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Miniaturized system of neurotrophin patterning for guided regeneration

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ABSTRACT

Understanding the fundamentals of cell behaviour is imperative for designing and improving engineering strategies for regenerative medicine. By combining the precision of confocal microscopy with photochemistry, nerve growth factor (NGF) was chemically immobilized on chitosan films either in distinct areas or as concentration gradients. Using rhodamine as a proxy for NGF, a series of immobilized concentration gradients were created, using the number of rastering scans within a defined area and the distance between each area as a way to control the resulting gradient. The same photochemistry was applied to create NGF patterns on chitosan films which were visualized by immunostaining, and the immobilized NGF remained bioactive as demonstrated with a neuron survival assay. Neuron survival was $73.2 \pm 1.3\%$ after 3 days of culture on chitosan films with 30 ng/cm^2 of homogenously immobilized NGF, which was comparable to $74.8 \pm 3.4\%$ neuron survival on chitosan with 50 ng/ml of soluble NGF present. Interestingly, when neurons were plated on a chitosan film that had distinct immobilized NGF-patterned areas surrounded by unmodified chitosan, the neurons remained predominantly as single cells in the NGF-patterned regions, but formed aggregates outside of these patterns on the plain chitosan film. Thus, the immobilized NGF pattern influenced neuron behaviour and can be used to further probe mechanisms of other neuron behaviour such as axon guidance. Importantly, the versatility of the confocal laser patterning technique reported here can be extended to other factors to elucidate fundamental cell functions, and hence design strategies in regenerative medicine.

1. Introduction

Regulation of cellular activities through signaling molecules such as neurotransmitters, hormones, and growth factors are complex events. They often involve multiple signaling cascades and cross talk between pathways. Analyzing the sequence of events in signaling cascades is important not only for our understanding of disease regulation, but in developing and improving strategies for regenerative medicine. We are particularly interested in identifying and studying key signaling molecules involved in path finding for axon guidance.

The growth cone is highly motile at the tip of the axon and continuously samples the extracellular environment for signaling molecules (Gordon-Weeks, 2005). It is capable of receiving and integrating multiple types of signals, and transducing them to direct axon growth and guidance. Thus, the growth cone responds to a combination of chemotactic and haptotactic cues which guide axons to their innervating target during development of the nervous system (Gordon-Weeks, 2005). Nerve growth factor (NGF) is one of the neurotrophic factors important for neuron survival and neurite growth. It is also one of the factors that has a chemotactic effect on growth cones. When presented with a concentration gradient of NGF, growth cones guide growing axons to their proper innervating target (Gundersen and Barrett, 1979; Tessier-Lavigne and Placzek, 1991). In a proof-of-concept study, a well-defined linear concentration gradient of soluble NGF of at least 133 ng/(ml mm) was required to guide neurites of PC12 cells and/or dorsal root ganglion neurons (DRGs) up to 7.5 mm towards the higher end of the concentration gradient (Cao and Shoichet, 2001, 2003). While this study showed that the axon guidance, which occurs during development, can be reproduced *in vitro*, this system is not practical for *in vivo* implantation, because it required constant concentrations of NGF in source and sink chambers in order to maintain the gradient. To overcome this limitation, a macroporous scaffold of poly(2-hydroxyethyl methacrylate) (PHEMA) was used to physically entrap a stable linear concentration gradient of NGF to guide axon growth (Kapur and Shoichet, 2004). While this method of gradient synthesis is simple and versatile, it is limited to scaffolds that are 18–23 cm long which is not physiologically relevant

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(Kapur and Shoichet, 2004; Moore et al., 2006). Moreover, PHEMA is not degradable. To overcome these limitations we were interested in immobilizing a neurotrophin concentration gradient in a biodegradable polymer scaffold with more physiologically relevant dimensions for regenerative medicine strategies.

Many studies in the past have focused on creating concentration gradients of various trophic factors to study axon guidance. For example, microscopic diffusible gradients of guidance cues were produced by repetitive injection of picolitre volumes of growth factor solution into the culture dish, controlled by an electrically gated pressure application system (Lohof et al., 1992). Several key signaling molecules involved in nerve growth cone guidance have been identified using this model system. However, the linearity and the diffusible concentration gradient produced can only be approximated at any one time (Lohof et al., 1992). Other common methods of producing surface patterns to influence cell behaviour are by lithography, micro-contact printing on self-assembled monolayers, and microfluidic techniques (Chen et al., 1998; Jiang et al., 2005; Johann et al., 2007; Nicolau et al., 1999). Although these methods provide spatial deposition of biological molecules to study fundamental questions of biology or cell behaviour, it is difficult to create linear concentration gradients using soft lithography techniques such as micro-contact printing. Photo-reactive moieties activated by a pulsed infrared or ultraviolet (UV) laser within the confocal microscope are particularly useful to create protein patterns on substrate surfaces. The clarity and resolution of images taken by the confocal microscope is of higher quality than those from a conventional fluorescence microscope. Thus, the desired patterns on a molecular scale can be produced with great precision when combined with photochemistry (Wosnick and Shoichet, 2008). This method of spatially controlled pattern deposition on substrate surfaces can be used to create various immobilized protein patterns in 2D, thereby mimicking those found *in vivo*, such as concentration gradient patterns. This system can then be used as a tool to investigate and identify the signaling molecules involved in cell behaviour and function. For example, surface patterns of cell adhesive peptides or concentration gradients of signaling molecules have influenced cell elongation, formation of neuron aggregates, and controlled axon guidance in 2D cultures (Chen et al., 1998; Henley and Poo, 2004; Karp et al., 2006; Lohof et al., 1992; Yuan et al., 2003; Zeng et al., 2007). Findings from these studies can enhance our understanding of developmental diseases and design strategies in regenerative medicine.

While 2D cultures have identified and elucidated many important signaling cascades controlling cell function, cell behaviour in 3D is also critical to regenerative medicine. By controlling stage movement of the confocal microscope through computer programs, 2D patterning can be extended to 3D patterning: complex protein patterns can be created using this new common analytical tool through depth control of the microscope stage (Wosnick and Shoichet, 2008). From the perspective of regenerative medicine, creating complex 3D patterns of biological molecules will be a powerful tool to control and guide cell growth and proliferation.

A wide variety of biodegradable materials have been reported for tissue engineering applications (Kim et al., 2007; Schmidt and Leach, 2003), including chitosan which has shown some benefit for neural tissue engineering (Zahir et al., in press). Chitosan is a natural material derived from the shells of crustaceans and the exoskeleton of arthropods (Domard and Rinaudo, 1983; Hirano, 1999). It has been widely exploited for various biomedical applications in recent years (Hirano, 1999; Kumar, 2000; Singh and Ray, 2000), including chemical modification for increased solubility at physiological pH. Moreover, chitosan has been used in a variety of applications, such as a mucoadhesive material for various drug delivery vehicles (Grabovac et al., 2005; Kafedjiiski et al., 2005; Langoth et al., 2006), and for neural tissue engineered scaffolds, where cell adhesion is enhanced by crosslinking extracellular matrix proteins such as laminin, or cell adhesive peptides (Ho et al., 2005; Suzuki et al., 2003). We previously reported a biodegradable methacrylamide-modified chitosan hydrogel scaffold that allowed neurite penetration, and promoted neural adhesion (Yu et al., 2007). Thus, chitosan was an obvious choice for further investigation, providing a scaffold on which to immobilize growth factors in a spatially controlled manner using laser photo-patterning to examine neuron behaviour.

