Microfluidic Device for Single Cell Studies of Spinal Cord Injury

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INTRODUCTION

Spinal Cord Injury is damage to the spinal cord due to physical trauma. Study of spinal cord injury has hardly been achieved at the single cell level. Polymer microfluidics devices may provide a unique *in vitro* platform for studying single neuronal responses to mechanical forces. For example, one device developed in our lab, called microchannel device, is made from poly (dimethylsiloxane) (PDMS) and has two parallel channels connected by numerous, smaller microchannels. The device geometry allows for compartmentalization of single neurons in each microchannel. Also, the orientation of neurons in microchannels allows for uniform compression studies and visualization of neuronal responses. By keeping axons linear and separated, the microfluidic device allows for easy visualization and characterization by eliminating the complex network of axons usually formed by neuron cultures. Additionally, the device geometry and material allows for easy compression of single cells through use of a simple home-made stretcher device. The cross-sectional dimensions of the microchannels can be reversibly controlled by varying the applied external strain through the home-made stretcher, thereby allowing different mechanical forces to be applied to neurons. Our microfluidics device subjects single neurons to mechanical forces via static pressure by microchannel confinement, which has never been done before.

Before beginning experiments in studying cell response to mechanical forces, the first challenge is designing the device and stabilizing cell growth, which includes optimizing device designs for cell viability, optimizing cell seeding and maintenance conditions, and characterizing neuron growth in devices. The objectives of this summer project are to optimize device conditions for neuron cell growth and to explore preliminary results of strain and compression experiments.

DEVICE DESIGNS AND GEOMETRY

The microfluidic devices are made from PDMS, a flexible polymer, and consist of a bottom PDMS slab containing a device pattern and a thin film of PDMS cover bonded tightly on top (Figure 1).

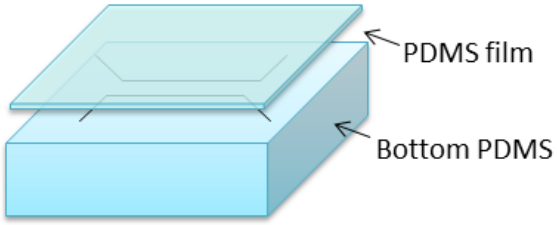

Figure 1: PDMS device structure

Two general device designs are used for study. Both have two parallel channels with reservoirs on the ends. The two channels are connected by either smaller microchannels or nanochannels (Figure 2).

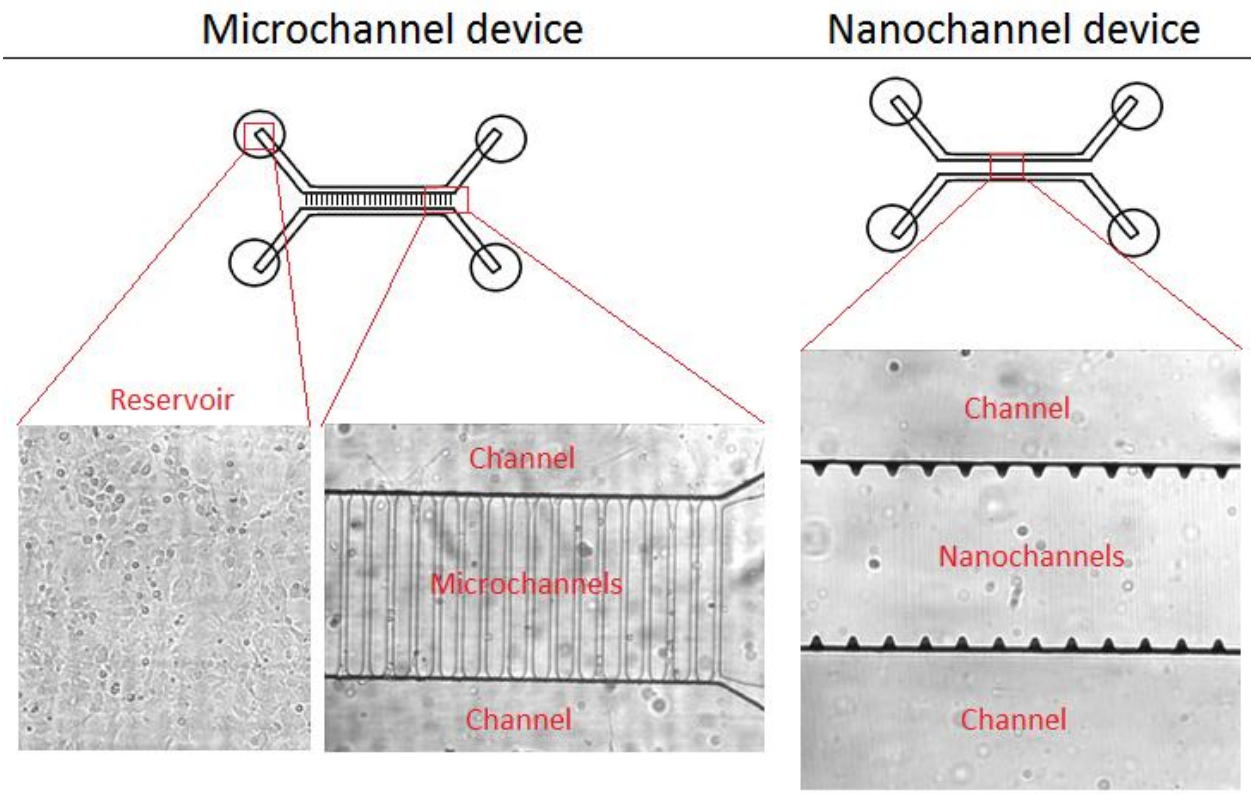

Figure 2: Microchannel device and nanochannel device

In the relaxed state, microchannels have a 10µm width, while nanochannels have a less than 1µm width. The width of microchannels and nanochannels can be adjusted by compressing or stretching the polymer devices using a home-made stretcher (Figure 3).

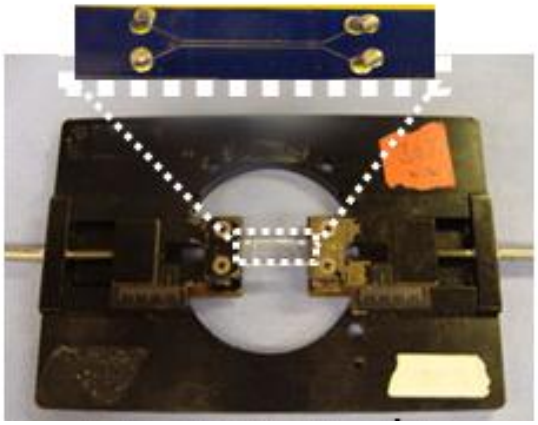

Figure 3: Home-made stretcher loaded with a microfluidic device

MATERIALS AND METHODS

Microfluidic device molds were fabricated using standard photolithography method. Poly (dimethylsiloxane) (PDMS) was poured onto the device molds to approximately 5mm thick at a 10:1 base to cross-linker ratio by mass. The PDMS was cured overnight at 60°C, cut into individual devices, and punched with 3-5mm holes for reservoirs. The PDMS devices were then plasma oxidized for 6 minutes and bonded to a thin film of PDMS as cover. The devices were then treated with poly-lysine and laminin extracellular matrix (ECM) coatings, washed carefully with distilled water and dried, and stored in the refrigerator until use.

