

A Rapid, Quantitative Method for Assessing Axonal Extension on Biomaterial Platforms

Jared M. Cregg,¹ Sherri L. Wiseman, B.S.,¹ Nicole M. Pietrzak-Goetze, B.S.,¹ Martyn R. Smith, Ph.D.,² David B. Jaroch, M.S.,³ Daniel C. Clupper, Ph.D.,^{2,3} and Ryan J. Gilbert, Ph.D.¹

Measuring outgrowth of neuronal explants is critical in evaluating the ability of a biomaterial to act as a permissive substrate for neuronal adhesion and growth. Previous methods lack the ability to quantify robust outgrowth, or lack the capacity to quantify growth on opaque substrates because they exploit the transparent nature of culture dishes to segregate neuronal processes from an image background based on color intensity. In this study, we sought to investigate the ability of opaque silica sol-gel materials to facilitate axonal outgrowth; therefore, a method was developed for quantifying outgrowth of neurites from dorsal root ganglion explants on these unique surfaces. Dorsal root ganglia were isolated from stage-nine chick embryos and cultured for 48 h on sol-gel materials presenting agarose and chitosan polysaccharides individually or in combination. Explants were then imaged, and basic image analysis software was used by three independent observers to obtain axonal length and axonal area measurements. Robust axon length and axonal spread measurements for ganglia cultured on agarose-chitosan sol-gel matrices yield an estimate of strong neural compatibility for these substrates over silica matrices presenting no polysaccharides, or silica matrices presenting chitosan or agarose individually. We suggest that this simple protocol for quantifying material biocompatibility offers an analysis strategy that can be used universally to the same end.

Introduction

BIO MATERIALS ARE CURRENTLY BEING DEVELOPED to improve the biocompatibility of devices implanted into the nervous system.¹ Typically, *in vitro* models utilizing dissociated neurons or neuronal explants are used to assess the ability of a material to promote axonal adhesion and extension. Dissociated neuronal cultures allow highly specific examination of neuronal plasticity and growth cone response to precisely controlled microenvironments. Unfortunately, *in vitro* bioactivity of individual neurons is not strictly indicative of the response a population of neurons may have on a biomaterial surface, as suspensions of dissociated neurons lack the neural organization and supporting neuroglial and connective protein elements found in normal nervous tissue.^{2,3} In initial estimates of biomaterial compatibility, therefore, it is important to evaluate the vitality of neuronal explants cultured on a biomaterial surface.

The polysaccharides agarose and chitosan have been extensively investigated for use in neural tissue engineering applications for their ability to support cell attachment and growth.⁴⁻⁹ Toward a coating for neural electrodes and probes, we sought to improve biocompatibility of sol-gel materials

with nervous tissue through incorporation of agarose and chitosan. Dorsal root ganglion (DRG) explants were cultured on sol-gel surfaces containing agarose and chitosan, and a robust response of axons was elicited. Growth was atypical in the degree of neurite fasciculation and the amount of emanating processes, making it impossible to discern individual neurites from ganglion to growth cone. Also, sol-gel matrices are not transparent in nature and induce significant nonspecific binding of antibody stains. Here, the unique characteristics of the growth observed as well as the unique properties of the biomaterial add a level of complexity to the quantification of explant vitality.

Currently, there are several methods in the literature for assessing explant vitality. Qualitative means have been utilized to visually rank explants based on the length and density of emanating processes^{10,11}; however, more quantitative measurements that limit user subjectivity are generally preferred. One simple quantitative method involves manual tracing of axons extending from a ganglion.^{3,12,13} In this method, as many as 50 axons emanating from each ganglion are approximated using a straight line, requiring a significant amount of user-dependent image processing that is impractical for large sets of data and may result in fatigue-related

¹Regeneration and Repair Laboratory, Department of Biomedical Engineering, Michigan Technological University, Houghton, Michigan.

²Department of Biomedical Engineering, Michigan Technological University, Houghton, Michigan.

³Department of Materials Science and Engineering, Michigan Technological University, Houghton, Michigan.

user bias. Additionally, these studies do not present viable options for quantification of dense explant outgrowth, as they target individual processes for measurement.

