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The use of immobilized neurotrophins to support neuron survival and guide nerve fiber growth in compartmentalized chambers

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ABSTRACT

We answered two major questions: (1) does retrograde signaling involve retrograde transport of nerve growth factor (NGF); and (2) is a gradient of immobilized NGF sufficient to promote and guide local axonal growth? To answer these questions, we developed a technique that resulted in stably immobilized NGF and combined this with compartmented chambers. NGF was photochemically-immobilized on a chitosan surface either in the cell body (CB) compartment, distal axon (DA) compartment, or both. Neuron survival and axon outgrowth were found to be insignificantly different from positive controls where soluble NGF was present. When NGF was immobilized on chitosan surfaces in the DA compartment, and in the absence of soluble NGF, neuron survival was observed, likely due to the retrograde signal of the activated TrkA receptor and NGF-induced signals, but not the retrograde signal of NGF itself. Axons were guided towards the higher end of the step concentration gradient of NGF that was photo-immobilized on the chitosan surface in the DA compartment by laser confocal patterning, demonstrating axonal guidance. These studies provide better insight into NGF signaling mechanisms which are important to both understanding developmental disorders and degenerative diseases of the nervous system, as well as improving design strategies to promote nerve regeneration after injury.
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1. Introduction

Understanding mechanisms of neuron survival, axon growth, and axon guidance are critical to elucidating developmental disorders and developing strategies for nerve regeneration. For example, neurodegenerative diseases such as Alzheimer's disease and Down's syndrome may have impaired NGF retrograde transport in cholinergic neurons rather than a decline in NGF production [1–3]. However, the causes for the failure of the NGF retrograde transport mechanism remain unclear. Having a research tool to investigate the retrograde transport mechanisms that contribute to degeneration would be very useful. Moreover, after nerve injury, tubular scaffolds have been implanted across a gap to promote regeneration [4]; however, regeneration is not complete. Incorporating a neurotrophin concentration gradient into the scaffold design may promote greater regeneration.

NGF promotes sympathetic neuron survival, axon outgrowth and axon guidance predominantly via the TrkA receptor [5,6] which is expressed on superior cervical ganglia (SCG) neurons [7], making them an excellent model system. Many research findings have shown that neuron survival is supported by the retrograde transport of signaling endosomes containing NGF and the activated TrkA receptor, which is known as the signaling endosome hypothesis [8–12].

A recent study using NGF-conjugated quantum dots demonstrated NGF is internalized at the distal axon into signaling endosomes that are transported along the axon to the cell body [12]. The speed at which the quantum dots appear in the cell bodies corresponds to a previous study using ^{125}I-NGF [13]. However, phosphorylated TrkA (pTrkA) appeared in the cell bodies within 1 min after the application to the distal axons of ^{125}I-NGF, which itself took 30–60 min to be observed in the cell body [13]. This suggests that the NGF-induced retrograde signal precedes the NGF-containing signaling endosomes. Moreover, MacInnis et al. demonstrated that neuron survival is not compromised when NGF, chemically bound to microbeads, cannot be taken up by the cells [14]. We also recently showed that sympathetic neurons can

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survive on chitosan surfaces to which NGF is chemically tethered, but not when it is only physically adsorbed [15].

Chitosan is a natural material derived from the shells of crustaceans and exoskeletons of arthropods [16,17]. It has been widely explored in recent years for biomedical applications [17,18]. While chitosan is biodegradable, it did not degrade during the timeframe of the current experiments [19].

Knock down of CREB by siRNA in the axon eliminated the accumulation of pCREB in the nucleus of cell bodies and compromised neuron survival in the presence of NGF in the distal axons [20]. These results contradicted earlier findings that NGF-pTrkA complex internalization and their transport to the cell body together is required to phosphorylate CREB and support neuron survival [21]. Hence, retrograde trafficking of NGF-induced signals without NGF internalization into signaling endosomes remains controversial despite continuous research efforts over the past decade. Herein we asked whether retrograde survival signaling involves retrograde transport of NGF using a new research tool with immobilized NGF.

NGF is also known to guide axon growth. Developing axons are guided to their innervating targets by proteins, such as neurotrophins, and surface-bound matrix molecules [19,22,23]. These molecules act by either repelling or attracting the growth cone. However, directing axonal growth is a complex event and crosstalk between these pathways adds to the complexity. Herein we asked whether a gradient of immobilized NGF was sufficient to promote and guide local axonal growth using a new photochemical gradient immobilization technology.

Patterned immobilization of biomolecules on surfaces is a powerful method for understanding and controlling cell behaviour [24–26]. We have demonstrated that a laser confocal patterning technique can be used to immobilize biomolecules in a 3D hydrogel [27], and that stably immobilized NGF patterns on a chitosan surface can influence neuron behaviour [15].

In the present study, we studied the biology of neuron survival, axon growth and guidance by combining our confocal laser patterning technique with the compartmentalized culture system. By selectively inhibiting NGF internalization in either the cell bodies, distal axons, or both, we asked whether neuron survival and axon outgrowth were affected by stably photoimmobilized NGF to chitosan surfaces using the compartmentalized chambers. Moreover, we studied whether a stably immobilized NGF gradient in the DA compartment guided axon growth up the gradient. Thus this new methodology allows us to ask some fundamental questions while at the same time allowing axons to be visualized as they grow on the neurotrophin-chitosan substrate. This may be useful to probe the effects, on either survival or guidance, of a series of different factors immobilized as gradients or at constant concentrations.

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. SCG dissection and compartmented culture

SCGs were explanted from post-natal day 2 Sprague–Dawley rats, and the ganglia dissociated into single neurons according to an established procedure [28]. General culturing procedures and medium formulations were as described in [29]. The dissociated neurons were re-suspended in Leibovitz's L-15 medium with no NGF. Neurons were plated in the centre compartment of the three compartment culture system of the Campenot chamber (Tyler Research Corp., Edmonton AB). Eight hours after cell plating, culture media, according to Table 1, were added all around the Campenot chamber in the 35 mm Petri dish outside of the cell body (CB) compartment. The Campenot chambers were prepared on the photoreactive chitosan surfaces after NGF had been immobilized or patterned (see below). The compartment in which the neurons were plated is referred to as the cell body (CB) compartment and the two side compartments in which axonaxons extend as the distal axon (DA) compartment from here on.

Table 1
Concentrations of soluble NGF added to the culture media for SCG neuron cultures in compartments.

		Day 1–2		Day 3		Day 4	
		CB (ng/ml)	DA (ng/ml)	CB (ng/ml)	DA (ng/ml)	CB (ng/ml)	DA (ng/ml)
Soluble NGF	Control 1	10	50	10	50	10	50
	Control 2	10	50	10	10	10	0
	Control 3	10	50	10	50	0	50
	Non-cond.	10	0	10	0	0	a
	Immob. 4d	10	50	10	10	0	b
	Cond. 50NGF	10	50	10	10	0	c
Immobilized NGF	CB	0	50	0	10	0	0
	DA	10	0	10	0	0	0
	CB + DA	0	0	0	0	0	0

400 μl of culture medium was added to each DA compartment.

[a.] Supernatant medium, containing no soluble NGF was collected from the DA compartments on day 4 before NGF withdrawal from the CB compartments. The supernatant medium was then fed to the DA compartments in the "Conditioned 50NGF" group.

[b.] Supernatant medium was collected, after 4 days of incubation, from compartment cultures where NGF was immobilized on chitosan surfaces in the DA compartment. The medium was then fed to the DA compartments in the "Conditioned 50NGF" group after NGF withdrawal from both CB and DA compartments on day 4.

[c.] Supernatant medium, containing 50 ng/ml of soluble NGF was collected from the DA compartments on day 3 before NGF withdrawal from both CB and DA compartments on day 4. The same supernatant medium was then fed back to the DA compartments after NGF withdrawal.

2.2. 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 prepared as described in [15]. A thin chitosan film was cast in a 35 mm Petri dish by drying the chitosan solution 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). 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 surface to improve cell adhesion using carbodiimide chemistry as previously described in [15]. Sulfo-SANPAH was dissolved in PBS at 0.25 mg/ml protected from light and sterilized by passing the solution through a 0.22 μm filter. Photoreactive chitosan was then produced by reacting the collagen-crosslinked chitosan surfaces with sulfo-SANPAH for at least 3 h at RT on a shaker in the dark. The surface concentration of sulfo-SANPAH on the chitosan surface was determined previously to be 64.2 ± 9.0 μg/cm^2 from [15]. The photoreactive chitosan surface was washed twice in ddH_2O to remove any salts and allowed to air dry. Using a pin rake (Tyler Research Corp., Edmonton, Alberta), 20 tracks about 5 mm long were scratched on the dried chitosan surface to direct axon extension from the CB compartment into the DA compartment underneath the Teflon barrier of the Campenot chamber (see Step 1 in Fig. 1).

