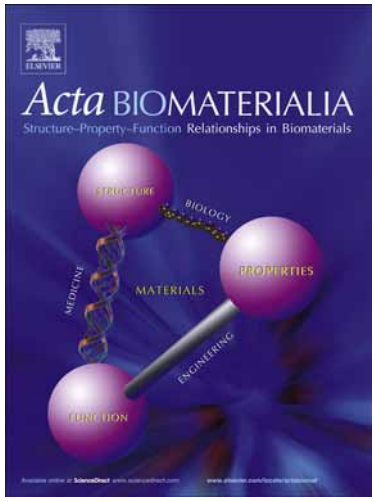

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Robust Neurite Extension Following Exogenous Electrical Stimulation within Single Walled Carbon Nanotube-Composite Hydrogels

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Short Title: Neurite Outgrowth in Conductive Hydrogels

Abstract

The use of exogenous electrical stimulation to promote nerve regeneration has achieved only limited success. Conditions impeding optimized outgrowth may arise from inadequate stimulus presentation due to differences in injury geometry or signal attenuation. Implantation of an electrically-conductive biomaterial may mitigate this attenuation and provide a more reproducible signal. In this study, a conductive nanofiller (single-walled carbon nanotubes [SWCNT]) was selected as one possible material to manipulate the bulk electrical properties of a collagen type I-10% Matrigel™ composite hydrogel. Neurite outgrowth within hydrogels (SWCNT or nanofiller-free controls) was characterized to determine if: 1) nanofillers influence neurite extension and 2) electrical stimulation of the nanofiller composite hydrogel enhances neurite outgrowth. Increased SWCNT loading (10-100-μg/ml) resulted in greater bulk conductivity (up to 1.7-fold) with no significant changes to elastic modulus. Neurite outgrowth increased 3.3-fold in 20-μg/mL SWCNT loaded biomaterials relative to the nanofiller-free control. Electrical stimulation promoted greater outgrowth (2.9-fold) within SWCNT-free control. The concurrent presentation of electrical stimulation and SWCNT-loaded biomaterials resulted in a 7.0-fold increase in outgrowth relative to the unstimulated, nanofiller-free controls. Local glia residing within the DRG likely contribute, in part, to the observed increases in outgrowth; but it is unknown which specific nanofiller properties influence neurite extension. Characterization of neuronal behavior in model systems, such as those described here, will aid the rational development of biomaterials as well as the appropriate delivery of electrical stimuli to support nerve repair.

Keywords: Nerve Regeneration, Nerve Tissue Engineering, Nanomaterial, Electrical Stimulation, SWCNT, Neuron

1. Introduction

Since the 1800's, electrical stimulation has been applied to the nervous system as a potent stimulus to both induce movement and produce visual sensations of light with more recent biomedical applications directed toward stimulation of neural tissue to promote repair following injury [1]. During embryogenesis and following injury, cells are subjected to an endogenous voltage gradient generated by ion transport across the epithelium which impacts directed migration [2-6]. The manual disruption of natural current gradients during avian embryogenesis results in malformed limb buds, indicating the importance of this biophysical guidance cue for proper patterning and development [3, 4, 6]. Adult lower vertebrates, such as amphibians, have an innate regenerative capacity and following amputation in a newt forelimb, a transient gradient originating from the disrupted epithelium of 10-100 $\mu A/cm^2$ exits the regenerating stump tip [5, 7, 8]. Together, these results indicate endogenous currents are present following injury and if local cells retain responsiveness to these signals post-natally, they may be manipulated, influencing regeneration.

Neurons exhibit varying sensitivity to exogenous electrical stimuli *in vitro*. While some studies indicate no cellular response [9], other reported responses include: increased outgrowth [10, 11], directional outgrowth (anode/cathode bias) [4, 12-15], or increased branching [13]. Many of these studies utilized lower order model systems with known

regenerative capacities (e.g. *Xenopus* limb regeneration), but limited knowledge of the mammalian neuronal response to electrical stimulation exists [16, 17]. In recent work, we report an isotropic increase in neonatal rat sensory neurite outgrowth following exogenous stimulation with 50 mV/mm (1 mA, 8 hr duration) relative to unstimulated controls [11, 18]. The heterogeneity in reported responses to exogenous electrical stimulation make it difficult to predict outcomes, and may be due to varied sensitivity to stimulation parameters (e.g. duration, current) and/or inherent differences attributed to species, age, or cell type [19, 20].

In vivo, oscillating electric fields have been used to treat canine spinal cord injuries and exhibited some promise in restoring ambulation. The beneficial effects, however, were not translated beyond human phase 1 clinical trials [21, 22]. Application of alternating current (AC) electrical stimulation was recently shown to improve functionality via accelerated re-growth following post-nerve crush in carpal tunnel syndrome resulting in enhanced sensory and motor nerve regeneration in humans [23, 24]. While promising, it remains unclear what factors are needed for translation to large-gap or more severe nerve injuries where natural structural and biochemical guidance cues are absent. Further, varying injury size and tissue composition likely impacts signal attenuation, providing the local cellular milieu with a variable electrical stimulus that may hinder translation from *in vitro* to *in vivo* platforms [21, 22, 25-27]. We hypothesize that the inclusion of an electrically-conductive biomaterial will mitigate signal attenuation and provide a more reproducible stimulus for a variety of wound geometries.

One possible method of manipulating bulk conductivity is to include an electrically conductive nanomaterial within the biomaterial. In this work, single-walled carbon nanotubes (SWCNT) were selected for their tailorable electrical, thermal, and mechanical properties to modulate conductivity within a model hydrogel [28-36]. The ability for carbon nanotubes to impact cell behavior in the absence of electrical stimulation has been of interest in recent years as the development of nano-bio interfaces gains traction in miniaturized medical applications. Both multi-walled carbon nanotubes (MWCNT) and SWCNT have been used for a variety of medical and clinical applications with promising results. However in the past these nanomaterials are generally used as coatings or within polymer composites for implants in applications such as bone regeneration, and with varying degrees of tissue response based on mode of application (inhalation vs. implantation or digestion) [37, 38]. SWCNT are smaller in diameter (~ 1 nm), compared to the larger diameter MWCNT (4-65+ nm) and electrical and mechanical properties of each should be considered in detail prior to selection for the application, including chirality, mechanical strength, functionalization, biocompatibility, and conductivity [38-45].

In tissue engineering, nanomaterials have been used as both a substrate as well as incorporated in composite biomaterials. In a 2012 study, Martinelli and colleagues demonstrated an increase in cardiomyocyte viability, proliferation, and electrophysiological maturation when cultured on multi-walled carbon nanotube (MWCNTs) films [46]. Similarly, other electrically active cells, such as neurons, may also benefit from contact with nanomaterial interfaces. Stout and colleagues utilized electrospun poly(lactic-co-

glycolic acid)(PLGA) carbon-nanofiber composites as a growth substrate for human cardiomyocyte and rat neuroblastoma cells, and found proliferation increased for both cell types [37]. In other work, both hippocampal and cortical neurons cultured on MWCNT films exhibited higher viability, growth, and synaptic output in the absence of applied electrical stimulation [47-49]. Hippocampal neurons increase postsynaptic currents and there is evidence of enhanced network efficacy, as well as increase action potential firing frequency on MWCNT films in comparison to both the nanomaterial free culture [50]. The inclusion of microscale features of conductive carbon nanotube ropes (1 mm diameter 1.5 cm length) with electrical stimulation elicited an enhanced neurite extension from neural stem cells on 2D surfaces [51]. In addition, tissue compliant MWCNT composites have shown benefit in neural interfacing applications, enabling acute recordings of neural activity [52]. Therefore, it is possible that electrically-conductive biomaterials may support or illicit more robust outgrowth or improved interfacing [53]. While the majority of the studies target cells grown on electrically conductive surfaces/biomaterials, the use of electrically-conductive injectable materials may be more readily translated for neural applications [54].

