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A Biomaterials Approach to Peripheral Nerve Repair

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy

By

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Network of Excellence for Functional Biomaterials

National University of Ireland, Galway

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# TABLE OF CONTENTS

TABLE OF CONTENTS ................................................................. ii

LIST OF FIGURES ................................................................. ix

LIST OF TABLES ........................................................................ xxiii

ACKNOWLEDGEMENTS .......................................................... xxv

ABSTRACT .................................................................................. xxvii

1. Introduction.............................................................................. 1

1.1 Peripheral Nerve Injury and Repair........................................ 2

1.2 Autograft: The Limited Gold Standard.................................. 2

1.3 The Development of Nerve Guidance Conduits....................... 3

1.4 Regeneration within a Hollow Nerve Guidance Conduit.......... 4

1.5 Guided Nerve Regeneration - The Use of Structural Guidance Cues
........................................................................................................... 6

1.5.1 Intra-luminal Guidance Structures – Replacing or Supporting
the Fibrin Cable ........................................................................... 8

1.5.2 Luminal Wall Guidance Features – Enhancing Porosity and
Increased Guided Cell Migration .................................................. 12

1.5.3 Optimising Conduit Design and the Introduction of External
Stimuli ......................................................................................... 19

1.5.4 Surface Modifications and Peptide Mimetics ....................... 21

1.6 Molecular Delivery Therapies: The Creation of Conductive Micro
Environment ............................................................................. 28

1.6.1 Neurotrophic Factor Mimetics – A Possible Alternative for
Molecular Therapies .................................................................. 33

1.7 Schwann Cells: The Gold Standard for Cell Based Repair ......... 37
1.7.1 Stem Cells: A Possible Alternative to Autologous Schwann Cells

1.7.2 Genetically Modified Cells

1.8 Improvements in Current Intraluminal Guidance Structures

1.9 Hypotheses and Objectives

1.9.1 Phase One: The Development of a Biomaterial Platform for Repair

1.9.2 Phase Two: Optimising Packing Density, Collagen Fibre Degradation and Assessing Functional Recovery

1.9.3 Phase Three: A Biomaterials-Induced Proteomic Response to Conduit-Mediated Nerve Repair

1.9.4 Phase Four: The Effect of Intraluminal-Collagen Fibres on the Biomaterials-Induced Proteomics Changes during Peripheral Nerve Repair across both Non-Critical and Critical Nerve Gaps

1.10 References

2. Intraluminal Collagen Fibres: A Suitable Platform for Repair?

2.1 Introduction

2.2 Materials and Methods

2.2.1 Proof of Concept – Structuring of PGCL Sutures

2.2.2 Extrusion and Cross-Linking of Collagen Fibres

2.2.3 Fabrication of Structured Collagen Fibres

2.2.4 Surface Characterisation of Structured PGCL and Collagen Fibres

2.2.5 Neuronal Cell Interaction with the Structured Fibres
2.2.6 Preparation of Intraluminal Conduits and Experimental Groups for In Vivo Implantation ........................................ 79

2.2.7 Surgical Procedure ............................................. 79

2.2.8 Nerve Histology and Ultrastructural Analysis ............... 80

2.2.9 Morphology and Stereology ................................... 80

2.2.10 Simultaneous Retrograde Tracing ............................. 82

2.2.11 Statistical Analysis ............................................. 88

2.3 Results ........................................................................ 88

2.3.1 PGCL Proof of Concept Fibres ................................. 88

2.3.2 Fabrication and Characterisation of Intraluminal Collagen Fibres ................................................................. 89

2.3.3 Neuronal Cell Interaction with the Structured and Unstructured Fibres ............................................................... 94

2.3.4 Surgical Outcome of Implanted Intraluminal Guidance Conduits ................................................................................. 96

2.3.5 Simultaneous Retrograde Tracing – Innervation of Distal Targets ................................................................. 96

2.3.6 Histological & Morphological Analysis of Implanted Conduits ................................................................................. 97

2.3.7 Incorporation of Intraluminal Fibres into the Host Regenerative Process ................................................................. 97

2.3.8 Ultra-Structural Appearance of the Implanted Fibre Groups .................................................................................. 100

2.4 Discussion ...................................................................... 100

2.5 Conclusion ...................................................................... 119
3. Increased Packing Density and Degradation Rate across a Non-Critical and a Critical Nerve Gap................................. 125

3.1 Introduction ................................................................. 126

3.2 Materials and Methods .................................................... 129

3.2.1 Extrusion and Cross-linking of Collagen Fibres ................. 129

3.2.2 Subcutaneous Implantation of Cross-Linked Collagen Fibres .... 130

3.2.3 Fabrication of Nerve Guidance Conduits ......................... 131

3.2.4 Surgical Procedure ..................................................... 131

3.2.5 Force Measurement Analysis ....................................... 133

3.2.6 Recovery from Muscular Atrophy ................................. 134

3.2.7 Analysis and Quantification of Nerve Morphometry ............. 135

3.2.8 Ranking of Nerve Regeneration Parameters ..................... 136

3.3 Results ................................................................. 136

3.3.1 Cross-Linking of Fibres and Subcutaneous Implantation ........ 136

3.3.2 Nerve Morphometry 12 Weeks Post Implantation ............. 139

3.3.3 Force Measurement .................................................... 144

3.3.4 Grouped Ranking of Multiple Regenerative Parameters ......... 144

3.5 Discussion ............................................................. 147

3.6 Conclusion ............................................................. 149

3.7 References ............................................................. 150

4. The Proteomic Response to Intraluminal Guidance, Type of Biomaterial Conduit and Gap Distance Treated in Peripheral Nerve Repair................................................................. 153
4.1 Introduction ................................................................. 159

4.2 Materials and Methods .................................................. 161

  4.2.1 Preparation of Conduit Groups for In Vivo Implantation .... 161
  4.2.2 Surgical Procedure .................................................. 163
  4.2.3 Isolation of the Sciatic Nerve .............................. 165
  4.2.4 Protein Isolation, Precipitation and Quantification .......... 165
  4.2.5 8-plex Protein Tagging ........................................... 166
  4.2.6 Multidimensional Protein Identification Technology (MudPIT) Analysis ....................................................... 167
  4.2.7 Peptide and Protein Identification and Statistical Analysis ... 169
  4.2.8 Functional Enrichment Analysis Based on the Conduit Material .............................................................................. 170
  4.2.9 Function Enrichment Analysis Based on the Gap Length .... 171
  4.2.10 Validation of Protein Expression by ELISA ................. 171
  4.2.11 Statistics ............................................................... 172

4.3 Results ............................................................................. 172

  4.3.1 Mass Spectrometry and Functional Enrichment Analysis .... 172
  4.3.2 The Material-Specific Proteomic Response at Two Weeks .... 179
    4.3.2.1 Autograft – Gold Standard Protein Expression ....... 179
    4.3.2.2 The Influence of a Natural ECM Material on Nerve Regeneration ................................................................. 181
    4.3.2.3 PLGA: A Synthetic Alternative to the Native ECM ........ 183
    4.3.2.4 Validation of Material Specific Protein Expression (ELISA) ........................................................................... 184
4.3.3 The Effect of Gap Distance on the Proteomic Response ………… 185

4.3.3.1 The Effect of Gap Distance on Autograft ……………… 185

4.3.3.2 The Effect of Gap Distance in a Collagen Conduit ……… 195

4.3.3.3 The Addition of Intraluminal Structure to a Hollow Conduit and the Effect of Gap Distance ……………………………….. 196

4.3.3.4 Validation of Expression at Different Gap Lengths ……… 198

4.4 Discussion ……………………………………………………………. 199

4.5 Conclusions ………………………………………………………… 206

4.6 References …………………………………………………………… 206

5. Summary and Future Studies ……………………………………… 215

5.1 Introduction ………………………………………………………… 216

5.2 Summary ……………………………………………………………. 218

5.2.1 Phase I: The Development of a Biomaterial Platform for Repair …………………………………………………………………... 217

5.2.2 Phase II: Optimising Packing Density, Collagen Fibre Degradation and Assessing of Functional Recovery ……………… 218

5.2.3 Phase III: A Biomaterials-Induced Proteomics Response to Conduit-Mediated Nerve Repair ……………………………….. 219

5.2.4 Phase IV: The Effect of Intraluminal-Collagen Fibres on the Biomaterial-Induced Proteomics Changes during Peripheral Nerve Repair across both Non-Critical and Critical Nerve Gaps ……… 219

5.3 Limitations …………………………………………………………… 221

5.3.1 Phase I …………………………………………………………… 221

5.3.2 Phase II …………………………………………………………… 222

5.3.3 Phase III …………………………………………………………… 223
5.3.4 Phase IV ................................................................. 223

5.4 Conclusions ................................................................. 224

5.4.1 Phase I ................................................................. 224

5.4.2 Phase II ................................................................. 225

5.4.3 Phase III ................................................................. 225

5.4.4 Phase IV ................................................................. 225

5.5 Future Studies ............................................................. 228

5.5.1 An Intraluminal Fibre Conduit as a Controlled Drug/Cell Delivery System ................................................................. 228

5.5.1.1 Optimising Packing Density and Fibre Distribution ................................................................. 228

5.5.1.2 Controlled Release and Local Gradients .......... 229

5.5.1.3 Triggered Release of Temporal Components for Early and Late Stage Nerve Regeneration .................. 230

5.5.1.4 Cellular Depots Releasing Molecules of Interest ................................................................. 231

5.5.2 The Identification of Novel Protein/Gene Targets ........ 231

5.5.3 The Potential of Transcriptional Regulators, Glycoprotein and Apolipoprotein Neural Therapeutics .................. 232

5.5.4 Targeting the Neuromuscular Junction or Dorsal Root Entry Zone ................................................................. 234

5.6 References ...................................................................... 236

Appendices ......................................................................... 242
Chapter 1

Figure 1.1 Regenerative sequence occurring within a hollow NGC. Figure adapted from Belkas et al. This regenerative process occurs in five main phases: (1) the fluid phase - plasma exudate fills the conduit resulting in accumulation of neurotrophic factors and ECM molecules; (2) the matrix phase - an acellular fibrin cable forms between the proximal and distal nerve stumps; (3) the cellular phase - Schwann cells, endothelial cells and fibroblasts migrate (from the proximal and distal nerve stumps), align and proliferate along the fibrin cable forming a biological tissue cable; (4) axonal phase - regrowing axons use this biological tissue cable to reach their distal targets; (5) myelination phase - Schwann cells switch to a myelinating phenotype and associate with regenerated axons forming mature myelinated axons. ……………………… 7

Figure 1.2 Summarised schematic of the structural repair strategies used for improving existing hollow nerve guidance conduits. Repair strategies include the use of intraluminal guidance structures and micro-grooved luminal designs to provide additional structure support and topographical guidance to regenerating axons and migrating Schwann cells. A similar strategy involves using electrospun fibrous conduits with the advantages of high flexibility and porosity, a high surface area-volume and fibres that can be aligned for guided Schwann cell migration and proliferation and axonal growth. Variations in conduit design include the use of multichannel conduits for control of axonal dispersion, as well as designs, which optimise nutrient exchange or introduce external stimuli. These designs may be used alone or in combination, but they also may require further surface functionalisation.
These surface modifications can increase cell adhesion, migration, alignment and proliferation. 

**Figure 1.3** Schematic diagram of cellular and molecular based therapies used for the creation of a more conductive nerve microenvironment. Examples of molecular therapies include growth factors (VEGF, bFGF) and neurotrophic factors (NT3, NGF). Likewise, cell therapies involve the use of Schwann cells, stem cells (ASCs and MSCs) and genetically modified cells (Schwann cells overexpressing GDNF). These can be delivered by a number of means including: (i) suspension within solution or a biomaterial matrix (hydrogel, sponge); (ii) released via a diffusion based systems (controlled released via cross-linking, slow degrading polymer coatings from luminal wall); (iii) the use of affinity based delivery systems (factors conjugated to a fibrin matrix); (iv) microsphere (e.g. collagen, fibrin) encapsulation which can either be suspended within the lumen or released from the luminal wall. 

**Chapter 2**

**Figure 2.1** Conceptual diagram of the incorporation of either structured or unstructured fibres into a biomaterial nerve guidance conduit. The incorporation of intraluminal fibres within a nerve guidance conduit will create a platform for repair, while simultaneously providing topographical guidance cues to regenerating axons and migrating glial cells, ultimately creating an inductive environment for repair.

**Figure 2.2** Images of unstructured and structured collagen fibres and their incorporation into a hollow collagen nerve guidance conduit. 
(A) SEM image of an unstructured collagen fibre with a diameter of approximately 50 µm. Scale bar, 10 µm. (B) SEM image of structured collagen fibre with four channels on the surface of the fibres (channel diameter 10 µm). Scale bar, 10 µm. (C) Photo of a conduit with 18 structured collagen fibres.
inserted into the lumen of a hollow collagen nerve guidance conduit. Fibres are shown to be protruding from the lumen of the conduit. For implantation, fibres are trimmed to a length of 10 mm and inserted into a 12 mm long hollow nerve guidance conduit. This allows the proximal and distal nerves to be implanted into the conduit without inducing axial compression on the intraluminal fibres. Scale bar, 1.5 mm.

Figure 2.3
Excimer laser rotary stage setup for structuring of the fibres and scanning electron microscope images of the SEM-FIB process. (A) Photograph of the fabricated rotary stage used to hold the collagen fibres in place during structuring. Stage worked readily with the excimer laser system’s software package to allow precise control of the rotation of the fibres and alignment of the laser. Scale bar, 5 cm. (B-D) SEM images of the sequence of events in the SEM-FIB process. A suitable region of interest was identified (B) and the sample was rotated to 54° for structuring. High focus ion beam currents were used to generate a cross-section and subsequent polishing of the cross section was performed at lower beam currents. The resulting cross section can be seen in (C). Quantification of the cross section parameters was performed at higher magnification as in (D). Scale bars, 50 µm (B and C), 1 µm (D). ................................................................. 76

Figure 2.4
Proof of concept micro-structured fibres (A) A PGCL fibre structured initially with a single 10 µm longitudinal groove. Scale bar, 20 µm. (B) The PGCL fibre in the SEM-FIB system for analysis of the structured fibre. Scale bar, 5 µm. (C) A PGCL fibre incorporating multiple longitudinal grooves along its surface demonstrating the ability of the excimer laser system to structure a fibrous construct. Scale bar, 50 µm. .. 84

Figure 2.5
Range of micro-structures produced on the surface of the extruded collagen fibres using the excimer laser system (A-D).
Features can be produced with a high degree of reproducibility and can be quantitatively measured using a combination of SEM-FIB analysis and white light interferometry. All scale bars, 2 µm.

Figure 2.6 Neuronal interaction of PC12 cells with structured and unstructured control fibres. (A-C) PC12 cells shown extending rhodamine phalloidin stained neurites (green) along the surface of the intraluminal collagen fibres. Scale bars, 2 µm. (D) Illustration of PC12 cells/dissociated DRG embedded in a collagen type I hydrogel interacting with the surface of the collagen fibre. (E+F) Quantification of neurite length and alignment using Image J image analysis software. Structured fibres demonstrated significantly longer and more parallel neurite outgrowth than the control unstructured fibres (*p<0.05). (G) Schematic of the parameters analysed in relation to the long axis of the collagen fibres. (n=3 per group)

Figure 2.7 Dissociated dorsal root ganglion seeded on the surface of both structured and unstructured collagen fibres. (A) NF160+ neurites/axons (green) were seen to grow across the surface of the collagen fibres from the gel (top left) and extend along the surface. Neurites can be seen to overlap in the bottom right hand corner of the field, suggesting lack of topographical guidance. (B) NF160+ axons can be seen to grow along the surface on the fibres primarily sticking to on axis of the fibres with minimal crossover at the center of the fibre. Results suggest more parallel growth than that of unstructured fibres. (C) Schematic diagram of DRGs embedded in collagen type I hydrogel. Scale bars, 50 µm.

Figure 2.8 Microscopic images of surgical implantation of conduit groups and autograft at Day 0 (A and B) and images of explanted NGCs after 16 weeks in vivo (C and D). (A)
Representative image of conduit groups implantation. (B) An implanted autograft is secured in place with two 10-0 nylon sutures on Day 0. (C) An explanted nerve guidance conduit showing a regenerated tissue cable running proximal to distal 16 weeks after implantation (D) In vivo image of structured fibre conduit showing a defined regenerated tissue cable after 16 weeks in vivo.

Figure 2.9 Retrograde tracing results. (A) Schematic drawing of the concept of simultaneous retrograde tracing. A diamidino yellow dye is applied to the peroneal branch of the sciatic nerve. The dye is applied to the nerve for a period of 30 minutes to allow adequate absorption of the dye. The dye is subsequently transported retrograde to the spinal column over a period of 1 week. The absorption and transport of the dye will label the nuclei of the associated neurons yellow. This confirms regeneration of axons down the peroneal branch of the nerve and that the same motor neurons are attempting to reinnervate peroneal nerve targets. The fast blue dye is simultaneously applied at the same initial time period to the tibial nerve and allowed to be transported retrograde in the same manner as the aforementioned dye. Fast blue dye absorption will label the cytoplasm of the neurons with a blue colour. This confirms regeneration down the tibial nerve branch and that the same neurons are correctly attempting to reinnervate tibial nerve targets. Finally if both dyes are present in the same neuron (yellow nucleus, blue cytoplasm) then it can be concluded that one neuron is incorrectly attempting to synapse with both peroneal and tibial nerve targets. (n=6 per group).

Figure 2.10 Histological overview of the regenerated nerve cables seen present at the mid-graft level of each of the experimental groups as follows: (A) Autograft (B) Hollow Conduit (C) Structured Fibre Conduit and (D) Unstructured Fibre Conduit.
In both of the collagen fibre groups, fibres can be seen to have successfully been incorporated into the regenerative process with little to no apparent foreign body response. Fibres appear to have been reduced in area from the original circumferential area it is likely that the fibres at this stage have begun to partially degrade. All scale bars: 100 µm.

Figure 2.11  Morphological analysis of axonal area, density and the axonal fibre distribution from the midgraft of the harvested neuronal tissue. (A) Autograft shows a significantly higher nerve regeneration area than all of the experimental conduit groups ($p<0.05$). (B) Graph details the distribution of axons within the regenerated tissue across all experimental groups. Autograft and fibre groups show a higher proportion of myelinated axons with diameters in the range of 3-4 µm than that of control hollow conduits. (C) Graph shows no significant differences seen between all experimental group for axonal density. (D) Figure detailing procedure for quantification of axonal area. The axonal area was traced along its extremities (red) using image analysis software (ImageJ) and the overall axonal area (including the collagen fibres) was quantified. Collagen fibres present in the neural tissue was similarly traced (green) and the overall fibre area was quantified. The final axonal area used in (A) was calculated by subtracting the fibre area from the overall axonal area. (n=8 per group for all measurements).