2.3. NGF immobilization for compartmented culture

To immobilize NGF to the DA compartment only, NGF (AbD Serotec, Raleigh, NC) solution (15 μg/ml, 50 μl) was added to the dried chitosan surface to cover an area of about 0.5 cm^2 lined by a sterile rubber gasket (sterilized in 70% ethanol) on the surface of chitosan where the Campenot chamber would later be placed (Step 1 in Fig. 1). The amount of NGF immobilized was determined by ELISA, as described in [15]. Similarly, 10 μl of 4.0 μg/ml of NGF solution was applied to the center of the tracks scratched on the surface of chitosan to immobilize NGF to the CB compartment. NGF was allowed to adsorb overnight at 4 °C and exposed to UV (350 nm) the next day using a UV reactor (Southern New England Ultra Violet Company, Connecticut) for 10 min. Markings were made at the bottom of the Petri dish to denote where NGF was immobilized on the chitosan surface to facilitate the placing of the Campenot chamber (Step 2 of Fig. 1). The rubber gaskets were removed and the chitosan surface was washed extensively in 20 mM Tris + 150 mM NaCl + 0.1% Tween 20 (TBST, pH 7.5) for at least 15 times by forcefully injecting TBST through a 20G syringe needle. The chitosan film was left in TBST for about 1 h shaking gently. This procedure was repeated 3 more times. The chitosan surfaces were then washed 3

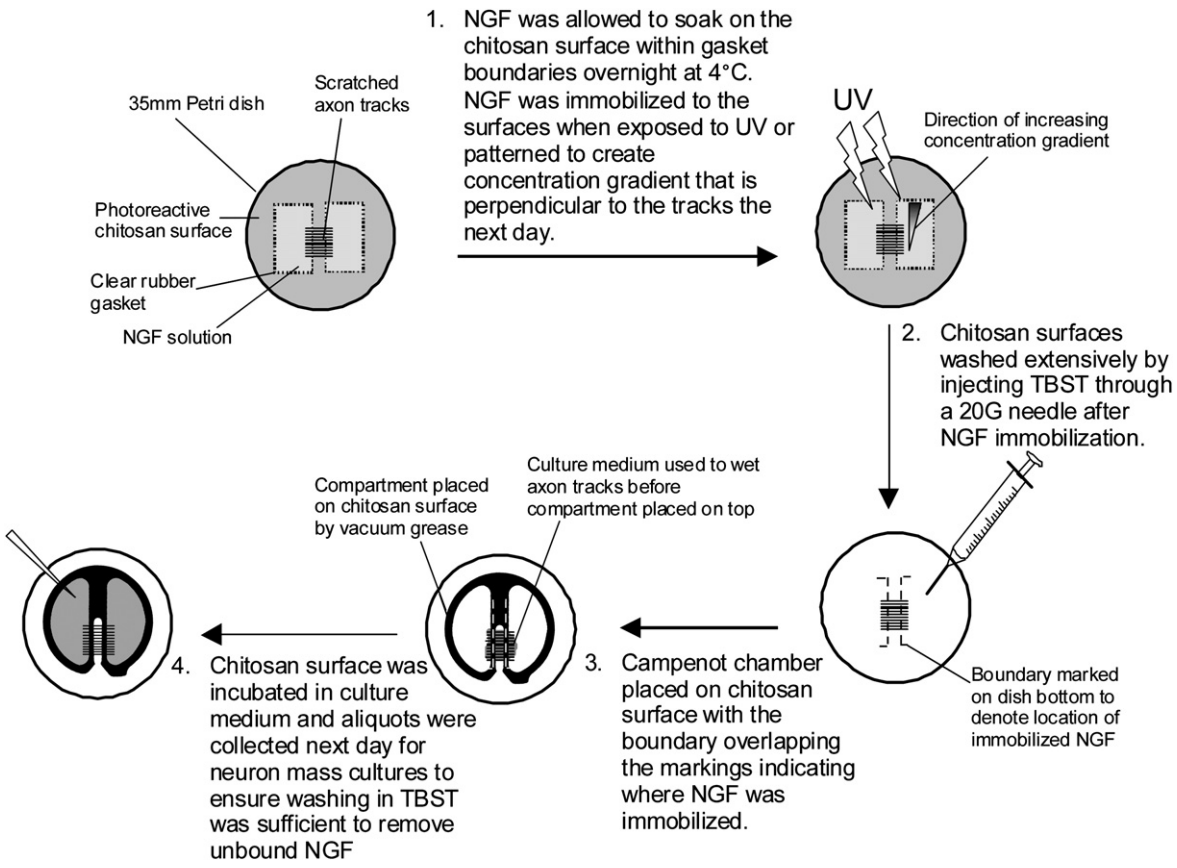

Fig. 1. A schematic diagram showing the preparation of NGF photo-immobilization on chitosan surface in a 35 mm Petri dish. Following NGF immobilization or patterning, unbound NGF was removed by extensive washing in TBST. The Campenot chamber was then positioned on the NGF immobilized chitosan surface for compartmented cultures.

more times in PBS to remove any residual Tween 20 and two more times in ddH_2O, prior to air-drying.

2.4. NGF concentration gradient patterning for axon guidance

NGF solution (15 μg/ml, 50 μl) was added to the dried chitosan surface and allowed to adsorb as above. Alexa Fluor 488 hydrazide (Alexa 488, 1.0 μg/ml, Invitrogen, Burlington, ON) was added as well to help visualize and locate the appropriate region on the chitosan surface for NGF patterning using the confocal microscope. NGF concentration gradient patterns were created on the photoreactive chitosan surface by confocal laser patterning as previously described in [15] at the end of the scratched tracks, so that the direction of the patterned concentration gradient was perpendicular to the tracks (Fig. 8B). Briefly, a 364 nm UV laser (Coherent Innova 90C, Santa Clara, CA) connected 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. A macro computer program written using the Leica software (Leica Microsystem v2.6.1, Heidelberg GmbH) was used to control the microscope stage movement and UV laser exposure as previously described in detail [15]. After NGF gradient patterning, the chitosan surfaces were washed extensively in TBST as described above.

2.5. Quantification of the step concentration gradient on chitosan surface

Alexa 488 was patterned on photoreactive chitosan. The steepness of the gradient profile was determined by comparing the gray scale of the immobilized Alexa 488 gradient profiles to known concentrations of Alexa 488. The immobilization yield of NGF on chitosan was assumed to be the same as Alexa 488, since both contain only one primary amine group which readily reacts with sulfo-SANPAH upon UV photolysis. After photo-patterning and subsequent washing in PBS to remove any unbound Alexa 488, the step concentration gradients were imaged in sections using a 10× objective on an upright microscope (BX61, Olympus Canada Inc., Markham, Ontario) and a monochrome camera (CoolSNAP HQ monochrome, Photometrics, Roper Scientific, Tucson, AZ). Sections of the gradients were stitched together using PhotoShop CS3 Extended. The gray scale profile was obtained and analyzed using the Image J software (NIH).

2.6. Mounting the campenot chamber on NGF immobilized chitosan surface

The Campenot chamber was mounted on the chitosan surface as described by Campenot [30] so that the Teflon barrier of the chamber aligned with the markings indicating where NGF had been immobilized (Step 3 of Fig. 1). The assembly was then incubated at 37 °C, 5% CO_2, and 100% humidity for 1 h. Following incubation, 400 μl of culture medium was placed in each DA compartment according to Table 1. The compartments were left in the incubator overnight for cell plating the next day.

2.7. Stability of immobilized NGF on photoreactive chitosan surfaces

To ensure any unbound NGF was sufficiently removed from the chitosan surfaces after NGF immobilization or NGF gradient patterning, several compartment controls were performed. SCG neurons were plated in 10 ng/ml soluble NGF in the CB compartment in these controls. The compartments were grouped into the "Immobilized", "Non-conditioned", and "Conditioned 50NGF" groups. For the "Immobilized" group, after NGF immobilization in the DA compartment alone and extensive washing in TBST to remove any unbound NGF, as described above, 400 μl of culture medium, containing no NGF, was added to the DA compartments where NGF had been immobilized (see Step 4 of Fig. 1). These compartments were incubated at 37 °C, 5% CO_2, and 100% humidity. The media in each DA compartment were collected at 1, 2, 3, and 4 d, and used as conditioned medium for SCG neurons in mass cultures grown on collagen-coated chamber slides. Similarly, 400 μl of medium containing no soluble NGF and 50 ng/ml soluble NGF were added to each DA compartment of the "Non-conditioned" and "Conditioned 50NGF" groups, respectively. Media from these DA compartments were collected after 4d (from the "Non-conditioned") and 3d (from the "Conditioned") of culture and fed to neurons in mass cultures as *conditioned* media (see Table 1).

SCG neurons in mass cultures were plated in medium containing 10 ng/ml soluble NGF. After 4d of cultures, NGF was withdrawn for 6 h and the Conditioned media collected from the above compartments were fed to these neurons. The cultures were fixed after 24 h to assess the effect of NGF desorption, if any, from the immobilized surfaces. To ensure neuron survival in the mass culture was not compromised by over-starving during NGF withdrawal, neurons fed with the "Non-conditioned" and "Conditioned 50NGF" media served as additional negative and positive controls, respectively. Starved neurons would be further compromised by

the lack of soluble NGF in the "Non-conditioned" media, and rescued by the "Conditioned 50NGF" media.

After 4d of culture, soluble NGF was withdrawn from both the CB and DA compartments in the "Conditioned 50NGF" control group for 6 h and then replaced with one of three conditioned media collected from the DA compartment (see Table 1) of the: "Immobilized", "Non-conditioned", and "Conditioned 50NGF" groups. The compartments were fixed after 24 h. Any soluble NGF accumulated due to desorption from the chitosan surfaces in the "Immobilized" group, after 4d of incubation, should allow retrograde transport of NGF via distal axons in the DA compartment to support neuron survival in the CB compartment. Neuron survival is compared among groups where the distal axons are fed with media collected at day 4 from the "Immobilized", "Non-conditioned" and "Conditioned 50NGF" groups.

2.8. Immunostaining in SCG neurons

Neurons were fixed in 4% paraformaldehyde (PFA) for 15 min at RT. The fixed cells were washed 3 times, 5 min each with PBS, and then permeabilized for 10 min in 0.5% TritonX-100. The cell bodies in compartmented cultures and mass cultures 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 (1:1000, Clone TUJ1, Abcam Inc., Cambridge, MA) in blocking solution and PBS (1:1) overnight at 4 °C. The cells were washed in PBS 3 times for 5 min each on the following day. Neurons were then incubated in goat anti-mouse Alexa 546 secondary antibody (1:500, Molecular Probes, Burlington, Ontario) for 1 h at RT, and washed as mentioned above following incubation. NGF immobilized on the chitosan surface was visualized by immunostaining with a rabbit polyclonal NGF antibody (1:500, Abcam Inc.) followed by goat anti-rabbit Alexa 546 secondary antibody. The nuclei were stained with Hoechst 33342 (5 μM, Invitrogen). Axons in the DA compartments were stained with rhodamine phalloidin (Invitrogen) for 25 min at RT and washed as above in PBS. All stained samples were mounted using GelTol (Fisher Scientific, Ottawa, Ontario). Cell bodies in compartmented cultures and mass cultures were imaged using a 20× objective on the upright microscope and a coloured camera (DP70, Olympus Canada Inc., Markham, Ontario). Axons in the DA compartments were imaged using a monochrome camera.

