DEVELOPMENT OF A NEW ORGANTYPIC CULTURE METHOD FOR SPINAL CORD AND OTHER NEURAL TISSUE.

BY

CHERYL P. HOLDEN (PARSLEY)

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fullfillment of the Requirments for the Degree of

MASTER OF SCIENCE

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

Culture methods for CNS tissue to date have consisted of dissociated cultures or explant cultures. Although dissociated cell culture allows for monolayer visibility, neural networks as formed *in situ* cannot be preserved, eliminating an understanding of the integrated functioning of various regions in the CNS. Previous explant methods were limited because they used thick explants that made it difficult to obtain monolayer visibility, and often required the use of serum (Crain and Peterson, 1963; Stoppini et al., 1991). Lastly, the roller tube method did not allow for time dependent observation and, since cultures were rolled, raised the question of whether intact neural networks were being maintained (Gähwiler, 1981). This thesis focuses on developing a method that is simpler for culturing CNS explants, and in a relatively inexpensive manner. Briefly, the culture procedure consisted of thinly sliced explant tissues placed on the bottoms of petri dishes. Cultures grown in serum free media were maintained for up to 5 weeks, and were amendable for histology procedures, electrophysiology studies, time-dependent observations, and other functional studies. The biggest advantage of a serum free environment was that it allowed for controlled observations of additives during our trophic factor studies. The use of our CNS culture method, which is described in this thesis, allowed us to demonstrate the following: cells within these cultures maintained viability, retained their basic cyto-architectural organization and produced functional interconnections. The method was used in preliminary trophic factor studies on neurite outgrowth. Data, although preliminary, reflected that seen in the literature on growth factor effects on neurons and slice outgrowth.

Acknowledgements

Foremost, I wish to thank my mother who has always supported me in my academic endeavors by saying that I could do anything I put my mind to, and then making sure I did it. She and my stepfather, Bruce, made it possible for me to do anything I wanted. At times, it was a long hard road, but no matter what they have always been rallying and supporting me.

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List of Abbreviations

Analysis of Variance	ANOVA
Artificial cerebrospinal fluid	ACSF
Basic fibroblast growth factor	bFGF
Brain derived nerve growth factor	BDNF
Central Nervous System	CNS
Ciliary neurotrophic factor	CNTF
Glial fibrillary acidic protein	GFAP
Interleukin-1	Il-1
Interleukin-6	Il-6
Microtubule associated protein	MAP-2
Nerve growth factor	NGF
Neurotrophin-4/5	NT-4
Neurotrophin-3	NT-3
Peripheral nervous system	PNS
Phaeochromocytoma	PC12
Phosphate-buffered saline-.3% Triton	PBS-T
Student-Newman-Keuls	SNK
Tumor necrosis factor	TNF
Tyrosine kinase receptor	Trk

1.0 Introduction

1.1 Goal of the thesis

The present thesis research has two major objectives: the first is to establish a novel method for central nervous system (CNS) slice culture that would allow maintenance of CNS tissue explant cultures, namely, spinal cord. This would be done by first replicating some of the past methods for explant culture and then utilizing some of the strong points of the culture methods to develop a new method. This includes experimenting with several media preparations, culture plates, and preparation procedures. The second goal is to demonstrate the usefulness of this new technique for studies in the field of neuroscience by trying various experimental applications: microscopic visualization, immunohistochemistry processing, electrophysiology, and trophic factor studies.

The introduction is divided into several sections to highlight the pertinent background information for the experiments undertaken. Thus, I first introduce the cell culture procedure: the establishment of explant cultures and the emergence of slice culture as a system to address network properties of CNS behavior. The limitations of previously established techniques will also be discussed. The second section deals with trophic factors, their cellular biological mechanisms, their observed actions on spinal cord and brain tissue, and their use in previous cell culture studies to elucidate factor-induced differentiation, survival and development. In particular, an emphasis on the properties of trophic factors on neurite outgrowth and trophic support of motor neurons will be presented in detail, in relation to the broader perspective of spinal cord injury and prospective repair mechanisms.

1.20 Cell culture

1.21 A history of cell culture

Cell culture has advanced the field of physiology significantly by allowing for long-term culture of cells in a chemically defined environment. Predominantly, dissociated cell cultures were used to study many of the individual properties possessed in various physiological systems, including the CNS. In addition, in dissociated cell culture neurons may lose their cell morphology, for example dendritic tree growth may damaged. This preparation includes separation of individual cells by various steps including trituration and enzymatic digestion to yield a final monolayer of a specific cell population. The cells' individual properties can then be studied in depth using various experimental methods including patch clamp electrophysiology and imaging. The drawbacks in using the dissociated cell preparation is that neural networks as formed *in situ* cannot be preserved, therefore eliminating an understanding of the integrated functioning of various regions in the CNS. Also, some cell types such as motor neurons survive only a few days in culture and have not recovered from the mechanical and chemical shock of dissociation when studies are performed (Askanas et al., 1991) questioning the reliability of extrapolating results to *in vivo* behavior.

1.22 Explant culture

Several investigators have demonstrated that different regions can re-establish functionally appropriate interconnections when cultured together (Crain and Peterson, 1963; Gähwiler and Brown, 1985; Bolz et al., 1990; Streit et al., 1996). Thus, explant co-cultures could be used as a powerful approach for the study of cellular interactions between adjacent or segregated CNS regions *in vitro*. Although explant culture has

become an asset as a method to study intact physiological systems, its potential has still not been fully exploited. Part of this may be due to the technical difficulties involved in explant culture (Crain, 1965) or to limitations in cell visualization.

Explant culture had its first success in the scientific community with Crain and Peterson in 1963 by culturing 1 mm^3 explants using a Maximov type system. Using this method, Crain and Peterson examined the *in situ* properties of functionally interconnected networks of CNS rat cells *in vitro* (Crain and Peterson, 1963; Crain, 1965). Seeing the potential in this type of culture, explant culture was made popular years later by Gähwiler who rejuvenated the roller tube method originally introduced in 1947 by Hogue, and later modified to become a well known technique by Hild in 1957. The roller tube method consists of a decreased explant thickness (500 μm), allowing for better oxygenation and nutrient diffusion. Then, similar to Crain and Peterson's method, a plasma clot is used to secure the explant to a glass slide. The slide is placed inside a roller tube and then rolled at a particular frequency. Eventually, a near-monolayer of cells in culture can be produced. This is advantageous for experiments requiring sophisticated techniques such as patch clamp electrophysiology and calcium (Ca^{++}) imaging (Gähwiler, 1981 and Knöpfel, 1990). Although popular due to the excellent visualization produced (comparable to a dissociated cell preparation), critics of the roller tube method (including Gähwiler himself) have raised the question as to whether the rotation of the cultures in the roller drum may cause disruption of the naturally formed physiological networks by causing unnatural cell migration (Gähwiler, 1981). Disadvantages of the roller tube method also include the following: first, since the slides are placed inside the plastic roller tubes, periodic microscopic evaluation is impossible if sterility is to be maintained

(Gähwiler, 1981). Second, the use of plasma is not always practical in all experimental studies since it may contain many undefined components.

Stoppini and colleagues developed the next advance in explant culture. They created a variation of the explant culture that used a porous membrane insert which acts as an interface between nutrient supply (media) and oxygenation, grown in a culture well (Stoppini et al., 1991). Although these cultures grew successfully, unlike Gähwiler's roller tube method, Stoppini's technique did not produce cultures thin enough (150 μm final thickness) to visualize individual cells. Therefore, only "blind" electrophysiological methods could be used (Stoppini et al., 1991; Xie and Ziskind-Conhaim, 1995).

1.23 Media alternatives

As science advanced and more controlled experimental environments were desired by researchers, the need for a serum free system was recognized, and serum free media alternatives began being marketed. These alternatives to the traditional serum containing media have eliminated many of the variables associated with serum and allowed total control over the culture environment. Using the serum free system, the effects of drugs and various chemical agents on particular cell properties can be studied in a completely defined medium. For example, an important focus in the field of neuroscience is the study of the effects of specific trophic factors on neurons (Jelsma and Aguayo, 1994).

1.30 Trophic factors

1.31 Mechanisms of action

Korsching, (1993) reviews several modes of action for trophic factors including retrograde transport; anterograde transport; local action including granule and purkinje cell release, nerve sheath synthesis, glial cell production, neuronal origin; and autocrine

actions. Retrograde transport involves the synthesis of the trophic factors in the target cells, secretion as a soluble form into the extracellular space, receptor-mediated uptake with retrograde axonal transport to the soma. Anterograde transport, though not as common, involves anterograde signaling mechanisms, an example of which occurs when bFGF is transported (anterogradely) in retinal ganglion cell axons to the superior colliculus (Ferguson et al., 1990). Glial cell actions are mediated locally via indirect mechanisms, and autocrine mechanisms may serve not to act on target-neuron interactions but to sustain neurons that have suffered loss of contact (axotomy) until contact can again be re-established.

1.32 Distribution

In the peripheral nervous system (PNS) an endogenous supply of trophic factors is produced from Schwann cells and other non-neuronal cells. Unlike the PNS, neurotrophins are secreted in the CNS by neurons under physiological conditions and can be regulated by neuronal activity and hormonal changes as discussed in a review by Thoenen 1995). This difference is often used to explain why PNS neuronal loss after axotomy is usually much less than in the CNS.

1.33 The neurotrophins

Neurotrophins are soluble polypetide factors that promote the survival and differentiation of neurons both in vivo and in cell culture environments (Askanas et al., 1991; Korshing, 1993; Lindholm et al., 1993; Oppenheim et al., 1991; Ray and Gage 1994; Sendtner et al., 1990). Neurotrophic factors influence a broad range of events including developmental regulation of neuronal survival, induction of fiber outgrowth, neuroblast cell division (DiCicco-Bloom et al., 1993), stimulation of glial cell precursor

division (Barres et al., 1994), protection from excitotoxic damage (Lindholm et al., 1993), and modulation of peptide expression patterns (Kitzman et al., 1998; Stoop and Poo, 1996). Recently neurotrophic factors have become the focus of research to investigate the neurotrophins' roles in regeneration and apoptosis. Several studies have identified and characterized the receptors for the neurotrophins and other trophic factors (Dechant et al., 1994; Escandón et al., 1994; Heumann 1994; Ip et al., 1993; Jelsma and Aguayo 1994; Knüsel et al., 1994).

1.34 The neurotrophins - High affinity receptors

The high affinity receptors for the neurotrophin family are a group of related transmembrane tyrosine kinase receptors (Trk): Trk A, the nerve growth factor (NGF) receptor; Trk B, the receptor for both brain derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT4) receptor; and Trk C, the neurotrophin-3 (NT-3) receptor. Each neurotrophin normally prefers its particular receptor but, there is sometimes cross reactivity with the other neurotrophin receptors (Escandón et al., 1994; Ip et al., 1993; Knüsel et al., 1994). Also present are a number of truncated forms of the receptor molecules that can bind neurotrophins, but vary in their course of action, being either agonists or antagonists of the neurotrophins when binding the ligand or once associated with the full length receptor (Tsoulfas et al., 1993; Valenzuela et al., 1993).

1.35 The neurotrophins - Low affinity receptor

NGF, NT-3, NT-4 and BDNF can also bind to a low-affinity neurotrophic receptor, p75. The p75 receptor is not related to the Trk family of receptors and its downstream pathway and effector molecules have yet to be fully characterized (Dobrowsky et al., 1994; Ip et al., 1993). There are two aspects of the function of the p75 receptor as

discussed in a review by Heumann (1994); first, it may interact with the Trk receptors by aiding in the formation of high-affinity binding sites, and by making this complex, may actually modulate trk tyrosine kinase activity (Dobrowsky et al., 1994; Ip et al., 1993). Second, p75 appears to activate a second trk independent pathway similar to that activated by tumor necrosis factor (TNF). Recent evidence illustrates p75 may also play an important role in neuronal cell death either by itself, or upon NGF activation (Barrett, 1994).

1.36 The neurotrophins - Their actions

Classifying the actions of neurotrophins is difficult because both their production and the response of cells to activation by binding of the ligand to its receptor vary during development. Therefore, here is a brief overview of some of the effects of the neurotrophins on neuronal populations. Trk A and its neurotrophin ligand NGF have been demonstrated to support superior cervical ganglion neurons. This population undergoes massive reduction in mice possessing Trk A or NGF gene knockouts. Mice genetically engineered with Trk A mutations are characterized by a loss of cholinergic neurons that project from the basal forebrain. NGF has also been studied due to its possible role with p75 in cellular apoptosis. Although there are many conflicting hypotheses regarding the role of NGF and the p75 receptor, one study performed theorizes that early in development binding of NGF causes massive cell death, while later in development NGF demonstrates cell saving properties (Barrett, 1994). Thus, depending on the cells functional state, neurotrophins may lead to cell death or cell survival.

Since BDNF and NT-4 share the Trk B receptor, its not surprising that they both have survival effects on motor neurons. Although BDNF and NT-4 both have survival effects

and bind to the same receptor, there are differences in the time frame that they become effective. BDNF tends to be upregulated increasingly after injury, while NT-4 is not upregulated until several days after the injury was sustained (Funakoshi et al., 1993; Meyer et al., 1992). BDNF has been shown to maintain survival of spinal motor neurons (Hughes et al., 1993), basal forebrain cholinergic cortical neurons, cultured dopaminergic neurons, retinal neurons, and enhance differentiation of CNS stem cell-derived neuronal precursors but not survival (Ahmed et al., 1995). On the other hand, NT-4 promotes the survival of spinal motor neurons, facial motor neurons, and cholinergic neurons (Hughes et al., 1993).

NT-3 has been demonstrated to promote the survival and differentiation of cells from different neuronal populations in both the PNS and CNS, including neural crest cells (Tessarollo et al., 1994). In addition to being imperative for the development and survival of muscle sensory neurons (proprioceptive), NT-3 has also been demonstrated to prevent death of adult central noradrenergic neurons and to enhance the sprouting of corticospinal tracts during development and after spinal cord lesion (Tessarollo et al., 1994). Although NT-3 has its highest affinity to the trkC receptor, it has also been reported to have a tendency to bind to both trkB and trkA receptors as well (Tessarollo et al., 1994; Escandón et al., 1994). The ramifications of the effects of these neurotrophins acting upon their respective receptors and their respective interactions have still not been fully elucidated.

1.37 Ciliary neurotrophic factor

Another common trophic factor that has undergone extensive investigation is ciliary nerve growth factor (CNTF). CNTF belongs to the cytokine family and acts on a wide

range of cell types including neuronal and glial cells. Although CNTF was named for its ability to rescue cultured ciliary neurons, its ability to rescue other neuronal cell types has also been demonstrated, as for example with spinal motoneurons (Oppenheim et al., 1991; Sendtner et al., 1990).

1.38 Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF) is a mitogenic factor that can act on a variety of cell types of both mesodermal and ectodermal origin (Ghosh and Greenberg, 1995, Korsching, 1993). It belongs to a family of growth factors that enhance survival and differentiation of many different cell types including motoneurons (Ghosh and Greenberg, 1995, Korsching, 1993), and neuroblasts (Ray and Gage, 1994).

2.00 Materials and Methods

2.10 Reproductions of Previous Methods

First, the culture methods of Stoppini et al. 1991, and Khan et al., 1990 were reproduced in order to develop the following method. Changes were then made by testing various culture dish types and media types to settle on the described method. Some of the variations included:

DMEM/F12 Media (GIBCO) + 20% Bovine serum

DMEM/F12 Media (GIBCO) + 12% Bovine serum

Antimitotics were also used in some serum cultures in concentrations discussed in the proceeding method.

Commercial collagen preparations.

2.20 Culturing procedure (spinal cord and brainstem)

Collagen solution was freshly prepared monthly from adult rat tails according to the procedure of Elsdale and Bard (1972), with slight modifications. Although rat tail collagen was available commercially, we preferred the quality of that prepared in our laboratory. For coating the plastic dishes and glass coverslips, rat tail collagen was used at a concentration of 50 μg/ml. Twenty-four hours prior to a culture experiment and under sterile conditions, 35 mm culture dishes (Corning) were coated with 0.5 ml of rat tail collagen and allowed to dry in a culture hood for 4-6 hours. The dishes were rinsed twice with 1 ml of sterile de-ionized water, and then soaked in 1 ml of Neurobasal medium (GIBCO) overnight in an incubator at 37 °C in an atmosphere of 5% CO_2, 95% air (Narco). On the day of the experiment the Neurobasal media was replaced with 0.5 ml of Neurobasal media and B27 supplement (GIBCO) (Brewer et al., 1993). Trophic factors were added either in combination or alone at the following concentrations(ng/ml): NT-3 (10), NT-4 (10), BDNF (2), NGF (2), CNTF (15), and bFGF (10). Culture dishes were then returned to the incubator until the tissue slices were ready to be placed in them. For electrophysiological studies, collagen-coated glass coverslips were prepared 48 hours prior to the experiment. The desired number of round 25 mm glass coverslips were cleaned according to the method of Fitzgerald (1989). Coverslips were sterilized by autoclaving, coated with 0.3 ml of rat tail collagen, placed on the bottom of 35 mm culture dishes and treated in the same as collagen-coated dishes (Figure $1A_2$).