Primary dorsal root ganglia (DRG) were dissected from chicken embryos (E8-E10) and dissociated to single cells. Cells were seeded in reservoirs and channels of PDMS devices pretreated with ECM coatings. Devices were then placed in an incubator at 37°C. Neuron cell culture media includes 89% Dulbecco's modified Eagle's medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Antibiotic-Antimycotic Solution, supplemented with 5-fluoro-2'-deoxyuridine (FUDR) and NGF (50ng/ml). Media was changed daily in devices by removing half of old media from reservoirs and adding fresh media. Fluorescent staining with CellTracker were used for better visualization of neurons inside devices during compression experiments.

RESULTS OF OPTIMIZING CONDITIONS

Cell attachment and viability inside PDMS devices depend upon conditions such as the type and concentration of extracellular matrix coating, cell seeding concentration and maintenance conditions, and device geometry. Those conditions were optimized below. Additionally, cell growth inside devices was characterized for microchannel devices and nanochannel devices. Finally, neuronal responses to compression and strain using the home-made stretcher were studied.

Extracellular Matrix Coatings

Extracellular matrix coatings allow neurons to attach on PDMS surfaces. Preliminary tests for optimal coating conditions were performed using SHSY5Y neuroblast cells due to the similarities between SHSY5Y and primary neurons. Cured PDMS in well plates were treated with different coating conditions, including poly-lysine (PK) overnight (100 µg/ml, 200 µg/ml), laminin for 4 h (25 µg/ml, 50 µg/ml), and combinations of poly-lysine overnight (100 µg/ml, 200 µg/ml) and laminin for 4 h (25 µg/ml, 50 µg/ml, 100 µg/ml). SHSY5Y cell attachment was compared one day after cell seeding.

It was found that combining poly-lysine and laminin produced the best cells attachment (Figure 4 and Appendix A). All combinations of poly-lysine and laminin concentrations tested produced comparable results in cell attachment, with the lowest effective concentrations being poly-lysine (100 µg/ml) and laminin (25 µg/ml).

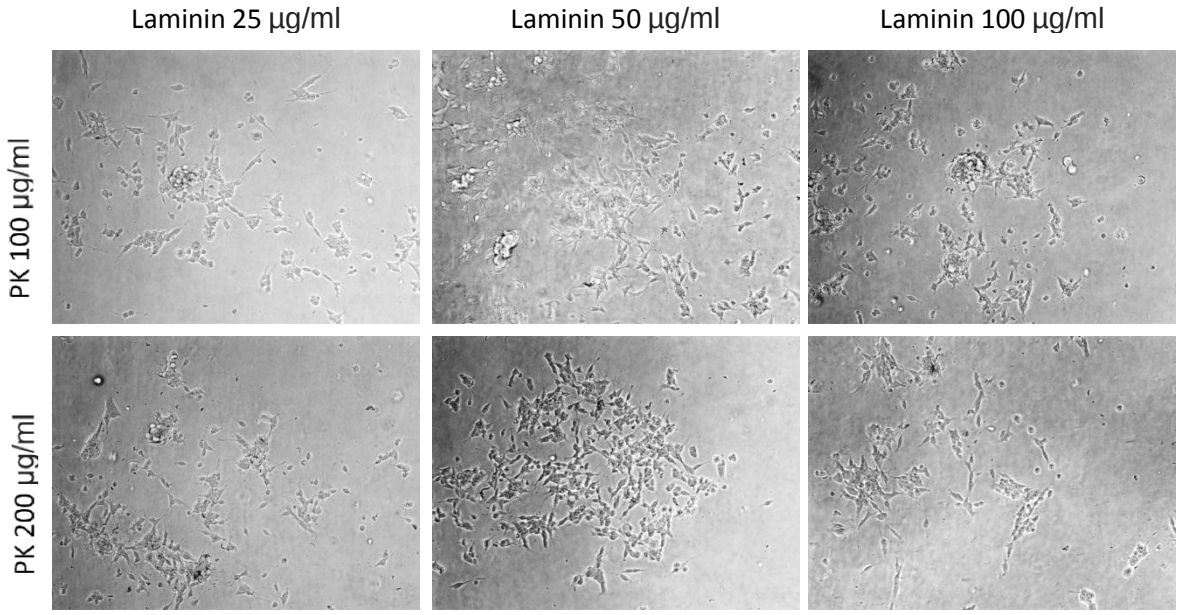

Figure 4: SHSY5Y cell attachment on PDMS treated with combinations of poly-lysine and laminin

Cell Concentration for Seeding and Maintenance Conditions

Neurons are healthiest when grown at a sufficiently high density. Different concentrations of SHSY5Y cells, in the order of magnitude 10^5, 10^4, 10^3 cells/ml, were tested in PDMS well plates and PDMS device reservoirs. In all cases, 10^5 cells/ml produced the cell attachment density for easiest visualization, since lower concentrations produced sparse cell attachment (Figure 5).

