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GRADIENTS OF STIFFNESS GUIDE NEURITE GROWTH IN 3D COLLAGEN GELS

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ABSTRACT

One approach to enhance nerve and spinal cord regeneration following injury is to implant a biomaterial scaffold to "bridge" the gap of the injury. Structural/mechanical anisotropy has been suggested as a means of orienting this growth axially. We have spatially varied the mechanical properties of a 3D collagen gel to direct growth axially and unidirectionally. Gradients of mechanical properties were generated in collagen gels by exposing the collagen to a 0-1mM gradient of genipin, a cell-tolerated crosslinking agent, for 12hrs via microfluidics. The gradient of stiffness was confirmed via a gradient of genipin-induced fluorescence intensity, which we have previously correlated to the storage modulus of collagen gels. The growth of neurites from isolated chick embryo dorsal root ganglia (DRG) in the presence of these gradients was evaluated after 5 days in culture. In control cases, neurites grew into the collagen gel and up either side of the cross-channel to approximately equal lengths. A 20% difference in differential growth was observed in control experiments. In contrast, when presented a gradient of shear modulus from ~365Pa – 60Pa, neurites elected to grow down the gradient of stiffness to the compliant side, with an almost 300% difference. Interestingly, the length of neurites in gels with gradients was significantly greater than the length of those grown in gels with uniform, untreated gels with high compliance. Control of neurite growth, cell migration, and other aspects of cell behavior in 3D scaffolds via mechanical properties offers vast potential for tissue engineering and other regenerative therapies.

INTRODUCTION

The mechanical stiffness of tissue substrates and/or surrounding network has now proven to be a crucial regulator of cellular functions. In several cell types, growth and movement of the cell can be dictated by the substrate/matrix stiffness. Lo et al first reported the preferential movement of fibroblasts with respect to mechanical stiffness and coined the term 'durotaxis' to describe the phenomenon [1]. Since then, quantitative differences in cell motility have been identified for neurites, astrocytes, smooth muscle cells, and epithelial cells, and many other phenotypic and functional phenomena have been observed for these and other cells that affect proliferation, differentiation, matrix synthesis and degradation, and traction-mediated events [2-5].

Directed cell migration is fundamental in many physiological and pathologic processes such as tissue morphogenesis, wound healing, and tumorigenesis, and is also desired frequently in several tissue engineering applications [6]. Of particular interest is the directed growth of neurites for regeneration of peripheral and central nervous system tissue [7, 8]. During development, axons are guided by attractive and repulsive, soluble, chemotactic cues and adhesion-based

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haptotactic cues, as well as contact guidance fields established by glia, aligned ECM proteins, and other axons, all of which are naturally presented in a three dimensional environment [9]. Approaches to regenerating peripheral nerves and spinal cord tissue have attempted to include these directional cues to orient neurite growth. While mechanical stiffness has shown to significantly affect neurite outgrowth on 2D substrates, and has been used to enhance growth isotropically by tuning matrix stiffness to entice neurite growth [10], durotactic gradients in a 3D, tissue-like system have not been employed.

Several approaches have been devised to probe the influence of substrate/network stiffness on cell behavior. Simple techniques involve functionalizing poly(acrylamide) gels of different concentrations and/or crosslinking density with proteins) that foster cell attachment[1], or coating these gels with a thin, albeit 3D layer of collagen [5]. Microfabrication techniques have been used to develop more elaborate systems comprising, for example, calibrated, elastomeric microposts of different dimensions, which maintain different bending properties to present a substrate of varying stiffness to the cells [11]. We have developed a system to generate stable, smooth gradients of mechanical properties through a 3D collagen gel. Microfluidic networks are pre-filled with a type I collagen solution, which is allowed to self-assemble. Gradients of solutions of genipin, a cell-tolerated, fast-acting crosslinking agent, are generated through the collagen gel for a defined period of time to establish a 1D gradient through the 3D gel. We have previously demonstrated that collagen crosslinked with genipin emits a red autofluorescence, the intensity of which fluorescence increases with increased crosslinking [12]. Greater crosslinking can be produced by higher concentrations of genipin and by increasing the duration of the exposure to genipin. The intensity is also well correlated to the mechanical properties of the crosslinked gels. As such, the gradients of crosslinks generated with genipin are directly visualized as a gradient of fluorescence, and interpreted as a gradient of stiffness. We demonstrate the functionality of these gradients in guiding outgrowth from chick dorsal root ganglia.