Figure 2.12  Microscope images (toluidine blue stained) of unstructured (A and B) and structured (C and D) shown to have been successfully incorporated into the host regenerative process. Vascular cells can be seen to be growing in close proximity to the intraluminal fibres as well as a number of regenerated myelinated axons. Scale bars, 25µm and 10 µm for 400x and 1000x images respectively.
Figure 2.13 Microscopic images (1000x magnification) of regenerated nerve at the midgraft level stained with 1% toluidine blue. Images shown represent the following groups: (A) Unstructured fibres (B) structured fibres, (C) autograft and (D) hollow conduit. All scale bars, 10 µm. (n=8 per group). … 108

Figure 2.14 Morphological analysis of regenerated nerve. (A) An unbiased counting frame was applied in a systematic random manner to multiple fields over the entire nerve regeneration area. Inclusion lines are represented in blue and exclusion lines are represented in red. Scale bar, 5 µm. (B) Axon counts show significantly more axonal profiles in autograft group versus all experimental groups (p<0.05). (C + E) No significant difference was seen in the mean myelinated/unmyelinated diameter between each experimental group (p<0.05). (D) G ratio was significantly lower in the autograft group versus all conduit groups. (F) Autograft displayed a significant higher myelin thickness versus all the conduit groups (p<0.05). (n=8 per group). ………………………………………………. 112

Figure 2.15 Morphological distribution of regenerating axons across the length of the nerve guidance conduit. (A) Axon densities were seen to be significantly lower at the proximal and distal components of the nerve in comparison to the midsection (p<0.05). Axons were more diffuse and wide spread and showed a tendency to be become more compact as they approached mid-graft. (B) Axonal area was significantly higher proximal to the midsection for both groups (p<0.05). This was due to the lower axonal densities seen in the proximal and distal components. (C) Myelinated axon diameters showed no significant differences from the proximal to distal nerve sections. (D) Diagrammatic representation of the regenerating nerve components showing the classical tapered profile associated with regeneration in a nerve guidance conduit. ………………………………………………….. 114
Figure 2.16 TEM micrographs of the structured and unstructured collagen fibres. (A) Structured fibres result in the formation of defined fascicular bundles with an outer boundary (white arrows) with native collagen being deposited within the bundles and the inter-fascicular space (black arrows). (B) Unstructured collagen fibres can be clearly seen in close proximity to regenerating axons (black arrow), however fascicular bundles are less organised with no apparent boundaries of the bundles. The deposition of native collagen filaments is less dense and scattered compared to that of the structured group. All scale bars, 4 µm. .......................................................... 116

Chapter 3

Figure 3.1 The transition from a low packing density (Chapter 2) to the high packing density (Chapter 3). Packing density is calculated by multiplying the total number of fibres by the cross-sectional area of an individual collagen fibre and dividing by the total cross-sectional area of the conduit. …. 128

Figure 3.2 Masson’s trichrome image of intraluminal collagen fibres implanted in a rat subcutaneous model. (A+B) Two week and four week images of control non-cross-linked fibres show intraluminal collagen fibres (black arrows) present two weeks post implantation. Dark purple zone at centre of the four week control represents the implanted suture (n = 3). (C+D) Two week and four week images of cross-linked fibres show collagen fibres present at both time points (black arrows) (n = 3). (E) Ninhydrin assay showed significant reduction in number of amines in the 30:10 mM EDC–NHS collagen fibres and 0.625% GTA fibres in comparison to control non-cross-linked collagen fibres (p < 0.05, n = 4 for all treatment groups, mean ± SD). .......................................................... 137

Figure 3.3 Assessment of nerve morphometric parameters at 12 weeks. (A) The 15 mm autograft was seen to be significantly greater,
in terms of axonal density, versus the conduit groups. No significant differences in axonal density were seen at the 15 mm gap length. (B) The 10 mm autograft showed significantly more fascicular area than the hollow conduit. No significant difference was seen versus the fibre group. At 15 mm, both the autograft and the fibre conduit showed significantly higher fascicular area than the hollow conduit group. The 10 mm autograft was significantly greater than the 15 mm autograft. (C+D) No significant differences were seen across the two gap lengths in terms of myelinated fibre and axon diameters, except in terms of the 10 mm hollow conduit which was significantly less than autograft in terms of myelinated fibre diameter. (E+F) At 10 mm autograft showed a significantly thicker myelin sheath and higher g-ratio than both the conduit groups. At 15 mm no significant differences were seen. (G) Autograft showed a significantly greater number of axons than both the fibre groups at both gap lengths. Scale bar, 5 µm (*p < 0.05, one-way ANOVA followed by Newman-Keuls post-hoc test; #p < 0.05, unpaired Student’s t-test; mean ± SEM).

Figure 3.4 Measurement of regenerated muscle force and recovery from muscular atrophy. (A) Photograph of rat placed in the automated functional assessment station. (B) Assessment of force recovery in the tibialis anterior (TA) muscle. The 10 mm autograft showed a significantly greater max tetanic force than that of the 10 mm hollow conduit (left). No significant differences were seen across the remaining groups. In terms of max twitch force (right), no significant differences were seen across groups. (C) Assessment of extensor digitorium longus (EDL) showed no significant differences in terms of max twitch and force across all experimental groups. (D) Photograph shows the isolated TA, EDL and gastrocnemius (GC) muscles isolated at 12 weeks from both the injured and
uninjured sides of the animal. (E) The TA showed significantly more recovery from muscular atrophy in the 10 mm autograft than the 10 mm hollow conduit group. Autograft significantly outperformed both the conduit groups at the 15 mm gap length. (F) The 10 mm autograft showed significantly greater recovery than the 10 mm hollow conduit and both the 15 mm conduit groups respectively. (G) In terms of GC muscle recovery, the autograft was significantly greater than the conduit groups at both gap lengths. The 10 mm autograft showed a significantly higher muscle mass ratio than that of the 15 mm graft (*p-value < 0.05, one-way ANOVA followed by Newman-Keuls post-hoc test; #p-value < 0.05, unpaired Student’s t-test).

Figure 3.5 Ranking of nerve regeneration parameters. (A) Ranking of grouped nerve morphometric components. Parameters of myelin thickness, number of myelinated fibres, and fascicular area were scored from 1 to 48 based on their performance in that particular category and grouped together to give an overall indication of performance across those parameters. The 10 and 15 mm autograft can be seen to significantly outperform both the conduit groups. No significant differences across gap lengths were seen. (B) For functional recovery, the 10 mm autograft significantly outperformed the 10 mm hollow conduit. Across gap lengths, the 10 mm autograft significantly outperformed the 15 mm autograft. No significant differences were seen across the remaining groups. (C) In terms of muscle mass recovery ranking, the 10 mm and 15 autografts significantly outperformed the conduit groups. The 10 mm autograft performed significantly better than its 15 mm counterpart. (D) Overall, the 10 mm and 15 mm autografts outperformed the conduit groups. And the 10 mm autograft significantly outperformed the 15 mm autograft (* p-value <
0.05, one-way ANOVA followed by Newman-Keuls post-hoc test; # p-value < 0.05, unpaired Student’s t-test). ………. 145

Chapter 4

Figure 4.1 Schematic diagram of the factors assessed which have an effect on peripheral nerve repair. The material used, gap distance treated, and the incorporation of intraluminal structure have been shown extensively to influence nerve regeneration. This study aims to understand this influence at the base proteomic level in the acute phase (first two weeks) of nerve repair as a function of time and their spatial expression. This analysis allows for the identification of key regulators which are important for successfully nerve repair, throughout the conduit, as a function of both the material and strategy used for repair. …………………………………… 162

Figure 4.2 Schematic diagram of the study layout (A) and workflow of ITRAQ tagging and MudPIT mass spectrometry analysis (B). ……………………………………………………………… 164

Figure 4.3 Biological network analysis of differentially regulated proteins as a function of the material used in the proximal component of the graft. (A) Top biological networks corresponding to each material and the most significant biological functions regulated in each. (B) Overview of the significantly identified and regulated proteins in the proximal graft. (C) Overlap of molecules in the top biological networks. (D) Snapshot of proximal regenerative environment – proteins that are synthesised locally in axons and Schwann cells, macrophages and blood borne components infiltrate the injured nervous area and are responsible for the changes in protein expression demonstrated herein. ………………… 173

Figure 4.4 Differences in biological function, networks, and focus molecules as a function of the material used. (A) Top
biological functions and the associated biological networks and comparative differences in their regulation. (B) Differences in regulation of focus molecules, red (upregulated) and green (downregulated). (C) Overlap of focus molecules in top networks. (D) Schematic of the contribution of cellular, neuronal, and debris components at midgraft.

**Figure 4.5** Material-specific differences at the distal end of the regenerating nerve. (A) Biological networks and top tier biological functions as a function of the material used. (B) Focus molecules and their regulation at midgraft. (C) Overlap of molecules in top biological networks generated by IPA software.

**Figure 4.6** Material Specific ELISA data validating core proteins in the proximal, middle and distal environments of the injured nerve two weeks post implantation. Data was compared using one-way ANOVA followed by the Newman-keuls post-hoc test (*p*-value < 0.05 was deemed significant).

**Figure 4.7** Pathway analysis of distance-specific proteomic changes at the proximal end of the graft/conduit. Differential networks are produced depending on the distance of the proximal graft from the distal end. Across all treatment groups, top biological functions show different degrees of biological significance depending on the gap distance treated.

**Figure 4.8** Pathway analysis of distance-specific proteomic changes at the midpoint of the graft. The figure shows the networks generated and significant biological networks of each treatment group and how they vary across increasing gap lengths.

**Figure 4.9** Changes in protein expression and associated changes in biological networks and top tier functions at the distal end of
the graft. The figure shows changes in the autograft, hollow, and filled collagen conduits as a function of the gap length treated. ……………………………………………………… 192

Figure 4.10 ELISA data for treatment groups based on the gap distance treated. All data was analysed by one-way ANOVA followed by a Newman Keuls post-hoc test for comparison of groups at different gap lengths and compared to the 10 mm ($) and 15 mm (*) autograft respectively (a \( p \)-value < 0.05 was deemed significant). ………………………………………………… 201

Chapter 5

Figure 5.1 Schematic diagram summarising the conclusions of the four main phases of the project. ……………………………………….. 220

Figure 5.2 Schematic showing concepts of future studies and their sites of application. ………………………………………………… 227

Appendices

Figure I-1 Isolation of sciatic nerve using a gluteal muscle splitting approach. ………………………………………………………… 254

Figure I-2 Removal of desired nerve segment using microscissors. .. 254

Figure I-3 Suturing of the collagen nerve guidance conduit using 10 – 0 Ethicon™ sutures. ………………………………………………………… 254

Figure L-1 Toluidine blue stained nerve section shown traced, for evaluation of the axonal area. Total axonal area is represented in red and fibre area is represented in green. Scale bar, 30 \( \mu \)m. ………………………………………………………… 258

Figure L-2 Unbiased counting frame for evaluation of axon number, density and diameter of fibers. Exclusion lines are represented in red and inclusion lines are represented in blue. Scale bar, 5 \( \mu \)m. ………………………………………………………… 259
Figure N-1  Rat placed within the automated functional assessment station. In the picture the tibialis anterior muscle is attached to the strain gauge (black arrow) and the sciatic nerve has been attached to the stimulating electrodes (blue arrow). ……. 261
LIST OF TABLES

Chapter 1

Table 1.1  Nerve Guidance Conduits – Clinically Approved and Under Development .................................................. 5

Table 1.2  The Use of Intraluminal Guidance Structures ......................... 14

Table 1.3  Luminal Guidance Features and Variations in Material Design ................................................................. 22

Table 1.4  Molecular Therapies for the Creation of a Conductive Microenvironment .................................................. 34

Table 1.5  Examples of Cell Based Approaches for Peripheral Nerve Repair ............................................................... 41

Chapter 2

Table 2.1  List of the Treatment Groups and Number of Animals Used for the Current In Vivo Study and the Experimental Procedures Carried out on Each Respective Group. ....................... 81

Table 2.2  Parameters for Excimer Laser Structuring of the PGCL Fibres and Resulting Diameters and Depths of the Micro-Structured Fibres ............................................................... 83

Table 2.3  Parameters for Excimer Laser Structuring of the Extruded Collagen Fibres and Resulting Diameters and Depths of the Micro-Structured Fibres. ............................................... 85

Chapter 3

Table 3.1  Design of Animal Study for Investigating the Effect of a Densely Packed Intraluminal Fibre Conduit on Nerve Regeneration across a Non-Critical and a Critical Nerve Gap. ........................................................................ 132
Chapter 4

Table 4.1 List of Abbreviations .............................................154
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Abstract

Peripheral nerve injuries are a major clinical problem which currently affect over one million people worldwide. Transection injuries caused by workplace and home injuries, road traffic accidents and by intentional harm lead to debilitating and painful injuries for those afflicted. Treatments are limited and largely ineffective. The patient’s own donor nerve (autograft) is the gold standard for repair but this treatment is largely unsuccessful, suffering from limited supply with donor site morbidity and pain. The use of hollow nerve guidance conduits (NGCs) is the current clinically approved alternative. However these have shown limited applicability in the clinic. Thus, the objective of this thesis was to improve and understand the use of these hollow NGCs and attempt to improve functional nerve regeneration using the concept of intraluminal guidance. Intraluminal guidance involves the incorporation of biomaterial structures (in this instance extruded collagen fibres) to replace the natural fibrin cable which forms during repair and guide regenerating axons to their distal targets. An intraluminal collagen fibre conduit was tested across both a non-critical (10 mm) and critical nerve gap (15 mm) in a rat sciatic nerve model for its ability to promote repair. Through sequential studies, it was shown that the incorporation of intraluminal structure reduced the misdirection of regenerating axons during repair and also achieved functional regeneration in a number of parameters similar to that of the gold standard. Additionally, the intraluminal fibre conduit demonstrated the ability to regenerate across, the aforementioned, critical nerve gap (a nerve gap where regeneration is minimal if not absent). Based on these results, a further understanding of the early molecular mechanisms resulting in these beneficial effects was warranted. Using proteomics analysis the effect of the biomaterial used, the incorporation of intraluminal structure and the influence of increasing gap distance were documented. From this understanding, the key molecular components governing the increased nerve regeneration seen in the intraluminal fibre conduit and the robust regeneration seen within autograft were revealed. Based on the conclusions of this study, an intraluminal fibre conduit or a hollow biomaterial conduit may be further functionalised to match the molecular profile of the gold standard for repair.
Chapter One

Introduction

Contents of this chapter have previously been published in:

1.1 Peripheral Nerve Injury and Repair
Peripheral nerve injury is a large scale problem annually affecting more than one million people worldwide. These injuries often result in painful neuropathies due to reduction in motor function and sensory perception. Peripheral nerve injuries are common in both civil and military environments and are primarily the result of transection injuries or burns, but may also arise from degenerative conditions (1, 2). Over relatively short nerve gaps, spontaneous natural regeneration may occur. However, over larger gaps, microsurgical repair is essential for nerve repair (3-5).

Currently there are a variety of microsurgical repair methods available, including direct repair, autograft/allograft transplantation, and the use of hollow nerve guidance conduit (NGC) repair (3-5). Direct nerve repair (also known as end-to-end suturing, end-end repair, end-to-end neurorrhaphy or end-to-end coaptation) is the preferred method of treatment for peripheral nerve repair (6). This method of treatment, however, is limited to the treatment of short nerve defects requiring tension-free suturing of the injury site (6). For optimal regeneration the nerve stumps must be correctly aligned and repaired with minimal tissue damage, using the minimal number of sutures. This repair method is limited to nerve gaps shorter than 5 mm (7). Beyond this relatively short gap, injuries are precluded from primary repair and alternative tissue engineering strategies are the main option.

1.2 Autograft: The Limited Gold Standard
For patients precluded from direct repair, autograft is the current gold standard and has remained so for the last 50 years (7-9). These grafts being taken primarily from the sural nerve of the treated patient and have demonstrated a success rate of only 50% on patients treated (6, 10). These grafts are primarily sensory, due to the unavailability of motor nerves, limiting their potential to repair a motor nerve deficit (tibial) and mixed nerve injuries (sciatic) and may be one of the primary reasons for the poor functional recovery rates associated with autografts (11-13). The use of sensory nerves for the treatment of motor nerve deficits causes morphometric mismatches in the native environments, mismatch in axonal size, distribution and alignment (12, 14). Motor neurons are primarily in the range of 3 - 20 µm, whereas
sensory neurons range from 0.2 - 15 µm (15). If sensory nerve grafts are therefore used to treat an injury composed primarily of motor axons, there is a potential for size mismatch, potentially limiting regeneration. Secondary to this limitation, the use of autograft has a number of disadvantages including donor site morbidity, the requirement for a second surgical site, a very limited supply, donor site mismatch, and the possibility of painful neuroma formation and scarring (16). The use of autograft also requires secondary removal of degenerated axons and myelin by the host from the graft itself, increasing the healing time (17). Similarly in recent studies, it has been shown that sensory and motor neurons have different Schwann cell modalities and if placed in the incorrect microenvironment, may limit their regenerative ability (18).

Autograft use is currently limited to a critical nerve gap of approximately five cm in length and beyond this distance requires the use of allograft (2). Allograft requires the use of extensive immune-suppression up to 18 months post implantation, and patients become susceptible to opportunistic infections, occasionally resulting in tumour formation (19). The combinatorial effects of these limitations may be the primary cause for the limited recovery associated with autograft and allograft treatment. In efforts to address the limitations of these nerve grafting techniques, the primary alternative is the use of hollow NGCs.

1.3 The Development of Nerve Guidance Conduits

The use of hollow NGCs was originally proposed for nerve repair as early as 1881 with the first successful application occurring in 1882, where a hollow bone tube was used to bridge a 30 mm nerve gap in a dog (20). Today, the use of hollow NGCs is the clinically approved alternative to autograft repair. These conduits have a number of advantages for nerve repair, including limited myofibroblast infiltration, reduced neuroma and scar formation, reduction in collateral sprouting and no associated donor site morbidity, and facilitates the accumulation of a high concentration of neurotrophic factors; ultimately guiding regenerating nerves to their distal targets (21). However, the use of hollow NGCs is currently limited to a critical nerve gap of approximately 4 cm (22). These NGCs allow the creation of a controlled micro-environment for the regeneration of nerve fibres and have shown some
clinical success (23, 24). Current clinically translated NGCs are primarily made from synthetic materials such as poly-glycolic acid (PGA), polylactide-caprolactone (PLCL), various combinations of the PGA or PLCL, or from animal extracted collagen (Table 1.1) (6, 23, 24).

Despite some success in nerve repair, these hollow NGCs fail to match the regenerative levels of autograft and show poor functional recovery (25). Early attempts of improvements for NGCs involved variations in material design and fulfilling a number of criteria for the ideal hollow conduit. These criteria included: (i) limiting scar infiltration, while allowing diffusion of nutrients into the conduit and wastes to exit the conduit; (ii) providing sufficient mechanical properties for structural support; (iii) exhibiting a low immune response; (iv) biodegradability, to remove the need for secondary surgery and to prevent chronic inflammation and pain caused by nerve compression due to the eventual collapse of implanted NGCs (26). For the first criterion, adequate nutrient exchange and waste removal in a NGC can be achieved, if the material is permeable with a molecular weight limit of approximately 50 kDa (27-29). For the remaining criteria, a number of different materials both biological (e.g. collagen, SIS) and synthetic (e.g. polyhydroxybuturate (PHB), PVA, PGA) have been considered throughout the years (3, 5, 30, 31). These past studies have shown some improvements in nerve regeneration and functional recovery; however, not to the full extent.