Timed-pregnant rats were anesthetized, and the ensuing procedures were performed under sterile conditions. E15 - E18 fetuses were removed, the brainstem and spinal cord from each fetus was dissected out (see Xie and Ziskind-Conhaim, 1995), embedded in

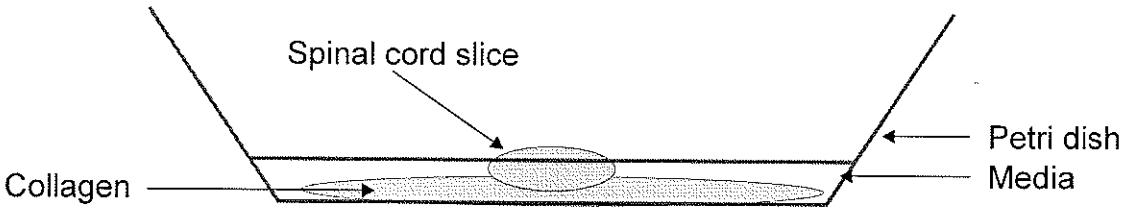
2.5% agarose and cut into transverse sections of 30, 50, 80 or 150 μm thickness using a Leica VT1000 vibrating blade microtome. Slices were transferred into sterile dishes containing dissecting media (in mM: NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 4; N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 4.2; and D-glucose, 11; at pH 7.3), where they were cleaned of agarose and meninges and placed into collagen-coated culture dishes. Co-culture slices were placed approximately 1 mm apart in a dish, while single cultures were placed in the center of an individual dish (Figure $1A_1$). Five or six individual culture dishes were then placed into sterile 150 mm culture dishes (VWR) in the incubator. Cultures were fed every 4-5 days by removing 0.2 ml, then adding 0.2 ml of fresh Neurobasal and B27 media and required trophic factors. For several electrophysiology experiments, antimitotics (fluorodeoxyuridine 10 μM and uridine 10 μM) were added at day 3 for 48 hours to reduce proliferation of non-neuronal cells (Banker and Goslin, 1991).

Photographs of the cultures were taken as often as every 3-4 days on a Nikon Diaphot 300 inverted microscope equipped with Hoffman modulation optics. As a comparison to cultures using thicker explants, we also cultured slices at a thickness of 300 μm. Further, postnatal hippocampal (P3-6) and spinal cord cultures (P8-16) were undertaken using an oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 125; KCl, 2.5; $NaHCO_3$, 26; NaH_2PO_4, 1.25; D-glucose, 25; $MgCl_2$, 1; and $CaCl_2$, 2.

Figure 1

Our simple method involves placing an explant on the bottom of a collagen coated petri dish (A_1). For electrophysiological studies, a collagen coated sterile glass coverslip is placed on the bottom of the petri dish and then the tissue slice is centered on the coverslip (A_2). The media is changed every 4 – 5 days.

A $_1$



A $_2$

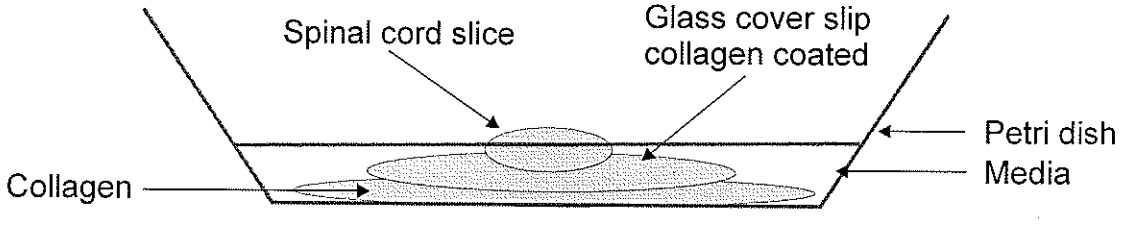


Figure 1

2.21 Hippocampal Slice procedure

L. Song in Parsley et al., 1998 utilized this method to culture postnatal rat hippocampus slices. The differences here were that postnatal (P4 and P8) animals were used, and the tissue was dissected in oxygenated warm ACSF. The hippocampus of the rats were isolated, embedded in agar and sliced at thicknesses of 80 μm, 100 μm and 150 μm.

2.30 Histochemistry (spinal cord and brainstem)

2.31 Live / Dead cell assay (spinal cord)

Using room temperature solutions, culture media are removed and cultures were washed 3 times for 5 minutes each in oxygenated ACSF. Then, 1.6 μl/ml of a 1% stock solution of propidium iodide and 2 μl/ml of a 10% stock solution of fluorescein diacetate was added (Jones and Senft 1985). After a 10 minute incubation at room temperature, cultures were then washed 3 times for 5 minutes each in oxygenated ACSF. The cultures were immediately photographed using standard fluorescein and rhodamine filter sets on either a Nikon Labophot-2 light/epifluorescence microscope, or Nikon Diaphot 300 inverted light/epifluorescence microscope.

2.32 Immunohistochemistry (spinal cord and brainstem)

The following antibodies were chosen due to their unique specificity. The Panaxonal primary antibody is a cocktail of antibodies that stain a variety of axonal proteins. MAP-2 stains for microtubule associated protein 2 found in dendrites and neuronal cell bodies. When both Panaxonal and MAP-2 primary antibodies were used in combination, it allows for all neuronal, axonal and dendritic structures to be visualized. GFAP was used to stain for glial fibrillary associated protein found in astrocytes.

Cultures were fixed in phosphate-buffered 4% paraformaldehyde at 4° C or at room temperature for 3 days. The cultures were then washed 6 times for 20 minutes each in phosphate-buffered saline - .3% Triton (PBS-T). The following primary antibody series were then added: mouse microtubule associated protein (MAP-2) at 1:1000µl (Sternberger), mouse Panaxonal at 1:1000µl (Sternberger), and rabbit glial fibrillary acidic protein (GFAP) at 1:1000µl (Chemicon). Cultures were placed in a sealed container at 4° C for another 3 days, then washed 3 times for 20 minutes each in PBS-T. The secondary antibody series of anti-mouse Cy3 at 1:100µl (Sigma) and anti-rabbit FITC at 1:100µl (Sigma) were incubated at room temperature for 1.5 hours, then washed 2 times for 20 minutes each in PBS-T, and 20 minutes in 50 mM Tris HCl (pH 7.4). Cultures were air-dried overnight inverted over a piece of tissue paper, and photographed under epifluorescence illumination as described above or coverslipped with a glycerol-based anti-fade media (Valnes and Brantzaeg, 1985) and stored in the freezer for future morphological studies.

2.40 Electrophysiology

A glass coverslip containing the explant culture was removed from an individual culture dish. The coverslip was then adhered to the bottom of a round Plexiglas ring (25 mm outer diameter) with high vacuum grease (Dow Corning), forming the experimental chamber. A perfusion system was attached to the chamber, and ACSF was perfused at approximately 2 ml/minute. Whole-cell patch clamp recordings were obtained (Hamill et al., 1981) with an Axopatch 1D amplifier (Axon Instruments) and data were acquired using pCLAMP software (v 6.0, Axon Instruments). Microelectrodes were pulled on a two-stage upright puller (Narishige PP-83) with resulting resistance values between 3 and

6 MΩ in a recording solution containing (in mM): K-gluconate or CsF, 140; ethylene glycol-bis(β-amino ethyl ether) N,N,N',N',-tetraacetic acid (EGTA), 11; KOH, 35; N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 10; $CaCl_2$, 1. pH was adjusted to 7.3.

2.50 Trophic factor studies (spinal cord)

2.51 Quantification method

Cultures were grown for 14 days in the various trophic factors. The cultures were then fixed with 4% Paraformaldehyde for three days, processed immunohistochemically using MAP-2 and Panaxonal antibodies (see Immunohistochemistry section for details). The coverslipped cultures that were treated with the anti-fade media and stored in the freezer were removed and allowed to warm to room temperature. Images of the slice cultures were digitized using a Neuroleucida 2.1 image analysis system (MicroBrightField) connected to a Nikon Optiphot light/epiflourescence microscope equipped with a CCD camera, and attached to an IBM compatible computer. Using a fluorescein filter and 40x magnification, MAP-2 and Panaxonal labeled axons were digitized and then traced manually. The computer program Morph (MicroBrightField) then calculated the area of the traced axons. Statistical comparisons using the ANOVA with an SNK test Control n=2 cultures; BDNF n=3 cultures; CNTF n=3 cultures; NGFa n=2 Cultures; NGFb n=2 cultures; bFGF n=3 cultures; NT4 n=3 cultures; NT3 n=3 cultures were then completed on mean neurite outgrowth, and total area outgrowth .

2.52 Reconstructions

Cultures followed the above preparation procedure outlined for quantifying the cultures. Once processed immunohistochemically and coverslipped with the Antifade

media, the cultures were photographed using a fluorescien filter and a flouro10 objective on a Nikon Diaphot 300 inverted microscope, equipped with a CDC camera and Axon Imaging Workbench data acquisition program installed on a 486 IBM compatible computer. Several sections of the slices were taken to compose a final montage of the slice. The montage was reconstructed using Corel PhotoPaint. Final images were imported into CorelDraw and printed.

2.53 Apoptosis assay for DNA laddering

(Protocol provided by Dr. L. Kirshenbaum - University of Manitoba)

NT-3 cultures were removed from the incubators at day 14, the media was withdrawn and slices were collected off the agar. The slices were either homogenized with a Polytron in a solutions of 250 µl homogenization buffer + 8 µl of 10% SDS solution, or just placed in the buffer/SDS solution. The tissue was then vortexed and incubated at 65°C for 30 minutes. Then, 35 µl of K-acetate (8M) was added and the tissue was frozen at –20°C overnight. Samples were then transported on ice. The tissue underwent a phenol chloroform extraction, and the DNA was precipitated in 2.5 volume of 100% ethanol. Samples were then loaded on a 1% agarose gel and run at 100 volts for one hour. (Homogenization Buffer 0.1M NaCl, 0.01M EDTA - pH 8.0, 0.3M Tris pH 8.0, 0.2M sucrose).

3.00 Results

3.10 Reproductions of Previous Methods

Spinal cord slices cultured by the method of Stoppini et al., 1991 grew well. The method produced cultures that were suitable only for 'blind' electrophysiological approaches due

to the final explant thickness. The membranes made immunohistochemical processing burdensome. Media contained serum so additives could not accurately be controlled. Spinal cord slices cultured using Khan et al., 1990 also grew well. The bundles of filaments made visualization difficult for electrophysiological procedures, and microscopic visualization also difficult.

3.20 Explant viability and topography (spinal cord)

CNS slices were cultured for periods of up to 5 weeks with a contamination rate under 1%. Sample results of spinal cell viability assessed with live and dead cell indicators for the different section thicknesses of 300, 150 and 80 μm at culture day 14 are presented in Figure 2. Using standard fluorescein and rhodamine filter sets, live cells fluoresce green while the nuclei of dead cells fluoresce red. A slice culture prepared at 300 μm thickness is presented in Figure 2A. There were a considerable number of living cells (Fig. $2A_1$), but at this thickness it was difficult to visualize individually labeled cells within a focal plane in the slice region. Cell viability assays confirmed widespread cell death due to a decrease in the fluorescein (green) signal which still overpowered the individual propidium (red) labeled cells (Fig. $2A_2$). Also, slices usually appeared mottled upon examination using interference optics. Nonetheless, electrophysiological recordings could be obtained from neurons using a 'blind' approach. Overall, 50 ± 7% of cells were viable at this section thickness after 14 days in culture (n=4).

A representative example of results on assessing cell viability from the 150 μm slices is shown in Figure 2B. In comparison to slices prepared at 300 μm thickness, the live cells present in 150 μm slices are well labeled and easy to visualize within the slice. The live cell assay is presented since only a minimally detectable fluorescent signal for dead cells

was obtained. Overall, 95 ± 3% of cells were viable at 150 μm section thickness after 14 days in culture (n=4).

Preparation of slice cultures at 80 μm thickness is presented in Figure 2C. Because of the reduced slice thickness following culture for 14 days, cell visualization was superior and the tissue integrity appeared to be generally well preserved. However, an increased number of dead cells were observed within the slice (Fig. $2C_2$), possibly due to the mechanical trauma of microtome slicing at this thickness. Overall cell viability after 14 days in culture was 78 ± 7% (n=4). A Nissl stain of an 80 μm thick slice is shown in Figure 2D demonstrating the topography of a cultured spinal cord slice. Note that general spinal cord topography appears to be retained and there was a dense labeling of cell bodies that were rather uniformly distributed throughout the slice.

Figure 2

Live/dead cell assays after 14 days in culture demonstrates an abundance of living cells within spinal cord slices. The live cell indicator, fluorescein diacetate, shows living cells in slice cultures at 300 μm (A_1), 150 μm (B), and 80 μm (C_1) thicknesses. The dead cell indicator, propidium iodide, identifies dead cell nuclei at 300 μm (A_2), and 80 μm (C_2) thicknesses. A Nissl stain of an 80 μm slice culture (D).

3.30 Time-dependent observation (spinal cord and brainstem)

The use of our culture method to study time dependent changes is represented in Figure 3. Photographs of cultures were taken to show the extension of processes at 2, 8 and 14 days in culture for spinal cord (Fig. $3A_1$-A_3) and brainstem (Fig. $3B_1$-B_3). At day 2 for both spinal cord (Fig. $3A_1$) and brainstem (Fig. $3B_1$) there is little process extension. By day 8, some processes can be observed to extend for over 1 mm (Fig. $3A_2$, $3B_2$). By day 14, continued process extension is observed to have distances >1.3 mm (Fig. $3A_3$) or 2.1 mm (Fig. $3B_3$), as well as having become much more extensively arborized. Panels $3C_1$ to C_3 demonstrate the development of a presumed axon bundle interconnecting a spinal cord-brainstem co-culture by culture day 14. Although general size and morphology may change slightly over time (Figure $3A_1$ vs. A_3, 3_1 vs. 3_3, or 3_1 vs. 3_2), Nissl stains demonstrate the preservation of the overall morphology (e.g. Figure 2D).

Figure 3

Slice culture allows assessment of time-dependent changes. All slices were sectioned at 150 μm thickness. $A_{1\text{-}3}$ Spinal cord. $B_{1\text{-}3}$ Brainstem. $C_{1\text{-}3}$ Brainstem – spinal cord co-culture. In all, note the time-dependent extension of processes, which are seen forming a bridge in co-culture ($C_{2 \text{ and } 3}$).

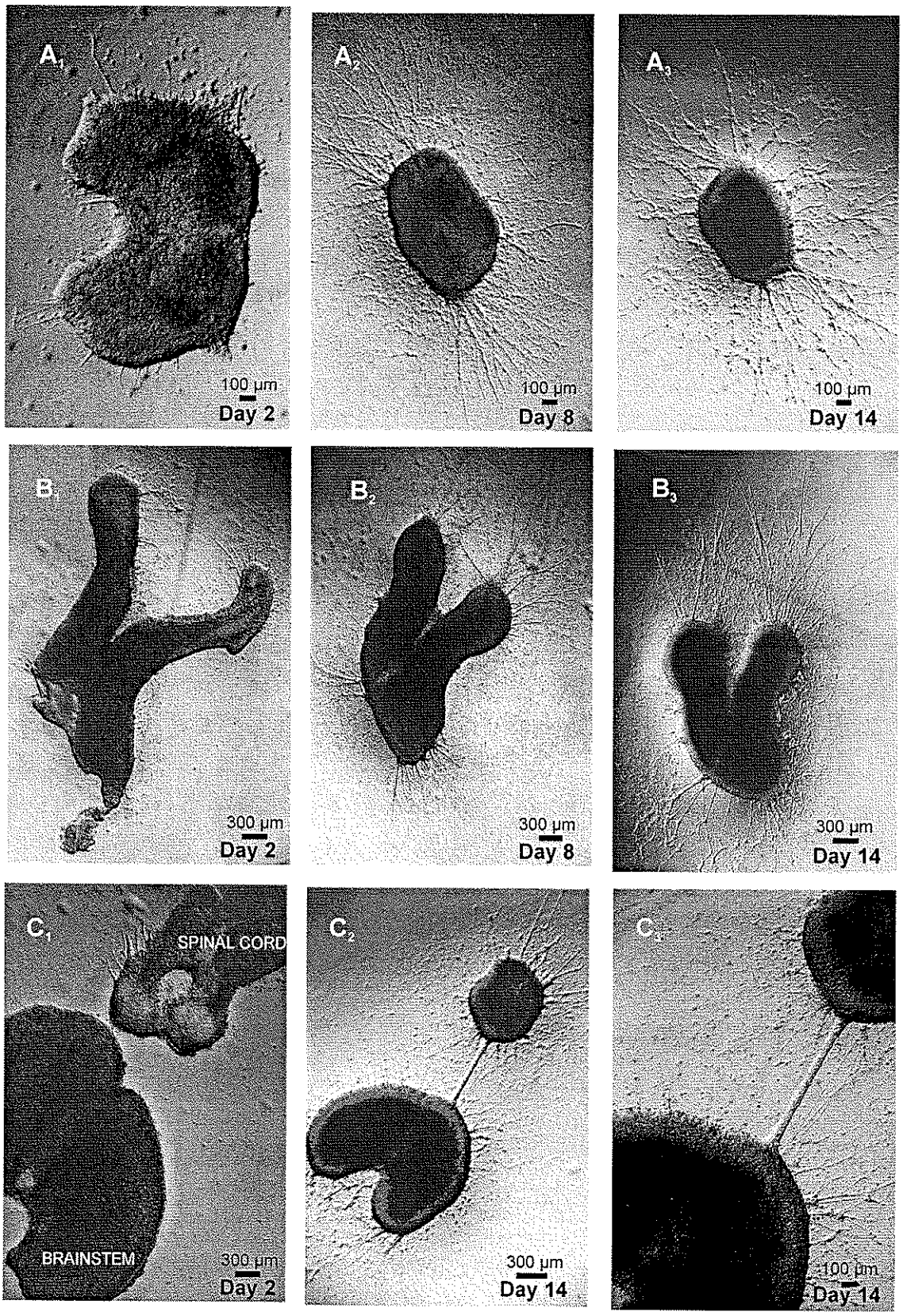

A1
100 µm
Day 2
A2
100 µm
Day 8
A3
100 µm
Day 14
B1
300 µm
Day 2
B2
300 µm
Day 8
B3
300 µm
Day 14
C1
SPINAL CORD
BRAINSTEM
300 µm
Day 2
C2
300 µm
Day 14
C3
100 µm
Day 14

Figure 3

3.40 Immunohistochemistry (spinal cord and brainstem)

Immunohistochemical staining of spinal cord slices with Panaxonal and MAP-2 antibodies demonstrates extensive outgrowth and arborizations within the slice (Fig. $4A_1$). Many neurites can be observed radiating from the slice explant proper (Fig. $4A_2$). A few types of axonal/dendritic patterning are illustrated throughout the cultures, which is either punctate (dotted) or continuous in appearance (Fig. $4B_1$). These neurites occurred in different ratios depending on the trophic factors. Neurites were observed that extended far out into the culture dish; while others extended out for some distance, and then curved back into the slice. Figure $4C_1$ shows an explant co-culture of spinal cord and brainstem containing axonal interconnections traveling as bundles (Fig. $4C_2$) or individually (Fig. $4C_3$). Neuronal cell bodies can be easily visualized within the slice proper, as exemplified in a brainstem slice (Fig. $4C_4$).