Several semiautomated or automated methods have utilized image computation to quickly assimilate data on neurite lengths and neurite outgrowth area.^{3,12,14–17} However, the methods and techniques presented in these studies are only suitable for explants with spacing between processes. In addition, the transparent nature of culture dishes is exploited to use thresholding for segregation based on color intensity. Analysis of literature suggests a need for an accurate method of quantifying dense neuronal explants on opaque two-dimensional substrates.

In the present study, we use calcein-AM live stain to view neuronal explants on opaque sol-gel surfaces eliciting non-specific antibody adsorption based on ambient surface charge. The results yield estimates of neural biocompatibility, where neural biocompatibility is defined in this article as the ability of neurons to extend processes on a biomaterial. Here, we present a method for consistently and accurately measuring axonal length and axonal area of neuronal explant outgrowth on these surfaces using basic image analysis software (for example National Institutes of Health sponsored ImageJ, Bethesda, MD). Using this method, output measurements are the longest axonal length aspect, and the area of axonal spread on the biomaterial. Using our method to obtain accurate outgrowth measurements from ganglia provides an analysis strategy for scenarios which prevent computer software from instantaneously assimilating data on growth parameters.

Materials and Methods

Bioglass preparation

Sol-gel materials were formed by the acid-catalyzed hydrolysis of tetraethylorthosilicate (TEOS) (Acros Organics, Geel, Belgium) and subsequent polycondensation of SiO_4^{4-} . Pure silica-sol material was formed by the reaction of TEOS (99.7% purity; Sigma-Aldrich, St. Louis, MO) in an acetic acid solution (5 wt% in H_2O) (1 mol TEOS:12 mol H_2O) at 65°C under rapid agitation.

Agarose sol-gel materials were made by dissolving Sea-Prep agarose (Lonza, Basel, Switzerland) into a solution of boiling acetic acid solution (5 wt% in H_2O) to form a final agarose concentration of 1 wt%. TEOS was then added to this solution (1 mol TEOS:12 mol H_2O) and allowed to react under rapid agitation at 65°C. Five milliliters of the resultant homogeneous gel was subsequently cast into the wells of tissue culture plates (BD Biosciences, Franklin Lakes, NJ). The plates were covered and allowed to age in an airtight container maintained at 37°C under 100% humidity for 3 days.

Chitosan composite materials were produced by forming a pure silica sol through the reaction of TEOS in an acetic acid solution (5 wt% in H_2O) (1 mol TEOS:6 mol H_2O) under vigorous stirring at 65°C. A 2 wt% chitosan (Sigma-Aldrich) solution was made by dissolving chitosan into a second solution of deionized water (1 mol TEOS:6 mol H_2O) and acetic acid (5 wt% in H_2O) at room temperature until the solution was clear and homogeneous. The reacted silica sol and chitosan solutions were combined and stirred for 5 min. Five milliliters of the resultant solution was plated and aged as described above.

Agarose-chitosan sol-gel matrices were formed by first dissolving chitosan into an acetic acid solution (1 wt% in H_2O) at

room temperature overnight. After complete dissolution of chitosan, agarose was dissolved into the chitosan solution at 65°C until homogenous and clear. TEOS (1 mol TEOS:12 mol H_2O) was added to the agarose/chitosan composite. Upon reaction, a viscous clear gel separated from solution, forming a silica bearing agarose/chitosan composite and an unreacted TEOS layer. The excess TEOS was separated from the gel by centrifuging the material at 2000 rpm for 2 min. Five milliliters of the agarose/chitosan gel was then cast into the wells of tissue culture plates. Plates were then aged as described above.

Sol-gel neutralization

As prepared, all glass types have a low pH. To neutralize sol-gel glasses, samples were rinsed in a 0.1 M basic phosphate-buffered saline (PBS) (Zymed Laboratories, San Francisco, CA). Glasses were submerged in 8.2 pH PBS and subsequently placed on a Forma Orbital Shaker (Thermo Fisher Scientific, Waltham, MA) at 75 rpm for 24 h. After 24 h, solution was removed and the pH was measured using a SevenEasy™ pH meter (Mettler; Toledo, Columbus, OH). After the first 24 h of neutralization, fresh 8.2 pH PBS was added to each of the samples. At 48 h, the solution was again removed, pH was measured, and basic solution was replaced. After 3 days, the pH of all glass types was near 7.4. Solutions were then removed and replaced with sterile 7.4 pH PBS and placed on the shaker overnight to assure that residual acid was removed from sol-gel glasses. After the last rinse period, solution was removed and glasses were submerged in sterile 7.4 pH PBS and stored at 4°C until use.