In previous work by Behan and DeWitt et al, nanomaterials, acid-treated single walled carbon nanotubes (SWCNT, 0.8-2 nm in diameter, 100-1000 μm in diameter; 0-50 μg/mL), were suspended throughout model 3D collagen-type I-35% Matrigel^{TM} hydrogels and reported no change in primary Schwann cell viability or morphology over 2 weeks *in vitro* [55]. It remains unknown, however, if the nanomaterial will impact neurite outgrowth and/or if the measurable benefit of electrical stimulation to neurite outgrowth

is lost [55]. In this work, neurite outgrowth from primary DRG was evaluated within a SWCNT-collagen type I:10% Matrigel™ composite hydrogels (0-100 µg/mL), in the presence or absence of electrical stimulation. Changes to neurite outgrowth were measured in response to both nanomaterial loading and electrical stimulation. Based on previous results demonstrating the benefits of electrically conductive materials with electrically excitable cells, we hypothesize that SWCNT-composite hydrogels will enhance neuronal outgrowth, and in combination exogenous with electrical stimulation we expect to see a further increase to neurite extension. To our knowledge this represents the first study to electrically stimulate primary neurons within a 3D, electrically conductive, nanomaterial-laden hydrogel.

2. Materials and Methods

2.1 Isolation and Culture of Dorsal Root Ganglia

Whole dorsal root ganglia (DRG) were isolated from P2 neonatal rats as described previously (Sprague Dawley, P2; Taconic Farms, Inc.) [11, 18, 56]. DRG isolation was approved by the Rensselaer IACUC committee, in accordance with NIH Guide for Care and Use of Laboratory Animals. The ganglia were collected from the lumbar region of the spine and stored for access on demand at 4℃ in Hibernate AB medium (BrainBits, Springfield IL) for up to 5 days using the manufacturer protocol [11, 56]. Previous work demonstrated no significant changes in outgrowth for DRG stored at 4℃ ≤5 days.

2.2 SWCNT Functionalization with Carboxyl Groups

Single-walled carbon nanotubes (SWCNT) produced via high-pressure carbon monoxide conversion (HiPco) synthesis over an Fe catalyst (HiPco Purified, NanoIntegris) were acid treated to functionalize the SWCNT with carboxyl groups and remove remain-

ing Fe catalyst [55]. 5M sulfuric acid with 5M nitric acid at a 3:1 (v/v) ratio and SWCNT at a concentration of 0.3 mg/mL prior to sonication for 15 min to disperse the nanomaterial, followed by boiling in deionized water (DIW) for 90 min. The acid-treated SWCNT were filtered using a 0.2 micron, hydrophilized Teflon membrane and neutralized with a 20% ammonium hydroxide solution prior to a second filtration with the Teflon membrane. The SWCNT suspension was then lyophilized. The dry SWCNT were weighed and resuspended in DIW at a concentration of 800 μg/mL. SWCNT in DIW solution were stored at 4° C for on demand use. The materials are 0.8-1.2nm in diameter, and 1-1000 nm in length (Supplemental Figure 1A & B), and further information including RAMAN spectrum and additional electron microscopy images can be found at NanoIntegris.

2.3 Dorsal Root Ganglia Outgrowth due to Electrical Stimulation in 3D

Phenol-free growth medium was used to reduce background fluorescence within the hydrogels [(Dulbecco's Modified Eagle Medium (DMEM; MediaTech, Inc.), 10% Fetal Bovine Serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (L-glut; Hyclone), 50 U/ml Penicillin/Streptomycin (P/S; MediaTech, Inc.), 10 μg/mL Bovine Pituitary Extract (BPE; BD Biosciences, San Jose, CA), and 6.6 mM Forskolin (Sigma Chemical)] [57]. On ice, nanomaterial-free 3D composite hydrogels (controls) were mixed containing a 2:1:1:1:5 ratio of 5x phenol-red free DMEM, phenol-red free growth medium, FBS, 0.1 N sodium hydroxide (Sigma Aldrich), and 4 mg/mL acid-solubilized bovine Collagen type I (MP Biomedicals, Solon, OH). Growth factor-reduced Matrigel™ (GFR-Matrigel™, BD Biosciences) was added (10% by volume) on ice and mixed thoroughly [57]. While

neurons can be cultured within collagen I hydrogels, the inclusion of GFR-Matrigel (10%) was necessary to support Schwann cell (peripheral nerve glial support cell) spreading, viability, and migration. The Collagen-GFR-Matrigel composite hydrogels will provide a common platform for future neuronal-Schwann cell co-culture experiments. While Collagen I is a predominant protein in the peripheral nerve, type IV collagen and laminin are the common matrix proteins in Schwann cell basal lamina, as well as Matrigel™ [58, 59]. Nanomaterial-laden constructs were created in a similar fashion. A stock (100 mg/mL in DI water) of acid-treated SWCNT as described above was sonicated to disperse SWCNT aggregates and manually mixed into composite nanomaterial-laden hydrogels with SWCNT loadings ranging from 0, 20, 50, and 100 µg/mL [55]. DI water was added to balance the varying amounts of SWCNT added. All the composite hydrogels had a final concentration of 1.8 mg/mL collagen I. SWCNT-Collagen-Matrigel hydrogels were previously described in detail [55], and exhibit increased pigmentation with increased nanomaterial content (Supplemental Figure 1C).

The samples were well-mixed and 200 µL of liquid hydrogel was pipetted into a cured Sylgard 184 poly (dimethyl siloxane) mold (PDMS; Dow Corning, Midland, MI) (7 mm x 15 mm x 2 mm), and allowed to partially gel for 10 minutes at 37°C. DRG were transferred by sterile forceps, evenly spaced along the midline of the hydrogel (3 DRG/gel), covered with an additional 200 µL of liquid hydrogel, and gelled at 37°C for an additional 60 minutes. This positioned the DRG in the center of the scaffold (both in depth and width of the construct) to ensure that neurite outgrowth could be quantified. The DRG-seeded biomaterials were transferred to a 12-well dish with fresh, phenol red free

growth medium supplemented with 50-ng/mL nerve growth factor (NGF; Invitrogen) and cultured overnight.

2.4 Rheological Properties of the Nanomaterial-laden Hydrogels

Composite hydrogels containing 0-100 µg/mL SWCNT were prepared as described above, but kept on ice prior to mechanical testing to prevent gelation. Methods utilized for hydrogel characterization are described in detail in Zuidema et al 2014 [60]. 300 µL of liquid hydrogel was plated onto a TA AR-G2 rheometer (TA Instruments, New Castle, DE) with a 20 mm steel plate at 4 ℃ at a height of 500 µm. The temperature was raised to 37 ℃ for 15 minutes to allow complete gelation, which was calculated from time sweeps to examine gelation kinetics conducted at 1% strain and a frequency of 1 Hz from 4-37℃ [60]. To calculate the storage and loss moduli of hydrogels (G' and G"), a strain sweep at an angular frequency of 1 Hz was conducted based on earlier frequency sweeps of nanomaterial free, control hydrogels over a larger range of 0.1-100 Hz. The elastic modulus was calculated by averaging the linear region of the strain sweep for G' at 1.0% strain, where the linear region spans from approximately 0.05-5% strain as shown in Figure 1A.

2.5 Conductivity Measurements of Composite Hydrogels

To measure the conductivity of the hydrogels, SWCNT-collagen hydrogels (0-100 µg/mL) were formed as described above and kept on ice in 50 mL tubes. A handheld 3-point conductivity probe (Oakton Instruments, Vernon Hills, IL) was submerged in the hydrogel precursor solution, and the hydrogel was allowed to gel at 37℃ for 15 minutes

prior to acquiring 3 conductivity readings per sample at room temperature for each gel.

