Neural Stem Cell Differentiation in Collagen Scaffolds for Retinal Tissue Engineering

by

Erica Ueda

B.S., Mechanical Engineering
University of California, Los Angeles, 2006

Submitted to the Department of Mechanical Engineering
in partial fulfillment of the requirements for the degree of

Master of Science in Mechanical Engineering

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
[June 2008]
May 2008

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Abstract

Rat neural stem cells (NSCs) were cultured in monolayer or in porous collagen scaffolds and exposed to neurogenic or non-neurogenic medium to determine the effects on neural differentiation and neurite growth. Nestin, βIII-tubulin, and GFAP expression were determined using immunofluorescent techniques, and the neurite length was measured.

NSCs differentiated into neurons, with actively growing neurites, and astrocytes when cultured in differentiation medium (DM) or neurogenic medium (NM). NSCs cultured in monolayer expressed more nestin and βIII-tubulin and had significantly longer neurite extensions than NSCs cultured in collagen scaffolds. Laminin coated scaffolds promoted the attachment of NSCs to the scaffold struts and resulted in a more even distribution of nestin and βIII-tubulin positive cells throughout the scaffold. Overall, NSCs cultured in DM for at least 14 days resulted in the most neuronal differentiation and neurite growth.

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Acknowledgments

I would like to thank my advisor, Professor Myron Spector, for all of his guidance, insight, and support throughout this project. I have truly enjoyed this exciting opportunity to explore tissue engineering and work in a great lab environment.

I would like to thank my VA Tissue Engineering labmates and lab manager, Alix Weaver, for teaching me all of the techniques needed for my experiments, but most of all for making the lab an enjoyable and friendly place to work.

I would like to thank my parents, especially my mom, who has always supported me and inspires me to always keep learning.

I would like to thank Cean for his constant encouragement and support, and for making me apply to MIT in the first place.

I would like to thank all of my friends who have made my MIT experience unforgettable.

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Chapter 1

Introduction

The ultimate goal of this research project is to develop a tissue engineered implant for the treatment of degenerative retinal disorders, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD). The implant consists of a thin, porous collagen scaffold seeded with select stem cells. In advance of starting an animal study, the optimal *in vitro* conditions necessary for the differentiation of various stem cells into neuronal cells in collagen scaffolds must be investigated. A 3D (collagen scaffold) rather than a 2D (monolayer) culture system is predicted to enhance the neuronal differentiation and synaptic potential of stem cells since collagen scaffolds can be designed to model the native tissue architecture and facilitate cell-cell interaction [11]. Collagen scaffolds also serve as a delivery vehicle to localize and organize the transplanted cells. To understand this tissue engineering based approach for the treatment of retinal diseases, one must first learn the basic structure and function of the retina and how it is affected by the various diseases.

1.1 Retina Structure and Function

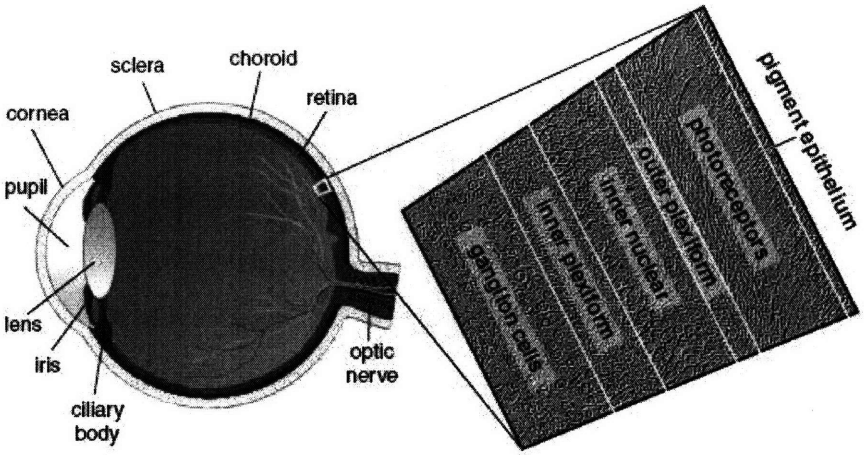
Light entering the eye is focused by the cornea and lens, and continues to pass through all of the retinal layers until it reaches the photoreceptors at the back of the retina. The photoreceptors are in contact with the retinal pigmented epithelium (RPE), which has two main functions. The first is the daily phagocytosis of rod and cone

outer segment fragments which are shed from their distal ends. The second function of the RPE is the uptake, processing, transport, and release of retinol (vitamin A) [2]. There are 125 million photoreceptors of which there are two types: rods and cones. Rods are used for low-light vision and cones are used for bright light, acute detail, black and white, and color vision. Cones are either green-, blue-, or red-sensitive. The fovea is a cone-dense region in the center of the macula containing more than half the total number of cones and is devoid of rods, thus generating focused, color vision.

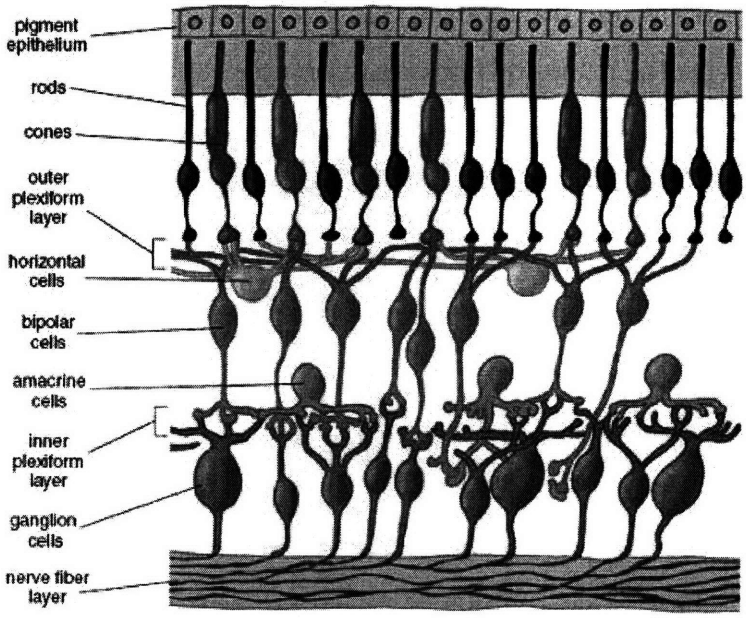
Figure 1-1 shows that the retina has a very layered structure. The inner nuclear layer consists of one of four types of horizontal cells, 11 types of bipolar cells, and 22 - 30 types of amacrine cells. Photoreceptors at the back of the eye form synapses with bipolar and horizontal cell dendrites in the outer plexiform layer. In the inner plexiform layer, bipolar and amacrine cells connect with ganglion cells at the surface of the retina. There are approximately 20 types of ganglion cells, and their impulses are sent through more than 1 million optic nerve fibers to the visual cortex in the brain. Blood vessels radiate from the optic nerve head to nourish the tissue. A very comprehensive and informative database on the anatomy of the retina and function of the visual system is presented by Helga Kolb, Eduardo Fernandez, and Ralph Nelson online at Webvision (webvision.med.utah.edu).

1.2 Retinal Diseases

In all forms of retinal degenerations, the photoreceptors eventually die via apoptosis [24]. Photoreceptor outer segments consist of approximately 1,000 stacked discs. The outer segments are renewed continually as discs at the distal end are shed and phagocytosed by RPE cells, and new discs are formed at the base of the outer segment. Two degeneration mechanisms are: (1) Disruption of photoreceptor outer segment morphogenesis and (2) RPE cell dysfunction.



(a) Eye



(b) Retinal layers

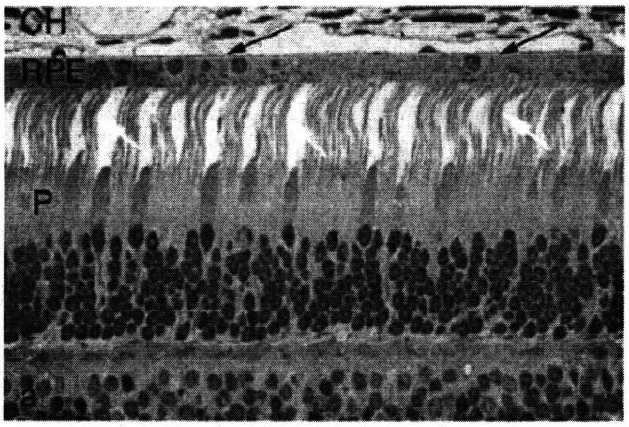
Figure 1-1: Retina [16]

1.2.1 Age-related macular degeneration

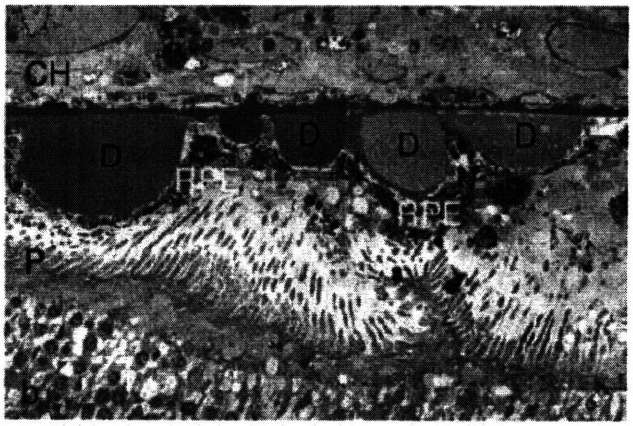
Age-related macular degeneration (AMD) affects the macula, a region approximately 6 mm in diameter located in the central axis of vision. Drusen (small, yellow deposits) forms between the RPE and Bruch's membrane, as shown in Figure 1-2(b). There are two forms of AMD: dry AMD and wet AMD. In dry AMD, hard drusen forms and the light sensitive cells in the macula slowly degenerate. There is no growth of new blood vessels from the choroid into the retina. Dry AMD accounts for about 85 - 90% of people with AMD. Wet AMD is characterized by soft drusen formation and new vessel ingrowth from the choroid toward the macula. These new vessels leak blood and fluid under the macula, causing rapid loss of central vision. In the normal retina, the photoreceptors are highly organized and their outer segments are in close contact with the RPE. In AMD retina, drusen formation causes distortion of the RPE and Bruch's membrane and disorganization of the photoreceptors. Drusen formation is thought to occur either due to inflammation after RPE injury and accumulation of monocyte and RPE cell debris, or accumulation of rod and cone outer segment fragments which are not properly phagocytosed by the RPE [3].

1.2.2 Retinitis pigmentosa

More than 45 genes for retinitis pigmentosa (RP) have been identified, but the most common cause of RP is a mutation in rhodopsin. RP is characterized by "bone-spicule" formations due to accumulation of pigment granules released by degenerating cells in the RPE. There are two forms of RP: rod-cone RP and cone-rod dystrophy. In rod-cone RP, the rods are predominantly affected and results in night blindness and loss of mid-peripheral vision. As the disease progresses, cones also begin to degenerate and day vision and acute central vision are compromised. In cone-rod dystrophy, the cones and rods degenerate simultaneously and more central vision is lost.

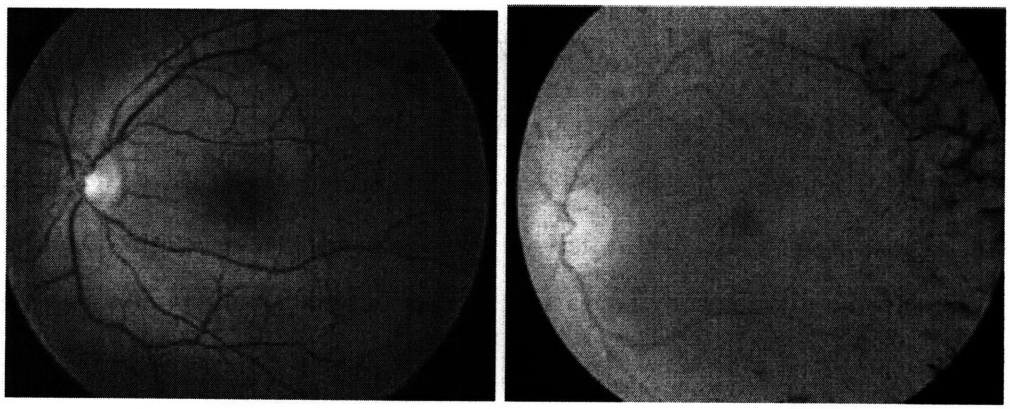


(a) Normal retina



(b) AMD retina

Figure 1-2: Normal retina vs. retina with age-related macular degeneration (AMD) [3]. CH = choriocapillaries, RPE = retinal pigmented epithelium, P = photoreceptors, D = drusen.



(a) Normal retina

(b) RP retina

Figure 1-3: Normal retina vs. retina with retinitis pigmentosa (RP) [15].

1.3 Animal Models and Stem/Progenitor Cells

The *rd* (retinal degeneration) mouse, *rds* (retinal degeneration slow) mouse, and RCS rat are a few animal models of spontaneous retinal degeneration. The *rd* mouse develops photoreceptors with outer segments, but they begin to rapidly degenerate beginning at postnatal day 8. The *rd* mutation primarily affects rods, and by age 21 the *rd* mouse only has a single row of cones in the outer nuclear layer. The *rds* mouse fails to develop photoreceptor outer segments and undergoes a degeneration of both rods and cones. In the RCS rat, irregular outer segments are first noticed at age 2 weeks and by age 3 months photoreceptor loss is complete. A defect in the Mertk gene reduces the ability of the RPE cells to phagocytose shed photoreceptor outer segments, resulting in accumulation of debris in the subretinal space.

The RCS rat is a good choice for an animal model because many studies using this animal model have been published and can be used for comparison. In one study, the investigators began by transplanting retinal tissue from rat fetuses into the subretinal space of RCS rats [32]. They later advanced to human clinical trials where they implanted human fetal retina and RPE subretinally [25, 27, 26]. Pigmentation characteristic of RP disappeared after 3 - 6 months, and no tissue destruction, fibrosis, or necrosis of the retina was observed. One patient reported to have a visual improvement from 20/800 prior to the operation to 20/160 12 months after the operation. This demonstrates that retinal tissue implants can have an impact and improve vision in patients, and motivates the investigation of using a stem cell-seeded collagen scaffold construct in place of the fetal retina and RPE.

The stem/progenitor cell selected for transplantation is critical to the success of tissue regeneration or repair. Brain-derived progenitor cells were reported to migrate into host retina and differentiate into neurons and glia, but they did not express retina-specific markers [31]. Whether Although rat neural stem cells obtained from the hippocampus were used in this study, future experiments using retinal progenitor cells (RPCs) should also be investigated for retinal tissue engineering applications. RPCs can differentiate into all six neuronal and one glial cell type of retinal lineage starting

with the development of retinal ganglion cells first, followed by overlapping phases of horizontal cells, cones, amacrine cells, rods, and bipolar cells [7]. RPCs can be obtained from the retinal ciliary margin and embryonic neuroretina. Unfortunately, there are only about 10,000 retinal stem cells per human eye making them difficult and expensive to obtain in large quantities [8, 10]. Using neural stem cells is still beneficial since they have the potential for controlled differentiation into all neural tissues and the capacity to home in on injury or inflammation sites and release trophic factors [30].

1.4 Objectives and Hypotheses

Previous studies have reported the differentiation of stem/progenitor cells in collagen gels and polymer scaffolds, but at this point no study to our knowledge has demonstrated the neural differentiation of stem cells in porous, sponge-like, collagen scaffolds [6, 13, 17, 18, 20, 23, 29, 31]. It is believed that these stem cell-seeded collagen scaffolds can replace lost tissue and/or stimulate a trophic effect to support the remaining surviving tissue. Prior to conducting an *in vivo* animal study where the stem cell-seeded collagen scaffold would be implanted into the subretinal space of an animal model, the tissue engineered construct must be developed and characterized *in vitro*.

Objectives:

- Develop a collagen scaffold construct for *in vitro* culture of stem cells
- Differentiate rat neural stem cells in monolayer and in collagen scaffolds toward the neural lineage
- Use immunofluorescent and confocal imaging to identify neural progenitors, neurons, and astrocytes
- Measure the percentage of nestin, βIII-tubulin, and GFAP staining and the neurite length

- Compare the effects of the following independent variables on neural differentiation:

  Cell type: Neural stem cell vs. Marrow-derived stromal cell

  Substrate: Monolayer vs. Collagen scaffold

  Medium: Neurogenic vs. Non-neurogenic

  Culture time

  Laminin vs. Non-laminin coated scaffold

Hypotheses:

- Rat neural stem cells will express a higher percentage of nestin, βIII-tubulin, and GFAP expression and grow longer neurite extensions than rat marrow-derived stromal cells.

- Collagen scaffolds will enhance cell interactions and thus upregulate neural differentiation and neurite extension compared to monolayer cultures.

- Cells cultured in neurogenic medium will express a higher percentage of nestin, βIII-tubulin, and GFAP expression and grow longer neurite extensions.

- The percentage of nestin, βIII-tubulin, and GFAP expression will increase and neurite extensions will grow with time.

- Laminin coated scaffolds will increase cell attachment and interactions and upregulate neural differentiation and neurite extension.

Chapter 2

Materials and Methods

2.1 Collagen Scaffold Fabrication

To study the behavior and differentiation potential of stem cells in three-dimensions, stem cells were seeded into porous collagen scaffolds. The scaffolds were composed of 0.5% Bio-Gide® porcine type I/III collagen powder obtained from Geistlich Pharma AG, Wolhusen, Switzerland. 10 ml of collagen slurry per plastic mold was cooled at 1°C/min to -40°C, and freeze-dried according to the program steps shown in Table A.1 on page 67 to produce collagen sheets approximately 0.5 - 1 mm thick. The collagen sheets were dehydrothermally (DHT) cross-linked in a vacuum oven at 105°C for 24 hours. This process creates covalent cross-links between the collagen fiber polypeptide chains without denaturing the collagen [22]. Dermal biopsy punches were then used to punch 8 mm diameter scaffolds. The collagen scaffolds were hydrated in decreasing concentrations of ethanol, and then further cross-linked chemically in a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) solution for 30 minutes at room temperature.

In earlier experiments, it was observed that the collagen scaffolds autofluoresced when viewed under a fluorescent microscope and sometimes made it difficult to distinguish the collagen scaffold fibers from the fluorochrome of interest. To resolve this issue, the collagen scaffolds were dyed with either Alexa Fluor 488 or 555 so that the collagen scaffold would fluoresce either green or red, respectively, to contrast the

fluorochrome. The protocol on page 71 was adapted from Brendan Harley's PhD Thesis [14]. The Alexa Fluor succinimidyl esters link the fluorochrome to the primary amines (R-NH_2) contained in the collagen scaffold. The Alexa Fluor dyes have proved to be bright and photostable.

2.2 Collagen Scaffold Pore Size Analysis

To measure the average scaffold pore size, six different collagen scaffolds fabricated under the same freeze-drying conditions given in Table A.1 were embedded in JB-4, sectioned at 6 μm on a microtome, and stained with aniline blue according to the *Collagen Scaffold Pore Analysis Protocols* on page 72. Six sections from each of the six scaffolds, except for one scaffold which only had five sections, were imaged at 4$\times$ using an Olympus light microscope with a digital camera. The TIFF files were converted to greyscale images and processed using Scion Image Software (Scion Corporation, Frederick, MD). The pore characterization macro calculated the distance between the pore walls along lines at various angles radiating from the center of the selected elliptical region, and then transformed the average distance between struts along each line into a best-fit ellipse representing the average pore cross-section. The minor and major axes of the best-fit ellipse are calculated using equations A.3 and A.4, respectively, which are then used to calculate the mean pore diameter using equation A.6. To account for the effect of pores sectioned at an arbitrary angle rather than through their maximal cross-section, the ellipse major and minor axes are corrected by multiplying by 1.5.

2.3 Stem Cell Culture

Adult rat hippocampal neural stem cells (NSCs) and rat marrow-derived stromal cells (MSCs) were used in 1D monolayer and 3D collagen scaffold experiments. NSCs labeled HCN-A94-2 P12 were obtained from Fred Gage, and were then expanded to P15 and stored at -80°C with 1.5 million cells/vial. The MSCs were isolated from the

femur and tibia of young rats less than 6 weeks old, expanded in T150 flasks at P0, passaged and replated in T150 flasks at P1, and stored in vials frozen at -80°C with 500,000 cells/vial.

2.3.1 Neural stem cell experiments

All cultureware used to grow NSCs were first coated according to the *Poly-L-ornithine and Mouse Laminin Coating Protocol* on page 86. The NSCs from each vial were thawed, plated at P16, and cultured in a T150 flask with 30 ml of rat NSC expansion medium (EM) in an incubator maintained at 37°C, 5% CO_2, and 95% relative humidity. The medium was changed every 2 - 3 days until the cells became confluent after 4 - 5 days. The rat NSC EM consisted of D-MEM/F12 (high glucose) containing L-glutamine, 1% N-2 supplement, 1% penicillin/streptomycin, and 20 ng/ml basic fibroblast growth factor (bFGF). The bFGF was added fresh each time.

For monolayer experiments, NSCs were counted, resuspended, and replated in 24-well plates at 5,000 cells/cm^2 at P17 with 0.5 ml/well of rat NSC EM medium. For collagen scaffold experiments, 12-well plates were coated with 500 μl/well of agarose. Hydrated collagen scaffolds were then placed flat against the bottom of each well, and the excess water in the scaffolds was absorbed by lightly placing the edge of filter paper strips on the scaffold. NSCs were seeded onto collagen scaffolds at 200,000 or 1 million cells/scaffold in a 10 or 20 μl suspension at P17. Wait at least 10 minutes to allow the cells to attach to the scaffold before slowly pipetting 1.5 - 2 ml/well of rat NSC EM. The NSCs were cultured for 2 days in rat NSC EM in the well plates or collagen scaffolds in an incubator maintained at 37°C, 5% CO_2, and 95% relative humidity to allow the cells to proliferate and infiltrate the collagen scaffold.

Next, the NSCs were cultured in either rat NSC differentiation medium (DM) or rat NSC neurogenic medium (NM) to induce neural differentiation in monolayer (1D) or in the collagen scaffolds (3D). The rat NSC DM was used to differentiate the cells into three neural lineages: neurons, astrocytes, and oligodendrocytes. It consisted of D-MEM/F12 (high glucose) containing L-glutamine, 1% N-2 supplement, 1% penicillin/streptomycin, 0.5% FBS, and 1 μM *all-trans*-Retinoic acid. The

rat NSC NM was used to differentiate the cells into only neurons, and consisted of D-MEM/F12 (high glucose) containing L-glutamine, 1% N-2 supplement, 1% penicillin/streptomycin, 1 μM *all-trans*-Retinoic acid, and 5 μM forskolin. Cells continued to be cultured in rat NSC EM were used as the control group. The media was changed every 2-3 days and the scaffold contraction was measured until the experiment was terminated at 6 or 14 days post induction.

2.3.2 Marrow-derived stromal cell experiments

The MSCs from each vial were thawed, plated at P2, and cultured in a T150 flask with 30 ml of rat MSC expansion medium (EM) in an incubator maintained at 37°C, 5% CO_2, and 95% relative humidity. The medium was changed every 2 - 3 days until the cells became confluent after 6 - 7 days. The rat MSC EM consisted of DMEM-LG (low glucose), 20% FBS, 1% antibiotic-antimycotic, and 10 ng/ml bFGF. The bFGF was added fresh each time.

For monolayer experiments, MSCs were counted, resuspended, and replated in 24-well plates at 8,000 cells/cm^2 at P3 with 0.5 ml/well of rat MSC EM medium. For collagen scaffold experiments, 12-well plates were coated with 500 μl/well of agarose. Hydrated collagen scaffolds were then placed flat against the bottom of each well, and the excess water in the scaffolds was absorbed by lightly placing the edge of filter paper strips on the scaffold. MSCs were seeded onto collagen scaffolds at 200,000 or 1 million cells/scaffold in a 10 or 20 μl suspension at P3. Wait at least 10 minutes to allow the cells to attach to the scaffold before slowly pipetting 1.5 - 2 ml/well of rat MSC EM. The MSCs were cultured for 2 days in rat MSC EM in well plates or collagen scaffolds in an incubator maintained at 37°C, 5% CO_2, and 95% relative humidity to allow the cells to proliferate and infiltrate the collagen scaffold, and the scaffold contraction was measured.