2.9. Neuron survival and axon density measurement

Neuron survival in compartmented cultures in culture conditions shown in Table 1 were counted blindly. The percentage of surviving neurons was determined by dividing the number of live neurons by the total number of live cells (neuronal and non-neuronal cells). Neuron survival in the mass culture controls were counted similarly.

Axon density was measured using the Image J software (NIH) by analyzing the fluorescent intensity in the DA compartment within a square region that has the same width as the track scratched on the chitosan surface. To demonstrate directional guidance of the axons, the DA compartment was re-created by stitching the images together using Photoshop CS3 Extended and divided into three regions, namely top, middle, and bottom. Axon density in each of the three regions was then analyzed. Axon guidance by the NGF gradient was also analyzed by drawing a line parallel to the NGF gradient in each of the three regions fixed at 200 μm from the end of the tracks scratched on the chitosan surface. Directionality was assessed by analyzing axons that crossed this line. Axons that aligned perpendicular to this line were considered to have no preferred directionality (neutral) and given a score of 0. Axons that deviated upward from neutrality were given a score of +1, whereas those axons that deviated downward from neutrality were given a score of −1. The total positive and negative scores are compared to the number of neutral axons as an estimate of axon directionality in each of the three regions. At least 100 axons were analyzed at random for each sample.

2.10. Statistical analysis

All neuron survival and axon outgrowth data are reported as the mean ± standard deviation ($n = 3$ experiments). The reported mean and standard deviation for all axon density assays to demonstrate directional guidance was normalized with respect to the top region of the DA compartment. Statistical significance between each culture conditions in the neuron survival, axon outgrowth and guidance assays were determined by single factor ANOVA with post-hoc Tukey HSD analysis ($p < 0.05$).

3. Results

3.1. Immobilized NGF stably bound

NGF was photochemically-immobilized to chitosan surfaces and tested for neuron survival and axon outgrowth (Fig. 1). To ensure that NGF was stably immobilized to chitosan, several controls were performed. In compartments where NGF was immobilized in the DA compartment, the surfaces were washed extensively in a Tris buffer with salt and Tween (TBST) and separated into 4 groups. These groups were then incubated in culture medium containing no soluble NGF at 37 °C for 1, 2, 3, and 4 days respectively. These were the "Immobilized" controls. Medium from the DA compartment of each group was collected at the respective time points to be used as culture medium in neuron mass culture (see below). In the "Non-conditioned" and "Conditioned 50NGF" controls, the DA compartment contains culture medium with no soluble NGF and 50 ng/ml of soluble NGF, respectively (see Table 1). Media from the DA compartments from these two controls were also collected and used as culture media for neurons in mass culture (see below).

Neurons in the mass culture were grown on collagen-coated surfaces and initially plated in medium containing 10 ng/ml soluble NGF. On day 4, neurons were starved for 6 h by NGF withdrawal. Media collected from the compartment controls, as described above, were fed to these neurons and cultured for another 24 h. Neuron survival in media collected from the "Immobilized", "Non-conditioned, and "Conditioned 50NGF" controls were compared to that in the positive control where neurons were plated and maintained in medium containing 50 ng/ml of soluble NGF (50NGF control).

After 5 days of culture, neuron survival in the positive 50NGF control was 82.7 ± 6.0%. In the four "Immobilized" control groups, where the supernatant media was collected from the DA compartments of photochemically-immobilized NGF on chitosan surfaces after 1, 2, 3, and 4d of incubation, neuron survival ranged from 19.9 to 26.0% (see Fig. 2). This was statistically different from that of the positive 50NGF control. Moreover, when the starved neurons in mass culture were fed with the "Non-conditioned" medium, neuron survival was 16.9 ± 2.5% which was not different from the low rate of neuron survival observed when neurons were left to starve for 24 h (22.4 ± 1.8%). In contrast, when starved neurons were fed with medium collected from the "Conditioned 50NGF" control, neuron survival was rescued to 84.2 ± 4.1% which was not statistically different from the 50NGF control (82.7 ± 6.0%). Thus, the neuron mass culture demonstrates that photo-immobilized NGF was stably bound to chitosan because, with extensive TBST washing, neuron survival was significantly lower than the positive control. Moreover, the possibility that NGF desorbed from NGF immobilized chitosan surfaces in the DA compartments and accumulated in sufficient quantities to support neuron survival is low, because neuron survival in the "Immobilized" controls remained significantly lower than the 50NGF control, yet not statistically different from the negative controls – "Non-conditioned" and "Starved." As neurons similarly starved were rescued with medium collected from the "Conditioned 50NGF" control, the low rate of neuron survival observed in the "Immobilized" controls could not be due to over-starving of the neurons.

3.2. NGF not internalized by neurons

To provide further evidence that immobilized NGF remained stably immobilized on the chitosan surface and was not internalized by axons, the fate of immobilized NGF in the DA compartment was tracked by immunostaining. SCG neurons that were plated in the CB compartment and supported by soluble NGF (10 ng/ml). This is to allow axon extension into the DA compartment where NGF had been chemically immobilized (The chitosan surface was washed extensively in TBST, as described, to remove any unbound NGF prior to cell plating). After 5 days of culture, neuron cell bodies were starved by NGF withdrawal for 24 h and then fixed in 4% PFA for NGF-immunostaining. As a positive control, neurons supported by soluble NGF in both CB and DA compartments were subjected to

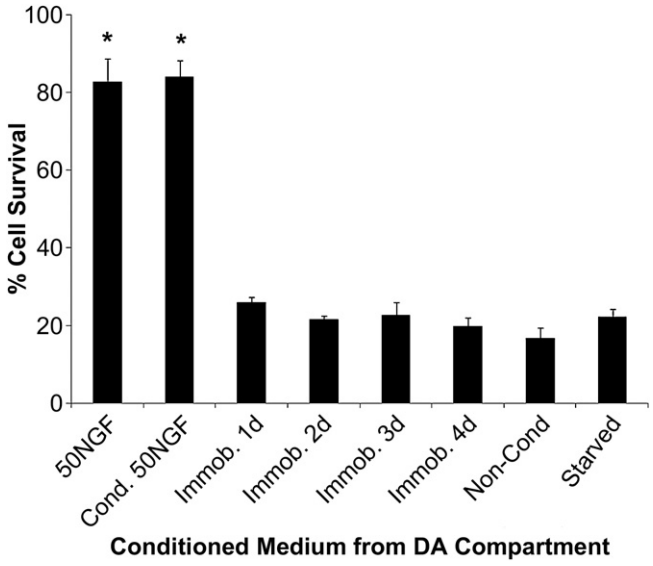

Fig. 2. After NGF immobilization on the chitosan surface, and sufficient washing to remove any unbound NGF in TBST, NGF immobilized on chitosan surface in the DA compartment was incubated in medium for 1, 2, 3, and 4d. These media were collected and used as the media in which to culture neurons on collagen-coated wells as the *Immbo. 1d, Immob. 2d, Immob. 3d,* and *Immob. 4d* to demonstrate NGF is stably immobilized on the chitosan surface. Culture media were also collected from the DA compartment containing 50 ng/ml (*Cond.* 50NGF) and 0 ng/ml of soluble NGF (*Non-Cond.*). Neurons were initially plated on collagen in medium containing 10 ng/ml of soluble NGF. Culture wells in which neurons were plated and maintained in medium containing 50 ng/ml of soluble NGF (50NGF) served as the positive control. Soluble NGF was withdrawn on day 4 of the culture. The collected supernatant media were fed to these neurons and cultured for 24 h. No conditioned medium was fed to a set of culture wells after NGF withdrawal and they served as a negative control. Neurons survival in the 50NGF and *Cond.* 50NGF positive controls were statistically higher than in culture wells where neurons were fed with supernatant media collected from DA compartments where NGF was immobilized (*, **$p < 0.05$). Neuron survival rescued using medium collected for the *Cond.* 50NGF control demonstrate that the low survival numbers in the *Immob.* conditions were not due to over-starving of the neurons to induce apoptosis. Neuron survival in the *Immob.* conditions were not statistically different from the *Non-Cond.* control demonstrating that little or no NGF desorbed from the NGF-chitosan surfaces to support neuron survival.

similar NGF withdrawal from both compartments, followed by stimulation of the axons with 100 ng/ml of soluble NGF in the DA compartment for 2 h before fixation. When NGF was available in the soluble form to axons in the DA compartment, NGF was clearly internalized by the axons (Fig. 3A), which is in stark contrast with the lack of NGF staining observed inside axons when it is chemically immobilized on the chitosan surface (Fig. 3B). For the latter, NGF immobilized on the chitosan surface in the DA compartment are seen as bright punctae. The majority of these punctae are not observed inside the axons; however, some NGF punctae overlap with the axons. Together with the series of wash, supernatant and rescue controls, these immunostaining data demonstrate that NGF remained stably immobilized on the chitosan surface and are unavailable for axons to internalize and transport by the retrograde mechanism to the cell body.

3.3. Sympathetic neuron survival and growth independent of NGF internalization

Having established a method to stably immobilize NGF, we asked whether NGF internalization is required for sympathetic neuron survival. NGF was selectively immobilized in either the CB compartment, DA compartment, or both CB and DA compartment regions using photochemistry. In the CB compartment, 24 ng/cm^2 (or 1.2 ng) of NGF was immobilized on the chitosan surface because lower concentrations of immobilized NGF resulted in poor survival (data not shown). NGF was immobilized to chitosan in the DA compartment at 44 ng/cm^2. After immobilization, the chitosan surfaces were washed extensively in TBST (as before) to remove any unbound NGF. Compartmented cultures were prepared with the Campenot chambers placed on the chitosan surfaces such that the NGF immobilized regions correspond with the appropriate compartments (Fig. 1). SCG neurons were plated in the CB compartment. Culture medium in the compartment where NGF was immobilized contained no soluble NGF from the day of cell plating. When NGF was immobilized in the CB compartment alone, 50 ng/ml of soluble NGF was present in the DA compartment to allow for axon extension. NGF was then withdrawn from the DA compartment and neuron survival was assessed, as elaborated below. When NGF was immobilized in the DA compartment alone, neurons were plated in the presence of 10 ng/ml soluble NGF. After axons extended into the DA compartment, NGF was withdrawn from the CB compartment. Neuron survival, supported by immobilized NGF in the DA compartment alone, was then determined. Similarly, when NGF was immobilized in both the CB and DA compartments, neurons were plated in the CB compartment in the absence of soluble NGF, and survival was measured five days later. Table 1 shows the concentration of soluble NGF in the culture medium over the course of the culture period.