2.6 Electrical Stimulation Culture Chamber Design and Construction

Electrical stimulation (ES) culture chambers used in this study have been previously described in detail [11]. For this study, DC stimulation was selected to (1) visualize any directional bias and (2) simplify parameter selection. Two chambers (7 mm x 30 mm x 1.5 mm) were first excised from cured non-conductive PDMS in rectangular polystyrene plates (Thermo Fisher Scientific, Waltham, MA). Next, 2% agarose salt bridges were formed within the chambers to minimize cell exposure to electrical by-products. A bench-top DC power system (Marlin P. Jones & Assoc., Inc., Lake Park, FL) supplied current through two platinum electrodes (Biological Grade, Sigma Aldrich). The voltage drop across the chambers was monitored continuously throughout the experiment using two references electrodes (platinum) placed within each chamber via SCC-FT01 feed through components and LabView (National Instruments, Austin, TX) [11]. The DRG hydrogels were placed within the chambers in fresh phenol-free growth medium supplemented with 50 ng/mL NGF and transferred to a chamber for exposure to 8 hr electrically stimulation at 50 mV/mm DC (1mA) or 0 mV/mm as unstimulated controls. The electrical stimulation parameters were chosen from previous 2D experiments where 50 mV/mm elicited the greatest and longest neurite outgrowth following a single 8 hr stimulation over field strengths ranging from 0-100 mV/mm [11]. Following treatment, the hydrogels were transferred to fresh medium and incubated for an additional 48 hours prior to fixation.

2.7 Immunofluorescent Staining

The DRG-laden composite hydrogels were fixed with a 4% paraformaldehyde-4% sucrose (Sigma Aldrich) solution in 2x PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgSO4, pH 7.0 with KOH in DI H2O; Sigma Aldrich) at room temperature for 60 minutes. Samples were rinsed three times with PBS, permeabilized for 30 minutes with 0.1% Triton X-100 in PBS (Sigma Chemical) and blocked with 2.5% goat serum in PBS (GS; Invitrogen) for 60 minutes at room temperature. To visualize neurite outgrowth within the hydrogels, the samples were incubated for 60 minutes at room temperature with mouse anti-β-III-tubulin primary antibody (1:500 in 2.5% GS), rinsed three times for five minutes in HBSS, and incubated for 60 minutes at room temperature with goat-anti-mouse Alexa Fluor 488 IgG2b secondary antibody (Invitrogen) (1:1000 v:v in 2.5% GS), rhodamine phalloidin to visualize filamentous actin (1:1000 in 2.5% GS), and 4', 6-diamidino-2-phenylindol (DAPI; Invitrogen; 1:1000 in 2.5% GS) to label all cell nuclei. Immunostained samples were rinsed three times for five minutes in HBSS prior to storage in sterile HBSS at 13℃ and imaged within 7 days.

2.8 Microscopy and Image Analysis

Neurite outgrowth was visualized using a Zeiss Axio Observer Z1 fluorescence microscope with a 20x dry objective and Zeiss AxioVision software (Carl Zeiss Microscopy, LLC, Thornwood, NY). ImageJ (NIH Freeware) was used to trace the perimeter of outgrowth emerging from the DRG as well as the DRG explant to quantify total neurite outgrowth area and longest neurite using methodology described in detail by Cregg et al. 2010 [61].

2.9 Statistics

Neurite outgrowth was calculated from four separate experiments, with triplicate DRG for each hydrogel (n=4) for a minimum of 12 DRG examined per condition. A minimum of 8 DRG per condition was required based on previous sample size calculations using a one-sample *t*-test (Excel) in order to have a 95% power to detect ($\alpha = 0.05$) variance between control DRG populations. Rheology measurements were calculated from three separate experiments (n=3). Conductivity measurements were conducted for three separate experiments with three readings for each sample (n=3). Statistical significance was determined using Excel 2010 Analysis ToolPak to perform a two-way ANOVA with replication. *p*-values < 0.05 were considered statistically significant.

3. Results

3.1 Rheological and Electrical properties of SWCNT Composite Hydrogels

Neurite outgrowth is known to be influenced by hydrogel stiffness [62-66]. Rheological measurements were performed to quantify any changes in the bulk materials by measuring the hydrogel storage and loss moduli (G' and G") (Figure 1A). Despite the inclusion of SWCNT, no significant differences in elastic moduli were detected for any of the hydrogel compositions (0-100 µg/mL SWCNT), with moduli ranging from 37.8-50.9 Pa (Figure 1; $p>0.05$).

FIGURE 1

The electrical conductivity of the SWCNT-laden hydrogels (0-100 µg/mL) was measured via a 3-point probe test. Due to electrolytes within the medium, control hydrogels (0 µg/ml) are conductive. SWCNT inclusion increases bulk conductivity with a 1.3-fold increase (1723 mS/m) for a 20-µg/mL SWCNT loading relative to the nanomaterial-free controls (1385 mS/m) (Figure 1B). Increased SWCNT doping further enhances bulk conductivity non-linearly with 1.5-fold and 1.7-fold increases with respective 50 and 100 µg/mL SWCNT loading (Figure 1B, $p<0.05$). While the inclusion of nanomaterial from 10-100 µg/ml did not significantly alter the bulk mechanical properties of the hydrogel, significant changes in electrical conductivity were measured (Figure 1).

3.2 Neurite Outgrowth is enhanced in SWCNT Composite Hydrogels

To evaluate changes to neurite outgrowth via the inclusion of electrically conductive nanomaterial, primary DRG were encapsulated within SWCNT-collagen I-10% Matrigel™ composite hydrogels (20-100 µg/mL) and nanomaterial-free collagen I-10% Matrigel™ controls (0 µg/mL). Regardless of SWCNT loading (20-100 µg/mL), neurite extension was not inhibited by nanomaterial (Figure 2). While neurite outgrowth appeared to be similar in higher SWCNT loadings (50 or 100 µg/mL) relative to control nanomaterial-free hydrogels (Figure 2 A-D); outgrowth within the 20-µg/mL composite appears be significantly more robust (Figure 2 A-D), exhibiting a significant 3.3-fold increase in total outgrowth (Figure 2 E; $p<0.05$) and neurite length was 1.6-fold greater within the 20 µg/mL SWCNT composite hydrogels relative the control hydrogels (Figure 2 F; $p<0.05$). In contrast, both neurite persistence length and total outgrowth within the 50 and 100 µg/mL SWCNT composites was not significantly greater relative to the control hydrogels (Figure 2 E-F). Based on these experiments, we can conclude that

neurite outgrowth is not impaired by the nanomaterial loading, but rather there appears to be a non-dose dependent benefit from SWCNT inclusion.

FIGURE 2

3.3 Neurite Outgrowth is more robust due to Electrical Stimulation within SWCNT hydrogels

To examine any benefit to the co-presentation of electrical stimulation within the nanomaterial-laden hydrogels, both DRG in SWCNT and nanomaterial-free hydrogels were subjected to electrical stimulation. 20 µg/mL SWCNT loading was selected for this study, since this loading supported the greatest neurite outgrowth (Figure 2). Electrical parameters (50 mV/mm DC, 8 hr, 1 mA) were chosen based on previous work in 2D demonstrating increased outgrowth and neurite length over 8 hr DC stimulation within 0-100 mV/mm of electrical stimulation [11]. In the electrically-stimulated, nanomaterial-free, control hydrogels (50 mV/mm, 8 hours, 1 mA), a 2.9-fold increase in outgrowth was observed in comparison to DRG cultured within unstimulated, nanomaterial-free hydrogels (Figure 3 A-B and E, $p<0.05$). The electrically-mediated increase is similar to that which was measured from the inclusion of the SWCT at the 20 µg/mL loading without electrical stimulation (3.3-fold increase, Figure 3 C and E, $p<0.05$). Combining both SWCNT-hydrogels with electrical stimulation results in a 7.0-fold increase in neurite outgrowth relative to unstimulated, nanomaterial-free control hydrogels (Figure 3 D and E, $p<0.05$). Co-presenting both electrical stimulation and nanomaterial-laden hydrogels resulted in a significantly greater outgrowth relative to either cue being pre-

sented individually. Similar trends are observed with the neurite persistence length with 1.6-fold and 1.7-fold increases due to inclusion of either SWCNT (20 µg/mL) or electrical stimulation (50 mV/mm) alone, respectively (Figure 3 E; $p<0.05$). Combining the nanomaterial-laden hydrogel concurrently with electrical stimulation (50 mV/mm) increased neurite length further, resulting in a significant 2.1-fold enhancement over the unstimulated, nanomaterial-free control (Figure 3 F; $p<0.05$).