Herein we describe a new technique to create various patterns of immobilized factors on chitosan surfaces using the UV laser within a confocal microscope. Patterning biological factors on a substrate can serve as a powerful tool to study cell response to various spatially resolved immobilized factors that are present *in vivo*, such as concentration gradients of growth factors. Tetramethylrhodamine cadaverine (Rh), as a substitute for NGF, was first immobilized to demonstrate this concept. This Rh molecule was chosen because it contains a single primary amine group which is specific to the photochemistry used. We demonstrate here that the immobilized NGF on chitosan remains stable and bioactive and that the NGF pattern created on chitosan affects neuron affinity for chitosan. Importantly, this method can be translated to 3D patterning.

2. Materials and methods

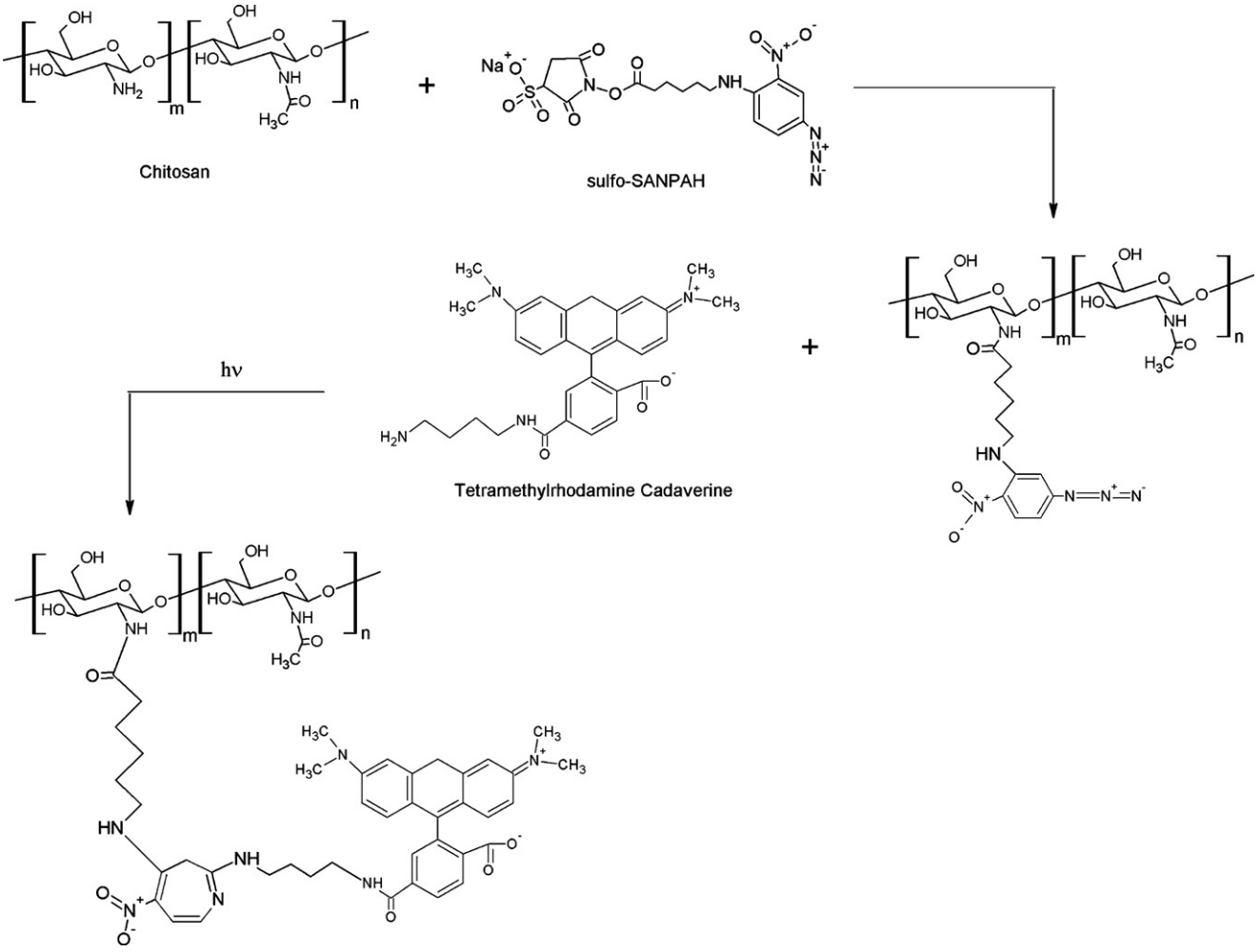
All chemicals were purchased from Sigma–Aldrich (Oakville, ON) and used as received unless otherwise stated. Distilled and deionized water (ddH_2O) was prepared using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedfore, MA) system, and used at 18 MΩ resistance.

2.1. Photoreactive chitosan film fabrication

Chitosan (FMC BioPolymer AS, Norway), with molecular weight 250,000 g/mol (manufacturer's specification) and a degree of deacetylation of 90% (by ^{1}H NMR, data not shown), was dissolved as a 3 wt% solution in 2 wt% acetic acid overnight on a shaker. Chitosan solution was poured into a Petri dish and allowed to air dry overnight. The dried film was neutralized in an ammonium hydroxide solution (NH_4OH:ddH_2O:MeOH = 3:7:90) for 30 min. The film was then neutralized by washing repeatedly in phosphate buffered saline (PBS, pH 7.4) and cut to have a dimension of 8 mm × 8 mm. The films were disinfected in 70% ethanol for 30 min and then washed in PBS to remove residual ethanol.

Collagen derived from rat tail was crosslinked to the chitosan films to improve cell adhesion using carbodiimide chemistry. Briefly, collagen was activated in 2.5 mM *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and 5 mM *N*-hydroxysulfosuccinimide (sulfo-NHS) for about 3 min, then the activated collagen was immediately added to PBS in a ratio of 1:25 (v/v). The cut films were added to the solution immediately and allowed to react at room temperature (RT) for 2 h on a shaker. As collagen precipitates in PBS, the films were washed extensively in PBS to remove any residual collagen before conjugating *N*-sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate (sulfo-SANPAH, Pierce, Rockford, IL) to the films to synthesize photoreactive chitosan.

Sulfo-SANPAH was dissolved in PBS at 0.25 mg/ml protected from light and sterilized by passing the solution through a 0.2 μm filter. Photoreactive chitosan was then produced by reacting the collagen-crosslinked chitosan films with sulfo-SANPAH for at least 3 h at RT on a shaker in the dark, as shown in Scheme 1. The films were washed thoroughly in PBS to remove any unreacted sulfo-



Scheme 1. The heterofunctional crosslinker, sulfo-SANPAH, is first reacted with chitosan to form photoreactive chitosan. After removal of excess sulfo-SANPAH, tetramethylrhodamine cadaverine (Rh) is then added to photoreactive chitosan and exposed to UV light (364 nm) resulting in Rh conjugated to chitosan.

SANPAH. The washes were monitored by UV at 480 nm until no more nitrophenyl azide groups were detected.

The amount of sulfo-SANPAH crosslinked to the chitosan film was quantified by measuring the absorbance of the photoreactive chitosan films at 480 nm compared to a calibration curve of the sulfo-SANPAH solution at known concentrations. The films ($n = 5$) were cut to fit snuggly into wells of a 96-well plate using a custom made stainless steel cutter. The calibration curve was prepared by adding 100 μl of the sulfo-SANPAH solutions, ranging from 0–500 μg/ml in serial dilution, to the well plate. Absorbances of the films and the sulfo-SANPAH solutions were recorded at 480 nm using a UV microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). The resulting surface concentration of sulfo-SANPAH crosslinked to the chitosan films was found to be 64.2 ± 9.0 μg/cm^2, as shown in Fig. S1.