METHODS

Microfluidic Networks: A simple 'source-sink' arrangement was used to generate gradients in a cross channel connecting source-to-sink. Channel dimensions were selected by simulating flow in networks with a computational fluid dynamics package (ESI-CFD Huntsville, AL) to achieve uniform gradients across the height of the cross channel, which showed that the source and sink channels should be (at least) 2x wider than the cross-channel. Source and sink channels were 500μm wide x 100 μm deep, and were connected by a 5mm long, 250μm wide, and 100μm deep channel. Microfluidic networks were fabricated using standard photolithography techniques [13]. The design was drawn with AutoCAD and a photomask was professionally printed (Cad-Art services, Poway, CA). A silicon wafer was spin-coated with SU-8 negative photoresist (Microchem, Newton, MA) and baked for 5min at 65°C followed by 10min at 100°C. The photoresist was exposed to UV light through the photomask using a Quintel 2001 CT Mask Alignment/Exposure system. The coated wafer was baked again and immersed in SU-8 developer for 12 min to clear un-reacted photoresist and form the final 'master'. A poly(dimethyl siloxane) (PDMS) (Dow Corning, Midland, MI) solution was poured over the master and baked overnight at 50°C to produce a negative relief. Then the PDMS was removed, the design was cut out of the mold, and holes were punched for the inlet and outlet using a blunt 19-gauge syringe. The design and a clean glass slide were plasma treated and bonded together to form the final device. The inlets were connected to a syringe pump (Harvard Apparatus, Cambridge, MA) using polyethylene tubing (Small Parts, Miami Lakes, FL).

Collagen Preparation: Type I collagen solutions were prepared as previously described [14] by mixing 20μl 1M Hepes buffer, 140μl 0.1N NaOH, 100μl of 10X PBS, 52μl of PBS (Invitrogen, Carlsbad, CA), and 677 μl of type I 3.0mg/ml collagen (Elastin Products, Owensville, Missouri) to make a 2.0mg/ml collagen solution. The collagen solution self-assembles into a gel upon incubation at 37°C.

Generation of Mechanical Gradients:
To generate gradients of mechanical properties, the microfluidic networks were first filled with type I collagen solution using a syringe operating at 0.1ml/min (Harvard Apparatus, Cambridge, MA) while viewing with an upright tissue culture microscope to ensure the network was properly filled with no bubbles. After inspection, the filled microfluidics network was transferred to a humidified 37ºC. incubator to facilitate self-assembly. The source and sink solutions were then changed to culture medium (DMEM + 10% FBS, 1% glutamine, 1% penicillin/ streptomycin) with and without genipin (Challenge Bioproducts Co., Taichung, Taiwan). These solutions were flowed gently through the fibrillar gel-filled network at 0.0003 ml/min for 12 hours at 37ºC. After 12 hours, inlets were switched to medium to rinse out remaining genipin in the network. Genipin gradients were made with 1, 5, and 10mM genipin solutions in medium, each crosslinked for 12 hours.

Gradients of genipin-mediated crosslinking were verified directly by quantifying the fluorescence intensity emitted by the crosslinked collagen (590nm Exc, 630nm Em), which indirectly verifies the gradient of mechanical properties. A previous study confirmed that fluorescence intensity was strongly correlated to the shear modulus. Following rinsing, networks were transferred to a computer controlled stage and imaged using an Olympus IX81 inverted microscope (Olympus, Melville, NY). The fluorescence intensity in the cross-channel was quantified using Olympus Microsuite Image Analysis Software (Olympus, Melville, NY).