Glass sterilization

To prepare samples for culture, sol-gel glasses were rinsed with Hank's balanced salt solution (HBSS) 1× (Media Tech, Herndon, VA) to remove any remaining PBS. Sol-gel glasses were placed in a sterile culture dish with 30 mL of HBSS, and all glass surfaces were irradiated under ultraviolet light for 30 min.

Cell culture

To assess material ability to support attachment and growth of neurons, DRG explants were isolated and cultured on glasses. DRG explants were isolated from stage-nine chick embryos using techniques approved by the Institutional Animal Care and Use Committee at Michigan Technological University. After isolation, DRGs were cleaned of excess tissue and split in half. Ganglia were then pipetted into a 0.6 mL micro-centrifuge tube and centrifuged for 2 min at 2000 rpm. After centrifugation, HBSS was removed leaving a cluster of ganglia. Two-hundred μL of neurobasal growth medium supplemented with L-glutamine and B27 (Invitrogen, Carlsbad, CA) was subsequently added to break up the ganglia. Neurobasal solution with ganglia was then carefully pipetted onto the sterile glass surfaces. Samples were then placed in the incubator at 37°C for 4 h to allow cell attachment to glass surfaces. After 4 h, cultures were supplemented with 3 mL of neurobasal media with nerve growth factor (Calbiochem, San Diego, CA) such that the final concentration of nerve growth factor was 50 ng/mL neurobasal media. The cultures were then incubated for 48 h.

Visualizing neuronal cultures

After culture, growth medium was removed from the sol-gel glasses and the samples were washed with PBS. Calcein-AM stock solution was made by dissolving 1 mg of calcein-AM (Sigma) into 250 μ L of dimethyl sulfoxide (Sigma). 5 μ L of the calcein-AM stock was added to 10 mL of PBS. The calcein-AM solution was added on top of each sol-gel glass and then placed within a tissue culture incubator for 30 min. After incubation, the glasses were washed several times with PBS. Images of explants cultured on the sol-gel glass surfaces were obtained using an upright Olympus BX51 microscope with Olympus DP Controller software (Olympus, Center Valley, PA).

Quantification of axonal growth

Axonal growth was measured to determine the material's potential to foster neural adhesion and neurite extension. Images were analyzed using Axiovision software v4.5 (Zeiss, Thornwood, NY). Upon opening an image in Axiovision, the following steps were taken to measure axonal growth:

1. The DRG explant was outlined using the outlining tool (Fig. 1A).

2. Straight lines were drawn through the outlined DRG (Fig. 1B). A criterion was established such that the lines must be drawn through the relative center of the DRG. Acceptable lines were defined as lines that bisect the DRG into two sections of roughly corresponding areas. This step is necessary to eliminate irregular, confounding DRG geometries. The shortest and longest lines (Fig. 1C) were kept on the figure, while the rest were deleted. The point at which these lines intersect becomes the adjusted center of the DRG.
3. The outlining tool was then used to outline the entire explant (see Fig. 1D). The area of the DRG (Fig. 1A) was subtracted from the area of the entire explant (Fig. 1D), to determine the total area of axonal growth (axonal spread).
4. The line tool was then used to draw multiple straight lines through the adjusted center of the DRG (Fig. 1E). The longest line in the DRG was subtracted from the longest line drawn through the center of the DRG (Fig. 1F), and the value was recorded as the longest axonal length aspect.

Axiovision software recorded all measurements in pixels, so pixel measurements were converted to microns using a micrometer scale bar image.