MAP-2 positive neuronal cell bodies can be observed migrating out from the slice explants (Fig. $4B_1$). Many of these neuronal bodies are presumed motorneurons due to their size and morphology including large soma diameter, as well as having multipolar properties. Again, the number of neurons and distance they migrated out of the slice also varied between the various trophic factors.

GFAP staining illustrated a large number of positive stained cells both within and outside the slices (Fig. $4B_2$).

Figure 4

Immunocytological procedures to assay slice organization and composition. All slices were sectioned at 150 μm thickness and processed at culture day 14. A_1 Immunostaining for Panaxonal and MAP-2 to demonstrate extensive arborizations within the spinal cord slice. A_2 Enlargement of boxed area in A1 to show projection extending out from the slice. B_1 Axons and neurons labeled with Panaxonal and MAP-2. Note the labeled cell bodies, which have migrated out from the slice. B_2 A spinal cord slice stained with GFAP to illustrate presence of glia (same region as B_1). C_1 Brainstem – spinal cord co-culture. $C_{2\text{-}4}$ Expanded regions as indicated with boxes in C_1 (but not in the same focal plane). $C_{2\text{-}3}$ Distinct patterns of axonal projections interconnecting slices are visualized using Panaxonal and MAP-2 immunostaining. C_2 Axons travel in bundles between the slices. C_3 Single axons projecting between the slices. C_4 Nuclei within the brainstem slice.

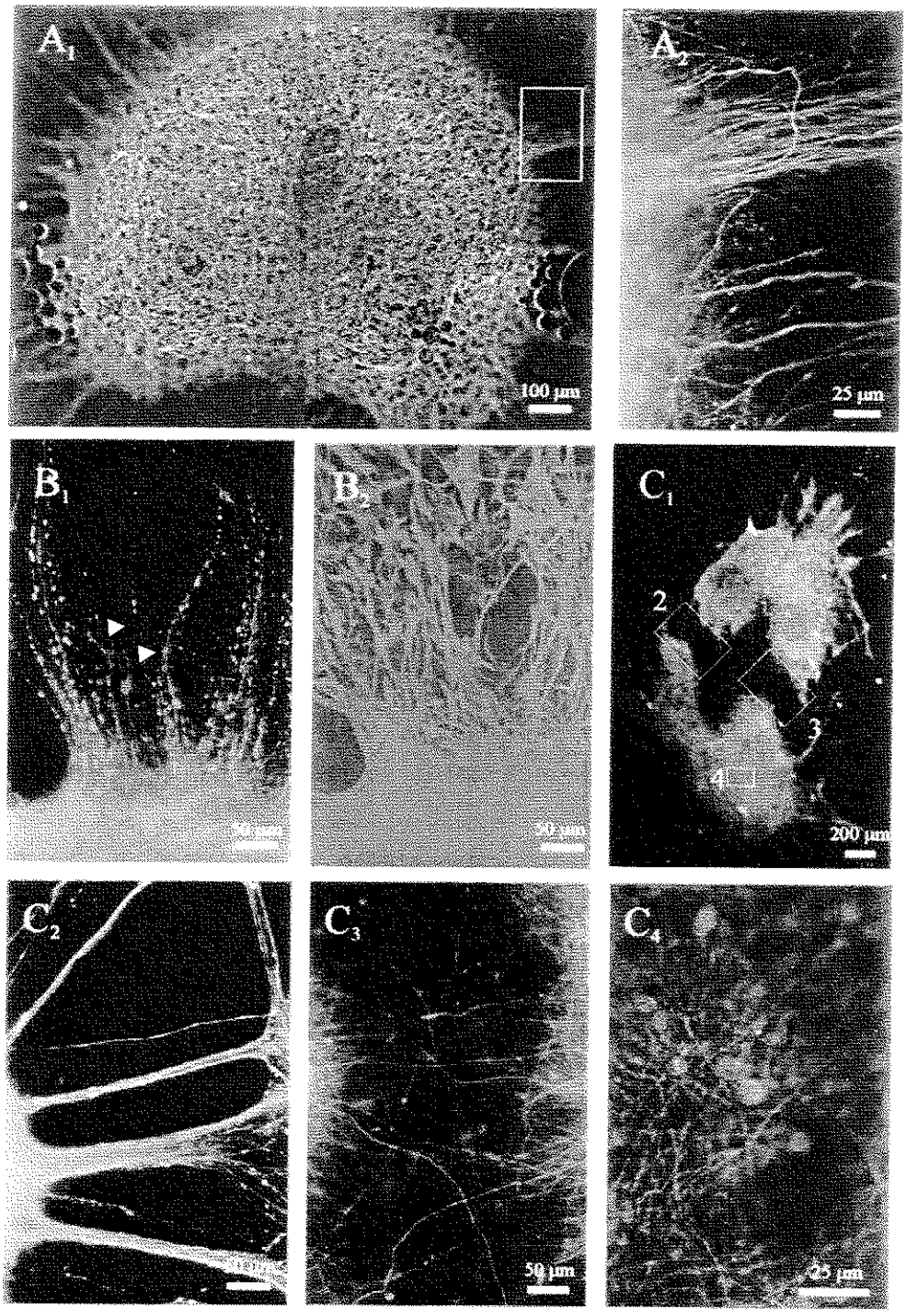


Figure 4

3.50 Hippocampal slice cultures

Figure 5($B_{1,2}$) illustrates that overall topography of the hippocampus was well maintained, as were interconnections and cellular visualization was easily performed. Panaxonal labeled axons were visualized for both CA1 pyramidal and dentate granule cell regions (Fig. 6). Also in Figure 6, GFAP staining of the CA1 region illustrated glia within the slice and neurons could be seen in the CA3 region. Cultures could be maintained for a period of at least four weeks. All results published in Parsley et al., 1998.

3.60 Electrophysiology

We have obtained several blind (from 300 μm slices) and visual (from 150 and 80 μm slices although 50 and 30 μm slices have much better visualization) patch clamp recordings from cultured neurons and glia in spinal cord and brainstem slice cultures. An example of the cellular resolution provided for visual patch recordings using a 80 μm spinal cord and hippocampal slice cultures is presented in Figure 5 A and B. Notice the ease of cellular visualization at both low (A) and high (B) magnification using Hoffman modulation optics. Sample recordings are presented in Figure 7 from two neurons in a spinal cord slice under both voltage and current clamp configurations (Fig. 7A and 7B). After 14 days in culture both single and repetitive firing can be observed in different neurons in response to depolarizing current steps and spontaneous synaptic events are observed (Fig. 7C).

Figure 5

Hoffman modulation optics images of spinal cord (A) and hippocampal slice cultures (B) to show the ease of use of cellular visualization for electrophysiological targeting. A. An 80 μm thick spinal cord slice after 14 days in culture at two magnifications. B. An 80 μm thick hippocampal slice culture (P8) after 27 days displays a well preserved overall topography (B_2) including neurons in the CA3 region (B_2). Figure originally published in the Journal of Neuroscience Methods (1998) by Parsley et al., with the hippocampal slice experiments performed by L. Song.

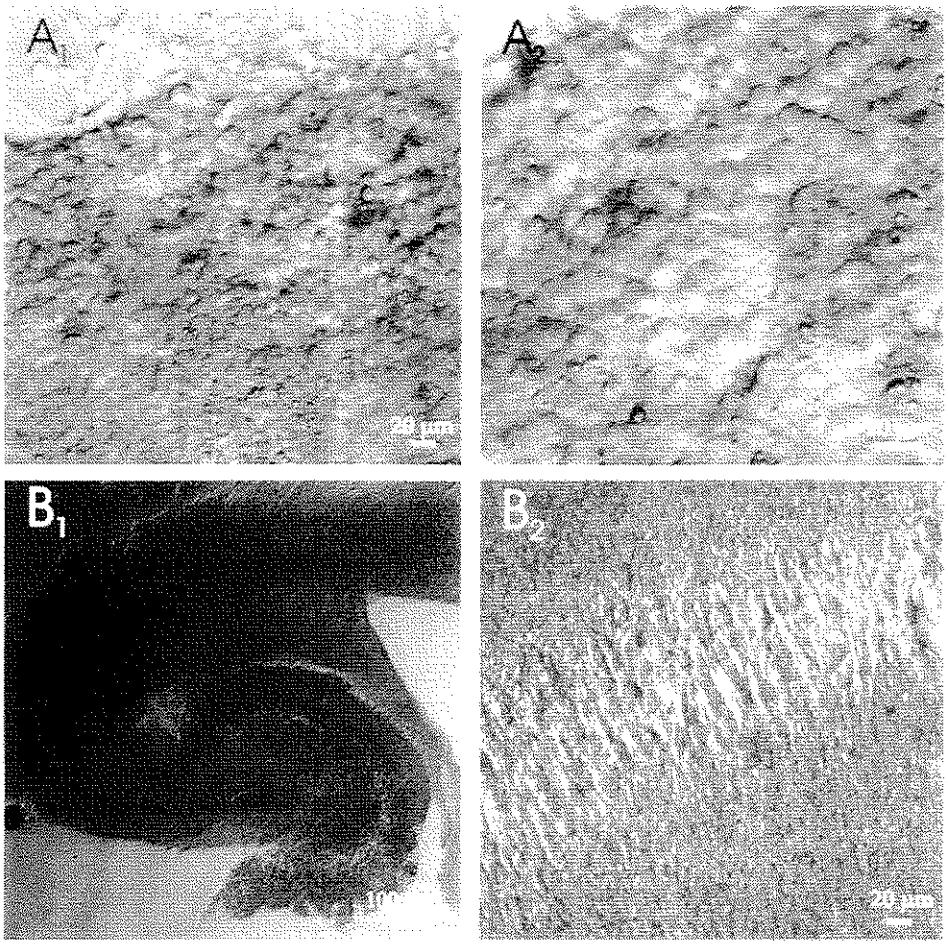


Figure 5

Figure 6

Hippocampal slice culture. A_1 Hippocampal slice (100 μm) after 6 days in culture (P4 animal. A_2 Immunostaining of CA1 neurons with Panaxonal and MAP-2. A_3 Immunostaining of dentate granule cells with Panaxonal and MAP-2. A_4 Immunostaining of CA1 region for glia using GFAP. B_1 Panaxonal and MAP-2 staining to demonstrate overall neuronal organization of a 150 um hippocampal slice culture maintained for 7 days (P3 animal). B_2 Neurons from subicular region have migrated out of the slice proper. Figure originally published in the Journal of Neuroscience Methods (1998) by Parsley et al., with the experiments performed by L. Song.

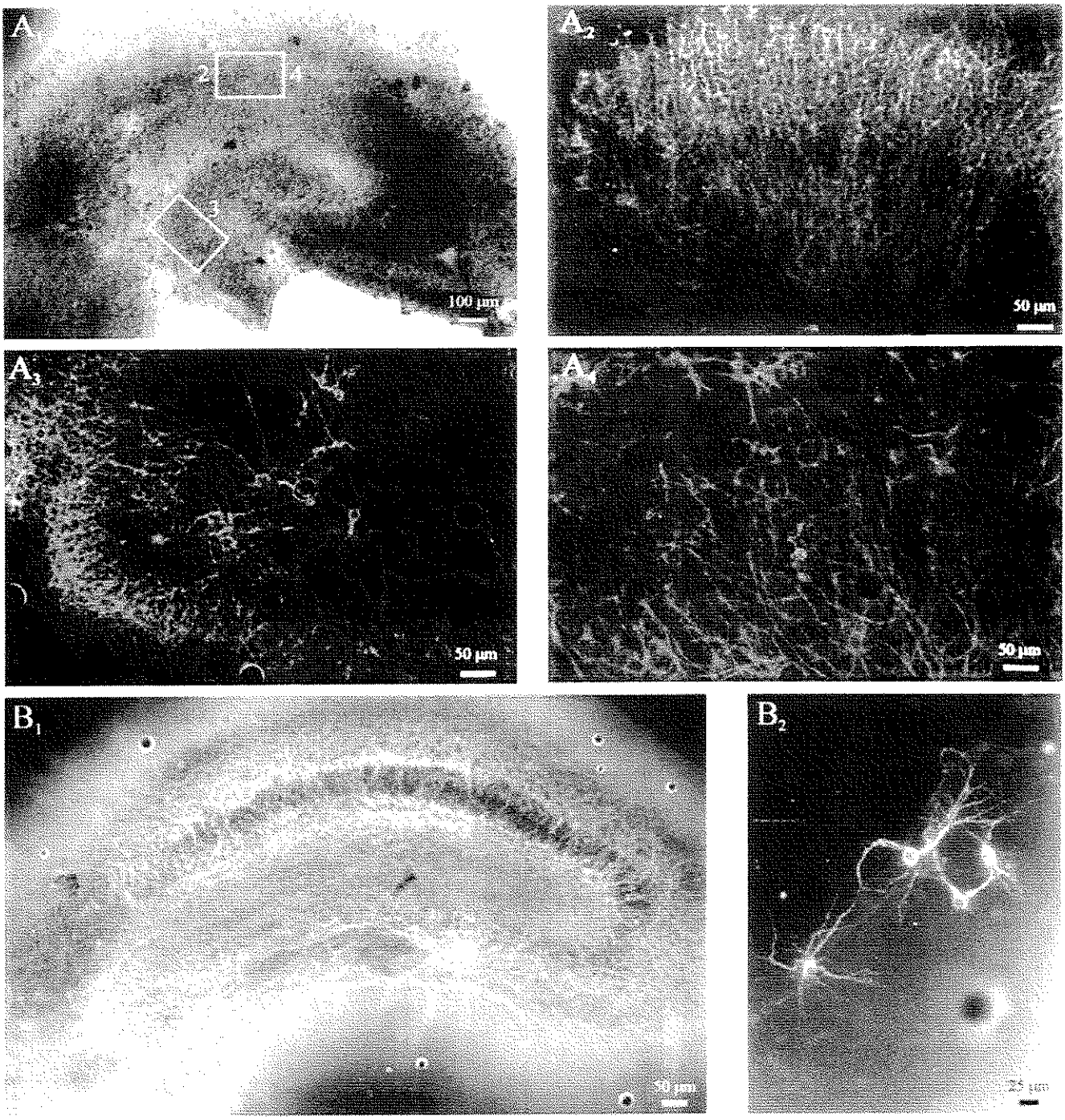


Figure 6

Figure 7

Response properties of two neurons (A and B) in the same spinal cord slice after 14 days in culture. A_1 and B_1 Voltage clamp recordings showing currents evoked from a voltage step protocol. A_2 and B_2 Current clamp recordings showing action potentials generated from current steps. C Spontaneous synaptic activity is observed in slice culture. Spontaneous inhibitory synaptic currents reverse at ~ –55mV.

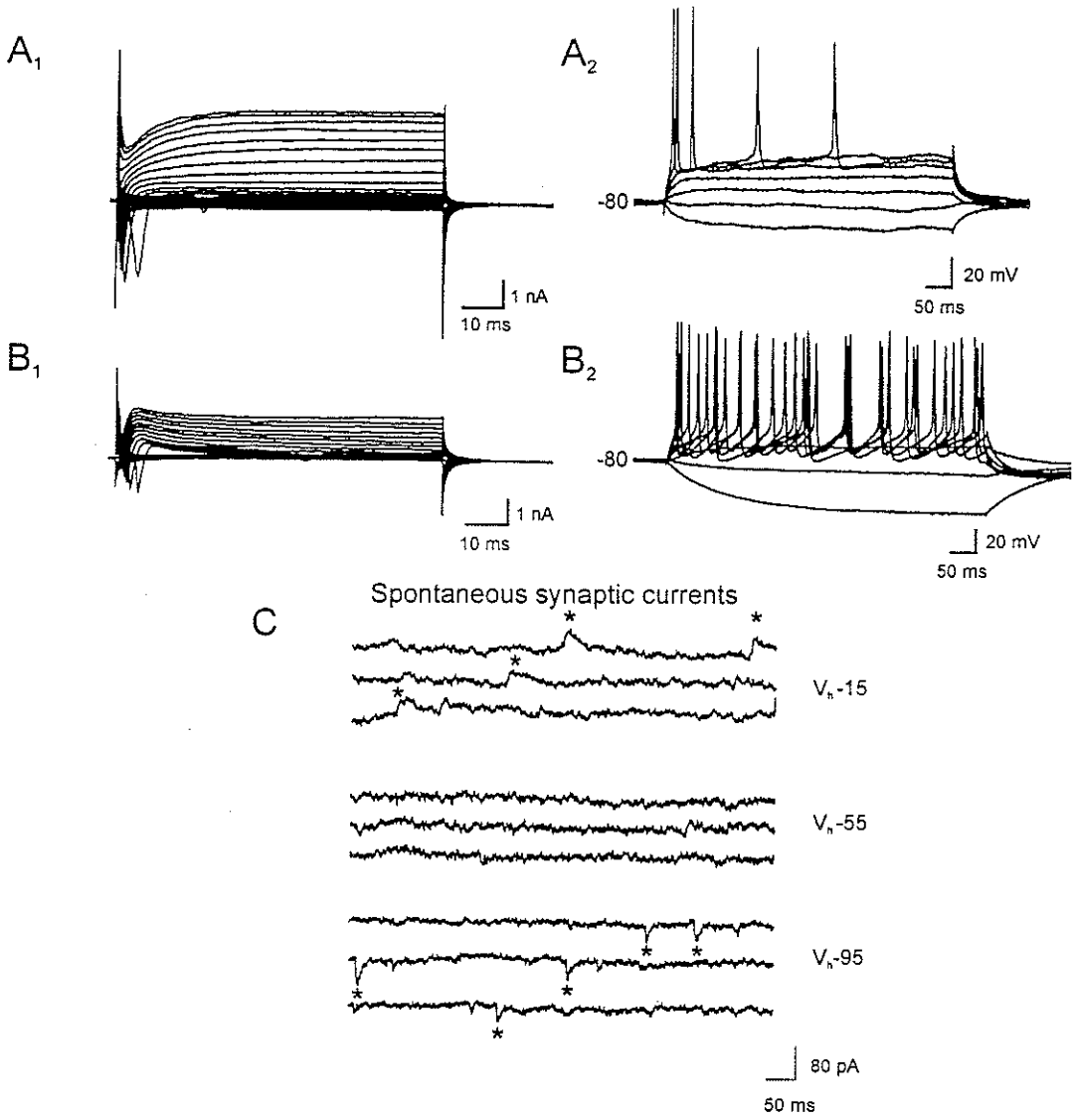

A1
A2
-80
1 nA
10 ms
20 mV
50 ms
B1
B2
-80
1 nA
10 ms
20 mV
50 ms
C
Spontaneous synaptic currents
Vh -15
Vh -55
Vh -95
80 pA
50 ms

Figure 7

3.70 Trophic factor studies (spinal cord)

3.71 Quantification studies (spinal cord)

Figures 8 and 9 demonstrate the results of two quantification studies comparing cultures with either NGF, BDNF, NT-4, CNTF or NT-3 to control cultures. In the first study (Fig.8), all cultures except the NT-3 cultures demonstrated growth. Instead, the addition of NT-3 to the cultures caused a significant decrease in neurite outgrowth from the control cultures ($p< 0.01$). There was no enhanced growth from any of the trophic factors vs. control cultures that was considered significant. In the second study (Fig. 9), a new lot of NT-3 was used. All cultures demonstrated growth however, NGF, BDNF and NT-4 were the only trophic factors to have a significant enhanced neurite outgrowth effect ($p< 0.01$).