2.2. Tetramethylrhodamine cadaverine patterning on photoreactive chitosan

Tetramethylrhodamine cadaverine (Rh, Invitrogen, ON) was dissolved in *N*, *N*-dimethylformamide (Caledon Laboratories Ltd., Georgetown, ON) and diluted to 0.25 mg/ml in carbonate buffer (pH 9.7) to ensure the single primary amine group is unprotonated. A 364 nm UV laser (Coherent Innova 90C, Santa Clara, CA) coupled to the confocal microscope (Leica, TPS SP2) was used to activate the nitrophenyl azide group of sulfo-SANPAH on photoreactive chitosan films at a location that is spatially controlled by the microscope. Patterns of Rh can be made by specifying a region of interest using the microscope software (Leica Microsystem v2.6.1, Heidelberg GmbH) and a macro computer program to control the stage movement and number of scans made by the laser. The macro was written using the Leica software to create concentration gradient patterns of the desired shape and dimension. The macro allows the user to specify the initial number of scans (a_1) made by the laser, the scan increment (d), the spacing between patterns at each scanning step, and the number of patterns desired (n). The total number of scans (a_n) made by the laser at each n^{th} scanning step follows the arithmetic sequence as shown in the following equation:

$$a_n = a_1 + (n-1)d \quad (1)$$

For example, the Rh patterns were created by programming both the coordinates and number of scans. The resolution of each scan was a 150 μm × 150 μm square using a 20× objective (Leica, NPlan 20×) with a 1.7 mm working distance. Multiple scans within the same defined area following equation (1) would result in increased number of Rh molecules immobilized. Unreacted Rh was removed by repeated washing in 50 mM phosphate buffer (pH 7.4) for at least 3 days with the buffer changed at least twice each day. The Rh patterns on chitosan were then imaged on an upright microscope

(Olympus, BX61) equipped with a monochrome camera (Photometrics Cool Snap HQ, Roper Scientific, Tucson, AZ).

2.3. NGF patterning on photoreactive chitosan

Photoreactive chitosan was soaked in NGF (AbD Serotec, Raleigh, NC) solution (1 μg/ml in PBS) overnight at 4 °C. NGF patterns were created on photoreactive chitosan by precise control of the microscope stage and UV laser using the same macro developed for the Rh patterns. After patterning, the films were washed thoroughly in 20 mM Tris–hydrochloride + 150 mM NaCl + 0.1% Tween 20 (TBST, pH 7.6). The NGF patterns were then visualized by immunostaining. The NGF-patterned films were blocked in 0.5 wt% bovine serum albumin (BSA) and 6% goat serum in PBS for 1 h at RT. Rat anti-NGF primary antibody (Promega, Madison, WI) was applied at 1:100 dilution in blocking buffer and PBS (1:1) overnight at 4 °C. The films were washed in TBST the next day for at least six times and Alexa Fluor 546 goat anti-rat IgG (H + L) (Invitrogen, ON) was applied in 1:500 dilution in blocking buffer and PBS (1:1) for 1 h at RT with gentle shaking. The films were washed at least six times again in TBST before imaging as above.

NGF squares were patterned on chitosan surfaces to study whether NGF patterns can influence neuron behaviour. To create NGF patterns on chitosan surfaces, photoreactive chitosan was soaked in 7.5 μg/ml NGF solution overnight and patterned on the confocal microscope using a 5× objective. Two square regions of immobilized NGF were created by scanning the specified region repeatedly for either 5 or 10 min side by side using the UV laser. The patterned films (NGF-pattern) were then washed extensively in sterile TBST (pH 7.5) for at least 15 times by injecting TBST forcefully from a syringe through a 20 gauge needle to remove any unbound NGF. The films were left in TBST on a shaker for about 1 h before they were washed again as above. This procedure was repeated two more times. The films were rinsed at least three more times in PBS to remove any residual Tween 20. The films were then soaked overnight at 4 °C for cell culture the next day in Leibovitz's L-15 medium (L15, Gibco-Invitrogen, Burlington, ON) modified as previously described (Campenot and Martin, 2001).

2.4. Bioactivity and stability of immobilized NGF on photoreactive chitosan surface

Photoreactive chitosan film was placed in a 48-well plate and NGF solutions (0.75 and 7.5 μg/ml) were added to the wells to produce a uniform surface concentration of approximately 3 and 30 ng/cm^2 of immobilized NGF as determined by enzyme-linked immunosorbent assay (ELISA, see below). The films were soaked overnight at 4 °C and exposed to UV (350 nm) using a UV reactor (Southern New England Ultra Violet Company, Connecticut) for 10 min the following day to homogeneously immobilize NGF to the photoreactive chitosan surface. The films were then subjected to the same extensive washing procedure in sterile TBST, as described in Section 2.3, to remove any unbound NGF and soaked overnight in L15 medium as above. This medium was collected the next day for cell culture on collagen-coated wells as one of the negative controls to demonstrate that the washing in TBST to remove any unbound NGF was sufficient. This will be referred to as the "*Wash*" control hereafter. As another control, NGF (7.5 μg/ml) was allowed to adsorb to the chitosan film as above but not exposed to UV to chemically bind NGF, and subjected to the same extensive washing procedure in TBST as above to remove the unbound NGF. This will be referred to as the "*Adsorption*" control from hereafter.

To test if the immobilized NGF remains stably bound on the chitosan surface, cultured medium from neurons where 30 ng/cm^2 NGF had been immobilized on the chitosan surface was collected and fed to neurons cultured on collagen-coated wells from which 10 ng/ml of soluble NGF (10NGF) had been previously withdrawn for 6 h, under serum free conditions. This was the "*Supernatant*" control. To ensure the neurons were not over-starved resulting in induced apoptosis, another set of cells was subjected to the same NGF withdrawal treatment as the *Supernatant* control, but serum free medium with 50 ng/ml of soluble NGF (50NGF) was added to rescue these neurons after the 6 h starvation. This was the "*Rescue*" control.

2.5. Quantifying immobilized NGF on surface of chitosan film using ELISA

NGF immobilized on the surface of chitosan films were quantified using ELISA. Photoreactive chitosan films were prepared and cut to fit snuggly into the wells of a 96-well plate, as described in Section 2.1. 100 μl of NGF solution was added to each well containing a chitosan film. The NGF concentrations applied to the films were 0, 5, 10, 20, 40, 60, 80 ng/ml ($n = 5$) at each concentration. The plate was sealed and the films were allowed to soak in NGF solution overnight at 4 °C. The films were then immobilized using the UV reactor as described in Section 2.4. Following immobilization, the films were washed extensively in TBST as described in Section 2.3. Control films were treated identically with NGF solutions of 10 and 60 ng/ml, but not exposed to UV. The control films served as controls to demonstrate that NGF was chemically immobilized on the chitosan surface and that the washing procedure was sufficient to remove any unbound NGF.

The surface concentrations of NGF immobilized on the chitosan films were determined using an ELISA kit following instructions provided by the manufacturer (Promega) with modification. Instead of flicking out contents in the wells after each wash, the films were washed by forcefully injecting TBST into the wells and the contents aspirated. After colour development was stopped by adding 1N HCl, the developed solution was transferred to a new 96-well plate. The absorbance reading from the plate was recorded at 450 nm using a UV microplate reader. The absorbance reading from the chitosan films were compared to a standard curve, prepared according to the manufacturer, to determine the total NGF immobilized on the chitosan surface and the resulting surface concentration. Assuming that all NGF added was available for immobilization, the yield of immobilization was approximately 3.5%. The immobilized surface concentration calculated in Section 2.4 was determined using this reaction yield. Fig. S2 shows that when the concentration of applied NGF increases, the resulting surface concentration of NGF also increases as expected. When NGF (10 and 60 ng/ml) was adsorbed on the chitosan surface but not photo-immobilized, there was significantly less NGF detected compared to the amount of NGF immobilized ($^*p < 0.05$).