1.4 Regeneration within a Hollow Nerve Guidance Conduit

Understanding the natural regenerative process occurring within hollow NGCs is a prerequisite for improved nerve regeneration. Briefly this regenerative process can be divided into five main phases: (i) the fluid phase; (ii) the matrix phase; (iii) the cellular migration phase; (iv) the axonal phase; and (v) the myelination phase (Figure 1.1) (7). This initial fluid phase is followed by the formation of an acellular fibrin cable between the proximal and distal stumps, formed from the former influxed ECM precursor molecules (7, 32, 33). This fibrin cable usually forms within one week of NGC repair and forms an ECM bridge for the next stage of regeneration.
<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Composition</th>
<th>Degradation Time</th>
<th>Available lengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurogen®</td>
<td>Integra Neurosciences, Plainsboro, NJ</td>
<td>Collagen Type I</td>
<td>4 years</td>
<td>3 cm</td>
</tr>
<tr>
<td>NeuraWrap™</td>
<td>Integra Neurosciences, Plainsboro, NJ</td>
<td>Collagen Type I</td>
<td>4 years</td>
<td>4 cm</td>
</tr>
<tr>
<td>NeuroMend™</td>
<td>Collagen Matrix, Inc., Franklin Lakes, NJ</td>
<td>Collagen Type I</td>
<td>4 – 8 months</td>
<td>2.5 cm</td>
</tr>
<tr>
<td>Neuromatrix™ / Neuroflex™</td>
<td>Collagen Matrix, Inc., Franklin Lakes, NJ</td>
<td>Collagen Type I</td>
<td>4 – 8 months</td>
<td>2.5 cm</td>
</tr>
<tr>
<td>Neurotube®</td>
<td>Synovis Micro Companies Alliance, Birmingham, AL</td>
<td>Woven polyglycolic acid (PGA)</td>
<td>6 – 12 months</td>
<td>3 cm</td>
</tr>
<tr>
<td>Neurolac®</td>
<td>Polyganics Inc., Netherlands</td>
<td>Poly((\text{DL})-lactic-co-(\varepsilon)-caprolactone)((PCL))</td>
<td>2 – 3 years</td>
<td>3 cm</td>
</tr>
<tr>
<td>Salubridge™ / Hydrosheath™ or Salutunnel™</td>
<td>Salumedica LLC, Atlanta, GA</td>
<td>Salubria™ - Polyvinyl alcohol (PVA) hydrogel</td>
<td>Non-biodegradable</td>
<td>6.35 cm</td>
</tr>
<tr>
<td>Surgisis® Nerve Cuff /Axoguard™</td>
<td>Cook Biotech Products, West Lafayette, IN</td>
<td>Porcine small intestinal submucosa (SIS) matrix</td>
<td>Not reported</td>
<td>4 cm</td>
</tr>
<tr>
<td>Avance® Nerve Graft</td>
<td>AxoGen®</td>
<td>Decellularised allograft</td>
<td>Not reported</td>
<td>3 – 4 cm</td>
</tr>
</tbody>
</table>
In the next phase of repair, Schwann cells from the proximal and distal nerve stumps, as well as some endothelial cells and fibroblasts, migrate along this fibrin cable (1, 7). These Schwann cells subsequently proliferate and align, forming an aligned Schwann cell cable i.e. the glial bands of Büngner. This biological tissue cable provides a trophic and topographical tissue cable for the axonal phase of repair. During this axonal phase of repair, new regenerative axonal sprouts, guided by their individual growth cones, use this biological cable tissue as a guidance mechanism to ultimately reach their distal target. These regenerating axons reach the aforementioned targets after approximately two to four weeks (1, 7, 32).

It is worth pointing out that during the period of the cellular and axonal repair phase, the fibrin cable, which has a degradation time of approximately two weeks, has degraded, having fulfilled its role for providing a structural cue for cellular migration (34, 35). Following the axonal phase, Schwann cells switch from the more proliferative “regenerative” phenotype to a presumably more mature “myelinating” phenotype (30). These mature Schwann cells subsequently wrap around the larger regenerated axons to form the myelin sheath (a mature myelinated axon), resulting in some functional repair of nerve fibres; this usually occurs six to sixteen weeks after repair and longer in some larger animal models (36, 37). This regenerative sequence takes place within hollow NGCs up to a critical nerve gap of approximately 4 cm in humans and approximately 1.5 cm in a rat sciatic nerve model, after which regeneration is limited or absent (3, 30, 38, 39). Functional recovery however remains poor across all nerve gaps (39, 40).

1.5 Guided Nerve Regeneration – The Use of Structural Guidance Cues

Insufficient levels of regeneration in a hollow NGC, especially across critical nerve gaps, may be attributed to the inadequate formation of extracellular matrix components during the initial stages of regeneration i.e. the formation of the fibrin cable (14, 41). Without the formation of this aligned ECM bridge, there is limited migration of native Schwann cells into the site of the lesion, from both proximal and distal nerve stumps and consequently a reduction in
Figure 1.1. Regenerative sequence occurring within a hollow NGC. Figure adapted from Belkas et al. (7). This regenerative process occurs in five main phases: (1) the fluid phase - plasma exudate fills the conduit resulting in accumulation of neurotrophic factors and ECM molecules; (2) the matrix phase - an acellular fibrin cable forms between the proximal and distal nerve stumps; (3) the cellular phase - Schwann cells, endothelial cells and fibroblasts migrate (from the proximal and distal nerve stumps), align and proliferate along the fibrin cable forming a biological tissue cable; (4) axonal phase - re-growing axons use this biological tissue cable to reach their distal targets; (5) myelination phase - Schwann cells switch to a myelinating phenotype and associate with regenerated axons forming mature myelinated axons.
Introduction

the formation of glial bands of Büngner, the essential trophic and topographical guidance structure for regenerating axons (1, 7, 14, 33, 42). In attempts to replace the support and guidance provided by this ECM tissue cable, a number of strategies for nerve repair have focused on the addition or manipulation of structure in NGCs (Figure 1.2).

1.5.1 Intra-Luminal Guidance Structures – Replacing or Supporting the Fibrin Cable

One current strategy for nerve repair is the addition of structural intra-luminal guidance cues, which may act as a replacement for the unformed or incomplete fibrin cable, or act as an additional anchor for its formation (39, 43). These intra-luminal guidance channels act as a platform for Schwann cell migration and proliferation and simultaneously can provide additional topographical guidance cues to regenerating axons. Ultimately, the addition of intra-luminal channels aim to recapitulate the hierarchical organisation and biological function of the native extracellular matrix (14). In early studies, by Matsumoto et al., the addition of laminin coated collagen fibres into a PGA NGC were shown to bridge a gap of eight cm within a canine peroneal nerve model far exceeding that of a critical nerve gap (37). However, functional recovery was not characterised. This concept was further explored, by Yoshii et al., using bundles of collagen fibres alone without the use of an external conduit structure (44, 45). Gaps of 20 mm and 30 mm were consecutively bridged in successive studies; however functional recovery remained poor. Despite this, the addition of intra-luminal fillers clearly showed the ability to extend the regeneration limits of hollow NGCs (44, 45). Over the years, a number of variations of these intra-luminal guidance structures have been used within hollow NGCs in attempts to bridge a critical nerve gap or to enhance functional recovery (Table 1.2). Similar studies by Ngo et al. highlighted the importance of “packing density” (or “void fraction”) (36), as well as the distribution of intra-luminal structures, as essential considerations for their incorporation within hollow NGC (40, 43, 46).

A study by Ngo et al. observed that high densities (approximately 15 - 30% of the cross sectional area) of poly(L-lactide) (PLLA) microfilaments inhibited nerve regeneration (47). At lower densities (approximately 3.75 -
7.5% of the cross-sectional area), regeneration was increased and the ability to bridge a critical nerve gap in a rat sciatic nerve model was demonstrated. These were taken as the optimal packing densities for the introduction of intra-luminal structures, as lower densities resulted in the fibres settling to the bottom of the conduit; while higher densities resulted in the inhibition of regenerating nerves (47). This inhibition was similarly seen, by Stang et al., where the addition of a dense collagen sponge within a hollow NGC was shown to inhibit regeneration entirely (46).

Ngo et al. demonstrated that axonal regeneration was further reduced when intra luminal fibres were juxtaposed (47). One instance showed that fibres clustered in the centre of the conduit resulted in complete failure in regeneration. This result highlights the necessity for the correct positioning of intra-luminal fillers within a hollow NGC and when used with the appropriate material combinations, as seen in a later study where PLLA intra-luminal filaments were incorporated into a permeable poly (\(\alpha\)-lactide) (PLA) NGC. A further increase in their regenerative potential was seen \textit{in vivo} in contrast to that of the original impermeable silicone NGC (48). A similar trend was seen in a study by Huang et al. where different quantities of Spidrex® silk fibre conduits were used. It was shown that approximately 200 silk fibres showed a significant increase in axonal regeneration and the ability to bridge both a 8 mm and 13 mm long nerve gap. The 13 mm silk fibre group showed similar axon regeneration to autograft in terms of axonal area at midgraft, however, at the distal end regeneration remained significantly higher in the autograft group (49).

A number of similar studies were carried out using different combinations of intra-luminal guidance structures and outer conduit materials and these are summarised in Table 1.2. These intra-luminal guidance structures include gels, sponges, films, filaments and fibres which have been used alone, or in combination with a number of supportive factors. One approach taken is the addition of nanoscale guidance cues to micron scale intra-luminal guidance structures. These nanoscale features were successfully incorporated into both film (40, 50) and filament guidance structures (14).
Figure 1.2. Summarised schematic of the structural repair strategies used for improving existing hollow nerve guidance conduits. Repair strategies include the use of intraluminal guidance structures and micro-grooved luminal designs to provide additional structure support and topographical guidance to regenerating axons and migrating Schwann cells. A similar strategy involves using electrospun fibrous conduits with the advantages of high flexibility and porosity, a high surface area-volume and fibres that can be aligned for guided Schwann cell migration and proliferation and axonal growth. Variations in conduit design include the use of multichannel conduits for control of axonal dispersion, as well as designs, which optimise nutrient exchange or introduce external stimuli. These designs may be used alone or in combination, but they also may require further surface functionalisation. These surface modifications can increase cell adhesion, migration, alignment and proliferation.
The use of aligned polymeric fibrous films serves an interesting alternative to the use of intra-luminal fibres/filaments.

A critical nerve gap of approximately 17 mm was bridged using aligned electrospun thin films of poly(acrylonitrile-co-methylacrylate) (PAN-MA) fibres (50). These aligned sub-micron scale fibres (400 - 600 nm in diameter) showed a significant increase in nerve regeneration in contrast to that of control unaligned films and in later studies showed the ability to be arranged into a variety of configurations.

These electrospun films have the advantages of a high surface area-to-volume ratio, a compact aligned topography, controlled packing configurations, and a low packing density (approximately 0.6% of the NGC cross sectional area) (40). These intra-luminal films allow controlled positioning of guidance structures, eliminating the problem of fibre overlap associated with the use of intra-luminal fibres/filaments. The use of these aligned films has also been shown to promote significantly increased and aligned fibronectin fibril deposition on the surface of the fibres forming an organised extracellular network versus a smooth solvent-cast film which had a more disorganised matrix. Fibronectin which has been shown to promote cell adhesion and outgrowth from neuronal cells and is one of the many ECM components synthesised by Schwann cells that contributes to the enhanced growth seen through the use of such films (42). It was similarly shown in this study that the deposition of this organised fibrin matrix significantly increased neurite outgrowth and schwann cell migration in vitro in comparison to the same substrate where fibronectin deposition has been inhibited. Despite the advantages of such a concept, a single film placed along the midline of the conduit showed the most promising results. The addition of further films (in various configurations) limited regeneration creating areas devoid of axonal growth. The author noted the disadvantage of creating zones within the conduit itself. These zones allowed symmetrical mismatches of migration of supportive cells from the proximal and distal nerve stumps. Schwann cells could be seen migrating in the upper zone proximally, while distilling
migrating in a lower zone of the configuration. This misalignment resulted in the incorrect formation of an aligned tissue cable (40). The use of such a system therefore requires careful positioning of each film within the conduit to create a controlled environment for repair.

The use of nano-scale topographies was similarly achieved by Koh et al. through the use of micron-scale intra-luminal filaments which were composed of aligned electro-spun nano-fibrous yarns (14). These intra-luminal filaments consisted of (PLGA) nano-fibres (between 200 - 600 nm in diameter) and were approximately 25 μm diameter (approximately 10 % of the NGC cross sectional area). These filaments, combined with surface functionalisation and growth factor delivery, successfully bridged a nerve gap of approximately 15 mm after a period of 12 weeks. Further such intraluminal structures combined with a bi-layered outer conduit were shown to achieve similar levels of regeneration and functional recovery to that of autograft across a large critical nerve gap (14).

1.5.2 Luminal Wall Guidance Features – Enhancing Porosity and Increasing Guided Cell Migration

A number of physical alterations to the luminal wall have also been considered to introduce physical guidance signals within a hollow NGC. These physical guidance features range from micron-scale features to the more biomimetic nano-scale topographies (Table 1.3) (4) and primarily involve either the incorporation of longitudinal micro-channels on the inner lumen of a NGC or luminal walls composed of orientated and non-orientated electro-spun micron-scale to nano-scale fibres. It has been shown that the use of micron-scale features induces a guidance effect on neurites of regrowing neurons. These neurites show increasing alignment as features approach the size of regenerating axons, which are of approximately the same width as glial bands of Büngner or smaller (8). Depending on nerve type and anatomical location, these axons may have a diameter of 2 μm to 5 μm (Aδ) or 15 - 20 μm (Aα) (8, 15). Overall, neurites show increasing alignment as features decrease in width from 500 μm to 5μm (4, 8, 33, 51-53). To take advantage of the guidance effect of the aforementioned topographical features micro and nano-scale structures are currently being incorporated into a number of NGCs.
designs with the luminal walls displaying longitudinally ordered guidance structures to regenerating axons and similarly to that of migrating and proliferating Schwann cells (Table 1.3).

One such example was shown by Rutkowski et al. across a nerve gap of approximately 10 mm in a rat sciatic nerve model using a micro-patterned laminin-coated (poly(D,L -lactic acid)) PDLLA conduit. This study highlighted that over a non-critical nerve gap the inclusion of micro-channels alone had no significant effect on the level of nerve regeneration, as against control hollow non-micro-grooved conduits (54).

However, the addition of micro-channels, when assessed over a critical 1.5 cm nerve gap, exhibited a significant increase in nerve regeneration and functional recovery versus control NGCs (55). Similarly, Hu et al., using a unidirectional freezing method, followed by freeze drying, produced a collagen - chitosan conduit with longitudinal orientated micro-channels, in the range of 25 - 55 µm, within the luminal wall. This produced a hollow NGC with topographical guidance features, while maintaining structural integrity and a high degree of porosity, and was successfully used to bridge a 15 mm critical nerve gap. This longitudinal micro-channelled conduit showed a similar level of regeneration and functional recovery to that of autografts at 12 weeks post implantation. It also showed the ability of a NGC with micro-scale topographical features to successfully bridge a critical nerve gap without the addition of neurotrophic factors, cells, or similar molecular therapies (55).

Another similar luminal wall guidance strategy involves the use of electro-spun fibrous conduit (Table 1.3) (26, 28, 41, 54-58). The use of these aligned electro-spun tubes has a number of advantages over continuous tube strategies: (i) the materials are highly flexible and porous, and these are well adapted for use within biological systems; (ii) nano- and micro-scale fibres have a high surface area-volume-ratio increasing the area available for protein absorption, Schwann cell migration, and regeneration of axons; (iii) fibres that can be preferentially aligned resulting in increased Schwann cell alignment, proliferation and growth, and the promotion of guided axonal growth (14, 41, 59).
### Table 1.2. The Use of Intraluminal Guidance Structures