3.711 Single cultures (spinal cord)

Initially, all cultures except NT-3 contained neurite outgrowth. In fact, in most NT-3 cultures there was no neurite outgrowth (Fig.10 A and B), instead there was massive necrotic cell death confirmed by the absence of DNA laddering which would have indicated apoptosis. When some neurite outgrowth did occur, it was not extensive (Fig. 10C). When spinal cord co-cultures were grown, the second slice had an interesting rescuing effect from the necrotic death (Fig. 23).

Single cultures performed after these experiments with a different lot of NT-3 were not able to reproduce similar necrotic death.

In control cultures, neurite outgrowth varied from considerable (Fig. 11A) to minimal (Fig. 11C). There are very few MAP-2 positive neurons that migrate out from the slice proper in the control cultures.

Figure 8

Neurite outgrowth is significantly decreased by NT-3 ($p<0.01$), and is not significantly enhanced by other trophic factors ($n=3$).

Figure 9

Quantification of neurite outgrowth shows that after 14 days in culture, NGF, BDNF and NT-4 were the only trophic factors to have a significant enhancing effect ($p < 0.01$)(n=3).

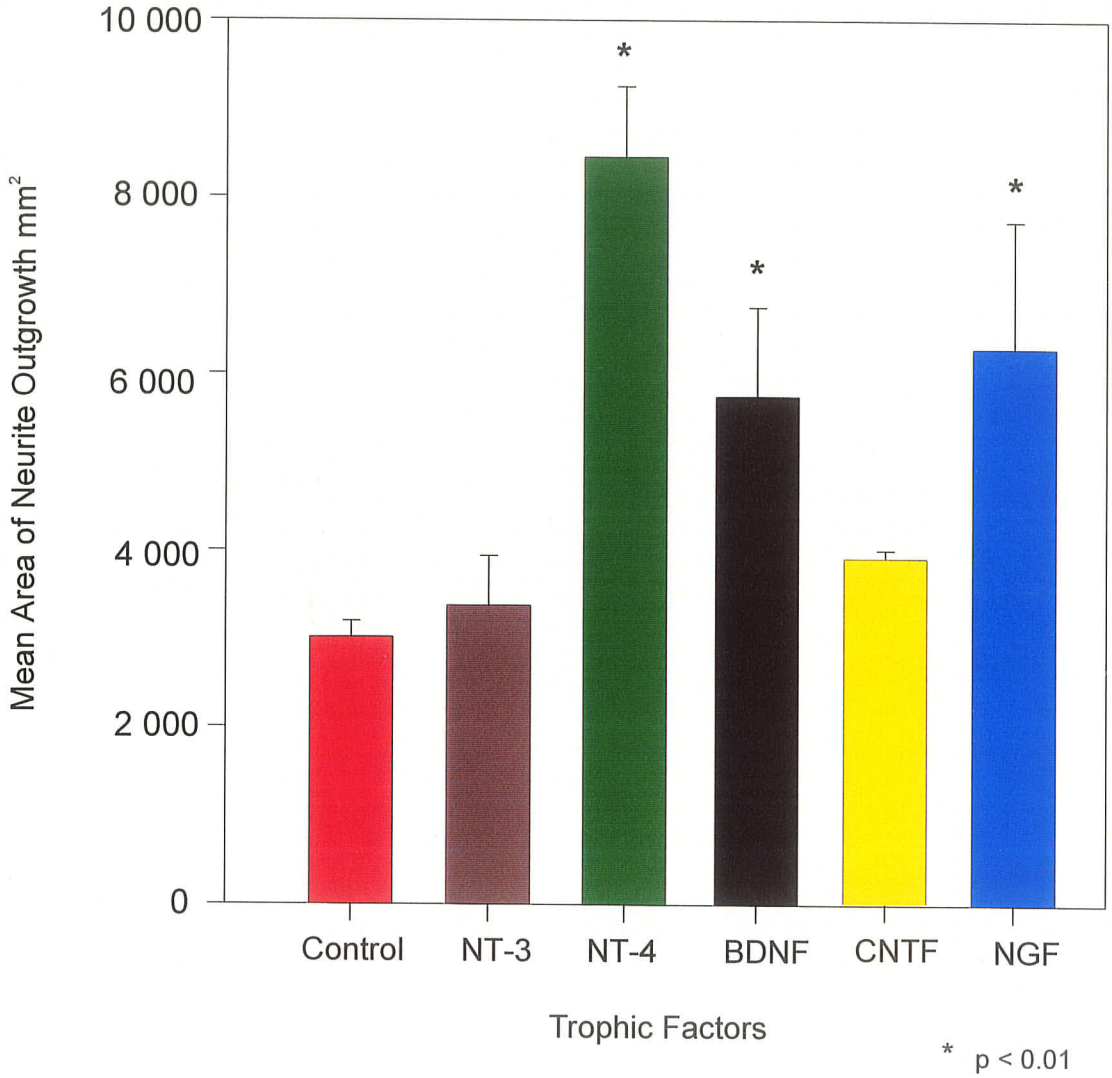

10 000
8 000
6 000
4 000
2 000
0
Mean Area of Neurite Outgrowth mm²
*
*
*
Control
NT-3
NT-4
BDNF
CNTF
NGF
Trophic Factors
* p < 0.01

Figure 9

Figure 10

A spinal cord culture processed at day 14 according to the described methods. Notice the extensive necrotic cell death in slices A and B when stained with propidium iodide. Even when neurite outgrowth did occur (C), it was not widespread.

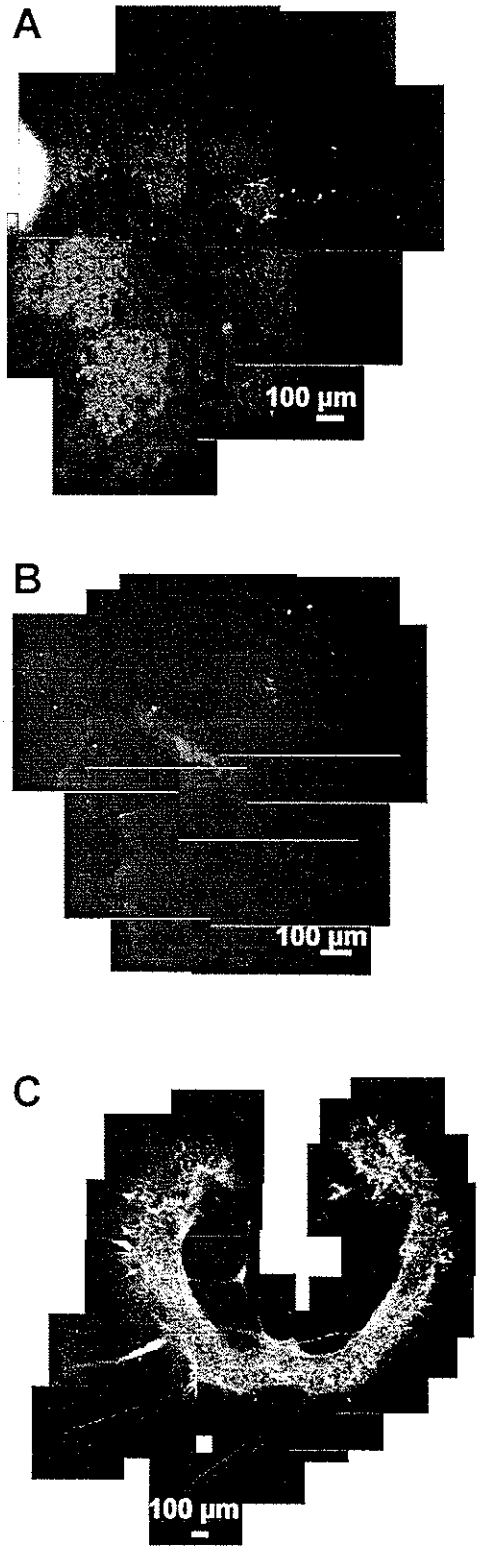


Figure 10

Figure 11

Single spinal cord cultures (controls) processed at culture day 14 according to the immunohistochemistry method described (A-C). Notice the variable patterns of neurite outgrowth. No distinct neurons are visible out of the slice proper.

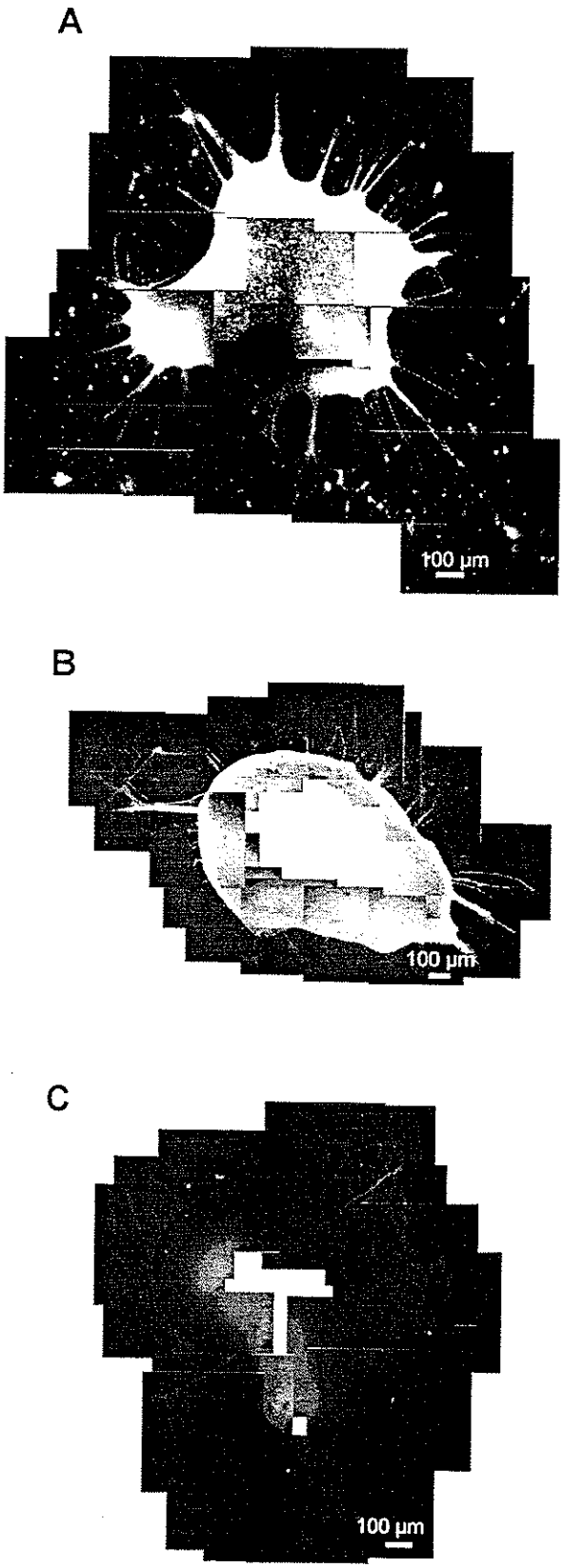


Figure 11

BDNF cultures contain numerous neurite outgrowths with some that grow long distances out from the slice cultures (Fig. 12A). In most cultures, MAP-2 positive neurons migrated out from the slice proper (indicated with arrowheads in Fig. 12 and B). Some of the neurons indicated by arrowheads in Fig. 12 are magnified in Fig. 13. The Arrowhead in Fig. 13 B_1 demonstrates an example of some of the large soma multipolar neurons presumed to be motor neurons found outside of the slice proper in BDNF cultures.

CNTF cultures created one of the most surprising results. Some cultures would produce extensive neurite outgrowth extending great distances out from the slice proper (Fig. 14A). In many CNTF cultures another typical pattern emerged in which neurite outgrowth would extend outward from the slice proper only to turn and grow back and re-enter the slice (Fig. 14B). Cultures in which CNTF was added are characterized by a number of MAP-2 positive neurons migrating out of the slice proper (indicated by arrowheads). Some of the neurons are large soma, multi-polar neurons presumed to be motor neurons. Figure 15 provides a magnification of the neurons indicated by arrowheads in Fig. 14.

Cultures grown in NT-4 are characterized by numerous short neurite outgrowths (Fig. 16 A-C), with some long neurite outgrowths extending away from the slice proper (Fig. 16 A and B). NT-4 cultures contained many MAP-2 positive neurons that migrated out of the slice proper (Fig. 16 A-C). Arrowheads in Figure 16 B indicate areas that are enlarged in Figure 17. Figure 17 $B_{3 \text{ and } 4}$ provide excellent views of some of the large soma multi-polar neurons found in NT-4 cultures.

Figure 12

Single spinal cord cultures (BDNF) culture day 14, processed according to the methods protocol (see methods section). Notice the variable patterns of neurite outgrowths ranging from long neurite outgrowth that extend far out into the culture dish in A, to shorter neurite outgrowths that only extend out a short distance from the slice proper in B, and C. B contains many neurons that migrated out of the slice proper, while C contained none. Some of the MAP-2 positive neurons are indicated by arrowheads in A and B.

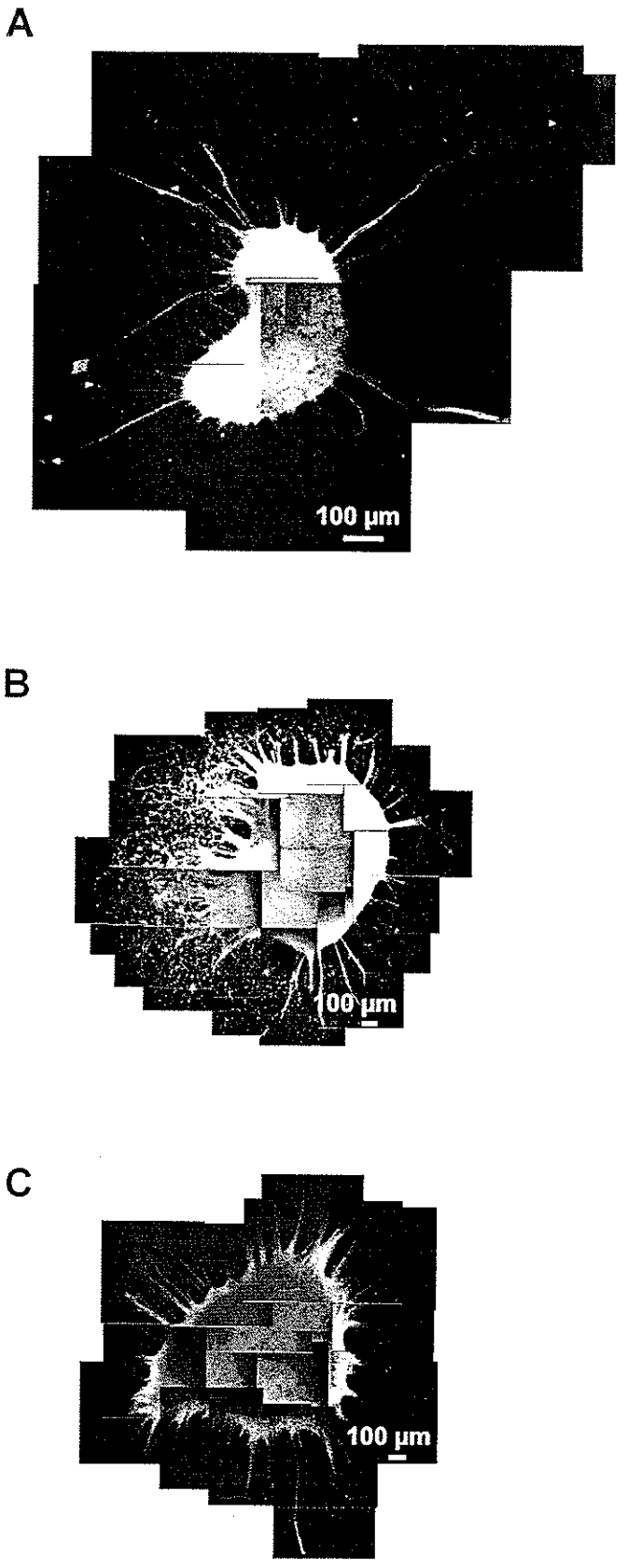
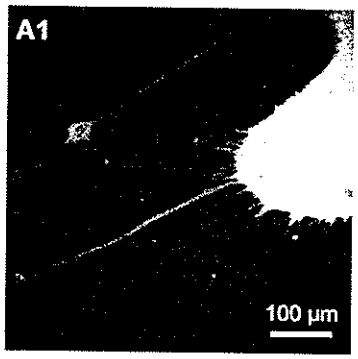
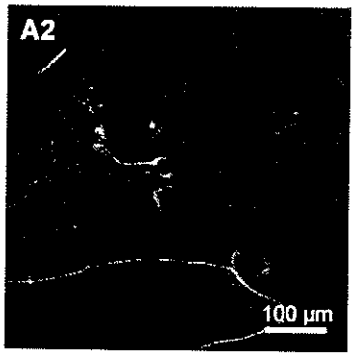


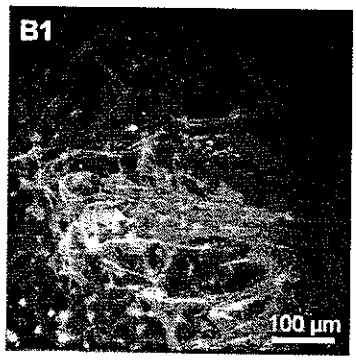
Figure 12

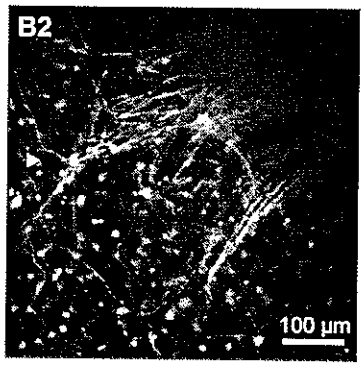
Figure 13

Enlargement of MAP-2 positive neurons from BDNF spinal cord cultures in Figure 14 A and B. The Arrowhead in B_1 demonstrates an example of some of the large soma multipolar neurons presumed to be motor neurons found outside of the slice proper in BDNF cultures.









