Published in final edited form as:

In Vitro Cell Dev Biol Anim. 2013 September ; 49(8): 608–618. doi:10.1007/s11626-013-9647-8.

Myelination and node of Ranvier formation on sensory neurons in a defined in vitro system

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Abstract

One of the most important developmental modifications of the nervous system is Schwann cell myelination of axons. Schwann cells ensheath axons to create myelin segments to provide protection to the axon as well as increase the conduction of action potentials. *In vitro* neuronal systems provide a unique modality to study a variety of factors influencing myelination as well as diseases associated with myelin sheath degradation. This work details the development of a patterned *in vitro* myelinating dorsal root ganglion culture. This defined system utilized a serum-free medium in combination with a patterned substrate, utilizing the cytophobic and cytophilic molecules (poly)ethylene glycol (PEG) and N-1[3 (trimethoxysilyl) propyl] diethylenetriamine (DETA), respectively. Directional outgrowth of the neurites and subsequent myelination was controlled by surface modifications, and conformity to the pattern was measured over the duration of the experiments. The myelinated segments and nodal proteins were visualized and quantified using confocal microscopy. This tissue-engineered system provides a highly controlled, reproducible model for studying Schwann cell interactions with sensory neurons, as well as the myelination process, and its effect on neuronal plasticity and peripheral nerve regeneration. It is also compatible for use in bio-hybrid constructs to reproduce the stretch reflex arc on a chip because the media combination used is the same we have used previously for motoneurons, muscle and for neuromuscular junction (NMJ) formation. This work could have application for the study of demyelinating diseases such as diabetes induced peripheral neuropathy and could rapidly translate to a role in the discovery of drugs promoting enhanced peripheral nervous system (PNS) remyelination.

Keywords

Myelination; Patterning; Sensory Neurons; Schwann Cells

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I. INTRODUCTION

In the peripheral nervous system Schwann cells ensheath individual neuronal axons to create myelin segments, a process known as radial sorting (Jessen and Mirsky, 2005). These sheaths not only provide protection to the axon but also allow the machinery responsible for action potential propagation to be concentrated at distinct sites, known as nodes of Ranvier. There are four specialized domains associated with the node of Ranvier: the internode, the paranodal region, the juxtaparanode, and the node of Ranvier itself. The node of Ranvier is an area of unmyelinated axon which contains clusters of voltage-gated sodium channels (vgsc's). By concentrating the sodium channels at the nodal region, it facilitates rapid conduction of action potentials known as saltatory conduction (Sherman and Brophy, 2005). In the paranode, which is directly adjacent to the node of Ranvier, the lateral edge of the myelin sheath spirals around the axon and within this spiral can be found the cell adhesion molecule contactin associated glycoprotein (CASPR) (Sherman et al., 2005). Extending approximately 10–15 μm from the paranode is the juxtaparanode which harbors the voltage-gated potassium channels (vgpc's). Further adjacent lies the internode where axons are ensheathed by tightly compacted myelin whose major component is myelin basic protein (MBP).

Myelination of neurons by Schwann cells has been extensively studied using dorsal root ganglia (DRG) cultures in a variety of serum containing and serum-free *in vitro* systems, with most systems relying on the use of biological substrates for cell culture (Callizot et al., 2011; Hlady and Hodgkinson, 2007; Liazoghli et al., 2011; Svenningsen et al., 2003; Wood et al., 1990). Less common has been node of Ranvier formation by Schwann cells on motoneurons (Pang et al., 2012; Rumsey et al., 2009) and by oligodendrocytes on CNS neurons (Pang et al., 2012) in *in vitro* systems. Among the important findings from previous work with *in vitro* myelinating systems are the requirement of ascorbic acid in basal lamina modification to facilitate myelination by Schwann cells, the key role played by the extracellular matrix protein collagen type IV, the importance of β -1 integrin activation by laminin, the role of cyclic AMP in activation of the myelin genes and the importance of factors such as insulin-like growth factor I (IGF-I) and neuregulin-1 in enhancing myelination (Cheng et al., 1999; Chernousov et al., 2006; Clark et al., 1998; Fernandez-Valle et al., 1994; Lemke, 2006; Podratz et al., 2001; Russell et al., 2000). However, in these studies, axonal outgrowth and Schwann cell myelination were random/variant/non-uniform in its organization.

