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Final Narrative Report

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Institution: Rutgers, the State University of New Jersey
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1. Specific Aims:

The original aims of the proposal were:

Specific Aim 1: Elucidate the relationship between neurite growth and the mechanical properties of a biomaterial scaffold.

Specific Aim 2: Evaluate the relationship between neurite growth and the gradient of cell adhesion sites on a substrate.

Specific Aim 3: Evaluate the effects of substrates with both mechanical and adhesion gradients on neurite growth and direction

These aims were to be accomplished by developing new microfluidics devices that would allow the control of the stiffness and adhesivity of a three-dimensional (3D) collagen gel system. Working in a 3D environment greatly improves the physiological relevance of the research, since any axon regeneration in vivo will naturally be in a 3D environment, but also introduces many complications.

2. Project successes

We successfully completed Aim 1 and are near completion of Aim 2. A number of complications arose (see below) which delayed our progress, which is not surprising since the project involved technology development and subsequent research, rather than use of a pre-existing system.

For Aim 1, we have demonstrated that neurites prefer to grow down a gradient of stiffness (to the 'softer' side in a 3D gel. This was accomplished by filling a source-sink microfluidics network with soluble collagen, allowing that collagen to gel, and then establishing a gradient of a cell-tolerated crosslinking agent – genipin – through the collagen gel. This generated a gradient of crosslinks, which we correlated to a gradient of mechanical properties, based on a rheological characterization we have published. Genipin has the unique characteristic of causing collagen to fluoresce upon crosslinking, and we were able to directly visualize the gradient of stiffness (Figure 1).

When neurites growing from a chick dorsal root ganglion experienced the gradient, the neurites preferentially grew down the gradient of stiffness. Neurites presented the control condition grew evenly (within error) towards either side. (Figure2).

For Aim 2, in the past 6 months we have modified and optimized a protocol that allows us to covalently graft extra peptides to collagen in solution. For example, we graft peptides from laminin (IKVAV or YIGSR) that have been shown to entice neurite growth, and then we create a gradient of these immobilized peptides in a collagen gel using the same source-sink arrangement. A collagen solution with peptides flows through the source and an untreated solution flows through the sink. The two solutions mix in the cross-channel to create a gradient of peptide-grafted collagen to untreated collagen, in solution. This gradient becomes 'permanent' when the microfluidics network is incubated at 37degC. We visualize the gradient by including a fluorescent tracer on one of the peptides/collagen solutions (Figure 3). Our recent results demonstrate that axons emanating from a chick embryo dorsal root ganglion will migrate up a gradient of either YIGSR or IKVAV within a 3D collagen gel (Figure 4).

Parallel control studies have revealed a particularly intriguing aspect of working with peptide-grafted gels. Typically, when working with small, bioactive peptides, a scrambled version of the peptide is applied as a control. For instance, studies of the ubiquitous cell binding domain RGD (often in the form GRGDS) use GRDGS as a control. When GRGDS is coated on an otherwise bio-inert substrate, many tissue cells will readily attach and extend processes, but cells will not attach to GRDGS-coated substrates. However, in our work, we are grafting these peptides to a 3D scaffold with a basal level of adhesion. As such, grafting the scrambled peptides actually *decreases* the adhesion of cells and, as a result, can increase cell migration. We

have demonstrated this with smooth muscle cells and will now work to control glial cell migration with these 'reverse' gradients.

We have also devised a protocol to enhance the efficiency of our grafting procedure. We use a 'hetero-bifunctional coupling agent', EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), which basically allows a carboxyl terminal to be bound to an amine. Since proteins have at least one of each, we can bind peptides to collagen. One problem, however, is that since the peptides have both carboxy and amino ends, we tended to get peptide-peptide grafting. We have overcome this by acetylating the peptide prior to the first 'activation' step. Acetylation converts a carboxy to an amine, which allows multiple sites to be activated on the peptide to increase grafting to the free amines in collagen.