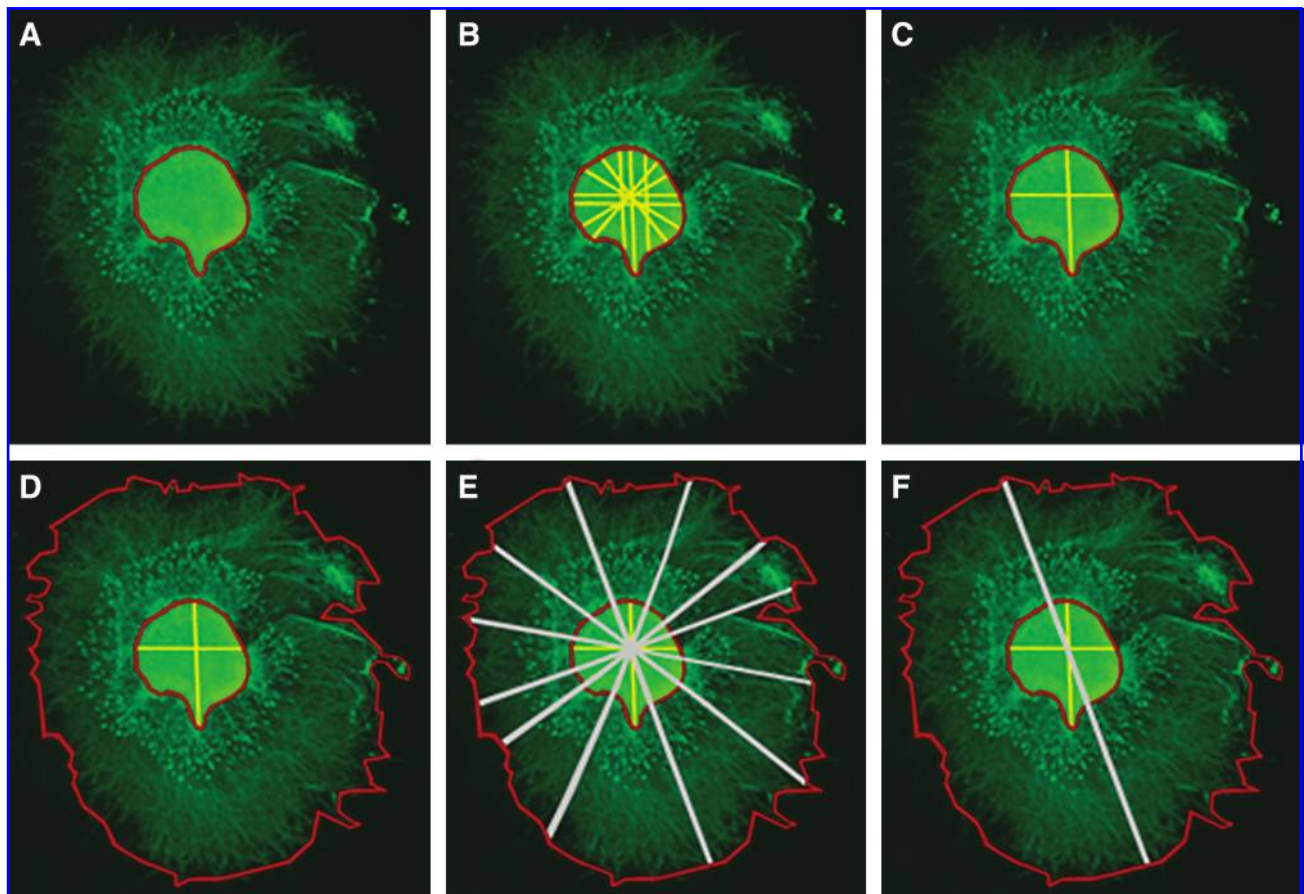


FIG. 1. Measurement of area of axonal spread and longest axon length aspect. The labeled image shows (A) the area of the ganglion, (B) lines depicting possible shortest and longest axis of the ganglion, (C) the long and short axis lines of the ganglion, (D) area of entire explant including axonal spread, (E) lines depicting the possible longest growth axis of axons, and (F) the longest axonal length aspect. Notice that the intersection point of the center lines in (C) is the adjusted center of the ganglion; therefore, axonal growth lines in (E) are measured through it. Color images available online at www.liebertonline.com/ten.

