Assessing mechanotactic axon pathfinding in induced Pluripotent Stem Cell-derived motor neurons

by

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Assessing mechanotactic axon pathfinding in iPSC-derived motor neurons

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Abstract

In the central nervous system, upper motor neurons and lower motor neurons, alongside spinal cord interneurons, coordinate all muscle contractions within the body. Axon guidance is a crucial process to the development of functional neuronal networks, as well as regeneration of proper neuronal function for treatment of neurological diseases. Biomedical engineering of scaffolds that promote the axon guidance and proper rewiring of neurons is an area being actively studied by many researchers. In this project, the effect of mechanical strength of fibrin-based hydrogel on the growth of spinal motor neuron axons is studied using a gradient fibrin hydrogel assay, combined with light microscopy and mathematical analysis of acquired images. The motor neurons showed no significant preference for softer or stiffer substrate.

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Introduction

Stretch-Activated Ion Channels and their possible roles in development:

During development of the nervous system, neurons grow extended axons toward specific targets to relay information across the body of an organism. The mechanism by which axons home onto the correct direction and target is traditionally explained by chemotaxis, where target tissues secrete various factors, which may form gradient in order to elicit differential growth in the axons (Bonanomi and Pfaff, 2010). However, during the extension of the axon, they may traverse through many tissue types with a large range of mechanical properties and exert force onto the surrounding matrix (Athamneh and Suter, 2015). The reactionary forces exerted onto the growth cones are sensed by cells using mechanoreceptors on growth cones, mainly stretch-activated ion channels (SAIC), which can respond to mechanical perturbations with varied sensitivity, and create transient Calcium influx to activate downstream signaling events (Kerstein *et al*., 2013). SAICs are found to be expressed in many neural and non-neural tissues throughout multiple developmental stages (Shibasaki *et al*., 2010). These receptors, classically, were first found as mechanical sensors for environmental stimuli, such as Piezo2, which was identified in the cochlear hair cells and convert air vibrations into electrical signals (Beurg and Fettiplace, 2017). Its homolog protein, Piezo1, was found to functions in homeostatic control by triggering cellular division in response to cell-crowding or stretch (Gudipaty *et al*., 2017). Recently, Piezo1 has been found to facilitate the pathfinding of Retinal ganglion cell axons during formation of the optic tract in *Xenopus* brain development (Koser *et al*., 2016, Thompson *et al*., 2019). Similar research exploring the effect of substrate stiffness and axon growth has been conducted on various different nervous systems with varied results (Table 1). Mice Cortical neurons were shown to be insensitive to substrate stiffness (Norman and Aranda-Espinoza, 2010), Dorsal Root Ganglions (DRG) were shown to grow axons slower and form fewer branches when TRPC1, a member of the Transient receptor potential (TRP) family channels, was activated (Kerstein *et al*., 2013). These TRP channels are also known for their function in

chicken sensory and motor neurons. Studies also demonstrated knock-out of another SAIC of the TRP family, TRPV2, resulted in reduced axon growth, while activation led to enhanced axon growth in sensory and motor neurons (Shibasaki *et al*., 2010). The lack of coherence in the results indicate that different lineages of neurons respond to substrate stiffness in fundamentally different manners.

Table 1: Summary of mechanotactic properties of different neurons in the literature**.**

Type of neural tissue	Stiff substrate or stretch	Soft substrate	Model system used	Reference
Dorsal Root Ganglion axons	Increase growth	Decrease growth	Chicken embryo	Shibasaki *et al*., 2010
Retinal Ganglion axons	Increase growth	More branching	*Xenopus* retinal explant	Koser *et al*.,2016
Cortical neurons	No effect	No effect	Dissociated cortical neurons	Norman and Aranda-Espino za, 2010
Hippocampal neuron	Increased branching	N/A	Dissociated hippocampal neurons	Previtera *et al*., 2010
Spinal cord neurons	Decrease growth	N/A	*Xenopus* spinal cord	Kerstein *et al*., 2013