3. Project challenges

This project represented a new research endeavor for the PI – BioMEMS (Biological microelectromechanical systems), and as such, a primary challenge was developing the intellectual and physical infrastructure.

A significant problem was bubble formation. Traditional microfluidics is performed with liquid or gas filled microchannels that hold picoliter volumes. In our application, we fill the channels with a fibrillar gel – collagen – that is porous, which allows flow to permeate through the gel. In traditional microfluidics, the introduction of a bubble into a microchannel can be problematic, in that it disrupts flow patterns that may have been specifically engineered, but the patterns generally return after the bubble has traversed out of the network. However, in our application, the pore size of the collagen gel is much smaller than any bubble. The introduction of any bubbles subsequently destroys the gel, deforming it beyond use. Our protocols called for several switches of inlet solutions, and each step increased the risk that air would be introduced to the system. A significant amount of time was spent developing a troubleshooting a protocol that has virtually eliminated bubbles.

A second major challenge involved overcoming diffusive limitations within the networks. Although the channels were only several millimeters in length, this was long enough to limit the availability of oxygen and nutrients from the inlet solutions, and cell viability suffered. Dissolving more oxygen into the medium was not an option because it would have dramatically increased the likelihood of introducing bubbles. We ultimately designed a multi-layered microfluidics assay that provided additional inlet and outlet ports for medium and oxygen.

A final major challenge was visualizing the cells and axons within the network. Immunohistochemically labeling the cells within the collagen gel inside the channels was not trivial. It again required several solution changes, each of which could again introduce bubbles or debris that could destroy an experiment. Additionally, while optically transparent, the PDMS polymers are not ideal for imaging. We are working on additional modifications to unseal the microchannels after gel formation to have them resemble river beds rather than enclosed channels, which would allow use to add solutions to the top of the gel for both nutrient supply and immunohistochemical labeling.

4. Implications for future research and/or clinical treatment.

During growth and development, axons are guided to their targets by gradients of molecules and potentially mechanical properties. By identifying gradients that entice and direct neurite growth through biomaterials, and incorporating these gradients on to a biocompatible material (collagen), we have provided a critical first step in improving tissue engineered grafts for spinal cord regeneration. The microfluidics platform is a valuable platform to test other molecules or gradients to further improve the grafts – we are not limited to one or two adhesive peptide sequences, but can mix and match at will. A major next step is scaling the technology to develop an implantable construct – in other words, designing a means to either extract the gradient gels from the microchannels and incorporate them into an implantable tube, or forming the gradients within a gel filled tube a priori.

We firmly believe that at a cure for spinal cord injury will require the careful combination and integration of multiple disciplines – neurobiology, engineering, materials science, etc. Many other modalitites that hold promise towards spinal cord regeneration can be incorporated, such as including stem cells directly in our graft.

5. We are actively continuing this research on several fronts. We have received an award from the Paralyzed Veterans of America Research Foundation to control astrocyte migration in hopes of alleviating the glial scar. We collaborate with Dr. Melitta Schachner (an NJ Professor of Spinal Cord Research), where we graft peptide-mimics of bioactive carbohydrates to further entice axon regeneration, and have received a good score on an R21 application to the NIH (revised proposal will be submitted in November). We are developing 3D microenvironments for stem cell differentiation into neural lineages, and have received small awards from the Charles and Johanna Busch Foundation and Johnson & Johnson for these endeavors. We are particularly interested in assaying the response of axons to multi-modal cues – combinations of soluble, adhesive, and mechanical gradients to recapitulate the in vivo environment in a controlled setting.

6. Publications

Peer-reviewed Journal Articles:

Sundararaghavan, H., Monteiro, G.A., Lapin, N.A., Chabal, Y., Miksan, J.R., and *Shreiber, D.I.* Characterization of genipin-mediated crosslinking of type I collagen gels: correlation of fluorescence to mechanical properties. J. Biomed. Mat. Res. A.

In preparation:
Sundararaghavan, H., Monteiro, G.A., Firestein, B., and *Shreiber, D.I.* Neurite response to gradients of mechanical properties in 3D collagen gels. Biotechnology & Bioengineering. Expected submission, Nov, 2007.