Neuron survival analysis demonstrated that SCG neurons survived on chitosan surfaces that had NGF immobilized, with extensive distal axon outgrowth observed in compartmentalized cultures after 5 days (Fig. 4). When NGF was *immobilized* to both the CB and DA compartments and maintained in the *absence* of soluble

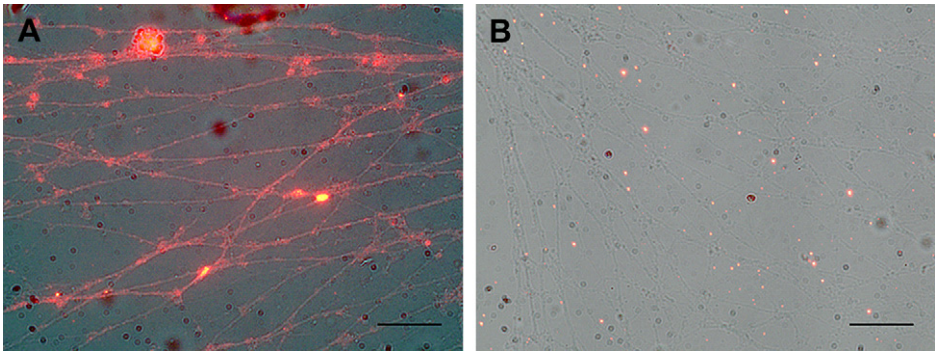

Fig. 3. SCG axons grown on chitosan surfaces with NGF present as (A) soluble and (B) immobilized in the DA compartment with NGF visualized by immunostaining. (A) When NGF is present in soluble form, NGF is internalized by axons; however, (B) the majority of the immobilized NGF (seen as red punctae) is not internalized by axons. Higher exposure was necessary in order to visualize and better detect the immunostaining signals in (B).

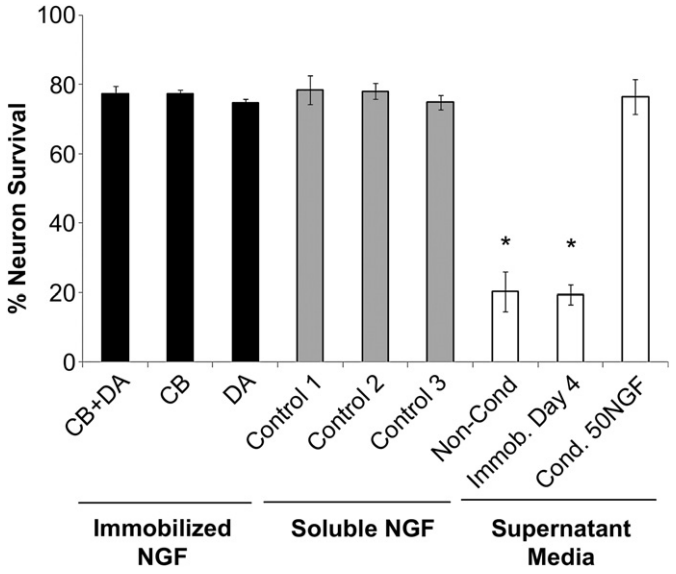

Fig. 4. Neuron survival in compartmented cultures where NGF is chemically immobilized on the chitosan surface was compared to the survival where soluble NGF is present in controls after 5 days of culture. Neuron survival in the presence of immobilized NGF in both CB and DA compartment was not statistically different from Control 1 where 10 ng/ml and 50 ng/ml of soluble NGF were present in the CB and DA compartment respectively. Where NGF was immobilized to the CB compartment only and 50 ng/ml of soluble NGF withdrawn from the DA compartment after axons had extended, neuron survival was similar to Control 2 where 10 ng/ml of soluble NGF was present in the CB compartment and soluble NGF likewise withdrawn from the DA compartment. Neuron survival was not compromised when NGF was immobilized to the DA compartment alone and soluble NGF from the CB compartment was withdrawn. Neuron survival in response to immobilized NGF in the DA compartment alone was not statistically different from Control 3 where 50 ng/ml of soluble NGF was present in the DA compartment and soluble NGF was similarly withdrawn from the CB compartment. There was no statistical difference between Controls 1–3. To demonstrate neuron survival in NGF-chitosan compartments is not due to NGF desorption from the chitosan surfaces, culture medium from the DA compartment where NGF was immobilized on chitosan surfaces after 4d of incubation (*Immob. Day* 4) were fed to the DA compartment and CB and DA compartments. As negative and positive controls, media containing no soluble (*Non-Cond*) and 50 ng/ml of soluble NGF (*Cond.* 50NGF) were collected from DA compartments where no NGF was immobilized to the chitosan surface. Neuron survival in the *Immob. Day* 4 and *Non-Cond.* was not statistically different from each other, but both were significantly lower than neuron survival in the Immobilized NGF and Soluble NGF groups. The lack of neuron survival in the *Immob. Day* 4 and *Non-Cond.* groups are not due to induced apoptosis by over-starving the neurons as neurons in the *Cond.* 50NGF group, that was similarly starved by NGF withdrawal, could be rescued where neuron survival was not statistically different from the neuron survival observed in Control 1.

NGF, neuron survival was $77.2 \pm 2.3\%$ after 5 days of culture, which was not statistically different from neuron survival in Control 1 ($78.4 \pm 4.2\%$, $p > 0.05$), where *soluble* NGF was maintained in both compartments (see Table 1). When NGF was immobilized to the CB compartment only and neurons plated in culture medium containing no soluble NGF, axons extended into the DA compartment where 50 ng/ml of soluble NGF was present after 3 days of culture. The NGF was then withdrawn from the DA compartment to starve the axons as detailed in Table 1. Neuron survival was found to be $77.2 \pm 1.3\%$. This survival number was not statistically different from that of Control 2 ($78.0 \pm 2.2\%$, $p > 0.05$) where soluble NGF was only present in the CB compartment. Immobilized NGF on the chitosan surface in the DA compartment alone was assessed for neuron survival by possible retrograde signaling. Neuron cell bodies were maintained with 10 ng/ml soluble NGF for the first 3 days while axons extended in the presence of immobilized NGF (44 ng/cm^2) in the DA compartment. NGF was withdrawn from the CB compartment on day 4, and neuron survival was measured 24 h later to be $74.6 \pm 1.0\%$ (see Fig. 4) which was not statistically different from the neuron survival of Control 3 ($74.8 \pm 2.1\%$, $p > 0.05$) where soluble NGF was added only to the DA compartment (and the CB compartment was starved by NGF withdrawal). These results suggest that cell survival does not depend upon NGF uptake by the growth cone but on the retrograde NGF-induced signal, which reaches the cell body, as previously shown by MacInnis [14]. Importantly, the survival numbers in compartments where NGF was immobilized are significantly greater than in the negative control, "Non-conditioned" group where no soluble NGF is present.

To provide further evidence that NGF remains stably bound to the chitosan surface and NGF desorption from the chitosan surface, if any, is unable to support neuron survival, compartment cultures were used. Neurons cultured in compartments from the "Conditioned 50NGF" group were starved by NGF withdrawal from the CB and DA compartments as per Table 1. Media collected from the "Immobilized", "Non-conditioned", and "Conditioned 50NGF" controls, at the time points shown in Table 1, were then fed to the DA compartments in the "Conditioned 50NGF" groups after NGF withdrawal and cultured for another 24 h. If NGF had desorbed from the NGF immobilized chitosan surfaces and accumulated in the culture medium, then neuron survival in the CB compartment could be rescued by the retrograde transport of any bioactive desorbed NGF, and axon growth in the DA compartment would be maintained.

The Supernatant Media group (Fig. 4) compares neuron survival in the CB compartment in the "Conditioned 50NGF" group after the conditioned media are fed to the DA compartment following NGF withdrawal. Compartments fed with media collected at day 4 from both "Immobilized" and "Non-conditioned" groups show neuron survival to be $19.3 \pm 2.9\%$ and $20.3 \pm 2.7\%$, respectively. These survival numbers were statistically different from neuron survival observed in the CB compartment when medium collected from the "Conditioned 50NGF" group was fed back to the DA compartment after neuron starvation ($76.4 \pm 5.0\%$). Neuron survival in the CB compartment of the "Conditioned 50NGF" group is not compromised by over-starving the neurons, as medium which was collected at day 3 from the DA compartment of the "Conditioned 50NGF" control (containing soluble NGF), rescued neuron survival.