Transduction of physical environmental signals in neurons

Cytoskeletal components are the driving force behind all cellular actions involving morphing of the cell membrane, such as formation of filopodia and neuronal growth cones (Kerstein *et al*., 2015). During axon growth, the surface adhesion molecules (SAM) attach to the ECM, creating traction force. Intracellular domain of the SAMs activate a series of intracellular signaling mechanism, such as Rho-GTPase, to start actin polymerization towards the direction of movement, and the actin filaments, if coupled to cell membrane components, can then generate force to pull the cell forward or extend the cell membrane (Nichol *et al*., 2016). Coupling between actin and cell membrane relies on various mechanisms, such as Talin which connects integrin on the cell membrane to the actin filaments, and myosin II motors which generates the mechanical force behind filopodia protrusion and contraction

(Xue *et al*., 2010). The myosin II proteins can therefore be considered as a Calcium-dependent component required for axon growth. Additionally, works done by Kerstein *et al* (2013) showed an alternative possible mechanism behind axon mechanosensing, which is mediated by Calcium-dependent protease calpain, which specifically cleaves talin and severs the linkage between actin filament and integrin. With these two Calcium-dependent cell-migration mechanisms in consideration, the presence of SAIC, which are usually cation-selective and calcium-permeable (Huynh *et al*., 2016; Gnanasambandam *et al*., 2015), can serve as the link between detecting mechanical forces and intracellular biochemical signalling. Another factor also required is the presence of proper cell-adhesion molecules and a compliant substrate that permits the attachment, as different compliant substrates can result in different neurite growth (Norman and Aranda-Espinoza, 2010). Integrins play an important role in sensing the properties of substrates (Tan *et al*., 2011)

Probing motor neuron axon mechanotactic properties using fibrin-based hydrogel with a stiffness gradient

Retinal ganglion cells extend axons towards softer substrate *in vitro* (Koser *et al*., 2016) and *in vivo* (Koser *et al*., 2016, Thompson *et al*., 2019). The experiment performed in similar studies all used two-dimensional media with primary tissue explant placed on top and cultured. For the application of engineered biocompatible materials being used in the human body as a scaffold for recovering nervous system injuries, human induced pluripotent stem cells (hiPSC) are more relevant Currently, tissue engineering using iPSC-derived neurons in combination with fibrin-based hydrogel has been proven to successfully support neuron growth and patterning (de la Vega *et al*., 2018). The advantages provided by bio-compatible materials such as fibrin include the ability to house cells in a three-dimensional construct, which mimics the matrix where cells naturally path-find through and reach targets. The microenvironment parameters includes porosity, stiffness, and molecules embedded inside which can all be defined by researchers when used in combination with 3D-bioprinting technologies. In this project, the mechanotactic properties of motor

neuron axons are assessed using a gradient fibrin hydrogel, and compared to NPC-induced neurons. Based on results found using chicken embryos (Shibasaki *et al*., 2010) motor axons should grow faster when growing towards stiff substrate environment if TRPV2 ion channels are activated due to high stiffness, and if TRPC1 is activated, neurons are expected to grow slower as described by Kerstein *et al.* (2013) in spinal cord explants.

Methods

iPSC-derived neural progenitor and motor neurons

Neural Progenitor Cells (NPC) were obtained from stocks generated as previously described using hiPSC-derived Neural stem cells from line 1-DL-01 hiPSC line (WiCell). NPCs culture were expanded by culturing in 6-well adhesion plates for one week with STEMdiff™ Neural Progenitor Medium (NPM) (05834, Stemcell Technologies, Vancouver, BC, Canada) with 1% Penicilin/Streptomycin (P4333, Sigma, St. Louis, MO, USA), coated with laminin (L2020, Sigma, St. Louis, MO, USA). NPCs were then induced using STEMdiff™ Neural Induction Medium (NIM) (05835, Stemcell Technologies, Vancouver, BC, Canada) for one week till observable axons form. Cells were removed from the culture dish using 0.25% trypsin - EDTA when seeding.