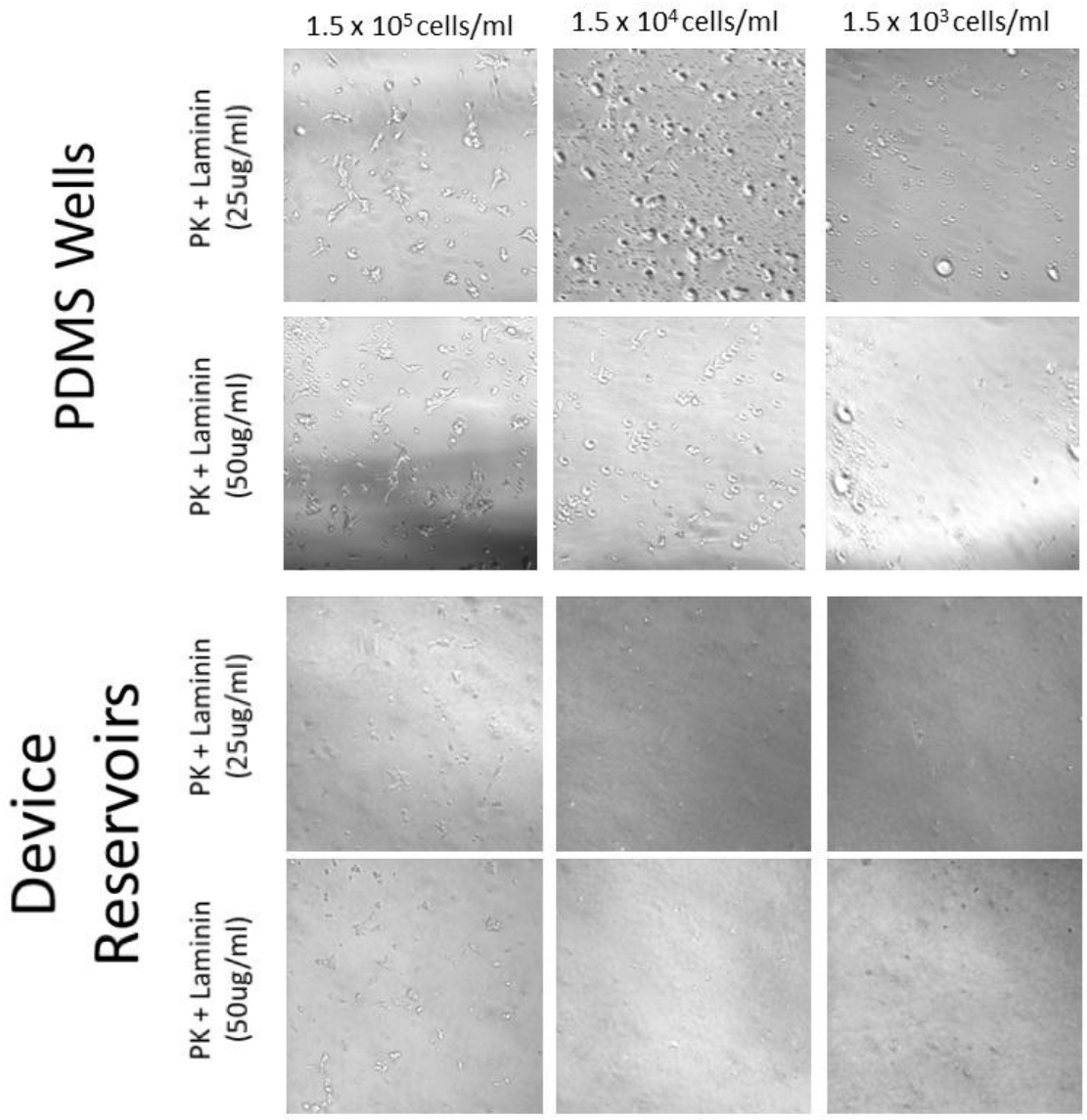

Figure 5: SHSY5Y cells seeded at different concentrations, in various settings. Note, poly-lysine (PK) was 100ug/ml in all cases.

Another optimized condition is seeding cells in only one channel of the PDMS device and then tilting the device at a slight angle inside the incubator to allow media and cell migration down to other channel for maximum seeding into microchannels for microchannel devices and along notch regions of nanochannel devices.

Device Alternations

Using the optimized conditions for ECM treatment and cell seeding, SHSY5Y cells were grown in PDMS devices. However, cells grew in the reservoir and channel near edge of reservoir but did not grow in the middle of channels (Figure 6).

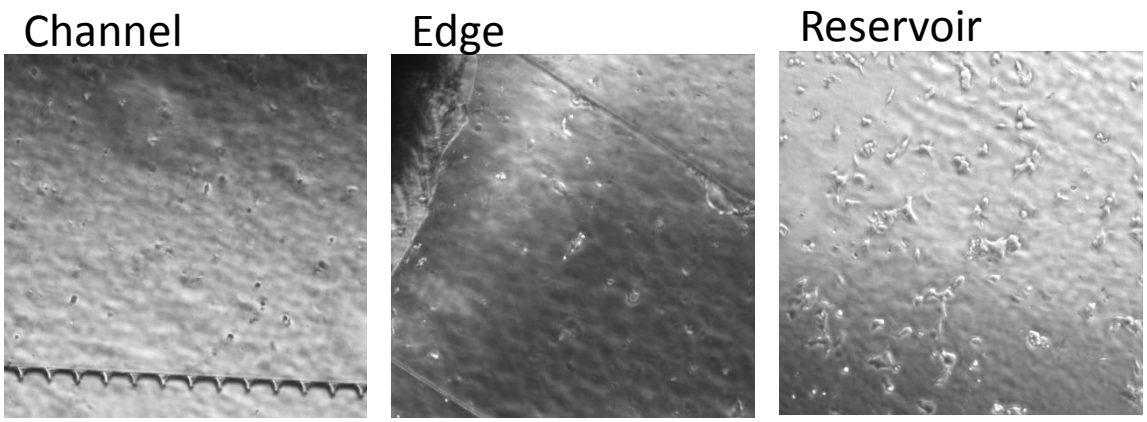

Figure 6: SHSY5Y cells in various regions of PDMS device

A possible explanation for the cells' failure to grow in the middle of channel may be lack of nutrients due to media volume limitation inside the channel and nutrient depletion due to slow diffusion from reservoir to channel. Two solutions to alter the device geometry were tested. In one solution, more reservoirs were created by punching multiple holes along the channel to facilitate nutrient diffusion into channels (Figure 7). As another solution, a new device mold was created with the channel height increased from 50 μm to 80 μm, providing more space for media. When tested with primary neurons in PDMS devices, both device geometry alternations were effective in enabling neuron growth in channels (Figure 8). The device geometry with 80 μm channel height was ultimately chosen for used in future experiments due to easier reproducibility and simplicity, and adopted for both the microchannel device and nanochannel device.

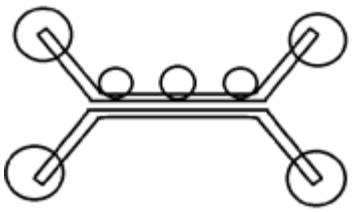

Figure 7: Altered device with multiple reservoirs

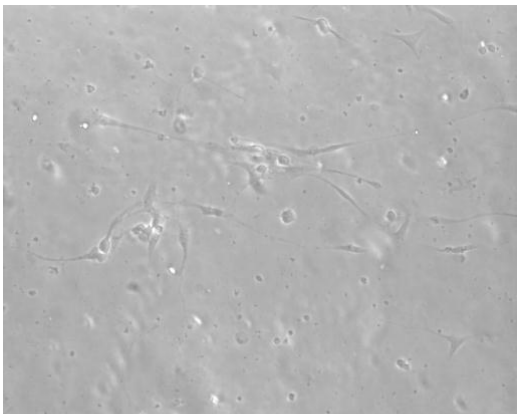

Figure 8: Primary neuron cells growing in new device with 80μm height, at 2 days after seeding

Also, in addition to increasing nutrients, the increased channel height provides easier washing of channels during the extracellular matrix coating procedure, which eliminates toxicity of excess, nonattached ECM proteins. Overall, the increased volume of media inside channel provides more nutrient availability, easier flow inside channel, and more seeding of cells, which all contributed to better neuron growth in the devices.

RESULTS FOR MICROCHANNEL DEVICES

Microchannel devices were studied over the course of one week to observe typical primary neuron growth inside the devices, under conditions of no compression or strain. Those characterizations guided

recommendations concerning timing of compression and strain experiments. Moreover, preliminary compression experiments were performed on microchannel devices using a home-made stretcher.

