearing cells, taken along the length of the cell culture region, were imported into ImageJ. These images were cropped to non-overlapping regions of interest (ROIs), positioned $470 \mu\text{m}$ from the left edge of the neurite micro-channel array (all $900 \mu\text{m} \times 350.47 \mu\text{m}$, except for top or bottom regions, which were $600 \mu\text{m} \times 350.47 \mu\text{m}$). A thresholding tool was used to convert the fluorescent images into binary black and white images, where the pixel intensity equaled 0 (black) for the background and 255 (white) for the fluorescent cellular regions, and the entire white area equaled total neuron area. Grayscale, fluorescence images of DAPI stained nuclei and anti-tubulin β -III labeled neurons, cropped in the manner previously described, were color corrected and merged to count the number of DAPI stained cells that were positive for anti-

tubulin β -III labeling. The number of neurite-microchannels, or channels containing neurites, and the length of the processes within these channels were also determined.

2.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (v 5.0, GraphPad Software, San Diego, CA) with statistical outliers determined using R (v 3.4.2, R Core Team, Vienna, Austria). One-way ANOVA, multiple t-test comparison, and unpaired t-test with Welch's correction was performed on the data to determine statistical significance between devices and regions.

2.9 NGF Simulations

Computer simulation models were run to determine location-specific depletion of NGF over 48 hours in both "Notch" and "Standard" devices. Simulations were performed with the commercially available finite element package, Comsol (v 5.2a, Burlington MA). Geometries of each device were created using a commercial 3D software tool (SolidWorks 2014, Waltham, MA) before they were imported into Comsol. These models were run using the 'transport of diluted species' physics module displayed as Equations 1–3, which incorporate parameters found in literature for NGF (Table 1).

$$\frac{\partial c}{\partial t} + \mathbf{u} \cdot \nabla c = \nabla \cdot (D \nabla c) + R \quad (1)$$

$$\mathbf{N} = -D \nabla c + \mathbf{u} c \quad (2)$$

$$-\mathbf{n} \cdot \mathbf{N} = 0 \quad (3)$$

Equations 1, 2, and 3 describe diffusion by Fick's Law, convection, and the chemical reaction rate constant accounting for the cellular consumption of nutrients within the model. The terms in the equations are as follows: c is concentration, D is the diffusion coefficient, R is the reaction rate coefficient, $\mathbf{u}$ is the velocity field, $\mathbf{N}$ is the molar flux, $\mathbf{n}$ is the unit normal vector, and t is time. Cellular uptake of NGF was extrapolated from the experimental data of Gunning et al. shown in the supplementary data [20]. Cell counts from day 2 of each device were averaged and multiplied by a theoretical volume of an average PC12 cell (Table 1) to calculate the total cellular volume. Cell sheets, representative of total cellular volume (Supplementary Data Section S3), were created in SolidWorks and migrated into Comsol where they were positioned in the appropriate cell-soma chamber. The initial NGF concentration and reaction rate for cell sheets of the "Notch" and "Standard" model were set to the values indicated in Table 1. A minimum geometric mesh size of 4.2×10^{-6} m with 29251966 total elements was used to generate the "Standard" device, while a minimum geometric mesh size of 2.7×10^{-6} m with 12847628 total elements was used to generate the

“Notch” device. These mesh values were necessary in order to recreate the finite structures of the device’s cell culture regions. The models were simulated to reflect a change in NGF concentration gradients up to 48 hours after the replacement of NGF-enriched medium on Day 2 of experimentation. Day 2 represents a time point in the experiment at which most of the PC12 cells bear neuronal processes.

3. Results

3.1 Morphometric Analysis to Assess Survival and Maturation of Process-bearing PC12 culture in “Standard” and “Notch” Devices

To probe for differences in neuron survival and maturation due to variations in device design, we cultured process-bearing PC12 cells in “Standard” and “Notch” devices, and subsequently performed morphological assessments on cell images acquired at various time points of culture. PC12 cells were primed for differentiation in NGF-enriched medium for 7 days prior to device seeding, as shown in Figure 1B. After priming, the PC12 cells exhibit an ability to re-sprout their processes within 24 hours of re-plating, a phenomenon identified in a previous report [19].

Cell counts were collected for images taken on days 2, 4, and 8 of device culture, as displayed in Figure 2. Regions 1 and 4 (R1 and R4) from the “Standard” device were plotted against each other and were compared to R1 and R2 from the “Notch” device. Cell numbers in the central region, R4, of the “Standard” device was significantly lower when compared to the device’s outer region, R1. There is no significant increase in cell number for R1 and R4 of the “Standard” device from days 2 to 8. Overall, the higher cell counts in both regions of the “Notch” device are comparable to those of the outer region, R1, of the “Standard” device.