2.6. SCG dissection and culture on NGF immobilized chitosan film

Superior Cervical Ganglia (SCG) were explanted from post-natal day 2 Sprague–Dawley rats, and the ganglia dissociated into single neurons according to an established procedure (Mahanthappa and Patterson, 1998). The dissociated neurons were re-suspended in L15 medium with no NGF. Neurons were then plated onto the surface of chitosan films or collagen-coated wells as follows: collagen + 50NGF (positive control), photoreactive chitosan (50NGF + no NGF immobilized), NGF-immobilized chitosan (3 and 30 ng/cm^2, no soluble NGF), NGF-pattern + 10NGF, *Adsorption* control + no soluble NGF, *Wash* control + no soluble NGF; and *Supernatant* and *Rescue* controls were both plated on collagen-coated wells with 10NGF for the first 2 days. All cells were incubated at 37 °C, 5% CO_2, and 100% humidity, and the media was changed every 2 days. Following 3

days of culture on the chitosan films, the neurons were fixed in 4% paraformaldehyde (PFA) for 15 min at RT followed by immunostaining for the neuron specific marker, βIII-tubulin as described Section 2.7. After 2 days of culture on the NGF-patterned chitosan, NGF was withdrawn and the neurons were starved for 6 h under serum free conditions before being fixed in 4% PFA and immunostained for βIII-tubulin. NGF in the *Supernatant* and *Rescue* controls was withdrawn for 6 h under serum free conditions 2 days after plating, and medium from the 30 ng/cm^2 NGF immobilized condition was fed to the *Supernatant* control, while the *Rescue* control received serum free medium containing 50NGF. These cells were fixed in 4% PFA and immunostained as below following two more days in culture.

2.7. Immunostaining for βIII-tubulin in SCG neurons

The fixed cells were washed three times, 5 min each with PBS, and then permeabilized for 10 min in 0.5% TritonX-100. The cells were blocked in 0.5% (w/v) BSA and 6% (v/v) goat serum for 1 h at RT. Neurons were incubated in mouse anti-rat βIII-tubulin monoclonal antibody (1:500, Clone TUJ1, Stem Cell Technologies Inc., B.C., Canada) in blocking solution and PBS (1:1) overnight at 4 °C. The cells were washed in PBS three times, 5 min each the following day. Neurons were then incubated in goat anti-mouse Alexa 546 secondary antibody (1:500) for 1 h at RT, and washed as mentioned above following incubation. The nuclei were stained with Hoechst 33342 (5 μM). All immunostained chitosan films were mounted using ProLong Gold Antifade reagent (Invitrogen, Burlington, ON). Neurons were imaged using a 20× objective on the upright microscope as described in Section 2.2. All surviving cells that showed positive Hoechst staining were counted. The percentage of surviving neurons was determined by dividing the number of live neurons by the total number of all live cells (neuronal and non-neuronal cells).

To identify the NGF square patterns immobilized on chitosan surface as described in Section 2.3, the location of the NGF patterns were marked on the coverslip after mounting the immunostained chitosan film using the antifade reagent mentioned above. The percentage of live neurons inside and outside of the NGF patterns were then imaged and quantified as described above.

2.8. Statistical analysis

All cell-related data are reported as the mean ± standard deviation. Statistical significance between each culture condition was determined by single-factor ANOVA ($p < 0.05$) in the neuronal survival assay. Two-factor ANOVA ($p < 0.05$) was used to determine whether patterning time and exposure of neurons to the patterns have an effect on neuron behaviour for each cell cluster group.

3. Results

3.1. Patterns of tetramethylrhodamine cadaverine (Rh) are photo-immobilized on chitosan by UV laser confocal patterning

When sulfo-SANPAH is exposed to UV light, the nitrophenyl azide group forms a nitrene that undergoes subsequent ring expansion to react with nucleophiles such as primary amines. Rh was specifically chosen to demonstrate this chemistry because it contains only one primary amine group, as shown in Scheme 1. After patterning Rh with the confocal UV laser and subsequent washing in phosphate buffer to remove any unbound Rh, the observed fluorescent pattern was due to Rh conjugation to chitosan via sulfo-SANPAH. Fig. 1a shows a series of oval patterns of Rh that are separated by approximately 100 μm and immobilized on the surface of chitosan, demonstrating that the confocal microscope UV laser can specifically modify chitosan in a region of interest specified by the user. The patterns seen in Fig. 1a were created by scanning the region 1, 5, 10 15, 20, and 25 times with the brighter ovals resulting from greater numbers of scans. The fluorescent intensities of the oval patterns are shown in Fig. 1b and c as grey levels, where 0 represents black and 255 represents white (or fluorescence). The grey levels increase with increasing number of scans over the specified region and correspond to increased numbers of immobilized Rh molecules on the surface of chitosan. Thus, the surface concentration of patterned molecules can be adjusted by the number of scans that the laser makes over the specified region of interest and this method can be used to create concentration gradients of immobilized Rh.

The macro computer program, written using the Leica software to control the stage movement and laser scans, follows the arithmetic sequence described by Eq. (1), where the user can specify a region of interest, and parameters of the arithmetic sequence. The resulting Rh concentration gradient patterns are shown in Fig. 2a and b. The resulting gradient patterns are step concentration gradients of Rh produced by moving the microscope stage and increasing the number of scans made by the laser by a specified number of times over the region of interest simultaneously. For example, the +3 symbol next to the gradient pattern indicates that the difference between each scanned region of 150 μm was three additional scans, i.e., first region of interest was scanned once, the second region was scanned four times, etc. Thus, when the spacing between each scanned area was set at 75 μm and the region of interest at 150 μm, the overlapped area between the first region and the next region would be scanned four times by the laser. The number of Rh molecules immobilized increased with each additional scan. The corresponding fluorescent intensities of the gradient patterns are shown as grey levels in Fig. 2c, where

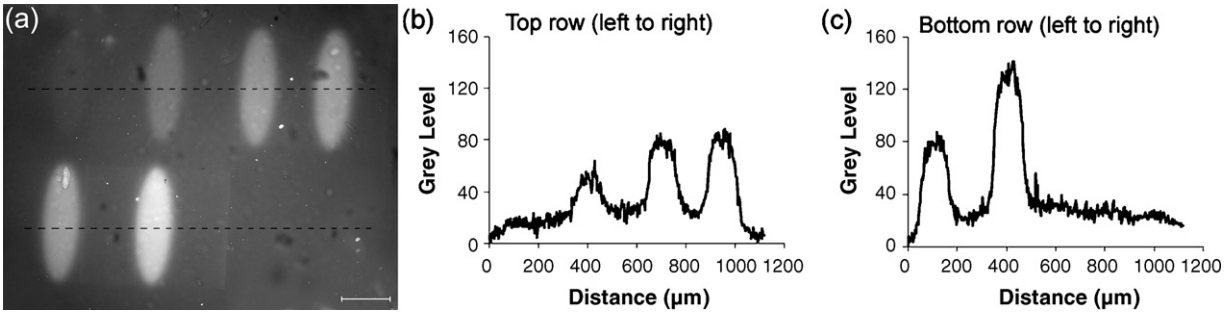


Fig. 1. Oval patterns of tetramethylrhodamine cadaverine (Rh) are immobilized to the chitosan surface by laser confocal patterning. Patterns on row 1 have been patterned by scanning the oval region of interest 1, 5, 10, and 15 times; and 20 and 25 times on Row 2. Fluorescent profiles of the oval patterns are depicted as grey levels in (b) and (c) according to the dotted line drawn in (a). As the number of scans increase the grey level increases correspondingly denoting high surface concentration of Rh immobilized on the surface of chitosan. Scale = 100 μm.