The neuron cell bodies remained healthy in conditions where NGF was immobilized in both the CB and DA compartments (Fig. 5A) which is comparable to those in Control 1 where soluble NGF was present in both compartments (Fig. 5D). Similarly, cell bodies exposed to immobilized NGF in the CB compartment remained healthy (Fig. 5B) which is comparable to the appearance of neuron cell bodies in the CB compartment in Control 2, where 10 ng/ml of soluble NGF was present (Fig. 5E). Morphologies of the neuron cell bodies where NGF was immobilized to the DA compartment alone and soluble NGF withdrawn from the CB compartment (Fig. 5C) are comparable to those observed in Control 3 where 50 ng/ml of soluble NGF was present in the DA compartment only where soluble NGF was similarly withdrawn from the CB compartment (Fig. 5F). Neuron morphologies in Fig. 5A–F are considerably healthier than those observed in Fig. 5G where NGF was withdrawn from the CB compartment and no soluble NGF present in medium in the DA compartment. The lack of neuron survival in the "Conditioned 50NGF" group fed with media collected from the "Non-conditioned" and "Immobilized" controls was also evident from the neuron morphologies shown in Fig. 5. Significant cell death is observed in Fig. 5G and H due to the lack of soluble NGF present in the media of the "Non-conditioned" and "Immobilized" controls. This observation is in contrast to the surviving neurons observed in Fig. 5I, which were rescued with medium collected from the "Conditioned 50NGF". Morphology of the neurons in Fig. 5I is similar to those observed in Control 1

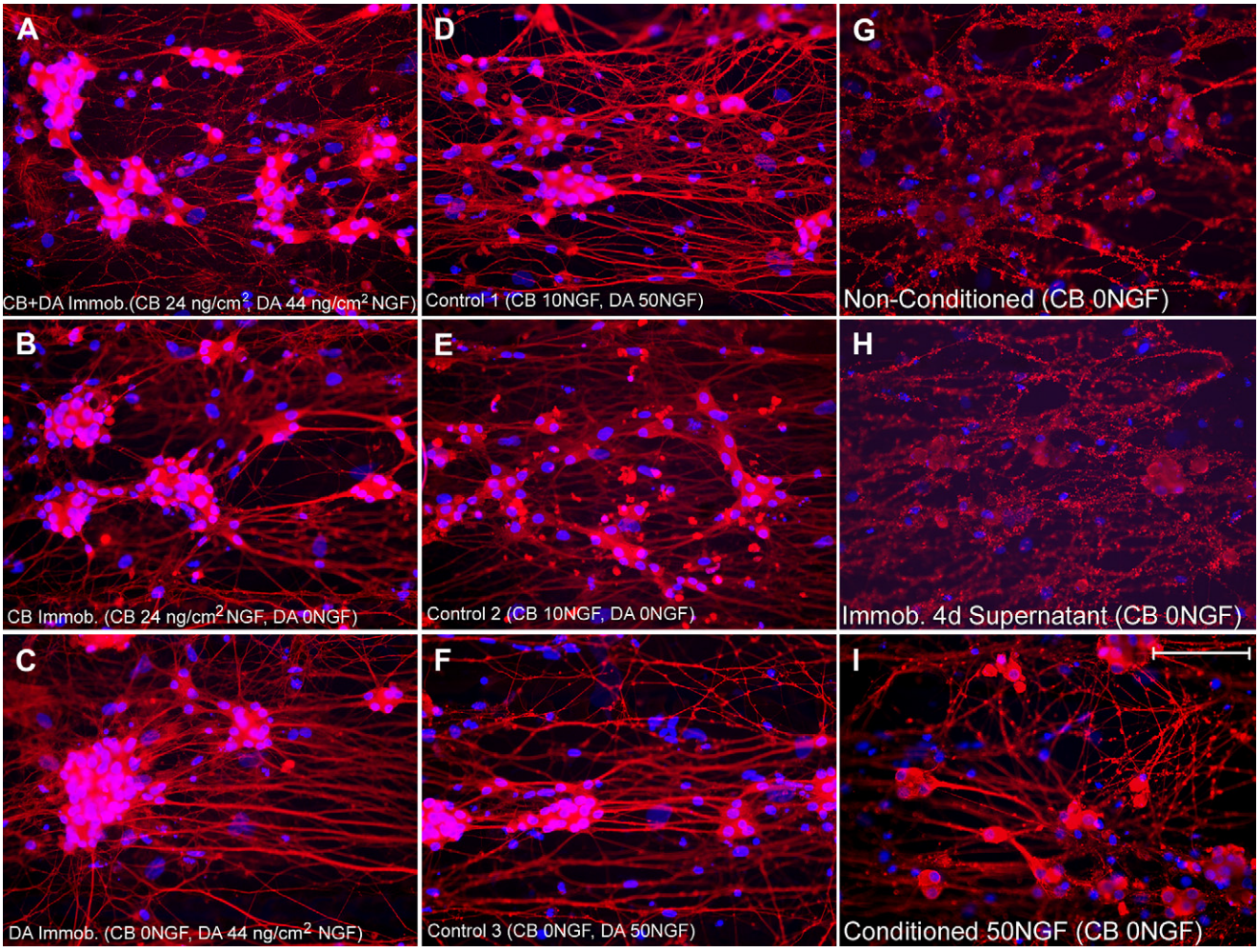

Fig. 5. Neuron survival in the CB compartment in response to NGF immobilized to both CB and DA compartments, and either in the CB or DA compartment alone were compared to controls where soluble NGF was present after 5 days in culture. (A) In the presence of immobilized NGF in both CB and DA compartments, neurons were plated and maintained in the absence of soluble NGF in the medium. Neuron cell bodies were healthy and comparable to (D) Control 1 where soluble NGF was present in both compartments (10 ng/ml and 50 ng/ml in the CB and DA compartments, respectively). (B) Neuron cell bodies remained healthy where NGF was immobilized to the CB compartment only and soluble NGF withdrawn from the DA compartment after axons had extended. Similar neuron morphology was observed in (E) Control 2 where 10 ng/ml of soluble NGF was present in the CB compartment only after NGF was similarly withdrawn from the DA compartment as in (B). (C) Neuron survival was also apparent when NGF was immobilized to the DA compartment only and 10 ng/ml of soluble NGF withdrawn from the CB compartment after axons had extended into the DA compartment. This observation was similar to the survival observed in (F) Control 3 where 50 ng/ml of soluble NGF was present in the DA compartment alone after soluble NGF was similarly withdrawn from the CB compartment as in (C). When the DA compartment, similarly starved, were treated with medium containing no soluble NGF, the morphology of neuron cell bodies indicate apoptosis (G). Medium collected from the DA compartments after 4 d of incubation, where NGF was immobilized was also fed to the DA compartment in cultures where soluble NGF was withdrawn from both CB and DA compartments. The cell bodies morphology also indicates apoptosis (H). However, neuron apoptosis observed in G and H are not the result of over-starving the neurons as the DA compartments of starved cultures could be rescued when the DA compartment was treated with medium collected from DA compartments containing 50 ng/ml soluble NGF (I). Scale = 100 μm.

(Fig. 5D) where soluble NGF is present in both CB and DA compartments. These observations together with the results shown in Fig. 4 suggest that neurons can be supported by immobilized NGF and the retrograde signal of NGF-induced effectors.

3.4. Axon outgrowth from sympathetic neurons on immobilized NGF

Having established that immobilized NGF does not prevent the retrograde survival signal, we investigated the impact of immobilized NGF on axon outgrowth. Extensive axon outgrowth was observed when NGF was immobilized on the chitosan surface in both the CB and DA compartments (Fig. 6A) and is similar to the growth observed in Control 1 (Fig. 6D). When NGF was immobilized to the CB compartment only and soluble NGF withdrawn from the DA compartment after axon extension, axon degeneration was observed (Fig. 6B). This demonstrates that NGF immobilized alone in the CB compartment cannot support distal axon outgrowth; however, it can support neuron survival (*cf.* Fig. 4). Axon degeneration was also observed where soluble NGF was withdrawn from the DA compartment in Control 2 (Fig. 6E), as expected. Importantly, extensive axon outgrowth was observed when NGF was immobilized in the DA compartment alone (Fig. 6C) with comparable axon morphologies to those observed in Control 3 (Fig. 6F), where NGF withdrawal in the CB compartment alone did not affect axon growth and maintenance. The extensive axon outgrowth observed in Fig. 6C suggests that neuron survival observed in Fig. 4 is supported by retrograde signal of other downstream effectors unaccompanied by NGF, as the NGF was stably immobilized and cannot be taken up by the axons.

Axon growth and maintenance were also compromised in the "Conditioned 50NGF" compartments where the DA compartments