Fluorescent images for day 8 of culture were evaluated for the total cellular area present in cell culture regions, in addition to the number of neurite-microchannels containing processes (Figure 3). Total cell area was significantly larger in the outermost regions (R1, R2, R7 and R8) of the “Standard” device when compared to its innermost regions (R4 and R5) (Figure 3A). No statistically significant difference was determined between cell areas in regions of the “Notch” device (Figure 3B). The fraction of grooves containing neurons were calculated for the regions of each device. Over the course of eight days, regions closest to the wells of the “Standard” device contained a larger number of neurite-microchannels that were replete with neurites. However, within the same time frame, cells in regions furthest from the wells began to appear emaciated and exhibit poor neurite outgrowth. The total area of neurons covering the notched cell region of the “Notch” device exceeds that of the cell culture surface of the “Standard” device (Figure 3C). The mean cell area and fraction of grooves containing neurites show no statistically significant differences among regions of the “Notch” device.

Overall, in the “Standard” device, hindrance of neurite growth seems to increase with movement, along the length of the channel, that is directed away from the medium wells and progresses mid-channel. The culture region of the “Standard” device contains a gradient of cell area and neurite outgrowth, dropping off at the center of the device. The “Notch” device

does not contain this gradient and each region is equivalent to all other regions of the same device with respect to cell area and neurite outgrowth. In the subsequent section, these inter-device variations in growth factor concentration gradients are modeled and further explored, using computer simulations, to more clearly discern the impact of device design on neuron survival. Although the fraction of grooves containing neurite processes show a correlation to variations in device geometry, the average length observed for neurites that have reached and entered the microchannels, whose dimensions are nearly identical for both devices, shows no significant difference between regions in the “Standard” and “Notch” devices (Supplementary Figure S1).

3.2 Simulation of NGF Diffusing in “Standard” and “Notch” Devices

A computational multiphysics simulation was designed to predict the uptake of NGF up to 48 hours after medium exchange on day 2 of experimentation. Both “Standard” and “Notch” microfluidic devices, cultured with neurite-bearing PC12 cells, were simulated. The aim of the simulation was to formulate a rationale for the spatially-dependent variation of cell viability and neuron maturation observed between the two devices. Each simulation outputs a time dependent and spatial representation of NGF concentration. Specific input parameters were required to fulfill the physics module chosen for the simulation (Table 1). To simplify modeling of the interaction between NGF and differentiated PC12 cells residing in a complex biological and chemical system, NGF depletion was interpreted as a combination of three variables: 1) NGF degradation, 2) internalization of NGF within PC12 cells and 3) cell-surface attachment of NGF to PC12 cells. NGF depletion, set as the reaction rate, was approximated based on data pulled from an NGF characterization study performed by Gunning et al. [20]. The initial reaction rate used in the computer simulations of the microfluidic devices was extrapolated from Gunning’s experiments, which were performed in cell culture dishes (Table S1, Supplementary Figure S2 and S3, Gunning et al., 1981). However, the reaction rate calculated resulted in complete depletion of NGF in the “Standard” device’s cell culture chamber after 6 hours (NGF concentration of 0 ng/mL) and determined that after 48 hrs a minimum NGF concentration of 73.1 ng/mL would be present in the “Notch” device (Supplementary Figure S6). The aggressive and unlikely depletion of NGF in the “Standard” device design suggests that the NGF depletion results from dish cultures cannot be directly extrapolated to microfluidic device cultures. Therefore, the reaction rate was adjusted by a factor of one-fourth, and in doing so, spatiotemporal differences in NGF consumption were more pronounced, clearly discerned, and in closer agreement to optical and fluorescent microscopy assessments. Given PC12 cells’ dependence on NGF for process formation and survival [20,27–29], the culture of NGF-treated PC12 cells inside microfluidic devices provides a useful system for simulating nutrient depletion.