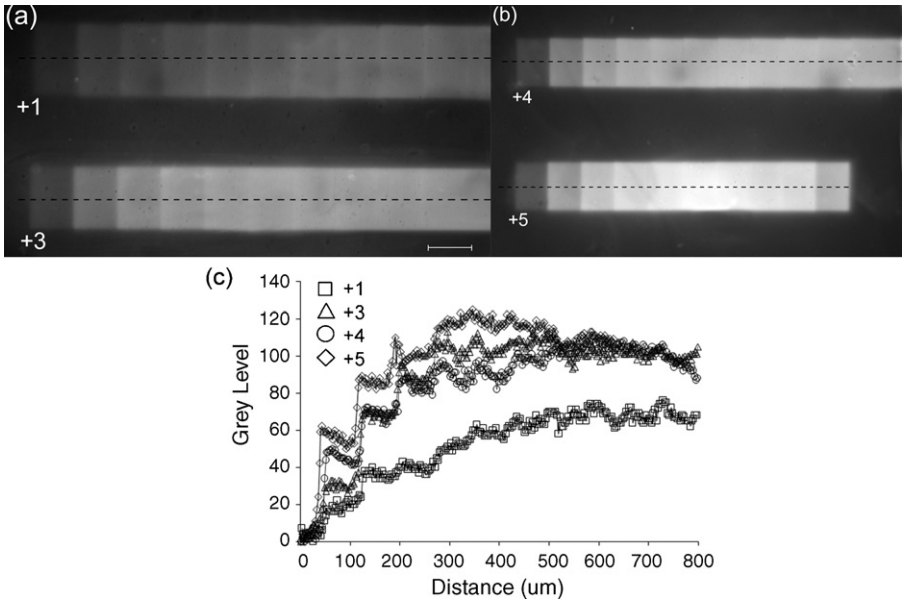


Fig. 2. Step concentration gradients of tetramethylrhodamine cadaverine (Rh) were created by laser confocal patterning and a user defined macro to control stage movement and UV exposure time (a and b). The numbers indicate the increase in the number of scans in subsequent patterns. The fluorescent intensity of the gradient patterns increases from left to right as the number of scans made by the laser over the photoreactive chitosan surface increases. The fluorescent intensity profiles of each of the gradients are shown as grey levels in (c) according to the dotted line drawn through the patterns in (a and b). Scale = 75 μm.

step gradients of Rh are evident. The length of the gradient was longest when the scan increment was set at +1, and shortest when the scan increment was set at +5 because the number of scans at the last scanning step was held constant at $a_n = 41$ (only partial gradient lengths of Rh are shown in Fig. 2). Importantly, the length of the gradient ranged from a few hundred microns to only a few millimeters in length, which is a significant reduction in gradient length over previously immobilized gradients of 18–23 cm, produced by a conventional gradient maker (Kapur and Shoichet, 2004; Moore et al., 2006). Thus, this technique offers great flexibility to create gradient patterns of various lengths by adjusting the number of scanning steps or specifying the dimension of the region of interest. Using Rh as a proxy for NGF, we demonstrated that the phenylazide chemistry is suitable for laser confocal patterning of immobilized concentration gradients and that distinct gradient patterns of desirable shapes and dimensions could be created using this technique.

3.2. Nerve growth factor is immobilized to photoreactive chitosan and visualized by immunostaining

To test whether the phenylazide chemistry developed with rhodamine could be used to create biological patterns of NGF, it was similarly immobilized on chitosan via laser confocal patterning. While a basic pH would increase the coupling efficiency of NGF to photoreactive chitosan, we were restricted to pH 7.4 because NGF denatures under basic conditions (Pignatti et al., 1975). The NGF patterns were only visible after excessive washing to remove adsorbed NGF and immunostained with rat anti-mouse NGF. A NGF pattern with increasing fluorescent intensity from right to left is clearly visible when the same macro computer program used for creating the Rh gradients was used, as shown in Fig. 3. However, the spacing between subsequent NGF patterns shown in Fig. 3 was chosen to be larger than the diamond and triangle shapes of the NGF pattern to demonstrate that desired shapes and dimensions can be spatially controlled using the macro computer program developed.

3.3. Immobilized NGF on chitosan surface remains bioactive and stably immobilized

To test the bioactivity of immobilized NGF on collagen-crosslinked photoreactive chitosan films, neuron survival and neurite outgrowth were examined relative to positive controls, where soluble NGF is present, by immunostaining the cell bodies and neurites with the neuron specific marker, βIII-tubulin, and the nuclei with Hoechst 33342, as shown in Fig. 4. The positive control (Fig. 4a) shows healthy SCGs and extensive neurite outgrowth on collagen in the presence of 50NGF (soluble) and serves as a qualitative benchmark for survival and outgrowth. Importantly, SCGs cultured on chitosan films modified with only the photochemical crosslinker, sulfo-SANPAH, in the presence of 50NGF, showed similar cellular behaviour to the positive control on collagen (Fig. 4b).

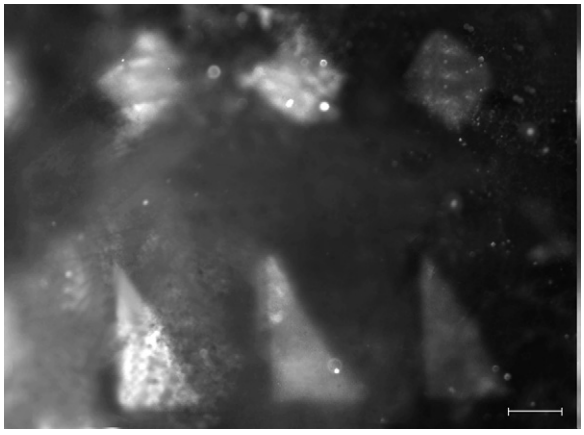


Fig. 3. Patterns of NGF immobilized on the surface of chitosan by activating the specified region of interest (i.e., diamond and triangle) using the UV laser and a user defined macro to control UV exposure time and stage movement. The patterns were visualized by NGF immunostaining, demonstrating spatial control of the described patterning technique (the contrast of the image was enhanced using Adobe Photoshop v. 7.0). Scale = 100 μm.

Thus, sulfo-SANPAH chitosan is not cytotoxic, which is consistent with previous studies where sulfo-SANPAH has been used to conjugate antibodies to either target specific protein ligands in cells or study protein interactions *in vivo* (Kota and Ljungdahl, 2005; Uckun et al., 1995). On the one hand, when a surface concentration of 3 ng/cm^2 of NGF was immobilized on the chitosan surface, the neuron cell bodies and neurite density were observed to be significantly lower than the positive controls where soluble NGF was present, as shown in Fig. 4c. In addition to low numbers of surviving neurons, neurite density was observed to be extensively reduced when the surface concentration of immobilized NGF was low. The arrows in Fig. 4c indicate the neurons that have undergone apoptosis. These observations demonstrate the importance of NGF as a survival and neurite outgrowth factor for SCG neurons. On the other hand, when 30 ng/cm^2 of NGF was immobilized to the chitosan surface, significant neurite outgrowth and healthy cell bodies were observed in (Fig. 4d) which are comparable to the two positive controls (*cf.* Fig. 4a and b). Interestingly, neurons that fell outside the chitosan surface, during plating in the culture wells, also sent neurites towards the film, indicated by the arrows in Fig. 4e, demonstrating the chemoattractive effect of NGF for these neurites.