Characterization of Cell Growth in Microchannel Devices

A sample size of five microchannel devices was studied over the course of one week. The first day after seeding, reservoirs generally showed good neuron attachment (Figure 9), though channels sometimes had attached cells and sometimes not. By the second day, cells were visibly attached and growing in channels if they were to grow at all (Figure 10).

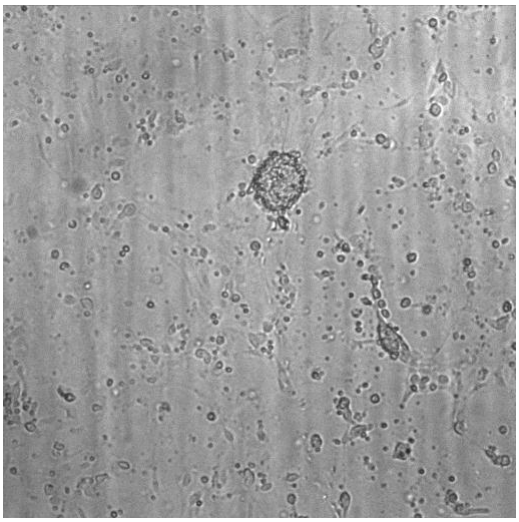

Figure 9: Primary neuron cells growing in reservoir, at 1 day after seeding

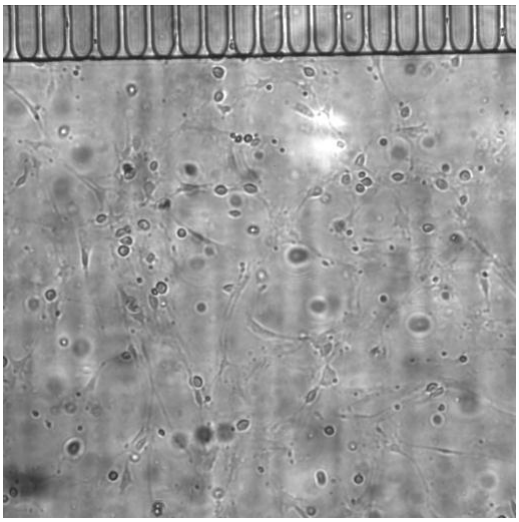

Figure 10: Primary neuron cells growing in channel, at 2 days after seeding

By the third day, many neurons had long axons in microchannels (Figure 11). Compression and strain experiments were recommended for day 2, 3, or 4, as soon as axons were long enough through the microchannels as determined visually. Neurons in devices generally survived up to one week. In some devices, only glia cells and no neurons were present by the seventh day.

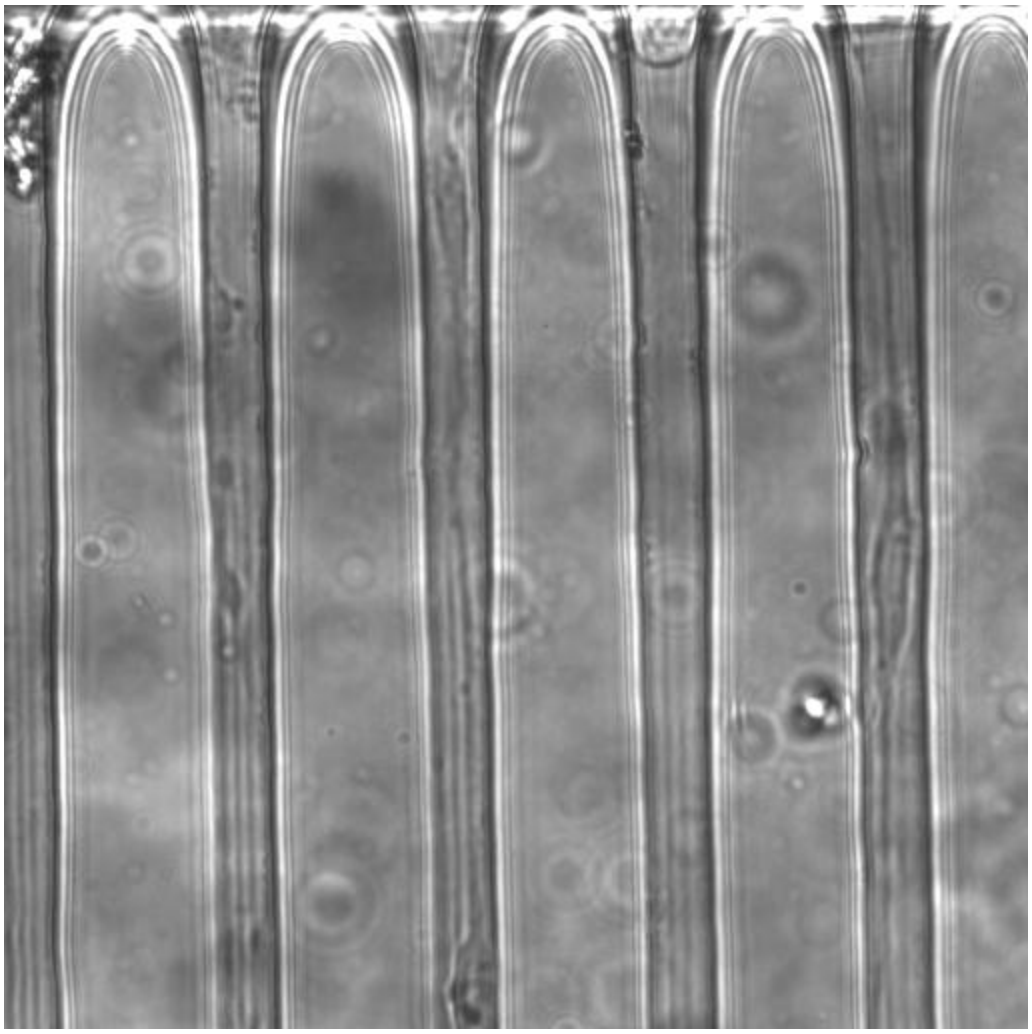

Figure 11: Primary neuron cells growing linearly in microchannels, at 3 days after seeding

Compression of Microchannel Devices

Two microchannnel devices were compressed four days after seeding, and the effect of 10% compression was observed and compared with a non-compressed control device. Briefly, the procedure involves labeling neurons with CellTracker, washing, imaging, and then applying compression using a home-made stretcher.

It was found that sheets of neurons in the reservoirs and channel started tearing in some places. Some axons in microchannels were washed away or became segmented and clumped together (Figure 12). Also, the position of some neurons shifted up or down after compression (Appendix B), but this phenomenon was observed in both compressed and control devices.