The development of *in vitro* culture systems where each variable is defined has enabled systems of multiple cell types to be established (Schaffner et al., 1995). This has led to defined systems for cardiac (Das et al., 2004; Natarajan et al., 2011), muscle (Das et al., 2006; Das et al., 2009), NMJ formation (Das et al., 2010; Guo et al., 2011; Guo et al., 2010a), sensory neurons and motoneuron co-culture (Das et al., 2007; Davis et al., 2012a) as well as for human stem cell derived culture models (Davis et al., 2012b; Guo et al., 2012; Guo et al., 2010b). The similar media system, non-biological substrate and defined cell preparation has enabled the integration of these models for body-on-a-chip applications.

Previously, we developed a serum-free DRG culture system utilizing the non-biological substrate N-1[3 (trimethoxysilyl) propyl] diethylenetriamine (DETA) (Liu et al., 2008). The utility of this substrate comes from its ability to form a self-assembled monolayer (SAM) on any hydroxylated surface and its non-degradability by cells. The photolithographic patterning of SAMs has also been used to control the directional outgrowth of axons (Ravenscroft et al., 1998; Stenger et al., 1998). In addition, the triamine functionality is also a structural analog to spermine, a growth factor known to promote cellular survival (Eisenberg et al., 2009; Kaeberein, 2009). All of these features had made DETA a useful substrate for bioengineering applications, a major goal in hybrid electronic systems, tissue engineering and cell-based biosensors.

Laser ablation photolithography of SAMs by deep ultraviolet (UV) radiation has been shown to modify organosilanes by photocleavage, rendering the surface amenable to additional SAM modification (Stenger et al., 1992). By rederivatizing with an organosilane resistant to protein adsorption and consequently cell adhesion, a patterned surface can be generated with regions that support cell adhesion and regions that do not support cell adhesion. Poly(ethylene glycol) (PEG) SAMs prevent the adsorption of proteins on glass surfaces by the entropy/hydrated surface hypothesis (Nogaoka et al., 1984; Prime and Whitesides, 1993; Yang et al., 1999). Therefore, a surface composed of alternating regions of PEG-DETA should facilitate the patterned adhesion of cells (Wilson et al., 2011).

In this study, we demonstrate myelination and node of Ranvier formation on sensory neurons in a chemically defined, serum-free medium on the biomimetic, non-biological substrate DETA. Additionally, this directional outgrowth of the axons and their subsequent myelination by Schwann cells was controlled using a photolithographic patterned PEG-DETA substrate. These findings are of interest in the area of myelinating *in vitro* tissue engineered systems and could play an important role in the development of cell-based biosensors and bio-hybrid devices where cell location and directional control of process outgrowth are critical to functionality. The compatibility of this media combination with other cell types also makes it amenable for the fabrication of multicellular constructs. The system would have significant applications for demyelinating diseases such as diabetes induced peripheral neuropathy and provide a high-content screening system for developing drugs to enhance PNS remyelination post-injury.

Experimental Setup and Methodology

A. PEG surface modification—Glass coverslips (VWR 48366067, 22×22 mm² No. 1) were first cleaned using 1:1 HCl-methanol followed by a concentrated H₂SO₄ soak for 2 hours. The coverslips were then coated with a PEG-terminated silane by a protocol modified from Papra et al. Dry toluene was prepared by distillation over metallic sodium to remove any water or other contaminants. The alkylsilane, 2-[Methoxypoly(ethyleneoxy)propyl]-trimethoxysilane (SiPEG) (Gelest, Tullytown, PA), was added to the toluene to a final concentration of 0.1% by volume in a MBraun glove box (MBraun, Stratham, NH). Concentrated HCl was added to a final volume of 0.08 % (0.8 ml HCl/L) to the SiPEG-toluene solution and the solution briefly stirred. The coverslips were then incubated in the SiPEG-toluene solution for 1 hour at room temperature. After 1 hour the samples were

removed and rinsed in serial washes of toluene (1x), ethanol (2x), and diH₂O (1x). The washed samples were blown dry under a stream of ultrapure nitrogen and were used immediately or stored in a desiccator until needed.