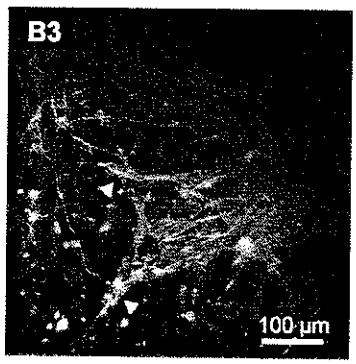


Figure 13

Figure 14

A-C demonstrates the variability among CNTF spinal cord cultures (cultured for 14 days and processed according to the methods protocol). A Illustrates the extensive growth seen in many CNTF cultures, where neurite outgrowth extends out great distances from the slices proper into the culture dish. In B, another common pattern is shown in which neurite outgrowth extends out into the culture dish only to turn back and re-enter the slice. Arrowheads indicate MAP-2 positive neurons migrating out of the slice proper.

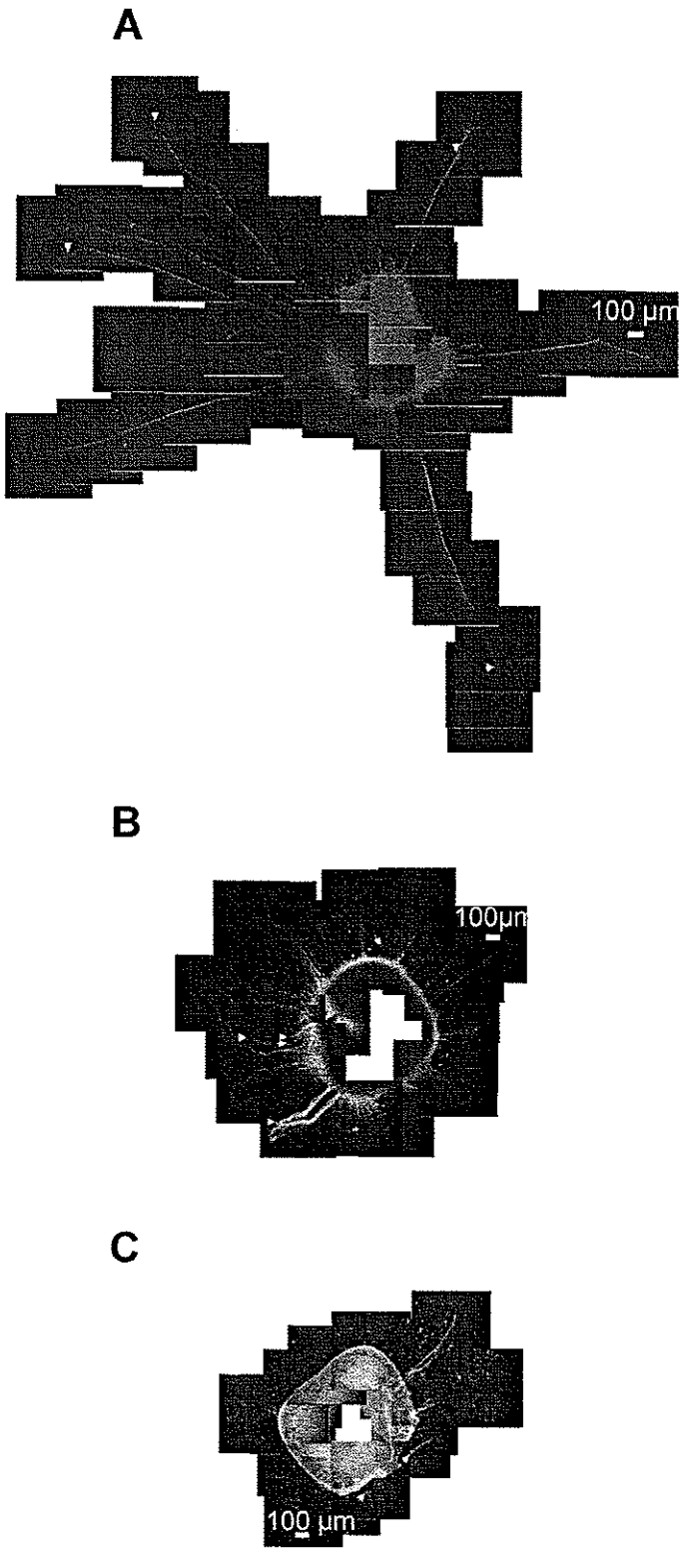


Figure 14

Figure 15

A-C Magnification of some of the arrowhead regions in figure 16 CNTF cultures. B_3 is a magnification of the area where the neurite outgrowth suddenly turned back on itself towards the slice proper. Large soma, multi-polar neurons presumed motor neurons where often found in CNTF cultures.

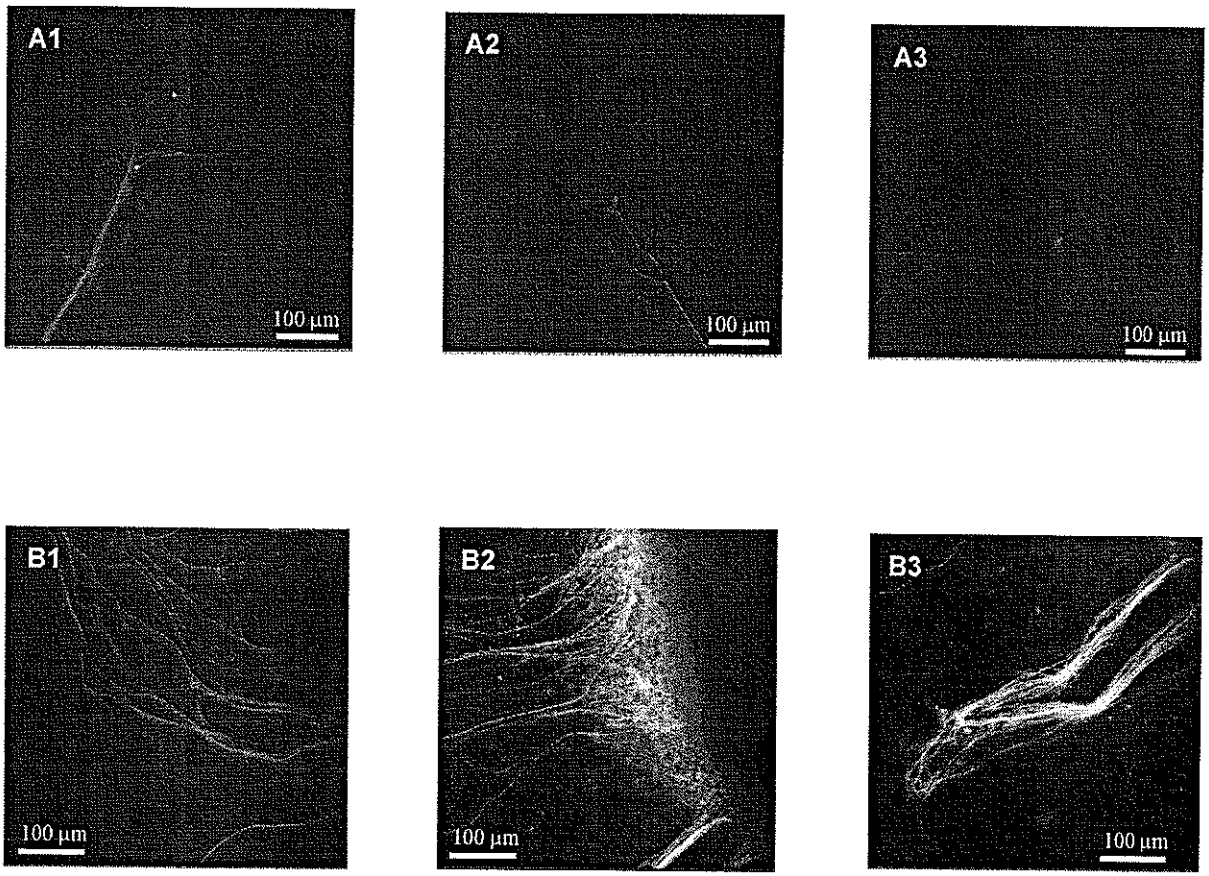

A1
100 µm
A2
100 µm
A3
100 µm
B1
100 µm
B2
100 µm
B3
100 µm
C1
100 µm
C2
100 µm

Figure 15

Figure 16

NT-4 spinal cord cultures processed at culture day 14 according to the standard method, are characterized by numerous short neurite outgrowths (A-C), with some long neurite outgrowths extending away from the slice proper (A and B). NT-4 cultures contained many MAP-2 positive neurons that migrated out of the slice proper (A-C). Arrowheads in B indicate particularly large soma, multi-polar neurons out of the slice proper that are presumed to be motorneurons.

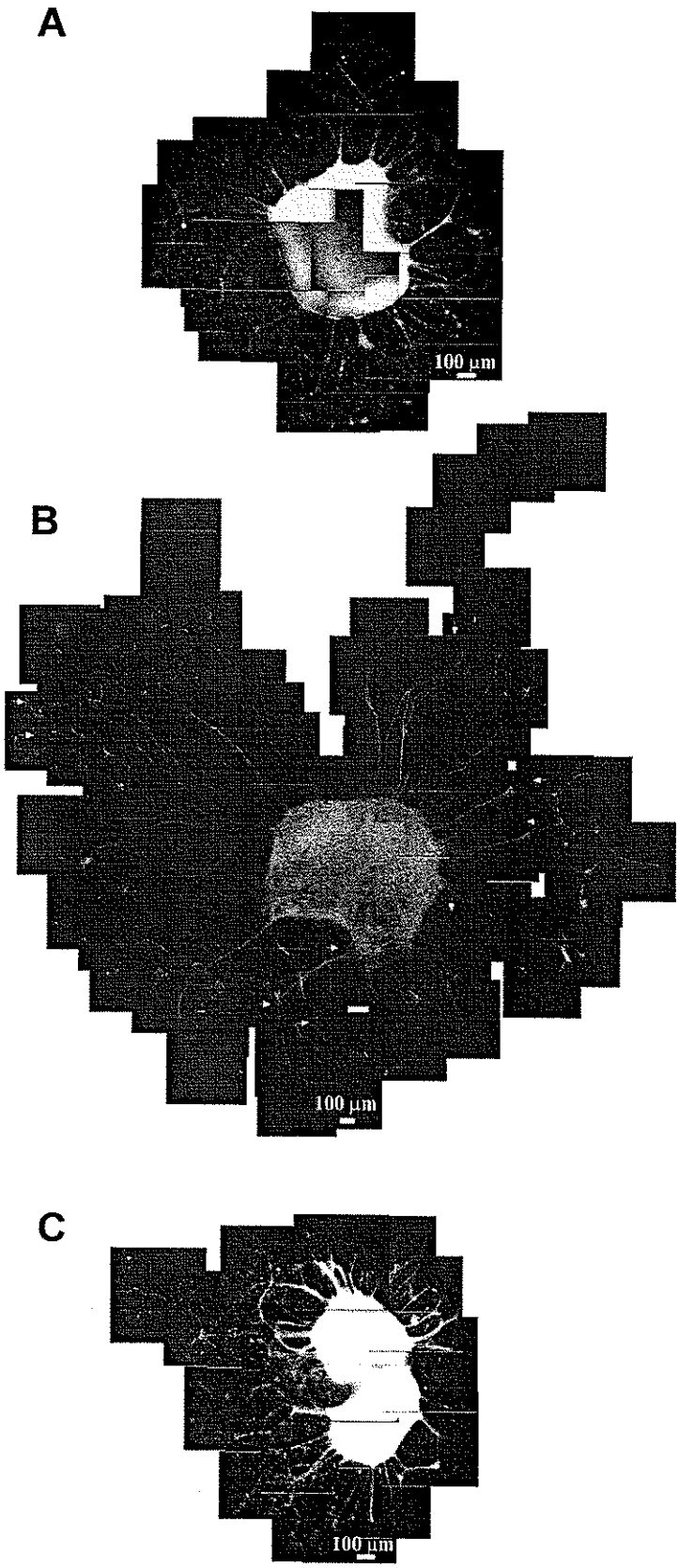


Figure 16

NGF cultures contain one of two patterns consistently. The first pattern is characterized by extensive neurite outgrowth long distances away from the slice proper that are solid staining (Fig. 18 A and B). The second pattern was characterized by short neurite outgrowths that stained punktate (dotted) in appearance (Fig. 18C). There is the presence of many MAP-2 positive neuronal cell bodies outside of the slice proper. Figure 19 provides a magnification of the areas indicated by arrowheads in Fig. 18.

One of the most impressive growth patterns occurred when bFGF was added to the slice cultures. In these cultures, there was extensive outgrowth, interconnection and migration of large clumps of cells away from the slice proper into the culture dish (Figs. 20 and 21). Co-cultures are used to illustrate this effect because differentiation was so great in single cultures that most cells migrated out of the slice proper with the loss of all cytoarchitecture, almost like a disintegration had occurred.

3.712 Spinal cord co-cultures

When co-cultures were grown, neurite outgrowth tended to be extensive between the slices, as well the total outgrowth for each individual slice was greater than that observed in a single culture exposed to the same trophic factor. This likely demonstrates the effect of other cell survival molecules produced by the second slice and indicated the complexity of tissue interactions between slices. A selection of co-cultures grown in different trophic factors provides a demonstration of this effect.

Figures 20 and 21 illustrate the extensive outgrowth and differentiation that occur when spinal cord slices are exposed to the mitogenic factor bFGF. These co-cultures support many MAP-2 positive neurons that migrate out if the slices proper.

Figure 17

The various MAP-2 positive neurons for NT-4 cultures are illustrated. B_3 and B_4 provide excellent views of some of the large soma multi-polar neurons found in NT-4 cultures. Other neurons are also visible in A-C.

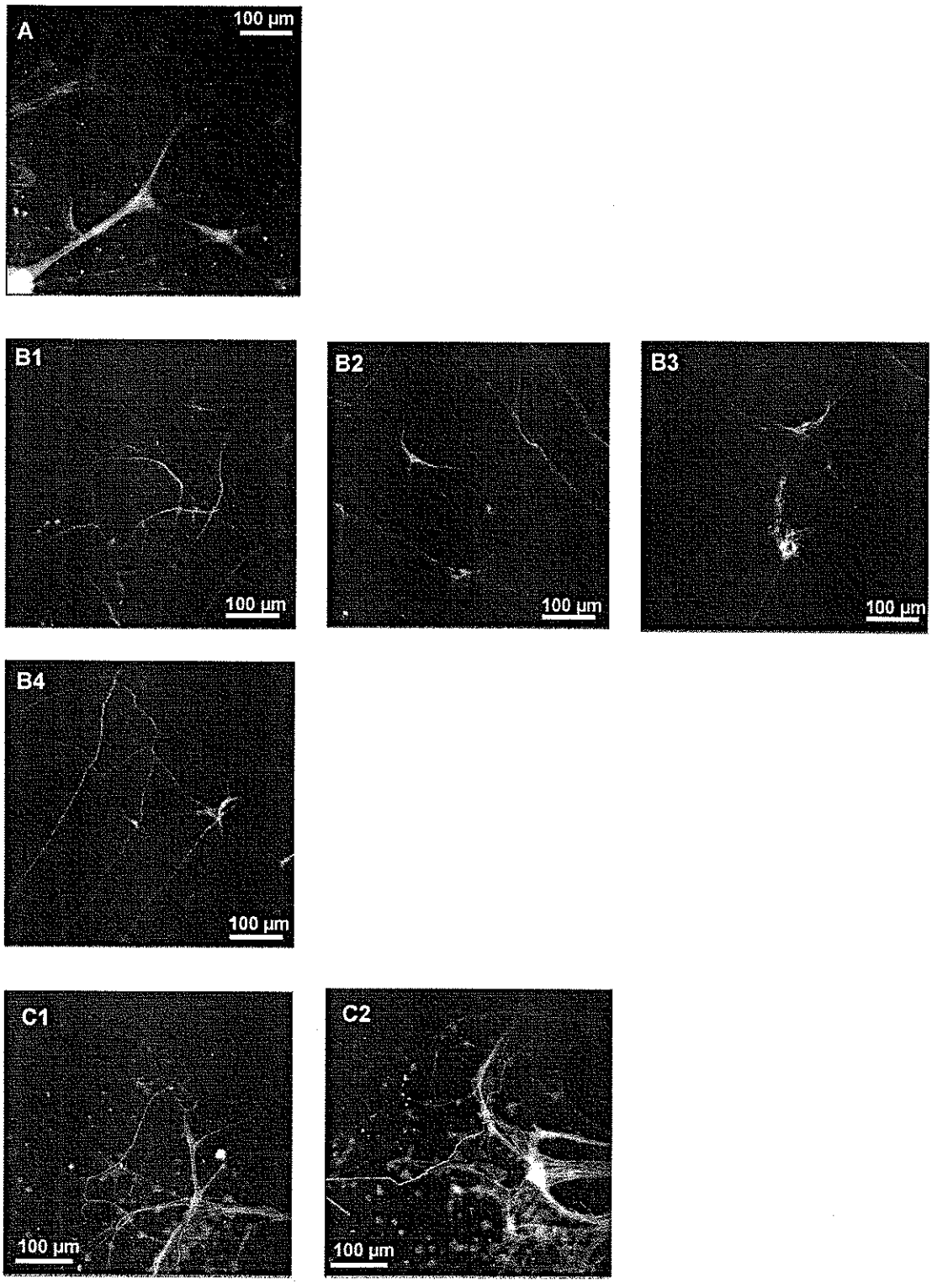


Figure 17

Figure 18

Two separate patterns are found when cultures are grown in NGF and then processed immunohistochemically according to the described methodology. The first pattern is solid staining pattern of neurite outgrowth that grows out long distances from the slice (A and B). The other pattern consists of numerous short outgrowths characterized by a punctate (dotted) like neurite outgrowth staining pattern (C). Arrowheads indicate MAP-2 positive neurons that have migrated out of the slice proper.