<table>
<thead>
<tr>
<th>Structure</th>
<th>Model</th>
<th>Gap (mm)</th>
<th>Time</th>
<th>Significant Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin/YIGSR collagen fibres (θ 100 - 150 µm)</td>
<td>Rat sciatic</td>
<td>15</td>
<td>8 weeks</td>
<td>Laminin/YIGSR coated fibre group significantly increased axonal density versus uncoated fibres.</td>
<td>(60)</td>
</tr>
<tr>
<td>2000 x collagen filaments (θ 20 µm)</td>
<td>Rat sciatic</td>
<td>20</td>
<td>4, 8 weeks</td>
<td>Critical gap bridged. No significant difference versus autograft at 8 weeks.</td>
<td>(61)</td>
</tr>
<tr>
<td>80 x laminin coated collagen fibres (θ 50 µm) / sponge</td>
<td>Canine peroneal</td>
<td>80</td>
<td>12 months</td>
<td>No significant difference in nerve regeneration or functional recovery seen between groups.</td>
<td>(62)</td>
</tr>
<tr>
<td>2,000 – 4,000 x collagen filaments with no outer conduit (θ 20 µm)</td>
<td>Rat tibial</td>
<td>30</td>
<td>4, 8, 12 weeks</td>
<td>Increased in number of filaments resulted in increased regeneration (4,000 V 2,000). Functional remains poor.</td>
<td>(45)</td>
</tr>
<tr>
<td>Various densities of PLLA (θ 40 - 100 µm) microfilaments</td>
<td>Rat sciatic</td>
<td>10, 14, 18</td>
<td>10 weeks</td>
<td>High filament densities inhibited nerve regeneration. Low filament densities increased nerve regeneration.</td>
<td>(47)</td>
</tr>
<tr>
<td>Collagen sponge in outer PGA conduit</td>
<td>Canine peroneal</td>
<td>15</td>
<td>1 to 24 weeks</td>
<td>15 mm nerve gap bridged. Functional recovery similar to autograft after 6 months.</td>
<td>(63)</td>
</tr>
<tr>
<td>16 x PLLA microfilaments (θ 60-80µm) in PLA conduit</td>
<td>Rat sciatic</td>
<td>14, 18</td>
<td>10 weeks</td>
<td>Both gaps bridged at 10 weeks. Outer permeable conduit showed increased myelination and recovery versus impermeable.</td>
<td>(48)</td>
</tr>
<tr>
<td>Structure</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Time</td>
<td>Significant Outcome</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>------------------------</td>
<td>----------</td>
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<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>2000 x PGA (θ 14µm) filaments in a chitosan conduit</td>
<td>Dog</td>
<td>30</td>
<td>6 months</td>
<td>Critical gap bridged with similar functional recovery to autograft.</td>
<td></td>
</tr>
<tr>
<td>10 x Bioglass™ fibres (θ 25 µm) in a Silastic conduit ®</td>
<td>Rat</td>
<td>5</td>
<td>4 weeks</td>
<td>Greater regeneration than a hollow Silastic® conduit. No statistical difference versus autograft at the early timepoint.</td>
<td></td>
</tr>
<tr>
<td>Collagen gel in PLGA-coated collagen conduit</td>
<td>Rat</td>
<td>15</td>
<td>12 weeks</td>
<td>A critical nerve gap bridged without the addition of neurotrophic factors.</td>
<td></td>
</tr>
<tr>
<td>20 x silk fibroin filaments (θ 10 µm) in a porous silk fibroin conduit.</td>
<td>Rat</td>
<td>10</td>
<td>24 weeks</td>
<td>No significant differences in axonal regeneration and functional recovery between silk fibroin and autograft.</td>
<td></td>
</tr>
<tr>
<td>Stacks of aligned/unaligned films of PAN-MA (θ 400-600 nm) in a tibial polysulfonate conduit.</td>
<td>Rat</td>
<td>17</td>
<td>16 weeks</td>
<td>Critical gap bridged with aligned showing significantly more regenerated axons across its length versus unaligned.</td>
<td></td>
</tr>
<tr>
<td>1 or 3 PAN-MA fibrous film configurations (θ 400-600 nm) in a tibial polysulfonate conduit.</td>
<td>Rat</td>
<td>14</td>
<td>6, 13 weeks</td>
<td>Functional nerve regeneration was significantly greater in the one film conduit versus that of the three film conduit.</td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Time</td>
<td>Significant Outcome</td>
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<tr>
<td>Fibrous (θ 2-20 µm) keratin hydrogel in silicone conduit.</td>
<td>Mouse</td>
<td>4</td>
<td>6 weeks</td>
<td>Keratin group showed significantly greater conduction delay than autograft group.</td>
<td>(68)</td>
</tr>
<tr>
<td>2,000 x PGA filaments (θ 14 µm) in an outer chitosan conduit.</td>
<td>Human</td>
<td>35</td>
<td>3 years</td>
<td>Increase in functional recovery seen across the three year recovery period.</td>
<td>(69)</td>
</tr>
<tr>
<td>100, 200 and 300 Spidrex® silk fibres (10 x 20 µm) in a silk fibroin conduit.</td>
<td>Rat</td>
<td>8, 11, 13</td>
<td>4, 8, 12 weeks</td>
<td>P200 showed significantly greater regeneration versus remaining silk fibre groups and the similar regeneration to autograft midgraft at a 13 mm nerve graft eight weeks post implantation.</td>
<td>(49)</td>
</tr>
<tr>
<td>Human keratin conduit in a Silastic® conduit.</td>
<td>Mouse</td>
<td>4</td>
<td>6, 12, 24 weeks</td>
<td>Significantly greater regeneration and functional recovery was in comparison to a hollow silastic conduit and was comparable to a sural autograft.</td>
<td>(70)</td>
</tr>
<tr>
<td>360 x PLGA microfilaments (θ 25 µm) composed of aligned nanofibres (θ 200-600 nm) releasing NGF in a bilayered PLLA conduit</td>
<td>Rat</td>
<td>15</td>
<td>12 weeks</td>
<td>Nerve regeneration and functional recovery significantly greater than autograft at the experimental endpoint.</td>
<td>(14)</td>
</tr>
<tr>
<td>Structure</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Time</td>
<td>Significant Outcome</td>
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<tr>
<td>Collagen gel and Chondrotin-6 sulphate in an Integra™ collagen conduit.</td>
<td>Rat</td>
<td>10</td>
<td>12 weeks</td>
<td>An Integra™ collagen conduit filled with a collagen-GAG hydrogel showed superior motor recovery to an empty collagen conduit and equivalent axonal counts to autograft.</td>
<td>(71)</td>
</tr>
<tr>
<td>Human keratin hydrogel in a Neurogen® conduit.</td>
<td>Rat</td>
<td>8</td>
<td>3, 7, 14 days</td>
<td>Increased early cellular infiltration versus saline filled conduits. Comparable cellular infiltration to Matrigel™ but significantly slower axonal regeneration.</td>
<td>(72)</td>
</tr>
<tr>
<td>Collagen and hyaluronic composite hydrogel in a nanofibrous PLCL conduit</td>
<td>Rat</td>
<td>10</td>
<td>3, 12 weeks</td>
<td>Improved sensory function in hydrogel groups. Autograft remained significantly greater in terms of force and EMG recovery. The hydrogel group did not significantly outperform an empty conduit.</td>
<td>(73)</td>
</tr>
<tr>
<td>Nanofibrous (251 ± 32 nm) or microfibrous (981 ± µm) PCL conduit</td>
<td>Rat</td>
<td>15</td>
<td>12 weeks</td>
<td>A nanofibrous PCL conduit showed significantly improved axonal counts, myelin thickness and CMAP versus microfibrous and control PCL conduits.</td>
<td>(74)</td>
</tr>
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</table>
The use of wall guidance avoids the problem of uneven fibre distribution, seen in the use of low density intra-luminal guidance structures (41, 47). This eliminates the problem of potential growth inhibition from overlapping fibres or compartmentalisation, which have adverse effects on nerve regeneration (40, 46, 47).

In a study by Chew et al. (41), the use of micro-scale electrospun copolymer of caprolactone and ethyl ethylene phosphate (PCLEEP) fibres successfully bridged a 15 mm nerve gap. The aligned electrospun fibres showed an increase in functional recovery versus control non-fibrous PCLEEP conduits showing an increasing trend in nerve regeneration with the subsequent addition of exogenous growth factors (41). Interestingly in this study, regeneration occurred at both the periphery and at the centre of NGC lumen (41). This was reported to be due to the slippage of PCLEEP fibres from the wall into the centre of the lumen and possibly highlights the need for additional intra-luminal guidance structures (41). In a recent in vitro study by Madduri et al., the effects of topographical guidance of electrospun fibres is elegantly shown, through the use of silk fibroin nanofibres (4). These electrospun fibres, in the range of 400 - 500 nm, successfully encapsulated neurotrophic factors (glial derived neurotrophic factor GDNF and nerve growth factor NGF) to provide synergistic topographical and trophic support to re-growing axons (4). The silk fibroin membranes were subsequently assessed with chick dorsal root ganglion cells (primarily sensory neurons and Schwann cells) and chicken embryonic spinal cord explants (primarily motor neurons and Schwann cells) (4). Interestingly, it was shown that there was a significant increase in neurite length and alignment, and promotion of glial cell migration and alignment, in the case of aligned electro-spun nano-scale fibres (4). This combination of topography and trophic support shows potential for the treatment of critical nerve gaps and increasing functional recovery. It also highlights the different modality of Schwann cells and axons which need to be targeted for mixed nerve repair.

However, luminal wall guidance alone does not exhibit similar levels of axonal guidance as do intra-luminal fillers when bridging a critical nerve gap (41). To complement these luminal wall guidance features and increase
regeneration across a critical nerve gap, a number of approaches need to be considered. A study by Koh et al. combined a number of strategies within their conduit design (14). A bi-layered laminin coated PLLA conduit, used in combination with intra-luminal PLGA fibres, was demonstrated to enhance the modified outer NGC. This bi-layered conduit consisted of an outer layer of randomly aligned electrospun nanofibres and an inner layer of longitudinally aligned nanofibres which were in the range 250 - 1000 nm in diameter. It was proposed that the longitudinally aligned inner layer provided topographical cues for regenerating axons and migrating Schwann cells while the outer layer provided structural support to the conduit structure while maintaining the porosity of the tube. This conduit was successfully used to bridge a critical nerve gap of 15 mm and exhibited functional recovery comparable to autografts (14).

1.5.3 Optimising Conduit Design and the Introduction of External Stimuli

Alternative strategies for enhancing nerve repair involve reconsidering the overall conduit design (Table 1.3). These approaches have been used to limit axonal dispersion (57), optimise nutrient exchange (26, 75) and to more closely resemble the micro-architecture of the peripheral nerve environment (76). Some of these designs have been successfully combined with non-invasive clinical approaches (i.e. ultrasound) and have shown potential to enhance peripheral nerve repair (26, 58).

The use of a multi-channel conduit is one promising alternative for peripheral nerve repair (22, 57, 76-78). A multichannel PLGA was originally investigated as an alternative to conventional NGC which was closely imitating native nerve’s architecture (76). Using a foam processing technique, conduits with multiple micro-channels were manufactured. The primary premise for this design was the controlled introduction of allogenic Schwann cells by increasing overall surface area for Schwann cell adherence and distribution. From this basis, a five channel conduit was then successfully used to bridge a short seven mm rat sciatic nerve gap. This design however had a low cross section available for nerve regeneration, making comparison with the control autograft group difficult (76). It was later put forward by de
Ruiter et al. and by Yao et al. that this multichannel design could be used to limit axonal dispersion within NGC (57, 79, 80). A single and seven channel PLGA NGCs were used to bridge a 10 mm nerve gap in a rat sciatic nerve model. At 12 weeks, there was no significant difference between single and multichannel conduits with regard to nerve regeneration. However, using a simultaneous retrograde tracing technique, there was a significant decrease in axonal dispersion versus control single channel conduits. The use of this conduit however showed these results in only 50% of the groups assessed, primarily due to swelling of the PLGA tube, resulting in occlusion of a number of the channels and the consequences of these results were not definitive (79). In order to improve this design, Yao et al., showed that the use of multi-channelled collagen nerve conduits could similarly be used to bridge a 10 mm rat sciatic nerve gap, without the structural instability seen in previous studies (57). This study showed similar results for nerve regeneration to previous work, however there was a significant decrease in overall axonal dispersion/misdirection using this multichannel design. Using this multichannel design in combination with additional factors, such as guidance structures or molecular/cell based therapies, could be an interesting approach for future nerve repair, and potentially reduce the misdirection of re-growing axons.

Another approach is the use of a bi-layered PLGA/Pluronic F127 asymmetrically porous conduit which has been shown to have a number of features (26, 75) to increase regeneration within a hollow nerve conduit. This conduit contains two distinct layers: an inner surface with nano-pores 50 nm in diameter asymmetrically aligned which allows the diffusion of nutrients and neurotrophic factors but reduces scar infiltration; and an outer surface consisting of micro-pores approximately 50 µm in diameter which permits vascular ingrowth into the conduit (26, 75). The use of asymmetric pores over non-asymmetric pores had previously been shown to increase early stage nerve regeneration (28, 75). This, in combination with the pluronic F127 coating increases the hydrophilicity of the conduit, resulted in an increase in the regeneration rate of regenerating axons versus that of control conduits (75, 81). Further to this, a PCL conduit coated with pluronic F127 with an inner
wall of nanopores (100 nm) showed aligned axonal growth and schwann cell migration in comparison to a coated conduit with inner micropores (200 \( \mu \text{m} \)) with faster nerve regeneration and muscular restoration at four weeks. Axons were seen to grow towards and cells to migrate into the walls of the microporous conduit (81).

These studies highlighted the importance of nutrient exchange and the structural properties of the inner conduit wall for nerve repair. In later studies, these bi-layered coated conduits when implanted were exposed to external ultrasound stimulation (US), as a novel non-invasive approach. The use of low intensity US resulted in a significant increase in nerve regeneration rates (0.72 mm/day in the US treated group versus 0.48 mm/day in the non-treated group) (26). Likewise, US resulted in increased myelination, axon diameter and thicker regenerative nerve cable (26). The effects of US stimulation have exhibited comparable results in a number of studies and may hold potential to improve current clinical nerve therapies especially when used in combination with additional regenerative factors i.e. neurotrophic factors, growth factors or cell based therapies.

1.5.4 Surface Modifications and Peptide Mimetics

The addition of topographical guidance cues and structural features to a conduit may require additional surface modifications of the biomaterial surface, depending on the base material. Numerous forms of surface modifications have been used with both synthetic biodegradable materials (poly(caprolactone) (PCL), PLA, PLLA, poly(\(\varepsilon\)-lactide)) and numerous natural materials (collagen, chitosan, fibrin) (37, 62, 82-86). These materials while they exhibit the required structural properties for guided cell growth, Schwann cell adhesion and migration, their surface characteristics may not be such as to induce the required effects; these materials tend to be hydrophilic or hydrophobic and are primarily surface functionalised by physical adsorption or chemical modification (87). Consequently, a number of surface modification techniques have been employed to increase cell adhesion, proliferation and migration. These modifications may take the form of full protein coatings, chemical and physical treatments, or the addition of protein mimetics onto the surface of the material (87).
Table 1.3 Luminal Guidance Features and Variations in Material Design

<table>
<thead>
<tr>
<th>Feature</th>
<th>Model</th>
<th>Gap (mm)</th>
<th>Time</th>
<th>Significant results</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>Luminal Guidance Features</strong></td>
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<td></td>
<td>Micro-Grooved/Micro-Channelled Luminal Features</td>
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<tr>
<td>Micro-channelled PDLLA NGC + Schwann cells (Groove width 10 μm, depth 4.3 μm)</td>
<td>Rat sciatic</td>
<td>10 weeks</td>
<td>Micro-channels had no significant effect on regeneration; addition of Schwann cells increased functional recovery</td>
<td>(54)</td>
<td></td>
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<tr>
<td>Micro-channelled collagen-chitosan conduit (Groove width 25 - 55 μm)</td>
<td>Rat sciatic</td>
<td>15 weeks</td>
<td>Similar level of regeneration and functional recovery to autograft at 12 weeks.</td>
<td>(55)</td>
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<tr>
<td><strong>Electrospun Nano- And Micro- Fibrous Conduits</strong></td>
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<tr>
<td>Electrospun PLGA/PCL fibrous conduit (θ 280 nm - 3 µm)</td>
<td>Rat sciatic</td>
<td>10 weeks</td>
<td>Conduits showed the ability to bridge a 10 mm gap and showed some restoration of functional recovery.</td>
<td>(59)</td>
<td></td>
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<tr>
<td>Electrospun PCLEEP fibrous conduit with GDNF (θ (3.96±0.14) µm)</td>
<td>Rat sciatic</td>
<td>15 weeks</td>
<td>Significant increase in functional recovery could be seen versus control conduits</td>
<td>(41)</td>
<td></td>
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<tr>
<td>Silk fibroin (P(LLA-CL)) fibrous conduits</td>
<td>Rat sciatic</td>
<td>10 weeks</td>
<td>Significant increase in functional nerve regeneration versus PLLA-CL NGCs.</td>
<td>(56)</td>
<td></td>
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<tr>
<td>Feature</td>
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<td>Gap (mm)</td>
<td>Time</td>
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<tr>
<td>Dual layer PLLA conduit (inner aligned and outer random PLLA fibers</td>
<td>Rat</td>
<td>15</td>
<td>12</td>
<td>Regeneration across a critical nerve gap and significantly greater axonal regeneration versus autograft. Functional recovery superior to autograft at the 12 week timepoint.</td>
<td>(14)</td>
</tr>
<tr>
<td>(θ 250-1,000 nm)) combined with intraluminal fiber structures.</td>
<td>sciatic</td>
<td></td>
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<tr>
<td>Aligned nanofibrous PCL conduits (θ 100 – 200 nm) with/without a laminin coating.</td>
<td>Rat</td>
<td>10</td>
<td>4, 6</td>
<td>Aligned PCL conduits significantly increase in motor recovery versus random and hollow conduits. The addition of laminin to the PCL conduits significantly increased sensory recovery.</td>
<td>(88)</td>
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<td></td>
<td>tibial</td>
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<tr>
<td>Dual layer PLCL nanofibrous conduit (inner aligned, outer random)</td>
<td>Rat</td>
<td>10</td>
<td>12</td>
<td>Gastrocnemius muscle force recovery and muscle mass reached 85% and 82% of autograft respectively. Regeneration was significantly higher in Autograft medially but similar distally.</td>
<td>(89)</td>
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<td>(inner aligned, outer random)</td>
<td>sciatic</td>
<td></td>
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<tr>
<td>Multichannel PLGA foam conduits containing Schwann cells (1 – 183 channel conduits)</td>
<td>Rat</td>
<td>7</td>
<td>6</td>
<td>In a 5 channel conduit regeneration was seen across a 7 mm nerve gap with axonal area comparable to autograft. Axon diameters were significantly greater than autograft.</td>
<td>(76)</td>
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<td></td>
<td>sciatic</td>
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**Variations In Conduit Design:**

Introduction
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<th>Feature</th>
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<th>Gap (mm)</th>
<th>Time</th>
<th>Significant results</th>
<th>Ref.</th>
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<tr>
<td>Multi-channel PLGA conduit (7-channel)</td>
<td>Rat sciatic</td>
<td>10</td>
<td>8, 12</td>
<td>Axonal dispersion was reduced (16.9%) versus single channel repair (21.4%). Autograft remained superior showing the lowest percentage of double projections (5.9%).</td>
<td>(79)</td>
</tr>
<tr>
<td>Multi-channelled collagen conduit (1-, 2-, 4-, 7-channel conduits)</td>
<td>Rat sciatic</td>
<td>10</td>
<td>16</td>
<td>Four-channel conduits significantly decreased axonal dispersion versus control single channel conduits.</td>
<td>(57)</td>
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<tr>
<td>PCL / Pluronic F127 NGC with either inner micropores (200 µm) or inner nanopores (100 nm)</td>
<td>Rat sciatic</td>
<td>10</td>
<td>4</td>
<td>A PCL conduit with inner nanopores showed aligned axonal growth and Schwann cell migration. Inner macropores resulted in growth into the conduit and disorganisation in the regenerative response.</td>
<td>(81)</td>
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**Enhancing Nutrient Exchange And The Introduction Of External Stimuli**

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<tr>
<td>PLGA conduit with Schwann cells and stimulated by ultrasound</td>
<td>Rat sciatic</td>
<td>10</td>
<td>6</td>
<td>Ultrasound stimulation combined with Schwann cell delivery resulted in significant increase in myelin thickness and axonal area versus autograft and hollow conduit repair.</td>
<td>(58)</td>
</tr>
<tr>
<td>Feature</td>
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<tr>
<td>PLGA conduit with asymmetric/symmetric pores</td>
<td>Rat</td>
<td>10</td>
<td>4, 8</td>
<td>PLGA conduits with asymmetric pores showed higher nerve regeneration than PLGA conduits with symmetric pores.</td>
<td>(28)</td>
</tr>
<tr>
<td>Bi-layered micro (50 µm) and nano (50 nm) porous PLGA conduit with Pluronic F127 coating</td>
<td>Rat</td>
<td>10</td>
<td>1 - 24</td>
<td>The introduction of a pluronic F127 coating increased the hydrophilicity of the PLGA conduit; and combined with improved nutrient exchange significantly increased regeneration and functional recovery versus uncoated PLGA NGCs.</td>
<td>(75)</td>
</tr>
<tr>
<td>Bi-layered micro and nano porous PLGA/Pluronic F127 NGC stimulated by ultrasound</td>
<td>Rat</td>
<td>10</td>
<td>1, 8</td>
<td>A bi-layered NGC + US increased nerve regeneration rates versus no ultrasound groups (0.72 mm/day V 0.48 mm/day)</td>
<td>(26)</td>
</tr>
<tr>
<td>Genipin cross-linked gelatin and β-tricalcium phosphate conduit stimulated by low level laser diodes.</td>
<td>Rat</td>
<td>15</td>
<td>12</td>
<td>Low-level-laser stimulation provided by gallium-aluminium-arsenide-phosphide excitation increased functional recovery and axonal regeneration versus non-treated animals. (once per day for 10 days)</td>
<td>(90)</td>
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</table>
Numerous ECM proteins have been considered as candidates for surface functionalisation including collagen, fibronectin and laminin (14, 91, 92). Laminin, a complex trimeric glycoprotein, is a major component of the basal lamina of Schwann cells and has positive effects on neurite extension and Schwann cell adhesion, proliferation and migration; and overall have exhibited the ability to improve nerve regeneration (14, 62, 85). This trimeric glycoprotein has been demonstrated to interact with Schwann cell integrins that may result in activation of myelination needed for successful growth and repair (14). Numerous studies have shown its ability to enhance Schwann proliferation and migration, as well as its direct effects on neurite outgrowth (14, 37, 85). Laminin has been used most frequently for surface modification for NGC and their respective structural components (14, 37, 54, 62, 83, 85, 88, 92, 93). Other ECM molecules such as collagen and fibronectin have the ability to significantly increase Schwann cell adhesion and proliferation and enhance neurite outgrowth however, although results have been shown to be significantly lower than that of laminin (14, 91, 92). A number of studies have conjugated laminin to their respective material or used them to enhance the aforementioned intraluminal fillers (37, 62, 83, 85, 94). Each respective study notably showed a significant increase in nerve regeneration compared to that of uncoated fibres (37, 62, 83, 85). Yu et al. presented a combination of laminin and slow releasing nerve growth factor (NGF) from an agarose hydrogel (92). The combined effect of these two factors yielded nerve regeneration and functional recovery similar to that of autograft eight weeks after injury across a 10 mm nerve gap at the middle of the graft (92). It would be interesting to see if these levels were maintained throughout the graft at a later timepoint (12 or 16 weeks). Similarly, in a recent study by Koh et al. the incorporation of a laminin coating, combined with PLGA intraluminal guidance structures, successfully bridged a critical nerve gap of 15mm, and showed superior functional recovery to that of autograft (14). These same ECM molecules can similarly be used as a luminal wall coating, increasing cell adhesion and proliferation as well as increasing guided axonal outgrowth and may serve to enhance some of the luminal wall guidance features mentioned previously (54, 82, 95).
Large ECM molecules, such as laminin, have a large molecular weight (about 900 KDa), making them quite difficult to synthesise (82, 85). One alternative to the use of these large glycoproteins is the use of short chain protein peptide mimetics (Table 1.4). These peptides have a number of advantages over large proteins including (i) high stability; (ii) low immune response; (iii) high surface density and orientation for ligand-receptor interaction and cell adhesion; (iv) a relatively low molecular weight; and (v) the ability to be used in high concentrations (85, 87). A number of these peptides have been used in the context of peripheral nerve repair, including; RGD (Arginine-Glycine-Aspartic acid), a peptide sequence found in fibronectin, laminin and other ECM molecules; IKVAV (Ile-Lys-Val-Ala-Val) and YIGSR (Tyr-Ile-Gly-Ser-Arg) of the laminin β chain, RNIAEIKDI peptides of the laminin γ chain, the primary cell binding domains of laminin; as well as similar peptide sequences such as HAV, a mimetic of the N-cadherin regulatory protein which is present on both neurons and glial cells (82, 85-87, 95).