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Neurite Outgrowth Assay: Microfluidic networks were modified to include a small well for chick dorsal root ganglion (DRG) culture (Fig. 1). A second network was generated that comprised a 1mm diameter well connected to a straight 250μm wide and 100μm deep channel. This network was place upside down (channels facing up) on a glass slide and filled with collagen solution. DRGs were isolated from E8 chicks, and a single DRG placed in the collagen-filled circular well. The network was transferred to a 37°C incubator to facilitate self-assembly, entrapping the DRG in the collagen gel. The 'source-sink' network was then plasma treated and bonded to the gel filled cell network such that the middle of the cross channel of the 'source-sink' intersected with the straight channel of underlying network approximately 300μm from the circular well. The top network was then filled with collagen solution, which was allowed to gel, and treated with genipin as above. After treatment with genipin, collagen gel-filled networks were rinsed generously with fresh medium. Gradients were again visualized through fluorescence microscopy, as above, and no differences in gradients were observed between these networks and the source-sink models bonded directly to glass slides.

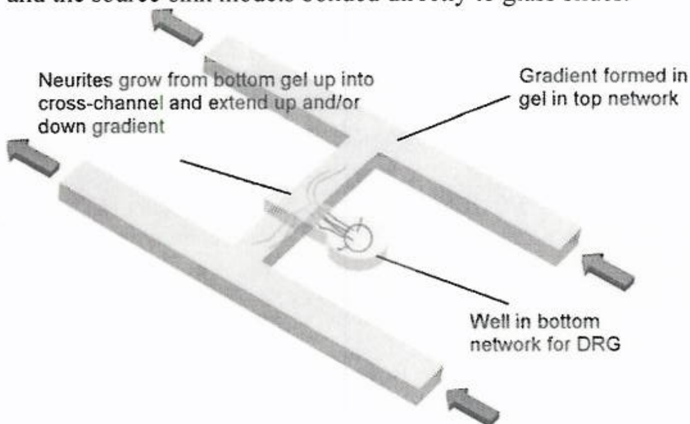

Fig. 1: Schematic of the PDMS network. Cells are allowed to grow through the cell channel and into the cross-channel. Projecting neurites then choose to grow up or down the stiffness gradient, or left or right for control conditions.

Networks were then transferred to an incubator and perfused with fresh medium (DMEM supplemented with 10% FBS and 100 ng/ml NGF (R&D Systems, Minneapoilis, MN)). via gravity flow. DRGs were cultured in the networks for five days. During this time, many neurites from neurons in the DRG grew up and into the cross-channel a significant distance, potentially in either or both directions. After 5 days, inlet solutions were changed to 4% paraformaldehyde to fix the collagen and cells, then to a rinse buffer comprising of 1% BSA + 0.5 % Triton in PBS. Inlet solutions were then changed to FITC-Phalloidin solution (1μg/ml) in rinse buffer, delivered at 0.0003ml/min, to label the F-actin cytoskeleton. Finally, devices were flushed with rinse buffer for 2 hours and then transferred to a fluorescence microscope for imaging. Neurite growth was evaluated in a gradient of mechanical properties generated by gradients from 1mM – 0mM genipin and untreated (0mM – 0mM) (n=4 each). One control experiment was conducted in collagen uniformly crosslinked (0.5mM – 0.5mM). Neurite growth was quantified as the length of neurites projecting up the stiffness gradient vs. down the stiffness gradient, or left vs. right for control conditions.