Sundararaghavan, H., Monteiro, G.A., and *Shreiber, D.I.* Adhesive gradients within 3D collagen gels guide neurite growth. Langmuir Expected submission, Nov, 2007.

Monteiro, G.A., Fernandes, A.V., Song, M., Uhrich, K.E., and Shreiber, D.I. Modulating the adhesive properties of collagen gels. Biomaterials, Expected submission Dec 2007.

Peer-reviewed conference proceedings:

Sundararaghavan, H.G. and *Shreiber, D.I.* Gradients of stiffness guide neurite growth in 3D collagen gels. 2007 ASME IMECE Conference, Seattle, WA.

Sundararaghavan, H.G. and *Shreiber, D.I.* Gradients of mechanical properties guide neurite growth in 3D collagen gels. 2007 ASME Summer Bioengineering Conference, Keystone, CO. (Received honorable mention in PhD Platform Presentation Competition)

Munikoti, V., Song, M, Monteiro, G.A., Uhrich, K.E., and *Shreiber D.I.* Modulating The Adhesive Properties Of Collagen Tissue Equivalents 2006 ASME Summer Bioengineering Conference, Amelia Island, FL.

*Shreiber, D.I.,*Sundararaghavan, H.G, Song, M, Munikoti, V., and Uhrich, K.E. Modifying the properties of collagen scaffolds with microfluidics. Winter meeting of the Materials Research Society, December, 2005, Boston MA .

Sundararaghavan, H.G., Miksan, J.R., and *Shreiber, D.I.,*. Characteriztion of genipin-crosslinked collagen gels. 2005 ASME Summer Bioengineering Conference, Vail, CO, June, 2005.

Abstracts:

Sundararaghavan, H.G., *Shreiber, D.I.* DRG growth in 3D mechanical gradients established using microfluidics. 2006 Biomedical Engineering Society Meeting, Chicago, IL.

Sundararaghavan, H.G., Song, M.J., Miksan, J.R., Uhrich, K.E, and Shreiber, D.I. Manipulation of biomaterial scaffold properties with microfluidics. 2005 BMES, Baltimore, MD, September, 2005.

Song, M., Sundararaghavan, H., *Shreiber, D.I.*, and Uhrich, K.E. Modifying cell adhesion to collagen gels for enhanced nerve regeneration. 1st Annual New Jersey Stem Cell Institute Symposium, November, 2004, New Brunswick, NJ.

Sundararaghavan, H., Song, M., Uhrich, K.E., and *Shreiber, D.I.* Using microfluidics to modify mechanical properties and adhesion sites in a 3D collagen gel. 1st Annual New Jersey Stem Cell Institute Symposium, November, 2004, New Brunswick, NJ.

Sundararaghavan, H., Song, M., Uhrich, K.E., and *Shreiber, D.I.* Using microfluidics to modify mechanical properties and adhesion sites in a 3D collagen gel. 2004 BMES Annual Meeting, October, 2004, Philadelphia, PA.

Song, M., Sundararaghavan, H., *Shreiber, D.I.*, and Uhrich, K.E. Modifying cell adhesion to collagen gels for enhanced nerve regeneration. 2004 BMES Annual Meeting, October, 2004, Philadelphia, PA.

Figures:

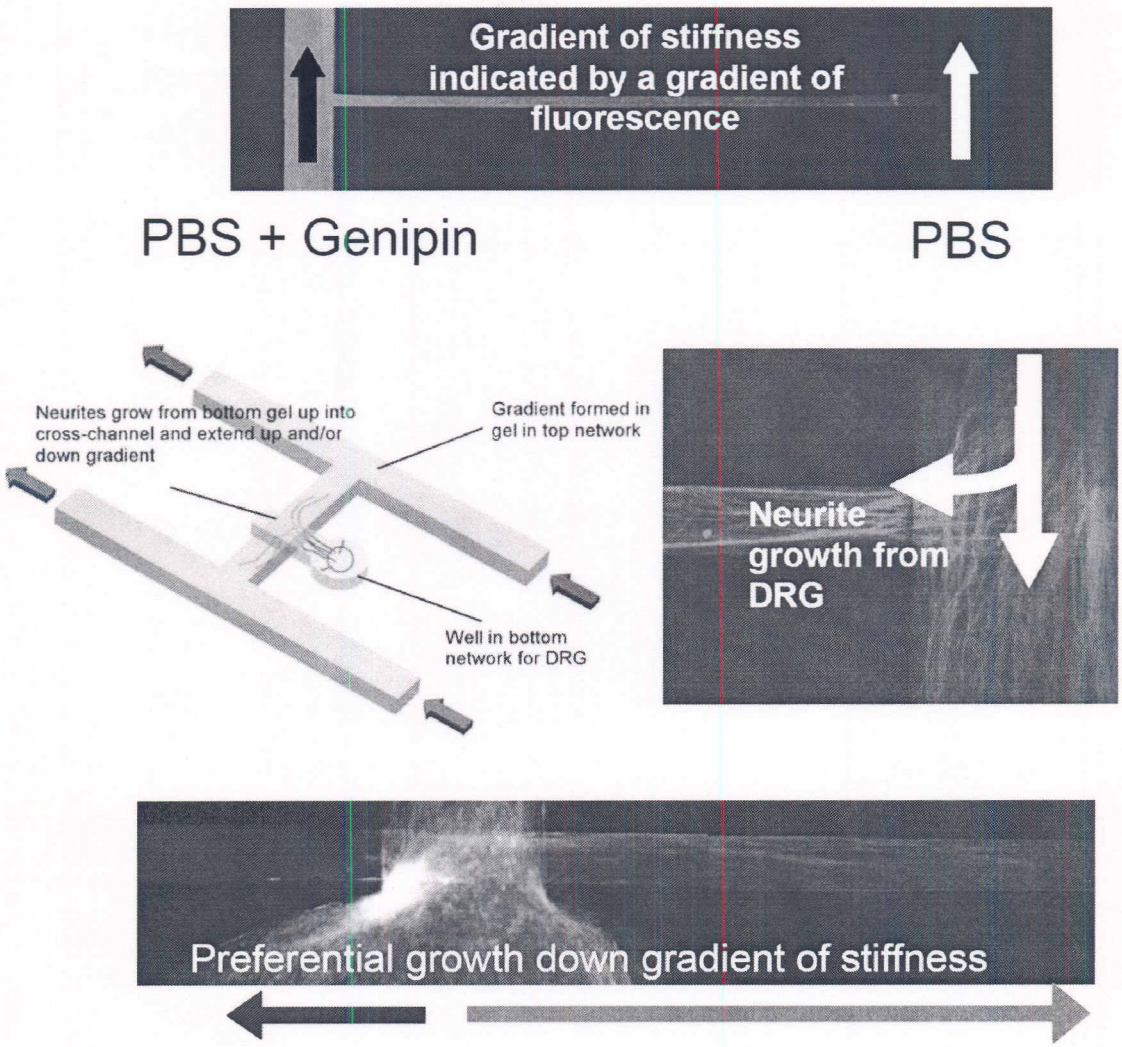


Figure 1: Neurite guidance by gradients of mechanical properties. A gradient of stiffness in a 3D collagen gel is established by exposing the collagen to a gradient of genipin, a cell-tolerated crosslinking agent that is a natural extract of the fruit of the *Gardenia Jasminoides*. Genipin has the unique advantage of causing the normally opaque collagen to turn blue and fluoresce upon crosslinking. Thus, we can visualize the gradient of crosslinks via fluorescence, and this indicates a gradient of stiffness (see attached paper, Characterization of genipin-mediated crosslinking of type I collagen gels: correlation of fluorescence to mechanical properties). We then culture a chick embryo dorsal root ganglion in a separate channel below the gradient. Neurites grow from the DRG into the gradient channel, and then choose to grow either up or down the gradient. When a gradient of stiffness is present, neurites preferentially grow down the gradient to the 'softer side'.