Next, the MSCs were cultured in rat MSC neurogenic medium (NM) to induce neural differentiation in monolayer (1D) or in the collagen scaffolds (3D). The rat MSC NM consisted of DMEM-LG (low glucose), 2% DMSO, 200 μM butylated hydroxyanisole, 10 μM forskolin, 1 μM hydrocortisone, 5 μg/ml insulin, 25 mM KCl,

and 2 mM valproic acid [33]. Cells continued to be cultured in rat MSC EM were used as the control group. The experiment was terminated at 5 hours post induction.

2.4 Immunofluorescent Staining

The cells in monolayer and in the collagen scaffolds were fixed in 4% paraformaldehyde-PBS for 15 and 30 minutes, respectively. The cell-seeded collagen scaffolds were transferred to tissue processor cassettes and processed according to the tissue processor program in Table A.2 on page 95. The cell-seeded scaffolds were then embedded in paraffin, sectioned at 6 μm on a microtome, and baked at 60°C for 1 - 2 hours on a slide warmer. Prior to immunofluorescent staining, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol.

The *Immunofluorescent Staining Protocols* in section A.7 were followed. On day 1, the cells were incubated in primary antibody diluted 1:100 in antibody diluent overnight at 4°C in a humid chamber. Mouse anti-nestin monoclonal antibody (Chemicon, Cat. #MAB353) is a marker for neural progenitor cells; mouse βIII-tubulin monoclonal antibody (TUJ1) (Covance, Cat. #MMS-435P) and rabbit βIII-tubulin monoclonal antibody (TUJ1) (Covance, Cat. #MRB-435P) are markers for immature neurons; rabbit anti-GFAP polyclonal antibody (Dako, Cat. #Z0334) is a marker for astrocytes. On day 2, the cells were washed with PBS for 3 $\times$ 5 minutes and then incubated in secondary antibody diluted 1:200 in PBS for 60 minutes at room temperature in the dark. Rhodamine (TRITC) anti-mouse (Jackson Immunoresearch, Cat. #715-025-150) fluoresces red; Fluorescein (FITC) anti-rabbit (Jackson Immunoresearch, Cat. #711-095-152) fluoresces green. The cells were washed with PBS for 3 $\times$ 5 minutes and then fixed in 4% paraformaldehyde-PBS. To stain the nuclei, the cells were incubated in 200 ng/ml of DAPI diluted in PBS for 60 minutes at room temperature in the dark. The DAPI-stained nuclei appear bright blue under a UV light. The cells were washed with PBS for 3 $\times$ 5 minutes. Glass slides were kept wet while coverslipping with Faramount Aqueous Mounting Medium, or well plates were kept moist with PBS. Fluorescent images were acquired using the

Olympus BX60 fluorescent microscope and DP71 digital camera located at the VA Tissue Engineering Laboratories, Room 118.

2.5 Immunohistochemical Staining

Cell-seeded collagen scaffolds were immunohistochemically stained with hematoxylin and eosin (H&E) and α-smooth muscle actin (SMA). Paraffin-embedded sections were baked at 60°C for 1 - 2 hours on a slide warmer, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. For H&E staining, the slides were dipped in Gills 2× Hematoxylin solution (Protocol, Cat. #245-654, filtered prior to use) for 3 minutes, rinsed in running tap water for 5 minutes, dipped a few times in acid alcohol, rinsed in running tap water for 5 minutes, and dipped in Eosin Y Solution Aqueous (Sigma, Cat. #HT110-2-128) for 1 minute. The slides were then dehydrated in 100% ethanol and xylene, and coverslipped with Cytoseal. The DAKO autostainer was used for α-SMA staining according to the protocol on page 104, and the slides were counterstained with Mayer's Hematoxylin (Sigma, Cat. #MHS16) for 1.5 minutes. The slides were kept wet while coverslipping with Faramount Aqueous Mounting Medium.

2.6 Statistical Analysis

Data were analyzed and plots were generated using StatView software (SAS Institute Inc., Cary, NC). An unpaired t-test compares the means of two groups and determines the likelihood that the observed difference occurred by chance. A significant difference between the means was assumed when $p < 0.05$, rejecting the null hypothesis of no difference between the groups. An analysis of variance (ANOVA) evaluates the effect of nominal independent variables on a continuous dependent variable, and determines the significance of each effect by calculating how much of the variability in the dependent variable can be explained by that effect. When $p < 0.05$, the null hypothesis was rejected and it was assumed that the effect was significant in helping

to explain the behavior of the dependent variable. The power is the probability of correctly rejecting a false null hypothesis. To control for the incorrect rejection of the null hypothesis when it was actually true, known as a type I or alpha error, the alpha value was set at $\alpha = 0.05$. The Fisher's protected least significant difference (PLSD) post hoc test is used to determine which mean values of the dependent variable for each factor are different from each other by evaluating all possible pair-wise comparisons with a multiple t-test. Bar and line plots are mean $\pm$ standard error of the mean (SEM).

Chapter 3

Results

3.1 Collagen Scaffold Pore Size Analysis

The mean pore diameter was calculated for six 8 mm diameter, 0.5 - 1 mm thick, 0.5% Bio-Gide® porcine type I/III collagen scaffolds fabricated via the same freeze-drying conditions. Measurements were made for six sections from each of the six scaffolds, except for one scaffold which only had five sections. The total mean pore diameter was 136 μm. An unpaired t-test showed a significant difference ($p < 0.05$) between the mean pore diameters of all scaffolds except scaffolds s2 and s4, s3 and s5, s4 and s5, s4 and s9, and s5 and s9 (Table B.1). Scaffolds 3 and 6 had the largest and smallest mean pore diameters, respectively.

Scaffold	Mean (μm)	Std. Dev.	Std. Error	Count	Min	Max	Median
Total	136.29	29.43	4.97	35	72.89	196.67	136.00
s2	124.22	9.52	3.89	6	107.22	134.27	125.52
s3	172.40	23.26	9.50	6	131.44	196.67	177.39
s4	131.64	33.05	13.49	6	72.89	164.05	136.34
s5	152.20	24.93	11.15	5	112.65	173.66	156.59
s6	99.71	2.16	0.88	6	97.27	103.17	99.59
s9	140.23	4.49	1.84	6	136.00	147.61	138.54

Table 3.1: Collagen scaffold mean pore diameter (μm)

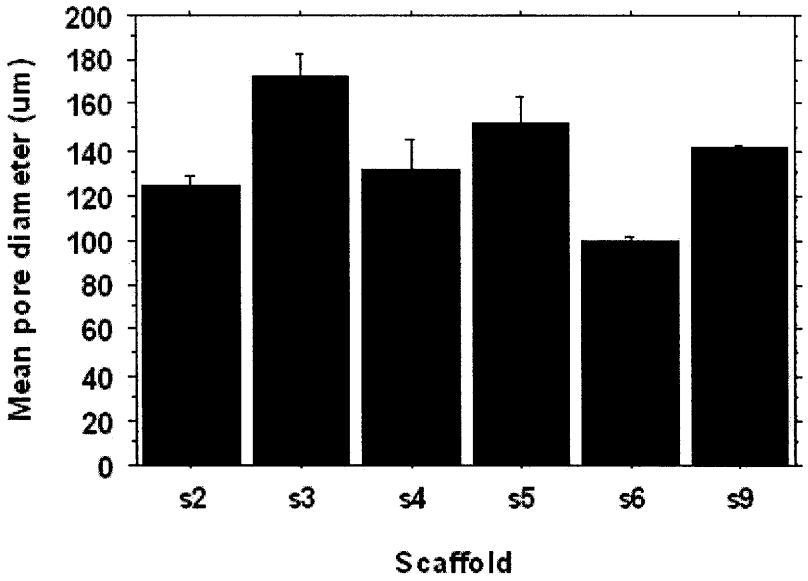


Figure 3-1: Collagen scaffold mean pore diameter (μm) $\pm$ SEM

3.2 Collagen Scaffold Contraction Measurements

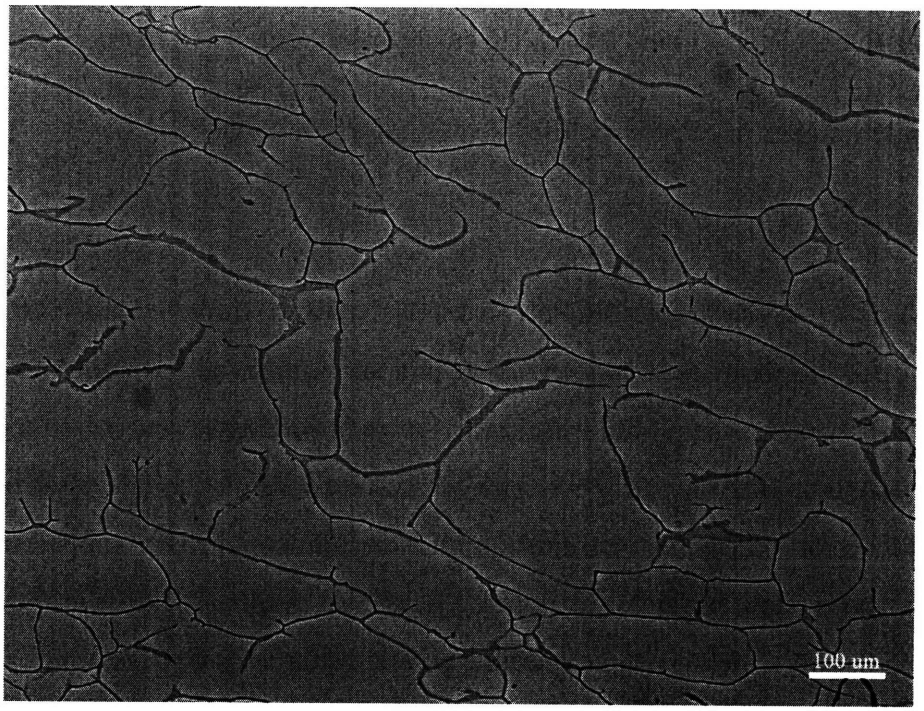
As the cells were cultured in the collagen scaffolds, the scaffold diameter was measured at various time points. Contraction was observed for the MSC-seeded collagen scaffolds, whereas no scaffold contraction was observed for any of the NSC-seeded collagen scaffolds. Scaffold contraction was recorded for three different experimental conditions.

Experiment 4 conditions:

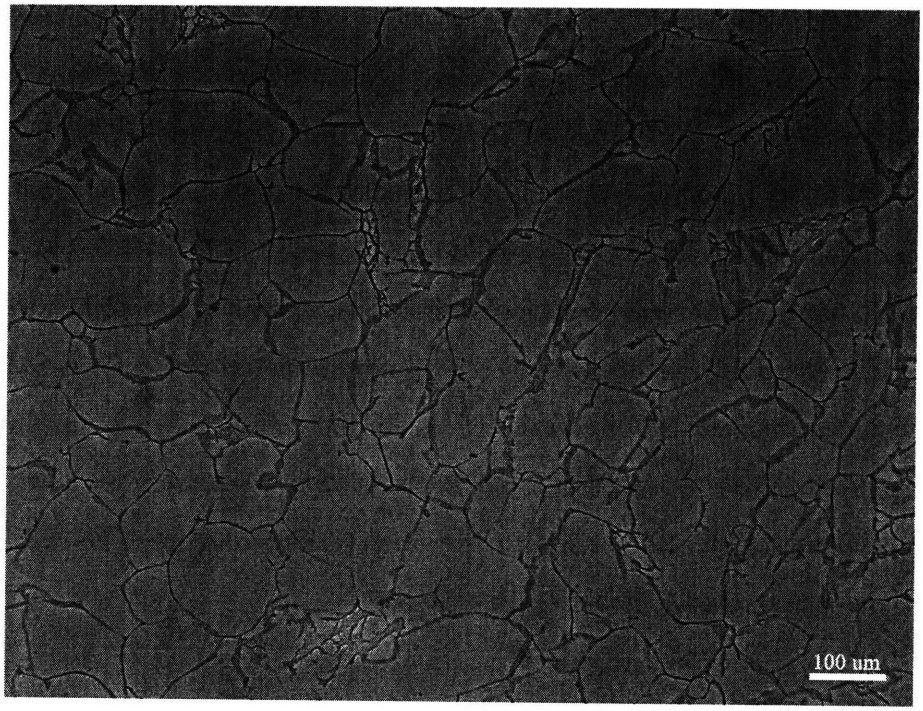
- 0.5% Bio-Gide® porcine type I/III collagen scaffold
- 9 ml of slurry in plastic mold cooled at 0.13°C/min to -20°C
- 200,000 cells/scaffold

Experiment 5 conditions:

- 0.5% Bio-Gide® porcine type I/III collagen scaffold
- 10 ml of slurry in plastic mold cooled at 1°C/min to -40°C
- 275,000 cells/scaffold



(a) Largest mean pore diameter = 172 μm, s3



(b) Smallest mean pore diameter = 100 μm, s6

Figure 3-2: Collagen scaffold largest and smallest mean pore diameter

Experiment 6 conditions:

- 0.5% Bio-Gide® porcine type I/III collagen scaffold
- 10 ml of slurry in plastic mold cooled at 1°C/min to -40°C
- 1 million cells/scaffold

The MSC-seeded collagen scaffold contraction data in Table 3.2 are split by days in culture post seeding, rat number, experimental condition, and type of medium. Figure 3-3 shows that the scaffolds, initially 8 mm in diameter, contracted with time in culture. A repeated measures ANOVA was used to study the effects of between-subject factors of experimental condition and rat number on the compact variable measuring the scaffold diameter at 0 and 2 days post seeding. Both the rat number ($p = 0.0008$, power = 0.965) and experimental condition ($p = 0.0018$, power = 0.925) had a significant effect on the scaffold diameter, but the time in culture post seeding had the most significant effect ($p < 0.0001$, power = 1.000). Fisher's PLSD for scaffold diameter showed a significant difference between rat MSC #1 and #3 ($p = 0.0046$), #3 and #4 ($p = 0.0398$), #3 and #5 ($p = 0.0002$), and #4 and #5 ($p = 0.0028$). Fisher's PLSD also showed a significant difference between experiments 4 and 5 ($p = 0.0010$) and experiments 4 and 6 ($p = 0.0011$).

Both MSC- and NSC-seeded collagen scaffolds were stained for α-SMA, a marker for contractile cells. Only the MSC-seeded scaffolds stained positive for α-SMA (Figure 3-5), whereas all of the NSC-seeded scaffolds in the various media stained negative for α-SMA (Figure 3-6). Most of the cells expressing α-SMA were located near the edge of the scaffold. This concurs with the observation that only the MSC-seeded scaffolds showed apparent contraction in culture.

3.3 Neural Stem Cell Experiments

NSCs were cultured in either rat NSC differentiation medium (DM) or rat NSC neurogenic medium (NM) to induce neural differentiation in monolayer (1D) or in

	Mean (mm)	Std. Dev.	Std. Error	Count	Min	Max
Diameter, d0, m5, exp4, EM	8.0	0.0	0.0	7	8.0	8.0
Diameter, d0, m1, exp4, EM	8.0	0.0	0.0	7	8.0	8.0
Diameter, d0, m1, exp6, EM	8.0	0.0	0.0	5	8.0	8.0
Diameter, d0, m3, exp5, EM	8.0	0.0	0.0	9	8.0	8.0
Diameter, d0, m4, exp5, EM	8.0	0.0	0.0	24	8.0	8.0
Diameter, d0, m4, exp6, EM	8.0	0.0	0.0	24	8.0	8.0
Diameter, d2, m5, exp4, EM	6.9	1.0	0.4	7	5.0	8.0
Diameter, d2, m1, exp4, EM	6.5	1.2	0.5	7	5.0	8.0
Diameter, d2, m1, exp6, EM	6.0	0.4	0.2	5	5.5	6.5
Diameter, d2, m3, exp5, EM	5.3	0.5	0.2	9	4.5	6.0
Diameter, d2, m4, exp5, EM	6.0	0.8	0.2	24	5.0	7.5
Diameter, d2, m4, exp6, EM	5.8	0.7	0.1	24	4.5	7.5
Diameter, d3, m5, exp4, EM	6.4	1.5	0.6	7	3.5	8.0
Diameter, d3, m1, exp4, EM	5.7	1.7	0.6	7	4.0	8.0
Diameter, d3, m3, exp5, EM	5.1	0.7	0.2	9	4.5	6.0
Diameter, d3, m4, exp5, EM	5.4	0.9	0.2	24	4.0	7.0
Diameter, d4, m5, exp4, NM	4.5			1	4.5	4.5
Diameter, d4, m1, exp4, NM	5.5			1	5.5	5.5
Diameter, d8, m1, exp6, EM	3.8	0.4	0.3	2	3.5	4.0
Diameter, d8, m4, exp6, EM	3.5	0.7	0.5	2	3.0	4.0

Table 3.2: MSC-seeded collagen scaffold mean diameter (mm). d0, d2, d3, d4, d8 = days post seeding; m5, m1, m3, m4 = Rat MSC #; EM = rat expansion medium; NM = rat neurogenic medium.

collagen scaffolds (3D). Cells continued to be cultured in rat NSC expansion medium (EM) were used as the control group. The % nestin, % βIII-tubulin (TUJ1), and % GFAP positively stained cells were counted and the neurite length was measured for both monolayer and NSC-seeded collagen scaffold cultures at 6 or 14 days post exposure to induction medium. All of the statistical data is provided in section B.3.

3.3.1 Monolayer vs. collagen scaffold

The substrate, either a laminin coated well or collagen scaffold, had a significant effect on the neural differentiation and neurite growth of NSCs cultured in DM or NM for 6 days under the experiment 4 conditions listed on page 32. The medium type ($p = 0.0027$, power $= 0.920$), substrate ($p = 0.0154$, power $= 0.719$), and their interaction ($p = 0.0103$, power $= 0.781$) all had a significant effect on the percent

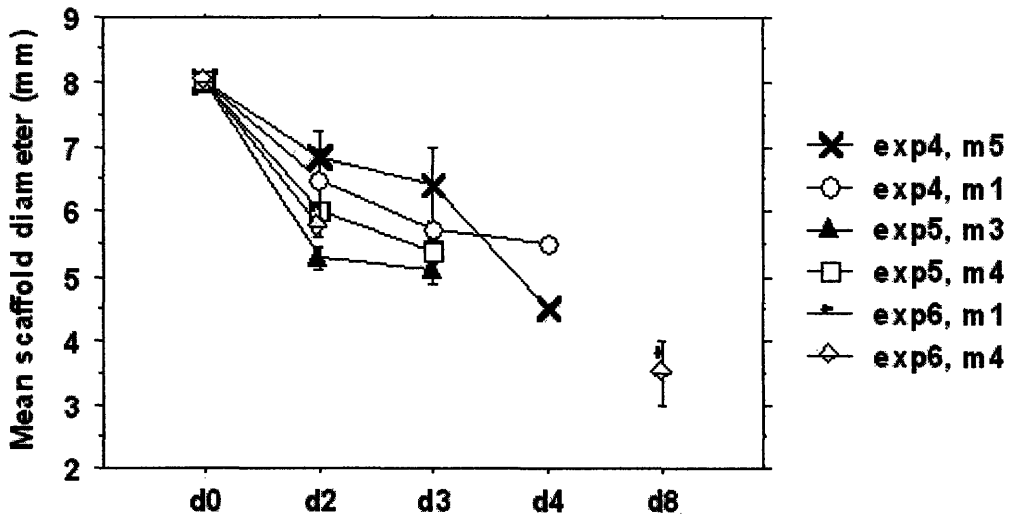
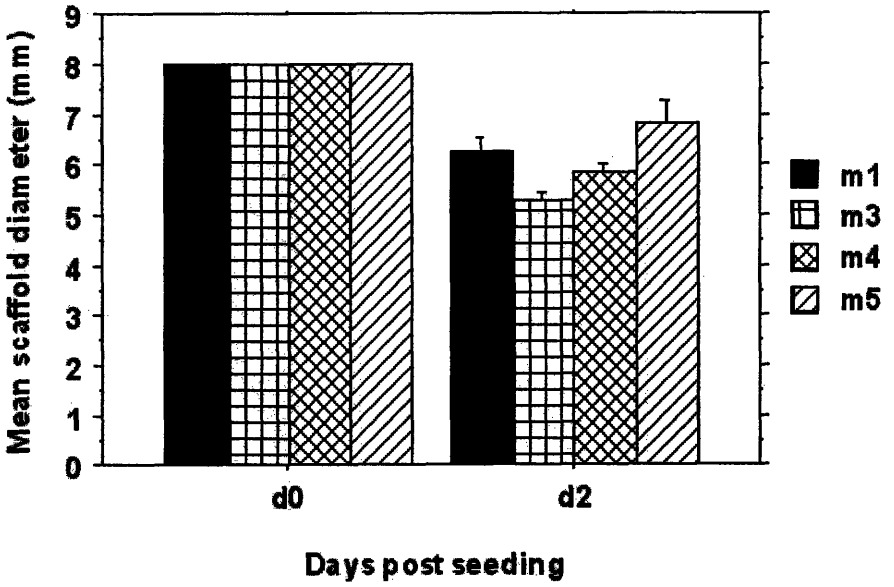


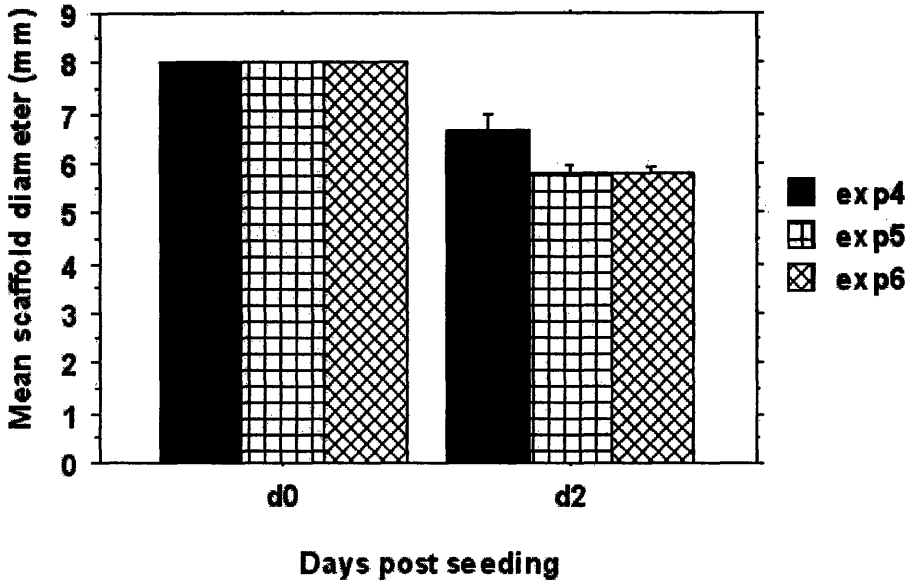
Figure 3-3: MSC-seeded collagen scaffold contraction. Mean diameter (mm) ± SEM.

of nestin positive cells. The NSCs in both monolayer and scaffolds expressed 25% nestin in DM, but in NM the NSCs in monolayer expressed more than twice as much nestin as those in scaffolds. The substrate ($p < 0.0001$, power = 1.000) significantly affected the neurite length of nestin expressing cells in both DM and NM, with NSCs in monolayer having processes twice as long as those cultured in collagen scaffolds.

Only the substrate had a significant effect on the percent of TUJ1 positive cells ($p = 0.0211$, power = 0.678) and on the neurite length of TUJ1 expressing cells ($p = 0.0075$, power = 0.840), with NSCs in monolayer having longer processes than those in collagen scaffolds. NSCs in monolayer and scaffolds expressed approximately 40 - 50% TUJ1 positive in DM, but in NM the NSCs in monolayer expressed more than twice as much TUJ1 as those in scaffolds. There were not enough samples to do a proper ANOVA on the GFAP expression. However, as expected, the GFAP expression was very low for NSCs cultured in NM since the NM was reported to differentiate cells down the neural and not glial lineage. Figures 3-7 and 3-8 show the interaction between these variables. The nestin, TUJ1, and GFAP expression in EM, DM, and NM were confirmed using the fluorescent and confocal microscopes (Figures 3-9 and 3-10).