IPSC-derived spinal motor neurons (BX-0100, BRAINXELL, 455 Science Drive Ste 210, Maddison, WI) were thawed and seeded into the hydrogel upon usage without pre-culturing, and changed to day 2 media as per manufacturer protocols (). The seeding media is made by mixing DMEM and Neural basal media 1:1, with additional B27, N2 supplement, and seeding supplement. Day 1 medium is the same with additional brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF).

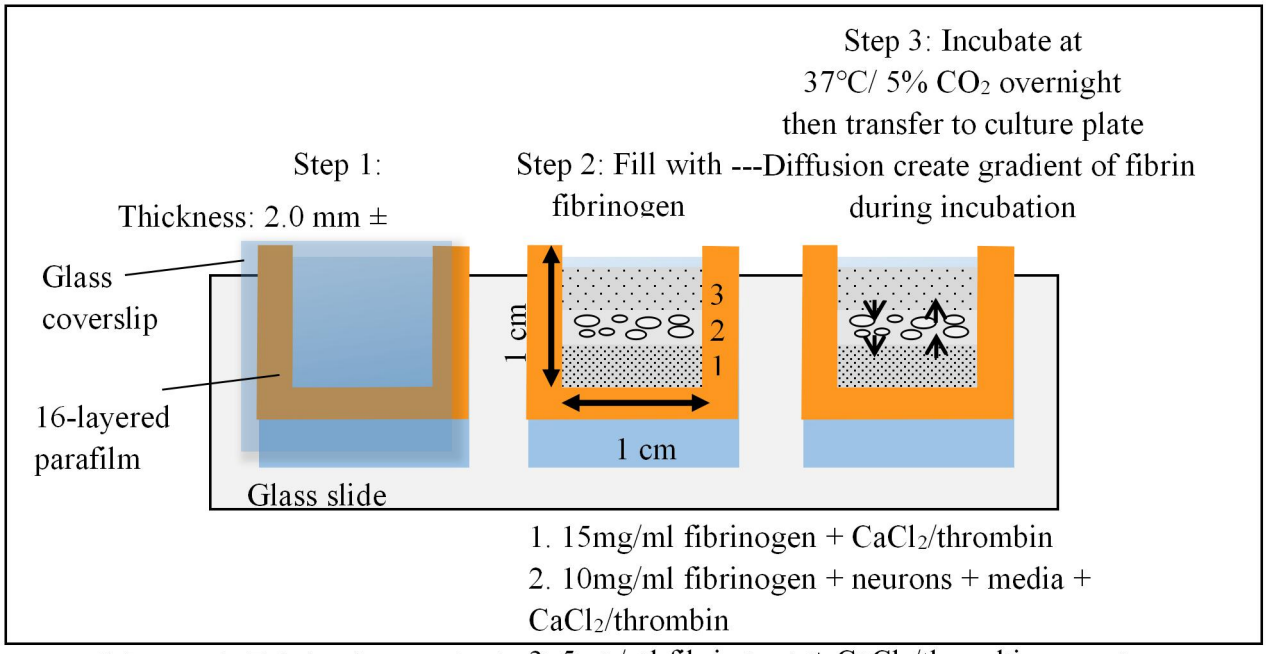
Fibrin hydrogel with stiffness gradient

Fibrin hydrogel with stiffness gradient is made by adapting the protocol from Koser *et al* (2016) but exchanging polyacrylamide with fibrin, and other necessary adjustments. Fibrinogen was dissolved, dialyzed overnight in Tris-buffered saline (TBS), then diluted in sterile-filtered TBS to make 15mg/ml and 5mg/ml stocks. To

fabricate the hydrogel, fibrinogen solution and $CaCl_2$/thrombin 1:1 mixture are added into a solidifying chamber at 9:1 ratio. Hydrogels for motor neuron studies have an additional 10µl of laminin supplemented. A chamber for solidifying the hydrogel gel is made by two microscope coverslip and an 16-layer parafilm cut to the dimension of 1cm x 1cm. 16-layered parafilm, when pressed together firmly, is measured to be 2.0 ± 0.1mm in thickness, thus the chamber is calculated to hold 200 ± 10µL of content. All glass components were sterilized by autoclaving, and parafilm was soaked in 70% ethanol and dried in the Biosafety Cabinet.