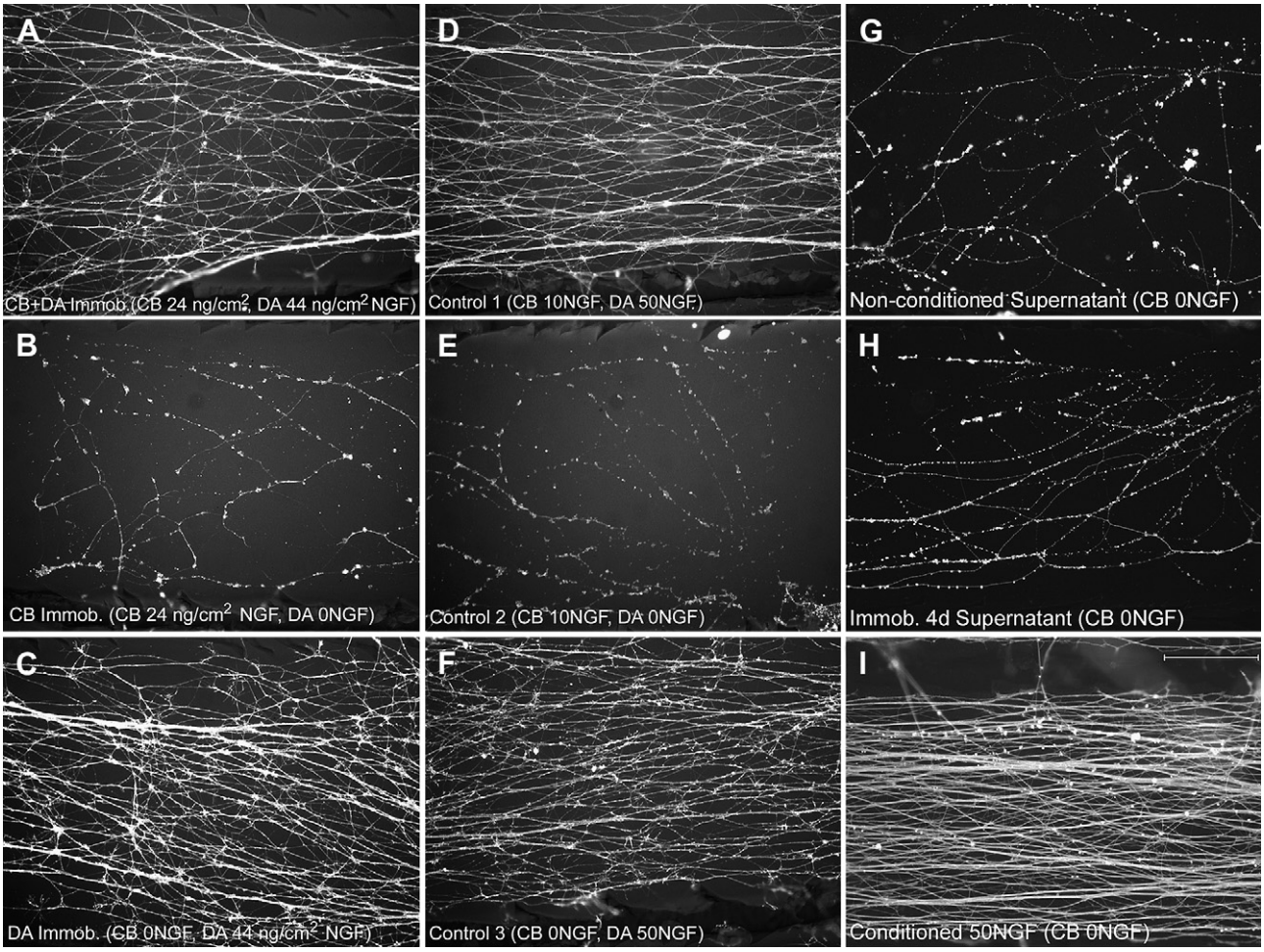

Fig. 6. Axon outgrowth in response to NGF immobilized the both CB and DA compartments, and either CB or DA compartment was compared to controls where soluble NGF was present after 5 days of culture. (A) Extensive axon outgrowth was observed where NGF was immobilized to both CB and DA compartments and neurons were maintained in culture medium containing no soluble NGF since the day of plating. Axon morphology was similar to (D) Control 1 where soluble NGF was present in the CB (10 ng/ml of soluble NGF) and DA (50 ng/ml of soluble NGF) compartments. (B) Axon degeneration was apparent where NGF was immobilized to the CB compartment only and soluble NGF withdrawn from the DA compartment after axons had extended. Similar axon degeneration was observed in (E) Control 2, as expected, where soluble NGF was present in the CB compartment only and soluble NGF likewise withdrawn from the DA compartment. Extensive axon outgrowth was observed in (C) where NGF was immobilized to the DA compartment only and soluble NGF withdrawn from the CB compartment. Axon outgrowth was maintained in (F) Control 3 with similar morphology to (C) where soluble NGF was present in the DA compartment only following NGF withdrawal from the CB compartment. Significant axon degeneration is observed, when the DA compartment was treated with medium collected from compartments containing no soluble NGF after NGF was withdrawn from both CB and DA compartments, as expected (G). Similar axonal degeneration is observed when medium collected from the DA compartments after 4 d of incubation, where NGF was immobilized, was also fed to the DA compartment in cultures where soluble NGF from both CB and DA compartments were similarly withdrawn. This indicates the lack of soluble NGF in the medium to sustain axon growth and maintenance (H). However, axon degeneration observed in (G) and (H) could not be the result of over-starving the axons as axons treated with medium collected from DA compartment of cultures containing 50 ng/ml of soluble NGF could sustain axon growth and maintenance similar to that observed in (D). Scale = 100 μm.

were fed with medium collected at day 4 from the "Non-conditioned" and "Immobilized" groups. Fig. 6G and 6H show the morphology of distal axons after media from the "Non-conditioned" and "Immobilized" controls were fed to the DA compartments in the "Conditioned 50NGF" group, respectively, following NGF withdrawal. Significant axon beading is observed showing signs of axon degeneration. This degenerate morphology is similar to that observed in Fig. 6E where NGF was similarly withdrawn from the DA compartment in Control 2. In contrast, axons treated with "Conditioned 50NGF" medium that was fed back to the DA compartment after NGF withdrawal remained healthy, and comparable to the morphology observed in Control 1 where soluble NGF is present in the DA compartment. These observations are consistent with the lack of neuron survival in the CB compartment when media collected from either the "Immobilized" or "Non-conditioned" compartments were fed to these neurons (see Figs. 4 and 5G, H). Hence, the above controls (Figs. 2, 4, 5, and 6) demonstrate that NGF is stably immobilized on the chitosan surface and that the accumulated NGF desorbed from the chitosan surface, if any, is not sufficient to support and maintain neuron survival and axon growth.

The axon density profile observed in Fig. 6 was quantified by comparing the measured axon densities to Control 1, which was set at $100 \pm 2.2\%$ (Fig. 7). When NGF was immobilized to both the CB and DA compartments, the measured axon density was found to be $75.7 \pm 18.4\%$ of Control 1. When NGF was immobilized to the CB compartment alone and soluble NGF was withdrawn from the DA compartment after axons had extended, there was a significant drop in axon density to $31.0 \pm 21.4\%$ ($p < 0.05$) compared to Control 1; however, there was no statistical difference in axon density when compared to Control 2 ($10.2 \pm 9.2\%$), where soluble NGF was similarly withdrawn from the DA compartment after axons had extended. The axon density measured in Control 2 was statistically lower than that measured in Control 1 (Fig. 7). Axon

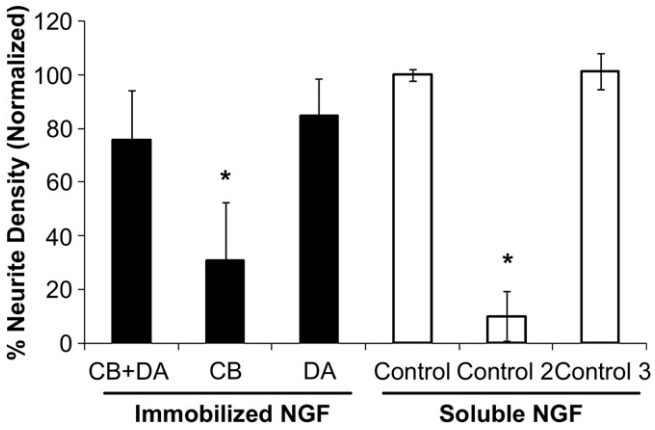

Fig. 7. Axon densities in compartmented cultures where NGF had been chemically immobilized were measured and compared to controls (Controls 1 and 3) after 5 days of culture. The average axon density and standard deviation was normalized against Control 1 where 10 ng/ml and 50 ng/ml of soluble NGF were present in the CB and DA compartments respectively. When NGF was immobilized to both CB and DA compartments, the measured axon density was similar to Control 1. When NGF was present either as immobilized or soluble form (Control 2) in the CB compartment and NGF withdrawn from the DA compartments after axons had extended, axon densities in both conditions were significantly lower than Control 1, as expected, but not statistically different from each other. When NGF was immobilized to the DA compartment alone where soluble NGF was withdrawn from the CB compartment, axon density was not statistically different from Control 3, where soluble NGF was present in the DA compartment only and NGF was likewise withdrawn from the CB compartment. (*$p < 0.05$ with respect to Control 1, mean ± standard deviation, $n = 3$ experiments).

density was 84.8 ± 13.8% of Control 1 when NGF was immobilized to the DA compartment which was also not statistically different from Controls 1 (100 ± 2.2%) and 3 (101.3 ± 6.6%), where, for the latter, NGF was withdrawn only from the CB compartment. There was no statistical difference in the axon density between Controls 1 and 3. Together, Figs. 4–7 demonstrate that SCG neuron survival and axon growth are possible even when NGF is immobilized and unavailable for internalization.

3.5. Axonal guidance by immobilized NGF concentration gradient

Having demonstrated that neuron survival and axon growth can be supported by immobilized NGF, we were interested in determining whether an immobilized step concentration gradient of NGF could guide axon outgrowth in compartmented cultures. The step concentration gradients were created by laser confocal patterning in a specified region of interest in the DA compartment. Profiles of the expected NGF step gradients patterned on the chitosan surface are shown in Fig. 8A. Axon tracks were deliberately made short (Fig. 8B) so that the axons were free to extend over the chitosan surface once they reached the DA compartment, from the CB compartment, and not confined to grow within the tracks. Axon densities were quantified and analyzed by dividing the DA compartment into three regions, namely bottom, middle, and top, reflecting the low, medium and high concentrations of NGF immobilized, respectively. When a shallow NGF gradient of 40.5 pg/130 μm of chitosan surface was patterned, axon density measurements in the three regions of the DA compartment were similar and comparable to the density distribution when a homogeneous concentration of NGF (44 ng/cm^2) was immobilized to the chitosan surface (Fig. 8C). A step NGF gradient of 68.4 pg/130 μm showed guidance towards the higher end of the concentration gradient by axon density analysis. Axon densities in the bottom and middle regions were not significantly different from each other, but were both statistically different from the top region ($p < 0.05$). When an even steeper gradient was immobilized, the NGF concentration increased by 84.3 pg/130 μm at each step before reaching a saturated level (see Fig. 8A). Axon densities were statistically different in all three regions, with the lowest density in the bottom region, followed by the middle region, and the highest axon density in the top region (Fig. 8C).

Fig. 8D shows the distribution of axons in the DA compartment when a shallow step gradient was immobilized. The uniform axon distribution agrees with the axon density measurement from Fig. 8C. This is also consistent with axon directionality analysis shown in Table 2. There were similar numbers of axons given a score of −1 (axons turning away from the direction of the gradient), 0 (axons showing no preferred directionality), or +1 (axons turning in the direction of the gradient) in each of the three regions. A similar trend was recorded when axons were exposed to a homogeneous surface concentration of NGF (i.e., "No Gradient" in Table 2). Axon density analysis observed in Fig. 8C shows axons were guided towards the top end of the 68.4 pg/130 μm concentration gradient. This finding agrees with the axon behaviour observed in Fig. 8E where more axons were observed in the high-end of the NGF gradient. Axon density appeared greater in the top region following the direction of the immobilized step gradient. Axons were also observed to turn towards the higher end of the concentration gradient in the top region. This observation is consistent with the axon directionality analysis shown in Table 2 where the percentage of axons in the top region given a score of +1 was at least twice the percentage of axons given a score of −1 or 0. Axon guidance was observed in all three regions when presented with a steep immobilized gradient of 84.3 pg/130 μm (Fig. 8F). The percentage of axons in each of the three regions with a score of +1 was consistently higher than those with a score of 0 or −1 (Table 2), suggesting axon guidance up the step gradient towards the higher NGF concentration. Axons in the bottom region of the gradient appear to turn up the gradient, as did those in the middle region. The most prominent guidance effect by the immobilized NGF gradient was observed in the top region where a cluster of axons turned and followed the gradient upwards (Fig. 8F). This guidance effect is not seen in Fig. 8G, where axons are uniformly distributed in the DA compartment that has a homogeneous surface concentration of 44 ng/cm^2 NGF immobilized to the chitosan surface. The percentage of axons given a score of −1, 0, or +1 was similar in each of the three regions, demonstrating a lack of axon guidance (Table 2). This observation is similar to that in Fig. 8D when a shallow step gradient was immobilized. Comparing the measured axon densities in Fig. 8C and observations shown in Fig. 8E and F, we can conclude that axons are guided by an immobilized NGF step concentration gradient.