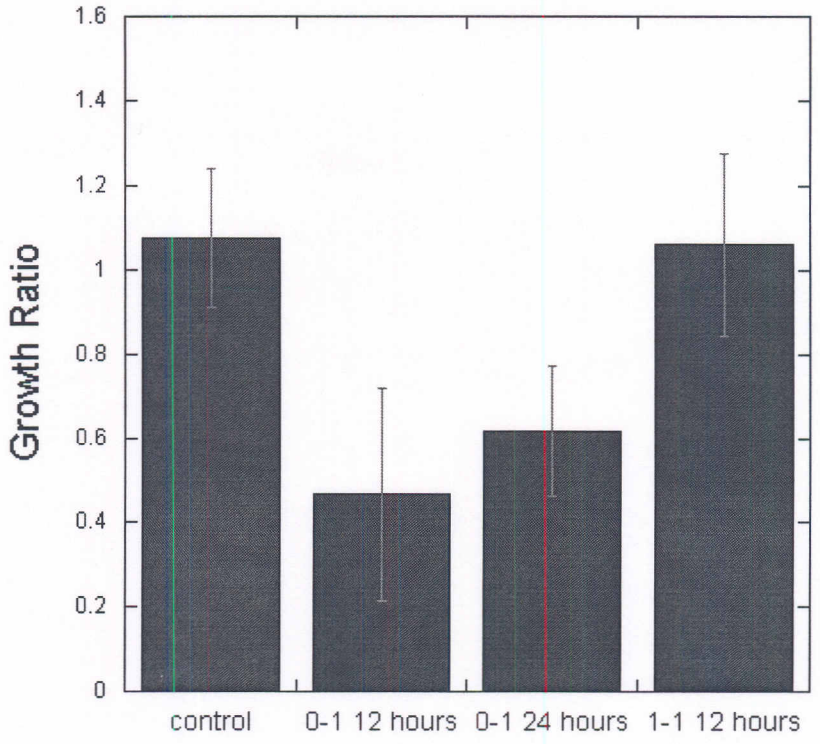


Figure 2: Neurites were exposed to either a uniform, untreated collagen gel (control), stiffness gradients formed by exposing collagen to a gradient of 0-1mM genipin for 12 hours or 24 hours, or a uniform collagen gel crosslinked with 1mM genipin. The ratio of the growth up the gradient to down the gradient was calculated based on the length of individual neurites. The ratio was close to one in the control and uniformly treated gels, indicating even growth in either direction. In gradient gels, the ratio was less than one, indicating biased growth.