To ensure that the extensive neurite outgrowth observed in Fig. 4d result from immobilized NGF and not residual unbound NGF, the chitosan films were washed extensively in TBST for at least 40 times after immobilization to remove any unbound NGF. The films were then soaked in culture medium overnight at 4 °C. This medium was then collected the next day and used as the culture medium for SCG neurons cultured on collagen-coated wells, as the *Wash* control shown in Fig. 4f. Here, the neuron cell bodies were smaller than those on the positive control, and there was little to no neurite outgrowth. However, non-neuronal cell survival was apparent in Fig. 4f. Our results are consistent with those of others, such as Ito et al. (1998) who showed that when epidermal growth factor (EGF) was photo-immobilized to polystyrene, the unbound EGF was removed with PBS. Importantly, in our studies when no NGF was immobilized to chitosan (and no NGF added in soluble form), the cell bodies were smaller and no neurite outgrowth was evident as shown in Fig. 4g. This underscores the importance of NGF as a survival factor for these neurons. When NGF was allowed to adsorb to the chitosan surface but not photo-immobilized (referred to as the *Adsorption* control), some adsorbed proteins inevitably remained on the chitosan surface even after extensive washing in TBST. Thus, neuron survival and neurite outgrowth was observed to some degree, but could not be sustained as neuron apoptosis and neurite degeneration were apparent as shown in Fig. 4h. The arrows in Fig. 4h indicate neurons undergoing apoptosis, and significant beading of the neurites and neurite fragments were observed. These observations were seen neither in the two positive controls (*cf.* Fig. 4a and b) nor when 30 ng/cm^2 of immobilized NGF was present (*cf.* Fig. 4d).

To demonstrate that the immobilized NGF on chitosan surfaces remains stably bound, the medium, in which SCG neurons were cultured on 30 ng/cm^2 of immobilized NGF, was removed and used to culture neurons on collagen, referred to as the *Supernatant* control. Neurons in the *Supernatant* control was initially plated in medium containing 10NGF and cultured for 2 days. NGF was then withdrawn in both controls for 6 h under serum free conditions. Supernatant medium from cultures where 30 ng/cm^2 of NGF was immobilized on the chitosan surface was collected and fed to neurons in the *Supernatant* control. Following two more days in culture, these neurons were immunostained and neuron survival was assessed, as shown in Fig. 4i. Neuron survival was compromised (arrows indicate apoptosed neurons) and significant neurite beading and fragments were observed due to neurite degeneration (Fig. 4i). Since neuron survival and neurite growth could not be sustained in the *Supernatant* control, these observations suggest that a negligible amount of NGF was present in the supernatant medium from the immobilized NGF. However, to ensure that the neuron death and neurite degeneration observed in Fig. 4i was not due to over-starving of the neurons due to prolonged NGF withdrawal, another set of cultures (initially plated in 10NGF and cultured for 2 days) was subjected to NGF withdrawal, as in the *Supernatant* control, but serum free medium containing 50NGF was fed to these neurons to rescue them. The rescued neurons shown in Fig. 4j remained healthy with extensive neurite outgrowth which are comparable to the two positive controls (*cf.* Fig. 4a and b). Hence, the contrast in neuron survival and neurite sustainability shown in Fig. 4i and j suggest that the immobilized NGF remains stably bound to the chitosan surface. The percentage of neuron survival from the different treatment groups shown in Fig. 4 is summarized in Fig. 5.

Neuron survival of 73.2 ± 1.3% when 30 ng/cm^2 of NGF is immobilized to chitosan, was similar to positive controls where 50NGF (soluble) was present: 74.8 ± 3.4% on chitosan and 76.7 ± 5.3% on collagen, which are not statistically different. When 3 ng/cm^2 of NGF was immobilized to the chitosan surface, neuron survival was only 38.8 ± 11.5%, which is statistically different from the survival observed on the chitosan surface with 30 ng/cm^2 of immobilized NGF. When NGF was allowed to adsorb to the chitosan surface (but was not photochemically immobilized), neuron survival was only 36.8 ± 6.9%. This is consistent with the results obtained by MacInnis and Campenot who observed similar neuron survival when their SCGs were cultured with microbeads that had NGF adsorbed but not chemically bound (MacInnis and Campenot, 2002). In the *Wash* control sample, only 11.8 ± 7.2% neuron survival was observed, clearly demonstrating the need of NGF for survival. When the medium used to culture neurons where NGF had been immobilized was fed to neurons in the *Supernatant* control, survival decreased to 29.6 ± 4.8% due to the lack of NGF in the medium. This confirms that the immobilized NGF is stably bound. Importantly, this reduction in survival is not due to induced apoptosis by over-starving of the neurons from NGF withdrawal before feeding these neurons with the supernatant medium, as the neuron survival in the *Rescue* control was 76.2 ± 4.2% which is comparable to the survival of 76.7 ± 5.3% on the positive control of collagen with 50NGF (but is statistically different from the percent survival obtained for the *Supernatant* control). The percent survival of neurons in the *Adsorption*, *Wash*, and *Supernatant* controls were all found to be statistically different from the percent neuron survival when 30 ng/cm^2 of NGF was chemically immobilized to the surface of photoreactive chitosan. Together, these neuron survival data allow us to conclude that NGF photo-chemically immobilized on chitosan, via the sulfo-SANPAH crosslinker, is stably bound and bioactive.

3.4. Spatially defined, immobilized NGF pattern on chitosan can influence neuron behaviour

To further examine whether spatially defined, immobilized NGF can influence cell behaviour, NGF was immobilized within 2 square patterns by scanning the region with the UV laser for either 5 or 10 min on the same chitosan film. On the one hand, neurons that were plated on the NGF pattern remained predominantly as single cells with very few neuron aggregates as shown in Fig. 6a. On the other hand, neurons that were plated outside of the NGF squares formed large aggregates (i.e., more than 10 neurons/aggregate) as seen in Fig. 6b. These data are summarized in Fig. 6c. By two-factor ANOVA, the scanning time was found not to be a contributing factor to the trends observed, however the location of the NGF pattern on the chitosan surface contributed to the formation of