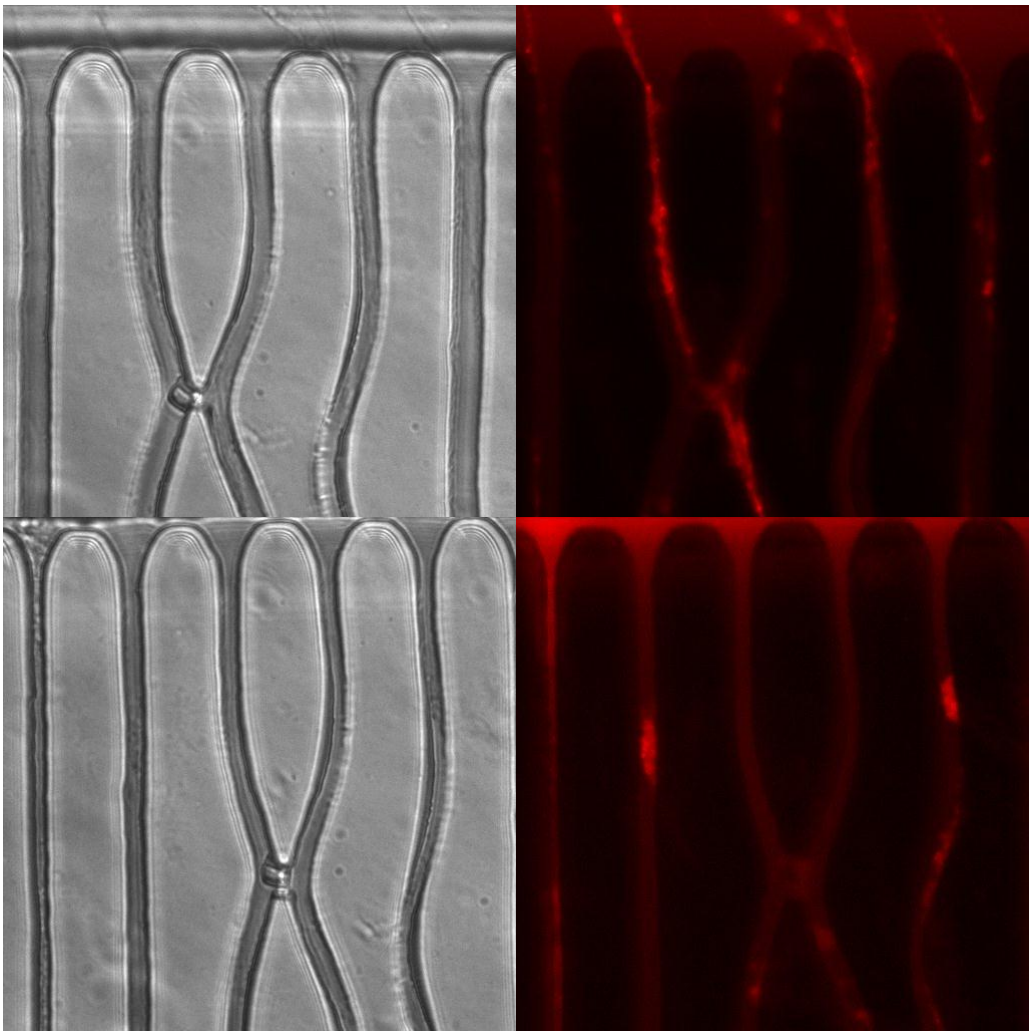

Figure 12: Top images were before compression. Bottom images were taken the following day in the compressed state

DISCUSSION FOR MICROCHANNEL DEVICES

The current microchannel device dimensions were not suitable for studying neuron compression. The 10% compression damaged many axons and neurons and interfered with the intention to study neuronal response to compression. Moreover, even the maximum compression at 10% did not provide a microchannel narrow enough for compressing axons, but any compression more than 10% would have cause deformation and bulging of the device above the horizontal plane. Perhaps if the initial microchannel widths were smaller, then less compression would needed and less cell damage would be incurred, since microchannels would be closer to the dimension necessary for compressing axons of single cells. Recommendation for future device includes fabricating microchannels with smaller width.

Moreover, smaller microchannel may enable better compartmentalization of cell bodies and axons. Current microchannels enclose entire neurons, including cell body and axons (Figure 12). Optimally, in

future devices, cell bodies will be positioned at the notch region and grow axons into microchannels, to achieve spatial separation of axons and cell bodies (Figure 13).

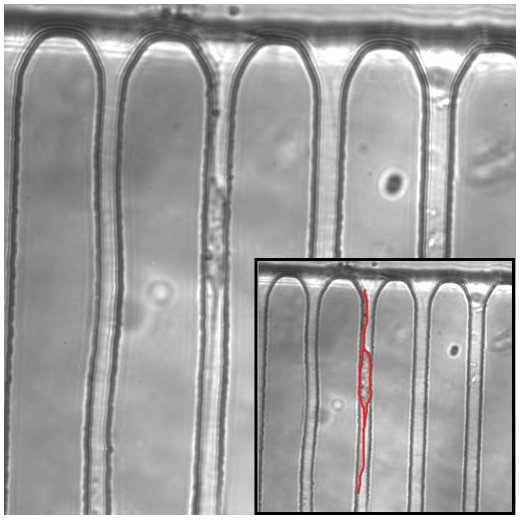

Figure 12: Microchannel enclosing a neuron. 400x magnification

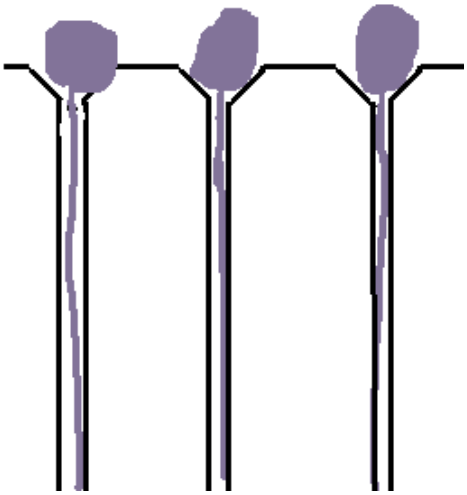

Figure 13: Design to allow spatial separation of cell bodies and axons

RESULTS FOR NANOCHANNEL DEVICES

Nanochannel devices were stretched using the home-made stretchers in order to open and maintain the nanochannels to allow axon growth into them. The procedure included stretching the PDMS devices on home-made stretchers, treating with ECM coatings and washing, and then seeding with primary neuron cells while still on stretchers. Cell growth for nonstretched and stretched devices were characterized.

Cell Growth in Nonstretched Nanochannel Devices

Nanochannel devices were compared with microchannel devices to demonstrate that primary neuron cells were equally compatible in both devices, when under conditions of no compression or strain. Three nanochannel devices and one microchannel device were seeded simultaneously and observed for three days. Under non-stretched condition, the neuron growth in microchannel devices and nanochannel devices were comparable. Both types of devices showed good growth of neuron cells and axons in reservoirs and channels (Figure 14).