A range of these peptides were successfully assessed, *in vitro* and *in vivo*, by Schense *et al.* within a fibrin matrix (86). These various peptide sequences exhibited a significant increase in regeneration compared to that of control uncoated fibrin matrix *in vitro*. Noteworthy was the synergistic effect of multiple peptides on neurite outgrowth where the combined effect of four individual laminin peptides was greater than the sum of neurite extension for individual peptide alone. In an *in vivo* study, using a four mm dorsal root model, a NGC filled with a peptide loaded fibrin matrix was successfully implanted. The incorporation of individual peptides within a fibrin matrix showed no significant difference versus fibrin alone; however the synergistic effects of the four laminin peptides, showed a significant increase in nerve regeneration versus control fibrin matrices (86). Similarly, in work carried out by Yao *et al.* a human laminin 5 peptide (PPFLMLLKKGSTR) coating was shown to exhibit similar levels of neurite outgrowth to that of a collagen coated substrate *in vitro* and successfully used in combination with micro-structured templates enhancing neurite growth and alignment (52).
These peptides have been used in a number of similar studies and results have shown levels of regeneration equivalent to whole proteins (60, 82, 96). Itoh et al. successfully coated collagen intraluminal fillers with either laminin or the YIGSR peptide and compared regeneration to uncoated collagen fibres (60). It was shown that both the laminin coated and peptide coated fibres indicated a significant increase in nerve regeneration versus uncoated collagen fibres. In particular, there was no significant difference in nerve regeneration between the whole glycoprotein and the peptide mimetic (60). In more recent studies, Santiago et al. modified the inner surface of a PCL scaffold with a peptide sequence (RGD) as a means to enhance axonal interactions, Schwann cell adhesion, and to increase adhesion of implanted ASCs (82). Wang et al., incorporated a CYIGSR peptide (YIGSR peptide with a glycine spacer) with a bi-layered micro/nano fibrous conduit resulting in increased nerve regeneration (96). These peptides are not without their limitations, it has been shown that when RGD sequence is isolated from it’s original protein structure, it loses its specificity and high affinity for cell attachment. The structure and modifications of this peptide are therefore important to maintain the high affinity necessary for clinical applications. The use of such unmodified peptides are also non-specific and can bind a large number of different cell types. Other important considerations include the addition of a spacer, e.g. glycine in Wang et al., to ensure the binding of the peptide to cell surface integrins needed for attachment (97, 98). These surface modifications including large glycoproteins or their peptide mimetics can be seen as key factors for enhancing structural features of current NGCs.

1.6 Molecular Delivery Therapies: The Creation of a Conductive Micro Environment

The addition of structural features to hollow NGCs is one approach to improve nerve regeneration, in particular across critical nerve gap (37, 45, 61, 66, 99). The addition of these features alone is insufficient to increase functional recovery. In efforts to improve functional regeneration in both critical and non-critical gaps, the creation of a more conductive micro-environment is of high importance. The reduction in functional nerve regeneration over these challenging nerve gaps can be attributed to a variety
of factors. These include inadequate ECM formation (mentioned earlier), insufficient neurotrophic support, inadequate Schwann numbers, reduction in Schwann cell migration and proliferation, and possible reduction in the neurotrophic effects of the distal nerve stump (1, 7, 32). In efforts to enhance functional nerve regeneration, advances have been made to create a more conductive environment for repair. Strategies include the use of exogenous growth factors (e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor (bFGF)), neurotrophic factors (e.g. neurotrophin-3 (NT3), nerve growth factor (NGF)) or cell based therapies (e.g. Schwann cells, stem cells) (Figure 1.3) (9, 100-112).

Neurotrophic factors enhance functional regeneration, by supporting axonal growth, Schwann cell migration and proliferation, and increasing neuroprotection through receptor mediated activation of specific intrinsic signalling pathways (113). These neurotrophic factors primarily belong to three distinct families: (1) the neurotrophins; (2) the glial cell-line derived neurotrophic factor family ligands (GFLs); (3) the neuropoietic cytokines (114). Each family has distinct functional characteristics with some overlapping cellular responses (114). Neurotrophins include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4) (115); GFLs include Glial cell-line derived neurotrophic factor (GDNF) and neuropoietic cytokines include ciliary neurotrophic factor (CNTF) (114). These neurotrophic factors have been used alone or in combination, to harness the most effective response for nerve regeneration (Table 1.4).

An ethylene vinyl acetate (EVA) conduit used for release of either NGF or GDNF, bridged a 15 mm nerve gap through the addition of these respective neurotrophic factors alone (100). The GDNF conduit group exhibited four times the number of myelinated axons than that of the NGF group, showing its potential for peripheral nerve repair (100). Neurotrophic factors delivered alone however have shown limited functional recovery and recent efforts have been made for the synergistic delivery of these neurotrophic factors (9). One such study, carried out by Madduri et al., involved co-delivery of both NGF and GDNF using a luminal diffusion-based delivery system (9).
Figure 1.3. Schematic diagram of cellular and molecular based therapies used for the creation of a more conductive nerve microenvironment. Examples of molecular therapies include growth factors (VEGF, bFGF) and neurotrophic factors (NT3, NGF). Likewise, cell therapies involve the use of Schwann cells, stem cells (ASCs and MSCs) and genetically modified cells (Schwann cells overexpressing GDNF). These can be delivered by a number of means including: (i) suspension within solution or a biomaterial matrix (hydrogel, sponge); (ii) released via a diffusion based systems (controlled released via cross-linking, slow degrading polymer coatings from luminal wall); (iii) the use of affinity based delivery systems (factors conjugated to a fibrin matrix); (iv) microsphere (e.g. collagen, fibrin) encapsulation which can either be suspended within the lumen or released from the luminal wall.
This study argued that co-delivery was essential for increased functional regeneration, as peripheral nerve contained different neuronal and glial subpopulations (both motor and sensory) (4, 9). NGF which acts through the high affinity Trk A receptor is primarily found on sensory neurons, shown to promote axon regeneration and re-innervate sympathetic axons following nerve injury (9).

The failure of single growth factor delivery may also be attributed to poor release kinetics, with some delivery systems exhibiting a high initial burst release (101). In efforts to improve release, delivery systems which alter these release kinetics are being considered. One such strategy involves the use of physical cross linking methods used in combination with a polymer coating (9). This combination was shown to limit the initial burst release of growth factors, indicating a significant increase in nerve regeneration versus a PLGA polymer coating alone. The effects of this system on late stage functional recovery remain to be seen, but early results seem promising.

One alternative, for controlled delivery of neurotrophic factors, is the use of an affinity based delivery system (104, 105). This system encloses growth factors within a fibrin based matrix for intraluminal delivery of growth factors. This avoids the initial burst release seen in some diffusion based systems and allows the controlled release of growth factors by cell based degradation of the delivery system and the surrounding fibrin matrix. Using this system, GDNF or NGF were successfully delivered within the lumen of a silicone conduit increasing the early stage regenerative response and successfully bridging a 13mm gap in a rat sciatic nerve model (104). For functional recovery, GDNF in combination with this diffusion based delivery system exhibited a higher level functional recovery than that of the control allograft groups. This may be partially attributable to an increase in the number of large myelinated axons, increased early stage regeneration and the ability of the fibrin matrix to act as an intraluminal guidance structure for early stage cell migration. This study successfully incorporated a fibrin based intraluminal guidance structure for enhanced contact guidance while synergistically creating a more conductive micro-environment for functional nerve regeneration (104). GDNF, in particular, contributes to this significant
increase in functional recovery, due to its ability to act on both motor and sensory neurons (106). This same combinatorial effect was seen in the use of nanofibrous constructs which were successfully combined with GDNF delivery – resulting in a similar increase in functional recovery (41). However, the nerve regeneration response as a whole is stimulated by a number of factors which act synergistically to improve nerve repair. If the development of the nervous system is considered in its entirety, there is a defined synergy between angiogenesis, and neurogenesis (107).

The addition of vascular endothelial growth factor (VEGF) indicated a significant increase in angiogenesis and also exhibited a similar increase in overall nerve regeneration (102). Although the addition of neurotrophic factors has shown advantages for nerve repair, their use has some limitations including unintentional activation of multiple signalling pathways resulting in undesired biological effects, e.g. aberrant sprouting associated with the use of NGF, and short half-lives and poor stability - lasting literally minutes upon release in serum conditions (115-117). These limitations may be overcome through increasing our knowledge of neurotrophic factor and growth factor delivery, reducing unintentional effects, and similarly by optimising the release kinetics of neurotrophic factors, using new technologies, the disadvantage of their limited half-lives may be overcome (115-117).

New emerging delivery approaches being developed include the use of biological collagen/fibrin microspheres, microfibers, and hydrogels, as well as synthetic polymeric carriers for the creation of a sustained system for viable growth factor delivery (118-120).

1.6.1 Neurotrophic Factor Mimetics – A Possible Alternative for Molecular Therapies

Molecular therapies using neurotrophic factor based therapies have shown potential for enhancing functional recovery, as well as for increasing nerve regeneration. These therapies may also hold the potential for functional critical nerve repair. The use of these factors has a number of limitations as mentioned earlier. One alternative to the use of large neurotrophic factor is the use of small molecule mimetics (115, 117, 121, 122).
<table>
<thead>
<tr>
<th>Factor</th>
<th>NGC</th>
<th>Delivery Method</th>
<th>Model</th>
<th>Gap (mm)</th>
<th>Time</th>
<th>Significant Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDNF</td>
<td>EVA conduit</td>
<td>Luminal release</td>
<td>Rat facial</td>
<td>5</td>
<td>6 weeks</td>
<td>NGC + GDNF showed highest nerve regeneration level.</td>
<td>103</td>
</tr>
<tr>
<td>or NT-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GDNF</td>
<td>EVA conduit</td>
<td>Luminal release</td>
<td>Rat sciatic</td>
<td>15</td>
<td>6 weeks</td>
<td>Gap bridged with both factor combinations.</td>
<td>100</td>
</tr>
<tr>
<td>or NGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GDNF conduit resulted in four-fold higher regeneration than NGF.</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Silicone</td>
<td>Suspension (Matrigel®)</td>
<td>Rat sciatic</td>
<td>10</td>
<td>1, 2, 4, 26 weeks</td>
<td>VEGF increased angiogenesis, Schwann cell migration and nerve regeneration.</td>
<td>102</td>
</tr>
<tr>
<td>bFGF</td>
<td>Bilayered PDLLA conduit</td>
<td>Luminal degradation based release</td>
<td>Rat sciatic</td>
<td>15</td>
<td>4, 10, 16 weeks</td>
<td>Gap was bridged using the PDLLA conduit.</td>
<td>123</td>
</tr>
<tr>
<td>NGF</td>
<td>PPE NGC/silicone</td>
<td>PPE microsphere</td>
<td>Rat sciatic</td>
<td>10</td>
<td>12 weeks</td>
<td>NGF increased nerve regeneration versus control conduits.</td>
<td>108</td>
</tr>
<tr>
<td>GDNF</td>
<td>Collagen + PLGA NGC</td>
<td>Luminal release (various rates)</td>
<td>Rat peroneal</td>
<td>3</td>
<td>12 weeks</td>
<td>Number of myelinated fibres tripled for all rates versus no GDNF group.</td>
<td>109</td>
</tr>
<tr>
<td>Factor</td>
<td>NGC</td>
<td>Delivery Method</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Time</td>
<td>Significant Results</td>
<td>Ref.</td>
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</tr>
<tr>
<td>NGF</td>
<td>Porous PCL conduit</td>
<td>Suspension / luminal release</td>
<td>Rat</td>
<td>12</td>
<td>4, 8</td>
<td>NGF luminal release showed superior regeneration to suspension.</td>
<td>(124)</td>
</tr>
<tr>
<td>GDNF</td>
<td>Collagen + PLGA NGC</td>
<td>Luminal release</td>
<td>Rat</td>
<td>10</td>
<td>2 weeks</td>
<td>Significant increase in early peripheral nerve regeneration.</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>Silicone + NGF NGF</td>
<td>Affinity-based fibrin matrix</td>
<td>Rat</td>
<td>13</td>
<td>4, 8, 12</td>
<td>12 weeks motor recovery of GDNF greater than isograft.</td>
<td>(104)</td>
</tr>
<tr>
<td></td>
<td>CNTF Chitosan / PLGA NGC</td>
<td>Luminal release</td>
<td>Dog</td>
<td>25</td>
<td>12 weeks</td>
<td>Functional recovery comparable to that of autograft.</td>
<td>(111)</td>
</tr>
<tr>
<td>NGF</td>
<td>PLLA-CL NGC</td>
<td>Core shell nano-fibrous release</td>
<td>Rat</td>
<td>10</td>
<td>12 weeks</td>
<td>NGC showed similar functional recovery regeneration to autograft.</td>
<td>(112)</td>
</tr>
<tr>
<td>NGF or GDNF</td>
<td>PLGA NGC</td>
<td>Microsphere release</td>
<td>Rat</td>
<td>10</td>
<td>6, 16 weeks</td>
<td>GDNF or NGF reached their maximum regenerative capacity quicker than control groups by six weeks.</td>
<td>(125)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Chitosan/ Gelatin Schwann cells NGC</td>
<td>Suspension + Schwann cells (collagen gel)</td>
<td>Rat</td>
<td>10</td>
<td>16 weeks</td>
<td>Combination of conduit and Schwann cells with TGF-β1 showed superior regeneration to empty conduit.</td>
<td>(126)</td>
</tr>
<tr>
<td>Factor</td>
<td>NGC</td>
<td>Delivery Method</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Time</td>
<td>Significant Results</td>
<td>Ref.</td>
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</tr>
<tr>
<td>NGF</td>
<td>Aligned PLCL NGC</td>
<td>Suspension (collagen and HA hydrogel)</td>
<td>Rat sciatic</td>
<td>10</td>
<td>12 weeks</td>
<td>Hydrogel addition enhance sensory but not motor recovery. The addition of NGF no effect in the system in vivo.</td>
<td>(73)</td>
</tr>
<tr>
<td>bFGF</td>
<td>Dual PLA:PCL layer conduit</td>
<td>GTA cross-linked gelatin microspheres (10 µm pore size) + IpSC neurospheres</td>
<td>Mouse sciatic</td>
<td>5</td>
<td>12 weeks</td>
<td>The addition of bFGF to the IpSC conduit significantly increased axonal regeneration in the conduit versus IpSCs alone. No significant differences in functional recovery were seen between groups.</td>
<td>(127, 128)</td>
</tr>
</tbody>
</table>
These neurotrophic factor mimetics have the advantages of low immunogenicity, low molecular mass with relatively low manufacturing costs, compared to the use of whole proteins (115). Research in this area concentrates primarily on the creation of mimetics of the neurotrophin factor, in particular mimetics of NGF, NT-3 and BDNF (115, 117, 121). These mimetics have primarily focused on creation of ligands of these neurotrophins with specific receptor targets (115, 117). In the case of NGF mimetics, one example is the use of a NGF mimic which selectively binds and activates the high affinity TrkA receptor, but not that of the low affinity p75 receptor. The activation of these signalling pathways is associated with quite different responses. TrkA activation has effects on neuronal survival and differentiation, and p75 activation has been associated with apoptosis (115, 117). These mimetics overcome the limitation of the larger neurotrophic factors as they have increased stability and controlled activation of known cellular pathways. However, these neurotrophin mimetics are currently in the early stages of research and are not as of yet being considered for peripheral nerve repair. Future strategies may include controlled release of these neurotrophin mimetics as a substitute for whole protein therapies, allowing for more controlled targeting of cellular responses.

1.7 Schwann Cells: The Gold Standard for Cell Based Repair

During nerve regeneration, Schwann cell migration and proliferation can be seen as prerequisites for successful nerve repair, seen in the cellular phase of successful nerve repair (1, 7, 29, 32). Schwann cells which remain after Wallerian degeneration migrate and proliferate to form aligned glial bands of Büngner, during the cellular phase of NGC repair (7, 29, 30, 32). At this stage, Schwann cells have switched to a regenerative phenotype, are actively secreting neurotrophic factors, and laying down basal lamina, and importantly their numbers have increased to 4 - 17 times the original number seen in normal nerve (approximately 20 x 10^6 cells/ml) (29, 38, 129, 130). However as gap distance increases, Schwann cell migration, proliferation and alignment decrease and Schwann cell numbers may be deemed insufficient for the creation of a conductive nerve environment (1). In attempts to aid the regenerative cellular response to injury, cellular based therapies are being
considered as an alternate means for repair (Table 1.5) (34, 58, 82, 99, 106, 131-133).

One suggested approach to improve functional recovery and nerve regeneration, and as an alternative to neurotrophic factor delivery, is the use of autologous or allogenic Schwann cells (38, 106, 132). If autograft is taken as the current gold standard for peripheral nerve repair, similarly the addition of autologous Schwann cells, to NGCs, can be taken as the current gold standard of cellular based therapies. The use of Schwann cells have the advantage of producing a number of neurotrophic factors, building their own basal lamina, expressing cell adhesion molecules, and at a later stage are actively involved in the re-myelination of regenerating nerve fibres (106, 132). The introduction of additional Schwann cells would therefore assist in the creation of a conductive nerve microenvironment, especially across a critical nerve gap (68, 106).