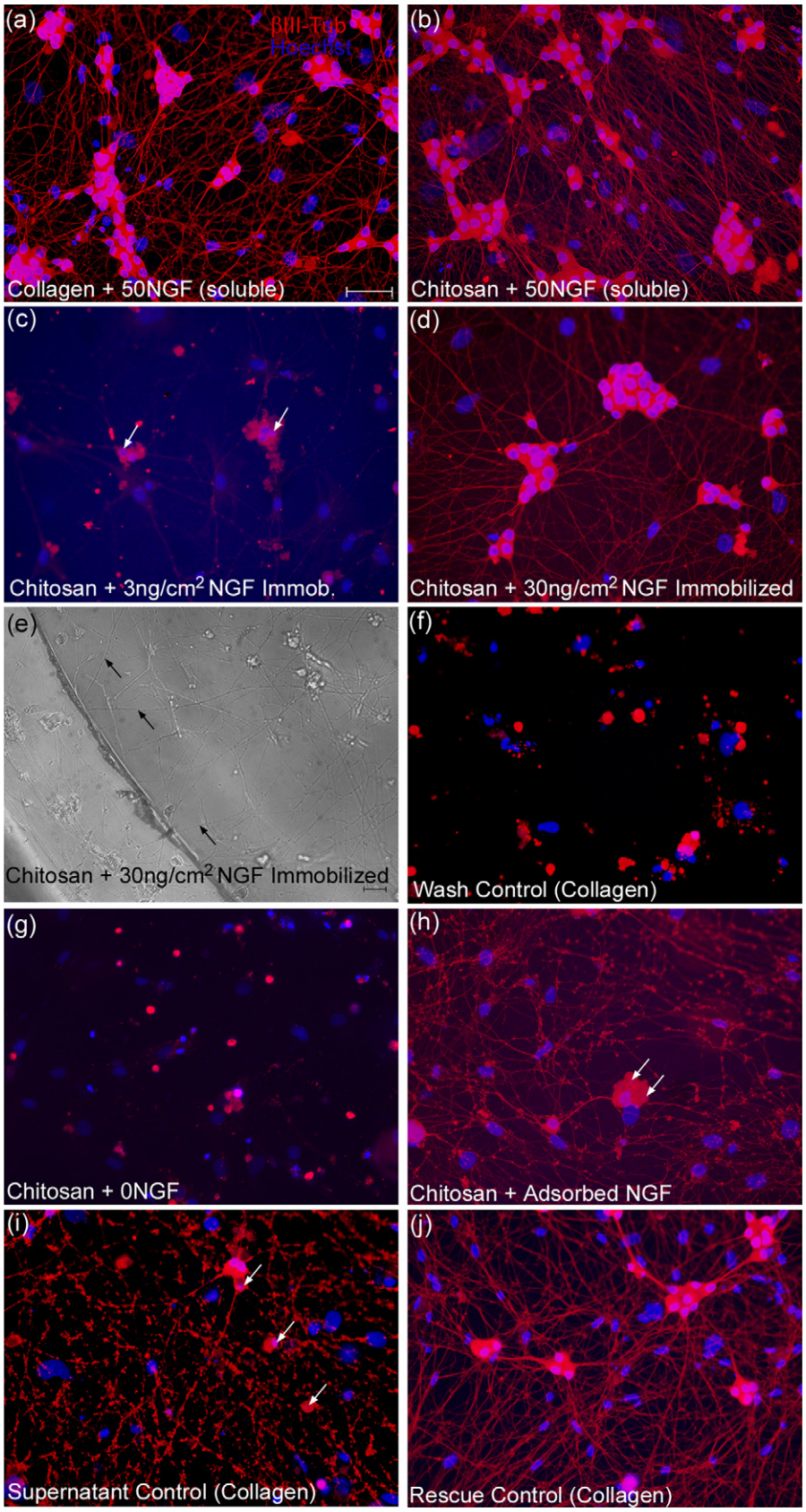


Fig. 4. Representative immunostained images of SCG neurons cultured on photoreactive chitosan (b–e, g, and h), and on collagen (a, f, i, and j). Neurons shown in (a–h) were cultured for 3 days and those in (i and j) were cultured for 4 days. Neuron survival on chitosan and collagen (a and b) show significant neurite outgrowth and survival when 50 ng/ml of soluble NGF (50NGF) is present. In contrast, when 3 ng/cm^2 of NGF is immobilized to the surface of chitosan, a significant decrease in neuron survival and neurite outgrowth are observed (c). When 30 ng/cm^2 of NGF is chemically immobilized, neurite outgrowth and neuron survival is comparable to the conditions where soluble NGF

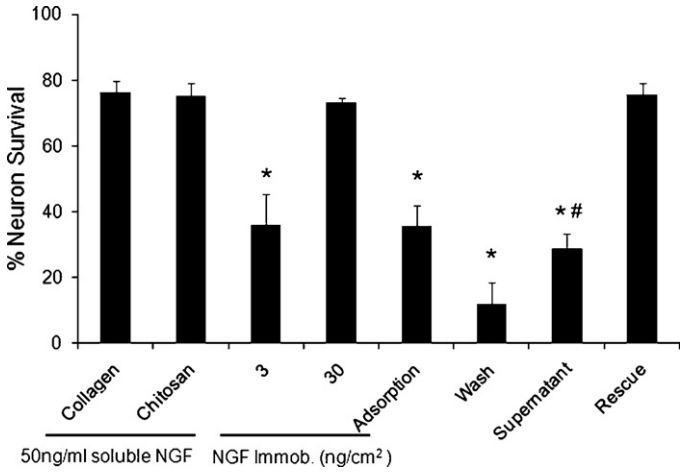


Fig. 5. Survival of neurons on chitosan when 30 ng/cm^2 of NGF was immobilized (quantified by Hoechst staining) is significantly different from negative controls, but not different from the positive controls where 50 ng/ml of soluble NGF (50NGF) is present, demonstrating that unbound NGF was sufficiently removed by washing (*Wash* control, *cf.* Fig. 4f) and that no covalently bound NGF was released into the media (*Supernatant* control, *cf.* Fig. 4i). When only 3 ng/cm^2 of NGF was immobilized to the surface of chitosan, neuron survival was significantly lower than when 30 ng/cm^2 of NGF was immobilized. When 30 ng/cm^2 of NGF was allowed to adsorb to the chitosan surface but not immobilized, neuron survival was significantly lower than when the NGF was immobilized within 3 days of culture. Neurons in the *Rescue* control were subjected to NGF withdrawal as in the *Supernatant* control, but neuron survival was rescued when serum-free medium containing 50NGF was fed to the neurons. Neuron survival between the *Rescue* and *Supernatant* controls was significantly different (*indicates statistical difference from 30 ng/cm^2 NGF immobilized condition, $p < 0.05$, #indicates statistical difference from *Rescue* control by one-way ANOVA, $p < 0.05$).

neuron aggregates outside of the NGF-patterned regions. Moreover, the same trends were observed regardless of plating density (~6400 or 19,200 cells/cm^2). There were more cell aggregates that contained 1–5 neurons for those neurons cultured on NGF squares compared to those cultured off of the squares. Significantly more neuron aggregates that contained 11–20 neurons or 20+ neurons were observed outside of the pattern than inside the squares; however, there was no difference in the percent of neuron aggregates that contain intermediate neuron numbers (i.e., 6–10 neurons/aggregate). Fig. 6c summarizes these observations when NGF was patterned for 5 or 10 min by confocal laser patterning and neurons cultured at a high density.

4. Discussion

The patterning method described here can be conveniently applied to materials that have a reactive moiety that can be activated by a UV or pulsed infrared laser. For example, agarose has been chemically patterned by specifically uncaging a coumarin-caged thiol with a pulsed infrared laser (Wosnick and Shoichet, 2008). While only 2D patterns created on chitosan films are shown here, the ability to control stage movement will allow 3D patterns to be constructed. Thus, by manipulating macros, complex patterns in 3D can be achieved (Wosnick and Shoichet, 2008). Moreover, confocal laser patterning of 2D substrates or 3D hydrogels is more flexible than creating patterns using photo-masks since a new mask is required each time a different size or shape of the pattern is needed. Thus, we were interested in combining the precise image quality offered by the confocal microscope together with the UV laser to create protein patterns that will spatially influence cell behaviour.

Various studies have shown that cells can respond to immobilized growth factors, such as either EGF (Ito et al., 1998) or NGF (Gomez and Schmidt, 2007) that have been chemically bound to substrate surfaces. Thus, investigations involving either cell–cell or cell–substrate interactions become possible using our method to spatially control the deposition of growth factors or proteins. In addition, mechanisms such as axon guidance can also be examined by chemically immobilizing concentration gradients of neurotrophic factors to substrate surfaces as shown in the present study. Results from these studies can directly influence design strategies for use in regenerative medicine.

This new method to immobilize factors allows physiologically relevant dimensions of axon guidance to be investigated. We anticipate step gradients of immobilized growth factors, shown in Fig. 3, can influence neurite behaviour as neurites are able to detect minute differences in growth factor concentrations. Since the distance between the steps in the gradient can be controlled by specifying the spacing between subsequent patterns, the gradient can be designed to be linear. A smooth linear concentration gradient can also be created by programming the microscope stage to move at a particular speed while the laser is making raster lines (Adams et al., 2005), or gradually adjusting the exposure time in consecutive raster lines while the stage is programmed to move at a constant speed (Hypolite et al., 1997). For example, Adams et al., 2005, demonstrated that a smooth linear concentration gradient of IKVAV peptide can be patterned by moving the motorized stage at the fastest speed possible (Adams et al., 2005).