Statistical analysis method

JMP IN software (Release 5.1.2; SAS, Cary, NC) was used to perform statistical analyses between groups. Thirty ganglia in two separate cultures were placed onto each glass type, and to account for differences in ganglion size and vitality, six ganglia per glass type were randomly selected for measurement. A one-way ANOVA test was run to determine statistical differences between sol-gel glass groups in length ($n=6$) and area ($n=6$) measurements. If groups were statistically different in the ANOVA test, Tukey-Kramer honestly significant difference (HSD) tests were then used to compare all pairs individually. A p -value less than 0.05 was considered to be statistically significant.

Results and Discussion

Ganglia were cultured on each glass type for 48 h. At 48 h, cells were stained and imaged. Representative images for ganglia in each group are presented in Figure 2. Ganglion cultured on agarose-chitosan sol-gel (Fig. 2B) had more robust outgrowth as compared with DRGs cultured on chitosan, agarose, and pure silica sol-gels (Fig. 2A, C, and D).

Axonal outgrowth area was found for six ganglia per group, and average area of axonal spread was plotted for each sol-gel group (Fig. 3). Outgrowth was significantly greater for ganglion cultured on the agarose-chitosan glass type. There were no statistically significant differences seen between any other groups.

The longest axonal length aspect was found for six ganglia in each group, and average lengths for each group were plotted (Fig. 4). Ganglia cultured on agarose-chitosan glass blends

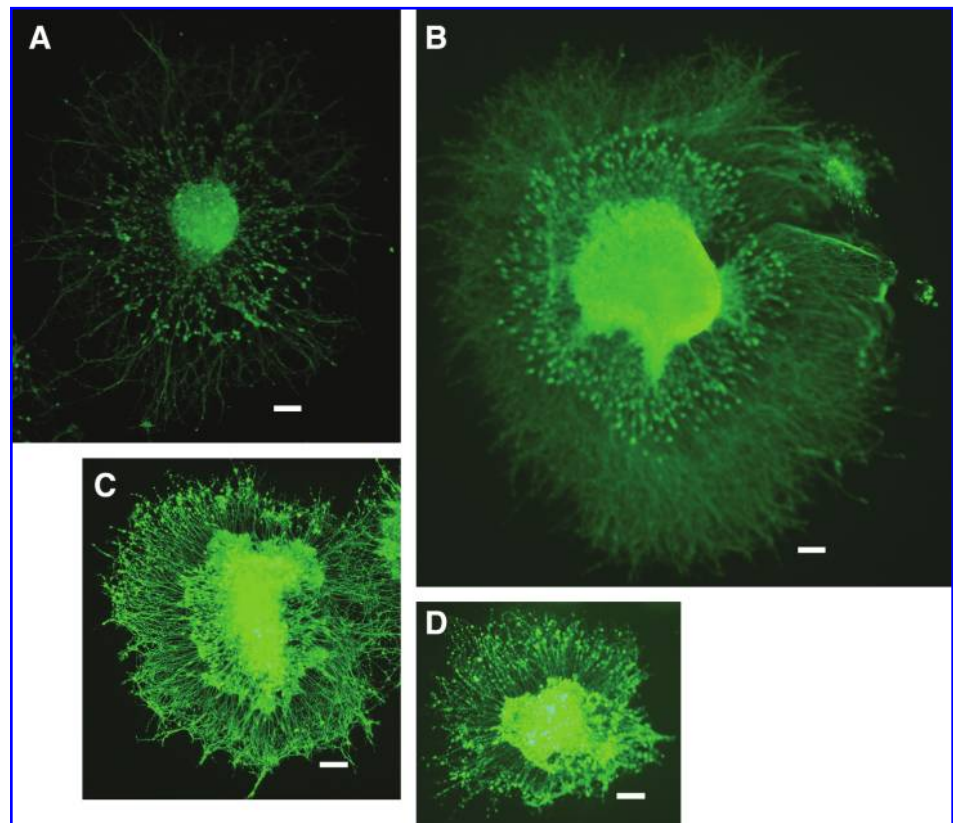
had the longest axon lengths when compared to ganglia cultured on other glass types.