FIGURE 3

4. Discussion

To our knowledge, this is the first study to demonstrate the synergy of electrical stimulation and electrically conductive 3D hydrogel on neurite outgrowth. Inclusion of the electrically-conductive SWCNT increased neurite outgrowth within the biomaterial even in the absence of the electrical stimulation. Similarly, the application of exogenous electrical stimulation in the absence of the nanofiller, resulted in an increase in neurite outgrowth within a 3D biomaterial. The complementary and inhibitory factors of complex combinational approaches for increasing regrowth are difficult to parse mechanistically *in vivo*, but *in vitro* models may serve to identify synergistic approaches that will promote more robust regeneration for more targeted *in vivo* studies.

While many nanofillers are available to manipulate the bulk conductivity of a hydrogel, SWCNTs were chosen due to reports of carbon nanotube films supporting cardiomyocyte maturation, neurite outgrowth, and synapse formation [47-50, 67, 68], as well as previous work in our group reporting their compatibility with peripheral glia

(Schwann cells) within 3D hydrogels [55]. In the absence of electrical stimulation, SWCNT inclusion supported neurite outgrowth in a non-dose dependent manner (0-100 µg/mL; Figure 3). Despite modest increases for all conditions tested, only the 20 µg/mL SWCNT loading resulting in a significant 3.3-fold increase in total neurite outgrowth compared to nanomaterial-free control hydrogels, along with a more modest increase in longest neurite length. Increases in neurite length are not due changes in mechanical stiffness as no significant increases in elastic moduli were reported for all SWCNT concentrations examined (Figure 1). In contrast to our work, Fukashima and colleagues reported a high weight-percent of SWCNT (up to 10%) significantly increased Young's Modulus 120-fold compared to polymer alone, due to crosslink network formation within the densely packed nanomaterial films [69]. In our hydrogels, concentrations of the nanomaterial are a small fraction, with a maximal ~0.01% by weight (100 µg/mL), consequently bulk material properties are not impacted. It is possible local changes in stiffness at the cellular level are present or cells sensing the nanomaterial topography itself may be important, resulting in the increased neurite outgrowth [70, 71]. Atomic force microscopy (AFM) nanoindentation has been employed previously to demonstrate changes in compressive mechanical properties and nanofiber fiber orientation on the microscale that may be indistinguishable at the macroscopic or bulk material level [70-74].

Recent work by Cellot and colleagues suggest tight contacts with neuronal cell membranes and MWCNT that may enable efficient electrical shortcuts across neuronal cell bodies [75]. Alternatively, other theories exist suggesting enhanced cellular communi-

cation via manipulation of a membrane potential on conductive scaffold blends of hyaluronic acid-3, 4-ethylenedioxythiophene (PEDOT)-collagen-I-gelatin-chitosan [76]. The depolarization of the neuronal cell membrane is required for initiation of action potential and it is plausible the SWCNT-hydrogel may also enhance cellular communication. Clearly more work is needed to determine what features of the nanofiller are beneficial to support greater outgrowth.

Other conductive materials, such as the polymer PEDOT, have gained interest in recent years as a coating for implantable neural prosthetics or electrodes. The integration of EDOT monomer (0.01M) has recently been shown to support neuroblastoma cell viability of to 3 days in culture [77-79]. Guarino et al. reported electrically conductive, macroporous PANi-polyethylene glycol diacrylate (PEGDA) sponge-like hydrogels were able to support PC-12 neuronal like cells as well as human mesenchymal stem cells for up to 5 days in culture [80]. In corroboration with our work, Tosun and colleagues reported that PC-12 cells seeded on top of or encapsulated within SWCNT-collagen hydrogels (5% w/w SWCNT:Collagen) up to 7 days remained viable and did not undergo SWCNT-induced DNA damage leading to apoptosis [81]. All of these conductive biomaterials supported cellular viability but did not explore exogenous electrical stimuli.

In this study, exogenous electrical stimulation was applied to both in the presence and absence of the SWCNT-laden hydrogel. Neuronal sensitivity to electrical stimulation has been reported *in vivo* [23, 24, 26] and *in vitro* for 2D [4, 9, 14, 20, 82, 83] since the early 1800's. We report a similar benefit to neurite outgrowth and length in 3D as was

observed in 2D [11, 84] in the absence of any nanomaterial. To our knowledge, this study represents the first work to examine changes in neurite outgrowth for neurons encapsulated within 3D biomaterials exposed to electrical stimulation. Other non-neuronal cells (e.g. fibroblasts, mesenchymal stem cells) seeded within 3D biomaterials are responsive to electrical stimulation and exhibit altered cell phenotype (adhesion and orientation) [85, 86]. To date, it is not understood the specific mechanism by which neurons sense the electrical stimulus supporting greater outgrowth, especially because electrophysiology measurements (e.g. patch clamp) are impossible in the presence of electrical stimulation with current technologies. Preliminary studies indicate voltage gated calcium channels are depolarized by the electrical stimulus, resulting in increased motility and release of neuro-supportive soluble neurotrophic factors (i.e. NGF) from the resident Schwann cells located within the DRG [84, 87]. Investigations are currently underway to more fully understand how both Schwann cells and neurons experience the electrical stimulus.

Interestingly, both the inclusion of nanofiller (20 mg/ml SWCNT) or electrical stimulation in a nanofiller free hydrogel individually resulted in a similar increase in neurite out-growth. The combination of both cues (SWCNT biomaterial and electrical stimulation) resulted in a further increase in neurite outgrowth (7.0-fold) and longest neurite (2.1-fold) beyond each individual cue alone (Figure 3). Increased neurite extension may be due to signal localization within the conductive SWCNT hydrogel, as doping nano-material into the hydrogels caused the electrical conductivity to increase significantly. Similar to findings reported by Voge and colleagues, the electrical conductivity of nano-

material-laden hydrogels was 1866 mS/m for 20 µg/mL SWCNT, a 1.3-fold increase over control nanomaterial-free hydrogels (Figure 1) [31, 55]. The collagen-Matrigel™ hydrogels are primarily aqueous, containing a high percentage of cell culture medium, and the resultant conductivity is near the expected value of culture medium alone of approximately 1400 mS/m. The acid-treated SWCNT are dispersed within the bio-material, but likely are bundled as previously described [55, 88]. Greater dispersion of the nanomaterial is expected to significantly increase conductivity of the hydrogel by creating a continuous network of SWCNT [89]. Fukashima and colleagues examined films of SWCNT-methacrylate blends with up to 10% SWCNT by weight, and found a significant increase in conductivity (up to 1000 mS/cm) in a temperature dependent manner, suggesting a charge carrier hopping mechanism that is likely allowing for the electrical percolation [31, 69, 89, 90]. Our more modest increases in conductivity from 0-100 mg/ml loadings with normal means of dispersion clearly suggest the nanomaterials are not fully percolated. This may work to our advantage, as a continu-ous network or sheet may shield the cells from the electrical stimuli, while a discontinuous network may provide greater exposure to electrical stimuli.