RESULTS

Gradients of stiffness: Stable 1D gradients of genipin-induced crosslinks were established in 3D collagen gels using microfluidics. The gradient was visualized via fluorescence changes induced by the genipin crosslinking (Fig. 2). Intensity profiles across the length of the cross-channel demonstrated steady changes in intensity (Fig. 3). We have previously characterized the rheological properties of collagen gels crosslinked with this regimen of crosslinking[12], and the fluorescence indicates a change in the storage modulus measured in shear at 1% strain and 0.1Hz from 265Pa – 60Pa for a gradient from 1mM-0mM, 570Pa – 60 Pa for 5mM – 0mM, and 800Pa – 60Pa for 10mM-0mM.

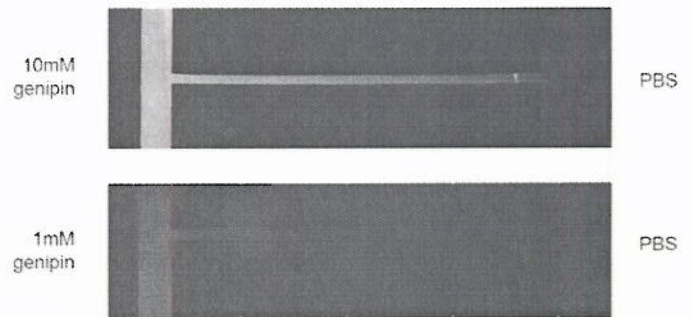

Fig. 2: Fluorescence produced by treatment of collagen with a gradient of genipin from 10mM-0mM (top) or 1mM-0mM (bottom) genipin for 12hrs.

Mosaic images of the cross-channels were built with computer controlled microcopy. In all cases, cellular processes were observed to grow in either direction upon entering the cross channel. In the control cases, growth was roughly evenly distributed in either direction. However, in the presence of a gradient from 265Pa – 60Pa (produced by exposing the collagen gel to a 1mM-0mM gradient of genipin for 12hrs, and then flushing the remaining genipin), the cellular response was strongly biased in the direction of increased compliance (Fig. 4). This response was quantified by determining the average distance of neurites to the left and to the right from the respective edge of the cross-channel. For the gradient condition, growth was always biased in the direction of greater compliance, and on average neurites grew 4X longer towards the compliant gel. For the control condition, growth was much more uniform, and on average (always expressing the direction of biased growth in the numerator, regardless of whether it was 'left' or 'right'), the difference in length was ~20% (Fig. 5). A similar result was observed for uniform presentation of 0.5mM

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genipin. Although the 20% difference in the control case significant (ANOVA, $P = 0.037$), the level of significance was dramatically greater for the experimental condition ($P = 1e\text{-}11$).

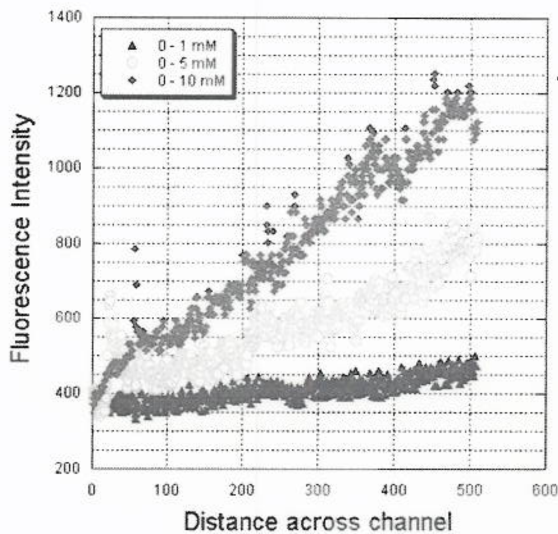

Fig. 3: The fluorescence intensity in the cross-channel demonstrates a steady increase towards the 'source' inlet. With identical exposure settings, the slope of the intensity curve is greater when a steeper gradient of genipin is established. Increased steepness can also be achieved by increasing the duration of incubation for a given concentration of genipin.