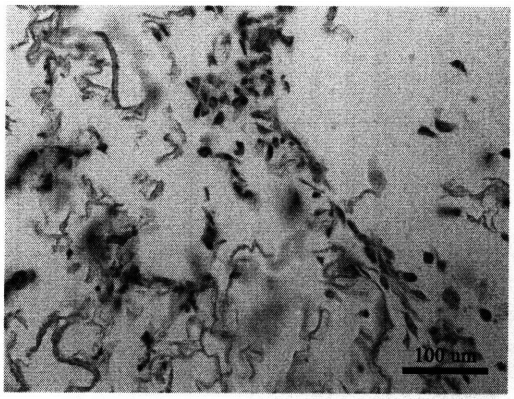


(a) Scaffold contraction split by rat number

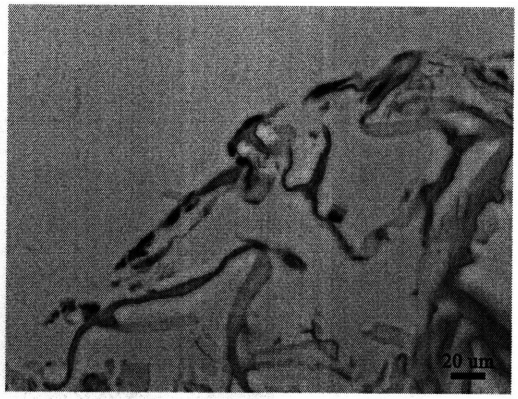


(b) Scaffold contraction split by experiment

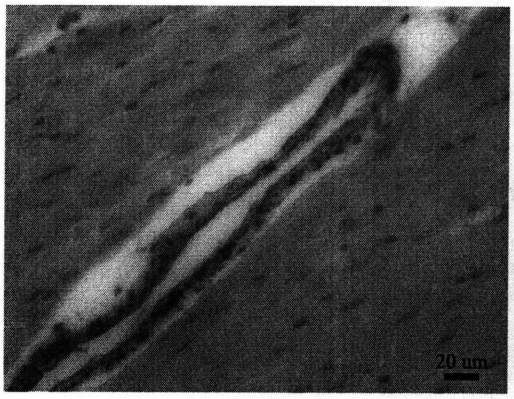
Figure 3-4: Repeated measures ANOVA for MSC-seeded collagen scaffold contraction. Mean diameter (mm) $\pm$ SEM.



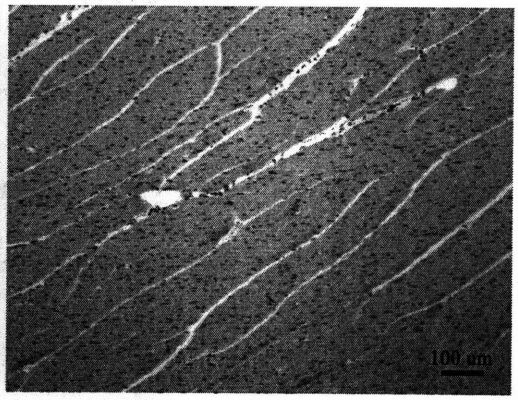
(a) α-SMA staining. Rat #1 MSC in neurogenic medium for 5 hours.



(b) α-SMA staining. Rat #4 MSC in expansion medium.



(c) α-SMA staining. Positive control goat heart tissue.

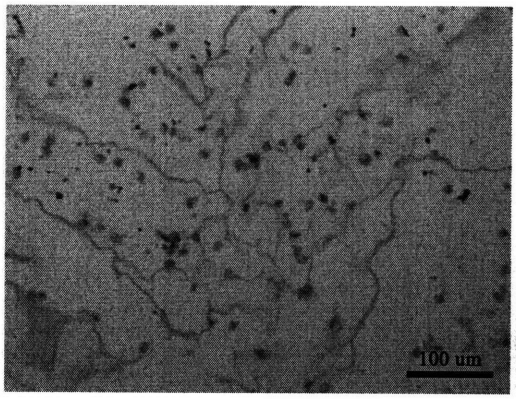


(d) α-SMA staining. Negative control goat heart tissue.

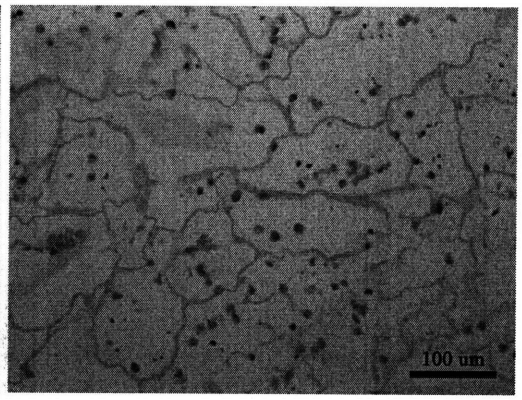
Figure 3-5: α-SMA stained MSC-seeded collagen scaffolds, counterstained with Mayer's hematoxylin.

3.3.2 Neurogenic vs. non-neurogenic medium

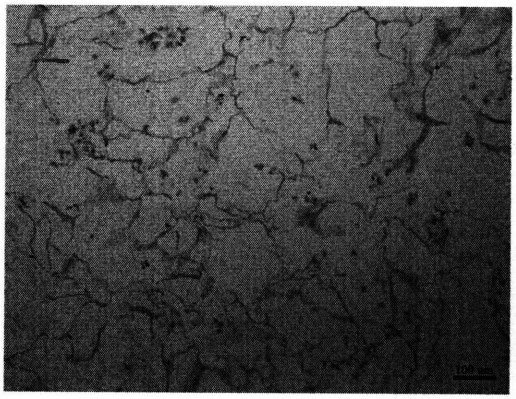
To test the effects of culturing the rat NSCs in neurogenic versus non-neurogenic medium, data from experiment 5 was used to compare the percent of immunofluorescent staining and process length in NSC-seeded collagen scaffolds cultured in EM, DM, or NM for 6 or 14 days. An ANOVA showed that the medium type significantly affected the % nestin ($p < 0.0001$, power = 1.000), and that some interaction between culture time and medium type had a significant effect on the % TUJ1 expression ($p = 0.0285$, power = 0.682). Further analysis using Fisher's PLSD post hoc test showed a significant difference in the % nestin staining between all three media (EM, DM, NM), which is apparent as shown in Figure 3-11. NSCs cultured in DM expressed the most nestin, and those cultured in EM expressed the least nestin. Fisher's PLSD



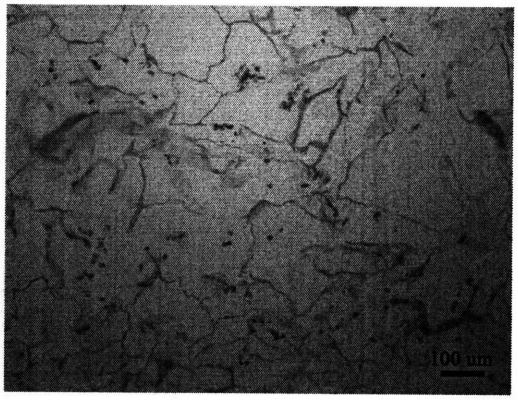
(a) α-SMA staining. Rat NSC in expansion medium.



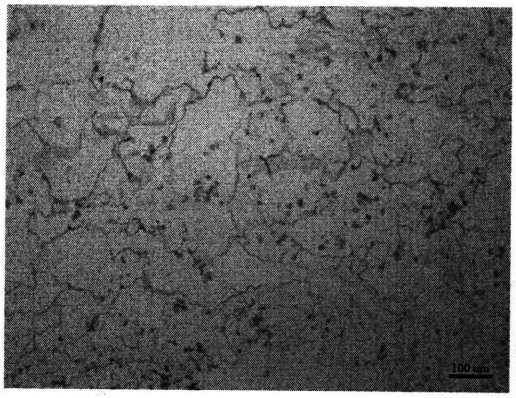
(b) α-SMA staining. Rat NSC in expansion medium, negative control.



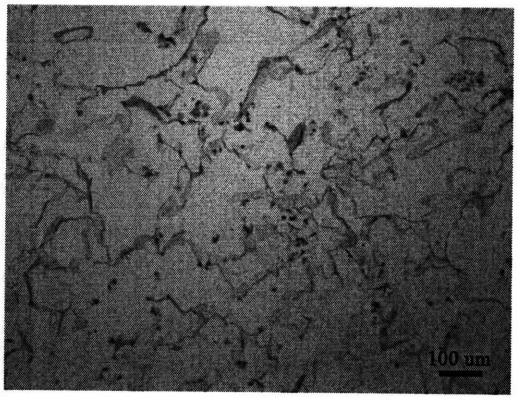
(c) α-SMA staining. Rat NSC in differentiation medium for 6 days.



(d) α-SMA staining. Rat NSC in differentiation medium for 6 days, negative control.

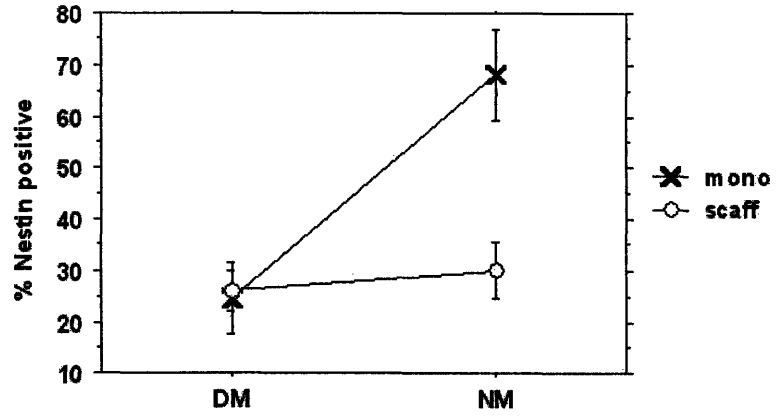


(e) α-SMA staining. Rat NSC in neurogenic medium for 6 days.

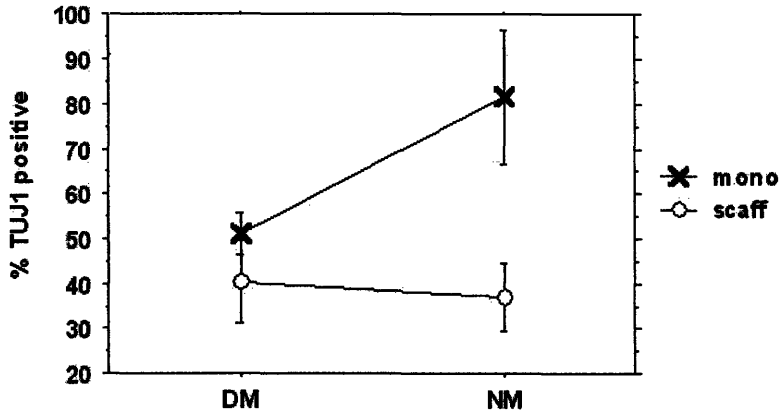


(f) α-SMA staining. Rat NSC in neurogenic medium for 6 days, negative control.

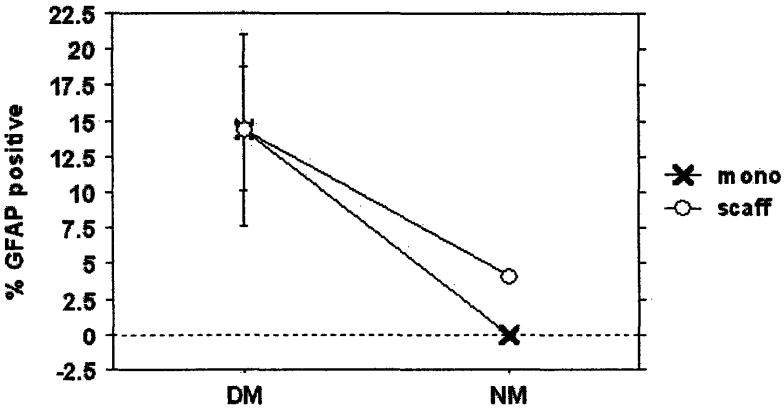
Figure 3-6: α-SMA stained NSC-seeded collagen scaffolds, counterstained with Mayer's hematoxylin.



(a) % Nestin positive

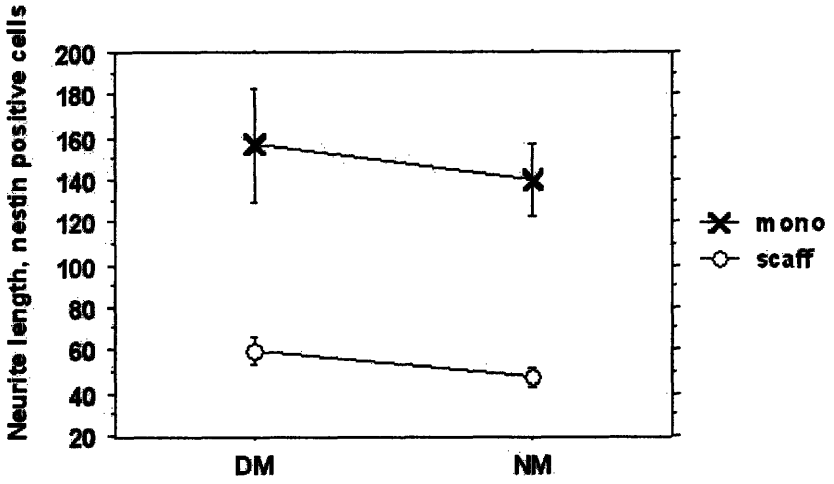


(b) % TUJ1 positive

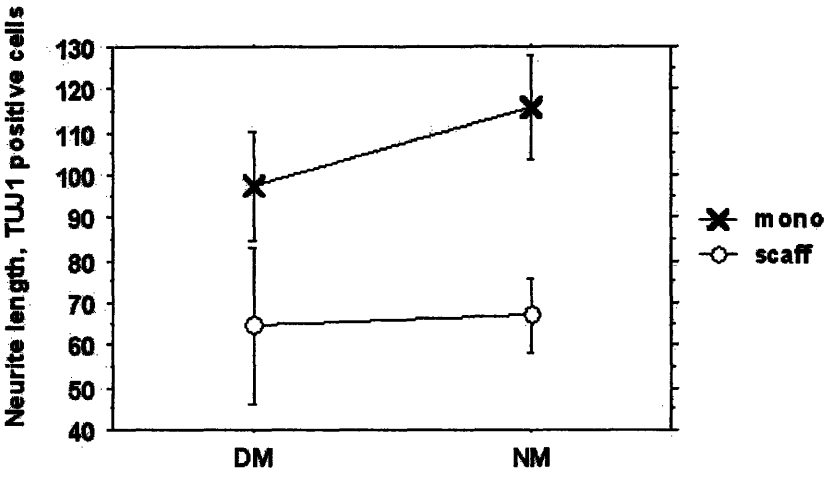


(c) % GFAP positive

Figure 3-7: Effects of monolayer vs. scaffold for rat NSCs cultured in DM or NM for 6 days. Mean ± SEM.

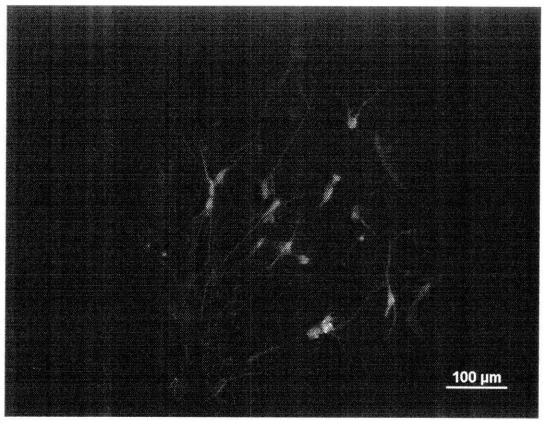


(a) Nestin positive neurite length (μm)

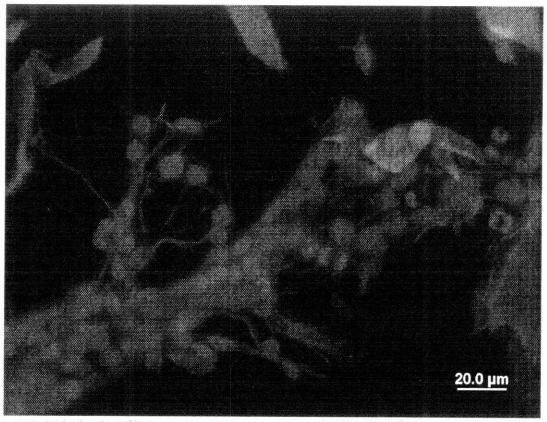


(b) TUJ1 positive neurite length (μm)

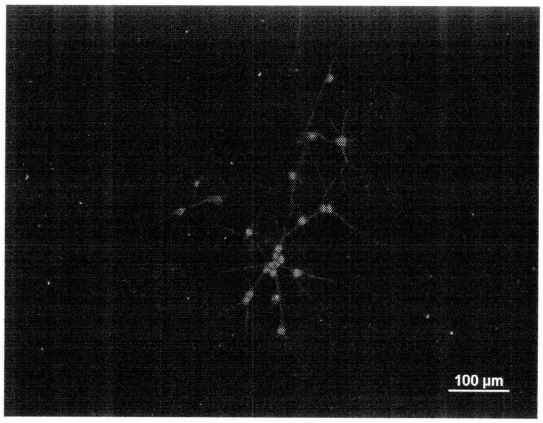
Figure 3-8: Effects of monolayer vs. scaffold for rat NSCs in DM or NM for 6 days. Mean (μm) $\pm$ SEM.



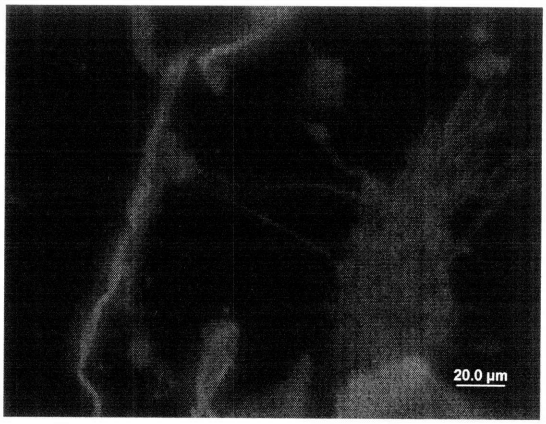
(a) Nestin stained rat NSC monolayer in NM.



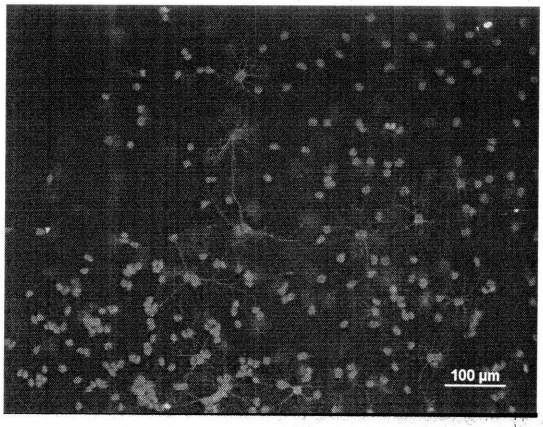
(b) Nestin stained rat NSC-seeded scaffold in NM.



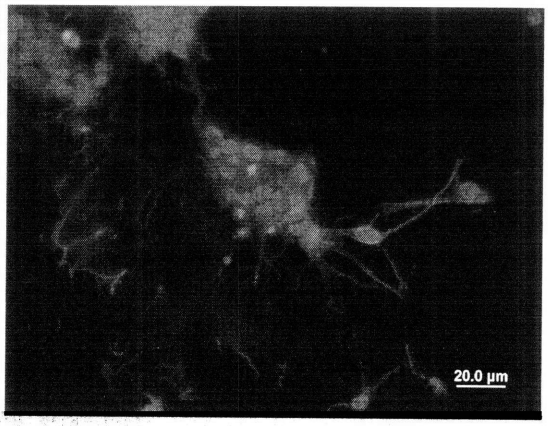
(c) TUJ1 stained rat NSC monolayer in NM.



(d) TUJ1 stained rat NSC-seeded scaffold in NM.

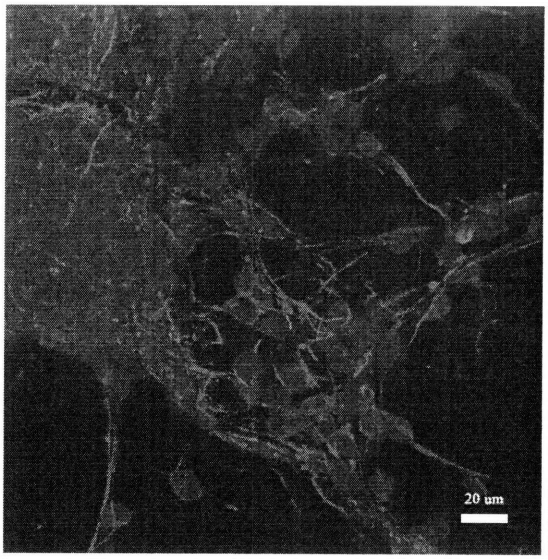


(e) GFAP stained rat NSC monolayer in DM.

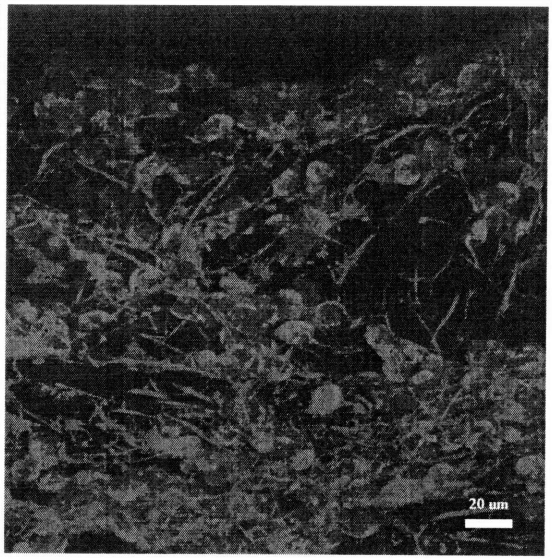


(f) GFAP stained rat NSC-seeded scaffold in DM.

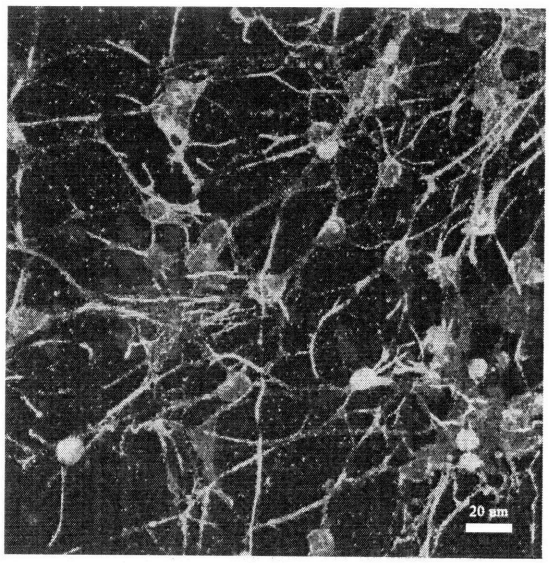
Figure 3-9: Nestin, TUJ1, GFAP stained rat NSC in monolayer or scaffolds cultured in DM or NM for 6 days.



(a) Confocal: Nestin stained rat NSC-seeded scaffold in NM.



(b) Confocal: TUJ1 stained rat NSC-seeded scaffold in NM.



(c) Confocal: GFAP stained rat NSC-seeded scaffold in DM.