Seeding cells into the fibrin hydrogel

IPSC-derived neurons were suspended in 1X NIM and motor neurons in 1X Seeding medium, then 15mg/ml fibrinogen is added in 2X the volume, to dilute to 10mg/ml fibrinogen. The solution is then added into the gel chamber on top of the 15mg/ml fibrinogen layer. Lastly, 5mg/ml fibrinogen is overlayed on top. Each layer was first mixed with sterile $CaCl_2$/thrombin mixture before adding into the chamber.



Scheme 1: Fabrication method of fibrin hydrogel with stiffness gradient, and method for seeding neurons. Three major steps are shown. In step 1, three individual chambers can fit into two microscope slides, and are clamped tightly before proceeding. In step 2, denser fibrinogen solution is first filled, then the gel layer containing fibrinogen, Neural Induction Media (NIM) for NPC-derived neurons or Seeding media for motor neurons. Later, axons were photographed on the transition zone between zone 1 and 2 (stiff zone), and between zone 2 and 3 (soft zone).

Analysis of motor axon growth

Motor neurons were photographed using Leica DMI3000B microscope under bright field settings. Photos were processed using ImageJ2 distribution platform FIJI (Rueden *et al*., 2017, Schindelin *et al*., 2012), photos were processed by improving contrast to contain 2% saturated pixels. Axons were manually traced using the plugin NeuronJ 1.4.3 (Meijering *et al*., 2004). The length of the longest axon from each motor neuron was measured from the soma to the tip. Directionality measurements were performed by first applying a band-pass filter to the photographs taken, removing features smaller than two pixels (2.5μm) and larger than five pixels (12.5μm), then measuring the angle of axons with respect to the defined 0° horizontal line, which is also the direction of the fibrin stiffness gradient. The measurement is automatically performed by the directionality function in ImageJ using Fourier Component mode, which is a global method that analyzes the photograph in pieces, and calculate the polar coordinates of image features. A histogram with bin size 10° is automatically generated to divide 90° into 9 bins (Liu, 1991). The 90° degree angle indicates neuronal processes lying either along the stiffness gradient (0°) or perpendicular to the stiffness gradient (90°).

Results

Neural progenitor cell induced neurons in gradient fibrin hydrogel

IPSC-derived neurons were unable to grow networks of neurons successfully in gradient fibrin hydrogel. Cells exhibited a round-shaped morphology for more than 3 days, and eventually grew processes (Figure 1c). These processes appear much thicker comparing to the processes seen on cultured cells. At the 6^{th} day, the hydrogel started to degrade, and cells started to show signs of lysis (Fig. 1d, arrows). The abnormal axon growth and low viability indicates the fibrin hydrogel, when used by itself, does not support axon growth well.