Schwann cells may be introduced into the conduit via a number of methods. These include injection, suspension within an intra-luminal hydrogel, distributed along intraluminal guidance structures or released from the luminal wall (34, 58, 82, 132). The implanted Schwann cells can be successfully incorporated into the regenerative process and is nicely shown through the use of retrovirally labelled allogenic Schwann cells (harvested from neonatal rats) (38). At the optimum concentration (80 x 10^6 / ml), these labelled cells were shown to be successfully incorporated into the host regenerative process, and furthermore doubled the rate of regeneration versus that of control hollow silicone NGCs (38). Based on these studies, Schwann cells have been successfully implanted in a number of studies with varying effects on nerve regeneration and functional recovery. In a study by di Summa et al., Schwann cells were seeded within a hollow fibrin conduit and implanted in a 10 mm rat sciatic nerve model (132).

These fibrin-Schwann-cell conduits showed a significant increase in nerve regeneration, versus control hollow conduits, and conduits seeded with differentiated bone marrow derived mesenchymal stem cells (dBMSCs), and differentiated adipose derived mesenchymal stems cells (dASCs) (132).
Recently, this same system, was used to bridge a 10 mm rat sciatic nerve gap over a period of 16 weeks (134). This later study highlighted the benefits of alternate cell therapies, showing functional recovery levels comparable to that of autograft (discussed below). A similar study using a PHB conduit filled with a fibrin matrix and seeded with Schwann cells or dBMSCs was shown to increase early nerve regeneration (2 weeks), unlike that of control hollow PHB conduits and conduits filled with matrix alone (34). However effects on late stage functional recovery remain to be seen. The use of autologous Schwann cells has a number of disadvantages associated with their use: culture times are long and difficult, the extraction of Schwann cells from the host is often painful and requires sacrifice of host nerve tissue (132).

The sacrifice of this tissue has the same disadvantages as those of autograft i.e. donor site morbidity, and the need for a secondary surgical site. Similarly for the use of allogenic Schwann cells, an extensive immune response, requiring further immune suppression, similar to that associated with the use of allografting, is exhibited (135). Alternative extraction methods and cellular therapies are now being considered including stem cells and the use of gene therapy approaches.

1.7.1 Stem Cells: A Possible Alternative to Autologous Schwann Cells

One cellular based alternative is the use of stem cells to enhance the host regenerative response. In a number of studies these cell types have been considered to enhance nerve regeneration (Table 1.5). These stem cells come from numerous sources but many studies are concentrating on the use of either bone marrow derived mesenchymal stem cells (BMSCs) or adipose derived stem cells (ASCs) (82, 99, 132). Autologous BMSCs can be easily derived by aspirating from the bone marrow of patients (99). Likewise ASCs can be easily extracted using conventional liposuction techniques (132). These cells conform to the criteria for ideal transplantable cells: are easily extracted, proliferate rapidly in culture, have a relatively low cost, raise no ethical issues associated with their use, and have the ability to differentiate along multiple cells lines, in particular neural and associated glial cell lineages (99, 131, 132, 136). Both ASCs and BMSCs have the advantage of exhibiting the ability to secrete multiple neurotrophic factors including
Introduction

GDNF, NGF, NT-3 and BDNF (137-139). These cells have been used in a number of studies in both the differentiated and undifferentiated states in order to investigate their effect on peripheral nerve regeneration (Table 1.5).

The advantage of using MSCs in their undifferentiated state in vivo allows these multi-potent cells to be stimulated by advancing axons and native Schwann cells, differentiating the MSCs along multiple pathways. This can aid in the creation of a conductive environment for nerve regeneration (131, 132). This differentiation in vivo may be caused by the fusion of implanted MSCs with host cells, rather than by directly differentiating into known cell types (99). These MSCs have the capacity to differentiate directly or indirectly into glial like cells, possibly secreting a variety of neurotrophic factors. Alternatively the implanted MSCs, have showed the capacity to differentiate into other supportive cells, such as endothelial-like cells, smooth muscle cells or pericytes (131). These endothelial-like cells can produce a variety of growth factors, such as VEGF, which has been shown to have a simultaneous effect on angiogenesis, neuritogenesis and neuroregeneration, which translates to positive effects on nerve regeneration in vivo (102, 107, 131).

In a very interesting study by Oliveira et al. the addition of undifferentiated BMSCs were shown to significantly increase functional recovery in a mouse median nerve model (131). Using the aforementioned model, a PCL NGC with suspended undifferentiated BMSCs was successfully implanted and regeneration was evaluated up to 12 weeks post implantation. At the defined end point there was a significant increase in the number of myelinated fibres, versus control conduits, and similarly a significant increase in angiogenesis (131). The authors hypothesised that this is attributed to the multipotent nature of the BMSCs and their known ability to secrete multiple neurotrophic factors. Similarly, in a study carried out by Ding et al., combining intraluminal fillers and undifferentiated BMSCs it showed that there was a significant increase in functional recovery across a critical nerve gap of approximately 50 mm in a canine nerve model versus that of control group, this functional recovery approaching that of autografts (99).
Table 1.5 Examples of Cell Based Approaches for Peripheral Nerve Repair

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>NGC</th>
<th>Method of Delivery</th>
<th>Model</th>
<th>Gap (mm)</th>
<th>Significant Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schwann cells</td>
<td>Hollow PLGA/silicone NGC</td>
<td>Wall release</td>
<td>Rat</td>
<td>10</td>
<td>In PLGA NGCs + Schwann cells + US nerve regeneration significantly increased.</td>
<td>(58)</td>
</tr>
<tr>
<td>and US</td>
<td></td>
<td>(3 x 10^5 cells/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schwann cells</td>
<td>Collagen conduit + collagen sponge</td>
<td>Suspension</td>
<td>Rat</td>
<td>20</td>
<td>Sponge inhibited axonal regeneration. The addition of Schwann cells with no sponge bridged a critical gap but showed poor functional recovery versus autograft.</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 x 10^6 cells/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSCs</td>
<td>Micro-grooved chitosan gold conduit</td>
<td>Injection into lumen</td>
<td>Rat</td>
<td>10</td>
<td>NSCS increased functional recovery and axonal regeneration versus a hollow conduit.</td>
<td>(140)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5 x 10^5 cells/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schwann cells</td>
<td>PHB conduit + fibrin matrix</td>
<td>Fibrin matrix</td>
<td>Rat</td>
<td>10</td>
<td>Increased nerve regeneration distance versus a hollow PHB and fibrin alone.</td>
<td>(34)</td>
</tr>
<tr>
<td>dBMSCs</td>
<td></td>
<td>(8 x 10^7 cells/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human uASCs</td>
<td>PCL conduit + RGD peptide</td>
<td>Suspension</td>
<td>Rat</td>
<td>6</td>
<td>Increased functional recovery and regeneration versus control groups.</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 x 10^8 cells/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schwann cells</td>
<td>Cellulose conduit + BD hydrogel</td>
<td>Suspension</td>
<td>Rat</td>
<td>10</td>
<td>Schwann cells + hydrogel increased regeneration distance. Effects lost at 16 weeks.</td>
<td>(106)</td>
</tr>
<tr>
<td>Cell Type</td>
<td>NGC</td>
<td>Method of Delivery</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Significant Results</td>
<td>Ref.</td>
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</tr>
<tr>
<td>uBMSCs</td>
<td>PCL conduit</td>
<td>Injection into lumen</td>
<td>Mouse</td>
<td>3</td>
<td>Increased number of myelinated fibres and angiogenesis versus control group.</td>
<td>(131)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5 x 10^8 cells/ml)</td>
<td>media n</td>
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<td></td>
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</tr>
<tr>
<td>uBMSCs</td>
<td>Chitosan conduit + PLGA fibres</td>
<td>Injection into lumen</td>
<td>Dog</td>
<td>50</td>
<td>Increased regeneration and functional recovery versus non BMSC group.</td>
<td>(99)</td>
</tr>
<tr>
<td>Schwann cells, dASCs</td>
<td>Fibrin glue conduit</td>
<td>Injection into lumen</td>
<td>Rat</td>
<td>10</td>
<td>dBMSCs/dASCs increase regeneration distance and Schwann cell migration versus hNGC.</td>
<td>(132)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4 x 10^7 cells/ml)</td>
<td>sciatic</td>
<td></td>
<td>Schwann cell treatment remained superior to all groups.</td>
<td></td>
</tr>
<tr>
<td>dBMSCs</td>
<td>Collagen conduit (Neuragen™)</td>
<td>Wall release</td>
<td>Rat</td>
<td>12</td>
<td>Increase in nerve regeneration in Schwann cell and dBMSC NGCs versus hollow group.</td>
<td>(133)</td>
</tr>
<tr>
<td>Schwann cells</td>
<td></td>
<td>(8 x 10^5 cells/ml)</td>
<td>sciatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human uBMSCs and cyclosporin</td>
<td>Fibrin glue conduit</td>
<td>Fibrin Matrix</td>
<td>Rat</td>
<td>10</td>
<td>The immunosuppressant cyclosporine increased human BMSC survival and nerve regeneration across the length of the conduit at 3 weeks.</td>
<td>(141)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8 x 10^7 cells/ml)</td>
<td>sciatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Type</td>
<td>NGC</td>
<td>Method of Delivery</td>
<td>Model</td>
<td>Gap (mm)</td>
<td>Significant Results</td>
<td>Ref.</td>
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<tr>
<td>GDNF transfected</td>
<td>Silicone conduit</td>
<td>Suspension (1 x 10^6 cells/ml)</td>
<td>Rat</td>
<td>12</td>
<td>GDNF transfected Schwann cells showed a significant increase in functional recovery and nerve regeneration versus unmodified Schwann cells</td>
<td>(142)</td>
</tr>
<tr>
<td>Schwann cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP-labelled dADSCs +</td>
<td>Silicone conduit</td>
<td>Matrigel suspension (2 x 10^8 cells/ml)</td>
<td>Mouse</td>
<td>10</td>
<td>dADSCs increased regeneration versus an empty silicone conduit. GFP-labelled cells were detected 12 weeks after injury. Iron oxide nanoparticles were detected but in only 25% of the implanted cells.</td>
<td>(143)</td>
</tr>
<tr>
<td>FeO nanoparticles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IpSC neurospheres and</td>
<td>Dual layer fibrous</td>
<td>Seeded for 14 days</td>
<td>Mouse</td>
<td>5</td>
<td>12 weeks after implantation axon regeneration was significantly greater in the IpSC + bFGF group versus IpSC alone and conduit alone. Autograft remained superior in functional recovery and regeneration.</td>
<td>(127)</td>
</tr>
<tr>
<td>bFGF (microsphere release)</td>
<td>conduit (Outer: PLA mesh, Inner: PLA:PCL sponge)</td>
<td></td>
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</table>
However, the mechanisms of the enhanced regenerative response are largely unknown, with very few BMSCs seen differentiating along a Schwann cell-like lineage (131). This variability was highlighted in an early study by Santiago et al., where the use of undifferentiated ASCs showed no transdifferentiation to a Schwann cell-like phenotype. A significant increase in nerve thickness was seen in the cell based group versus that of hollow NGCs (82). The variability of using these undifferentiated MSCs may limit their future clinical applications and further characterisation is needed to assess their suitability for peripheral nerve repair.

Due to these possible limitations of undifferentiated MSCs, one interesting alternative is the use of their differentiated counterparts. BMSCs and ASCs can be differentiated in vitro through combinations of various neurotrophic and growth factors, into a more glial or neural cell lineage (133, 135-137, 139). These differentiated MSCs (dMSCs) have the advantage of being differentiated in a controlled manner, with both bone marrow and adipose derived cells showing the ability to differentiate into Schwann cell-like cells (132, 133, 135, 137). These Schwann cell-like cells have a positive effect on neurite outgrowth on sensory dorsal root ganglion neurons in vitro (135, 137) and in recent studies have been shown to have beneficial effects in vivo (133). In the case of dASCs, these positive effects on neurite outgrowth may be attributed to increased level of BDNF and NGF versus that of their undifferentiated counterparts (139). The controlled differentiation of MSCs may also reduce concerns of adverse effects associated with undifferentiated MSCs, effects such as differentiation along a tumorogenic cell line (132). Similarly, this controlled differentiation allows the creation of a less variable cellular based treatment for peripheral nerve repair.

In a study, by di Summa et al. differentiated ASCs and BMSCs were tested in a 10 mm rat sciatic nerve model where they exhibited a significant increase in nerve regeneration versus that of control hollow conduits at an early stage of nerve regeneration (two weeks) (132). In a subsequent study, dASCs showed a significant increase in functional recovery and similar nerve morphometry to that of autograft 16 weeks post implantation (134). This study emphasizes the benefits of pre-differentiating MSCs to a Schwann cell-
like phenotype before implantation. It may yet prove a valid alternative for autologous Schwann cell implantation.

Ladak et al. in a later study showed dBMSCs implanted within a hollow collagen NGC, have a similar increase in levels of regeneration, with similar increases in vitro neurite outgrowth (133). Despite promising in vitro results and an increase in levels of nerve regeneration in vivo, functional recovery remained poor and significantly lower than that of autograft groups (133). This contrary result underlines the importance of correct cell-material combinations. Future studies in this area may consider the use of combinatorial approaches e.g. utilising structural contact guidance in the aforementioned intra-luminal guidance structures and variations in conduit design. These structures may be used to control the distribution of the implanted cells within the NGC and simultaneously interacting with native Schwann cells. In addition these same differentiated and undifferentiated MSCs may be subjected to ex vivo modulation discussed below.

1.7.2 Genetically Modified Cells

Current modification of Schwann cell cultures is primarily carried out ex vivo and a relatively few amount have been implanted in vivo. In an initial study by Timmer et al. Schwann cells were transfected to overexpress basic high molecular weight fibroblast growth factor (FGF-2) (144). The transfected cells were enclosed within a Matrigel™ filled silicone tube and implanted in a 15 mm rat sciatic nerve model. The transfected cell group showed a significant increase in the number of myelinated axons versus the control group. Later a similar study was carried out by Haastert et al. using two isoforms of FGF-2 (either high or low molecular weight FGF-2) (145). Schwann cells were transfected to overexpress each isoform of FGF-2 and similarly enclosed within a Matrigel™ filled silicone tube and implanted in a 15 mm rat sciatic nerve model. The high molecular weight FGF-2 supported functional sensory recovery; however, the low molecular weight FGF-2 was shown to have an inhibitory effect on myelination (145).

In a study by Li et al., allogenic rat Schwann cells were transfected with a retrovirus encoding for enhanced expression of GDNF and implanted within
a rat sciatic nerve model (142). The use of these GDNF modified Schwann cells showed a significant increase in myelination, nerve regeneration and functional recovery compared to that of hollow conduits or similarly conduits seeded with unmodified Schwann cells. It was also reported that this enhanced expression could be maintained for up to six weeks post injury, peaking roughly at four weeks post transduction (142). The use of genetically modified Schwann cells requires further in vivo studies to be carried out to assess their potential benefit for functional nerve regeneration and another interesting alternative may be the ex vivo modification of undifferentiated or differentiated MSCs; possibly increasing their potential for peripheral nerve repair.

Of note was a study carried out by Schmitt et al. suggesting that the use of adult canine Schwann cells (cSCs) is a more clinically relevant model for translational research. In particular for the translation of cellular based therapies, this cell type unlike those of rodent cells displays characteristics that are similar to primate cells (146). These characteristics include the stable expression of the low affinity binding receptor, p75NTR; the ability to grow for long periods in the absence of mitogens and no spontaneous immortalisation of cultured Schwann cells. These canine Schwann cells were successfully transfected by nucleofection, an alternative means to retrovirus transduction and showed the ability to express enhanced green fluorescent protein in vitro and in vivo. However, transfection by non-viral plasmids was seen as quite transient and expression levels became minimal after a period of one week in vivo. This suggests the need for prolonged release of this non-viral plasmid within the cell, if it is to show potential for peripheral nerve repair.

In a recent study, GFP in combination with iron oxide nanoparticles has been used to track the implantation of mouse dADSCs in a 10 mm mouse sciatic nerve model. GFP was shown to be detectable up to four weeks after injury with expression decreasing over the implanted time period. Iron oxide nanoparticles could be used to track implanted cells using MRI imaging, however only 25 % of the iron oxide nanoparticles remained within the implanted cells 12 weeks after injury (143). In order to truly harness the
benefits of MRI and iron oxide nanoparticle technology, the residency time of the iron nanoparticles would need to be improved for future live cell imaging experiments.

1.8 Improvements in Current Intraluminal Guidance Structures
One possible limitation is the use of slow degrading intraluminal guidance structures. Current intraluminal guidance structures are made of slow degrading polymers and this often results in inhibition of regenerating axons or the formation of axonal and Schwann cell depleted zones (40, 43, 47, 65). One interesting alternative to this approach is the use of a faster degrading polymer within the lumen of the conduit (14, 147). In a study by Nichterwitz et al. poly-p-dioxanone (PDO) filaments enclosed within a host epineural tube were implanted in a rat sciatic nerve model and assessed after a period of six weeks for Schwann cell alignment and axonal regeneration (147). This study highlights the capacity of Schwann cells to form glial bands of Büngner and to maintain these bands despite the fact that the underlying PDO filaments had begun to degrade. These regenerating axons were shown to follow these glial cell bands during repair. This can be seen to match the regeneration in the conduit over a relatively short gap. Noteworthy - during the weeks of the cellular and axonal phase, the fibrin cable which has a degradation time of approximately two weeks, has most likely degraded having fulfilled its role for cellular migration (34, 35) and has probably formed the trophic and topographical biological tissue which is a key factor for successful nerve repair. This could be seen as an essential pre-requisite for the use of intraluminal fillers. However, increases in toxicity levels must be accounted. Similarly in a study by Wood et al. the use of a fibrin matrix which degrades after a period of 4 weeks has beneficial effects on nerve regeneration (104). This further supplements the idea that intraluminal fillers are beneficial, but only at the early stages of growth, after which it can be hypothesised that they become an inhibitory molecule.

1.9 Hypotheses and Objectives
The overall aim of this project was to develop a suitable biomaterial platform to promote regeneration and functional recovery of the injured peripheral nerve. It was hypothesised that intraluminal collagen fibre conduit can
synergistically act as a platform for cellular migration and topographical guidance of axons, while creating a functional microenvironment for nerve regrowth, with the ultimate aim of treating long both non-critical and critical nerve injuries.

The development of such a system occurred in two parts: first was the development of suitable tissue-engineered platform for repair, the second was to elucidate the biomaterial-induced host response at a molecular level to the proposed repair strategy. The development of this system was carried out over four main phases, each with specific objectives and hypotheses.

1.9.1 Phase One: The Development of a Biomaterial Platform for Repair

**Overall aim:** To develop, characterise and assess a natural ECM bridge for its ability to promote repair in the injured peripheral nerve.

**Hypothesis:** The use of structured or unstructured collagen fibres, enclosed within a hollow collagen nerve guidance conduit, will promote regeneration across a non-critical nerve gap.