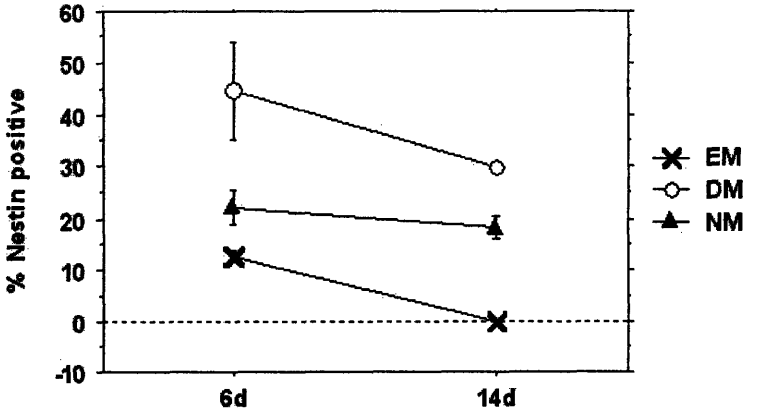
Figure 3-10: Confocal images of nestin, TUJ1, GFAP stained rat NSC-seeded collagen scaffolds cultured in DM or NM for 6 days.

also showed a significant difference between the % TUJ1 expression for cells in EM and NM ($p = 0.0263$). There were not enough samples to do a proper ANOVA on the GFAP expression, however, NSCs cultured in DM generally the highest percent of GFAP stained cells.

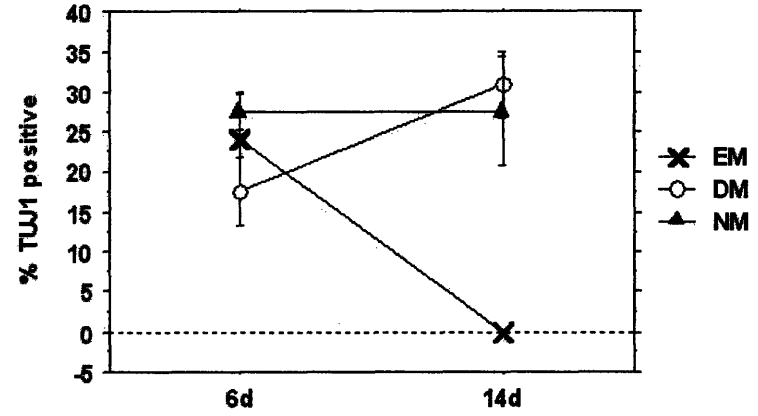
The medium type had a significant effect on both the nestin ($p < 0.0001$, power = 1.000) and TUJ1 ($p < 0.0001$, power = 1.000) positive neurite length. The measured lengths of nestin expressing cells were significantly different between those cultured in EM compared to those cultured in DM ($p < 0.0001$) and NM ($p < 0.0001$). The nestin positive neurite length measured in both the DM and NM were approximately three times as great as the length measured in EM. For TUJ1 expressing cells, a significant difference in neurite length was measured between all three medium types. Overall, NSCs cultured in DM had the greatest neurite growth and those in EM the least.

3.3.3 Culture time

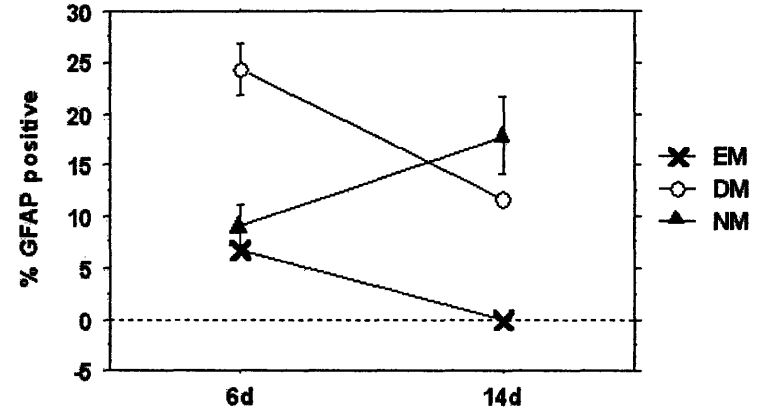
The same data set and analysis used in section 3.3.2 to determine the effects of neurogenic versus non-neurogenic medium were also used to study the effects of culture time on immunofluorescent expression and neurite length in NSC-seeded collagen scaffolds. The neural induction medium culture time had a significant effect on the percent of nestin positive cells ($p = 0.0079$, power = 0.805), and the interaction between time and medium type had some effect on the percent of TUJ1 positive cells ($p = 0.0285$, power = 0.682). Nestin expression decreased with time for NSCs cultured in all three media. NSCs cultured in DM expressed the most nestin (45% at 6 days and 30% at 14 days), and those cultured in EM expressed the least nestin (13% at 6 days and dropping to 0% by 14 days). The % TUJ1 positive cells in DM increased from 17% at 6 days to 31% at 14 days, whereas the % TUJ1 positive cells in NM remained at 28% for both time points. For NSCs cultured in EM, the % nestin, % TUJ1, and % GFAP expression dropped to zero by 14 days in culture. There were not enough samples to do a proper ANOVA on the GFAP expression. Unexpectedly, the % GFAP expressed by NSCs cultured in NM increased with time while the % GFAP for the EM and DM



(a) % Nestin positive

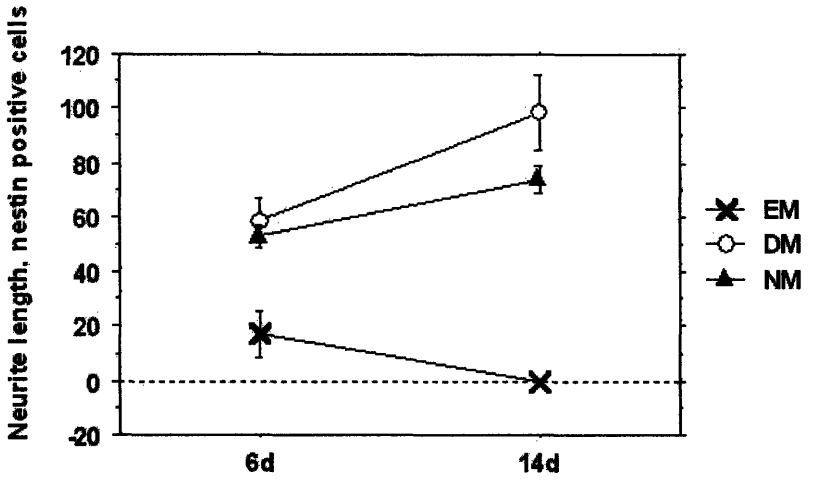


(b) % TUJ1 positive

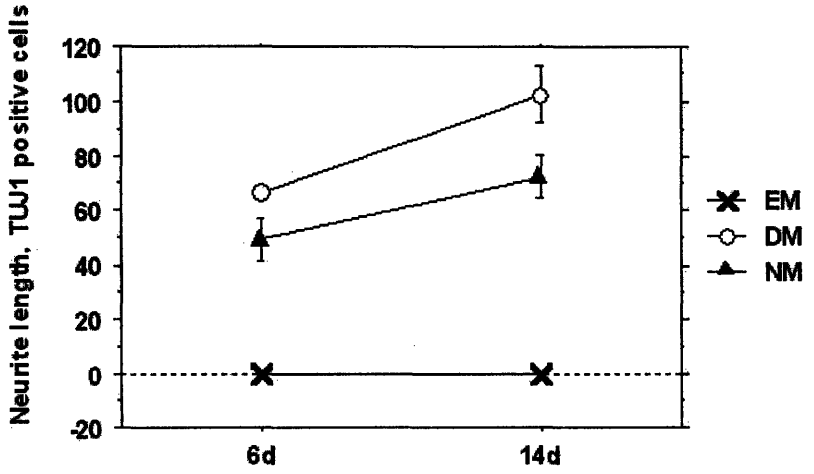


(c) % GFAP positive

Figure 3-11: Effects of neurogenic vs. non-neurogenic medium and culture time for rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days. Mean ± SEM.



(a) Nestin positive neurite length (μm)



(b) TUJ1 positive neurite length (μm)

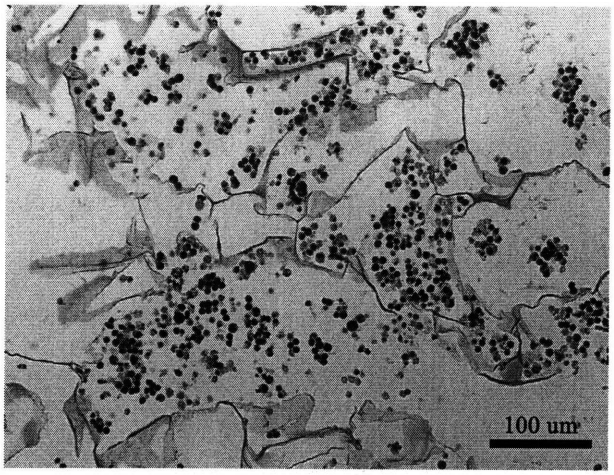
Figure 3-12: Effects of neurogenic vs. non-neurogenic medium and culture time for rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days. Mean (μm) $\pm$ SEM.

cultures decreased with time.

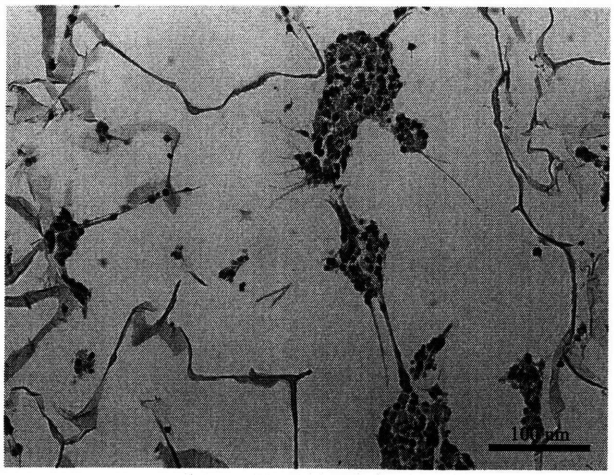
According to Fisher's PLSD, culture time also had a significant effect on the neurite length of cells expressing nestin ($p = 0.0011$) and TUJ1 ($p = 0.0018$). The neurites in DM and NM were significantly longer than those in EM and continued to grow with time. Nestin positive neurite length in DM increased from 59 μm at 6 days to 99 μm at 14 days, and TUJ1 positive neurite length increased from 67 μm at 6 days to 103 μm at 14 days.

3.3.4 Laminin coated collagen scaffolds

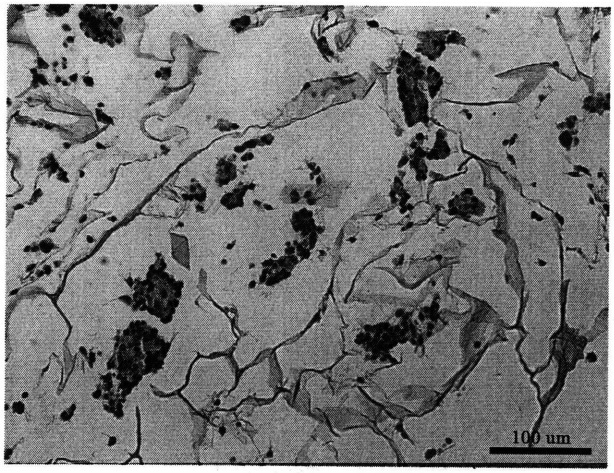
During the cell-seeded collagen scaffold experiments, cells appeared to float out of the scaffolds and aggregate at the bottom of the well. H&E immunohistochemical staining showed that cell-seeded scaffolds cultured in rat NSC DM and NM appeared to have more clustered cells than those cultured in rat NSC EM (Figure 3-13). An experiment using laminin-coated collagen scaffolds was performed to test if the laminin would facilitate cell attachment and possibly upregulate neural differentiation. An ANOVA showed no significant effects of the laminin coated scaffold on the percentage of nestin ($p = 0.153$, power $= 0.278$) or TUJ1 ($p = 0.675$, power $= 0.068$) positive cells, nor any significant effects on the neurite length at 6 days in the neural induction media. However, the Fisher's PLSD showed some significance of laminin ($p = 0.0465$) on the nestin positive neurite length. There were not enough samples to do a proper ANOVA on the GFAP expression. This analysis was done with data from experiment 6 conditions described on page 34. An interesting finding was that the cells in the non-laminin coated collagen scaffolds grew in clusters within the scaffold pores, but the nestin and TUJ1 positive cells were concentrated near the edge of the scaffold (Figure 3-14). In contrast, the cells in the laminin coated collagen scaffolds grew primarily along the scaffold struts, and the nestin and TUJ1 positive cells were spread throughout the scaffold.



(a) H&E staining. Rat NSC in expansion medium.

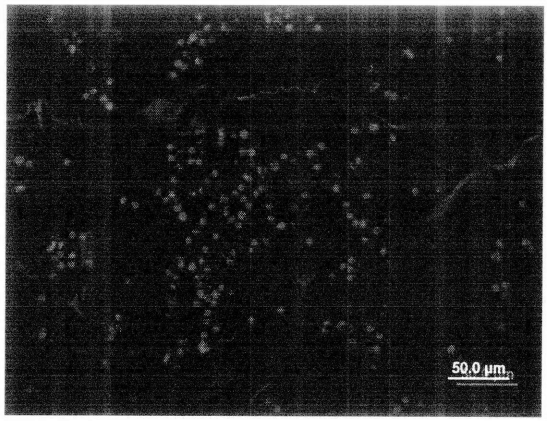


(b) H&E staining. Rat NSC in differentiation medium for 6 days.

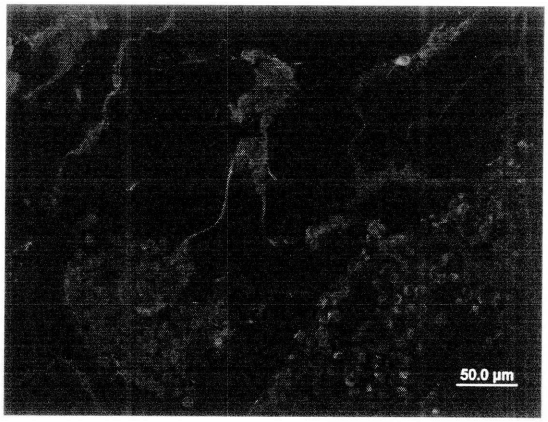


(c) H&E staining. Rat NSC in neurogenic medium for 6 days.

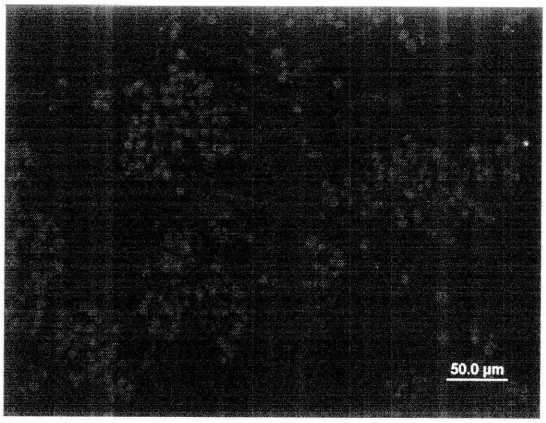
Figure 3-13: H&E stained NSC-seeded collagen scaffolds.



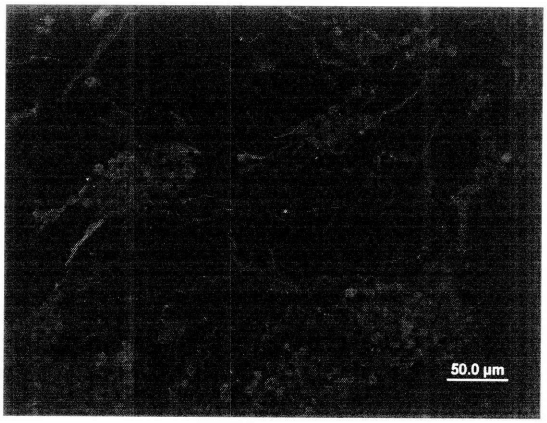
(a) No nestin staining at center of rat NSC-seeded, non-laminin scaffold in DM.



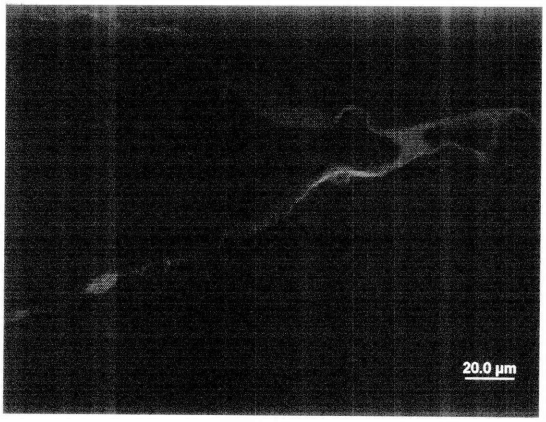
(b) Nestin staining at edge of rat NSC-seeded, non-laminin scaffold in DM.



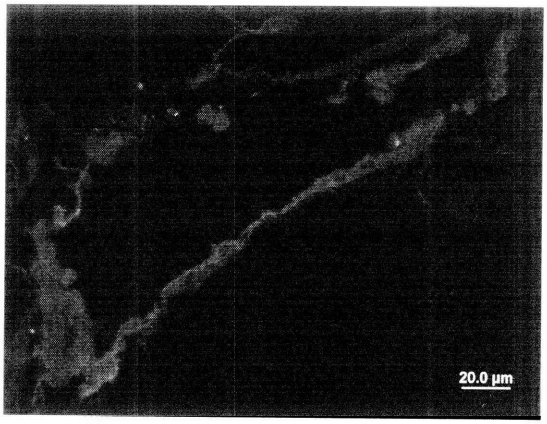
(c) Slight TUJ1 staining at center of rat NSC-seeded, non-laminin scaffold in DM.



(d) TUJ1 staining at edge of rat NSC-seeded, non-laminin scaffold in DM.

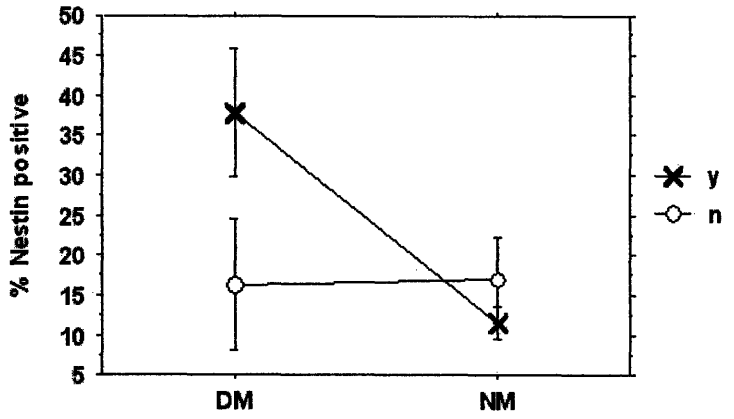


(e) Nestin staining of rat NSC along laminin coated scaffold in DM.

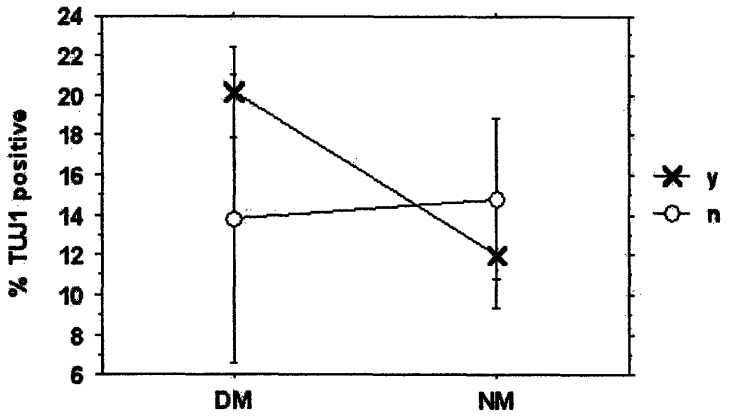


(f) TUJ1 staining of rat NSC along laminin coated scaffold in DM.

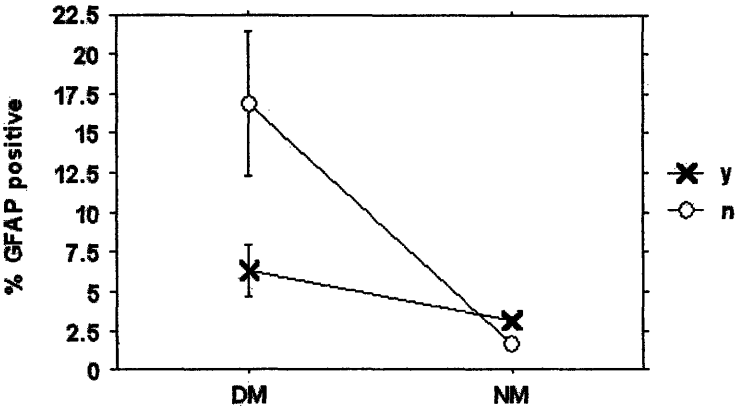
Figure 3-14: Nestin and TUJ1 stained rat NSC-seeded scaffolds in DM for 6 days, with or without laminin. Blue = nuclei, green = scaffold.



(a) % Nestin positive

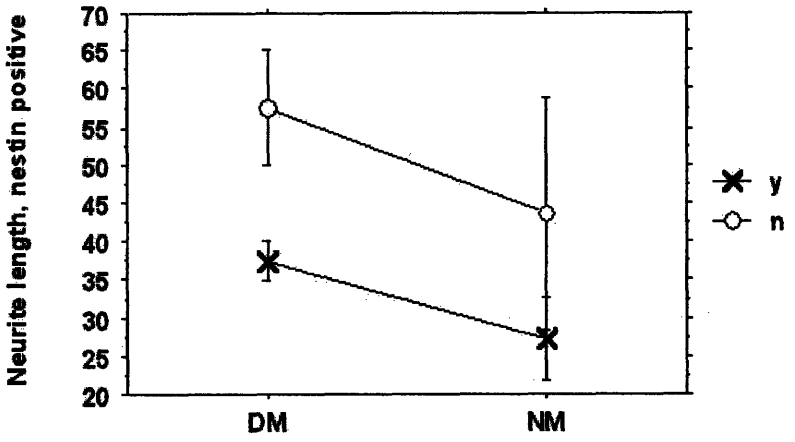


(b) % TUJ1 positive

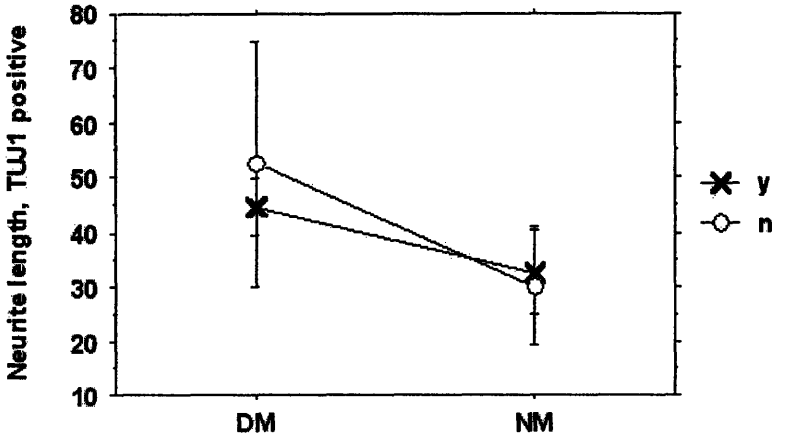


(c) % GFAP positive

Figure 3-15: Effects of laminin for rat NSC-seeded scaffolds cultured in DM or NM for 6 days. Mean ± SEM. y = laminin, n = no laminin.



(a) Nestin positive neurite length (μm)



(b) TUJ1 positive neurite length (μm)

Figure 3-16: Effects of laminin for rat NSC-seeded scaffolds cultured in DM or NM for 6 days. Mean (μm) $\pm$ SEM. y = laminin, n = no laminin.