The simulation results are shown in Figure 4 for both the “Standard” and “Notch” devices, each containing an initial NGF concentration of 100 ng/mL. A depletion of NGF at the innermost regions within the soma compartment of the “Standard” device occurs initially at 24 hours, showing a minimum NGF concentration of $5.03 \times 10^{-7} \text{ mol} \cdot \text{m}^{-3}$ (67.87 ng/mL), and progressively worsens and spreads towards wells as the simulation approaches 48 hours, the point at which NGF reaches a minimum concentration of $3.26 \times 10^{-7} \text{ mol} \cdot \text{m}^{-3}$ (44.01 ng/

mL). In general, a gradient of the NGF is visible over the 8 regions of the “Standard” device, with significantly higher levels at the outermost regions (Figure 4). NGF levels in the “Notch” device are maintained at a relatively high concentration of $7.08 \times 10^{-7} \text{ mol} \cdot \text{m}^{-3}$ (95.58 ng/mL) in all regions of the soma chamber, for up to 48 hours (Figure 4A), where a minimum concentration of $6.92 \times 10^{-7} \text{ mol} \cdot \text{m}^{-3}$ (93.37 ng/mL) is present. Overall, these results show that the “Standard” device is not able to efficiently transport NGF from the wells to replenish depleted NGF levels at innermost channel regions, while the “Notch” device is able to replenish its soma chamber with NGF over a period of 48 hours.

To observe the effects of incorporating a higher and lower cell population in each device, four additional simulations were performed, shown in Figures 4B and 4C. In these simulations, cell densities were tested at both one order of magnitude higher and one order of magnitude lower than experimental counts on day 2. High levels of NGF concentrations in both devices were maintained when the devices contained the lower theoretical cell density, whereas NGF concentrations were almost completely depleted when the devices contained the higher theoretical cell density. Large NGF concentration gradients are also present in both devices when plated with the high theoretical cell density, a case reminiscent to seeding the experimental cell density into the “Standard” device. Additionally, it was found that the NGF concentration present in the neurite chamber is minimally affected by cellular uptake in the opposing soma chamber (Figure 4A), but substantial increasing NGF gradients do emerge along the length of the neurite-microchannels when advancing from soma to neurite compartments.

4. Discussion

It is generally accepted that NGF is required for neuron differentiation and survival. The neurotrophic factor hypothesis states that neurons require NGF for both survival and cell maintenance, and that neurons compete with one another for the limited NGF produced by the target tissue [30]. Numerous studies have shown that a critical range of NGF concentrations is required for healthy neurite outgrowth, specifically in peripheral sensory, sympathetic postganglionic, and selective dorsal root ganglion neurons [27–29,31–33]. In optimal conditions, with NGF concentrations around 100 ng/mL, PC12 cell processes can grow as much as 0.5 to 1 mm per day [25]. The availability of lower NGF concentrations, 25 or 50 ng/mL, has been shown to impede PC12 neurite growth [20]. However, at the upper limit of the spectrum, NGF concentrations surpassing 995 ng/mL will saturate trkA and p75^{NTR} receptors and act to inhibit PC12 neurite growth as well [26].

Experimental data from the present study shows that PC12 neurite growth at the “Standard” device’s inner regions is hindered when compared to the device’s outer regions. The simulated “Standard” device shows that NGF concentration at the innermost regions will continuously drop over 48 hours, reaching 68% of the initial concentration (100 ng/mL) within 24 hours, and 44% at 48 hours. In contrast, NGF concentrations in the “Notch” device were maintained at more stable levels over 48 hours (93%). This was expected as the “Notch” device’s cell culture region is bordered by an NGF-enriched medium reservoir. Collectively, these observations suggest that NGF depletion, through cellular consumption and sequestration or enzymatic degradation, within the cell culture region of the “Standard”

device – coupled with absent flow of bulk medium and low diffusion-driven movement of NGF molecules – may be the mechanism that explains the differences in process formation across both devices.

Cells require sustained levels of metabolites and growth factors to maintain homeostasis, proliferate, and grow [34,35]. Results from the experimental assessment of PC12 cell morphology and computer simulations of mass transport suggest that nutrient diffusion and cellular uptake is the leading factor impeding healthy PC12 growth, and these phenomena are governed by device geometry. With an increase in neuron seeding density, simulations reveal an exacerbation of NGF nutrient depletion in both devices. These simulation results support the recommendation that researchers should take into account cell plating density, along with features of device design, when performing neuron assays using microfluidic cell culture vessels.