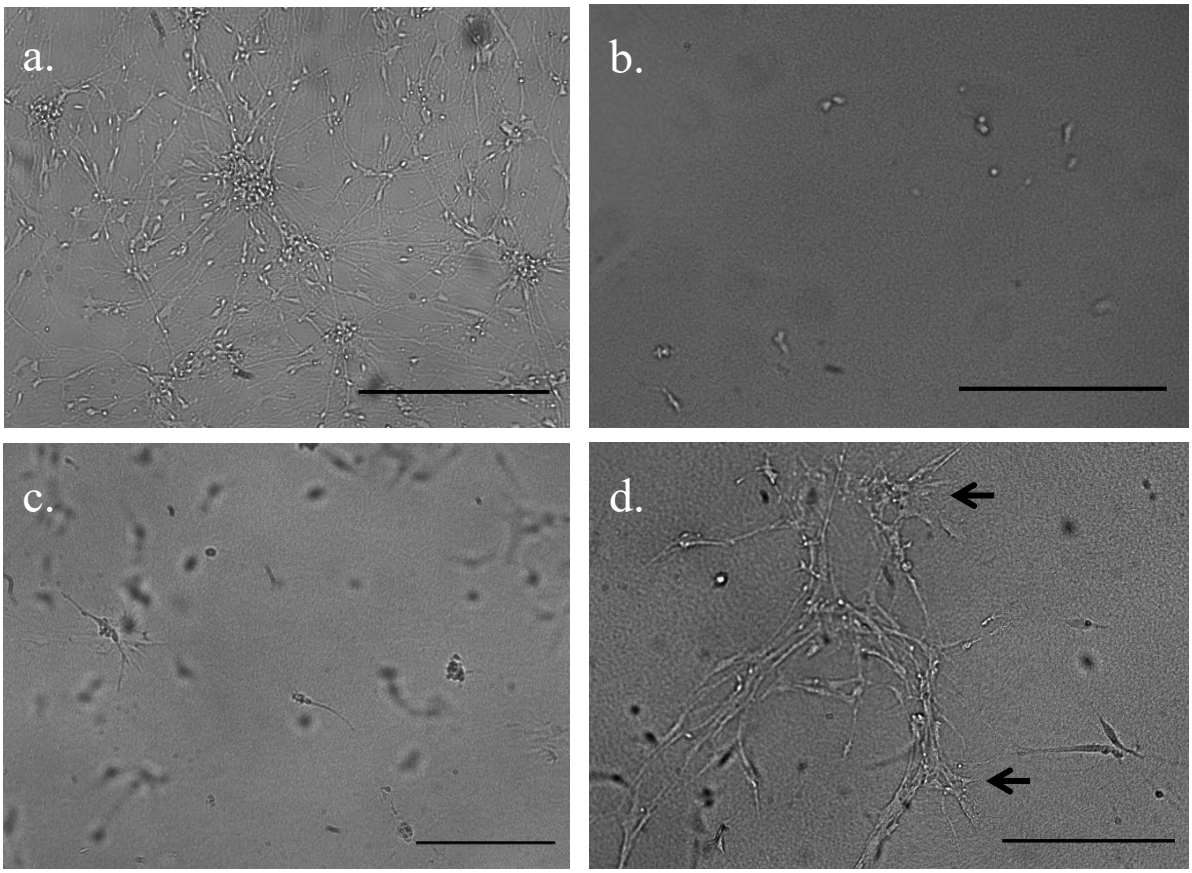


Figure 1: iPSC-derived NPC induced neurons do not survive past day 6 in gradient fibrin hydrogel, in all figures, scale bar is 500μm. a) Induced neurons cultured and differentiated using NIM in a tissue culture plate, neurons can survive till day 45, and appears to form network connections; b) Induced neurons seeded in fibrin hydrogel initially, cells appears spherical; c) At day 4, some neurons has migrated off the hydrogel, see in the background as out-of-focus, cells still on the gel shows limited axon growth; d) At day 6, aggregates of neurons are observed, indicating either activated proliferation or migration of cells. Signs of cell death were also observed (arrows).

iPSC-derived motor neurons on gradient fibrin hydrogel with laminin supplemented

After switching to Day 1 medium for one day, fibrin hydrogels were imaged with bright field microscopy. The axons extended from motor neuron cell bodies showed no significant difference (One-way ANOVA p-value = 0.230) in maximal axon length when grown in a stiff or soft part of the gradient gel (Figure 2c). After 48 hours of growth, images acquired with bright field microscopy show low contrast in neurons and the gel (Appendix. Fig. S1), and were processed in ImageJ by enhancing contrast to saturate 2% of the pixels.