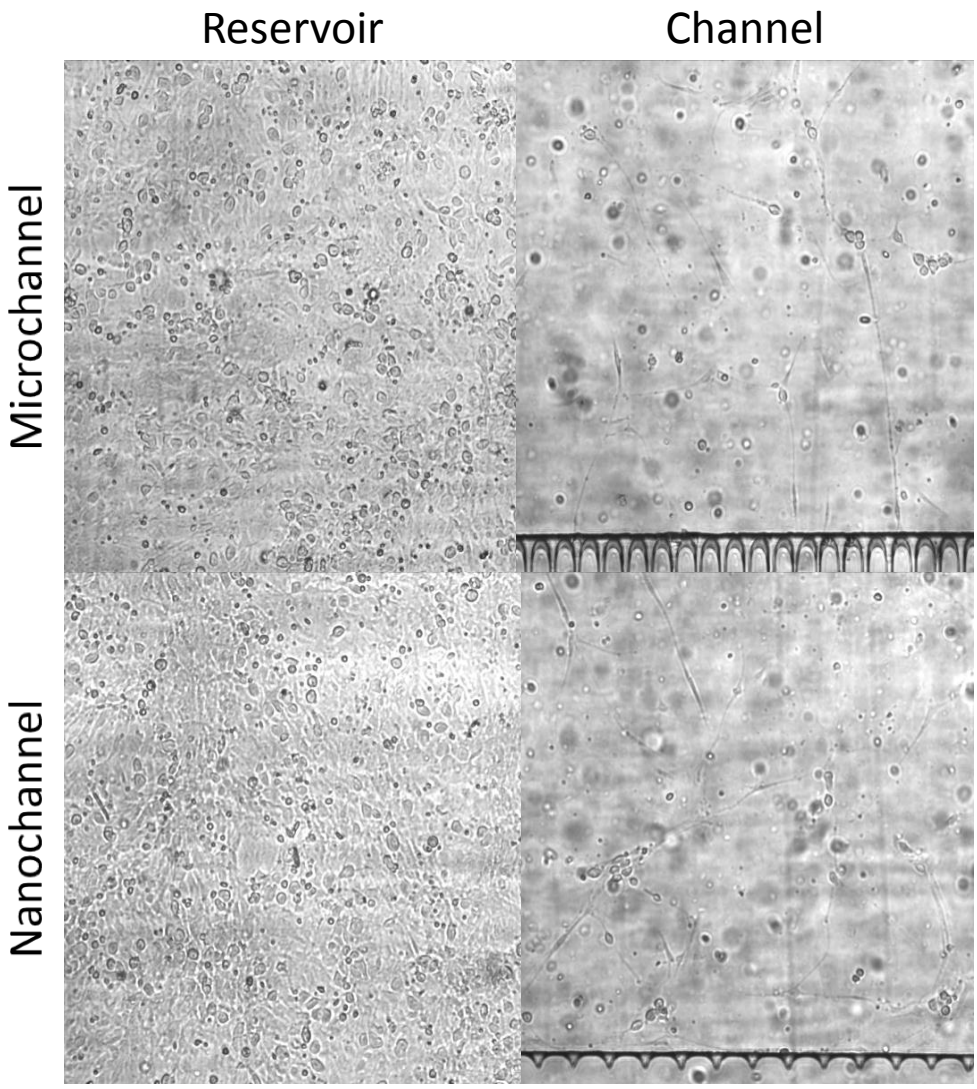

Figure 14: Microchannel and nanochannel devices show comparable growth in reservoirs and channels on day 2

Cell Growth in Stretched Nanochannel Devices

Growth of primary neuron cells in stretched and nonstretched nanochannel devices were compared. Three nanochannel devices were stretched on home-made stretchers with 10% strain for 3 days. One nonstretched nanochannel device served as the control. The control showed cell attachment in both the reservoir and channel by day 2, but the stretched devices showed cell attachment in only the reservoir (Figure 15). Moreover, for the stretched devices with reservoir cell growth, the cells in the reservoirs became unhealthy over the course of three days (Figure 16). In contrast, the nonstretched control showed neuron growth in both reservoir and channel, with progressively more growth over time (Figure 17).

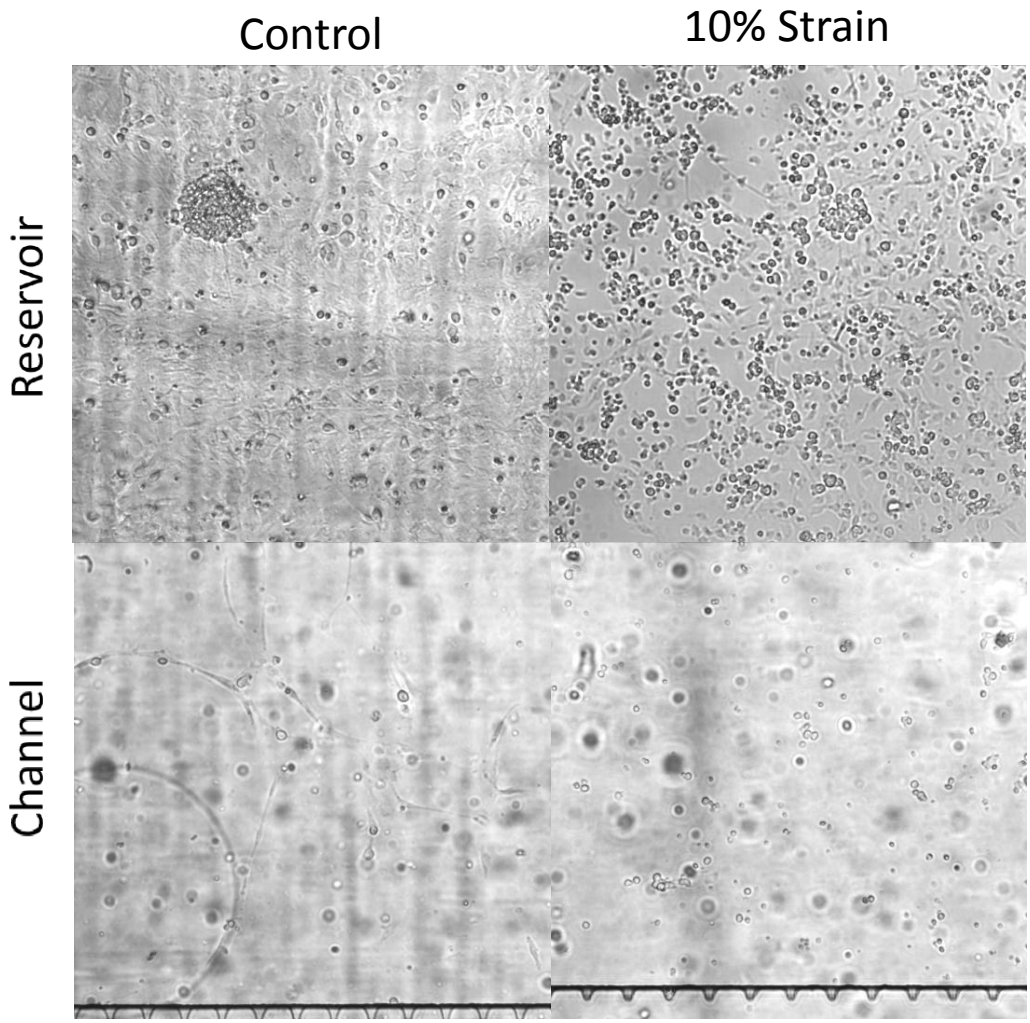

Figure 15: Similar reservoirs growth for control and stretched devices. Only control channels has growth

The stretched devices were removed from the stretchers after day 3 and then observed for one more day. Interestingly, for one of the previously stretched devices, the cells in the reservoir became healthier after removal of strain (Figure 16).