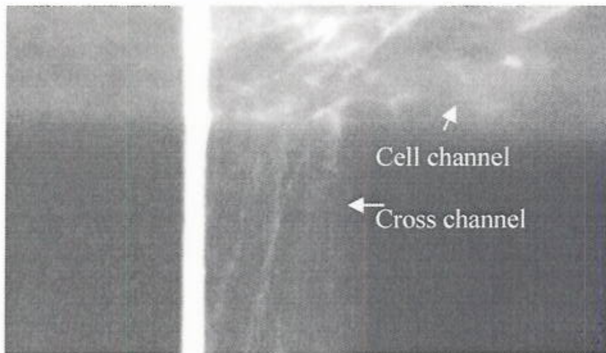

Fig. 4: Representative image of neurite growing through the cell channel into the cross channel. Cells are stained with phalloidin for visualization.

In addition to biasing growth towards greater compliance, the gradient of stiffness also accelerated growth in that direction. The average distance of neurites from the bottom channel was calculated for controls, for neurites growing up the gradient of stiffness, and for neurites growing down the gradient of stiffness. The growth up the gradient of stiffness was significantly less than controls, whereas the growth down the gradient of stiffness was significantly greater than controls (ANOVA followed by pairwise comparisons with Scheffe's post hoc test, $P < 0.05$), even though the stiffness at any particular location in the gradient experiments is greater than the stiffness in control experiments (except as the sink inlet is approached) (Fig. 6).

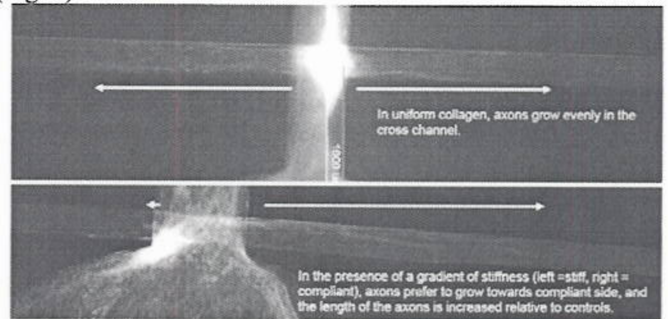

Fig. 5: Response of DRGs to a gradient of stiffness. (Top) In the controls, where cells experience a gel with uniform mechanical properties (G~60Pa), growth is nominally uniform in either direction. (Bottom) When presented a gradient of stiffness from ~265Pa – 60Pa, growth is strongly biased in the direction of greater compliance.

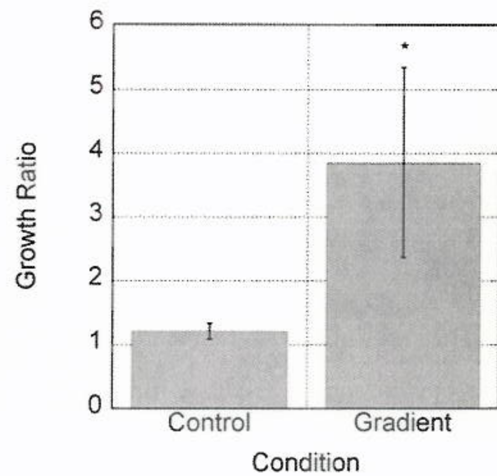

Fig. 6: Average ratio of differential growth in the cross-channel (direction with greater growth as numerator). Growth in the presence of a gradient was significantly biased towards greater compliance than the random differences measured in controls (ANOVA, $P < 0.05$).

DISCUSSION

Herein we have presented techniques for generating smooth gradients of stiffness in a 3D collagen gel for investigating the influence of mechanical properties on growth and migration. Lo first coined the term durotaxis to describe the preferred migration of fibroblasts on stiff 2D substrates vs. compliant ones. Many cell types have since been shown to demonstrate significant phenotypic changes in response to substrate mechanical properties. Neural cells have been of particular interest, and several studies have demonstrated that neurite extension and branching is enhanced on compliant 2D

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substrates [2, 15]. Efforts have been made to induce these changes for improved tissue engineering therapies. For instance, Balgude et al observed that compliant agarose gels encouraged neurite outgrowth vs. stiff ones [10]. The response of these cells to uniform grades of stiffness suggests that the direction of extension may be controlled by directional mechanical properties, which is a highly desired feature for engineering peripheral nerve grafts and therapies for spinal cord repair.