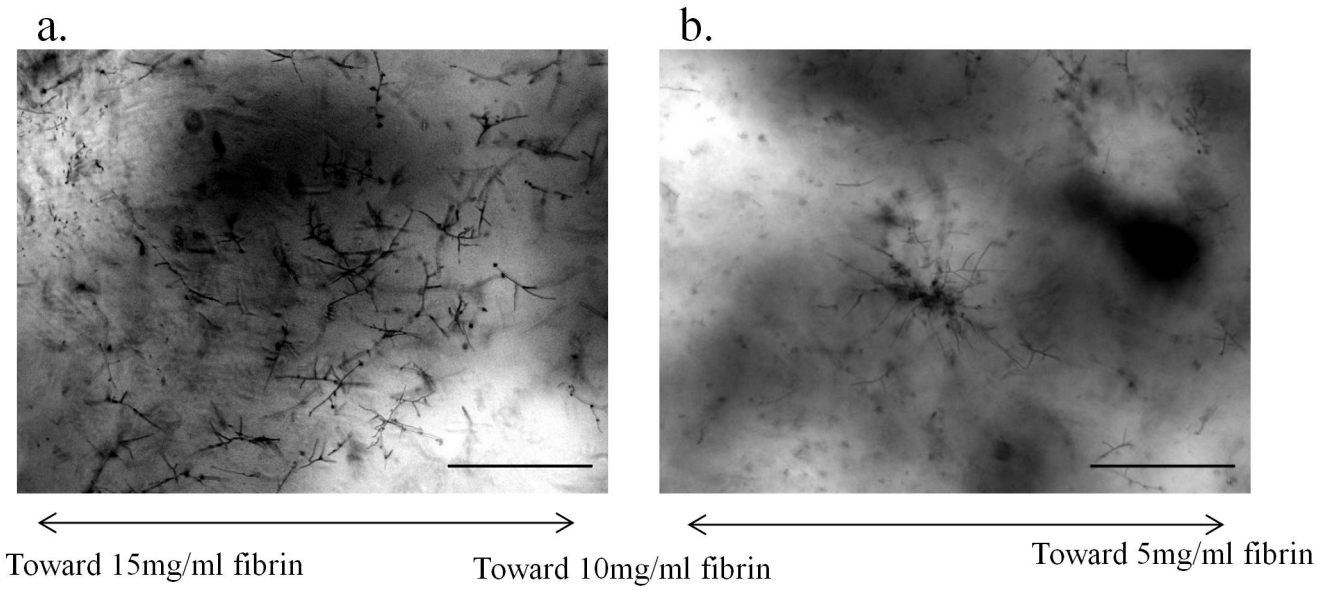
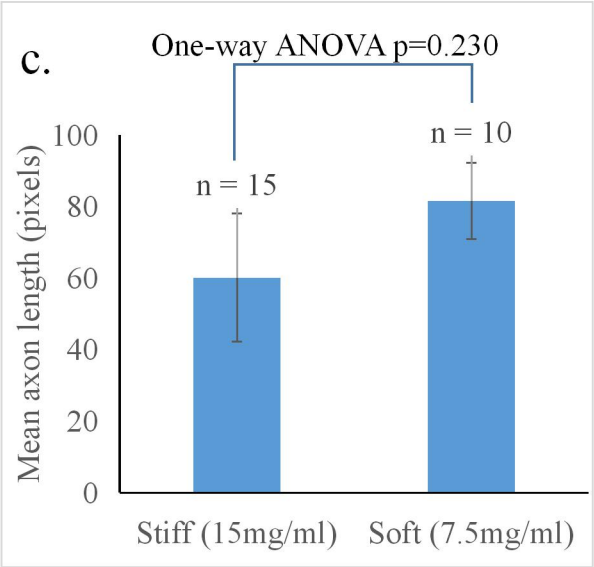


Figure 2: iPSC-derived motor neurons in gradient fibrin hydrogel. Axons were measured from cell body to the tip of the longest axon. Measurements were made only on cells with clearly visible cell body and axons extended from it. a) Sample picture of motor neurons on the stiff zone of the fibrin hydrogel; b) Sample picture of motor neurons on the soft zone of the fibrin hydrogel; c) Comparison on length of the axons measured in ImageJ using NeuronJ plugin. Error bars in the image is standard deviation in axon length



Directionality analysis of Motor neuron axons in gradient fibrin hydrogel

Despite the lack of difference in length, the processes of motor neurons appear to align favourably with the stiffness gradient, which is defined as 0˚ (Figure 3). A histogram of directions of motor neuron processes peaks around 0˚ in both soft and stiff zone. The histogram shows no significant difference between soft and stiff zone directionality.