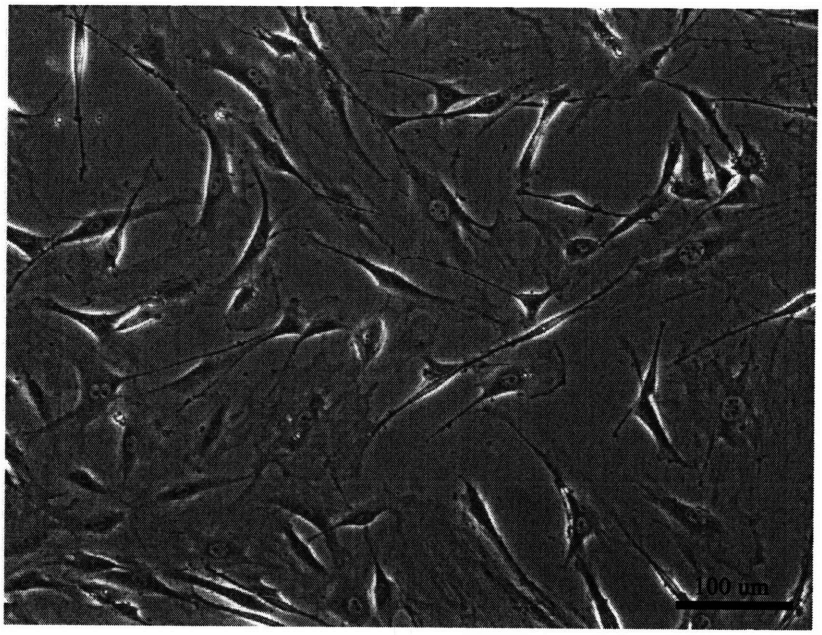
3.4 Marrow-Derived Stromal Cell Experiments

MSCs were cultured in rat MSC neurogenic medium (NM) to induce neural differentiation in monolayer (1D) or in collagen scaffolds (3D). Cells continued to be cultured in rat MSC expansion medium (EM) were used as the control group. The % nestin, % βIII-tubulin (TUJ1), and % GFAP positively stained cells were counted and the cell process length was measured for both monolayer and MSC-seeded collagen scaffold cultures at 5 hours post exposure to induction medium. All of the statistical data is provided in section B.4.

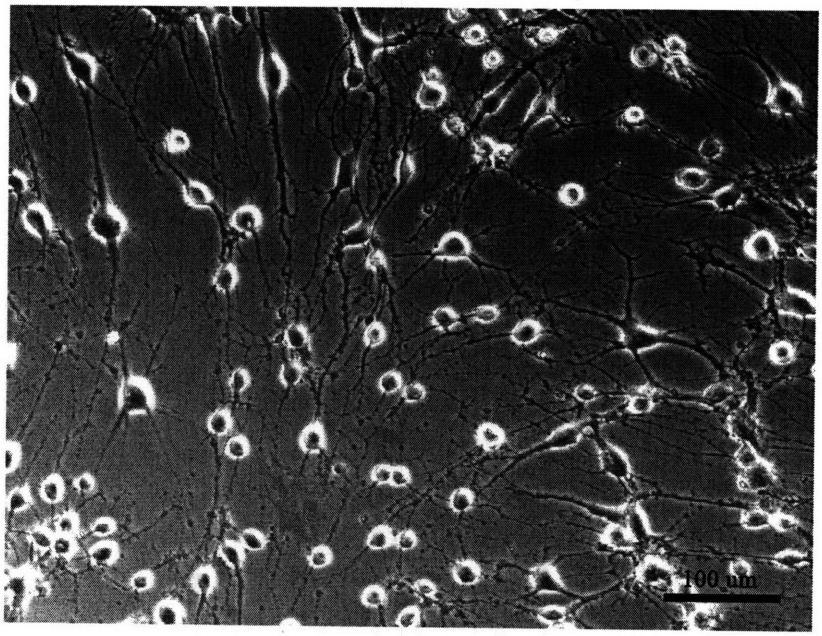
MSCs cultured in EM had a spindly, fibroblastic shape. After exposure to NM, morphological changes were observed as rapidly as 30 minutes after neural induction. The cytoplasm seemed to collapse into the cell body, giving the cells a "neuron-like" and phase-bright appearance (Figure 3-17). In monolayer, the MSCs began to detach from the well plate as early as 3 hours after exposure to NM. The MSCs also detached from the collagen scaffolds when cultured in NM. Many of the nestin and TUJ1 positively stained cells appeared to have two nuclei in the cytoplasm or a very large cell body (white arrows in Figure 3-18).

Only the medium type had a significant effect on the % nestin ($p = 0.0002$, power = 0.990) and % TUJ1 ($p = 0.0324$, power = 0.583) expressed by MSCs. MSCs cultured in monolayer and in collagen scaffolds both showed a higher percentage of nestin and TUJ1 staining for cells in NM compared to those in EM. MSCs in monolayer expressed 13% nestin and 21% TUJ1 after being exposed to NM for 5 hours. Almost no GFAP was expressed by the MSCs in any of the culture conditions.

The medium type, substrate, and their interaction all significantly affected the measured neurite length of nestin and TUJ1 positive cells. Cells cultured in monolayer had a much greater difference in process length between the EM and NM measurements compared to cells in scaffolds (Figure 3-20). For nestin positive cells, the process length was 166 μm and 34 μm for monolayer and cell-seeded scaffolds in NM, respectively. For TUJ1 positive cells, the process length was 120 μm and 20 μm for monolayer and cell-seeded scaffolds in NM, respectively.

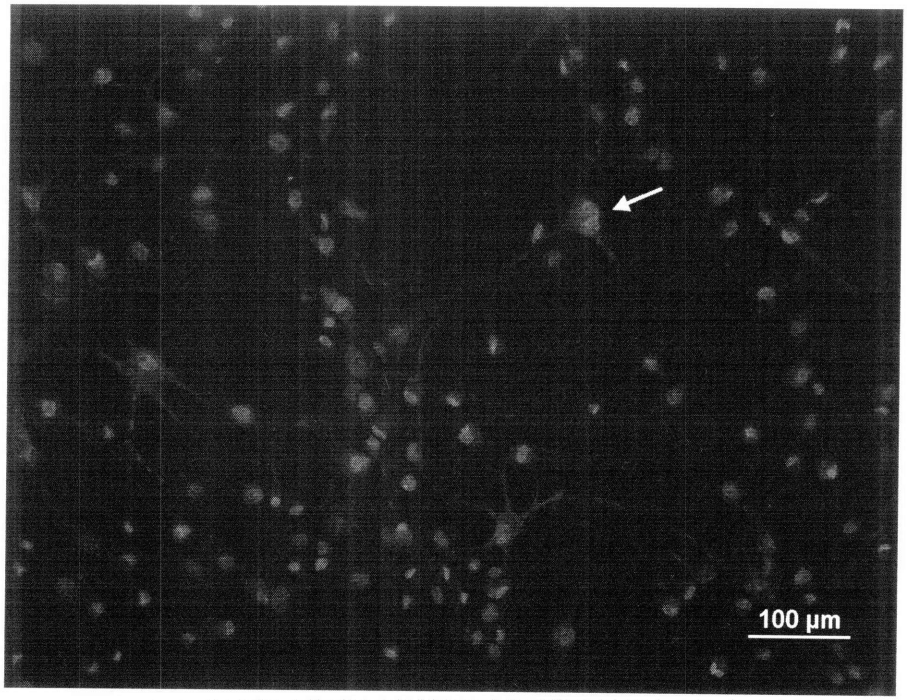


(a) MSC monolayer in EM

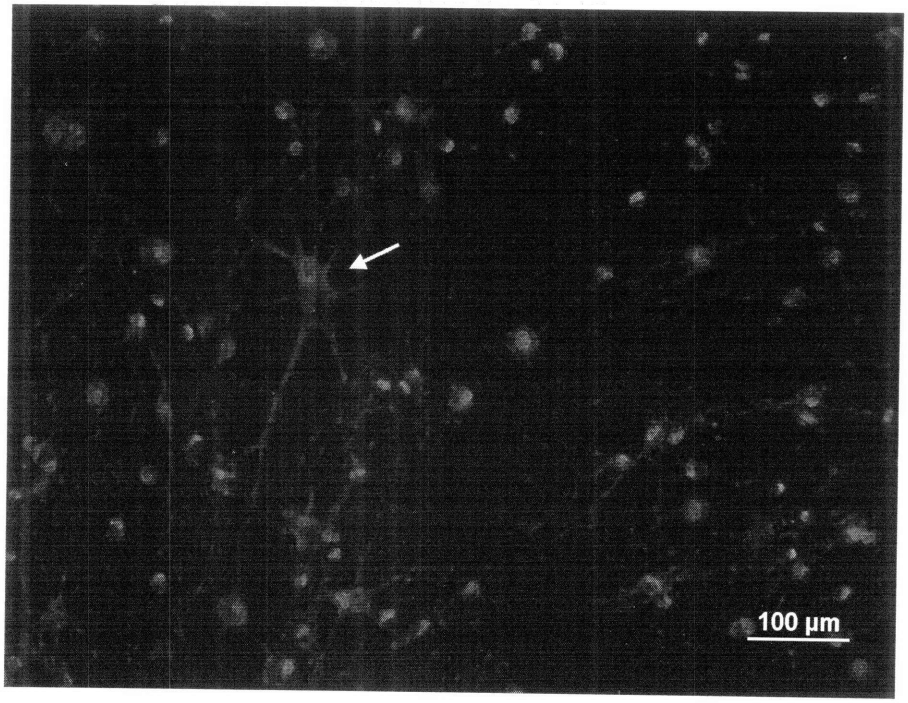


(b) MSC monolayer in NM

Figure 3-17: Rat MSC monolayer cultured in EM and NM for 5 hours.

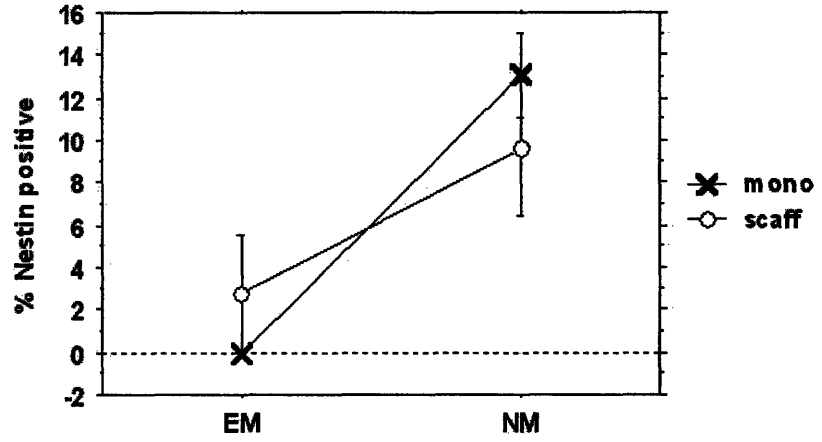


(a) Nestin stained rat MSC monolayer in NM

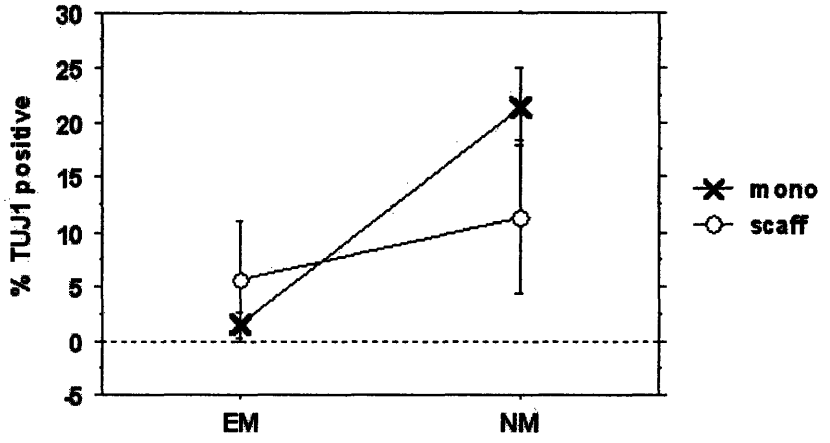


(b) TUJ1 stained rat MSC monolayer in NM

Figure 3-18: Nestin and TUJ1 stained rat MSC monolayer cultured in NM for 5 hours.

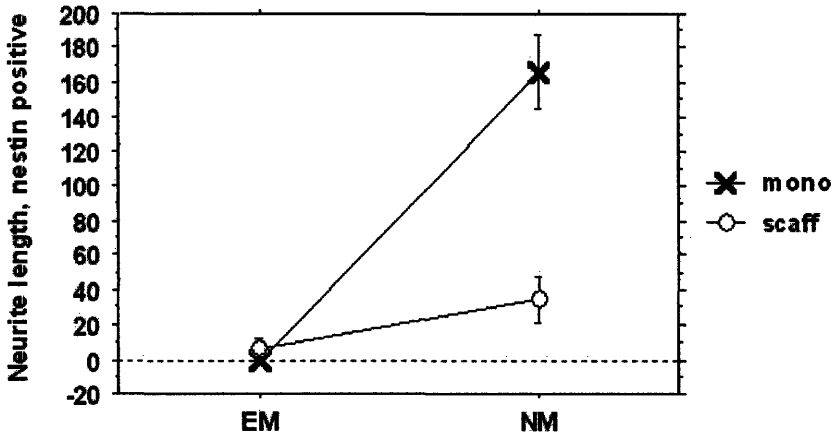


(a) % Nestin positive

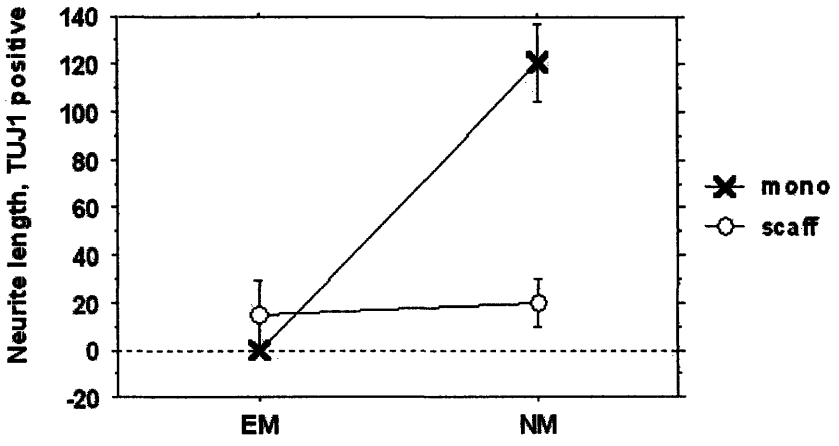


(b) % TUJ1 positive

Figure 3-19: Effects of monolayer vs. scaffold and neurogenic vs. non-neurogenic medium for rat MSCs in EM or NM for 5 hours. Mean ± SEM.



(a) Nestin positive neurite length (μm)



(b) TUJ1 positive neurite length (μm)

Figure 3-20: Effects of monolayer vs. scaffold and neurogenic vs. non-neurogenic medium for rat MSCs in EM or NM for 5 hours. Mean (μm) $\pm$ SEM.

Chapter 4

Discussion

4.1 Collagen Scaffold Pore Size

The scaffold pores must be large enough to allow the stem cells to infiltrate the collagen scaffold, as well as allow diffusion of oxygen, nutrients, and waste to and from the cells. Since the MSCs (20 μm diameter nucleus) are approximately twice as large as the NSCs, the pore size should be at least 100 μm to facilitate the migration of MSCs into the scaffold during seeding. The mean pore diameter of the scaffolds used in these experiments was 136 μm. Since the scaffold pores are much larger relative to the size of the stem cells, especially the NSCs, a true 3D environment may not be created but this porous construct still allows more interaction between the cells than in monolayer.

The pore size inhomogeneity between the different scaffolds could have been due to the use of plastic molds instead of metal pans, as well as variation with location in the mold. For example, the pore size is more likely to vary near the edge of the mold due to temperature gradients near the wall. For future experiments, collagen scaffolds should be fabricated using small, metal pans to achieve more homogeneous pores and to prevent warping of the metal pan that can occur with large pans. Also, a constant cooling rate of 1°C/min should continue to be used as this results in more uniform pore size, structure, and orientation compared to the quenching method [22].

4.2 Collagen Scaffold Contraction

Scaffold contraction was observed in all of the MSC-seeded scaffolds, but none of the NSC-seeded scaffolds. This observation was verified by positive α-SMA staining in the MSC-seeded scaffolds, mostly around the scaffold edge, and negative α-SMA staining in all of the NSC-seeded scaffolds. α-SMA is a protein expressed in many contractile cells such as myofibroblasts. The fact that MSCs are twice as large as NSCs could explain why these larger cells are able to contract the scaffold, but it could just be a difference in the cell phenotype. This difference in contractile behavior is important for applications such as retinal tissue engineering where the implanted construct must be very thin and undistorted. Experiments using smaller scaffold pores or decreased scaffold stiffness (less cross-linking) could be performed to test the contractile ability of the NSCs.

4.3 MSC Differentiation

The "neural differentiation" of MSCs has been previously reported, but many have questioned these claims [1, 5, 28, 33]. It is argued that differentiation toward a neuronal lineage is a gradual process involving specific genetic events, and it cannot be achieved within hours of neural induction [19]. Studies have also reported the apparent contraction of the cell body and rapid change in cell morphology occurring within hours of exposure to the neurogenic medium, and attribute this to collapse of the actin cytoskeleton leaving neurite-like processes rather than true differentiation [21]. MSCs, as well as other cells, exposed to cytotoxic chemicals exhibited this same behavior. The cell processes did not show any sign of growth such as that observed with the NSCs in DM or NM. Thus, only the results for the NSC experiments will be further discussed.

4.4 Monolayer vs. Collagen Scaffold

The substrate that the stem cells were cultured on affected the neuronal differentiation and neurite growth potential of the NSCs. Culturing in monolayer resulted in significantly longer cell processes which stained positive for nestin or TUJ1. A higher percentage of nestin and TUJ1 was observed for monolayer versus collagen scaffold cultures, but monolayer cultures also resulted in larger increases in the percent of nestin and TUJ1 positive cells than scaffolds when comparing between the different media.

These results were unexpected since it was hypothesized that the porous collagen scaffold would increase cell interactions and upregulate neural differentiation and neurite growth. One possible reason why this was not the case is that the cells in monolayer were exposed to higher concentrations of chemicals in the medium than the cells in scaffolds. At the start of the experiment, cells in monolayer were exposed to 50 μl medium/cell whereas cells in scaffolds were exposed to 7.5 μl/cell. Thus, the amount of medium each cell is exposed to might make a difference for a sensitive process such as neural differentiation. Two other possible reasons why this was not the case are: (1) The collagen scaffold could have increased the cell-cell interactions such that they did not need to extend their processes far to make connections with nearby cells or (2) The scaffold pores were too large to simulate a true 3D environment. H&E staining showed that the NSCs were usually clustered between the scaffold struts rather than attached to and spread along them, possibly indicating that the pores were too large to facilitate cell interaction and that they preferred to form "neurospheres" rather than spread along the scaffold. These neurospheres were 3D, at least more so than monolayer, and the clustered cells interacted amongst each other, although the NSCs were still observed to protrude their processes from the neurospheres to bridge connections with nearby cells. Another possible explanation for the monolayer versus scaffold effects could be that as the cells are differentiating into neurons they require a certain protein, such as laminin, to bind to the substrate, which the laminin coated well plates had but the collagen scaffolds did not. Thus,

a greater percentage of neuronal cells would be attached to the laminin coated well plate whereas the non-neuronal cells would be still be trapped within the scaffold pores. An experiment in which the scaffolds were coated with laminin before cell-seeding is discussed in section 4.7. It could also simply be the case that since the cells in collagen scaffolds are clustered, it is hard to accurately count the number of positively stained cells and measure their full neurite length. In this case, using the confocal microscope to generate 3D images at 100 μm sections would be more useful than analyzing 6 μm paraffin-embedded sections.

4.5 Neurogenic vs. Non-neurogenic medium

There were apparent differences between the neurogenic (DM and NM) and non-neurogenic medium (EM) in their ability to differentiate the NSCs into neuronal or glial cells and to stimulate neurite growth. Both neurogenic media produced significantly higher percentages of nestin, TUJ1, and GFAP positive cells and longer neurites than the EM by 14 days post induction, indicating that neuronal differentiation and nerve cell growth had been induced. This is supported by viewing the immunofluorescent and H&E images which show that the NSCs in DM and NM had long neurites which seemed to extend toward each other, while the NSCs in EM remained round without any sprouting processes. Cells grew neurites up to 10 and 7 times that of their 10 μm nuclear diameter in DM and NM, respectively, by 14 days post induction. Except for the % TUJ1 expression, DM produced the highest percentage of nestin and GFAP positive cells and the longest measured neurite lengths at 6 days post neural induction. Even at 14 days post induction, NSCs cultured in DM showed the highest level in all categories except for the percentage of GFAP. Thus, culturing the NSCs in DM gave the best results in terms of neuronal differentiation and neurite growth.

Although it was expected that the neurogenic media would induce neural differentiation and nerve cell growth more than the non-neurogenic medium, it was unclear whether the DM or NM would have a more significant effect. Even though both neu-

rogenic media are successful at differentiating the NSCs into neurons, the DM is also able to produce glial cells which are important for supporting and protecting the neurons and could be the key to ensure neuron survival whether in a tissue engineering construct or *in vivo*.

4.6 Culture Time

As expected and observed, nestin expression decreased with time and TUJ1 expression increased with time, indicating that the NSCs were differentiating from neural progenitors into more mature neurons between 6 and 14 days post DM induction. For NSC-seeded scaffolds cultured in NM, the levels of % nestin and % TUJ1 positive cells remained steady at the 6 and 14 day time points possibly suggesting that a "differentiation equilibrium" had already been reached. The steady-state NM levels of % nestin and % TUJ1 were 20% and 28%, respectively. It would be interesting to continue this experiment for longer time points to determine the final % nestin and % TUJ1 DM levels and see if the DM and NM levels converge. The measured nestin and TUJ1 stained neurite lengths increased with time in culture in both the DM and NM. It is unclear why the % GFAP decreased for DM cultures but increased for NM cultures. However, the sample size was fairly small so the experiment should be performed again to gain a better understanding of this response.

4.7 Laminin Coated Collagen Scaffolds

Since the NSCs cultured in DM and NM in collagen scaffolds tended to cluster together rather than attach and spread along the scaffold struts, laminin coated scaffolds were tested to see if they could increase cell attachment to the scaffold and improve the measured neural differentiation and neurite length relative to non-laminin coated collagen scaffolds. Although laminin was found to have no significant effect on the nestin and TUJ1 expression and neurite length for this experiment, there was a definite change in cell distribution in the scaffold. Many more NSCs attached to the

scaffold struts and lined the pores, and the nestin and TUJ1 positive cells were distributed throughout the laminin coated scaffold as opposed to being located mostly near the edge as observed in the the non-laminin coated scaffold. Perhaps the cells clustered together and grew as neurospheres because they did not have the ligands to attach to collagen. Adding laminin provided the NSCs with a favorable surface to grow on [4, 9, 12]. However, the total number of cells in the laminin-coated scaffold was less than that in the non-laminin coated scaffold probably because the pores were too large and there was limited surface area for the NSCs to attach. To reevaluate the effect of a laminin coated collagen scaffold, it would be interesting to reduce the scaffold pore size to increase the laminin coated surface area available for cell attachment and neurite growth and to better simulate a 3D environment for the NSCs. In addition, laminin coated oriented pore channels may further guide neurite extension.

Chapter 5

Conclusions

Rat neural stem cells (NSCs) were much more successful than rat marrow-derived stromal cells (MSCs) at differentiating into neurons and astrocytes and growing neurite extensions when cultured in neurogenic medium. The rapid change in cell morphology and the neuron-like appearance of MSCs was due to collapse of the actin cytoskeleton caused by the cytotoxic effect of the MSC neurogenic medium rather than true differentiation toward a neural lineage. NSCs which stained positive for nestin or βIII-tubulin developed and actively grew extended neurites, and those which stained positive for GFAP developed the star-like glial morphology when cultured in NSC differentiation medium (DM) or neurogenic medium (NM).

NSCs extended their processes and bridged connections with nearby cells both in monolayer and in collagen scaffolds, but culturing NSCs in monolayer resulted in a higher percentage of nestin and TUJ1 expressing cells and significantly longer neurite extensions than those formed in collagen scaffolds. Although this result is different from what was hypothesized, it was suggested that decreasing the pore size and coating the scaffold in laminin could increase cell attachment and interaction by providing a laminin coated surface in a 3D environment. This combination may significantly upregulate neural differentiation and neurite extension.

The differentiation of neural stem/progenitor cells into neurons increased with longer times in culture since the percent of nestin positive cells decreased while the percent of βIII-tubulin positive cells increased. The neurite length also increased

with time. Overall, culturing NSCs in DM for at least 14 days resulted in the most neuronal differentiation and neurite growth.