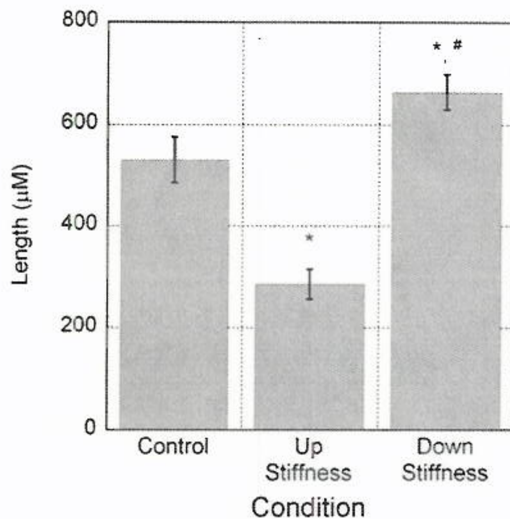

Fig. 6: Growth in the presence of a stiffness gradient is biased and accelerated in the direction of greater compliance. The length of neurites extending down the gradient of stiffness was significantly greater than the length in the presence of uniform stiffness (equal to the stiffness at the sink condition in gradient experiments - 0mM genipin, *G*~60Pa).

We have demonstrated that the neurites can respond to continuous changes in stiffness within a 3D gel. We created gradients of stiffness by exposing collagen gels to a gradient of genipin, which crosslinks collagen, for a defined period of time using microfluidics. When neurites from a DRG were placed at the center of the gradient grew into the cross-linked gel, they preferentially grew in the direction of greater compliance, which is consistent with the previous observations described above. It is perhaps even more noteworthy that the average extension of neurites down the stiffness gradient was greater than the average extension in uniform, control conditions. While it is possible that an intermediate, optimal stiffness exists between the center of the cross channel (~210Pa) and the stiffness at the sink condition (~60Pa) [15], we believe that the presence of the gradient drives neurite extension. We are currently conducting expanded experiments with a broader range of gradients, both in terms of the average value and the steepness, to evaluate this hypothesis.

We have used genipin to generate a gradient of crosslinks n collagen. Genipin has distinct advantages over many crosslinking agents. It is significantly less toxic than traditional aldehydes, and is much more cost-effective than enzymatic compounds such as lysyl oxidase. It also crosslinks on a much shorter time scale than glycation[3]. For our application, genipin is particularly beneficial because it generates fluorescence changes that are well correlated to stiffness, and, therefore, offers a non-contact means of evaluating mechanical properties. However, the 3D durotactic assay we have developed is somewhat limited because of the cytotoxic effects of genipin [12]. To avoid these effects, concentrations are maintained at 1mM or lower, which limits the magnitude of crosslinking that can be achieved. Fortunately, we have found that prolonging exposure to genipin increases crosslinking without a concomitant increase in cytotoxicity [12], so that steeper gradients may be achieved by extending the crosslinking time. Additionally, we indirectly measure stiffness based on prior characterization of gel rheology and the observed fluorescence intensity. We are currently assessing the properties within the networks directly by including magnetic microparticles in the gel, exposing the system to a controlled magnetic field, and measuring the resultant movement of the microparticles[16].