There are a number of protocols for axonal growth quantification in the literature; however, these methods suffer from specific drawbacks we sought to overcome: (1) qualitative rather than quantitative^{10,11}; (2) ability to quantify growth on opaque biomaterials eliciting nonspecific protein adsorption^{3,12-17}; (3) ability to quantify dense neuronal outgrowth^{3,12,14-17}; and (4) large amounts of manual tracing impractical for large sets of data.^{3,12,13} The method presented overcomes these obstacles by requiring minimal user tracing to translate pictorial images into quantitative data representing the longest axonal length aspect and the area of axonal spread. While calcein-AM live stain does not specifically probe neuronal markers, it allows observation of neurons on a material normally eliciting nonspecific antibody adsorption that cannot be accounted for by blocking agents. Here, the repertoire of biomaterials for which neural growth parameters may be assessed is expanded; the method retains the utility in measurement of DRGs cultured on transparent culture dishes or hydrogels, while expanding the types of biomaterials for which quantitative information concerning neural adhesion and growth properties can be obtained.

Determining the longest axonal length aspect of explants can present challenges, as explants can have variable shape. For example, explants can be circular or oblong in dimension. In this technique explants were normalized by finding long and short axes. This process was necessary to generate consistent, repeatable measurements between users and to eliminate subjectivity of the method.

Neuronal explants were cultured on various sol-gel matrices for 48 h. After culture, explants were stained, imaged,

FIG. 2. Representative images of neuronal cultures on various glass types. (A) Growth on chitosan glass matrices, (B) growth on agarose-chitosan glass matrices, (C) growth on agarose glass matrices, and (D) growth on pure silica-sol glass matrices. Ganglia cultured on agarose-chitosan glass matrices (B) demonstrate more robust growth as compared with other groups. Note that the differences in ganglion size between groups is reflected by the degree to which dorsal root ganglion are able to attach, adhere, and spread on the surfaces rather than differences in the size of initially cultured dorsal root ganglion halves. Scale bars = 100 μ m.



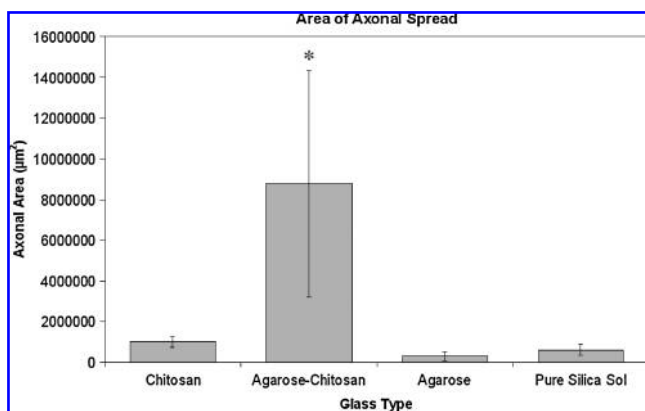


FIG. 3. Comparison of axonal spread area for ganglia on various glass materials. One-way ANOVA, followed by Tukey Kramer HSD yields statistical significance (indicated by *) between agarose-chitosan sol-gel matrices and all other groups.

and measured for the longest axonal length aspect, and area of axonal spread. Figure 2 presents representative images of these culture groups. From these images, ganglia on agarose-chitosan sol-gels show robust neurite extension in comparison with ganglia on chitosan, agarose, and pure silica sol-gel glass types. From Figure 2, it is also noted that the ganglion size is greater for agarose-chitosan than for chitosan, agarose, and pure silica-sol matrices, reflecting the increased attachment, adherence, and spreading of DRG on agarose-chitosan matrices (data not shown). Figure 3 graphically presents areas of axonal spread for ganglia cultured on different glass types using this technique. Statistical significance is seen between agarose-chitosan cultures and cultures in other groups. Figure 4 graphically presents the longest axonal length aspect for ganglia on different glass types. Statistical significance is seen between the agarose-chitosan ganglia and all other groups. The goal of this study was to develop a simple procedure to assess the ability of a biomaterial to foster axonal extension.