In recent years, there has been an interest in mediating a variety of cellular responses via conductive fibers or films (e.g. Polypyrrole, Polyaniline) with or without electrical stimuli [91-93][Review [94]]. Olfactory ensheathing glial cells, support cells that bridge the peripheral and central nervous systems, cultured on conductive polypyrrole/chitosan polymers exhibited enhanced release of neurotrophic factors and surface ligands following electrical stimulation (50-1000 mV/mm) [91]. Neuronal-like PC-12 cells exhib-

ited elongated morphology in response to electrical stimulation on either polypyrrole/chitosan films or polypyrrole-PLGA nanofibers [92, 93]. Huang (2012) has shown that utilization of conductive polypyrrole tubes within nerve guidance channels combined with electrical stimulation (3 V, 20 Hz) increases neurite regrowth and functional recovery across 15 mm rat sciatic nerve deficits [95]. However, Prabhakaran et al. reported exogenous electrical stimulation of immortalized rat neural stem cells (C17.2) (100 mV/mm, 1 hr) cultured on electrospun fibers of PANi:Poly-L-Lactide did not impact neurite extension compared to unstimulated controls [96]. This highlights the need to better understand how electrical stimulation, electrical conductive materials, and material topology may contribute both individually and in concert to influence cell behavior. Further research is needed to understand what features of the nanomaterial-laden hydrogel influence neurite outgrowth and refined presentation of nanofiller or other more biocompatible, electrically-conductive biomaterials (e.g. size range, electrical properties, dispersion). Increasing neuronal extension in 3D as reported in this work may be elicited in a variety of conductive materials and hydrogels, which could open the door for tailorable therapies for a variety of wound healing applications [77-79, 91-93].

The use of electrically conductive materials has also gained interest beyond neural engineering, in the fields of cardiac and skeletal muscle engineering to aid restoration of function following myocardial infarct [35]. Qazi and colleagues fabricated biodegradable polyaniline (PANi) patches that are conductive and cytocompatible [35]. Chen and colleagues utilized a combinatorial approach of aligned, conductive electrospun polycaprolactone (PCL):PANi fibers and found enhanced skeletal myotube formation

and orientation in the absence of an external electrical stimulus [97]. While these results are promising, the 2D nature of conductive films precludes their effective use and translatable results to more clinically relevant models using injectable, minimally invasive hydrogels, motivating the development of model 3D conductive materials [98, 99]. Therefore, recent works such as Li and colleagues use of a poly (N-isopropylacrylamide) (PNIPAAm) hydrogel containing SWCNT for a conductive stem cell carrier for cardiac repair, found enhanced cell adhesion and proliferation of the cardiac precursors [100]. These advances and work described herein with 3D conductive biomaterials containing CNT, concurrently with novel materials processing techniques such as 3D printing for organ and tissue fabrication may result in enhanced functionality for regenerative medicine and neural engineering applications [101].

5. Conclusion

The application of an exogenous electrical stimulus to enhance neurite outgrowth following nervous system injuries may improve functional regeneration. Utilization of a three-dimensional electrically conductive biomaterial within the injury site may minimize signal attenuation and allow for a reproducible electrical stimulus to be applied across a wide range of injury geometries and model systems. Additionally, electrically conductive materials appear to provide some inherent benefit to electrically active cells in the absence of electrical stimulation. This study investigated the inclusion of single walled carbon nanotubes (SWCNTs) as a model nanofiller to create an electrically conductive 3D composite hydrogel. Results indicate that all SWCNT loadings within the range of 10-100 µg/mL) supported neurite outgrowth, but only the 20 µg/mL loading significantly enhanced total neurite outgrowth and longest neurite relative to nanomaterial-free

control. Increases in neurite outgrowth do not appear to be influenced by mechanical properties as the elastic modulus does not significantly change despite an increase in conductivity. DRG outgrowth was greater when exogenous electrical stimulation (50 mV/mm) was presented in conjunction with the conductive hydrogels (20 μg/mL), resulting in a 7.0-fold increase in neurite outgrowth compared to control nanomaterial-free hydrogels. Continued characterization of neural cell behaviors in model systems, such as those described herein, will allow for the optimization and characterization of electrical stimulus parameters and development of novel conductive biomaterials to aid in nerve repair.

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Disclosure

The authors declare no conflicts of interest in this work.

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FIGURE AND TABLE LEGENDS

Figure 1. Inclusion of SWCNT within collagen I hydrogels does not alter bulk elastic modulus, however does increase conductivity. (A) Strain sweeps of hydrogels containing 0-100 µg/mL SWCNT show no difference in storage modulus. (B) At even low concentrations of SWCNT, electrical conductivity of collagen type I hydrogels is significantly enhanced. * = $p < 0.05$ compared to control 0 µg/mL hydrogels, # = $p < 0.05$ compared to all conditions, n=3, standard deviation shown.

Figure 2. Neurite outgrowth from dorsal root ganglia encapsulated within collagen type I - single walled carbon nanotube composite hydrogels. Neurite outgrowth within control (A) hydrogels appears modestly reduced compared to composite materials containing 100 or 50 µg/mL SWCNT (C,D), while those encapsulated in 20 µg/mL hydrogels appear to have enhanced outgrowth (B). Green = β-III-Tubulin neurites, Red=Phalloidin labeled actin, Blue= DAPI nuclei. Bar= 500 µm, 20x. Total neurite outgrowth (E) and neurite persistence length (F) is only significantly enhanced within 20 µg/mL SWCNT composites compared to control (0 µg/mL) composite hydrogels. * = $p < 0.05$ compared to all conditions, n=3, standard error shown.

Figure 3. Electrical stimulation enhances neurite outgrowth in SWCNT composite hydrogels. Neurite outgrowth is promoted by either electrical stimulation with 50 mV/mm (DC 8 hours, 1 mA) (B) or inclusion of 20 µg/mL SWCNT within hydrogels (C) compared to control (0 µg/mL) hydrogels (A) with significant increases in outgrowth (E) and neurite length (F). When SWCNT are combined with 50 mV/mm, a robust increase of neurite outgrowth is observed compared to either singular cue alone (D) and significant increases are observed in both total neurite outgrowth (E) and neurite persistence length (F). Green = β-III-Tubulin neurites, Red=Phalloidin actin, Blue= DAPI nuclei, Bar= 500 µm, 20x. * = $p < 0.05$ compared to all conditions, n=3, standard error shown.