Appendix A

Protocols

A.1 Collagen Scaffold Fabrication and Preparation Protocols

A.1.1 0.5% Type I/III Collagen Slurry Preparation Protocol

Materials

1. Cooling system
2. Hydrochloric Acid (HCl) (Fisher Scientific)
3. Stir bar
4. Stir plate
5. pH meter
6. Bio-Gide® porcine type I/III collagen powder (Geistlich Pharma AG, Wolhusen, Switzerland)
7. Ultra Turrax IKA T18 blender
8. Centrifuge
9. Spatula

Protocol

1. Turn on cooling system and wait until water cools to 4°C.
2. Prepare 200 ml HCl solution at 0.001 N.
   (a) Add 50 l of 6 N HCl to 3 ml sterile distilled water to make 0.1 N HCl.
   (b) Add 2 ml of 0.1 N HCl to 198 ml sterile distilled water to make 0.001 N HCl.
   (c) Stir using stir bar on stir plate.
   (d) Adjust to pH = 3 by adding drops of 6 N HCl.
3. Progressively add 1 g Bio-Gide® powder while stirring.
4. Adjust to pH = 3 by adding drops of 6 N HCl.
5. Pour collagen slurry into cooling container.
6. Blend at 15,000 rpm for 90 min.
7. Adjust to pH = 3 by adding drops of 6 N HCl.
8. Blend at 15,000 rpm for 90 min (same as step 6).
9. Pipette collagen slurry into 50 ml tubes.
10. Centrifuge at 1,000 rcf for 3 min to degas.
11. Remove bubbles on top with a spatula.
12. Store at 4°C.

A.1.2 Collagen Scaffold Fabrication via Freeze-Drying Protocol

Materials

1. Spatula

2. Plastic mold

3. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

4. Kimtech Science Kimwipes

5. VirTis adVantage freeze-dryer and vacuum pump

6. Tweezers

7. Aluminum foil

Protocol

1. Continuously pipette the slurry up and down using a 25 ml pipette until the slurry is well mixed. Try not to create air bubbles.

2. Remove air bubbles using a spatula.

3. Clean the plastic mold with 70% ethanol and Kim-Wipes.

4. Pipette 10 ml of collagen slurry evenly into the plastic mold.

5. Remove air bubbles using a 200 μl pipette tip.

6. Place the plastic mold in the freeze-dryer. Close the door and check the vacuum seal around the door.

7. Select and run the following freeze-drying program:

Temperature	Time		Pressure
15°C	10 min	Hold	
-40°C	55 min	Ramp	
-40°C	120 min	Hold	
-40°C	30 min	Hold	200 mTorr
-5°C	1020 min	Hold	200 mTorr
15°C	25 min		200 mTorr
	21 hours		

Table A.1: Collagen scaffold freeze-drying program

8. At the end of the freeze-drying program, release the vacuum and remove the plastic mold in a sterile manner using gloves.

9. Gently remove the collagen scaffold using tweezers and place it in an aluminum foil packet.

10. Store in a dessicator.

A.1.3 Dehydrothermal Cross-linking Protocol

References: Yannas and Tobolsky, 1967; Yannas, Lee, et al., 1989; Harley, Spilker, et al., 2004

Materials

1. VWR vacuum oven with BOC Edwards vacuum pump

2. Dessicator

Protocol

1. Place the aluminum foil packet containing collagen scaffolds in the vacuum oven with the top of the packet open.

2. Make sure the oven temperature is at 105°C.

3. Start with the *Vacuum* knob closed and the *Purge* knob open.

4. Turn on the vacuum pump.

5. Slowly open the *Vacuum* knob while simultaneously closing the *Purge* knob.

6. Make sure a vacuum has been pulled by watching the vacuum oven dial.

7. Dehydrothermally (DHT) cross-link the collagen scaffolds for 24 hours.

8. After 24 hours, turn off the vacuum pump. Slowly close the *Vacuum* knob while simultaneously opening the *Purge* knob.

9. Once the vacuum has been released, take out the aluminum foil packets and close them immediately in a sterile manner.

10. Store the DHT cross-linked collagen scaffolds in a dessicator.

A.1.4 Collagen Scaffold Hydration Protocol

Materials

1. 8 mm diameter dermal biopsy punch (Miltex, Cat. #52442)

2. Centrifuge tubes

3. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

4. Nutator

Protocol

All steps should be done in a sterile manner.

1. After DHT cross-linking the collagen scaffolds, punch collagen scaffolds using an 8 mm diameter dermal biopsy punch.

2. Hydrate in 50 ml tubes containing 30-40 scaffolds per tube.

100% ethanol	30 min on the nutator
80% ethanol	30 min on the nutator
50% ethanol	30 min on the nutator

3. Rinse 2× with sterile distilled water.

4. Leave in sterile distilled water for 48 hours on a nutator.

5. Store the collagen scaffolds in sterile distilled water at 4°C.

A.1.5 Carbodiimide Cross-linking Protocol

References: Olde Damink et al., 1996; Lee et al., 2001

Materials

1. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Sigma, Cat. #E-7550, MW = 191.7 g/mol, stored in dessicator at -20°C)
2. N-hydroxysuccinimide (NHS) (Sigma, Cat. #H-7377, MW = 116 g/mol, stored in dessicator at room temp.)
3. Dulbecco's Phosphate Buffered Saline (PBS) 1× (Gibco, Cat. #14190)
4. Sterile filter
5. Nutator

Protocol

1. Allow the EDAC to warm to room temperature for approximately 30 minutes to prevent condensed moisture inside the container.
2. Calculate the amounts of EDAC and NHS needed for the molar ratios of EDAC:NHS:COOH using the following equations:

$$\#Scaffolds \times \frac{g\ collagen}{scaffold} \times \frac{mol\ COOH}{g\ collagen} \times \frac{mol\ EDAC}{mol\ COOH} \times \frac{g\ EDAC}{mol\ EDAC} = g\ EDAC \tag{A.1}$$

$$\#Scaffolds \times \frac{g\ collagen}{scaffold} \times \frac{mol\ COOH}{g\ collagen} \times \frac{mol\ NHS}{mol\ COOH} \times \frac{g\ NHS}{mol\ NHS} = g\ NHS \tag{A.2}$$

   Assume the scaffolds are primarily collagen, and that an 8 mm collagen scaffold weighs 2 mg. Assume there are 1.2 mmol COOH per gram of collagen (Olde Damink et al., 1996). For EDAC 1, the molar ratios of EDAC:NHS:COOH are 1:0.4:1.

3. Dissolve the EDAC and NHS in distilled water. The final volume should be 1 ml of solution per collagen scaffold.

4. Sterile filter the EDAC+NHS solution.

5. Aspirate the sterile distilled water from the hydrated collagen scaffolds.

6. Add the EDAC+NHS solution to the collagen scaffolds, and cross-link at room temperature for 30 minutes.

7. Aspirate the EDAC+NHS solution, and rinse the collagen scaffolds with sterile PBS.

8. Aspirate the PBS and add sterile PBS to remove residual EDAC+NHS solution by rocking on a nutator for 1 hour.

9. Aspirate the PBS and rinse in sterile distilled water for 2 $\times$ 10 min.

10. Store the collagen scaffolds in sterile distilled water at 4°C for up to one week (effects of longer storage unknown, usually used within 1 day of cross-linking).

A.1.6 Alexa Fluor Fluorescently Stained Collagen Scaffold Protocol

Adapted from Brendan Harley PhD Thesis, 2006

Materials

1. Dimethyl Sulfoxide (DMSO) (Sigma, Cat. #D2650)

2. Alexa Fluor 488 carboxylic acid, succinimidyl ester (Molecular Probes Inc., Cat. #A20000, MW = 643.41 g/mol)

3. Alexa Fluor 555 carboxylic acid, succinimidyl ester (Molecular Probes Inc., Cat. #A20009, MW $\approx$ 1250 g/mol)

4. Dulbecco's Phosphate Buffered Saline (PBS) 1$\times$ (Gibco, Cat. #14190)

Preparing the stock solution

1. Add 250 μl of DMSO to the 1 mg vial of Alexa Fluor 488 or 555.

2. Make 10 μl aliquots.

3. Store at -20°C.

Protocol

All steps should be done in a sterile manner.

1. Make a 1:2000 dilution of the Alexa Fluor stock solution in PBS.

2. Aspirate the sterile water from the hydrated collagen scaffolds.

3. Add 1 ml of the Alexa Fluor+PBS solution per collagen scaffold, and soak for 20 minutes at room temperature.

4. Aspirate the Alexa Fluor+PBS solution and wash the scaffold disks 2× in PBS.

5. Store the collagen scaffolds in PBS at 4°C.

A.2 Collagen Scaffold Pore Analysis Protocols

A.2.1 Collagen Scaffold JB-4 Embedding and Sectioning Protocol

References: Freyman, 2001; O'Brien, Harley, et al., 2004; O'Brien, Harley, et al., 2005

Materials

1. JB-4 A monomer solution (Polysciences, Cat. #0226A-800)

2. JB-4 A catalyst (Polysciences, Cat. # 02618-12, Benzoyl Peroxide, plasticized)

3. JB-4 B embedding solution (Polysciences, Cat. #0226B-30)

4. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

5. Nutator

6. Plastic mold tray

7. Tweezers

8. Plastic block holders

9. Ammonium hydroxide (Sigma)

10. Thermo Shandon Finesse Microtome

11. Superfrost PLUS Gold slides (Thermo Scientific, Cat. #4981)

Solutions

1. Infiltration solution: Dissolve 0.625 g of JB-4 A catalyst in 50 ml JB-4 solution A.

2. Equilibration solution: 50:50 solution of infiltration solution and 100% ethanol.

3. Embedding solution: Dissolve 2 ml of JB-4 solution B in 50 ml catalyzed solution A.

Protocol

1. Dehydrate the collagen matrices in increasing concentrations of ethanol in water as follows:

Solution	Time
Distilled H_2O	10 min
50% EtOH	10 min
70% EtOH	10 min
80% EtOH	10 min
95% EtOH	10 min
95% EtOH	10 min
100% EtOH	10 min
100% EtOH	10 min
100% EtOH	10 min

2. Equilibrate the collagen matrices for 12 hours at 4°C in a solution of 50% ethanol + 50% catalyzed JB-4 solution A.

3. Infiltrate the collagen matrices with 100% catalyzed JB-4 solution A for 1-4 days at 4°C on a nutator. Change the solution every 24 hours. Infiltration is complete when the matrices appear translucent and sink to the bottom of the container.

4. Embedding

   (a) Combine the JB-4 catalyzed solution A and the JB-4 solution B at a ratio of 25:1. Mix well and pipette into plastic molds.

   (b) Place the matrices flat against the bottom of the plastic molds. Use tweezers to flatten the matrices as they float up while the solution begins to harden for approximately 30 minutes. After the solution becomes viscous enough that the matrices do not float, place plastic block holders onto each well and store the plastic mold tray at 4°C overnight.

5. Pop the JB-4 blocks from the mold.

6. Let the samples dry at room temperature for 1 day.

7. Microtome sectioning

   (a) Add a few drops of ammonium hydroxide to a water bath, and use it to moisten the surface of the JB-4 block.

   (b) Slowly microtome 6 μm sections and drop into the water bath to unwrinkle the sections.

   (c) Set sections on glass slides.

   (d) Let the slides dry overnight.

A.2.2 Aniline Blue Staining Protocol

Materials

1. Aniline Blue (Fisher, Cat. #A-967)

2. Glacial acetic acid (Fisher, Cat. #A A507-500)

3. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

4. Cytoseal 60 (Electron Microscopy Sciences, Cat. #18006)

Solutions

1. Aniline Blue solution: 2.5 g aniline blue + 2 ml glacial acetic acid + 100 ml distilled water. Filter before use.

2. 1% (v/v) acetic acid: 1 ml acetic acid + 99 ml distilled water

Protocol

1. Dip slides in aniline blue solution for 2 min.

2. Dip slides in 1% acetic acid solution for 1 min.

3. Dip 5-10× in 95% ethanol until most of the background staining disappears.

4. Dip 5-10× in 100% ethanol.

5. Mount slides with Cytoseal 60 and coverslip.

6. Dry slides.

A.2.3 Collagen Scaffold Pore Size Analysis Protocol

References: Brendan Harley PhD Thesis, 2006; Freyman, 2001; O'Brien, Harley, et al., 2004; O'Brien, Harley, et al., 2005

Materials

1. Olympus light microscope with digital camera

2. IrfanView software (Irfan Skiljan)

3. Scion Image software (Scion Corporation, Frederick, MD)

Protocol

1. Acquire images of the aniline blue stained collagen scaffold sections using a light microscope. Save as TIFF files.

2. Convert images to greyscale TIFF files.

   (a) Open IrfanView. Press "*b*" to open the "*Batch conversion*" window.

   (b) Set the output directory to "*Desktop*" and the output format to "*TIF-Tagged Image File Format*".

   (c) Choose "*Convert to greyscale*" and "*Resize to 1024 × 768*" in the advanced options.

   (d) Add the input files. Start the batch conversion.

3. Prepare the greyscale images for pore size analysis.

   (a) Open Scion Image. Open the greyscale images.

   (b) Under the "*Options*" menu, select "*Threshold*". Adjust the threshold until an optimal image of struts is visible and background spots are minimized.

   (c) Under the "*Process*" menu, select "*Binary*" and then "*Make Binary*". This transforms the thresholded image into a permanent binary image.

   (d) Clean up any remaining black spots with the erase tool. The pore analysis macro will not count any artifact under 5 pixels across, so it is not necessary to remove every spot.

4. Run the pore analysis macro.

   (a) Under the "*Analyze*" menu, select "*Set Scale*". For the Olympus light microscope at the VA Tissue Engineering Laboratories in room D1-147, 1 mm equals 538 pixels at 4× magnification.

   (b) Using the oval drawing tool, select a significant portion of the image for pore analysis.

   (c) Under the "*Special*" menu, select "*Load Macros*" and open the "pore characterization macros" file.

(d) Under the "*Special*" menu, select "*Linear Intercept*". The distance between the pore walls along lines at various angles radiating from the center of the selected region is calculated.

(e) Under the "*Special*" menu, select "*Plot Intercepts*". This macro will transform the average distance between struts along each line into a best-fit ellipse, and will calculate linear intercept coefficients C0, C1, and C2 for that ellipse.

(f) Copy the C0, C1, and C2 values to an Excel spreadsheet for each collagen scaffold sample.

(g) Calculate the minor (a) and major (b) axes of the best-fit ellipse describing the average collagen scaffold pore, as well as the aspect ratio, using the following equations:

$$a = \frac{1}{\sqrt{C_o + \sqrt{{C_1}^2 + {C_2}^2}}} \tag{A.3}$$

$$b = \sqrt{\frac{\sqrt{{C_1}^2 + {C_2}^2}}{C_o\sqrt{{C_1}^2 + {C_2}^2} + {C_2}^2 - {C_1}^2}} \tag{A.4}$$

$$Aspect\ ratio = \frac{a}{b} \tag{A.5}$$

(h) Calculate the mean pore diameter (d) from the major and minor axes of the best-fit ellipse. To account for the effects of pores that were not sectioned through their maximal cross-section, but rather at an arbitrary angle, the ellipse major and minor axes are corrected by multiplying by 1.5 (Gibson and Ashby, 1997). The mean pore diameter is calculated from the average radius by multiplying by a factor of 2.

$$d = 1.5 \times 2 \times \sqrt{\frac{a^2 + b^2}{2}} \tag{A.6}$$

A.3 Cell Culture Protocols

All cell culture protocols should be done in a sterile tissue culture hood.

A.3.1 Thawing Frozen Cells Protocol

Materials

1. Water bath
2. Cell culture medium
3. Centrifuge tubes
4. Centrifuge
5. Cell culture flasks
6. Incubator

Protocol

1. Warm the cell culture medium in a 37°C water bath.
2. Quickly remove the frozen cell vial from the liquid nitrogen tank.
3. Gingerly agitate the vial in a 37°C water bath for 20 seconds.
4. As the cell suspension begins to defrost and liquid forms around the outer edges, add a drop of medium.
5. Wait a minute and then add another drop of medium. Repeat until approximately 5 drops are added so that the cells thaw into the medium.
6. Pipette 10 ml of medium into a 50 ml tube. Transfer the cell suspension into the 50 ml tube.
7. Centrifuge the cell suspension. Centrifuge the rat neural stem cells at 1000g for 2 minutes. Centrifuge the rat marrow-derived stromal cells at 1500 rpm for 10 minutes.

8. Carefully aspirate the medium without disturbing the cells at the bottom of the tube.

9. Add 10 ml of fresh medium. Pipette the cell suspension up and down until the cells are homogeneously mixed.

10. Count the cells according to the *Cell Counting Protocol* in section A.3.2.

11. Pipette the 10 ml cell suspension into a T150 flask. Add 20 ml of medium for a total of 30 ml of medium.

12. Store the T150 flask in an incubator maintained at 37°C, 5% CO_2, and 95% relative humidity.

13. Change the medium, according to the *Media Changing Protocol* in section A.3.3, every 2-3 days until the cells reach approximately 80% confluency.

A.3.2 Cell Counting Protocol

Materials

1. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

2. Bright-Line Hemacytometer (Sigma, Cat. #Z359629)

3. Eppendorf tubes

4. Trypan Blue solution (Sigma, Cat. #T8154)

5. Inverted light microscope

Protocol

1. Clean surface of hemacytometer and coverslip with 70% ethanol. Place the coverslip over the silver counting area.

2. Beginning with a cell pellet, suspend the cells in a known amount of medium. Pipette the cell suspension up and down until the cells are homogeneously mixed.

3. Dilute a 100 μl sample of the cell suspension with Trypan Blue in an Eppendorf tube (1:2 dilution if few cells are expected, 1:5 or 1:10 if a large number is expected).

4. Mix well. Collect 15 μl of cell suspension+Trypan Blue in a micropipette tip.

5. Pipette the cell suspension+Trypan Blue between the glass coverslip and the hemacytometer, allowing it to be drawn under the coverslip by capillary action. Pipette until just enough cell suspension reaches the edges of the silvered surface. Do not overfill as this may change the volume and make the count inaccurate.

6. Repeat steps 4 and 5 for the other counting chamber.

7. Place the hemacytometer on an inverted microscope stage, and view with a standard 10× objective.

8. Count the cells in each of the four corner and central squares (bright cells are viable, blue stained cells are dead). Count cells that lie on the top and left lines, but not those on the bottom or right lines of each square to avoid double-counting cells in adjacent squares.

9. Repeat step 8 for the other counting chamber.

10. A maximum cell count of 20-50 cells per 1×1 mm^2 square is recommended.

11. Calculate the total number of cells using the following equation:

$$T = \frac{N_c}{N_s} \times D \times 10^4 \times V \tag{A.7}$$

T = total number of cells in suspension
N_c = number of cells counted
N_s = number of squares counted
D = dilution factor
V = volume of media used to suspend cell pellet

12. Plate the cells for expansion according to the *Thawing Frozen Cells Protocol* in section A.3.1.

A.3.3 Media Changing Protocol

Materials

1. Water bath
2. Cell culture medium
3. Vacuum pipette
4. Incubator

Protocol

1. Warm the cell culture medium in a 37°C water bath.
2. Tilt the cell culture flask such that the cell-coated surface is facing up. Aspirate medium from a corner not touching the cell-coated surface using a vacuum pipette. Use a new pipette for flasks containing cells from different animals or flasks containing different media.
3. Pipette the correct volume of fresh medium into the flask. Add 30 ml of medium for T150 flasks.
4. Cap the flask and return it to the incubator maintained at 37°C, 5% CO_2, and 95% relative humidity.
5. Change the medium every 2-3 days until the cells reach approximately 80% confluency.

A.3.4 Passaging Cells Protocol

Materials

1. Water bath

2. Cell culture medium

3. Trypsin-EDTA (Invitrogen, Cat. #25300)

4. Dulbecco's Phosphate Buffered Saline (PBS) 1× (Gibco, Cat. #14190)

5. Vacuum pipette

6. Centrifuge tubes

7. Centrifuge

8. Incubator

Protocol

1. Warm the cell culture medium, trypsin-EDTA, and PBS in a 37°C water bath.

2. Aspirate the medium from the cell culture flask with a vacuum pipette (change pipettes for different animals and media).

3. Rinse the flask with PBS. Trypsin-EDTA will not detach cells if it has come in contact with medium.

4. Aspirate the PBS and add trypsin-EDTA. Pipette 5 ml of Trypsin-EDTA into T150 flasks with rat neural stem cells. Pipette 10 ml of Trypsin-EDTA into T150 flasks with rat marrow-derived stromal cells.

5. Place the flask in the incubator for 5 minutes to allow the cells to detach. Skip this step when using rat neural stem cells.

6. Remove the flask from the incubator and tap on the sides of the flask to loosen the cells. Use a microscope to check that the cells are detached.

7. Once the cells are floating, return to the hood and add medium to inactivate the trypsin-EDTA. Pipette 15 ml of medium into T150 flasks with rat neural stem cells. Pipette 30 ml of medium into T150 flasks with rat marrow-derived stromal cells.

8. Using a sterile plastic pipette, transfer the trypsin-EDTA+medium cell suspension to a centrifuge tube. Cell suspensions from the same animal can be combined.

9. Centrifuge the cell suspension. Centrifuge the rat neural stem cells at 1000g for 2 minutes. Centrifuge the rat marrow-derived stromal cells at 1500 rpm for 10 minutes.

10. Aspirate the trypsin-EDTA+medium with a vacuum pipette while avoiding the cell pellet at the bottom of the tube.

11. Resuspend the pellet in a known volume of medium. Pipette the cell suspension up and down until the cells are homogeneously mixed.

12. Count the cells according to the *Cell Counting Protocol* in section A.3.2.

13. While counting, centrifuge the cell suspension a second time to ensure all of the trypsin-EDTA has been removed.

14. Resuspend the cells at the desired seeding density. Transfer cells to new flasks, well plates, or seed into collagen scaffolds according to the *Cell-Seeding Collagen Scaffolds Protocol* in section A.3.6.

15. Add fresh medium. Place flasks, well plates, or collagen scaffolds in the incubator maintained at 37°C, 5% CO_2, and 95% relative humidity.

16. Change the medium every 2-3 days.

A.3.5 Agarose Coating Well Plates Protocol

Adapted from D. Hastreiter PhD Thesis, 2002

Agarose coating prevents cells from attaching and growing on the bottom of well plates.

Materials

1. Beaker

2. Stir bar

3. Stir plate

4. Seaplaque Agarose (Cambrex, Cat. #50100)

5. Plastic wrap

6. Microwave

7. 12-well cell culture plate, tissue-culture treated polystyrene (BD Falcon)

8. Parafilm

9. Sterile bags

Protocol

1. Choose a beaker that is 2-4 times the volume of the solution.

2. Add room temperature sterile distilled water and a stir bar to the beaker.

3. Slowly sprinkle in the agarose powder while the solution is rapidly stirred. Use 1 g of Seaplaque Agarose per 25 ml of water.

4. Remove the stir bar.

5. Cover the beaker with plastic wrap. Pierce small holes in the plastic wrap for ventilation.

6. Heat the beaker in the microwave on high power until bubbles appear.

7. Remove the beaker from the microwave oven. Caution: Any microwaved solution may become superheated and foam over when agitated.

8. Gently swirl the beaker to resuspend any settled powder and gel pieces.

9. Reheat the beaker on high power until the solution comes to a boil.

10. Hold at the boiling point for 1 minute or until all of the particles are dissolved.

11. Remove the beaker from the microwave.

12. Gently swirl the beaker to thoroughly mix the agarose solution.

13. After dissolution, add sufficient hot distilled water to obtain the initial weight. Mix thoroughly.

14. Coat 12-well plates with 500 μl of liquid agarose per well. This should be done quickly as the agarose solidifies as it cools.

15. Wrap Parafilm around the well plates and put them in a sterile bag.

16. Store the bag in a cold room overnight. Do not use the plates after more than 1 day in the cold room because the agarose will crack.

17. Warm the plates for 1-2 hours in the incubator prior to use.

18. Change agarose-coated well plates every two weeks because the agarose breaks down.

A.3.6 Cell Seeding Collagen Scaffolds Protocol

Materials

1. Incubator

2. Water bath

3. Cell culture medium

4. Tweezers

5. Filter paper

Protocol

1. Warm the agarose-coated well plates for 1-2 hours in the incubator prior to use.

2. Warm the cell culture medium in a 37°C water bath.

3. Transfer the hydrated collagen scaffolds into the agarose-coated wells using sterile tweezers. Orient them flat against the bottom of the well.