1. Deep-UV photolithography of PEG-silane monolayers: PEG-silane modified silica substrates were patterned using deep ultraviolet (DUV) photolithography. The samples were patterned in a photolithography system of our own design, which was based on a mask aligner, 193 nm ArF excimer laser (Lambda Physik, Santa Clara, CA) with an in-line beam homogenizer. Samples were placed on the stage of the mask aligner under a 5×5 inch chrome plated photomask, which contained the pattern, to be ablated. The pattern consisted of 15 lines each of 12 μm, 22 μm, 48 μm, and 96 μm width lines (Fig 1 D). The masks were written in dark-field polarity such that the areas corresponding to the ablated pattern were transparent and the remaining areas were opaque. When necessary, the substrate was precision aligned using the aligner stage to ensure micrometer precision placement of the pattern. The substrate was then brought into contact with the mask and a vacuum applied between the stage and mask to ensure a hard contact. A hard contact was needed to minimize the gap between the substrate and mask to ensure a high contrast pattern with minimal edge effects due to refraction of the laser light. The SiPEG substrates were then exposed to 193 nm ultraviolet laser light for 30 seconds with a pulse intensity of 200 mJ/pulse and a frequency of 10Hz. After ablation the samples were removed from the aligner stage and stored for subsequent processing.

2. Back-fill of patterned PEG-silane monolayers with DETA-silane: After ablation, the patterned PEG-silane substrates were backfilled with the alkylsilane (3-Trimethoxysilyl propyl) diethylenetriamine (DETA). Fresh distilled dry toluene was prepared as above. DETA was added to the toluene to a final concentration of 0.1% (vol:vol) inside a glove box. The DETA-toluene solution was removed from the glove box and transferred to a beaker and the samples were immersed in the solution. To drive the reaction forward the solution was gently heated to no more than 65°C for 30 minutes. After reaction with DETA the samples were allowed to cool to room temperature, washed 3 times with dry toluene and heated to 65°C for an additional 30 minutes. Surfaces were characterized by static water contact angle measurements using a Rame-Hart Model 250 goniometer, and by X-ray photoelectron spectroscopy (XPS) using an Escalab 200i spectrometer (VG Scientific) by monitoring the N 1s peak (Spargo et al., 1994; Stenger et al., 1998; Stenger et al., 1993). The values are reported as the mean ± SEM.

B. Animals—Dated pregnant Sprague-Dawley rats were housed in an animal facility at the University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines. Pregnant rats were anesthetized and sacrificed at embryonic day 15, embryos were removed by caesarean section and fetuses dissected under a stereomicroscope (Carl Zeiss, Stemi, 2000).

C. Dorsal Root Ganglion Culture and Myelination of Sensory Neurons—Timed pregnant rats were euthanized by inhalation of an excess of CO₂ and embryonic day 15

(E15) rats were collected in cold Hibernate E + B27 + Glutamax + antibiotic/antimycotic (dissection media). The rat embryos were dissected and DRGs collected in cold dissection media. The ganglia contained both the DRG neurons as well as the Schwann cells. Two mL of 0.05% trypsin-EDTA was added to the tissue and then placed in a water bath for 10 minutes at 37°C. After 10 minutes, the trypsin was removed and the reaction stopped by addition of 5 mL of Hibernate E + 10% FBS. The DRG tissue was manually triturated using a 1000 µL pipette until a homogenate was obtained and then centrifuged for 5 min at 200×g. Next, the medium was removed and 1mL of DRG plating media (Table 1) was added, and the cells gently suspended. A cell count was conducted and the cells were plated on 22×22 mm² DETA coated coverslips as controls as well as on the patterned coverslips at a density of 200 cells/mm². DRG neurons were grown for two weeks *in vitro* prior to myelination induction. After two weeks, the medium was supplemented with 50 µg/mL ascorbic acid to induce Schwann cell myelination of axons. The cultures were grown an additional two weeks to facilitate myelination and node of Ranvier formation.

D. Immunocytochemistry & Laser Scanning Confocal Microscopy—The co-cultures were fixed in fresh 4% paraformaldehyde in PBS for 5 minutes and then rinsed twice with PBS. Next, cells were permeabilized with a solution of 0.5% Triton-X 100 in PBS + 5% bovine serum albumin (BSA) for 5 minutes, rinsed once with PBS and then blocked with permeabilization solution + 5% donkey serum. The cells were then incubated with primary antibody solutions in blocking buffer overnight at 4°C. The following primary antibodies were obtained commercially from Millipore: anti-neurofilament heavy chain (1:12,000) (AB5539), anti- τ crC (07-226), anti-parvalbumin (MAB1572), anti-voltage-gated sodium channel α (1:200) (AB5210), and anti-MBP (1:40) (MAB382). The anti-CASPR antibody (1:300) (sc-14340) was obtained from Santa Cruz Biotechnology, Inc. The next day primary antibody solutions were aspirated and the cells rinsed three times with PBS. Then, AlexaFluor® 488 nm, 594 nm and 647 nm secondary antibodies diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was then aspirated and the coverslips rinsed three times in PBS and allowed to dry. Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (Vector Labs, H-1200) and fixed using clear nail polish.