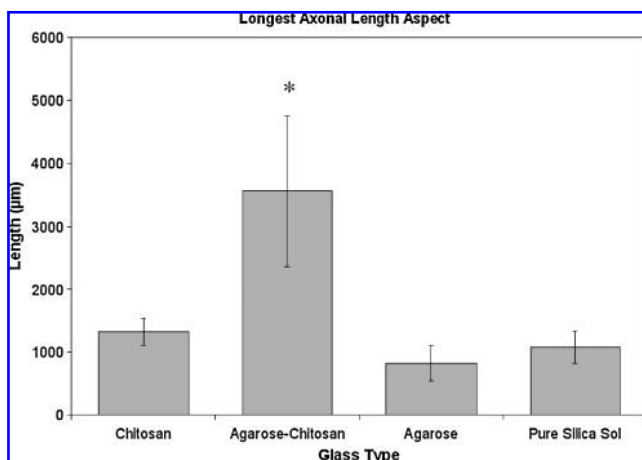


FIG. 4. Comparison of longest axonal length aspect for ganglia on various glass materials. One-way ANOVA, followed by Tukey Kramer HSD yields statistical significance (indicated by *) between agarose-chitosan sol-gel matrices and all other groups.

Thus, a procedure was developed to quantitatively compare DRG explant spread behavior on different materials. These figures exhibit the ability of our method to quantify axonal length and spread differences in neuronal explant images.

All length and area measurements were taken by three separate, blinded observers to determine the level of variability involved in the measurement method. Results among observers were compared using a linear regression test where measurements from all four groups were simultaneously compared between two users. For each line, Student's *t*-test was used to determine if the slope of the line was equal to 1 and the *y*-intercept of the line was equal to 0, where $n = 24$ and the α value selected was 0.05.¹⁸ Results from these tests (data not shown) indicate similarity of measurements between users based on high R^2 values (greater than 0.95) for the linear regressions, and limited rejection of null hypotheses (a slope equal to 1 and an intercept equal to 0), where rejection of the null hypotheses was found to be caused by a profound influence of the largest measured value on the regression. Results from linear regression tests on length and area measurements demonstrate that these methods are highly repeatable and that user subjectivity does not influence the effectiveness of the measurement.

The method presented yields results that translate into meaningful biological information. It is well established that the negative charge associated with sulfated proteoglycans can inhibit neurite extension.^{19–22} Additionally, it has been shown that the polycationic polysaccharide chitosan can enhance neurite extension when covalently coupled to agarose hydrogel scaffolds.^{9,19} Here, agarose-chitosan glasses support robust neuronal attachment and axon extension as compared with chitosan, agarose, and silica-sol glasses (Figs. 2–4), leading us to estimate the neural biocompatibility of agarose-chitosan glasses over that of chitosan, agarose, and silica-sol glasses. The robust growth of DRG explants on agarose-chitosan glasses suggests a synergism between agarose and chitosan that enables greater functionalization of the material over glasses presenting agarose or chitosan alone. We propose that the relationship may be explained by an increase in the ratio of ethyl alcohol functional groups present in both agarose and chitosan to the amine groups presented by chitosan, changing the character of the ambient surface charge. Emerging evidence has implicated heparan sulfated proteoglycans in axon guidance.^{23,24} It is conceivable that cationic polysaccharides interact with negatively charged heparan sulfate proteoglycans on syndecan coreceptors, acting directly or indirectly to establish focal adhesions at the axon growth cone.

Summary

In this study, two new measurements were introduced as a means of quantifying axonal outgrowth. These measurements, longest axonal length aspect and area of axonal spread, were measured by three blinded observers. The results obtained using the method yield an estimate of strong neural compatibility of agarose-chitosan matrices over other types of sol-gel materials while demonstrating the method as being effective in quantifying the differences observed between images and in overcoming drawbacks of current protocols. In future work, we suggest the use of this method as a standard method for measuring axonal growth on two-dimensional surfaces.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Ryan J. Gilbert, Ph.D.

Regeneration and Repair Laboratory

Department of Biomedical Engineering

Michigan Technological University

309 Minerals and Materials Engineering Building

1400 Townsend Drive

Houghton, MI 49931-1295

E-mail: rgilbert@mtu.edu

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