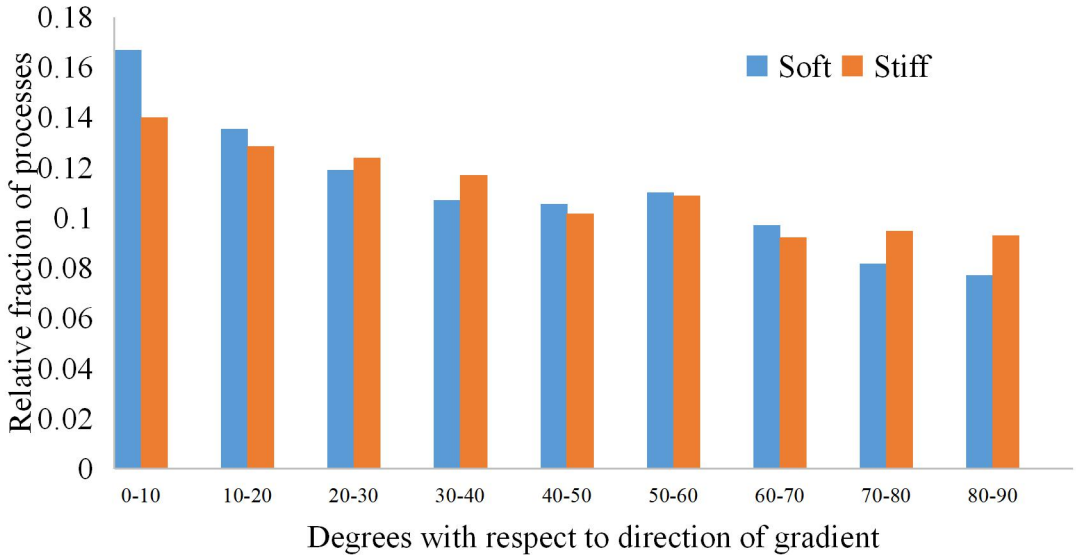


Figure 3: Analysis of the directionality of motor neuron processes with respect to fibrin hydrogel stiffness gradient. Data shows in both stiff and soft zones, more axons aligned with the stiffness gradient, shown by the increased amount of processes with angle around 0-10 degrees.

Discussion

Length of axon do not differ in substrate with different mechanical properties

Measurement of axon length after seeding motor neurons into the hydrogel for a define time period (48 hours) do not show significant difference in axon length (Figure 2c). Axons did not grow during the 24 hours period after seeding, but grown 24 hours after changing to day 1 media, the precise rate of axon growth was not determined. In contrast to the hiPSC-derived neurons, motor neurons showed increased abilities to extend processes. The data demonstrate the gradient hydrogel approach is a valid assay for studying growth and morphology of neurons.

The lack of axon growth in neural progenitor cell-derived neurons was not expected, as they can be reliably cultured to form neural networks in laminin-coated culture plates (Figure 1a) using commercial cell culture media. Neural progenitors cells (NPCs) are a common lineage of transiently-amplifying cells (TAC) important in the generation of differentiated neurons at specific time points (Kohwi and Doe,

2013). During neural development, TACs of different lineages form tissue boundaries and differentiate under the effect of diffused factors and cell adhesion signaling (Miyamoto *et al*., 2015).

Motor neuron processes preferentially align with the stiffness gradient

By analyzing the angle of motor neuron processes with respect to the stiffness gradient, the processes preferentially align with the stiffness gradient. This result suggests a mechanism that promotes the growth of axons towards either stiff or soft substrate, but no conclusion can be made about the actual preference, since there is no significant difference between the peaks in soft zone and stiff zone directionality trend in Figure 3. In theory, if increased stiffness can activate SAICs, there must be a lower threshold, and an upper limit of saturation for the detection of membrane stretch. Thus if the substrate stiffness is at threshold level, the orientation towards stiffness gradient should be diminished, and motor neurons should theoretically spread towards all directions equally. The same can be inferred for the lower limit of substrate stiffness. If there is no difference in sensitivity to stiffness gradient between the stiff and soft zones, it means either the stiffness of the substrate used is too similar, or there are multiple SAICs or other mechanosensory mechanisms working to cover a large range of stiffness levels. Indeed, there seems to be at least two distinct types of mechanosensors within cells: One is cell surface adhesion molecules and their intracellular signal transducers, such as talin, which are the linker molecule between actin filaments and the integrins in the membrane (Kerstein *et al*., 2015), which is suggested to be mechanosensitive, and upon experiencing stretch force, will strengthen the connections between actin and integrin (Margadant *et al*,, 2011). Another method of mechanosensation used by neurons is SAICs such as Vanilloid Receptor, or Transient Receptor Potential(TRP) family of mechanosensory ion channel. There are a large varieties of different TRP ion channels found to locate on growth cone of neurons. Studies using *Xenopus* spinal cord explant (Jacques-Fricke *et al*., Kerstein *et al*., 2013) demonstrated spinal axons grows slower due to SAIC activation. TRPC1, a SAIC that has been showned to decrease spinal axon growth by promoting the proteolytic degradation of talin (Kerstein *et al*.,2013). However,