RESULTS AND DISCUSSION

A. SiPEG-DETA line pattern surface preparation and cellular pattern conformity

Two control coverslips were used in order to test the quality of the SiPEG-DETA patterns: (1) one SiPEG coverslip was ablated without the photomask and rederivatized with DETA, and (2) a second SiPEG coverslip was rederivatized with DETA only. Laser irradiation and DETA rederivatization were done in the same conditions as for the SiPEG-DETA patterns.

The XPS measurements of the control coverslips indicated that SiPEG formed a self-assembling monolayer (SAM) on the glass coverslips (Fig. 1A). Additionally, DETA formed a SAM on ablated SiPEG, but it was not incorporated (or only incorporated in traces amounts) in the unexposed SiPEG regions (Fig. 1 B–C). Further, static water contact angle measurements of $92 \pm 2^\circ$ validated the hydrophobicity of the LASER exposed SiPEG after

DETA rederivatization. However, the non-ablated SiPEG monolayer was not affected by the reaction with DETA, as indicated by the contact angle value of $45 \pm 3^\circ$ for the unexposed SiPEG control coverslip, values that were close to that found for pure SiPEG. The mask used for these experiments resulted in lines of varying width and separation distance and the pattern uniformity was verified by palladium catalyzed copper reduction metallization (Fig. 1D). These results were consistent throughout the study indicating the reproducibility of the line patterns using this methodology.

B. Myelination Promoting Medium Formulation, Immunocytochemical Evaluation and Quantification of Myelination on Control DETA Surfaces

The defined medium formulation described in Table 1 supports the growth and development of sensory neurons and Schwann cells (Fig. 2A). In this medium, type Ia sensory neurons exhibited a mature phenotype as indicated by expression of the calcium binding protein parvalbumin and axonal extension (Fig. 2B–D) (Carr et al., 1989; Ernfors et al., 1994; Liu et al., 2008). These sensory neurons were also electrically active; displaying inward Na^+ currents and outward K^+ currents as well as firing repetitive action potentials (Fig.3).

The major component of compact myelin sheaths in the internode is MBP. MBP was evaluated using immunocytochemistry between days 25 and 30 as an indication of myelin sheath formation. The DRG axons were imaged using anti-neurofilament H (NF-H) antibody. Sheath formation around the axon was determined by the co-localization of the two antibodies. Myelin segments were observed in the DRG cultures and imaged using Z-stack confocal microscopy (Fig.4). After staining, myelin segments were quantified in order to determine the efficiency of Schwann cells myelination in the culture system. As shown in Table 1, 192.17 ± 2.74 myelinated segments were identified in the DRG culture. Additionally, functional myelination should result in rearrangement and clustering of vglut1's, vglut2's, and wrapping of CASPR in the axonal segment. This clustering would represent firm evidence of the formation of physiologically functional nodes of Ranvier.

C. Node of Ranvier Formation

In order to visualize nodal development in this system, immunocytochemistry was used to stain the vglut1's and CASPR localized at the nodes of Ranvier. As shown in Figure 5, vglut1's were found clustered between two myelinated segments of a sensory neuron axon, verifying node of Ranvier formation (Fig. 5 A–E). Additionally, clusters of CASPR (Fig. 5) were also seen in this culture system. The presence of these nodal proteins indicates maturation of the myelination process in this culture system.

D. Myelination of Sensory Neurons on Patterned SiPEG-DETA Surfaces

The cytophilic DETA foreground surrounded by cytophobic PEG background resulted in pattern formation supporting differential cell adhesion to the surface. The cellular adhesion to the line patterns was analyzed throughout the study and, as shown in Table 2, conformity was stable throughout the duration of the culture. At two days in culture 60 of 60 lines (100%) were isolated and had no cellular overgrowth (Fig 6); with 44.72 of 60 line patterns with no overgrowth after 20 days (Fig.7). Overall, the cells of respective DETA line patterns remained isolated, with no cell growth deviating from the original pattern design. Multiple

bi-nodal myelin basic protein segments, indicative of node formation, were observed consistently along axons grown within the 96 μm PEG-DETA patterns (Fig. 8). The patterning not only provided axonal guidance but further promoted Schwann cell myelination of the sensory neurons.