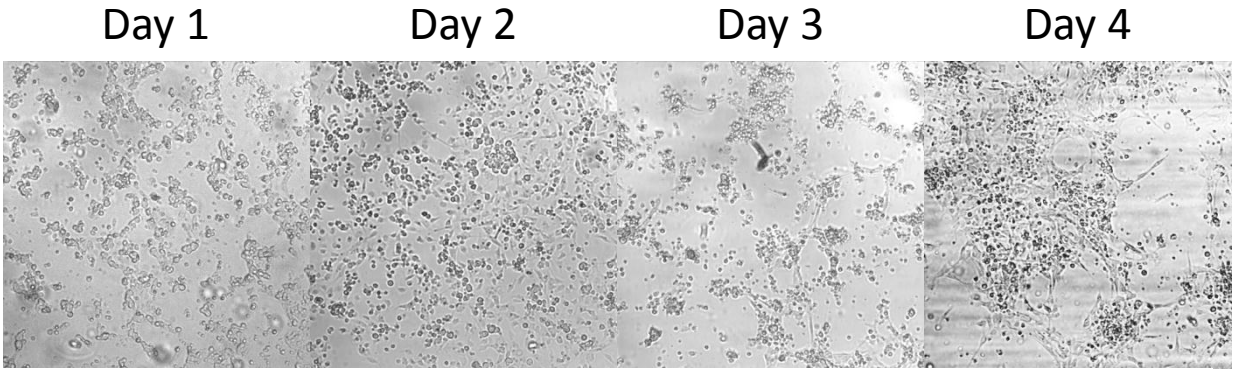

Figure 16: Reservoirs region with 10% strain for day 1, 2, 3, and no strain for day 4

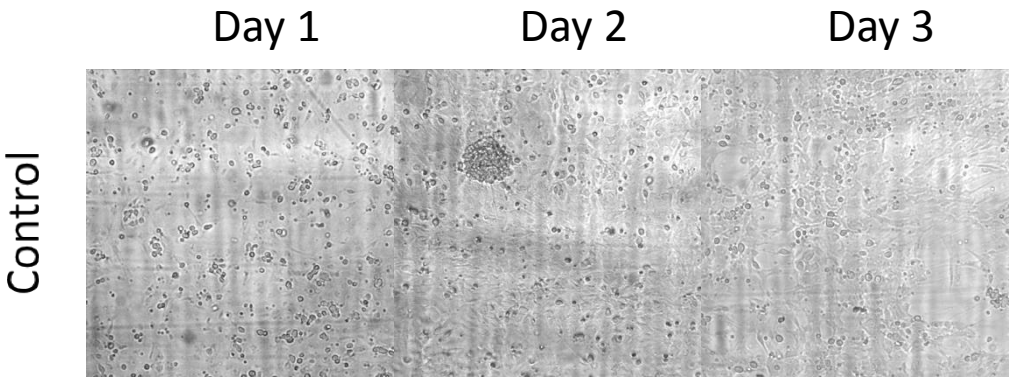

Figure 17: For control device, reservoir cells continually grew well

To further investigate the effect of percent strain on primary neuron cell growth, another experiment was performed that subjected nanochannel devices to no strain, 3% strain, and 8% strain. Similar to previous results, the control showed good cell growth in both the reservoir and channel. In contrast, the 3% stained device showed good cell growth in reservoir but not channel, and the 8% strained device did not show cell growth at all (Figure 18).

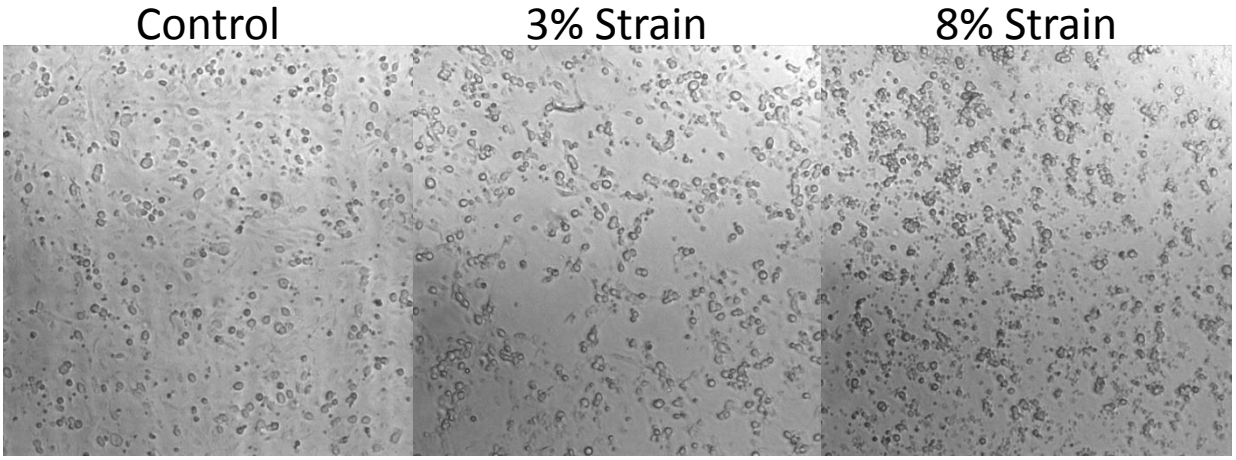

Figure 18: Reservoir region of control, 3% strain, and 8% strain devices.

DISCUSSION FOR NANOCHANNEL DEVICES

Stretched nanochannel devices had poor cell viability in channels, though any difference in cell growth could not have been intrinsic to the nanochannel device, as demonstrated by the comparable results of the nanochannel device and microchannel device in the unstretched state. The difference must have been resulted from the stretching process.

A possible explanation is that stretching causes the device to lengthen and decreases the height of channels, therefore causing the previous problem of nutrition deficiency, as discussed previously in the Device Alterations section.

But, another observation must be taken into account. In the experiment with control, 3%, and 8% strain, the control and lower strain showed good cell growth, while the higher strain showed low cell viability. Cells growing poorly in the reservoir for the stretched device cannot be explained by nutrient deficiency, since nutrient is plentiful in reservoir, regardless of the stretched or nonstretched state.

A more feasible explanation is the difference in topography of the stretched PDMS, which may be altering glia cell growth. By observing figures comparing stretched device reservoirs and control reservoirs, as in Figure 15 and 18, it seems that glia cells are not as prevalent in the reservoirs of the stretched device, as indicated by the absence of a layer of opaque, flat, triangular-shaped glia cells layer below the neurons. For some reason, perhaps due to difference in topography of stretched device, the stretched PDMS surface may effect growth of glia cells which then affects growth of neurons.