**Specific Objectives:**

- To develop a platform suitable for cellular migration and for guided regeneration of re-growing axons.
- To evaluate both structured and unstructured fibres *in vitro* as a platform for cellular migration and guidance of regenerating axons.
- To implant the intraluminal fibre conduits in a 10 mm rat sciatic nerve model and investigate their influence on the regenerative response during peripheral nerve repair.

1.9.2 Phase Two: Optimising Packing Density, Collagen Fibre Degradation and Assessing Functional Recovery

**Overall Aim:** To optimise the packing density and degradation properties of the implanted intraluminal fibre conduits and assess their ability to promote functional nerve regeneration across a critical and non-critical nerve gap.
Hypotheses:

- Optimising the packing density of the intraluminal fibre conduit will increase levels of nerve regeneration and promote functional recovery across both non-critical and critical nerve gaps.
- Improving the degradation properties of the implanted intraluminal fibres will allow further axonal regeneration at a late stage of repair by removing areas of growth inhibition.

Specific Objectives:

- To optimise the degradation properties of the intraluminal collagen fibres in vitro and assess their degeneration in vivo in a rat subcutaneous model.
- To implant the optimised intraluminal fibre conduit in vivo in a rat sciatic nerve model and assess its ability to promote regeneration across both non-critical and critical nerve gaps.
- To assess the level of functional recovery achieved using an intraluminal fibre conduit and compare it to the gold standard autograft.

1.9.3 Phase Three: A Biomaterials-Induced Proteomic Response to Conduit-Mediated Nerve Repair

Overall Aim: To assess the proteomics changes that occur during peripheral nerve regeneration, as a function of the treatment method and biomaterial used.

Hypothesis: Treatment of peripheral nerve injuries elucidates biomaterial-induced proteomic changes during peripheral nerve repair which is specific to the biomaterial and treatment method used.

Specific Objectives:

- To investigate the early biomaterial-induced proteomic changes two weeks after injury using a eight-plex multi-dimensional proteomics analysis.
Introduction

- To compare the changes in protein expression in both a biological (collagen) and synthetic (PLGA) nerve guidance conduit versus the current gold standard for repair (autograft).
- To elucidate proteins which may be over/under expressed in the current gold standard for repair in comparison to conduit–mediated repair.

1.9.4 Phase Four: The Effect of Intraluminal-Collagen Fibres on the Biomaterial-Induced Proteomics Changes during Peripheral Nerve Repair across both Non-Critical and Critical Nerve Gaps

**Overall Aim:** To investigate the proteomic changes that occur using an intraluminal fibre conduit across a non-critical and critical nerve gap and compare it to the gold standard for repair

**Hypotheses:**

- The use of an intraluminal guidance conduit promotes recovery in comparison to a hollow nerve guidance conduit, this increase in recovery is due to changes in the early proteomic response to nerve regeneration.
- Autograft shows superior regeneration and functional recovery in comparison to an intraluminal fibre conduit and these changes in recovery may be directly related to differences in protein expression between the conduit and the graft.
- Nerve regeneration decreases as gap length increases, across all treatment groups. This decrease in regeneration may be as a result of a differential proteomic response across increasing gap lengths.

**Objectives:**

- To implant an intraluminal fiber conduit and compare the proteomic changes which occur during the early stages of peripheral nerve repair.
- To compare the implanted intraluminal fiber conduit to the current gold standard and elucidate the missing components for repair.
- To assess the effect of gap length across all treatment groups and elucidate possible treatment strategies for repair.
1.10 References


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Introduction


Introduction


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Chapter Two

Intraluminal Collagen Fibres: A Suitable Platform for Repair?

Contents of this chapter have previously been published in:

2.1 Introduction

Treatment of peripheral nerve injuries are currently limited to a small number of microsurgical treatment methods. These treatments are ineffective as these interventions often lead to painful neuropathies as result of loss in motor control and sensory perception (1, 2). Over relatively short nerve gaps, spontaneous natural regeneration may occur. However, over larger gaps, microsurgical repair is essential for nerve regeneration (3-5). The primary methods of repair include direct/primary repair, transplantation of autografts or allografts, and the use of hollow nerve guidance conduits (6). Direct repair is limited to treating short nerve defects (less than five millimetres in length) and requires tension-free suturing of the damaged nerves (7, 8).

Beyond this relatively short gap, autografting is the current gold standard for repair. Despite providing a number of advantages for repair, success in the clinic has been limited. This is partly due to a number of the intrinsic limitations of the gold standard, particularly nerve and axonal size mismatches between the donor nerve and the targeted injured nerve (6, 9). In addition there is a requirement for a second surgical site i.e., donor site that has a limited supply of nerve. Donor site complications often leads to morbidity and pain. Despite these limitations, autograft healing is characterised by some intrinsic ideal components for peripheral nerve repair. Autograft provides a natural architecture for guided nerve regeneration, as well as being readily incorporated into the regenerative process; due to the graft being derived from the host’s own tissue. The intrinsic network of extracellular matrix proteins and cell adhesion molecules provides regenerating axons, and migrating and proliferating Schwann cells (from the proximal and distal nerve stumps) with appropriate topographical and biological guidance cues to achieve functional nerve regeneration (10). To recreate this natural internal architecture for repair, an extracellular matrix derived platform (i.e. one made from collagen, fibronectin or laminin) often proves useful for repair (11-13). In efforts to reconstruct this architecture, this study proposes the use of a natural extracellular matrix protein (collagen) based construct.
Figure 2.1 Conceptual diagram of the incorporation of either structured or unstructured fibres into a biomaterial nerve guidance conduit. The incorporation of intraluminal fibres within a nerve guidance conduit will create a platform for repair, while simultaneously providing topographical guidance cues to regenerating axons and migrating glial cells, ultimately creating an inductive environment for repair.
This construct displays topographical features and structural characteristics which are beneficial for repair. This investigation incorporates such a system in the form of intraluminal collagen fibres into the lumen of a hollow nerve guidance conduit. Hollow nerve guidance conduits are commercial alternatives to grafting and have a number of advantages for repair. These advantages include limiting myofibroblast infiltration, a reduction in scar and neuroma formation, and no associated donor pain (14). However, hollow conduits provides only limited structural support (in the form of a fibrin cable) and guidance to regenerating axons and as a result functional recovery remains poor (6, 8, 15). To overcome the limitations of the use of hollow conduits, intraluminal guidance structures (e.g. fibres, films, gels) are used to supplement/replace the fibrin cable and/or to recreate the natural topographical features of autograft (16-20).

This study uses intraluminal biologically derived collagen fibres for such a purpose (Figure 2.1). Ultimately, the addition of these extracellular matrix (ECM) derived components aims to recreate the hierarchical organisation and biological function of the native extracellular matrix. This investigation thoroughly analyses the incorporation of these components into the host regenerative process and assesses their feasibility for improving or enhancing conduit mediated nerve repair. In particular this study investigates the influence of intraluminal fibres on the levels of axonal dispersion within the biomaterial conduit.

In addition this study incorporates a number of longitudinal grooves on the surface of the collagen fibres for additional topographical guidance (Figure 2.2). The use of topographical guidance cues has shown to have beneficial effects for nerve repair. Topographical cues have been shown to introduce complex signalling responses within the growth cone of a regenerating and have resulted in profound changes in the neuronal response to injury (21). Structural cues have been incorporated on a number of substrates in vitro resulting in a significant increase in aligned neurite outgrowth (13, 21, 22).
Figure 2.2 Images of unstructured and structured collagen fibres and their incorporation into a hollow collagen nerve guidance conduit. (A) SEM image of an unstructured collagen fibre with a diameter of approximately 50 µm. Scale bar, 10 µm. (B) SEM image of structured collagen fibre with four channels on the surface of the fibres (channel diameter 10 µm). Scale bar, 10 µm. (C) Photo of a conduit with 18 structured collagen fibres inserted into the lumen of a hollow collagen nerve guidance conduit. Fibres are shown to be protruding from the lumen of the conduit. For implantation, fibres are trimmed to a length of 10 mm and inserted into a 12 mm long hollow nerve guidance conduit. This allows the proximal and distal nerves to be implanted into the conduit without inducing axial compression on the intraluminal fibres. Scale bar, 1.5 mm.
This increase in neurite alignment and growth is accompanied by a significant reduction in the number of neurites and neurite branching. Mahoney et al. demonstrated increased alignment and growth of PC12 cells on micro-structured polyimide for features in the range of 20-30 \( \mu \text{m} \) versus features which had a width of 40 – 60 \( \mu \text{m} \) (23). Previous work by Yao et al. demonstrated increased aligned growth of PC12 neurites on micro-structured PLGA film with features ranging from 5 – 10 \( \mu \text{m} \) in width (24). Based on these results, topographical guidance features have been combined with the afore-mentioned intraluminal guidance structures in the form of structured collagen fibres to assess their combined ability to enhance peripheral nerve repair.

The hypothesis of this chapter is that structured/unstructured collagen fibres will provide a three-dimensional platform for guidance of regenerating axons, \textit{in vitro} and \textit{in vivo}, to promote enhanced regeneration within a hollow collagen nerve guidance conduit. To investigate this hypothesis, this study had the following objectives: 1) to create a platform for cell migration and topographical guidance of axons, 2) to assess the effects of topographical guidance, of the structured/unstructured collagen fibres, on neurite outgrowth using a three dimensional \textit{in vitro} model, 3) to implant the structured/unstructured fibres, enclosed within a hollow collagen conduit, \textit{in vivo} and evaluate their effect on peripheral nerve regeneration using a 10 mm rat sciatic nerve model.

2.2 Materials and Methods

2.2.1 Proof of Concept – Structuring of PGCL Sutures

For initial proof of concepts, Monocryl™, a synthetic monofilament absorbable suture composed of a copolymer of glycolide and epsilon-caprolactone (PGCL) (70 \( \mu \text{m} \) diameter, Johnson and Johnson, Dublin, Ireland) was used to assess the ability of the excimer laser system to create longitudinal grooves along the surface of a fibrous substrate. A custom-made rotary stage was used was used to hold the PGCL fibres in place and to facilitate controlled rotation of the fibres. The rotatory stage was controlled...
using Optec Micromaster® Software (Optec MicroMaster®, Frameries, Belgium). The rotary stage was subsequently placed into the excimer laser system. The system consisted of an excimer laser (ATL Atlex®, Wermelskirchen, Germany) used in conjunction with a machining centre (Optec MicroMaster®, Frameries, Belgium). The active medium for the laser was argon fluoride gas (Spectra Gases, UK) with a 3-7 ns pulse duration. Argon fluoride gas allows operation of the laser at a wavelength of 193 nm, which results in the clean ablation of a large range of materials in particular biological substrates. This allows structuring of the initial PGCL fibres and later the extruded collagen fibres without damage to the underlying substrate (25). A constant pulse repetition frequency of 100 Hz with a variable speed of 250 - 500 µm/sec was selected to give the optimal compromise of process speed and feature accuracy. The microgrooves were generated using a standard mask projection machining approach. For each parameter, a different dimensioned mask was used with a varied demagnification and surface fluence (i.e. laser energy per unit area), to produce topographical features of defined width and depth (Table 2.2).

Structuring of the surface was confirmed using a scanning white light interferometric surface profiler (NewView 100, Zygo, Middlefield, CT, USA) and Scanning Electron Microscopy (SEM) (Phenom™ Desktop scanning electron microscopy (FEI®, Eindhoven, The Netherlands).

### 2.2.2 Extrusion and Cross-linking of Collagen Fibres

The extrusion procedure and cross-linking were carried out in the same manner as reported by Zeugolis et al. (26, 27). A 5 ml syringe (BD Scientific, UK) containing 5 mg/ml bovine type I atellocollagen was extruded at a rate of 0.3 mL/min by a syringe pump (KD-Scientific 200, KD-Scientific Inc., Massachusetts, USA) through 0.03 mm inner diameter silicone tubing (Polymer Technologies Ltd, Warwickshire, UK) into a fibre formation buffer (118mM phosphate buffer and 20% of polyethylene glycol, Mw 8000 (pH 7.50 and 37°C). Fibres were allowed to remain in the fibre formation buffer for five minutes and transferred into a fibre incubation buffer (6.0 mM
phosphate buffer and 75 mM sodium chloride; pH 7.10 and 37°C) for a further five minutes.

The extruded collagen fibres were subsequently cross-linked with N-(3-dimethylaminopropyl)-N-ethylcarbodiimide and N-hydroxysuccinimide (EDC and NHS respectively), at a ratio of 30 mM : 10 mM respectively, in 50 mM MES buffer (pH 5.5), for 24 hours. After this period, the fibres were rinsed three times with sterile distilled water and allowed to air-dry, under the tension of their own weight. Similarly, for non-cross-linked collagen fibres, fibres were briefly dipped in distilled water for approximately one minute and then allowed to dry under the tension of their own weight for 24 hours. The diameter of the collagen fibres were measured using an Olympus® IX-81® inverted microscope (Mason Technology, Dublin, Ireland). The degree of crosslinking was confirmed via the ninhydrin assay.

2.2.3 Fabrication of Structured Collagen Fibres

Microgrooves were produced on the surface of the collagen fibres using the excimer laser system mentioned above. Argon fluoride gas (Spectra Gases, UK), with a 3-7 ns pulse duration, was similarly chosen as the active laser medium for the laser. A pulse repetition frequency of 100 Hz with a variable speed of 250 - 500 µm/sec was similarly used. The microgrooves were generated using a standard mask projection machining approach. For each parameter, a different dimensioned mask was used with a varied demagnification and fluence, to produce topographical features of defined width and depth (Table 2.3).

A rotary stage was used in conjunction with the aforementioned excimer laser system to create micro-structured fibres with multiple grooves orientated longitudinally across its surface (Figure 2.3). Structuring of the surface was confirmed using a scanning white light interferometric surface profiler (NewView™ 100, Zygo®, Middlefield, CT, USA) and scanning electron microscopy (SEM) (Phenom™ Desktop SEM (FEI®, Eindhoven, The Netherlands).
2.2.4 Surface Characterisation of Structured PGCL and Collagen Fibres

Characterisation of the micro-structured fibres was carried out in the Advance Microscope Facility (Centre of Research on Adaptive Nanostructures and Nanodevices, Trinity College, Dublin) on a Carl Zeiss Auriga® focused ion beam (FIB) system. Depth measurements and groove widths were quantified using this SEM – FIB system. Briefly, samples were sputter-coated (Cressington® 208 HR sputter coating system) with a protective 10 nm palladium layer and grounded with silver paint. Samples were mounted in the FIB mounting chamber and a SEM image of each section was taken. The sample stage was tilted from 0º to 54º to determine a suitable site for cross-sectioning. Upon location of a suitable region of interest, FIB cross-sections were taken at high beam currents, and subsequent polishing of the surface performed at lower beam currents and SEM images taken of the sectioned region (Figure 2.3). This technique allowed for an accurate analysis of groove width and depth measurements from the cross-section (Table 2.2 and Table 2.3).

2.2.5 Neuronal Cell Interaction with the Structured Fibres

The interaction of neuronal cells with the structured and the unstructured collagen fibres were assessed in vitro. The interaction was initially assessed using the the PC12 cell line. PC12 cells derived from rat pheochromocytoma cells when treated with NGF becoming phenotypically altered and display many characteristics of a neuronal phenotype. Among these, the ability to extend neurites which can respond to topographical guidance cues was used for the initial proof of concept studies (28-31).

Control unstructured collagen fibres were compared to 5 µm and 10 µm structured fibres for their ability to guide neuronal outgrowth. Rat PC12 cells were cultured in dulbeco modified eagles media (DMEM) supplemented with 10% heat inactivated horse serum (HS), 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S). Cells were cultured at 37º in a cell culture incubator (5 % CO2 and 95 % relative humidity).
The Effect of Intraluminal Guidance
Figure 2.3 Excimer laser rotary stage setup for structuring of the fibres and scanning electron microscope images of the SEM-FIB process. (A) Photograph of the fabricated rotary stage used to hold the collagen fibres in place during structuring. Stage worked readily with the excimer laser system’s software package to allow precise control of the rotation of the fibres and alignment of the laser. Scale bar, 5 cm. (B-D) SEM images of the sequence of events in the SEM-FIB process. A suitable region of interest was identified (B) and the sample was rotated to 54 ° for structuring. High focus ion beam currents were used to generate a cross-section and subsequent polishing of the cross section was performed at lower beam currents. The resulting cross section can be seen in (C). Quantification of the cross section parameters was performed at higher magnification as in (D). Scale bars, 50 µm (B and C), 1 µm (D).
Growth media was changed every three days and PC12 cells were split/seeded once a confluence of 80% was reached. PC12 cells were seeded within a collagen gel solution (4,000 cells per 20 µl gel) at one end of the structured or unstructured collagen fibres. The collagen gel solution allowed the cells to interact within a three dimensional (3D) environment (Figure 2.6). The fibres were laid down onto a glass substrate to reduce interaction with the underlying substrate and held in place using sterile autoclaved silicone vacuum grease (Dow Corning, Midlands, MI). Before cell seeding, fibres and all substrates were sterilised with 70% ethanol for two hours, and subsequently rinsed three times with sterile 0.1 M PBS solution. Once suspended PC12 cells were differentiated to a neuronal cell phenotype through the addition of NGF to diluted cell culture media (50 ng/ml NGF in 1% HS, 0.5% FBS, 0.1% P/S) and allowed to interact with the collagen fibres for a period of seven days. Media was changed every three days during this time period. At the end of the culture, cells were fixed with 4% paraformaldehyde, washed, permeabilised with 0.1% Triton X-100 and stained with rhodamine-phalloidin solution (1:100, Sigma Aldrich, Dublin, Ireland). Samples were subsequently mounted and viewed under an Olympus IX-81® inverted microscope (Mason Technology, Dublin, Ireland). Outgrowth of the growing neurites was compared using the following parameters: neurite length, number of neurites and neurite orientation.

Dorsal root ganglion (DRG) cells were similarly used to compare the interaction of the structured and unstructured fibres with a primary neuronal cell line. Dissociated DRG cells were isolated as per the protocol in Appendix R and seeded within a collagen gel (4,000 cells per 20 µl gel respectively) as above. The cells were allowed to interact with the fibres for a period of 72 hours. At the end time-point, cells were fixed with 4% paraformaldehyde for one hour, rinsed twice with 0.1 M PBS solution and permeabilised with 0.1% Triton X-100. The samples were rinsed twice with 0.1 M PBS solution and blocked in 10% bovine serum albumen (BSA) solution for a period of one hour. Samples were rinsed twice with 0.1 PBS and exposed to rat neurofilament primary antibody (anti-NF160, 1:200) for approximately two hours. Samples were rinsed and exposed to donkey anti-
rat antibody for a period of one hour (1:100 in blocking buffer). Samples were rinsed twice, counterstained with DAPI (1:10,000) for two minutes, mounted and viewed under an Olympus® IX-81® inverted microscope (Mason Technology, Dublin, Ireland).

2.2.6 Preparation of Intraluminal Conduits and Experimental Groups for In Vivo Implantation

Intraluminal conduits were constructed by enclosing 18 collagen fibres within a hollow collagen conduit (approximately 2.2 % of the total cross-sectional area). These conduits were then filled with structured and non-structured collagen fibres respectively. Structured fibres of approximately 50 µm diameter and four longitudinal channels (10 µm width and a high depth) were used for all in vivo experimentation. Samples were sterilised by a two hour incubation period in 70 % ethanol, rinsed in sterile 0.1 M phosphate buffer and stored until use. 56 adult female Lewis rats weighing between 220 – 250 g were randomly assigned to one of four experimental groups (Table 2.1). Two experimental procedures were carried out for each experimental group: both nerve morphometry (n=8) and simultaneous retrograde tracing (n=6) analyses. All experimental procedures were carried out in accordance to National and Institutional guidelines set out by the Cruelty to Animals Act 1876 as amended by the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations 2002 and 2005.