The development of a defined *in vitro* system guiding axonal outgrowth and their subsequent myelination using photolithography represents a significant technological development. These experiments indicate that a PEG-DETA surface prepared by laser ablation photolithography is sufficient to drive organization of axonal outgrowth and subsequent myelination and node of Ranvier formation in a defined DRG culture system. This patterned myelination system has important implications for the development of cell-based biosensors and other bio-hybrid devices where the direction of cell growth and development plays a key role in device functionality. Furthermore, this system could be rapidly integrated into a high-throughput screening platform to support drug discovery processes for peripheral demyelinating diseases.

In this study, cellular overgrowth of the DETA lines was monitored over time to determine the effectiveness of PEG as a negative, cell repulsive surface. As indicated by Table 2, pattern conformity remained stable throughout the time in culture. In contrast, a previous study conducted using DETA-13F (tridecafluoro-1,1,2,2-tetrahydrooctyl- 1-trichlorosilane) patterns showed a significant decrease in pattern conformity over time in culture. This data indicates that PEG is an effective cell repulsive surface both during initial plating and during maturation of the culture. This observation is likely due to the fact that PEG resists protein adsorption while in culture, rendering cell motility onto the PEG region unlikely (Wilson et al., 2012).

The patterned mask used in these experiments, one with varying line widths and distances, resulted in significantly different cellular morphologies as the cells conformed to the cytophilic DETA regions of the substrate as shown in Figure 6. Myelin segments and node of Ranvier development was only observed on lines of 96 μm width in this system. This observation is of interest as it begins to shed light on the minimum spatial dimensions necessary for the cellular interactions that result in functional myelination in the PNS. It is also of interest to note that dissociated cultures grown on a pattern width of 12 μm did not survive past DIV 4.

DETA is a silane molecule that forms a covalently bonded monolayer to almost any hydroxylated surface. It has the advantage over biological substrates in that it is easily patterned using photolithography and it is not degraded by the proteases secreted by the cells that are plated on it. Therefore, a patterned cell repulsive/cell adhesive surface, SiPEG/DETA, respectively, on the surface of a microelectrode array (MEA) can provide high-throughput data of axonal conductivity of myelinated versus demyelinated neurons. The fact that DETA is a non-biological substrate provides the ability to study the ECM secretion and basal lamina formation necessary for compact myelin formation (Fernandez-Valle et al., 1993). Finally, the fact that this medium is compatible with the growth, differentiation and maintenance of primary cells as well as cells derived from human stem cells makes it

integratable with body-on-a-chip systems (Sung et al., 2013; Sung et al., 2010; Sung et al., 2011).

CONCLUSION

In conclusion, cellular adhesion, axonal outgrowth and myelination were spatially controlled using a patterned substrate of SiPEG-DETA. This work represents the first spatially directed development of DRG myelination using tissue-engineering principles in a defined, serum-free system compatible with other cell types. The model provides a unique platform to study the substrate cues resulting in cellular adhesion and the development of myelinated axons as well as soluble molecules influencing the myelination process. Furthermore, this substrate directed developmental system could be used in tissue engineering applications where the direction axonal outgrowth and myelination were important such as peripheral nerve regeneration models and an *in vitro* model of the stretch reflex arc as well as in demyelinating diseases.

Acknowledgements

We would like to acknowledge that support for this research was provided by NIH grant numbers R01NS050452 and R01EB009429. We confirm that any aspect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval of all relevant bodies.

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Fig. 1. XPS analysis and metallization reaction for PEG-DETA patterns

A) XPS survey spectrum of a PEG-coated glass coverslip (insert shows high resolution C 1s spectrum), **(B)** XPS survey spectrum of DETA on PEG-coated glass coverslip (insert shows high resolution N 1s spectrum), **(C)** XPS survey spectrum of DETA on ablated PEG-coated glass coverslip, **(D)** image of the lines pattern visualized using palladium catalyzed copper reduction metallization (dark lines indicate the DETA regions). Scale bar = 90 μm .

Fig.2. Type Ia sensory neurons on DETA coverslips

(A) phase contrast image, (B) parvalbumin expression immunocytochemistry, (C) TrkC expression immunocytochemistry, (D) merge image also indicating NF-H immunocytochemistry (green). Scale bar = 60 μ m.

Fig.3. Patch-clamp electrophysiology of a day 10 type Ia sensory neuron on DETA coverslips
(A) whole cell currents traces (10 mV steps), **(B)** repetitive firing of action potential traces,
(C) phase contrast image of the patched sensory neuron.