The results lead to several recommendations for future study. One recommendation is to use two fluorescent labels to distinguish glia and neurons to study the ratio of those cells in the stretched and nonstretched devices. Also, further study should be performed to verify that increasing strain indeed causes progressively worse cell growth. As a control, a nanochannel device should be placed on a stretcher but with 0% strain, to eliminate any differences from set-up differences due to stretcher.

Finally, further study can be done to study neuron/glia ratio when device is moved from stretched to nonstretched state and vice versa. Those studies may confirm that removing device from stretcher promotes a recovery of neuron cell growth. If results confirm that stretched PDMS surfaces inhibit glia cell growth, then stretching of PDMS devices may be a good way to control glia cell growth for neuron studies or brain tumor studies, since tumor is usually uncontrolled cell growth of glia cells.

CONCLUSION

PDMS microfluidics devices provide a useful platform for studying spinal cord injury at the single neuron level through compression and strain studies. Conditions such as extracellular matrix coating treatment, cell seeding concentration, and device channel height were optimized to promote primary neuron viability, cell attachment, and growth in PDMS devices. Neuron growth characteristics and timing were studied in microchannel and nanochannel devices. Compression experiments with microchannel devices revealed that current microchannel dimensions were not suitable for neuron compression, because compression damaged many axons and neurons in the process. Recommendation for future microchannel devices includes fabricating microchannels with smaller widths, to better enable compression studies and provide spatial separation of cell bodies and axons. Stretching experiments with nanochannel devices reveal that neurons grow poorly on stretched PDMS devices. Additionally, stretched PDMS surface seems to inhibit glia cell growth, though more experiments are required for confirmation. A recommendation for future experiments is to study neuron to glia ratio changes caused by topography or surface differences of the stretched PDMS device.

APPENDIX A:

Extracellular Matrix Coatings

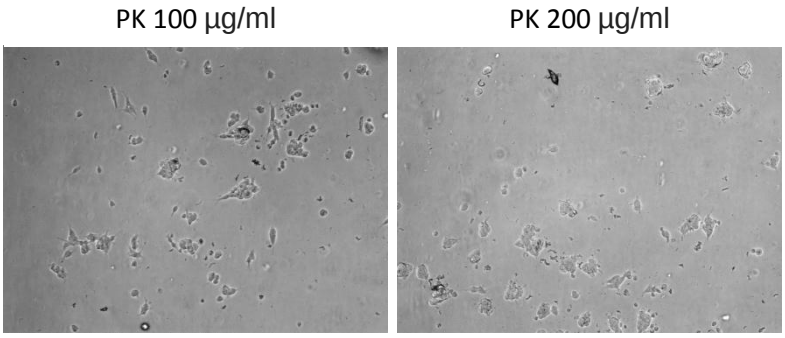

Figure A1: Poly-lysine (PK) produces mediocre attachment of SHSY5Y cells

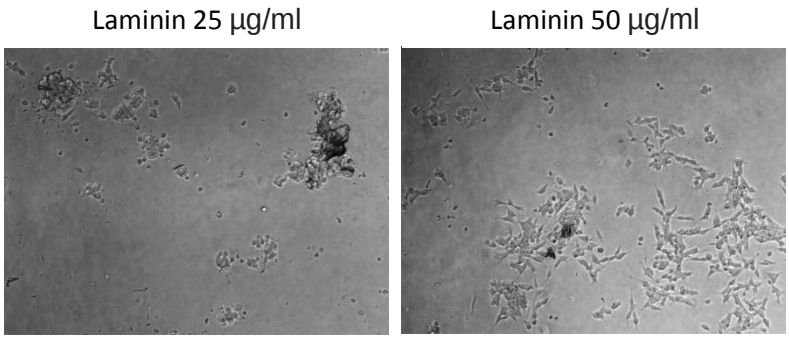

Figure A2: Laminin at 25 µg/ml is not sufficient, but higher concentration of laminin at 50 µg/ml produces excellent attachment.

APPENDIX B:

Compression of Microchannel Devices

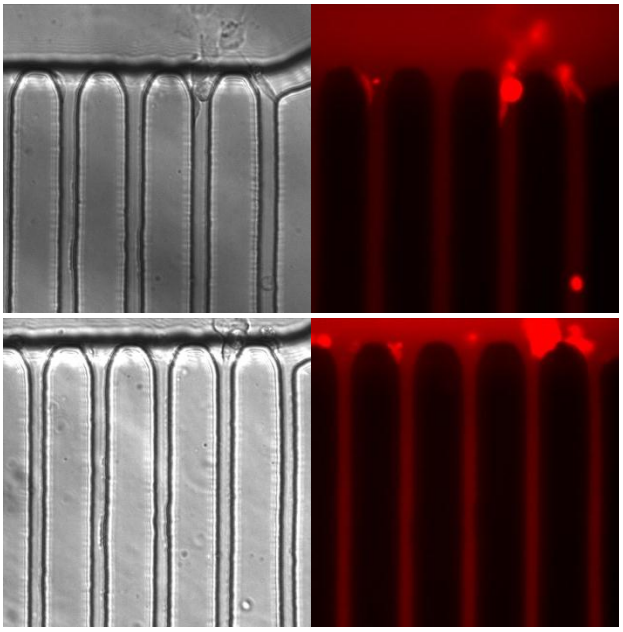

Figure B1: Top images from before compression and bottom images from after 1 day compression

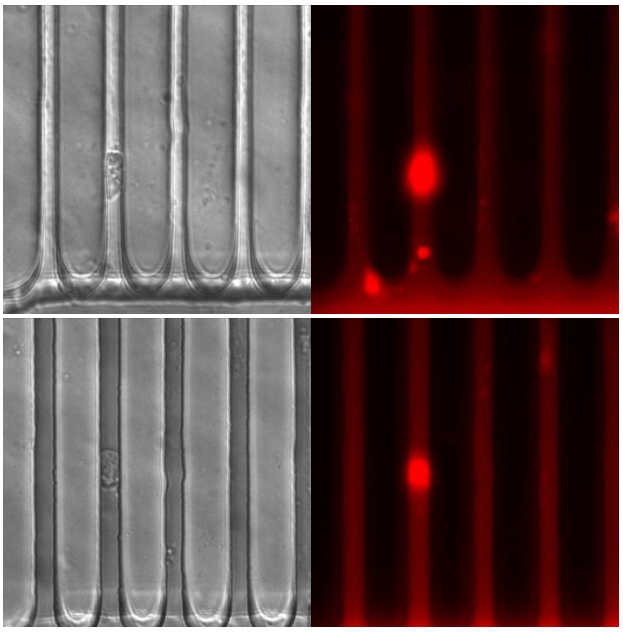

Figure B2: Top images from control device, bottom images taken next day