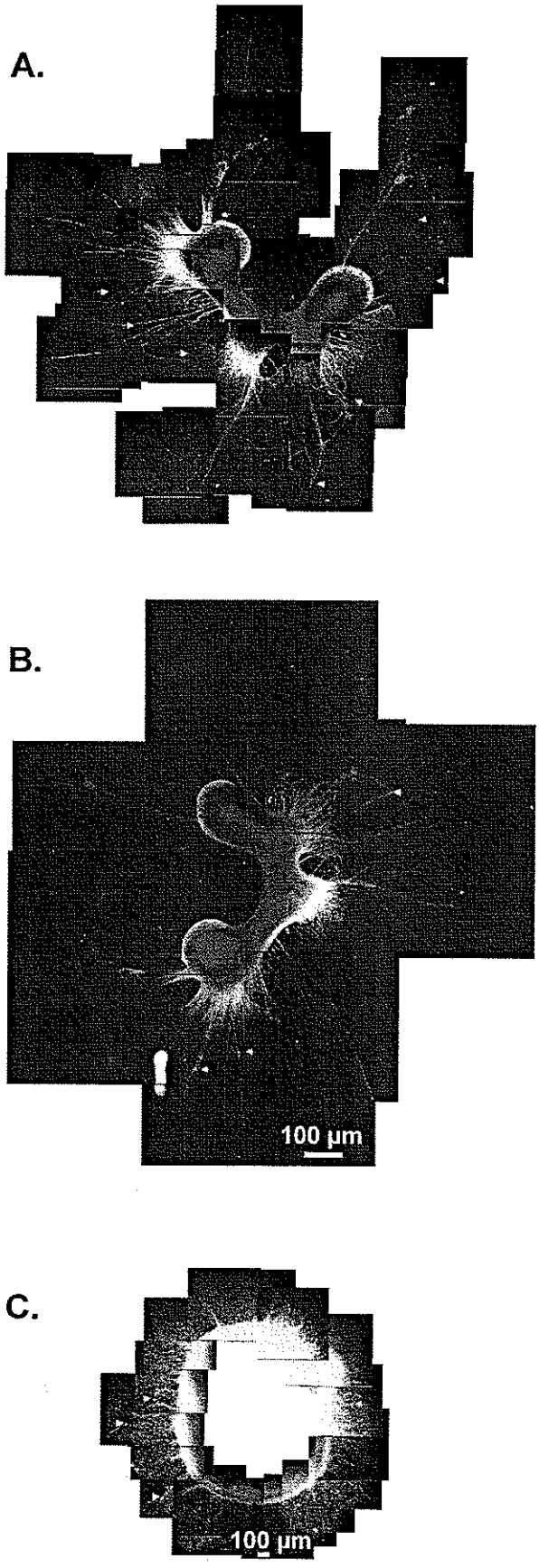


Figure 18

Figure 19

A magnification of the arrowhead regions in Figure 20 A and C. Figures shown in A_{1-5} demonstrate the variability of neurite outgrowth within one slice. Panels A_{1-3} are illustrative of the long neurite outgrowths seen in some cultures grown in NGF. Notice the neuronal networks/ganglia found in A_1 and A_2, and the smaller individual neurons found migrating out of the explant slice. A_{4-5} Illustrate the short neurite outgrowths seen in some NGF cultures. C_{1-4} Illustrate the punktate appearance of some neurite outgrowths in when cultures are grown in NGF. Map-2 positive small soma neurons are seen in all panels A-C.

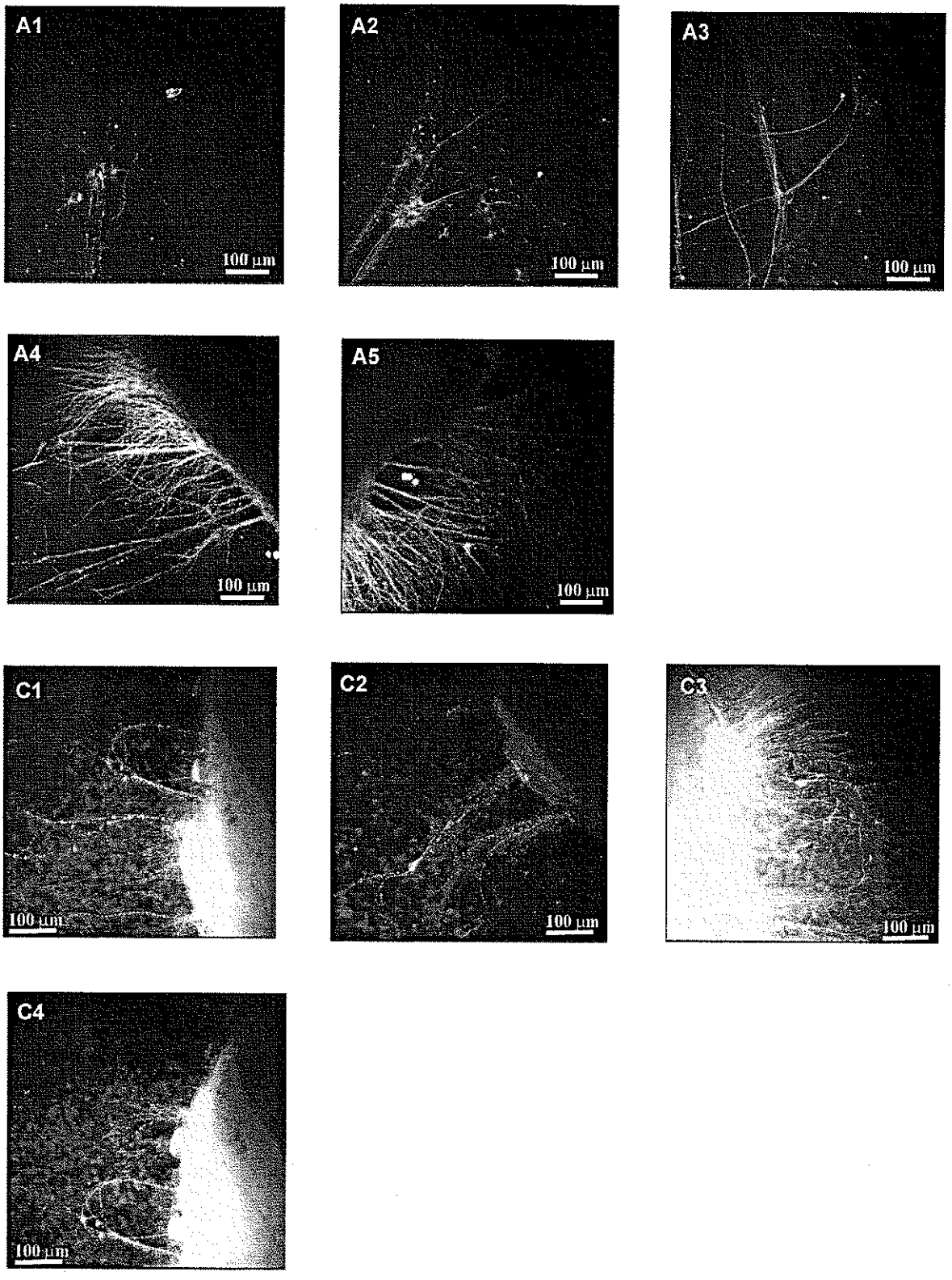

A1
100 µm
A2
100 µm
A3
100 µm
A4
100 µm
A5
100 µm
C1
100 µm
C2
100 µm
C3
100 µm
C4
100 µm

Figure 19

Figure 20

A spinal cord co-culture with bFGF added taken at culture day 14. Notice the immense neurite outgrowth. Due to its mitogenic effect, large clumps of tissue have broken off and migrated distances far away from the slices proper. Large axonal bundles of interconnections are found between the two spinal cord slices and the migrated tissue clumps. Many MAP-2 positive neurons are see outside of the slice proper.

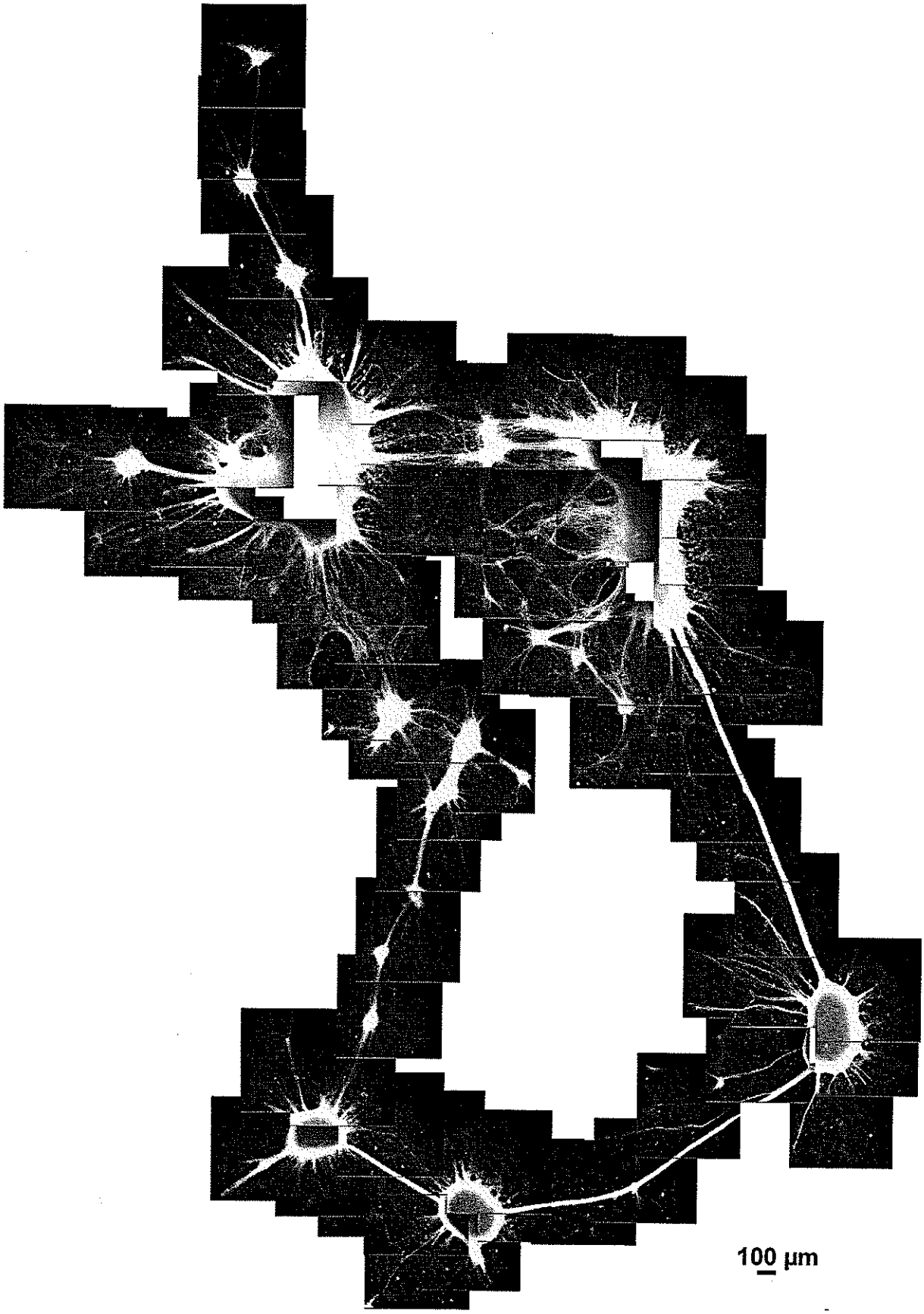


Figure 20

Figure 21

Another illustration of the massive differentiation seen in cultures grown in bFGF at culture day 14. The two spinal cord slices have lost their basic topography and share many interconnections between the slices and tissue clumps. Many MAP-2 positive neurons are visible outside of the slice proper, several that are presumed motorneurons due to their soma size and multipolarity.

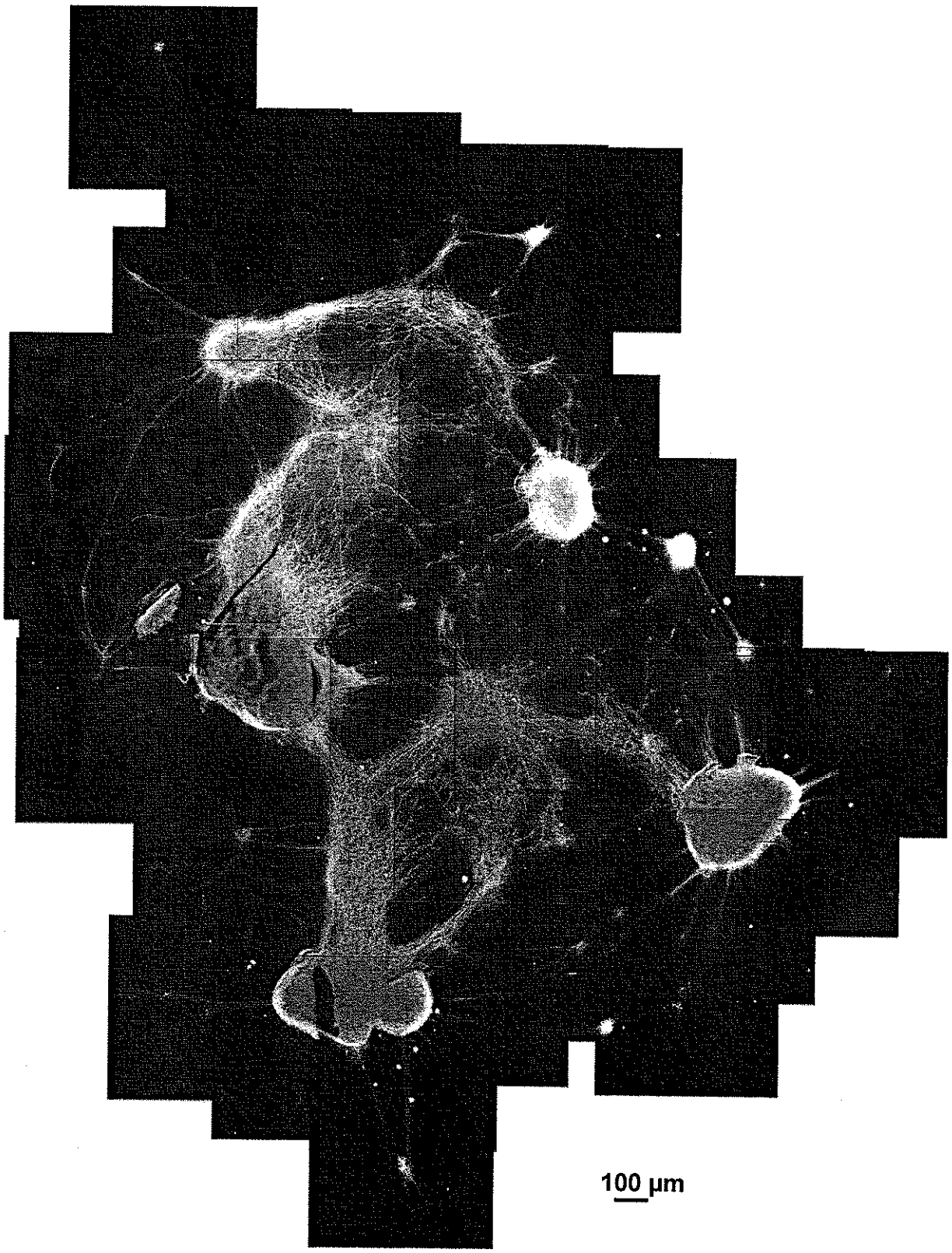


Figure 21

Figure 22 is an example of a spinal cord control co-culture. Notice the enhanced outgrowth and production of axonal bundles between the slices which does not occur in single slice cultures. Co-cultures have a few MAP-2 positive neurons that migrate out of the slices proper.

Figure 23 demonstrates the rescuing effect that the second spinal cord slice has in co-cultures exposed to the trophic factor NT-3. The massive necrotic death is not seen in these co-cultures. In co-cultures, MAP-2 positive neurons are visible outside of the slices proper.

Figure 24 demonstrates the extensive outgrowth that occurs in co-cultures exposed to NT-4. Neurite outgrowth and differentiation are so great that tissue topography of one slices is almost completely lost visually, and the boundaries between the slices are difficult to distinguish. There are some MAP-2 positive neurons visible in between the slices.