Supplemental Information

Scanning Electron Microscopy (SEM) Imaging of Carbon Nanotubes

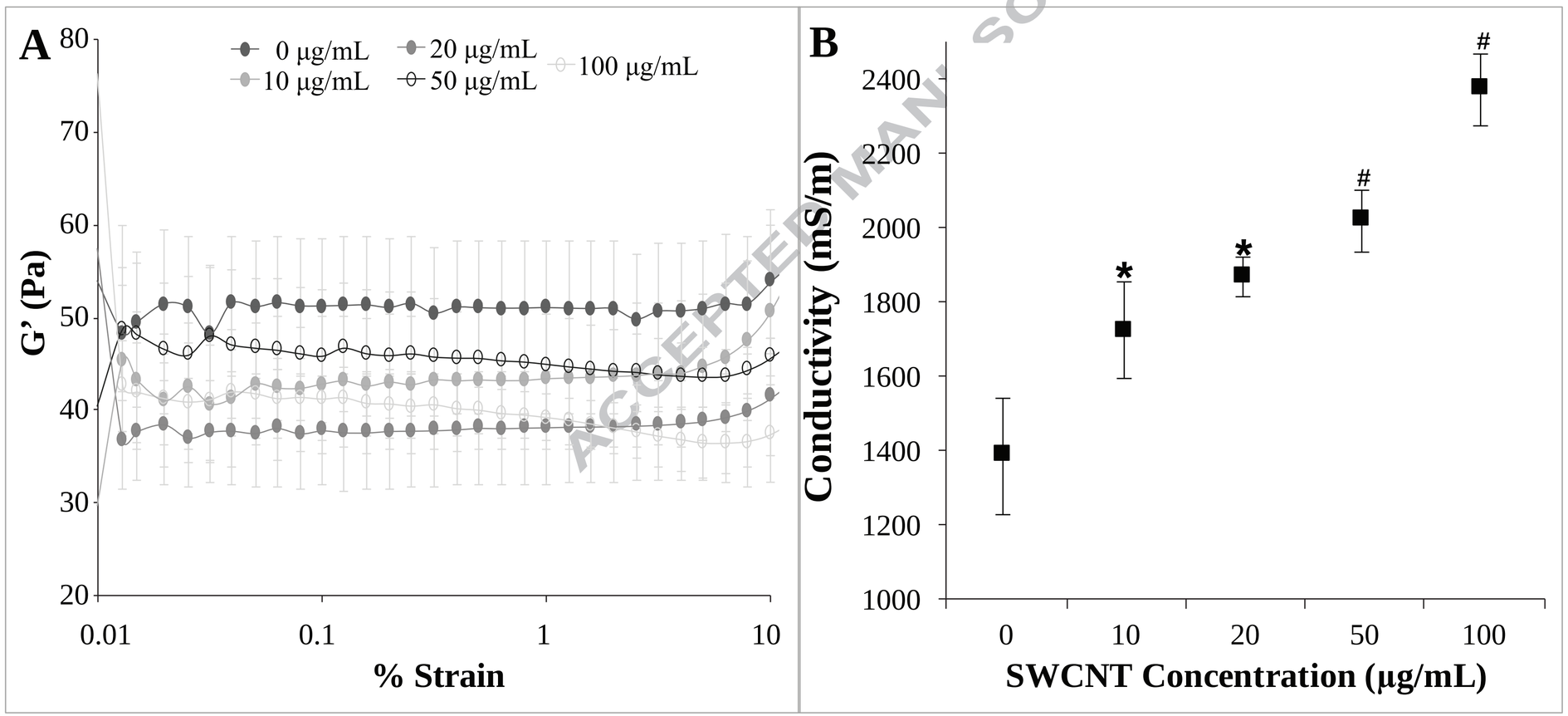
To prepare samples for SEM analysis, single wall carbon nanotubes (in DI water) were sonicated for 1 hour and then centrifuged at 1000 rpm (Eppendorf 5810 Centrifuge). Samples were then dried on clean coverslips and adhered to pin mounts using carbon tape. A 0.5 nm platinum coating was applied to the sample using a Technics Hummer V Sputter Coater (Union City, CA) prior to SEM analysis. Carbon nanotubes were imaged using an FEI Versa 3D DualBeam (Hillsboro, Oregon) under high vacuum mode. The SEM was operated using a 5.3 mm working distance, a 4.0 spot size, and an accelerating voltage of 5 kV. Images were captured at 50,000x and 80,000x magnification. Due to their small diameter (Optical images of the hydrogel composites were also taken using a Nikon camera at ambient room temperature. (Supplemental Figure 1).

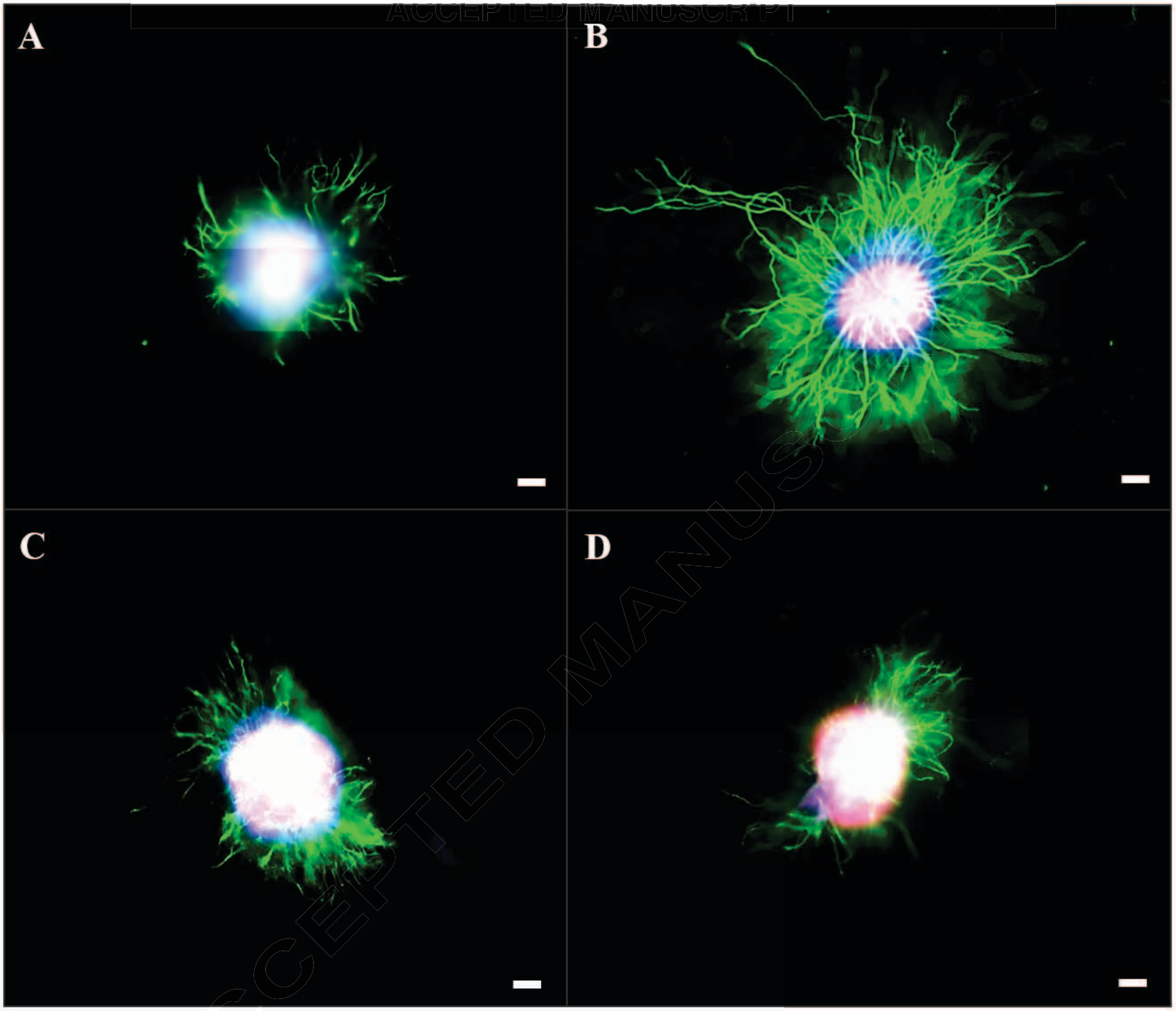
INSERT SUPPLEMENTAL FIGURE 1

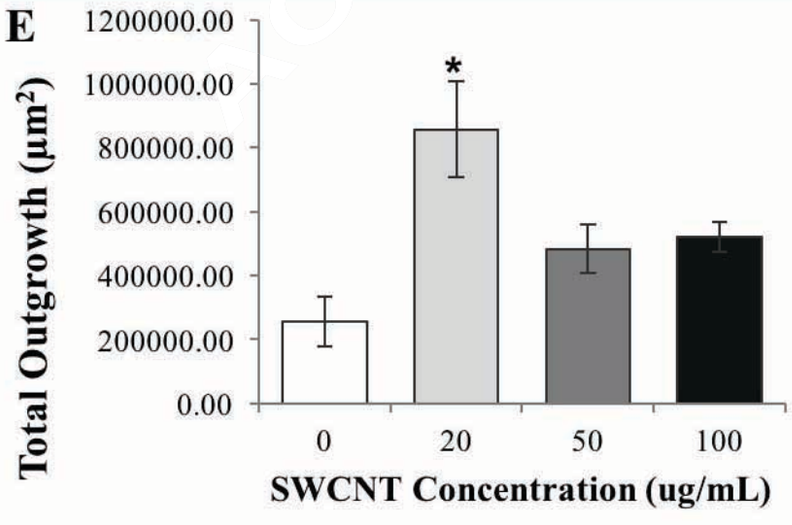
Supplemental Figure 1. Scanning Electron Microscopy images of SWCNT used in this work show filamentous structure and some bundling of the acid treated nanofiller (A) bar = 1 µm, (B) bar = 500 nm The SEM was operated using a 5.3 mm working distance, a 4.0 spot size, and an accelerating voltage of 5 kV. (C). 3D SWCNT-collagen type I composite hydrogels exhibit increased pigmentation with increased nanomaterial content. Control hydrogels with no nanomaterial inclusion are transparent (right), while hydrogels containing SWCNT exhibit a fairly uniform distribution of SWCNT within the range used in this study (left). There is aggregation visible in higher concentrations of nanomaterial and this is . Bar = 500 µm.