Recently it has been shown that NGF gradients may be important for directing neurite growth and survival [25,26,36,37]. The growth cone has been shown to be responsive to steep concentration gradients, with sensitivity falling within three orders of magnitude [26]. Tse et al., 2007 used a genetic algorithm to write a mathematical model to simulate the maximum distance for guided nerve growth within PC12 cell culture. They determined that a maximum of 4.5 cm of guided growth was theoretically possible. Similarly, patterned plasmid encoding for nerve growth factor has been shown to produce NGF concentration gradients and induce spatially dependent neurite outgrowth [38]. Furthermore, research using NGF gradients in 3D poly(ϵ -caprolactone)-block-poly(L-lactic acid-co- ϵ -caprolactone) (PCLA) fibrous scaffolds both *in vitro* and *in vivo* have shown success in promoting dorsal root ganglion (DRG) neuron regeneration in mice [39]. While we observed an NGF gradient along the length of cell culture chamber in the “Standard” device, we found that in both devices, steep gradients exist along the length of neurite-microchannels, or the neurite passageways connecting soma and neurite compartments. The highest NGF concentration was present at the microchannel end nearest the neurite chamber. It is possible that these universal steep gradients guide the growth of neurite process along the microchannels, and thereby account for the lack of significant variation observed in the length of processes existing within neurite-microchannels for both devices (Supplementary Figure S1). Computational simulations demonstrate how gradient formation within each device can be fine-tuned based on initial NGF concentration and cell plating density. Overall, the present work suggests that critical design criteria for selecting a compartmentalized neuron culture device include growth factor diffusion and uptake, which consequentially affect both neuron health and survival in a spatially dependent manner.

It is possible that undesirable NGF transport issues, exacerbated with increased cell density, may be overcome by introducing a sustained flow to the device; a pump or gravitational flow may negate diffusion limitations intrinsic to some device designs. To our knowledge, a NGF depletion analysis using computational modeling has never been performed for neurons cultured within a microfluidic device. Due to the challenges of measuring NGF uptake by PC12 cells within a microfluidic environment (limited by small sample volume and subtle changes in concentration over minute distances), we approximated NGF uptake by a PC12 cell using data generated from dish cultures (Supplementary Figure S3). However, we found

that the uptake rate determined from the dish culture was too large to accurately describe experimental results (Supplementary Figure S6). A theoretical uptake value was implemented to better illustrate the spatial dependence of NGF concentration within the two devices. As such, these simulations are meant to illustrate spatiotemporal trends in NGF concentration for the “Standard” and “Notch” devices when both are implemented for standard neuron culture assays, rather than predict exact values for NGF concentration. Future computational analysis could be improved by using sensitive fluorometric labeling and ELISA-based methods [40,41] to determine the uptake or degradation of NGF within a microfluidic device culture. It is clear that NGF concentration within the “Standard” and “Notch” devices is quick to respond to both changes in cell plating density and the uptake rate of NGF by neurons. Bearing in mind this study, researchers should consider the parameters outlined above before undertaking microfluidic neuron culture analyses *in vitro*.

Conclusion

Using a combinatorial analysis approach, we are not striving to endorse one device design over another. Instead, we are encouraging scientists to consider that subtle variation in the design of the microfluidic Campenot-like chamber can yield vastly different nutrient transport profiles and ultimately effect cell survival, in the absence of flow. Certain tactics, such as the inclusion of gravitational or continuous flow, to force-fit a microfluidic device design to a given application may counteract nutrient deprivation issues. Cell type and density, medium exchange frequency, and substrate coating are all among a handful of factors that should also be carefully weighed against a prospective device design. This manuscript serves as a technical note to encourage researchers to become more equipped with proper knowledge for selecting the most appropriate device design for a specific neuronal assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge the financial support of Dr. Brian Pierchala (Biologic & Materials Sciences Department) and guidance of Dr. Sandrine Martin and Dr. Pilar Herrera-Fierro in the use of the University of Michigan Lurie Nanofabrication Facility for device mold fabrication. We are grateful to Dr. Christin Carter-Sue and Ray Joe, from the Department of Molecular & Integrative Physiology, for donation of PC12 cells and helpful discussions. We thank Trey Lundmark and Priyan Weerappuli for their technical efforts and consultation with early stages of project development.

A.R.D. was a fellow of the University of Michigan Hearing Balance and Chemical Sciences research training program (NIH, T32 DC00011). Supplementary research funds were provided by the University of Michigan Undergraduate Research Opportunity Program (UROP), and J.G. was a UROP scholar. This work was supported by DOD OCRP Early Career Investigator Award W81XWH-13-1-0134 (G.M.) and DOD Pilot award W81XWH-16-1-0426 (G.M.).

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Figure 1. Geometry of “Standard” and “Notch” Microfluidic devices and morphology of neurite-bearing PC12 cells.
A. Comparison of geometric measurements for the “Standard” and “Notch” Device Design.
B. Prior to their addition into the microfluidic devices, the PC12 cells were preconditioned with 100 ng/mL NGF and half of the medium was replaced with fresh NGF medium every other day. Neurite-bearing PC12 cells were visible one day following NGF treatment, and substantial neurite formation was present after seven days. D = culture day. Scale bar = 50 μm.