We have used a simple microfluidic network to generate the gradient of genipin solution with the collagen gel. Collagen gels are fibrillar hydrogels with a large volume fraction of solution phase. We used syringe pumps to gently force the genipin solution (and control media) through the porous collagen gel and create very stable gradients. However, the gel-filled networks present a number of experimental difficulties, particularly the destructive potential of bubbles. These difficulties have been especially prevalent in visualizing and analyzing the growth within the network after the incubation period. We have, thus far, visualized cells using fluorescently-tagged phalloidin, which binds to the F-actin cytoskeleton. Labeling with phalloidin is a simple, one-step process following fixation, and therefore limits the number of times the fluid inlets are changed, thereby decreasing the risk of bubbles. However, Since all cells maintain a degree of F-actin, we have relied on the morphology of cellular process to indicate neurites, and we are unable to confirm based on phenotypic expression of markers, such as neurofilament proteins. It is highly likely that our labeling includes Schwann cells, which are in high numbers in DRGs. Since neurite growth is guided by Schwann cells (and vice versa) [17-19], the gradients of stiffness may be affecting Schwann cell migration, which in turn affects neurite extension. We are currently developing immunohistochemical techniques to label cells in networks, and we are performing experiments with isolated Schwann cells and peripheral neurons to confirm our findings.

Controlling neurite behavior with patterned gel stiffness offers intriguing possibilities for engineering therapies for spinal cord repair. A major impediment to spinal cord regeneration is the glial scar, which is a physical and chemical barrier that prevents axons from entering the injury site. Reactive astrocytes are the primary cell responsible for forming

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the glial scar. Since astrocytes prefer stiff substrates, and neurons prefer compliant ones, it may be possible to pattern mechanical properties in a scaffold to direct astrocytes and regenerating neurites away from each other to enhance the regenerative potential of biomaterial therapies. We have used microfluidics to generate simple 1D gradients in a 3D gel, but other patterns are also possible, including stripes, which have thus far only been employed in 2D systems [20].

We have focused our studies on the response of neurons to gradients of stiffness, but the devices should be easily amenable to studying similar phenomena in other cell types. The role of mechanotransduction, and particularly, the sensing of the stiffness of the extracellular environment has been increasingly recognized as a critical contributor on cellular[5] and even molecular[21] behavior. The techniques presented herein have the potential to investigate behaviors in a 3D tissue equivalent environment with control over not just gel stiffness, but also the simultaneous presentation of soluble and insoluble cues [22].

CONCLUSION

We have used microfluidics to generate stable 1D gradients of mechanical properties through a 3D collagen gel. The stiffness gradient, which was verified optically via crosslinking-induced changes in fluorescence, directed the growth of neurites through the gel in the direction of increased compliance, which is consistent with previous reports of neurite behavior on 2D substrates and in agarose gels of uniform stiffness [2, 10]. Importantly, the length of the neurites was also greater in the presence of a stiffness gradient, suggesting that the gradient drives neurite extension. These techniques and findings offer intriguing potential for engineered therapies for nerve and spinal cord repair as well as examination of durotactic behavior in other cell types.