A BDNF co-culture pictured in Fig. 25 illustrates few interconnections between the slices, but overall good neurite outgrowth. MAP-2 positive neurons are visible in between the two slices.

The NGF co-culture illustrated in Fig. 26 demonstrates a similar neurite outgrowth pattern to the in the single culture pictured in Fig. 18C. There are a few MAP-2 positive neurons visible outside of the slices proper.

3.80 Cultures of cord slices at thicknesses less than eighty microns

When spinal cord tissue was sliced at thicknesses of 30-50 microns and then cultured, the cord tended to migrate into 4 separate quadrants of tissue roughly representing what appeared to be the right and left dorsal and ventral divisions of the spinal cord. Outgrowth then developed both out of the quadrants and between the quadrants.

Figure 22

In Control spinal cord co-cultures (culture day 14), there is significant neurite outgrowth between the slices and a tissue clump, forming bridges of interconnecting axons between the slices and the tissue clump that has migrated away from the main slices.

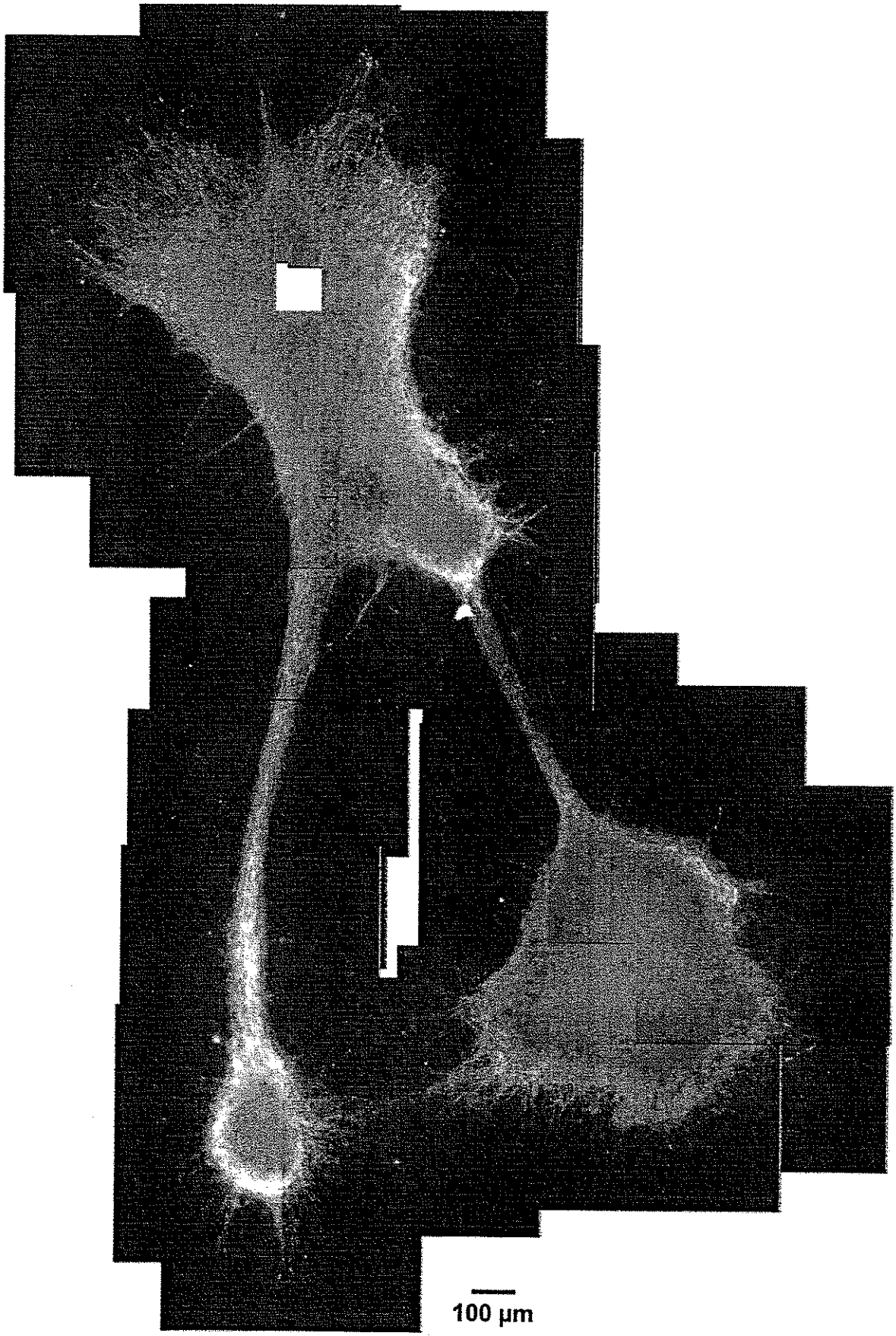


Figure 22

Figure 23

When NT-3 spinal cord co-cultures are grown (culture day 14), the second slice has a "rescuing effect" from the massive necrosis seen in figure 12 A and B. Notice that neurite outgrowth resulting in the formation of interconnections occurs primarily between slices, with little neurite outgrowth occurring in other areas of the slices.

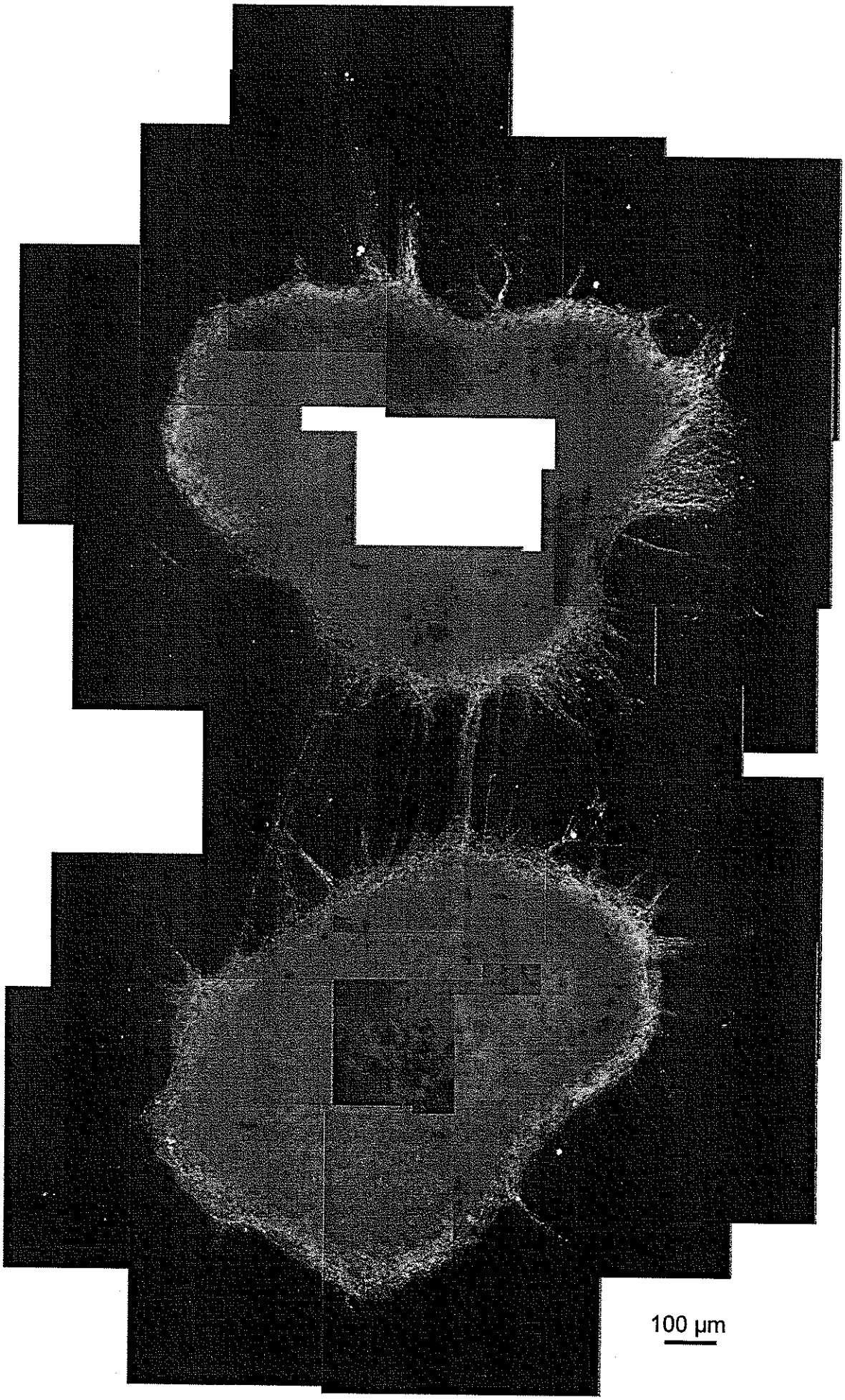


Figure 23

Figure 24

Spinal cord co-culture with NT-4 added and processed immunohistochemically at culture day 14. Notice the majority of neurite outgrowth occurring between the slices. In fact, the neurite outgrowth is so extensive between the slices that it is difficult to see the basic topography of the lower spinal cord slice. There are a few MAP-2 positive neurons that have migrated out of the slice proper seen throughout the culture.

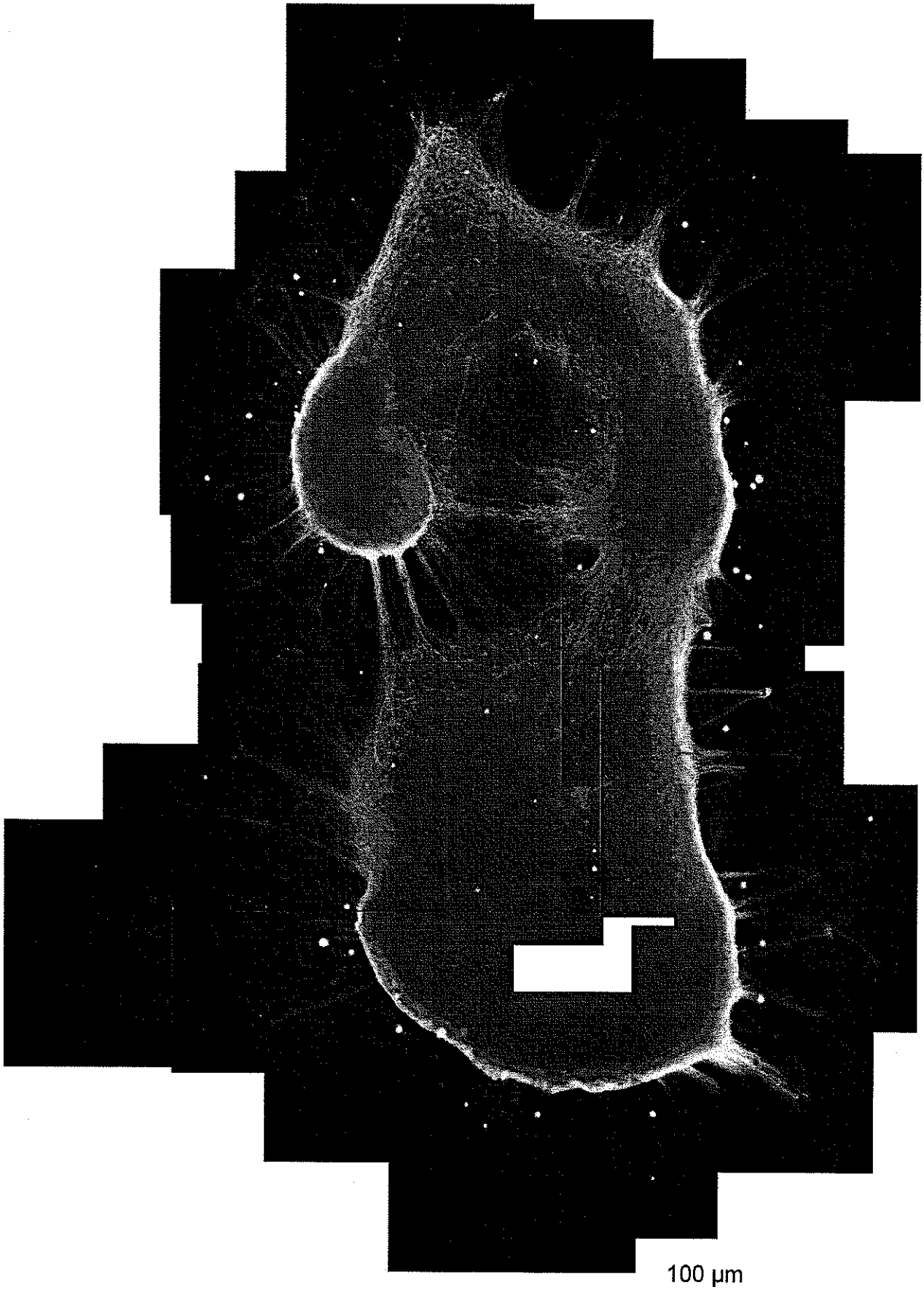


Figure 24

Figure 25

Spinal cord co-cultures grown in BDNF and processed at culture day 14 have some neurite outgrowth interconnecting the slices but do not have as extensive growth as NT-4 cultres shown in Figure 26 or CNTF co-cultures (not shown).

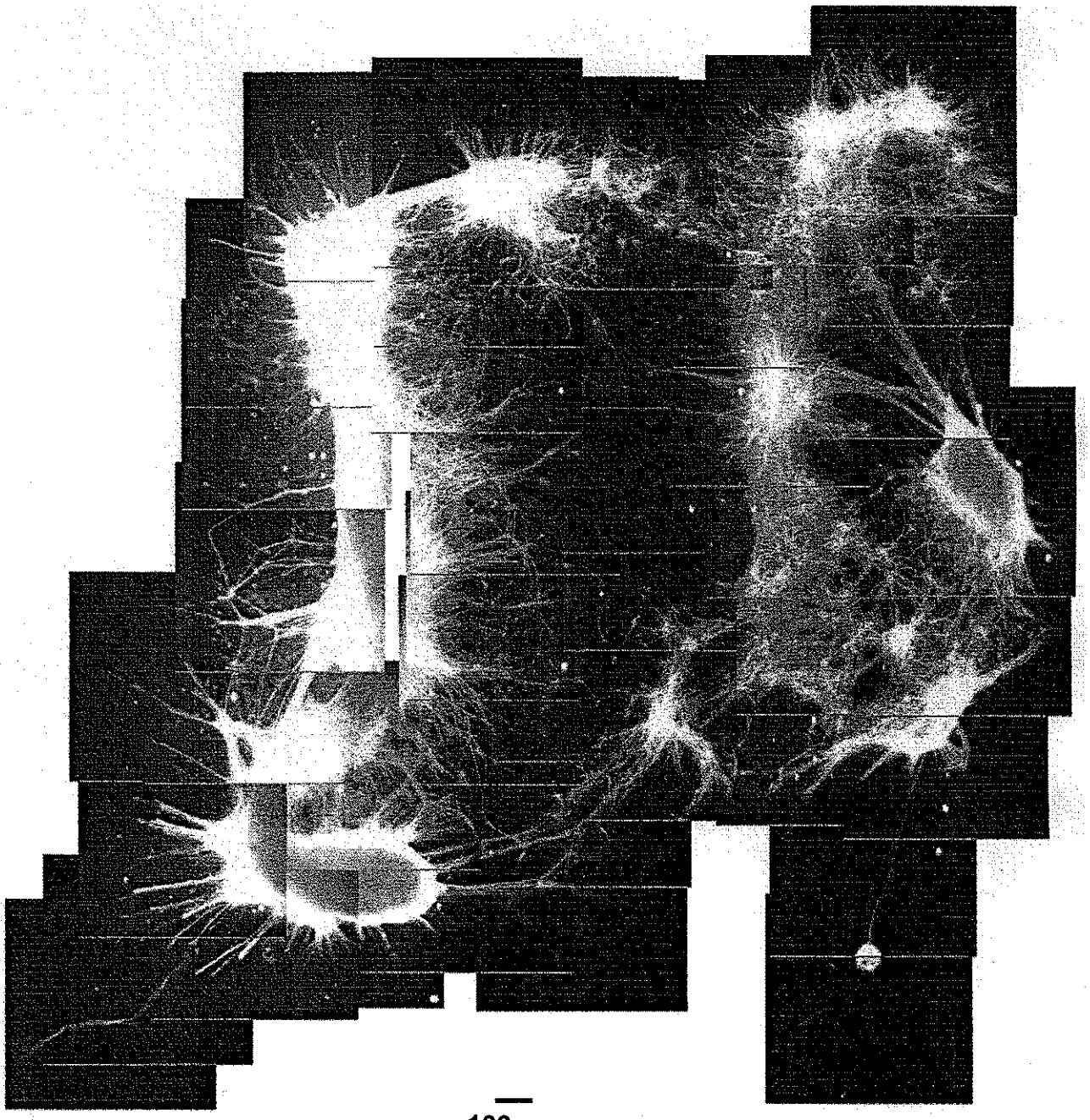


Figure 25

Figure 26

This spinal cord co-culture grown in the presence of NGF shows little neurite outgrowth by culture day 14. There are a few MAP-2 positive neurons that have migrated out close to the slice proper. No interconnections between the slices are seen in this culture. Neurite outgrowth is more prevalent from areas of the slices facing each other than from other areas of the slices.