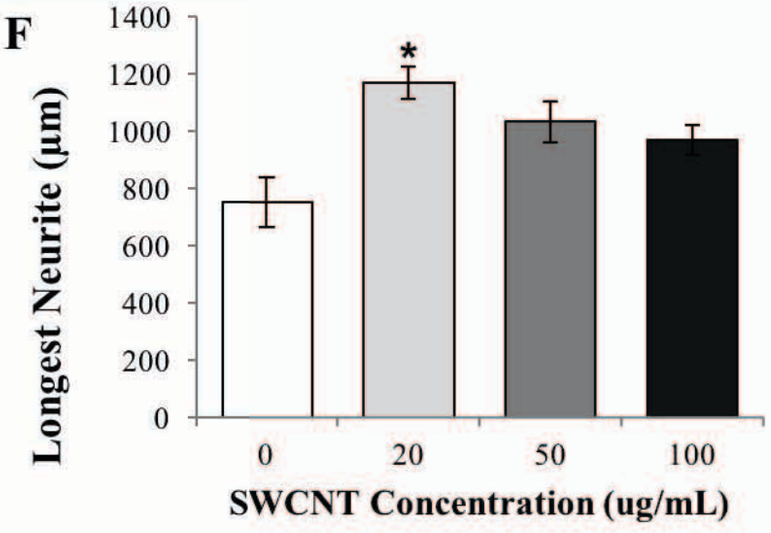
The acid-treated material was estimated to be greater than 9 nm in diameter, indicative of bundling [88, 102, 103]. Carboxylation is known to allow the SWCNT to aggregate or bundle [102]. The acid treatment can also result in shorter SWCNT in length [103].

Furthermore when the nanomaterial is placed in cell culture medium or embedded within the hydrogels, it is expected that the SWCNT will aggregate further [55, 88].


A
0 µg/mL
10 µg/mL
20 µg/mL
50 µg/mL
100 µg/mL
G' (Pa)
80
70
60
50
40
30
20
0.01
0.1
1
10
% Strain
B
Conductivity (mS/m)
2400
2200
2000
1800
1600
1400
1200
1000
*
*
#
#
0
10
20
50
100
SWCNT Concentration (µg/mL)