ACKNOWLEDGMENTS

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REFERENCES

1. Lo, C.M., H.B. Wang, M. Dembo, and Y.L. Wang, *Cell movement is guided by the rigidity of the substrate.* Biophys J, 2000. **79**(1): p. 144-52.
2. Flanagan, L.A., Y.E. Ju, B. Marg, M. Osterfield, and P.A. Janmey, *Neurite branching on deformable substrates.* Neuroreport, 2002. **13**(18): p. 2411-5.
3. Girton, T.S., T.R. Oegema, E.D. Grassl, B.C. Isenberg, and R.T. Tranquillo, *Mechanisms of stiffening and strengthening in media-equivalents fabricated using glycation.* J Biomech Eng, 2000. **122**(3): p. 216-23.
4. Wissink, M.J., M.J. van Luyn, R. Beernink, F. Dijk, A.A. Poot, G.H. Engbers, T. Beugeling, W.G. van Aken, and J. Feijen, *Endothelial cell seeding on crosslinked collagen: effects of crosslinking on endothelial cell proliferation and functional parameters.* Thromb Haemost, 2000. **84**(2): p. 325-31.
5. Discher, D.E., P. Janmey, and Y.L. Wang, *Tissue cells feel and respond to the stiffness of their substrate.* Science, 2005. **310**(5751): p. 1139-43.
6. Shreiber, D.I., P.A. Enever, and R.T. Tranquillo, *Effects of PDGF-BB on rat dermal fibroblast behavior in mechanically stressed and unstressed collagen and fibrin gels.* Exp Cell Res, 2001. **266**(1): p. 155-66.
7. Fry, E.J., *Central nervous system regeneration: mission impossible?* Clin Exp Pharmacol Physiol, 2001. **28**(4): p. 253-8.
8. Schmidt, C.E. and J.B. Leach, *Neural tissue engineering: strategies for repair and regeneration.* Annu Rev Biomed Eng, 2003. **5**: p. 293-347.
9. Gallo, G. and P.C. Letourneau, *Axon guidance: A balance of signals sets axons on the right track.* Curr Biol, 1999. **9**(13): p. R490-2.
10. Balgude, A.P., X. Yu, A. Szymanski, and R.V. Bellamkonda, *Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures.* Biomaterials, 2001. **22**(10): p. 1077-84.
11. Tan, J.L., J. Tien, D.M. Pirone, D.S. Gray, K. Bhadriraju, and C.S. Chen, *Cells lying on a bed of microneedles: an approach to isolate mechanical force.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1484-9.
12. Sundararaghavan, H.G., G.A. Monteiro, N.A. Lapin, Y. Chabal, and D.I. Shreiber, *Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence.* J. Biomed Mater Res A, Submitted.
13. Whitesides, G.M., E. Ostuni, S. Takayama, X. Jiang, and D.E. Ingber, *Soft lithography in biology and biochemistry.* Annu Rev Biomed Eng, 2001. **3**: p. 335-73.
14. Enever, P.A., D.I. Shreiber, and R.T. Tranquillo, *A novel implantable collagen gel assay for fibroblast traction and proliferation during wound healing.* J Surg Res, 2002. **105**(2): p. 160-72.
15. Leach, J.B., X.Q. Brown, J.G. Jacot, P.A. Dimilla, and J.Y. Wong, *Neurite outgrowth and branching of PC12 cells on very soft substrates sharply decreases below a threshold of substrate rigidity.* J Neural Eng, 2007. **4**(2): p. 26-34.
16. Lin, D.C., B. Yurke, and N.A. Langrana, *Use of rigid spherical inclusions in Young's moduli determination: application to DNA-crosslinked gels.* J Biomech Eng, 2005. **127**(4): p. 571-9.
17. Thompson, D.M. and H.M. Buettner, *Schwann cell response to micropatterned laminin surfaces.* Tissue Eng, 2001. **7**(3): p. 247-65.

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18. Tuszynski, M.H., N. Weidner, M. McCormack, I. Miller, H. Powell, and J. Conner, *Grafts of genetically modified Schwann cells to the spinal cord: survival, axon growth, and myelination.* Cell Transplant, 1998. **7**(2): p. 187-96.
19. Rosner, B.I., R.A. Siegel, A. Grosberg, and R.T. Tranquillo, *Rational design of contact guiding, neurotrophic matrices for peripheral nerve regeneration.* Ann Biomed Eng, 2003. **31**(11): p. 1383-401.
20. Ryan, A.F., J. Wittig, A. Evans, S. Dazert, and L. Mullen, *Environmental micro-patterning for the study of spiral ganglion neurite guidance.* Audiol Neurootol, 2006. **11**(2): p. 134-43.
21. Vogel, V., W.E. Thomas, D.W. Craig, A. Krammer, and G. Baneyx, *Structural insights into the mechanical regulation of molecular recognition sites.* Trends Biotechnol, 2001. **19**(10): p. 416-23.
22. Shreiber, D., H.G. Sundararaghavan, M. Song, and K.E. Uhrich. *Modifying the properties of collagen scaffolds with microfluidics*. in *Fall Symposium of the Materials Research Society (MRS)*. 2005. Boston, MA.

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