Immobilized NGF has been demonstrated here and by others (Gomez and Schmidt, 2007; MacInnis and Campenot, 2002) to support neuron survival and neurite outgrowth. The immobilized NGF pattern can stimulate neuron behaviour and will be studied for axon guidance when presented as an immobilized concentration gradient either for use in regenerative medicine strategies or as tool to investigate signaling mechanisms involved in axon guidance. When NGF squares were patterned on the chitosan surface, large neuron aggregates formed outside of the pattern while single neurons and small aggregates were found mostly within the NGF squares. This observation demonstrates that the SCG neurons can detect and respond to the NGF patterns. Thus, it is possible to use the methodology described here to create various patterns of NGF that will influence or control neuron behaviour, as was shown in Fig. 6 by the simple NGF squares. Moreover, as the photochemistry used is versatile, other proteins can be patterned on chitosan

is present (d). Neurons that were plated outside of the films also has neurites extend towards the film demonstrating the chemoattractive effects of NGF (indicated by the black arrows) after 2 days in culture as shown in the phase contrast image (e). After NGF immobilization and removal of unbound NGF by extensive washing in TBST, the films were soaked in culture medium overnight at 37 °C and this medium used the next day as the medium in which to culture neurons on collagen. This demonstrates that any unbound NGF was removed from the chitosan surface and that the lack of soluble NGF in the medium of the *Wash* control clearly cannot sustain neuron survival (f). This is comparable to the lack of neuron survival when no NGF is present in immobilized or soluble form shown in (g). However, when NGF is allowed to adsorb to the surface of chitosan but not exposed to UV for chemical immobilization and subject to the same extensive washing procedure, neuron survival and neurite outgrowth were not sustained (h). Beading of neurites was observed which indicates neurite degeneration. The white arrows indicate neurons that have undergone apoptosis in (h). The *Supernatant* control shows that the immobilized NGF remains stably bound to the chitosan surface (i). These neurons were cultured in 10 ng/ml of soluble NGF (10NGF) for 2 days after which NGF was withdrawn. Supernatant medium from the 30 ng/cm^2 NGF immobilized condition were then fed to these neurons and cultured for two more days. Neuron survival significantly decreased compared to the positive controls (a and b). Neurons that have undergone apoptosis are apparent (as indicated by the arrows). Fragmentation and beading observed in the neurite morphology indicate significant degeneration. However, this effect is not due to over-starving of the neurons as neurons in the *Rescue* control (j) can be rescued by giving them serum free medium containing 50NGF after the neurons have undergone the same starvation conditions as those in (i). Scale = 50 μm.

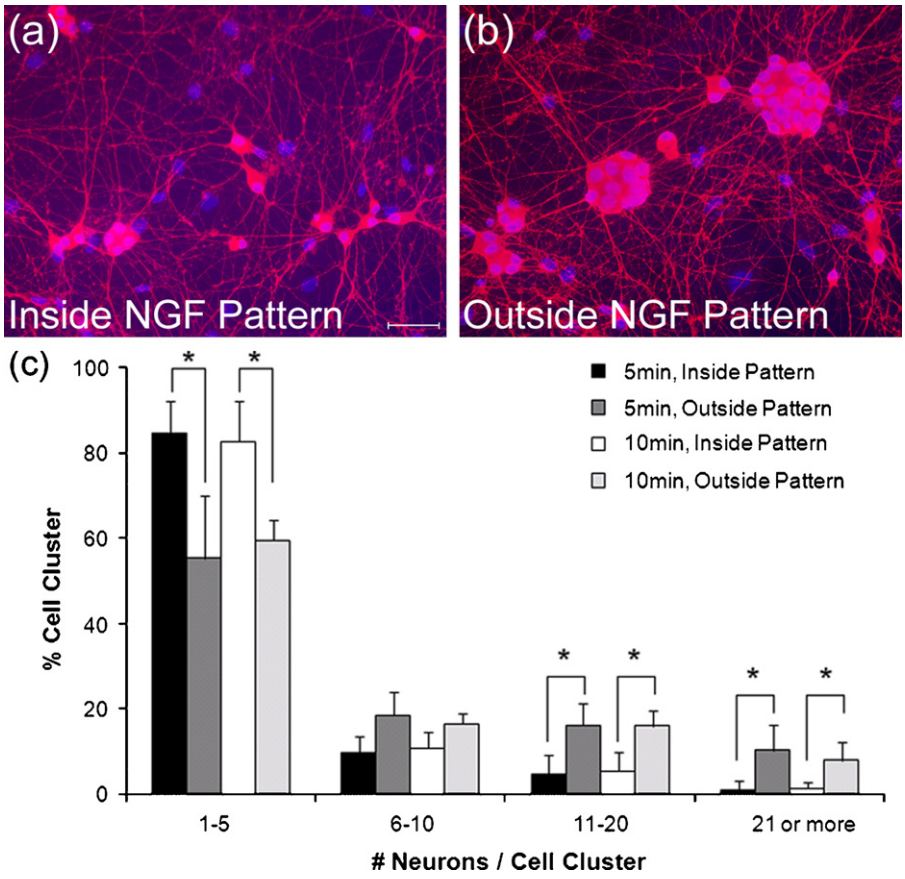


Fig. 6. Square NGF patterns created on photoreactive chitosan films by laser confocal patterning affected the affinity of the neurons for the chitosan surface. (a) Neurons tend to remain as single cells or form small cell aggregates when exposed to the NGF pattern on the photoreactive chitosan. (b) They form large cell aggregates in other regions of the chitosan where no NGF patterns are present. (c) Neurons were counted that were observed either inside (■) or outside (■) of the NGF pattern which was created by repeatedly scanning a specified region of interest for 5 min by laser confocal patterning. Similarly, neurons were counted that were observed either inside (□) or outside (□) of the NGF pattern, created by scanning the region of interest for 10 min. There was a significant difference in the % of cell aggregates that contain 1–5, 11–20, and 21 or more neurons ($^*p < 0.05$, $n = 5$ experiments). The neurons were stained red with βIII-tubulin, and the nuclei blue by Hoechst 33342. Scale = 50 μm.

surfaces. For example dual gradient patterns of NGF and brain-derived neurotrophic factor (BDNF) can be created on the same chitosan surface using the described methodology. Dual patterning has already been demonstrated with two fluorescent dyes in an agarose gel using a pulsed infrared laser combined with a confocal microscope (Wosnick and Shoichet, 2008). The methodology of confocal laser patterning described here opens new doors to create "biological tools" to study fundamental neuron behaviour.

The SCG neurons' response to the NGF square pattern on chitosan film also suggests that cell–cell and cell–substrate interactions may be influenced by a change in surface affinity due to the immobilized NGF squares. Many studies have found that neuron adhesion and neurite growth are affected by surface hydrophilicity, charge, and stiffness of the substrate (Cheng et al., 2003a,b; Freier et al., 2005; Haipeng et al., 2000; Mingyu et al., 2004). For example, cell adhesion of DRG neurons depended on the degree of deacetylation of chitosan, i.e., the presence of more amine moieties provided greater number of positive charges on the surface which enhanced neuron adhesion (Freier et al., 2005). Since NGF has a p*I* of 9.0 (Castellanos Ortega et al., 2001) (and collagen has a p*I* of 7.5 (Freudenberg et al., 2007)), at physiological pH 7.4, the bound NGF provides a net positive charge to promote greater neuron spreading and affinity in the NGF-patterned regions than the unmodified chitosan alone. In addition, the immobilized NGF also favours neuron spreading as NGF promotes neuron survival and neurite outgrowth. Hence, more single neurons or small neuron aggregates were observed within the NGF-patterned regions versus outside of these patterned regions, as quantified in Fig. 6.

We have shown here that patterns of NGF can be created in a spatially controlled manner using photochemical immobilization techniques made possible with UV confocal laser patterning. The bioactivity of NGF was conserved after chemical immobilization, and neurons were able to detect and respond to different surface concentrations of NGF. Importantly, neurons responded to the NGF patterns created on chitosan films. Given the versatility of the photochemistry used, other growth factors and proteins could be easily immobilized in spatially defined areas using confocal laser patterning and used to answer fundamental biological questions while advancing regenerative medicine strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2008.03.023.

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