0 mV/mm 50 mV/mm

0 µg/mL

20 µg/mL

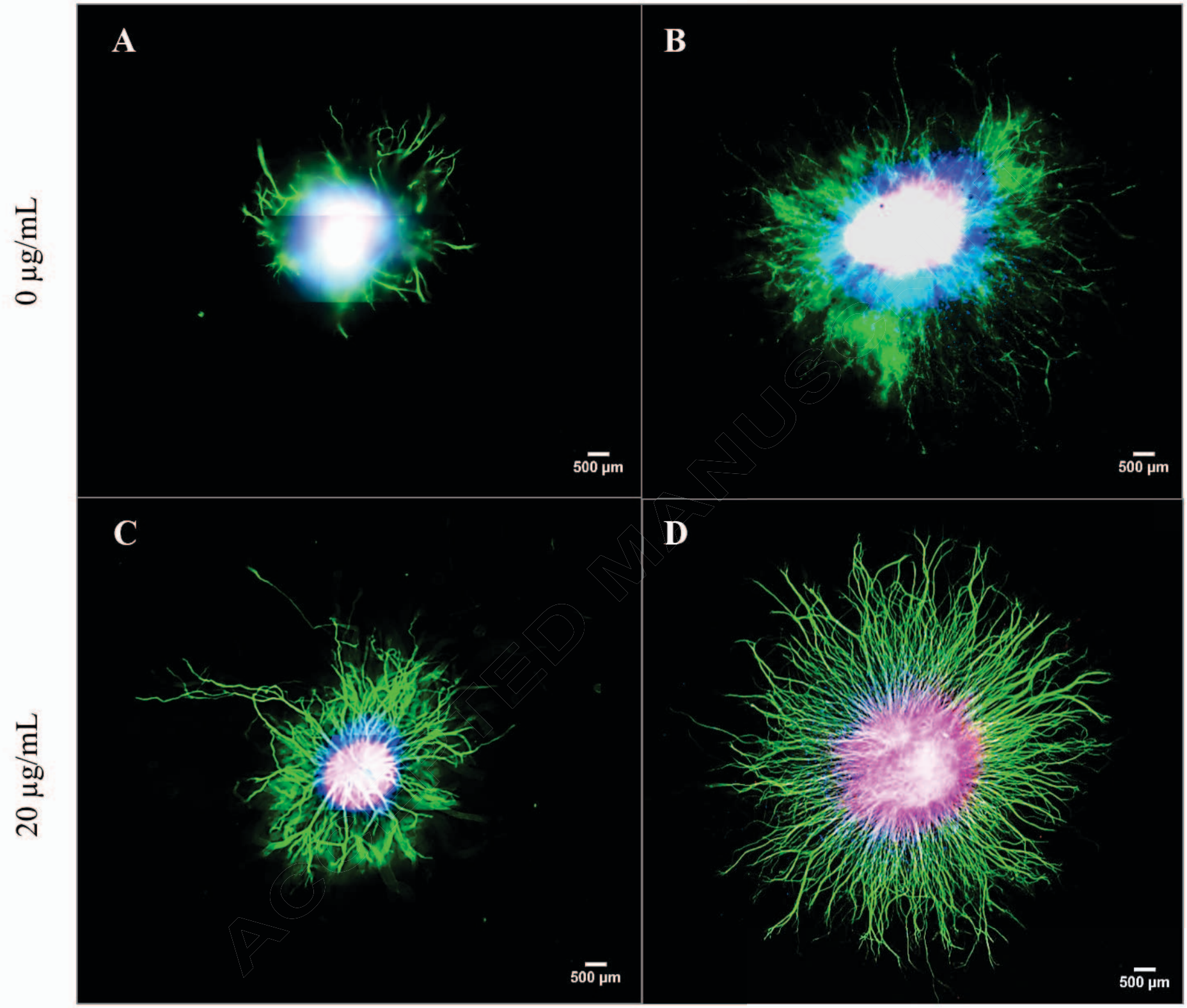
A

B

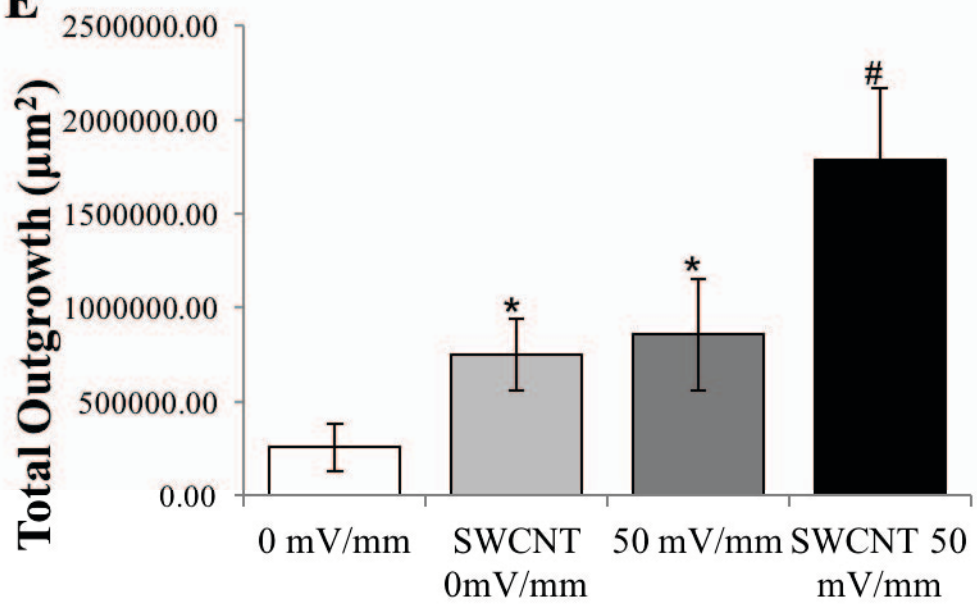
C

D

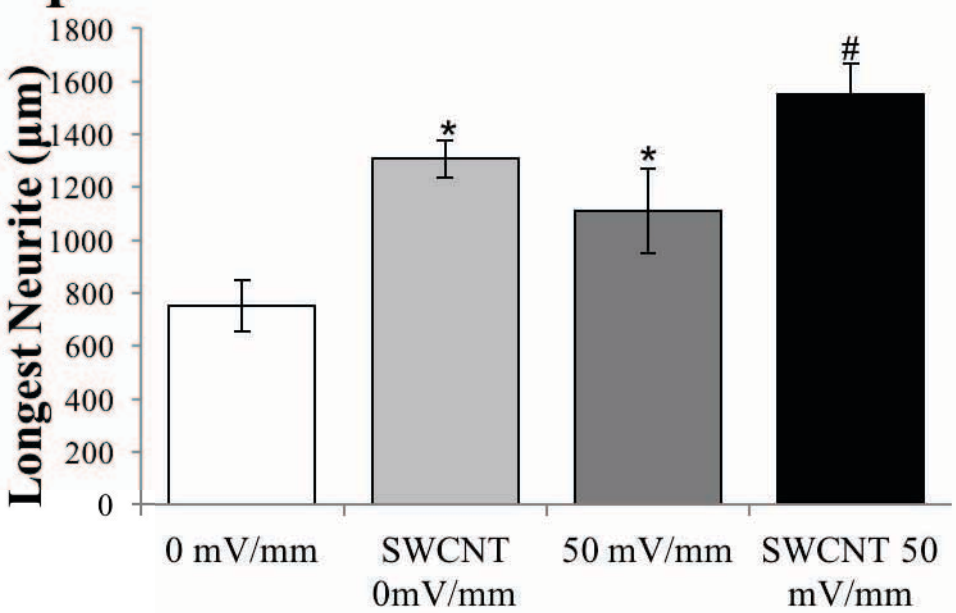
500 µm

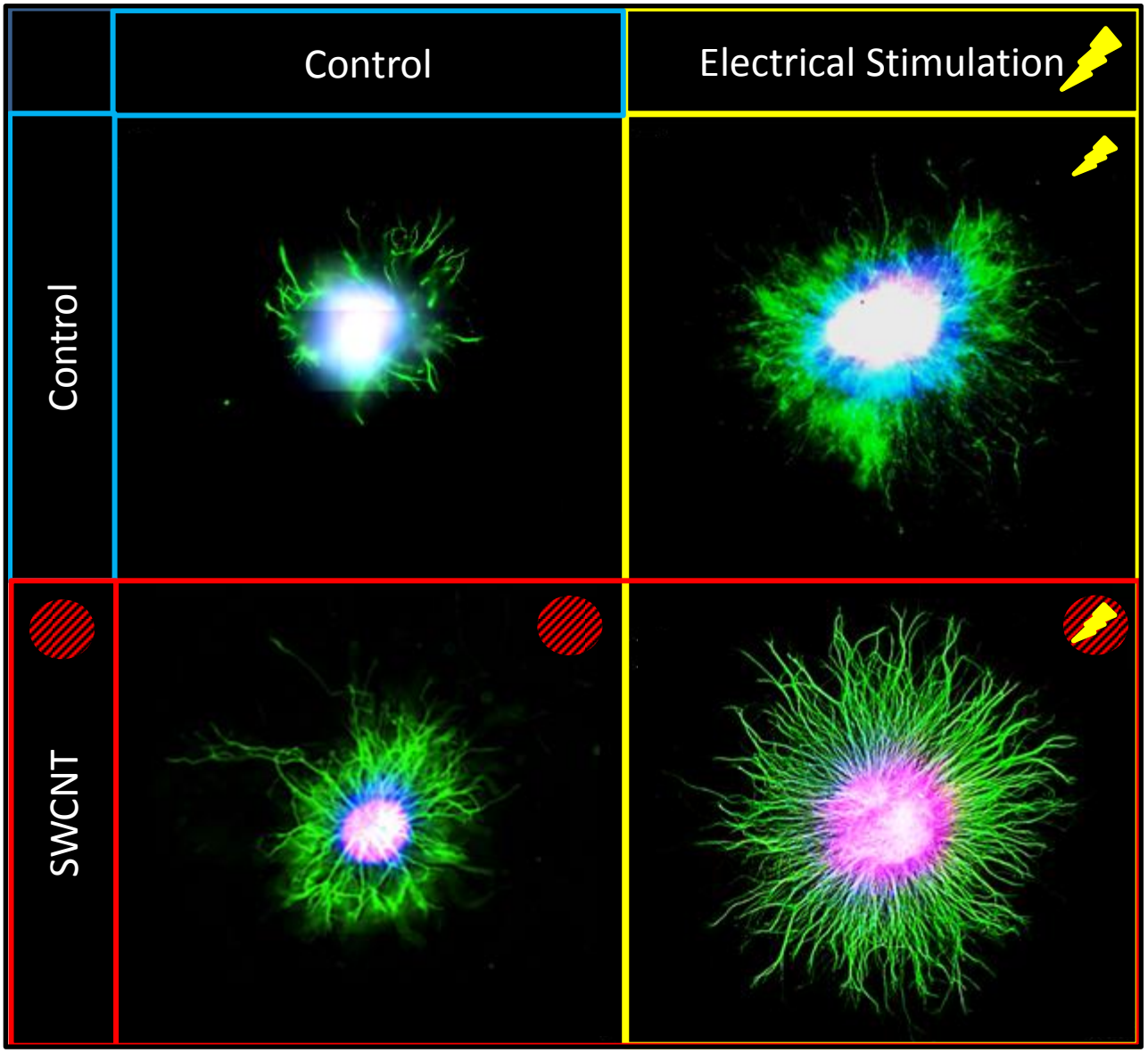


E



F




Control
Electrical Stimulation
Control
SWCNT

Robust Neurite Outgrowth Following Exogenous Electrical Stimulation within Single Walled Carbon Nanotube-Composite Hydrogels

Novel biomedical devices delivering electrical stimulation are being developed to mitigate symptoms of Parkinson's, treat drug-resistant depression, control movement or enhance verve regeneration. Carbon nanotubes and other novel materials are being explored for novel nano-neuro devices based on their unique properties. Neuronal growth on carbon nanotubes has been studied in 2D since the early 2000's demonstrating increased outgrowth, synapse formation and network activity. In this work, single-walled carbon nanotubes were selected as one possible electrically-conductive material, dispersed within a 3D hydrogel containing primary neurons; extending previous 2D work to 3D to evaluate outgrowth within nanomaterial composites with electrical stimulation. This is the first study to our knowledge that stimulates neurons in 3D composite nanomaterial-laden hydrogels. Examination of electrically conductive biomaterials may serve to promote regrowth following injury or in long term stimulation.