considering the large amount of different mechanosensitive channels that exist, more in-depth analysis on their distribution and timing of expression is needed.

Future directions:

To follow through with studying mechanosensing axon guidance in motor neurons, the best approach would be live-imaging of GFP-labeled motor neurons. With this method, the rate of axon extension, as well as direction up or down the stiffness gradient, can be determined and compared to neurons grown under the effect of SAIC inhibitors, such as Probenecid. Furthermore, using a genetic editing approach to selectively knock-out different types of mechanosensor proteins, can give useful insights as to which one of the many different mechanosensors is contributing to the axon growth of motor neurons.

Since there exists such large variety of different types of mechanosensory ion channels, it may be interesting to study the timing of expression and requirements for induction using hiPSC-derived neuronal model systems. Currently, expression profile analysis showed TRPC1 started expressing in *Xenopus* during the end of the bastula stage, alongside TRPC3 and TRPV2, while TRPV4 expression started diminishing (Silina *et al*., 2015). Currently, two of these ion channels: TRPV2 and TRPC1, have been found to contribute to axon guidance. The further refining of timing and location of these ion channels may provide more insights and guidance towards studying the role of mechanical forces in development of the nervous system, and the implications for engineering tissue scaffolds.

Conclusion

In conclusion, Human iPSC-derived motor neurons have some mechanotactic properties when grown in fibrin stiffness-gradient hydrogel. The precise preference by motor neurons is unclear. This result is unexpected and warrants further study to validate.

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Appendix

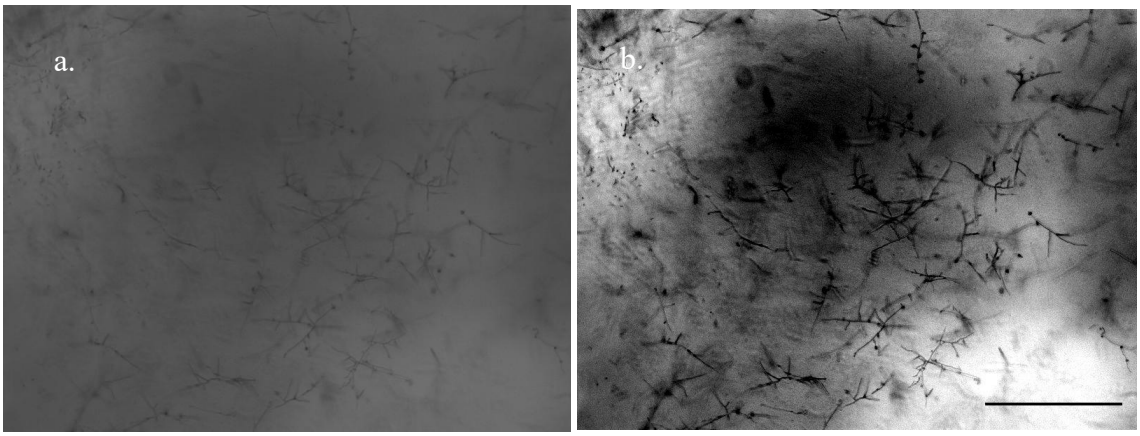

Figure S1. Raw microscope photographs (a) and ImageJ-processed photographs for neurons in gradient fibrin hydrogel.