The axon density distribution observed in Fig. 8E and F increased gradually from the low-end towards the high-end of the NGF gradient, which was not observed in Fig. 8D and G (with a shallow NGF gradient and homogeneous NGF concentration, respectively), where no axon guidance was observed. The increased axon density (in Fig. 8E and F) likely reflects greater branching of axons in response to the immobilized NGF at the higher end of the gradient. High concentrations of NGF have been demonstrated in the past to induce branching in axons [31,32]. Thus, it is possible that the immobilized NGF gradient elicited two effects: turning of the axons up the NGF gradient (as quantified in Table 2 and observed in Fig. 8F), and branching of the axons (especially at the high-end of the gradient where the absolute concentration of NGF is highest) leading to a "gradient" distribution of axons in the DA compartment measured in Fig. 8C. Hence, our results and observations demonstrate that axons can detect and be guided by immobilized NGF, which reinforces our findings that immobilized NGF can support axon growth (*cf.* Figs. 6 and 7).

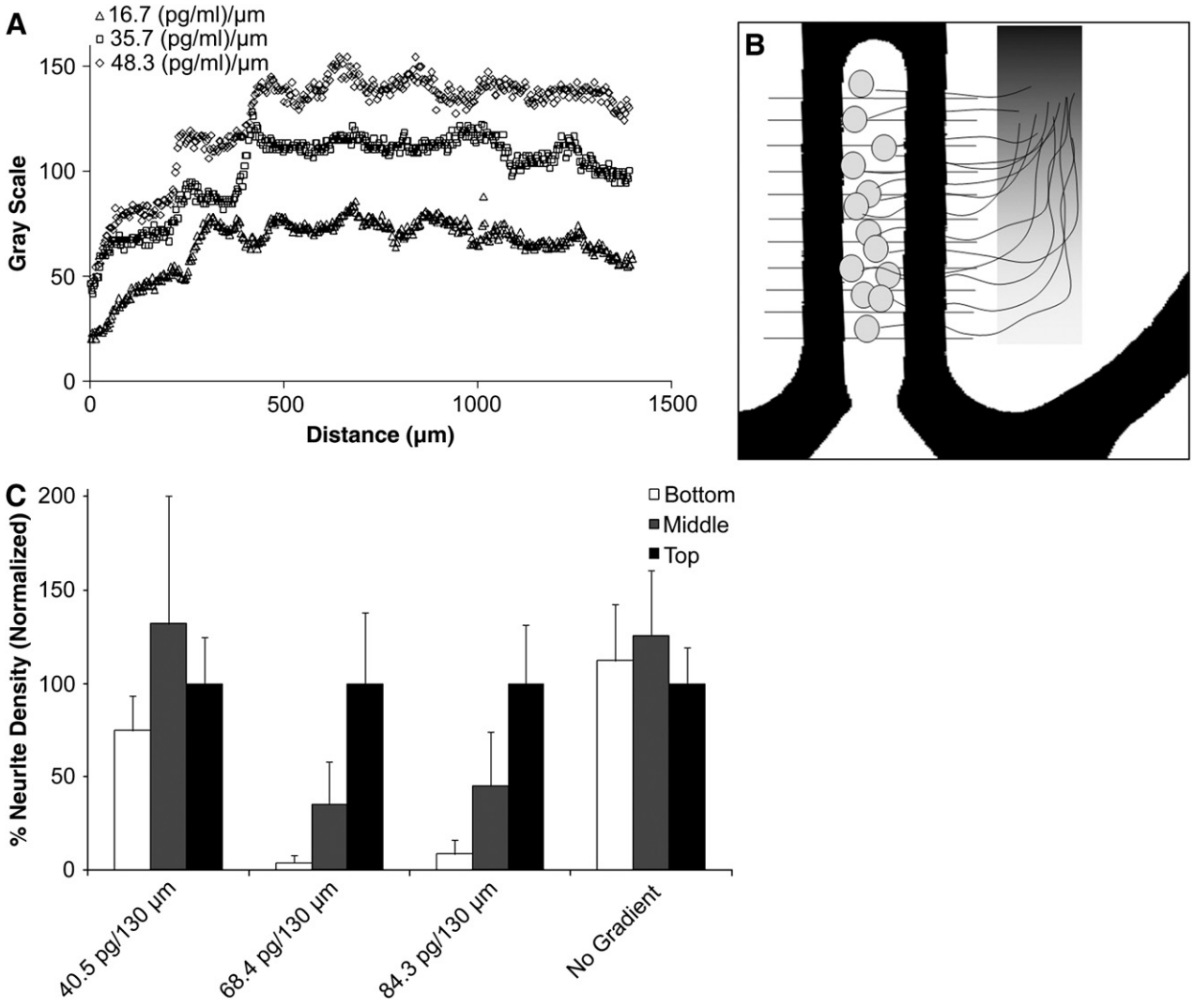

Fig. 8. (A) Step concentration gradient profiles in gray scale patterned on the chitosan surface for (△) 40.5 pg/130 μm, (□) 68.4 pg/130 μm, and (◇) 84.3 pg/130 μm are shown. (B) Tracks scratched on the surface of the Petri dish were deliberately made short so that axons are free to extend over the surface of the immobilized concentration gradient of NGF. (C) Axon guidance by the NGF step gradient profiles shown in (A) was analyzed by measured axon densities (normalized with respect to the top region) and compared between the bottom (□), middle (▩), and top (■) regions. No statistical difference in axon densities were observed when a shallow step gradient of 40.5 pg/130 μm was patterned on the chitosan surface. When a steeper step gradient (68.4 pg/130 μm) was created, axon density measured in the top region alone was statistically different from both the middle and bottom regions ($p < 0.05$) but no statistical difference between the middle and bottom regions. A steep NGF gradient of 84.3 pg/130 μm showed statistical difference between all three regions ($p < 0.05$) demonstrating axon guidance. (D) A shallow step gradient of 40.5 pg/130 μm did not elicit any guidance response in axons as they were observed to be distributed uniformly in the DA compartment and agrees with the axon density measurement in (C). (E) When a steeper step concentration gradient of 68.4 pg/130 μm was patterned on the chitosan surface, only axons in the top region were observed to turn and follow the direction of the gradient. (F) When an even steeper step gradient, 84.3 pg/130 μm, was patterned on the chitosan surface, axons in all three regions were observed to turn and following the direction of the immobilized NGF gradient. Axon turning observed in (E) and (F) was not observed in (G) where axons distributed uniformly across the DA compartment when a homogeneous concentration of NGF (44 ng/cm^2) was immobilized to the chitosan surface (arrows in F show the direction of axon turning). The brighter background near the top region resulted from an increased amount of Alexa 488 immobilized at the higher end of the concentration gradient, which was used to locate the region of interest during laser confocal patterning. Scale = 100 μm.

4. Discussion

Neuron survival, axon growth and axon guidance during development are complex events involving an intricate network of signaling pathways and crosstalk. Although significant research has been devoted to understand these mechanisms, they have yet to be fully resolved. To elucidate the complex mechanisms involved in neuron development and axon guidance, we developed a novel research tool that combines laser confocal patterning with the compartmentalized culture system. The laser confocal patterning is particularly important because it allows NGF immobilization to be spatially resolved to one of the CB compartment, DA compartment, or both. We have shown for the first time that SCG neurons can survive (*cf.* Figs. 4 and 5) and elicit extensive axon outgrowth (*cf.* Figs. 6 and 7) by immobilized NGF alone (in the absence of soluble NGF) from the day of cell plating, most convincingly in compartmented cultures where NGF was chemically immobilized in either or both the CB and DA compartments. When NGF is chemically immobilized in the DA compartment, or both CB and DA compartments, neuron survival and axon outgrowth remained comparable to the positive controls where soluble NGF was present. We have shown in mass and compartment cultures, using negative controls, that neuron survival and axon outgrowth observed is not due to NGF desorption, and that NGF remains stably immobilized on chitosan surfaces (*cf.* Figs. 2 and 4).