4. Absorb excess liquid contained in the scaffolds by holding strips of sterile filter paper with tweezers and gently placing the paper edge on the scaffold.

5. Resuspend the cells at the desired seeding density according to the *Passaging Cells Protocol* in section A.3.4.

6. Micropipette 10 μl of cell suspension onto the collagen scaffold.

7. To allow the cells to infiltrate the scaffold, wait approximately 10 minutes until adding the cell culture medium. Slowly add 2 ml of medium per well for 12-well plates.

8. Place the 12-well plates in the incubator maintained at 37°C, 5% CO_2, and 95% relative humidity.

9. Change the medium every 2-3 days.

A.4 Rat Neural Stem Cell Protocols

A.4.1 Poly-L-Ornithine and Mouse Laminin Coating Protocol

All plastic or glassware used to grow the neural stem cells must be coated with poly-L-ornithine and mouse laminin.

Materials

1. Poly-L-ornithine (Sigma, Cat. #P3655)

2. Mouse laminin (Fisher, Cat. #354232, 1 mg, 1.64 mg/ml)

3. Dulbecco's Phosphate Buffered Saline (PBS) 1× (Gibco, Cat. #14190)

4. Cell cultureware

5. Parafilm

6. Sterile bags

Protocol

Day 1

1. Make the poly-L-ornithine stock solution.

   (a) Dissolve the poly-L-ornithine in sterile distilled water to make a 10 mg/ml stock solution.

   (b) Store at -20°C.

2. Dilute the poly-L-ornithine stock solution. Use 10 μg/ml for polystyrene and other plastics, and 50 μg/ml for glass.

3. Add enough poly-L-ornithine solution to cover the cell cultureware surface. Pipette 10 ml into T150 flasks.

4. Wrap the cell cultureware in Parafilm and put in sterile bags.

5. Leave overnight (12-24 hours) at room temperature.

Day 2

1. Wash the cell cultureware 2× with sterile distilled water.

2. Dilute the mouse laminin in sterile PBS at 5 μg/ml.

3. Add enough laminin+PBS solution to cover the cell cultureware surface. Pipette 10 ml into T150 flasks.

4. Wrap the cell cultureware in Parafilm and put in sterile bags.

5. Leave overnight (24 hours) at room temperature.

Day 3

1. Store the cell cultureware in the laminin+PBS solution at -20°C for up to 6-8 months.

A.4.2 Rat Neural Stem Cell Differentiation Protocol

Materials

1. Recombinant Human Fibroblast Growth Factor (FGF) basic (R&D Systems, Cat. #233-FB/CF)
2. Bovine serum albumin (BSA)
3. *all-trans*-Retinoic acid (Sigma, Cat. #R2625, MW = 300.44 g/mol)
4. Dimethyl Sulfoxide (DMSO) (Sigma, Cat. #D2650)
5. Forskolin (Sigma, Cat. #F6886, MW = 410.5 g/mol)
6. D-MEM/F-12 (high glucose) containing L-glutamine (Invitrogen, Cat. #11320)
7. N-2 supplement 100× (Gibco, Cat. # 17502-048)
8. Penicillin-Streptomycin (Gibco, Cat. #15140)
9. Fetal Bovine Serum (FBS) (Invitrogen, Cat. #16140)
10. Centrifuge tubes

Preparing the stock solutions

1. 10 μg/ml bFGF stock solution
   (a) Pipette 2.5 ml of sterile PBS into a 15 ml tube.
   (b) Micropipette 33.3 μl of 7.5% g/ml sterile BSA into the 15 ml tube.
   (c) Add 1 ml of the BSA+PBS solution to the 25 μg bFGF vial and vortex.
   (d) Transfer the solution from the vial to the 15 ml tube.
   (e) Rinse the vial with more solution from the 15 ml tube.
   (f) Aliquot and store at -70°C for up to 3 months. Avoid repeated thawing and freezing.

(g) Add 1 μl per 1 ml of medium for a concentration of 10 ng/ml bFGF. Add 2 μl per 1 ml of medium for a concentration of 20 ng/ml bFGF.

2. *all-trans*-Retinoic acid stock solution

   (a) Dissolve 0.60088 mg of *all-trans*-Retinoic acid in 2 ml of DMSO.

   (b) Aliquot and store at -20°C.

   (c) Add 1 μl per 1 ml of medium for a concentration of 1 μM *all-trans*-Retinoic acid.

3. Forskolin stock solution

   (a) Dissolve 4.105 mg of forskolin in 2 ml of DMSO.

   (b) Aliquot and store at -20°C.

   (c) Add 1 μl per 1 ml of medium for a concentration of 5 μM forskolin.

Preparing the rat neural stem cell culture media

1. Rat neural stem cell expansion medium (rNSC EM)

   (a) D-MEM/F-12 (high glucose) containing L-glutamine

   (b) 1% N-2 supplement

   (c) 1% penicillin/streptomycin

   (d) 20 ng/ml bFGF (add fresh each time)

2. Rat neural stem cell differentiation medium (rNSC DM)

   (a) D-MEM/F-12 (high glucose) containing L-glutamine

   (b) 1% N-2 supplement

   (c) 1% penicillin/streptomycin

   (d) 0.5% FBS

   (e) 1 μM *all-trans*-Retinoic acid

3. Rat neural stem cell neurogenic medium (rNSC NM)

   (a) D-MEM/F-12 (high glucose) containing L-glutamine

   (b) 1% N-2 supplement

   (c) 1% penicillin/streptomycin

   (d) 1 μM *all-trans*-Retinoic acid

   (e) 5 μM forskolin

Protocol

1. Thaw the rat neural stem cells according to the *Thawing Frozen Cells Protocol* in section A.3.1. Use the rNSC EM as the cell culture medium.

2. Change the rNSC EM every 2-3 days according to the *Media Changing Protocol* in section A.3.3. The rat neural stem cells become confluent in approximately 4 days when cultured in T150 flasks.

3. When the cells are approximately 80% confluent, passage the cells according to the *Passaging Cells Protocol* in section A.3.4. Transfer the cells to well plates for monolayer experiments, or seed the cells into collagen scaffolds according to the *Cell Seeding Collagen Scaffolds Protocol* in section A.3.6 for 3D experiments.

4. Continue to culture the cells in rNSC EM for 2 days to allow the cells to attach to the well plate or collagen scaffold.

5. After 2 days, culture the cells in rNSC EM, rNSC DM, or rNSC NM. The rNSC EM is used as a control. The rNSC DM differentiates the cells into three lineages: neurons, astrocytes, and oligodendrocytes. The rNSC NM differentiates the cells into only neurons.

6. Change the media every 2-3 days. Differentiation is complete after approximately 6 days.

A.5 Rat Marrow-Derived Stromal Cell Protocols

A.5.1 Rat Marrow-Derived Stromal Cell Differentiation Protocol

Materials

1. Butylated Hydroxyanisole (Sigma, Cat. #B1253, MW = 180.24 g/mol)
2. Ethanol, 200 proof (American Bioanalytical, Cat. #AB00138)
3. Forskolin, from Coleus forskohlii (Sigma, Cat. #F3917, MW = 410.5 g/mol)
4. Dimethyl Sulfoxide (DMSO) (Sigma, Cat. #D2650)
5. Hydrocortisone (Sigma, Cat. #H0888, MW = 362.46 g/mol)
6. Dulbecco's Modified Eagle Medium (DMEM) (1×), low glucose (Invitrogen, Cat. #11885)
7. Insulin, from bovine pancreas (Sigma, Cat. #16634, MW = 5733.5 g/mol)
8. Acetic Acid, Glacial (Fisher, Cat. #A507)
9. Potassium Chloride (KCl) (Sigma, Cat. #P5405, MW = 74.55 g/mol)
10. Valproic Acid Sodium Salt (Sigma, Cat. #P4543, MW = 166.19 g/mol)
11. Fetal Bovine Serum (FBS) (Invitrogen, Cat. #16140)
12. Antibiotic-Antimycotic (Invitrogen, Cat. #15240)
13. Recombinant Human Fibroblast Growth Factor (FGF) basic (R&D Systems, Cat. #233-FB/CF)

Preparing the stock solutions

1. Butylated hydroxyanisole
   (a) Dissolve 72 mg of butylated hydroxyanisole in 2 ml of ethanol.

(b) Aliquot and store at -20°C.

(c) Add 1 μl per 1 ml of medium for a concentration of 200 μM butylated hydroxyanisole.

2. Forskolin

(a) Dissolve 8.21 mg of forskolin in 2 ml of DMSO.

(b) Aliquot and store at -20°C.

(c) Add 1 μl per 1 ml of medium for a concentration of 10 μM forskolin.

3. Hydrocortisone

(a) Dissolve 1 mg of hydrocortisone in 1 ml of ethanol + 1.76 ml of DMEM-LG.

(b) Aliquot and store at -20°C.

(c) Add 1 μl per 1 ml of medium for a concentration of 1 μM hydrocortisone.

4. Insulin

(a) Mix sterile distilled water and glacial acetic acid. Adjust to pH = 2.5.

(b) Dissolve 10 mg of insulin in 5 ml of the sterile distilled water + glacial acetic acid mixture.

(c) Aliquot and store at -20°C.

(d) Add 2.5 μl per 1 ml of medium for a concentration of 5 μg/ml insulin.

5. Potassium chloride (KCl)

(a) Dissolve 3.727 g of KCl in 30 ml of sterile distilled water.

(b) Aliquot and store at -20°C.

(c) Add 15 μl per 1 ml of medium for a concentration of 25 mM KCl.

6. Valproic acid

(a) Dissolve 0.665 g of valproic acid in 20 ml of distilled water.

(b) Aliquot and store at -20°C.

(c) Add 10 μl per 1 ml of medium for a concentration of 2 mM valproic acid.

Preparing the rat marrow-derived stromal cell media

1. Rat marrow-derived stromal cell expansion medium (rMSC EM)

   (a) DMEM-LG

   (b) 20% FBS

   (c) 1% antibiotic-antimycotic

   (d) 10 ng/ml bFGF (add fresh each time)

2. Rat marrow-derived stromal cell neurogenic medium (rMSC NM)

   (a) DMEM-LG

   (b) 2% DMSO (sterile filter prior to use)

   (c) 200 μM Butylated hydroxyanisole

   (d) 10 μM Forskolin

   (e) 1 μM Hydrocortisone

   (f) 5 μg/ml Insulin

   (g) 25 mM KCl

   (h) 2 mM Valproic acid

Protocol

1. Thaw the rat marrow-derived stromal cells according to the *Thawing Frozen Cells Protocol* in section A.3.1. Use the rMSC EM as the cell culture medium.

2. Change the rMSC EM every 2-3 days according to the *Media Changing Protocol* in section A.3.3. The rat marrow-derived stromal cells become confluent in approximately 6 days when cultured in T150 flasks.

3. When the cells are approximately 80% confluent, passage the cells according to the *Passaging Cells Protocol* in section A.3.4. Transfer the cells to well plates for monolayer experiments, or seed the cells into collagen scaffolds according to the *Cell Seeding Collagen Scaffolds Protocol* in section A.3.6 for 3D experiments.

4. Continue to culture the cells in rMSC EM for 2 days to allow the cells to attach to the well plate or collagen scaffold.

5. After 2 days, culture the cells in rMSC EM or rMSC NM for 5 hours.

A.6 Cell-Seeded Collagen Scaffold Protocols

A.6.1 Tissue Processor Protocol

Materials

1. Eppendorf tubes
2. 4% Paraformaldehyde-PBS (usb, Cat. #19943)
3. Tweezers
4. Blue sponges
5. Tissue processor cassettes
6. Tissue processor
7. Ethyl alcohol, 200 proof (PHARMACO-AAPER)
8. Paraffin

Protocol

1. Fill each Eppendorf tube with 0.5 ml of 4% paraformaldehyde-PBS.
2. Transfer the cell-seeded collagen scaffolds to Eppendorf tubes using tweezers.

3. Fix the cell-seeded collagen scaffolds in 4% paraformaldehyde-PBS for 30 min.

4. Using tweezers, place the cell-seeded collagen scaffolds between two blue sponges in the tissue processor cassettes.

5. Label the cassettes with pencil.

6. The tissue processor used is located at the VA Tissue Engineering Laboratories, Room 116. Place the cassettes in the tissue processor basket. Check the solution levels.

7. Run *Program 3*, which goes through the following steps:

Step	Solution	Time	Temperature
1	70% ethanol	10 min	Room
2	80% ethanol	90 min	Room
3	95% ethanol	90 min	Room
4	95% ethanol	90 min	Room
5	100% ethanol	90 min	Room
6	100% ethanol	90 min	Room
7	100% ethanol	90 min	Room
8	Xylene	90 min	Room
9	Xylene	90 min	Room
10	Xylene	90 min	Room
11	Paraffin	180 min	58°C
12	Paraffin	180 min	58°C
		19.5 hrs	

Table A.2: Tissue processor program

A.6.2 Embedding Collagen Scaffolds in Paraffin Protocol

Materials

1. Thermo Shandon Histocentre 2

2. Paraffin

3. Plastic or metal molds

Protocol

1. Turn on heat to melt paraffin.
2. Turn on cold plate.
3. Transfer the paraffin-embedded cassettes from the tissue processor to the paraffin bath in the histocentre.
4. Open a cassette and remove the blue sponge.
5. Dispense a small amount of paraffin at the bottom of a plastic or metal mold.
6. Put the mold on the cold plate.
7. Quickly transfer the collagen scaffold from the cassette to the bottom of a plastic or metal mold using tweezers. Press the collagen scaffold flat against the bottom of the mold as the paraffin solidifies to keep the sample in place.
8. Place the labeled cassette onto the mold.
9. Fill the rest of the mold with paraffin up to the level of the cassette top.
10. Place the mold on the cold plate for at least 30 minutes to allow the paraffin to solidify.
11. Store the mold at -20°C for at least 1 hour before taking off the mold.
12. Store paraffin blocks at -20°C before sectioning on the microtome.

A.6.3 Sectioning Paraffin Blocks Protocol

Materials

1. Water bath
2. Thermo Shandon Finesse Microtome
3. Tweezers
4. Superfrost PLUS Gold slides (Thermo Scientific, Cat. #4981)

5. Slide warmer

Protocol

1. Warm the water bath.
2. Remove excess paraffin around the cassette to ensure a correct fit in the sample holder.
3. Place the cassette in the holder and tighten the top screw.
4. Unlock the flywheel and move the sample close to the blade using the arrow keys on the control board. Align the sample surface parallel to the blade using the adjustment knobs.
5. Switch to *Trim* mode (10 μm) and start sectioning the paraffin block.
6. Check at the beginning that the sections include the surface of the whole block. If necessary, adjust the block orientation using the knobs.
7. When reaching the region of interest, switch to *Cut* mode (6 μm).
8. At first, the section will be curled. Use small tweezers to pick up one paraffin section while keeping it in contact with the cutting edge.
9. The next section will stick to this one and will allow a continuous number of sections to be obtained at once.
10. Carefully transfer the sections to the water bath using pencils or tweezers. Attach the end section(s) to the wall of the water bath.
11. The warm water will smooth the sections by warming the paraffin. Choose the best sections, usually in groups of three, and isolate them using pencils or tweezers.
12. Carefully position the glass slide under the water under the sections, allow the end of a section to stick to the top of the slide, then gently pull the slide out of the water as the rest of the sections stick to the slide.

13. Label the slide and let it dry.

14. Place the dry slide on the slide warmer (60°C) for 1-2 hours.

A.7 Immunofluorescent Staining Protocols

A.7.1 Immunofluorescent Staining for Paraffin-Embedded Sections Protocol

Materials

1. Xylene (Fisher Scientific)

2. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

3. Donkey Serum (Sigma, Cat. #D9663)

4. Tris-Buffered Saline (TBS) (Dako, Cat. #S3001)

5. Antibody Diluent (Dako, Cat. #S0809)

6. Mouse βIII-tubulin Monoclonal Antibody (TUJ1) (Covance, Cat. #MMS-435P)

7. Rabbit βIII-tubulin Monoclonal Antibody (TUJ1) (Covance, Cat. #MRB-435P)

8. Rabbit Anti-GFAP Polyclonal Antibody (Dako, Cat. #Z0334)

9. Mouse Anti-Nestin Monoclonal Antibody (Chemicon, Cat. #MAB353)

10. Universal Negative Control Mouse (Dako, Cat. #N1698)

11. Universal Negative Control Rabbit (Dako, Cat. #N1699)

12. Dulbecco's Phosphate Buffered Saline (PBS) 1X (Gibco, Cat. #14190)

13. Rhodamine (TRITC) Anti-mouse (Jackson Immunoresearch, Cat. #715-025-150)

14. Fluorescein (FITC) Anti-rabbit (Jackson Immunoresearch, Cat. #711-095-152)

15. 4% Paraformaldehyde-PBS (usb, Cat. #19943)

16. 4',6-diamidino-2-phenylindole (DAPI), dihydrochloride (Molecular Probes, Cat. #D1306)

17. Faramount Aqueous Mounting Medium (Dako, Cat. #S3025)

Preparing the stock solutions

Adapted from ihcworld.com.

1. DAPI stock solution

   (a) Dissolve DAPI in PBS to get a concentration of 5 mg/ml.

   (b) Aliquot and store at -20°C. Protect from light.

2. DAPI working solution

   (a) Thaw DAPI stock solution aliquot.

   (b) Micropipette 2 μl of DAPI stock solution into 50 ml of PBS to get a concentration of 200 ng/ml.

   (c) Store at 4°C. Protect from light.

Protocol

Day 1

1. Deparaffinize and rehydrate the paraffin-embedded sections on glass slides.

Xylene	2 × 5 min
100% ethanol	2 × 3 min
95% ethanol	2 × 2 min
80% ethanol	1 min
Rinse in running tap water	5 min

2. (Optional) Incubate slides in 2% donkey serum diluted in TBS for 30 minutes at room temperature. Aspirate out the blocking serum, but do NOT wash!

3. Incubate slides in primary antibody diluted 1:100 in antibody diluent overnight at 4°C in a humid chamber. NOTE: For negative controls, do NOT put any primary antibody, only negative control mouse or rabbit.

Day 2

1. Wash slides with PBS at 4°C for 3 × 5 minutes.

2. Add the secondary antibody diluted 1:200 in PBS and incubate for 60 minutes at room temperature in the dark.

3. Aspirate out the secondary antibody.

4. Wash with PBS at 4°C for 3 × 5 minutes.

5. Wash briefly with PBS at room temperature.

6. Fix the sections in 4% paraformaldehyde-PBS at room temp for 30 minutes.

7. Wash with PBS at room temperature for 3 × 5 minutes.

8. Incubate in DAPI working solution for 60 minutes at room temperature in the dark. Nuclei appear bright blue when viewed under a UV light on a fluorescent microscope.

9. Wash with PBS at room temperature for 3 × 5 minutes.

10. Coverslip slides with Faramount Aqueous Mounting Medium.

A.7.2 Immunofluorescent Staining in Monolayer Protocol

Materials

1. Donkey Serum (Sigma, Cat. #D9663)

2. Tris-Buffered Saline (TBS) (Dako, Cat. #S3001)

3. Antibody Diluent (Dako, Cat. #S0809)

4. Mouse βIII-tubulin Monoclonal Antibody (TUJ1) (Covance, Cat. #MMS-435P)

5. Rabbit βIII-tubulin Monoclonal Antibody (TUJ1) (Covance, Cat. #MRB-435P)

6. Rabbit Anti-GFAP Polyclonal Antibody (Dako, Cat. #Z0334)

7. Mouse Anti-Nestin Monoclonal Antibody (Chemicon, Cat. #MAB353)

8. Universal Negative Control Mouse (Dako, Cat. #N1698)

9. Universal Negative Control Rabbit (Dako, Cat. #N1699)

10. Dulbecco's Phosphate Buffered Saline (PBS) 1X (Gibco, Cat. #14190)

11. Rhodamine (TRITC) Anti-mouse (Jackson Immunoresearch, Cat. #715-025-150)

12. Fluorescein (FITC) Anti-rabbit (Jackson Immunoresearch, Cat. #711-095-152)

13. 4% Paraformaldehyde-PBS (usb, Cat. #19943)

14. 4',6-diamidino-2-phenylindole (DAPI), dihydrochloride (Molecular Probes, Cat. #D1306)

15. Faramount Aqueous Mounting Medium (Dako, Cat. #S3025)

Protocol

Day 1

1. Aspirate out the medium from the wells or the chamber slides.

2. Fix the cells in 4% paraformaldehyde-PBS at room temperature for 15 minutes.

3. Wash the cells with PBS at 4°C for 3 × 5 minutes.

4. (Optional) Incubate in 2% donkey serum diluted in TBS for 30 minutes at room temperature. Aspirate out the blocking serum, but do NOT wash!

5. Incubate in primary antibody diluted 1:100 in antibody diluent overnight at 4°C in a humid chamber. NOTE: For negative controls, do NOT put any primary antibody, only negative control mouse or rabbit.

Day 2

1. Wash the wells or chambers with PBS at 4°C for 3 × 5 minutes.

2. Add the secondary antibody diluted 1:200 in PBS and incubate for 60 minutes at room temperature in the dark.

3. Aspirate out the secondary antibody.

4. Wash with PBS at 4°C for 3 × 5 minutes.

5. Wash briefly with PBS at room temperature.

6. Fix the cells in 4% paraformaldehyde-PBS at room temp for 15 minutes.

7. Wash with PBS at room temperature for 3 × 5 minutes.

8. Incubate in DAPI working solution for 60 minutes at room temperature in the dark. Nuclei appear bright blue when viewed under a UV light on a fluorescent microscope.

9. Wash with PBS at room temperature for 3 × 5 minutes.

10. Keep wells moist with PBS, or remove the chambers and coverslip slides with Faramount Aqueous Mounting Medium.

A.8 Immunohistochemistry Protocols

A.8.1 Hematoxylin & Eosin (H&E) Staining Protocol

Materials

1. Xylene (Fisher Scientific)

2. Ethyl alcohol, 200 proof (PHARMACO-AAPER)

3. Gills 2× Hematoxylin (Protocol, Cat. #245-654)

4. Eosin Y Solution Aqueous (Sigma, Cat. #HT110-2-128)

5. Hydrochloric Acid (HCl) (Fisher Scientific)

6. Cytoseal 60 (Richard-Allan Scientific, Cat. #8310)

Protocol

1. Deparaffinize and rehydrate the paraffin-embedded sections after they have been baked for 1-2 hours.

Xylene	2 × 5 min
100% ethanol	2 × 3 min
95% ethanol	2 × 2 min
80% ethanol	1 min
Rinse in running tap water	5 min

2. Dip in Gills 2× Hematoxylin for 3 minutes. Note: Filter hematoxylin prior to use.

3. Rinse in running tap water for 5 minutes.

4. Few quick dips in acid alcohol (0.5% HCl diluted in 80% ethanol).

5. Rinse in running tap water for 5 minutes.

6. Dip in Eosin Y Solution Aqueous for 1 minute.

7. Dehydrate.

100% ethanol	2 × 3 min
Xylene	2 × 3 min

8. Coverslip with Cytoseal.

A.8.2 α-Smooth Muscle Actin (SMA) Staining Protocol

Materials

1. Tris-Buffered Saline (TBS) (Dako, Cat. #S3001)
2. Wash Buffer with Tween 20 (Dako, Cat. #S3006)
3. Protease XIV (Sigma, Cat. #P5174)
4. Peroxidase Blocking Reagent (Dako, Cat. #S2001)
5. Goat Serum (Sigma)
6. Monoclonal Anti-Actin, α-Smooth Muscle antibody (Sigma Cat. # A2547)
7. Antibody Diluent (Dako, Cat. #S0809)
8. Universal Negative Control Mouse IgG and IgM Cocktail (Dako, Cat. #N1698)
9. Universal LSAB2 Kit/HRP, Rabbit/Mouse (Dako, Cat. #K0675)
10. (3-amino-9-ethylcarbazole) AEC Substrate-Chromagen (Dako, Cat. #K3464)
11. Mayer's Hematoxylin (Sigma, Cat. #MHS16)
12. Faramount Aqueous Mounting Medium (Dako, Cat. #S3025)
13. Dako Autostainer

Preparing the solutions

1. Tris-Buffered Saline (TBS)
   (a) Dissolve 1 package (stored at room temperature) in 1 L of distilled water.
   (b) Store at 4°C in solution.
2. 10× Wash Buffer with Tween 20
   (a) Dilute 400 ml of 10× (stored at 4°C) in 3600 ml of distilled water.