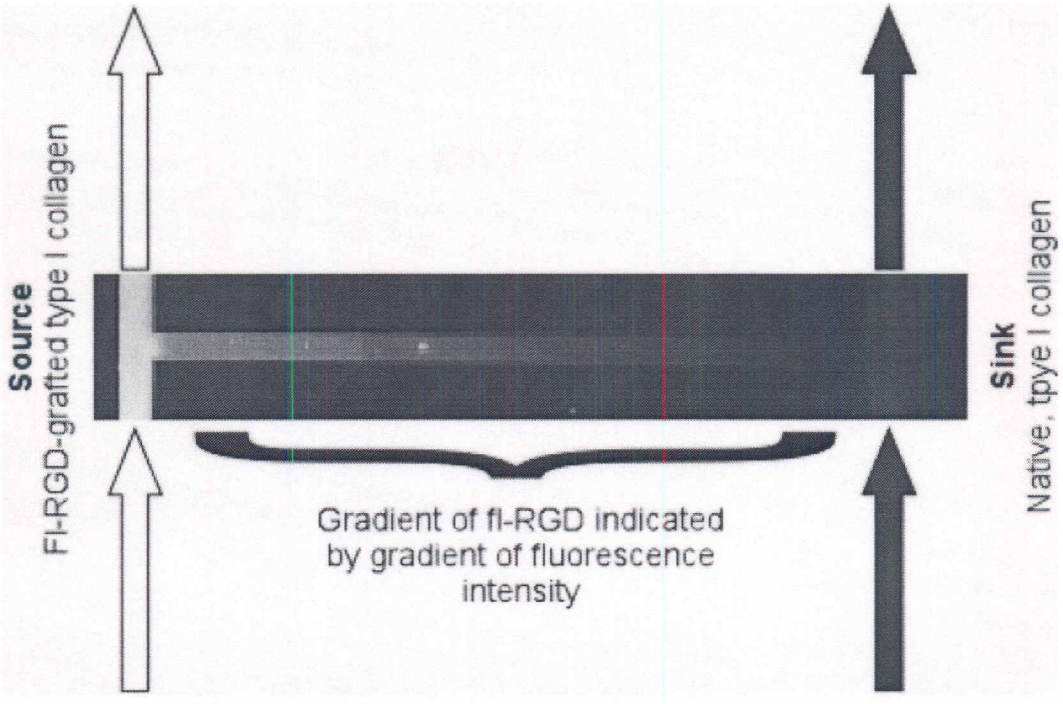


Figure 3: Gradient of RGD-grafted collagen. Fluorescently labeled RGD is grafted to collagen and mixed in a microfluidics network with untreated collagen. When the temperature of the mixture is raised, the gradient becomes 'locked in'.

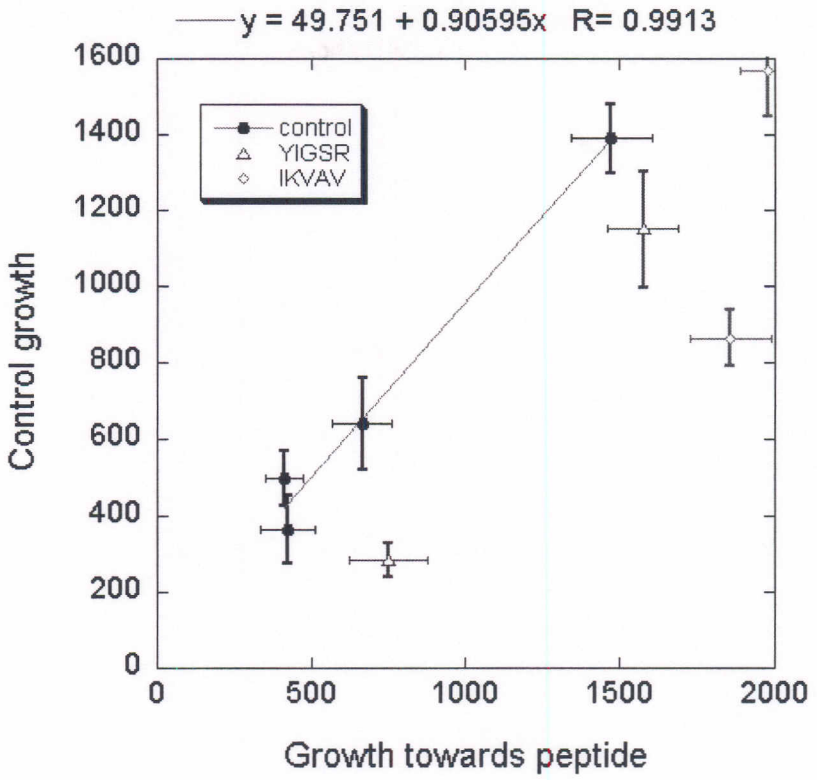


Figure 4: Gradients of laminin-derived peptides direct neurite growth. Each point represents the average of directional growth on neurites from a separate experiment. The growth towards the peptide 'source' (in microns) is on the x-axis; growth away is on the y-axis. For control experiments, both source and sink are untreated collagen. Control growth falls on a line with a slope near 1, which indicates uniform growth in either direction. Growth in gradient gels always falls below this line, indicating biased growth. We are now optimizing each peptide concentration as well as combinations of peptides and gradients of mechanical properties.