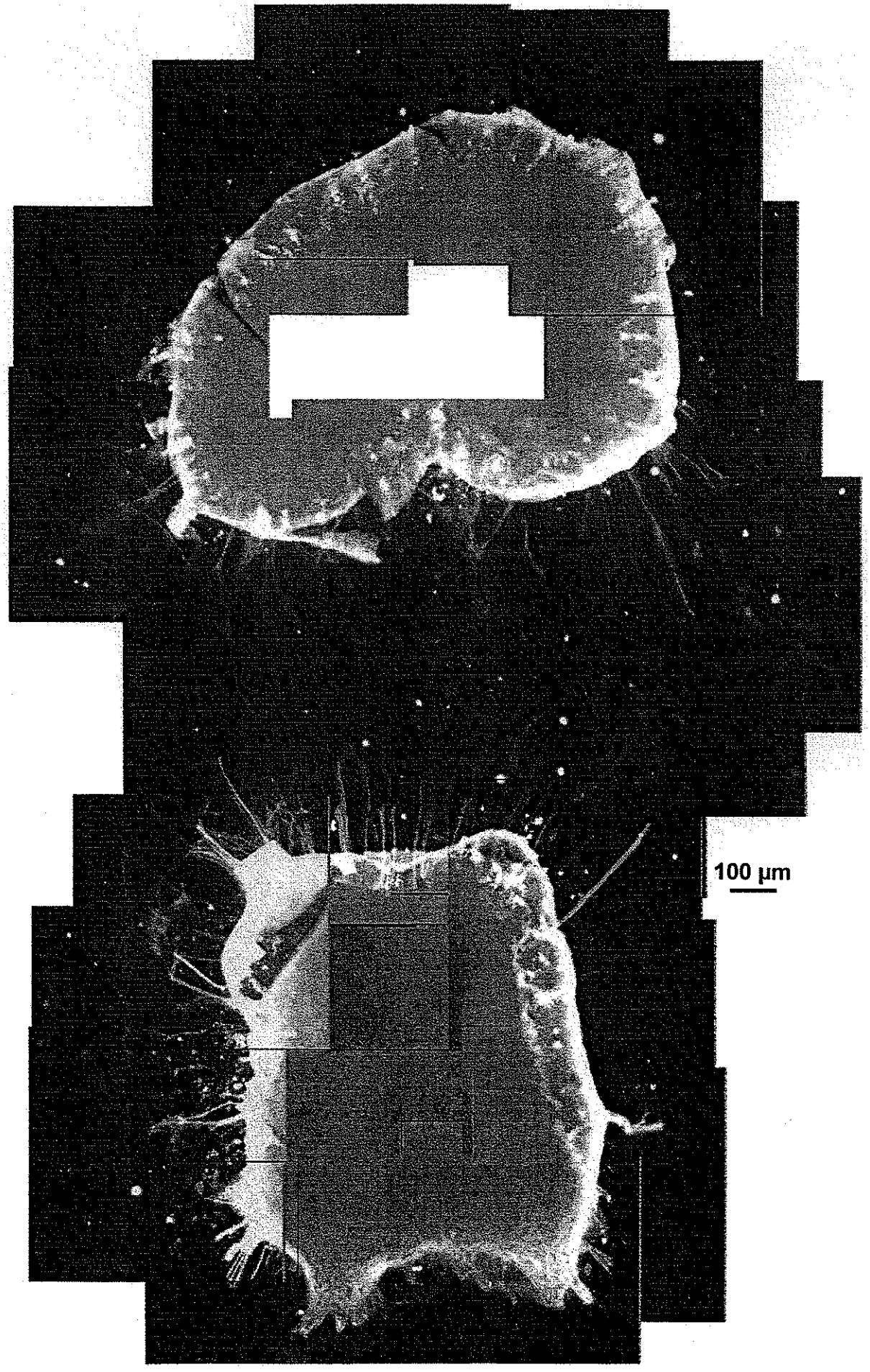


Figure 26

The technique of using such a thin slice also allowed for a monolayer appearance and allows for optimal visualization within the slice proper.

4.0 Discussion

After reproducing and researching several of the past CNS culture methods, my research produced a method for CNS culture that is relatively simple, inexpensive, and does not require the use of serum additives. I demonstrated that it produced cultures that were easily used for several experiment procedures in CNS research namely, immunohistochemistry, time-dependent studies, electrophysiology and trophic factor studies.

4.1 Past Published Methods

After reproducing two methods that were relatively simple for culturing CNS explants I found that there were a few drawbacks with the methods that could be improved upon. Stoppini's technique involves the membranes used to support the cultures. Firstly, the membranes required for this technique are expensive. Secondly, since the explants attach directly to the membranes, immunohistochemical processing becomes burdensome. Also as previously mentioned, the method produces cultures amenable for 'blind' electrophysiology experiments. Khan's method utilized carbon filament bundles that are sometimes difficult to work with. As well, the bundles impede microscopic visualization, and therefore make electrophysiological experiments difficult.

4.2 Section thickness is critical

We have demonstrated the importance of slice thickness by culturing explants of 300 μm, and then compared them to explants of 150 μm and 80 μm thicknesses. Although

many cells were viable in the 300 μm spinal cord slice cultures after 14 days (Fig. 3), visualization of these individually labeled cells was difficult. The 300 μm spinal cord slice cultures also contained large areas of pronounced cell death, which coincided with the darker areas of the slice observed with interference optics. These darker areas occurred preferentially in the center of the slices, presumably where the exchange of nutrients and/or gases was most compromised. Slice cultures produced at thicknesses of 80 μm or less were optimal for cell visualization, and therefore for visual electrophysiological recording techniques. Although thin slice cultures for both spinal cord and brainstem (30, 50, or 80 μm and 100 μm respectively) are optimal for studies requiring cell visualization, there was a slight increase in cell death within the slice proper probably due to trauma suffered from slicing at such a reduced thickness. The incidence of cell death seen in 150 μm spinal cord slices was minimal with cell viability being 95%. Therefore, future studies employing our method should use 150 μm slices if visualization at a near monolayer is not required, while slices of 80 μm or less are suggested for studies requiring more optimal cellular visualization.

4.22 Topography of hippocampus cultures

Since hippocampus topography has been well-studied in organotypic cultures (Stoppini et al., 1991, Gahwiler 1981 and 1988,Gahwiler & Brown 1985), the ability to produce comparable slices utilizing this method allows a direct comparison between methods. Song maintained postnatal hippocampus slices for periods of four weeks (Song et al., 1996; Parsley et al., 1998). The results were reported in Parsley et al., 1998 and demonstrated that overall topography of the hippocampus slices were well maintained, with many MAP-2 positive cell bodies and dendrites, and panaxonal labeled axons visible

in both the CA1 pyramidal, and dentate granule cell regions. The ease of visualization of neurons within the CA3 region makes these cultures suitable for electrophysiology studies. In conclusion, this culture method produced postnatal hippocampus slices equal to or superior in quality for physiological studies as produced by other culture methods.

4.3 Electrophysiological studies

The ease of cellular visualization using spinal cord or hippocampal slices of 80 μm or less thickness (Fig. 5) makes our method advantageous for visual patch recordings which are unattainable using the interface method (Stoppini et al., 1991). After 14 days in culture, a spinal cord slice had formed synapses, heterogeneity of neuronal firing properties was observed (Fig. 7A and B) and the observation of spontaneous synaptic events in neurons (Fig. 7C) suggests that the physiological properties of the neuronal population were maturing *in vitro* (Xie and Ziskind-Conhaim, 1995). The idea that the cultures were maturing during the culture period also appears to be supported by morphological changes observed by time-dependent viewing (Fig. 3). As single slices remain in culture over time (spinal cord and brainstem), the elongation of processes, and the continued arborization of processes can be visualized. In co-cultures, maturation was also demonstrated by the development of bundles (presumably axonal) that interconnect the slices.

Since it has already been demonstrated that *in vitro* connections follow the correct *in situ* pathways (e.g. Crain and Peterson, 1963; Gähwiler and Brown, 1985; Bolz et al., 1990; Streit et al., 1996), our method may be an asset for regeneration studies using spinal cord explants.

4.4 Trophic factor studies

This portion of the study was extremely difficult due to several influencing factors that include the complexity of growth factor interactions and since full explants were used, all tissue and cell types were represented, as were all types of inhibitory and regenerative trophic factors that naturally occur *in vivo* in the spinal cord. Of these, Cytokines play an important role since they regulate several cellular functions such as proliferation, differentiation and regulation of expression of cell surface proteins. A majority of cell damage and death during a spinal cord injury comes secondary to the cord injury itself. This damage is likely mediated by inflammatory processes including the release of proinflammatory cytokines (Xu et al., 1998) such as Il-1β, TNFα and Il-6 (Steit et al., 1998). It becomes crucial to control the levels of these inflammatory mediators in order to promote cell survival. *In vivo*, a steroid may be infused to control the inflammatory response. As pharmaceutical companies continue to develop new and better steroids, a potential therapy for use in spinal cord injured patients may be to use a therapy that alters the inflammatory cytokine cascade at the gene level. This is currently being done with mometasone furoate which functions by suppressing the inflammatory cytokines in particular Il-1, Il-6 in the pathogenesis of skin and allergic diseases (Barton et al., 1991).

In order to isolate the effect of a specific growth factor, single cultures were primarily utilized. The natural trophic factor balance within the cord was then offset by adding an excess of one growth factor type. It is possible, though, that the cord slices compensated for the additives by modulation, up regulating endogenous trophic factors skewing the true effects and perhaps explaining the similarities seen between growth patterns in our cultures when different trophic factors were added. Even small amounts of naturally

occurring growth factors, produced in response to the bolus addition of one growth factor, could still be enough to activate receptors and create a false impression of growth from one growth factor, which instead is actually growth caused by an indirect pathway of interaction. This could explain the variability in growth patterns within trophic factor groups (Figs. 10, 11, 12, 14, 16 and 18). The quantification studies confirmed the variability in the cultures, as more consistent growth results were expected. Therefore, growth may have occurred due to the addition of a single growth factor or, it may be occurring due to the regulatory mechanism of that growth factor on another growth factor's cycle. Since our method involved changing the media every 4-5 days, this difficulty was only semi-controlled, although future experiments may involve a complete daily media change to prevent accumulation of the negative trophic factor mediators, for example tumor necrosis factor (TNF). This was also dramatically demonstrated by cultures that were not changed on time but left for 7 days. The accumulation of damaging molecules that mediated tissue death caused a pH change in the media (observed by a yellowing of color of the pink media). These tissue cultures underwent large scale necrosis. This is only applicable in *in vitro* environments and would not model the natural *in vivo* cord environment directly. To try a more natural approach, a steroid and growth factor combination may be a solution to test and see if the cord would survive and regenerate without the removal of accumulating inflammatory mediators (absence of media changes).

The other difficulty in using this method to compare growth factors lies in the fact that the same section of the cord used is problematic to isolate. This means that different ratios of glial and neuronal populations will be present in the different explant slices, and

may respond differently to the various trophic factors, making it extremely difficult to make true comparisons both within and between growth factor groups. Therefore, conclusions from these types of experiments were difficult to determine.

The extensive death demonstrated in NT-3 single cultures (both spinal cord and brainstem) was extremely interesting since it conflicted with several past results which demonstrated that NT-3 could significantly increase cell survival *in vitro* (Hughes et al., 1993, and McTigue et al., 1998). Our results demonstrated that NT-3 somehow was triggering massive necrosis in our explant culture system. These results have also been demonstrated by Koh et al., 1995. The pathway of activation is not known, but one possible mechanism may be that since NT-3 can also bind to neurotrophic receptors other that Trk C, it may enhance cytotoxicity through these pathways (Koh et al., 1995). The NT-3 effects become further intriguing and complicated when added to co-cultures. The dramatic death seen in the single cultures was not seen when co-cultures were grown. This could implicate the production of an important molecule by the second slice that rescues the first slice from death. Another possibility is that the second slice could be producing enough trophic factors that NT-3 does not have the chance to bind to all of the neurotrophic receptors, and therefore does not exhibit its potential cytotoxic effects.

After numerous duplications of the results obtained by the addition of NT-3 to cultures and the possible negative impact on spinal cord regeneration these experiments produced, a new batch of NT-3 was ordered to confirm our results. The results became further complicated at this point as the new batch of NT-3 did not produce the same dramatic cell death. This could suggest that the first batch may have been contaminated, but unfortunately was disposed of before further testing could be conclusive.

The limited growth seen in control cultures was not surprising considering trophic support derived from the slice itself was limited. This may be also due to the fact that cultures had to recover from the trauma obtained during tissue slice preparation. In addition, control cultures must offset any inhibitory mediators being produced during or following tissue slicing. The absence of neurons out of the slice proper is therefore not unanticipated.

CNTF, which is classified as a cytokine, produced extremely varied results (Fig. 14 A-C). Some cultures produced such an excessive increase in neurite outgrowth that it was visually noticeable with the naked eye (Fig. 14A). Unfortunately, quantification studies did not reveal a significant increase in neurite outgrowth versus the control cultures. In all cultures (Fig. 14A-C) there were several neurons that had migrated out of the slice proper (indicated by arrowheads). The results were not expected in that I anticipated a greater ratio of cultures producing excessive outgrowth similar to past results reported by Oppenheim et al., 1991, Sedtner et al., 1990 and Korsching 1993 which demonstrated the ability of CNTF to support several neuronal cell populations including spinal motor neurons. Instead, the ability to support motor neurons may not correlate with increased outgrowth. Since in most cultures many neurons were present that had migrated out of the slice proper, I suggest instead that in the slice culture environment when conditions occur that are advantageous to neuronal survival, a greater number of neurons will migrate out of the slice proper. CNTF cultures appeared to support both large and small soma neurons. The large soma, multi-polar neurons most likely are motor neurons.

Since both BDNF and NT-4 activate the same Trk B receptor, it is not surprising that they both produced a significant increase in neurite outgrowth as compared to control

cultures (Fig. 9), and supported both large and small soma neurons (Figs. 12 and 16). They both supported neurons that were presumed to be motorneurons due to their large soma, and multi-polarity (Figs. 13 B_1 and 17 B_{3and4}). This is consistent with evidence showing that BDNF supports survival of rat motor neurons (Hughes, 1993). These results agree with the work of past researchers who have demonstrated BDNF and NT-4 to possess survival effects and are therefore expected to enhance neurite outgrowth.

When NGF was added to the cultures one of two growth patterns were consistently produced. The first pattern illustrated by Fig. 18 A and B involved numerous extensions with a mix of both long and short neurites, solid in pattern. The second pattern demonstrated in Fig 18C, contained numerous short neurite extensions that could be described as punctate like (dotted) in appearance, with a few long neurite extensions. This is extremely interesting and could possibly indicate two different binding patterns associated with NGF in that it can either bind to the Trk A receptor primarily, or the p75 receptor primarily. The first pattern of neurite outgrowth most likely occurs through a higher expression and thus binding of Trk A receptors. Chao, 1994, discusses studies that demonstrate that when Trk A receptors are expressed in greater numbers than p75 receptors, an increased amount of neurite outgrowth occurs. When this occurs, I suggest the first pattern of neurite outgrowth seen in Fig 18 A and B is obtained. Barrett, 1994, discusses the theory that suggests that in early development the binding of NGF to the p75 receptor could cause massive cell death. Since the rats used in these experiments were not fully developed I suggest that the second pattern (Fig. 18C), characterized by short neurite outgrowth, was produced by NGF binding of the p75 receptors, primarily by a higher ratio of p75 receptors being expressed. Therefore neurite outgrowth was not supported.

A future experiment to test this hypothesis would be to perform an experiment where NGF was not added to the cultures right away. Instead, a competitive antagonist for p75 receptors, that binds irreversibly, would be added to one set of cultures, while a competitive antagonist for Trk A receptors that binds irreversibly is added to the other set of cultures. The next day, NGF could then be added to all of the cultures. The growth pattern that predominates will be produced by NGF binding to the receptor that had not been blocked.

NGF tended to support many small soma neurons that remained close to the slice proper. These small neurons could be interneurons, which NGF is know to support in culture (Korsching, 1993), or small sensory neurons.

Whenever good neurite outgrowth occurred, ganglia (clumps of neurons) tended to be present. This could suggest that the number of neurons increased with cell survival. In cultures where there is increased outgrowth and thus probably more autocrine trophic support available to the neurons, the presence of ganglia was noticed (Figs.11A, 12A, 14A, and 16A). These ganglia tend to be composed of presumed motor neurons (large soma and multi-polar) and appear more prevalent in cultures with trophic factors known to support motor neuron survival, such as BNDF, CNTF and NT-4.

The results obtained by the addition of bFGF (Figs. 22 and 23) showing enhanced growth confirm results obtained by past researchers (Ghosh and Greenberg, 1995) demonstrating the ability of bFGF to enhance survival and differentiation of many different cell types. In fact, cell differentiation was so great that the tissue could not maintain its basic tissue architecture, instead breaking up into sections of tissue clumps which were interconnected.

4.5 Implications of using a spinal cord slice thickness between 30-50 microns

The ability of the spinal cord tissue to segregate into what appears to be 4 anatomical quadrants may provide a valuable tool for future studies focussing on developmental cues within spinal cord tissue. It could also allow for the isolation of a particular area of the cord for functional and pharmacological studies.

4.6 Advantages of this culture method

This thesis has focussed on the development of a procedurally simple and inexpensive explant culture technique that allows CNS slices to be maintained in organotypic culture. The technique relies on the use of comparatively thin tissue sections (30,50, 80 or 150 μm) as compared to those used in other culture methods (250 to 500 μm; see Gähwiler, 1981; Stoppini et al., 1991). Tissue viability was verified both by live/dead cell assay and electrophysiological recordings. These explants appear to maintain at least some of the *in situ* tissue organization, contain neurons that demonstrate mature neuronal properties and flatten to a final thickness that allows visualization of individual cells for visual patch clamp recordings on an inverted microscope. While this method offers a visual resolution already available with the roller tube method, it may offer additional advantages. Firstly, because thinner tissue sections are used, reduced topographical distortion due to slice flattening would be expected. Secondly, immunostaining procedures are performed easily, and are not hampered by difficulties associated with attachment to a porous membrane (Stoppini et al., 1991). Thirdly, unlike the roller tube method, this method allows for continuous microscopic evaluation of cultures throughout the culturing period while maintaining sterility. This is particularly useful for experiments that assess time-dependent changes. Since these cultures can be studied daily as either single cultures or co-cultures,

this method may be useful for studies on development or regeneration. Finally, due to the use of serum-free media, this culture method provides a controlled environment, one necessary to study the effects of specific molecules and/or neurotrophic factors on development and functionality of CNS neurons.

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