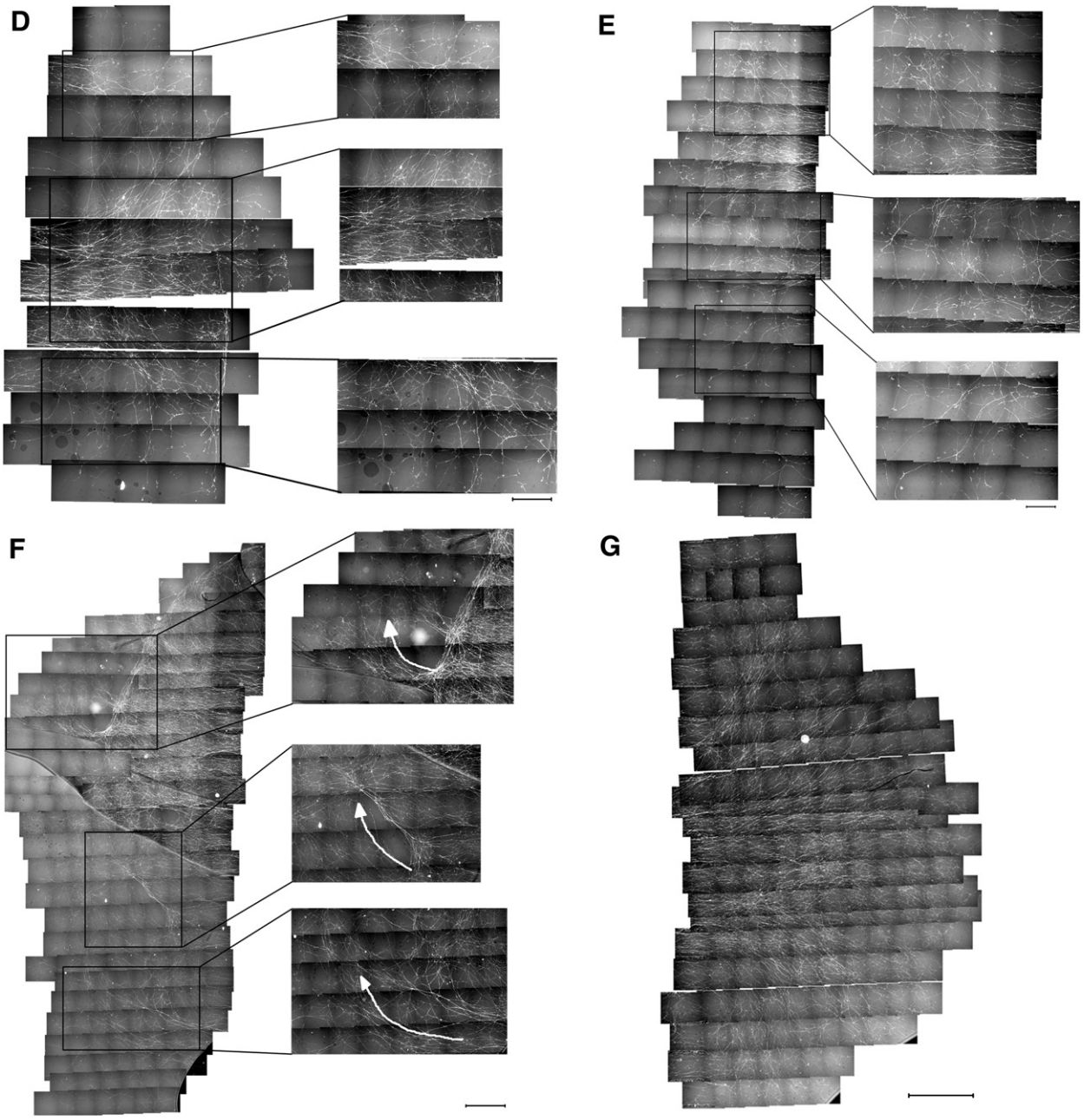

Fig. 8. (*continued*).

Table 2
Preferred axon directionality in response to the immobilized step concentration gradient of NGF. A score of −1 indicates axons turn away from the direction of the step gradient, 0 indicates axons have no preferred directionality, and +1 indicates axons are guided in the direction of the step gradient (Axon counts are representative of the images shown in Fig. 8. At least 100 axons were counted for each sample).

Step Gradient	40.5 pg/130 μm			68.4 pg/130 μm			84.3 pg/130 μm			No Gradient		
Region	−1 (%)	0	+1	−1 (%)	0	+1	−1 (%)	0	+1	−1 (%)	0	+1
Bottom	58	8	34	46	23	31	17	15	68	44	25	31
Middle	26	30	44	38	20	42	29	19	52	32	30	38
Top	28	31	41	26	17	57	15	19	66	39	23	38

We have also reported previously that when NGF is homogeneously immobilized on a chitosan surface at 30 ng/cm^2, neuron survival was 73%, while neuron survival as only 37% when 3 ng/cm^2 of NGF was immobilized on the surface. When NGF was adsorbed on the chitosan surface and then extensively washed in TBST, as described above, neuron survival was 39% which was statistically lower than when 30 ng/cm^2 of NGF was photochemically-immobilized on the chitosan surface [15]. These results are consistent with the findings shown in Figs. 2 and 4 that NGF remains stably immobilized on chitosan surfaces. NGF (if any) that accumulated in the culture media by NGF desorption from the chitosan surface, is insufficient to support neuron survival.

We also showed by immunostaining (Fig. 3) that there is no biologically significant uptake of NGF into distal axons that were grown on the NGF-chitosan surface. We provided evidence in

neuron compartmental cultures that neuron survival does not involve internalization and retrograde transport of NGF. These results suggest that cell body survival is maintained by the retrograde transport of the activated TrkA signal and other NGF-induced signaling molecules in signaling vehicles unaccompanied by the retrograde transport of NGF, since NGF is chemically bound to the chitosan surface and cannot be internalized by the axons in the DA compartments.

Our findings agree with those obtained by MacInnis et al. in their microbead experiments where the NGF-tethered microbeads also supported neuron survival by retrograde signal transduction without the uptake of soluble NGF by the axons [14]. Hence, our data demonstrates that patterned immobilization of NGF combined with compartmented cultures is a reliable research tool to investigate the retrograde signaling mechanism to support neuron survival and axon growth. Our results also support previous findings by others. K-252a, a protein kinase inhibitor, has been demonstrated to exert its own pro-survival side effect despite the fact that it blocks the phosphorylation of TrkA [33,34]. Mok et al. [34] showed partial neuron survival when TrkA phosphorylation was blocked by K-252a regardless of whether it was applied to either the cell bodies and proximal axons or the distal axons. Their results demonstrate that other retrograde signals to support neuron survival may exist which does not require the internalization of the NGF-TrkA complex. Thus, it is likely that there is more than one way to produce retrograde signals.

MacInnis et al. [35], and recently Mok et al. [34] showed that kinase inhibitors that block the phosphorylation of TrkA in the cell bodies and proximal axons do not block the ability of NGF, applied to the distal axons, to support local axon growth and retrograde survival. These results suggest that other downstream signals, activated by the phosphorylation of TrkA, carry retrograde survival signals to the cell bodies.

Another recent study by Wu et al. [36] provides evidence that the dynein-microtubule network is required for NGF signaling in PC12 cells and rat DRG neurons. Disruption of the microtubules by nocodazole inhibits the sustained phosphorylation of MAPK by NGF without interfering with TrkA activation. However, intracellular trafficking of TrkA was inhibited and sustained activation of MAPK was significantly suppressed. These results suggest that the microtubule network is important in trafficking the retrograde signal initiated by NGF, possibly in transporting the signaling endosome from the distal axons to the cell bodies.

Our results reported here and those described by others [13,14] suggest that signaling vehicles containing phosphorylated TrkA, but not necessarily NGF, may also provide a local axon growth signal and retrograde survival signal to support neuron survival. Hence, elucidating the mechanism of retrograde transport of survival signals to support neuron survival remains one of the impetuses for current research with significant implications for neurodegenerative diseases.

To gain further insight into the mechanisms of axon guidance, we chemically immobilized a step concentration gradient of NGF in the DA compartment and showed that axons can be guided towards the higher end of the concentration gradient. This is consistent with previous findings [6,37–39]. While growth cone guidance by soluble concentration gradients has been studied extensively, guidance by surface-bound molecules has not been as thoroughly investigated even though many guidance molecules are confined within tissues, such as those found in the extracellular matrix (ECM) including laminin [25], VEGF and FGF-1 which are associated with heparin sulfate proteoglycans [40]. The immobilized gradient system developed here mimics the *in vivo* cellular microenvironment closely, allowing relevant mechanisms to be elucidated and translation to disease or regenerative studies possible.

Axon density measured in the DA compartment, where the NGF concentration gradient was immobilized on the chitosan surface, was lowest in the bottom region, where the absolute NGF concentration is lowest, and gradually increased towards the top region, where the absolute NGF concentration is highest. This pattern of axon distribution observed in the DA compartment may be due to increased axon branching in the top region in response to the higher NGF concentration at the high-end of the gradient, in addition to axons turning in the direction of the NGF gradient observed in Fig. 8F (and quantified in Table 2). Increased axon branching from SCG neurons has been observed *in vivo* in the past when endogenous NGF was expressed in the SCG ganglia in transgenic mice [32]. Collateral axon branching from sensory neurons has also been demonstrated using NGF-coated polystyrene beads [31]. Axon shafts rapidly sprout filopodia at sites of contact with the NGF-coated bead. Thus, in addition to axons turning to follow the immobilized NGF gradient observed in the DA compartment, the observed axon distribution is likely due to axon branching at the higher end of the concentration gradient where the amount of NGF immobilized is at its maximum. Increased axon branching provides further evidence that the axons can detect and respond to different surface concentrations of immobilized NGF (as was demonstrated in the axon guidance studies).

Growth cones are exposed to multiple guidance cues, both attractive and repulsive, in their microenvironment to navigate the developing axons to their proper innervating target. The ability to create multiple gradients with different directionality *in vitro* will be useful for understanding the interaction or synergistic effects between different guidance molecules and possibly the signaling pathways involved. Dual patterning of different fluorescent dyes was recently demonstrated by Wosnick et al. in a 3D hydrogel using the laser confocal patterning technique described here [27]. As the photochemistry used is versatile, any amine-containing molecule or protein can be chemically immobilized on the chitosan surface [15]. Furthermore, the length and steepness of the concentration gradient can be tuned by patterning with laser confocal scanning [15]. Thus, synergistic effects of multiple guidance molecules can be studied simultaneously by creating multiple gradients of guidance molecules (both attractive and repulsive) using the methodology described here. The steepness of either gradient can also be varied by simply changing the parameters in the macro computer program that controls the stage movement and laser exposure. Pharmacological studies may also be pursued in conjunction with this methodology to identify possible signaling molecules or cytoskeletal networks that may be important for axon growth and guidance.

Elucidating the signaling molecules and mechanism involved in axon guidance will have significant implications for regenerative medicine. Biomaterials can be patterned with specific cues in concentration gradient patterns to promote and guide the regenerating nerves. We have demonstrated synergistic guidance effects of growth factors patterned in synthetic hydrogels [39]. We have reported techniques here and in the past that various gradient patterns of biologics can be immobilized on substrates in a concise and controlled manner [15,27].

5. Conclusions

We have presented evidence in this study that neuron survival can be supported by retrograde signals unaccompanied by NGF internalization, in accordance with findings by others. Moreover, immobilized step concentration gradients of NGF resulted in axon guidance with an increase in axon density with the amount of NGF immobilized. Combining laser confocal patterning and compartmented cultures, a new research tool has been developed to study *in vitro* models of neuron survival, axon growth and axon guidance.

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