Figure 2. Temporal visualization of neuron-like PC12 cells in “Standard” and “Notch” devices. **A.** Eight regions of interest (ROI, R1-R8) defined along the length of the soma chamber in the “Standard” device are shown in the schematic (i). The change in cell density over time for R1 (near entrance well) and R4 (~midway of channel length) is graphically represented (ii). Optical micrographs were acquired from R1 and R4 (iii). The cells near the cross-channels appear emaciated at day eight, as can be observed by degenerated neurites. **B.** Due to the shorter length of the cross-channel section of the device, only three regions of interest (R1-R3), all boarding the same well, were collected in the notched soma region of the “Notch” device (i). The change in cell density over time in R1 and R2 is graphically represented (ii). Optical micrographs were acquired from R1 and R2 (iii). Neurite formation is more homogenous in the regions along the grooved wall, and cells near the grooves appear healthy with more extensive neurite formation after eight days of culture. R = region. Scale bar = 50 μm .

Figure 3. Region-specific viability of neurite-bearing PC12 cells in “Standard” and Notch” devices.

A. Stitched representative image of fluorescent micrographs (i) and graphical mapping of cell area and fraction of grooves containing neurites (ii), spanning the length of the soma containing region within the “Standard” device. **B.** Stitched representative image of fluorescent micrographs (i) and graphical mapping of cell area and fraction of grooves containing neurites (ii), spanning the length of the soma containing region within the “Notch” device. No significant differences were found between regions for both mean cell area and fraction of grooves containing neurites in the “Notch” device. **C.** Graphical comparison of mean cell area and neurite growth fraction for the outermost and innermost regions of the “Standard” (S) and “Notch” (N) devices. Innermost regions = R2 for N and average of R4 and R5 for S. Outermost regions = average of R1 and R3 for N and average of R1 and R8 for S. The S device shows significantly less total cell area than the N device, while the S device’s innermost regions show significantly less area covered by neurons and

fewer neurite containing-grooves than its outermost regions. One-way ANOVA was used to determine any statistically significant differences among the mean cell area (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, $n = 3$) and fraction of grooves containing neurites (# $p < 0.05$, ## $p < 0.005$, ### $p < 0.0005$, $n = 3$) for the indicated device regions. Scale bar = 100 μm .

Figure 4. Finite element analysis of region-specific depletion of NGF in “Standard” and “Notch” devices over a two-day period and dependency on cell seeding density.

A. “Standard” and “Notch” devices show differential spatially-dependent depletion of NGF over a period of 48 hours, where less NGF is distributed to innermost culture regions of the “Standard” versus “Notch” device. **B.** Cell seeding density impacts rate of NGF depletion within outmost regions over a 48-hour period. Graphical representation of simulation results reveals that at high cell densities, when compared to low and mid-range (experimental) densities, greater NGF depletion occurs in the outermost regions of the N and S devices. Outermost regions = R1 and R8 for “Standard” (S) and R1 and R3 for “Notch” (N) devices. **C.** When considering the innermost device regions, the S device will be completely depleted of NGF by 48 hr with high cell densities, while the NGF will be readily accessible at all tested cell densities for the N device. Mid-range densities produce steeper rates of NGF depletion in the innermost regions of the S device. Innermost regions = R2 for N and R4 + R5 for S devices. S cell densities (δ): low = 1.27×10^5 cell/mL, mid (experimental) = 1.27×10^6 cells/mL, and high = 1.27×10^7 cells/mL; N cell densities (δ): low = 7.65×10^4 cell/mL, mid (experimental) = 7.65×10^5 cells/mL, and high = 7.65×10^6 cells/mL.

Table 1.

Parameters used for NGF Uptake Simulations

Parameters	Value	Data Source
Initial Concentration NGF	100 ng ml ⁻¹	Methodology Section
PC12 Diameter	15 μ m	[21–23]
Cell Surface Density of Notch Device on Day 2	391.02 cells mm ⁻²	Methodology Section
Cell Surface Density of Standard Device on Day 2	236.79 cells mm ⁻²	Methodology Section
Theoretical Reaction Rate	-7.5×10^{-10} mol m ⁻³ s ⁻¹	Calculated as shown in Supplementary Data
Reaction Rate	$-3.07372097 \times 10^{-9}$ mol m ⁻³ s ⁻¹	Calculated as shown in Supplementary Data
Neurite Diameter	1 μ m	[24]
Diffusion Coefficient D _c	7.8×10^{-7} cm ² s ⁻¹	[25,26]
Ave. Neurite Length	27 μ m	[24]
MW NGF	135 kDa	Millipore NC010 Product Sheet