Fig. 4. Confocal image Z-stack of a myelinated sensory neuron axon

(A) phase contrast image, (B) bottom image of the stack, (C) middle image of the stack, (D) top image of the stack. (NF-H = green, MBP = red, colocalization = yellow) Scale bar = 60 μm .

Fig. 5. Node of Ranvier formation on a sensory neuron axon on DETA coverslips
(A) phase contrast image, (B) MBP immunocytochemistry, (C) CASPR immunocytochemistry, (D) Na_v cluster immunocytochemistry, (E) color composite image. Scale bar = 50 μ m.

Fig. 6. Dissociated DRG cells in culture on PEG-DETA line patterns DIV 2
 (A–C) 10× phase contrast images of DRG cells on the different line widths, (D–F) 20× phase contrast images of DRG cells growing on the different line widths, (G) 30× phase contrast image of DRG cells growing on line pattern, (H–I) 40× phase contrast images of DRG cells growing on different line widths. Scale bars = 50 μ m.

Fig. 7. Dissociated DRG cells in culture on PEG-DETA line patterns DIV 20
10× phase contrast image of DRG cells on (A) 22 μm , (B) 48 μm , and (C) 96 μm line widths.
Scale bars = 100 μm .

Fig. 8. Bi-nodal myelin basic protein segments on sensory neuron axons on PEG-DETA patterned coverslips

A) Neurofilament staining of DRG axons at 20× magnification, B) MBP staining indicating formation of bi-nodal myelin segments at 20× magnification C) Composite image of NF and MBP co-localization representing myelin nodal formation of DRG axons on 96 μm patterns (D–F) 10× magnification

Table 1

Serum-free medium composition for growth and myelination of sensory neurons by Schwann cells..

Component	Amount / Concentration	Company	Catalog Number
Neurobasal	500 ml	Invitrogen	21103-049
B27	50 µl/ml	Invitrogen	17504-044
Glutamax	10 µl/ml	Invitrogen	35050-061
Antibiotic / Antimycotic	10 µl/ml	Invitrogen	15240-062
aFGF	20 ng/ml	Invitrogen	13241-013
VEGF 165	20 ng/ml	Invitrogen	P2654
CD Lipid mixture	10 µl/ml	Invitrogen	11905-031
h BDNF	20 ng/ml	Cell Sciences	CRB 600B
h GDNF	20 ng/ml	Cell Sciences	CRG 400B
r CNTF	50 ng/ml	Cell Sciences	CRC 401B
h CT-1	20 ng/ml	Cell Sciences	CRC 700B
NT-3	20 ng/ml	Cell Sciences	CRN 500B
NT-4	20 ng/ml	Cell Sciences	CRN 501B
Heparan sulfate	80 ng/ml	Sigma	D9809
Vitronectin	100 ng/ml	Sigma	V0132
h β-NGF	10 ng/ml	R&D Systems	256-GF
^I L-ascorbic acid	50 µg/ml	Sigma-Aldrich	396-HB

^I Supplemental component added only at indicated medium changes.

Table 2

Quantification of myelin segments and nodes of Ranvier on 96 μm width patterns.

	Culture 1	Culture 2	Culture 3
Myelinated segments	191.00 $\pm$ 7.93	187.67 $\pm$ 4.20	193.67 $\pm$ 6.08
Nodes of Ranvier	66.33 $\pm$ 6.94	70.33 $\pm$ 5.36	69.00 $\pm$ 4.73

The data shown is a mean of three coverslips evaluated per culture. The values are the mean $\pm$ the standard error of the mean (SEM).

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Table 3

DRG culture conformity to PEG-DETA line patterns.

Culture Day	Number of lines	Conforming Patterns	Percent Conformity
Day 2	60.00	60.00 $\pm$ 0.00	100.00
Day 10	60.00	44.72 $\pm$.19 ^a	74.53 ^a
Day 20	60.00	42.55 $\pm$.27 ^a	70.92 ^a

Conformity was quantified by counting the number of lines with cells attached to only the DETA surface. Data is the average $\pm$ SEM of 3 experiments performed on 6 coverslips/experiments at each time point.

^a It is important to note that the percent conformity to the pattern is altered due to the lack of long-term survival of cultures on 12 μ m patterns. Adjusted for the remaining patterns available at Day 10, and Day 20, the percent conformity is 99.34 and 94.56, respectively.