(b) Pour into the wash buffer container located under the autostainer.

3. Protease XIV

   (a) Dissolve 10 mg of protease XIV (stored at -20°C) in 10 ml of TBS for 0.1% (w/v) Protease XIV.

4. 5% Goat Serum

   (a) Thaw goat serum aliquot stored at -20°C.

   (b) Dilute 0.5 ml of goat serum in 10 ml of TBS.

5. anti-α-SMA primary antibody

   (a) Thaw anti-α-SMA antibody aliquot stored at -20°C.

   (b) Dilute 15 μl of anti-α-SMA antibody in 6 ml of antibody diluent for a 1:400 dilution.

Setting up the autostainer

Use goat heart-muscle tissue or aorta for positive and negative controls.

1. On the computer desktop, click on *DAKO Autostainer.*

2. Login: *Dong.* Password: *Dong.*

3. Under *Main Menu*, select *Program.*

4. Click on *File.* In the pull-down menu, click on *Open* and choose *SMA.*

5. The template will show two slides: #1 for positive SMA staining and #2 for negative control staining.

6. To add slides, click on *Slides* and type in the number of slides to be stained including all controls.

7. Copy and paste the staining protocol by first clicking on *Copy*, then click on the slide to be copied. Clicking on any slide will automatically paste the protocol to any desired row.

8. Click on *Next.*

9. *Rinse Missing* window will appear. Click on *Yes.* It is important to make sure that there is no rinse after the serum block to reduce unspecific background staining.

10. The program then shows the *Program Slides* screen. The standard setting uses 150 μl per treated section of each slide. The standard setup of the machine will treat 3/3 of the slide (marked yellow). To change this, click on the slide while holding *Ctrl*, or click on the slide in the top left corner to change all the slides the same way.

11. Click on *Next.*

12. The program will show how much wash buffer is needed to run the program. Check the wash buffer container under the autostainer. If necessary, make some new wash buffer and pour it into the container.

13. Click on *OK.*

14. The *Load Reagents* screen will show the amount and the location in rack #1 of reagents used in the protocol. The autostainer will calculate the amount of reagent required according to the number of slides being stained.

15. Prepare the solutions, load them in the rack, and click on *Next.*

16. Deparaffinize and rehydrate the paraffin-embedded sections on glass slides.

Xylene	2 × 5 min
100% ethanol	2 × 3 min
95% ethanol	2 × 2 min
80% ethanol	1 min
Rinse in running tap water	5 min

17. Load the slides as shown on the *Load Slides* screen. Use the TBS squeeze bottle to keep the slides wet.

18. Click on *Next.*

19. Click on *Prime Pump (Buffer)* and *Prime Pump (Water)*, and check if the autostainer is running.

20. Click on *Start Run.*

Autostainer steps

Rinse with TBS+Tween	
Add 0.1% Protease	45 min
Rinse with TBS+Tween	
Peroxidase blocking reagent	10 min
Rinse with TBS+Tween	
5% goat serum	30 min
NO RINSE	
Anti-α-SMA or negative mouse control	30 min
Rinse with TBS+Tween	
Secondary reagent: Biotinylated link antibody	15 min
Rinse with TBS+Tween	
Tertiary reagent: Streptavidin-Horseradish Peroxidase (SA-HRP)	15 min
Rinse with TBS+Tween	
Switch	
Substrate: AEC	10 min

Counterstaining

1. When the program is finished, immediately remove the slides and put them in TBS to keep wet.

2. Counterstain with Mayer's Hematoxylin for 1.5 minutes. Note: Filter hematoxylin prior to use.

3. Rinse in running tap water for 3 minutes.

4. Transfer the slideholder into a container with tap water.

5. Immediately coverslip with Faramount Aqueous Mounting Medium. Do NOT use Cytoseal, as this will take off the AEC staining.

Appendix B

Data

B.1 Collagen Scaffold Pore Size Analysis Data

Six 8 mm diameter, 0.5 - 1 mm thick, 0.5% Bio-Gide® porcine type I/III collagen scaffolds were fabricated via the same freeze-drying conditions. 10 ml of slurry in plastic molds were cooled at 1°C/min to -40°C. An unpaired t-test was used to compare the mean pore diameters of all six scaffold samples.

Scaffolds	Mean Diff.	DF	t-Value	P-Value	
s2, s3	-48.176	10	-4.695	0.0008	S
s2, s4	-7.417	10	-.528	0.6089	
s2, s5	-27.981	9	-2.557	0.0308	S
s2, s6	24.509	10	6.148	0.0001	S
s2, s9	-16.006	10	-3.724	0.0040	S
s3, s4	40.758	10	2.470	0.0331	S
s3, s5	20.195	9	1.389	0.1983	
s3, s6	72.685	10	7.621	<0.0001	S
s3, s9	32.170	10	3.326	0.0077	S
s4, s5	-20.563	9	-1.143	0.2826	
s4, s6	31.927	10	2.361	0.0399	S
s4, s9	-8.588	10	-.631	0.5424	
s5, s6	52.490	9	5.192	0.0006	S
s5, s9	11.975	9	1.166	0.2734	
s6, s9	-40.515	10	-19.900	<0.0001	S

Table B.1: Unpaired t-test for collagen scaffold mean pore diameter. S = statistically significant.

B.2 Collagen Scaffold Contraction Data

Note that no scaffold contraction was observed for any of the NSC-seeded collagen scaffolds. Tables B.2 and B.3 compare the contraction of MSC-seeded scaffolds by rat number and time in culture.

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Rat	3	5.745	1.915	6.212	0.0008	18.635	0.965
Days	1	82.742	82.742	268.401	<0.0001	268.401	1.000
Days * Rat	3	5.745	1.915	6.212	0.0008	18.635	0.965
Exp	2	4.425	2.212	6.868	0.0018	13.736	0.925
Days	1	120.321	120.321	373.511	<0.0001	373.511	1.000
Days * Exp	2	4.425	2.212	6.868	0.0018	13.736	0.925

Table B.2: Repeated measures ANOVA for collagen scaffold mean diameter.

	Mean Diff.	Crit. Diff.	P-Value	
m1, m3	0.507	0.345	0.0046	S
m1, m4	0.208	0.253	0.1045	
m1, m5	-0.283	0.372	0.1343	
m3, m4	-0.299	0.284	0.0398	S
m3, m5	-0.790	0.394	0.0002	S
m4, m5	-0.491	0.317	0.0028	S
exp4, exp5	0.438	0.255	0.0010	S
exp4, exp6	0.443	0.260	0.0011	S
exp5, exp6	0.005	0.204	0.9614	

Table B.3: Fisher's PLSD for collagen scaffold mean diameter. S = statistically significant.

B.3 Neural Stem Cell Experimental Data

B.3.1 Monolayer vs. collagen scaffold

	Count	Mean	Std. Dev.	Std. Err
% Nestin: Medium * Substrate effect				
DM, mono	3	24.640	11.958	6.904
DM, scaff	7	25.937	10.652	4.026
NM, mono	6	68.176	21.768	8.887
NM, scaff	7	30.050	14.690	5.552
% TUJ1: Medium * Substrate effect				
DM, mono	4	51.164	9.630	4.815
DM, scaff	4	40.608	18.347	9.174
NM, mono	5	81.587	33.758	15.097
NM, scaff	5	37.108	16.899	7.558
% GFAP: Medium * Substrate effect				
DM, mono	9	14.485	13.235	4.412
DM, scaff	4	14.414	13.428	6.714
NM, mono	2	0.000	0.000	0.000
NM, scaff	1	4.082		

Table B.4: % nestin, %TUJ1, and % GFAP means table for rat NSCs in monolayer or collagen scaffolds in DM or NM for 6 days.

(a) ANOVA for % nestin

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	2889.594	2889.594	11.856	0.0027	11.856	0.920
Substrate	1	1726.254	1726.254	7.083	0.0154	7.083	0.719
Medium * Substrate	1	1977.947	1977.947	8.115	0.0103	8.115	0.781

(b) Fisher's PLSD for % nestin

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	-22.099	13.744	0.0033	S
mono, scaff	25.670	13.961	0.0011	S

Table B.5: ANOVA and Fisher's PLSD for % nestin positive rat NSCs in monolayer or collagen scaffolds in DM or NM for 6 days. S = statistically significant.

(a) ANOVA for % TUJ1

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	805.454	805.454	1.613	0.2247	1.613	0.209
Substrate	1	3365.447	3365.447	6.742	0.0211	6.742	0.678
Medium * Substrate	1	1278.610	1278.610	2.561	0.1318	2.561	0.307

(b) Fisher's PLSD for % TUJ1

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	-13.462	22.731	0.2247	
mono, scaff	29.402	22.590	.0144	S

Table B.6: ANOVA and Fisher's PLSD for % TUJ1 positive rat NSCs in monolayer or collagen scaffolds in DM or NM for 6 days. S = statistically significant.

	Count	Mean	Std. Dev.	Std. Err
Nestin positive neurite length: Medium * Substrate effect				
DM, mono	3	156.667	46.458	26.822
DM, scaff	7	59.286	18.016	6.809
NM, mono	6	140.000	41.110	16.783
NM, scaff	7	47.286	12.945	4.893
TUJ1 positive neurite length: Medium * Substrate effect				
DM, mono	4	97.500	25.981	12.990
DM, scaff	4	64.750	37.125	18.562
NM, mono	5	116.000	27.019	12.083
NM, scaff	5	67.000	19.786	8.849

Table B.7: Nestin and TUJ1 positive neurite length (μm) means table for rat NSCs in monolayer or collagen scaffolds in DM or NM for 6 days.

(a) ANOVA for nestin positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	1045.899	1045.899	1.264	0.2749	1.264	0.178
Substrate	1	45991.527	45991.527	55.589	<0.0001	55.589	1.000
Medium * Substrate	1	27.717	27.717	0.034	0.8567	0.034	0.053

(b) Fisher's PLSD for nestin positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	-1.577	25.323	0.8977	
mono, scaff	92.270	25.721	<0.0001	S

Table B.8: ANOVA and Fisher's PLSD for nestin positive neurite length (μm) for rat NSCs in monolayer or collagen scaffolds in DM or NM for 6 days. S = statistically significant.

(a) ANOVA for TUJ1 positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	478.403	478.403	0.629	0.4409	0.629	0.111
Substrate	1	7425.625	7425.625	9.765	0.0075	9.765	0.840
Medium * Substrate	1	293.403	293.403	0.386	0.5445	0.386	0.088

(b) Fisher's PLSD for TUJ1 positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	-10.375	28.054	0.4409	
mono, scaff	41.778	27.881	0.0062	S

Table B.9: ANOVA and Fisher's PLSD for TUJ1 positive neurite length (μm) for rat NSCs in monolayer or collagen scaffolds in DM or NM for 6 days. S = statistically significant.

B.3.2 Neurogenic vs. non-neurogenic medium, Culture time

	Count	Mean	Std. Dev.	Std. Err
% Nestin: Time * Medium effect				
6d, EM	3	12.600	1.676	0.967
6d, DM	4	44.653	18.696	9.348
6d, NM	4	21.967	6.444	3.222
14d, EM	2	0.000	0.000	0.000
14d, DM	6	29.589	2.315	0.945
14d, NM	11	18.052	7.674	2.314
% TUJ1: Time * Medium effect				
6d, EM	2	23.960	8.370	5.918
6d, DM	4	17.430	8.495	4.247
6d, NM	3	27.514	3.940	2.275
14d, EM	2	0.000	0.000	0.000
14d, DM	5	30.916	9.318	4.167
14d, NM	5	27.598	15.290	6.838
% GFAP: Time * Medium effect				
6d, EM	1	6.667		
6d, DM	3	24.375	4.469	2.580
6d, NM	2	9.127	2.806	1.984
14d, EM	1	0.000		
14d, DM	2	11.556	0.629	0.444
14d, NM	4	17.866	7.366	3.683

Table B.10: % nestin, %TUJ1, and % GFAP means table for rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days.

(a) ANOVA for % nestin

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Time	1	626.782	626.782	8.382	0.0079	8.382	0.805
Medium	2	3342.098	1671.049	22.348	<0.0001	44.695	1.000
Time * Medium	2	177.253	88.626	1.185	0.3229	2.370	0.227

(b) Fisher's PLSD for % nestin

	Mean Diff.	Crit. Diff.	P-Value	
EM, DM	-28.055	9.775	<0.0001	S
EM, NM	-11.536	9.216	0.0163	S
DM, NM	16.519	7.286	<0.0001	S
6d, 14d	7.866	6.762	0.0245	S

Table B.11: ANOVA and Fisher's PLSD for % nestin positive rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days. S = statistically significant.

(a) ANOVA for % TUJ1

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Time	1	54.430	54.430	0.510	0.4860	0.510	0.100
Medium	2	653.650	326.825	3.064	0.0766	6.128	0.497
Time * Medium	2	970.768	485.384	4.550	0.0285	9.101	0.682

(b) Fisher's PLSD for % TUJ1

	Mean Diff.	Crit. Diff.	P-Value	
EM, DM	-12.942	13.229	.0545	
EM, NM	-15.586	13.481	.0263	S
DM, NM	-2.644	10.697	0.6060	
6d, 14d	-2.138	9.707	0.6455	

Table B.12: ANOVA and Fisher's PLSD for % TUJ1 positive rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days. S = statistically significant.

	Count	Mean	Std. Dev.	Std. Err
Nestin positive neurite length: Time * Medium effect				
6d, EM	3	17.000	14.731	8.505
6d, DM	4	58.750	16.378	8.189
6d, NM	4	52.750	8.421	4.211
14d, EM	2	0.000	0.000	0.000
14d, DM	6	98.833	33.820	13.807
14d, NM	11	74.091	17.467	5.266
TUJ1 positive neurite length: Time * Medium effect				
6d, EM	2	0.000	0.000	0.000
6d, DM	4	66.750	4.113	2.056
6d, NM	3	49.333	14.012	8.090
14d, EM	2	0.000	0.000	0.000
14d, DM	5	102.600	23.755	10.624
14d, NM	5	72.600	17.300	7.737

Table B.13: Nestin and TUJ1 positive neurite length (μm) means table for rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days.

(a) ANOVA for nestin positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Time	1	1240.494	1240.494	2.913	0.1008	2.913	0.359
Medium	2	16230.880	8115.440	19.055	<0.0001	38.111	1.000
Time * Medium	2	2606.937	1303.469	3.061	0.0655	6.121	0.529

(b) Fisher's PLSD for nestin positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
EM, DM	-72.600	23.329	<0.0001	S
EM, NM	-58.200	21.995	<0.0001	S
DM, NM	14.400	17.388	0.1003	
6d, 14d	-28.923	16.137	0.0011	S

Table B.14: ANOVA and Fisher's PLSD for nestin positive neurite length (μm) for rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days. S = statistically significant.

(a) ANOVA for TUJ1 positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Time	1	1762.074	1762.074	6.781	0.0199	6.781	0.685
Medium	2	19810.230	9905.115	38.118	<0.0001	76.236	1.000
Time * Medium	2	888.878	444.439	1.710	0.2142	3.421	0.294

(b) Fisher's PLSD for TUJ1 positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
EM, DM	-86.667	20.647	<0.0001	S
EM, NM	-63.875	21.040	<0.0001	S
DM, NM	22.792	16.695	0.0108	S
6d, 14d	-26.889	15.151	0.0018	S

Table B.15: ANOVA and Fisher's PLSD for TUJ1 positive neurite length (μm) for rat NSC-seeded scaffolds cultured in EM, DM, or NM for 6 or 14 days. S = statistically significant.

B.3.3 Laminin coated collagen scaffolds

	Count	Mean	Std. Dev.	Std. Err
% Nestin: Medium * Laminin effect				
DM, y	4	37.828	15.967	7.983
DM, n	3	16.267	14.137	8.162
NM, y	9	11.500	6.357	2.119
NM, n	3	17.013	9.350	5.398
% TUJ1: Medium * Laminin effect				
DM, y	3	20.160	3.938	2.274
DM, n	3	13.770	12.564	7.254
NM, y	6	11.998	6.417	2.620
NM, n	3	14.818	6.936	4.005
% GFAP: Medium * Laminin effect				
DM, y	3	6.325	2.762	1.595
DM, n	5	16.918	10.210	4.566
NM, y	2	3.218	0.200	0.141
NM, n	1	1.662		

Table B.16: % nestin, %TUJ1, and % GFAP means table for rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin.

(a) ANOVA for % nestin

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	636.762	636.762	5.745	0.0300	5.745	0.608
Laminin	1	250.622	250.622	2.261	0.1534	2.261	0.278
Medium * Laminin	1	713.199	713.199	6.434	0.0228	6.434	0.660

(b) Fisher's PLSD for % nestin

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	15.709	10.673	0.0068	S
y, n	2.962	11.075	0.5771	

Table B.17: ANOVA and Fisher's PLSD for % nestin positive rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin, S = statistically significant.

(a) ANOVA for % TUJ1

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	43.373	43.373	0.735	0.4095	0.735	0.119
Laminin	1	10.924	10.924	0.185	0.6752	0.185	0.068
Medium * Laminin	1	72.716	72.716	1.233	0.2905	1.233	0.167

(b) Fisher's PLSD for % TUJ1

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	4.027	8.909	0.3412	
y, n	0.425	8.909	0.9183	

Table B.18: ANOVA and Fisher's PLSD for % TUJ1 positive rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin.

(a) ANOVA for % GFAP

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	165.819	165.819	2.685	0.1453	2.685	0.285
Laminin	1	40.164	40.164	0.650	0.4465	0.650	0.105
Medium * Laminin	1	72.589	72.589	1.175	0.3142	1.175	0.151

(b) Fisher's PLSD for % GFAP

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	10.246	12.581	0.0955	
y, n	-9.293	11.252	0.0918	

Table B.19: ANOVA and Fisher's PLSD for % GFAP positive rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin.

	Count	Mean	Std. Dev.	Std. Err
Nestin positive neurite length: Medium * Laminin effect				
DM, y	4	37.500	5.260	2.630
DM, n	2	57.500	10.607	7.500
NM, y	9	27.333	16.325	5.442
NM, n	3	43.667	26.407	15.246
TUJ1 positive neurite length: Medium * Laminin effect				
DM, y	3	44.667	8.737	5.044
DM, n	2	52.500	31.820	22.500
NM, y	6	32.500	18.727	7.645
NM, n	3	30.000	19.079	11.015

Table B.20: Nestin and TUJ1 positive neurite length (μm) means table for rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin.

(a) ANOVA for nestin positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	482.233	482.233	1.814	0.1995	1.814	0.230
Laminin	1	1105.209	1105.209	4.157	0.0608	4.157	0.465
Medium * Laminin	1	11.256	11.256	0.042	0.8399	0.042	0.054

(b) Fisher's PLSD for nestin positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	12.750	17.486	0.1402	
y, n	-18.738	18.403	0.0465	S

Table B.21: ANOVA and Fisher's PLSD for nestin positive neurite length (μm) for rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin.

(a) ANOVA for TUJ1 positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	901.333	901.333	2.472	0.1470	2.472	0.285
Laminin	1	21.333	21.333	0.059	0.8138	0.059	0.055
Medium * Laminin	1	80.083	80.083	0.220	0.6494	0.220	0.070

(b) Fisher's PLSD for TUJ1 positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
DM, NM	16.133	23.733	0.1608	
y, n	-2.444	23.733	0.8231	

Table B.22: ANOVA and Fisher's PLSD for TUJ1 positive neurite length (μm) for rat NSC-seeded scaffolds in DM or NM for 6 days, with or without laminin. y = laminin, n = no laminin.

B.4 Marrow-Derived Stromal Stem Cell Experimental Data

	Count	Mean	Std. Dev.	Std. Err
% Nestin: Medium * Substrate effect				
EM, mono	10	0.000	0.000	0.000
EM, scaff	4	2.778	5.556	2.778
NM, mono	13	13.064	7.166	1.988
NM, scaff	8	9.647	8.967	3.170
% TUJ1: Medium * Substrate effect				
EM, mono	5	1.476	2.810	1.257
EM, scaff	3	5.556	9.623	5.556
NM, mono	11	21.484	11.790	3.555
NM, scaff	7	11.290	18.427	6.965
% GFAP: Medium * Substrate effect				
EM, mono	4	1.823	3.646	1.823
EM, scaff	1	0.000		
NM, mono	7	0.000	0.000	0.000
NM, scaff	4	.281	0.562	0.281

Table B.23: % Nestin, % TUJ1, and % GFAP means table for rat MSCs in EM or NM for 5 hours.

(a) ANOVA for % nestin

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	719.913	719.913	17.549	0.0002	17.549	0.990
Substrate	1	0.742	0.742	0.018	0.8939	0.018	0.052
Medium * Substrate	1	69.544	69.544	1.695	0.2025	1.695	0.230

(b) Fisher's PLSD for % nestin

	Mean Diff.	Crit. Diff.	P-Value	
EM, NM	-10.969	4.507	<0.0001	S
mono, scaff	0.027	4.652	0.9906	

Table B.24: ANOVA and Fisher's PLSD for % nestin positive rat MSCs in EM or NM for 5 hours. S = statistically significant.

(a) ANOVA for % TUJ1

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	863.894	863.894	5.215	0.0324	5.215	0.583
Substrate	1	48.717	48.717	0.294	0.5931	0.294	0.080
Medium * Substrate	1	265.580	265.580	1.603	0.2187	1.603	0.216

(b) Fisher's PLSD for % TUJ1

	Mean Diff.	Crit. Diff.	P-Value	
EM, NM	-14.514	11.342	0.0145	S
mono, scaff	5.661	10.760	0.2870	

Table B.25: ANOVA and Fisher's PLSD for % TUJ1 positive rat MSCs in EM or NM for 5 hours. S = statistically significant.

	Count	Mean	Std. Dev.	Std. Err
Nestin positive neurite length: Medium * Substrate effect				
EM, mono	10	0.000	0.000	0.000
EM, scaff	4	6.250	12.500	6.250
NM, mono	13	166.000	75.783	21.018
NM, scaff	8	34.625	36.641	12.955
TUJ1 positive neurite length: Medium * Substrate effect				
EM, mono	5	0.000	0.000	0.000
EM, scaff	3	14.667	25.403	14.667
NM, mono	11	120.455	52.700	15.890
NM, scaff	7	20.000	27.099	10.242

Table B.26: Nestin and TUJ1 positive neurite length (μm) means table for rat MSCs in EM or NM for 5 hours.

(a) ANOVA for nestin positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	68454.541	68454.541	26.936	<0.0001	26.936	1.000
Substrate	1	28366.753	28366.753	11.162	0.0022	11.162	0.916
Medium * Substrate	1	34317.537	34317.537	13.504	0.0009	13.504	0.960

(b) Fisher's PLSD for nestin positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
EM, NM	-114.167	35.475	<0.0001	S
mono, scaff	68.659	36.613	0.0006	S

Table B.27: ANOVA and Fisher's PLSD for nestin positive neurite length (μm) for rat MSCs in EM or NM for 5 hours.

(a) ANOVA for TUJ1 positive neurite length

	DF	Sum of Squares	Mean Square	F-value	P-value	Lambda	Power
Medium	1	20626.515	20626.515	13.558	0.0013	13.558	0.955
Substrate	1	9594.009	9594.009	6.306	0.0199	6.306	0.671
Medium * Substrate	1	17276.628	17276.628	11.356	0.0028	11.356	0.912

(b) Fisher's PLSD for TUJ1 positive neurite length

	Mean Diff.	Crit. Diff.	P-Value	
EM, NM	-75.889	34.372	0.0001	S
mono, scaff	64.412	32.608	0.0005	S

Table B.28: ANOVA and Fisher's PLSD for TUJ1 positive neurite length (μm) for rat MSCs in EM or NM for 5 hours.

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