2.2.7 Surgical Procedure

Rats were anesthetised by intraperitoneal injection under aseptic conditions. The left sciatic nerve was exposed and isolated at the mid-thigh level using a dorsal gluteal muscle splitting approach. The nerve was resected 5 mm proximally from the distal bifurcation of the tibial and peroneal branches and a five mm nerve segment was removed, resulting in the creation of a 10 mm nerve gap. The proximal and distal ends of the transected nerve were inserted 1 mm into 12 mm long collagen tubes (with/without intraluminal fillers) and secured to the epineurium using Ethilon™ 10-0 monofilament nylon sutures (Johnson and Johnson, Dublin, Ireland). The wound was finally closed in
layers and the animals received buprenorphine hydrochloride for the treatment of pain. Animals were continuously monitored for signs of distress or pain. For autograft repair the nerve were similarly transected at two different sites, approximately one cm apart, rotated 180º and re-attached to the transected nerve stumps, using Ethilon™ 10 – 0 monofilament nylon sutures (Johnson & Johnson, Dublin, Ireland).

2.2.8 Nerve Histology and Ultrastructural Analysis

Sixteen weeks post implantation, the sciatic nerve was re-exposed under anaesthesia. The exposed sciatic nerve was fixed in situ using Trump’s fixative solution (4 % formaldehyde, 1 % glutaraldehyde in 0.1 M phosphate buffer saline solution) for 30 minutes (32). The fixative solution was removed from the injury site, and the sciatic nerve was resected and divided into three one mm sections: one mm proximal to graft, mid-graft and one mm distal to the graft. The sections were placed in the Trump’s fixative solution and stored for sectioning. Sections from the midsection of the nerve were post-fixed in 1 % osmium tetroxide solution and passed through of series of graded alcohol solutions for serial dehydration of the samples. Sections were embedded in spur resin (EMS, Fort Washington, USA) and 1 µm semi-thin sections were cut using a glass knife on a Leica UltraCut E® Microtome (Rankin Biomedical Corp., Michigan, USA), stained with 1% toluidine blue and mounted for light microscopy and subsequent electron microscopy. For high resolution analysis of the regenerated axons and their associated myelin sheaths, ultrathin (50 – 60 nm thick) sections were taken from both the structured and unstructured collagen fibre groups and examined using a transmission electron microscope (TEM).

2.2.9 Morphology and Stereology

The proximal, midsection and distal sections of the resected nerve was quantified for axonal area, number of axons, axonal density, diameter of axons, and diameter of myelinated fibres, myelin thickness, axonal size distribution and g-ratio.
Table 2.6 List of the Treatment Groups and Number of Animals Used for the Current *In Vivo* Study and the Experimental Procedures Carried out on Each Respective Group.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Nerve Morphometry</th>
<th>Retrograde Tracing</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Hollow collagen conduit</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Unstructured fibres</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Structured fibres</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>32</strong></td>
<td><strong>24</strong></td>
<td><strong>56</strong></td>
</tr>
</tbody>
</table>
G-ratio was taken as the ratio of inner axonal diameter to the total axonal diameter and was used as a functional index of myelination. The ideal g-ratio was taken to be approximately between 0.6 and 0.7 (33).

All parameters were quantified using an upright florescent microscope (BX51 Upright Fluorescent Microscope®; Olympus Inc., Center Valley, USA) and analysed using ImageJ image analysis software (NIH, Bethesda, USA). The nerve area was quantified via tracing of the circumference of the regenerated tissue at 100 x magnification. Likewise, the number of blood vessels per section was evaluated at this magnification. Axonal counts were quantified stereologically at 1000 x magnification using the area fraction method (34). This method allows all axons to be equally sampled from the total nerve area. A 1600 µm² area was taken and counted using an unbiased counting frame and repeated in a systematic and random manner (Figure 2.7). The number of axons per unit area was calculated and extrapolated to the total nerve area to give total nerve counts for the section. Similarly, the fibre and axon size distribution, average axon and myelinated fibre diameters, and myelin thickness were counted similarly. A minimum of 50 axonal profiles per sample were evaluated using this method. Samples for the proximal and distal sections were only quantified for the fibre conduit groups due to the unavailability of samples from the autograft and hollow conduit group.

2.2.10 Simultaneous Retrograde Tracing

At week 16, the sciatic nerve was re-exposed, at the location where the tibial and peroneal nerves branch off the sciatic nerve (Figure 2.8). Distal from this branching point, the tibial and peroneal nerves were transected. The proximal end of the cut peroneal nerve was initially placed in a 5 % diamidino yellow solution (Sigma Aldrich, Co. Wicklow, Ireland) for 30 minutes. Subsequently the cut end of the tibial nerve was exposed to 5 % fast blue solution (Sigma Aldrich, Co. Wicklow, Ireland) for further 30 minutes. After exposure, the cut ends of the nerves were washed with 0.9 % saline solution and were sutured back into the surrounding tissue to prevent leakage of the dye.
Table 2.7 Parameters for Excimer Laser Structuring of the PGCL Fibres and Resulting Diameters and Depths of the Micro-Structured Fibres

| Targeted Groove Diameter (µm) | Actual Mean Diameter (±SD) (µm) | Mean Diameter (±SD) (µm) | Mean Depth (±SD) (µm) | Fluence Used (mJ/cm²) | Speed (µm/s) | Rep. Rate (Hz) | Spot Size (µm²) |
|-------------------------------|---------------------------------|--------------------------|-----------------------|-----------------------|--------------|---------------|----------------|-----------------|
| 2.5                           | 2.77 ± 0.18                     | 2.06 ± 0.62              | 176                   | 250                   | 100          | 340           |                |
| 5                             | 4.98 ± 0.54                     | 2.85 ± 0.64              | 120                   | 500                   | 100          | 1500          |                |
| 10                            | 9.55 ± 0.54                     | 10.01 ± 1.75             | 233                   | 500                   | 100          | 3000          |                |
Figure 2.4 Proof of concept micro-structured fibres (A) A PGCL fibre structured initially with a single 10 µm longitudinal groove. Scale bar, 20 µm. (B) The PGCL fibre in the SEM-FIB system for analysis of the structured fibre. Scale bar, 5 µm. (C) A PGCL fibre incorporating multiple longitudinal grooves along its surface demonstrating the ability of the excimer laser system to structure a fibrous construct. Scale bar, 50 µm.
### Table 2.8 Parameters for Excimer Laser Structuring of the Extruded Collagen Fibres and Resulting Diameters and Depths of the Micro-Structured Fibres.

<table>
<thead>
<tr>
<th>Groove Targeted Diameter (µm)</th>
<th>Actual Mean Diameter (±SD) (µm)</th>
<th>Mean Depth (±SD) (µm)</th>
<th>Fluence Used (mJ/cm²)</th>
<th>Speed (µm/s)</th>
<th>Rep. Rate (Hz)</th>
<th>Spot Size (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>3.45 (±0.32)</td>
<td>3.81 (±0.52)</td>
<td>340</td>
<td>250</td>
<td>100</td>
<td>340</td>
</tr>
<tr>
<td>5 (low depth)</td>
<td>5.09 (±0.50)</td>
<td>3.17 (±0.94)</td>
<td>86</td>
<td>500</td>
<td>100</td>
<td>1500</td>
</tr>
<tr>
<td>5 (high depth)</td>
<td>4.80 (±0.44)</td>
<td>5.92 (±0.82)</td>
<td>120</td>
<td>500</td>
<td>100</td>
<td>1500</td>
</tr>
<tr>
<td>10 (low depth)</td>
<td>9.65 (±0.55)</td>
<td>6.77 (±0.34)</td>
<td>130</td>
<td>500</td>
<td>100</td>
<td>3000</td>
</tr>
<tr>
<td>10 (high depth)</td>
<td>11.08 (±1.11)</td>
<td>8.55 (±0.72)</td>
<td>233</td>
<td>500</td>
<td>100</td>
<td>3000</td>
</tr>
</tbody>
</table>
The Effect of Intraluminal Guidance

A: 2.5 um Structured Fibres
B: 5 um Structured Fibres
C: 10 um Low Depth Structured Fibres
D: 10 um High Depth Structured Fibres
Figure 2.5 Range of micro-structures produced on the surface of the extruded collagen fibres using the excimer laser system (A-D). Features can be produced with a high degree of reproducibility and can be quantitatively measured using a combination of SEM-FIB analysis and white light interferometry. All scale bars, 2 μm.
Seven days after tracer application, the animals were transcardially perfused with 4% paraformaldehyde and 10% sucrose in phosphate buffered solution (PBS) and the spinal cord segments L1 to L6 were removed and post-fixed overnight. Sagittal longitudinal sections were embedded in Cyro-gel™ (Instrumedics Ltd, St Louis, MO) and 30 µm thick sections were cut on a cryostat (Microm HM505E Cryostat®, Walldorf) at -20°C. Sections were immediately evaluated under an upright fluorescent microscope (BX51 Upright Fluorescent Microscope®, Olympus Inc.). Neuronal profiles with blue cytoplasm and a dark nucleus were counted as FB-labelled neurons, profiles with a yellow nucleus and dark cytoplasm as DY-labelled neurons, and profiles with a yellow nucleus and blue cytoplasm as FB/DY-double-labelled neurons. All neurons in each section were counted.

2.2.11 Statistical Analysis

All graphical data is presented as mean ± standard error of the mean (S.E.M.). Graphpad™ v5.1 (Graphpad Software, California, USA) statistical analysis software was used for all statistical analysis. Statistical analysis included a one way analysis of variance (ANOVA) followed by a Tukey post hoc test for multiple comparisons. A $p$-value of 0.05 was deemed statistically significant.

2.3 Results

2.3.1 PGCL Proof of Concept Fibres

Monocryl™ PGCL sutures were structured using the excimer laser system and characterised using a combination of white light interferometry and SEM-FIB analysis. 70 µm monofilament fibres were sutured with a range of topographical features with groove diameter's of 2.5 µm, 5 µm and 10 µm respectively. Feature diameters quite accurately represented the intended feature dimensions for all parameters and a range of groove depths could be produced by varying the fluence of the excimer laser (Table 2.2). Groove diameters were quantified using SEM-FIB analysis and groove depths by white light interferometry. On the polymeric substrate a distinct and sharp
architecture was produced in comparison to the smoother more rounded architecture seen in the structured collagen fibres. The PGCL grooves were triangular in appearance and narrowed in diameter from the outer to the inner surface of the fibre. By controlled rotation of the fibre a number of longitudinal grooves could be incorporated onto the fibre surface; confirming the function of the manufactured rotary stage (designed specifically for this project) (Figure 2.4).

2.3.2 Fabrication and Characterisation of Intraluminal Collagen Fibres

Intraluminal collagen fibres were fabricated using a fibre extrusion process (Figure 2.1). These fibres were approximately 50 µm in diameter and were cross-linked using EDC: NHS at a ratio of 30 mM: 10 mM to increase their strength, lower their degradation rate and to maintain their structural integrity in vivo. This allowed the fibres to be held rigidly in place in the rotary stage for subsequent structuring. The degree of cross-linking was confirmed using a ninhydrin assay. This assay showed a significant reduction in the number of free amines in the cross-linked group versus the control non cross-linked collagen fibres group ($p < 0.05$).

The cross-linked fibres were subsequently structured using an excimer laser system that was linked to a machining centre that produced the defined topographical features (Figure 2.2 and Figure 2.3). For qualitative assessment and quantification of groove diameter SEM combined with FIB cross-sectioning was used (Figure 2.3). For quantification the actual groove depth white light interferometry was used. This analysis provided accurate measurements of the surface architecture of the PGCL and collagen fibres after ablation of the surface (Table 2.2 and Table 2.3). Using the excimer laser system, a range of fibre diameters and depths were produced. These results assessed the ability to produce reproducible topographical features of controlled depth and width. Surface features were evaluated qualitatively (for reproducibility, control of feature location and appearance of the structured groove) and quantitatively (for comparison of targeted and actual groove diameters, and groove depth) (Figure 2.2 – Figure 2.5 and Table 2.2- Table 2.3 respectively).
The Effect of Intraluminal Guidance

A: Control Fibre  
B: 10 um Structured Fibre  
C: 5 um Structured Fibre  
D: PC12/DRG Embedded in Hydrogel

E: Quantification of Neurite Length  
F: Quantification of Neurite Orientation  
G: Parameters Analyzed

Length of Longest Neurite (µm)

- Control
- 5 µm
- 10 µm

Orientation (%)

- Parallel
- Perpendicular

Control
5 µm
10 µm

Neurite Orientation

Neurite Length
Figure 2.6 Neuronal interaction of PC12 cells with structured and unstructured control fibres. (A-C) PC12 cells shown extending rhodamine phalloidin stained neurites (green) along the surface of the intraluminal collagen fibres. Scale bars, 2 µm. (D) Illustration of PC12 cells/dissociated DRG embedded in a collagen type I hydrogel interacting with the surface of the collagen fibre. (E+F) Quantification of neurite length and alignment using Image J image analysis software. Structured fibres demonstrated significantly longer and more parallel neurite outgrowth than the control unstructured fibres (*p<0.05). (G) Schematic of the parameters analysed in relation to the long axis of the collagen fibres. (n=3 per group)
The Effect of Intraluminal Guidance
Figure 2.7 Dissociated dorsal root ganglion seeded on the surface of both structured and unstructured collagen fibres. (A) NF160+ neurites/axons (green) were seen to grow across the surface of the collagen fibres from the gel (top left) and extend along the surface. Neurites can be seen to overlap in the bottom right hand corner of the field, suggesting lack of topographical guidance. (B) NF160+ axons can be seen to grow along the surface on the fibres primarily sticking to on axis of the fibres with minimal crossover at the center of the fibre. Results suggest more parallel growth than that of unstructured fibres. (C) Schematic diagram of DRGs embedded in collagen type I hydrogel. Scale bars, 50 µm.
2.3.3 Neuronal Cell Interaction with the Structured and Unstructured Fibres

Collagen fibres were seeded with two cell types to study the neural interaction with the intraluminal collagen fibres: one neural-like cell line (PC12) and one primary sensory neuron cell line (dissociated dorsal root ganglion). PC12 cells were originally cloned from a rat pheochromatoctoma in 1976, and showed phenotypical and morphological characteristics of postmitotic sympathetic neurons in the presence of nerve growth factor (NGF) (35). The most salient and relative properties of this cell line was the cessation of mitosis and the generation of long neurites which had the ability to respond to local changes in their environment and in particular respond to local changes in topography (21, 36, 37). PC12 cells were seeded onto control and structured collagen fibres of two different groove diameters: 5 µm and 10 µm respectively. The neurite length and their orientation were quantified with respect to the long axis of the collagen fibre and compared between groups after a period of seven days. It was seen that neurite length was significantly increased for both groove diameters versus that of control fibres ($p<0.05$).

The structured fibre groups showed significantly more parallel fibres than the control fibre group ($p<0.05$). No significant differences were seen between the 5 µm and 10 µm groove diameters (Figure 2.6).

The primary limitations of the PC12 cell line, however, is that it is a tumor-derived cell line and is not considered an exact model for quantifying the neural interaction (38, 39). For confirmation of the neural interaction with both the structured and unstructured fibres, primary embryonic dorsal root ganglion were used. These cells were derived from day 13 -15 rat embryo’s when they have reached the post-mitotic stage of their development (38, 39). DRG explants were disassociated and seeded onto both the structured (10µm) and unstructured collagen fibres (control) respectively. Neurite length and their orientation were quantified after a period of 72 hours. 72 hours was considered more appropriate than the seven days used in the PC12 study.
Figure 2.8 Microscopic images of surgical implantation of conduit groups and autograft at Day 0 (A and B) and images of explanted NGCs after 16 weeks in vivo (C and D). (A) Representative image of conduit groups implantation. (B) An implanted autograft is secured in place with two 10-0 nylon sutures on Day 0. (C) An explanted nerve guidance conduit showing a regenerated tissue cable running proximal to distal 16 weeks after implantation (D) In vivo image of structured fibre conduit showing a defined regenerated tissue cable after 16 weeks in vivo.
DRG cultures show faster growth of longer neurites but also contain a number of non-neuronal cells (Schwann cells, fibroblasts, endothelial cells) which will proliferate over longer culture periods. DRGs were similarly cultured on the surface of both structured and unstructured fibres and regenerating NF160+ neurites were seen to grow along the length of the both the unstructured collagen fibres.

2.3.4 Surgical Outcome of Implanted Intraluminal Guidance Conduits

Conduits were implanted in all animals; with only 10% of animals displaying minimum signs of irritation. 16 weeks post-implantation, all animals survived the entire period of implantation. At the defined endpoint, all conduits showed no signs of collapse (with minimum signs of premature degradation) and a tissue cable was seen to be present throughout the conduit for all implanted groups (Figure 2.8). One instance of neuroma was noted for the autograft group and was excluded from further analysis.

2.3.5 Simultaneous Retrograde Tracing – Innervation of Distal Targets

One week after exposure to the retrograde tracing dyes, the spinal column was removed and the L1-L6 segments of the spinal cord were isolated and sectioned for assessment. After sectioning, the number of fast blue (FB), diamidino yellow (DY) and double labelled (DL) motor neurons were counted to assess the number of axons approaching their distal targets and the degree of misdirection of regenerated nerves. The total number of neurons was calculated by summing all the labelled profiles (i.e. FB + DY + DL) and comparing these between groups. Retrograde tracing results showed a significantly higher (approximately 2.5 to 3 fold) total number of labelled neurons in both the structured fibres and unstructured groups versus the control hollow conduit ($p < 0.05$).

This suggests that statistically more regenerating axons are growing through their respective branches towards their distal targets versus control hollow conduits, at the point of the retrograde dye application (Figure 2.9). The regenerating axons may not however have reached their distal targets. The total number of neurons in the structured fibres group was not statistically
different to that of the autograft group. The number of misdirected axons was calculated by dividing the number of dual labelled motor neurons by the total number of motor neurons. This allowed calculation of the percentage of misdirected axons. A significant reduction in the number of misdirected axons was seen in both the structured and unstructured fibre groups (0.84 % ± 1.19 % and 2.42 % ± 2.33 %) respectively versus the autograft group (17.83 % ± 13.57 %) ($p < 0.05$).

### 2.3.6 Histological & Morphological Analysis of Implanted Conduits

Tissue samples were explanted and prepared for tissue sectioning. Samples were embedded in spur resin and 1 µm thick sections were stained with 1 % toluidine blue for visualisation under light microscopy. Images were taken initially at 100 x magnification at the midpoint for general observations of nerve regeneration and for quantitative evaluation of nerve regeneration (Figure 2.10 and Figure 2.11).

### 2.3.7 Incorporation of Intraluminal Fibres into the Host Regenerative Process

Initial qualitative analysis at 100 x magnification, showed a nerve tissue cable to have grown within the outer conduit in all conduit groups (Figure 2.10). For both fibre groups, the structured and un-structured fibres were seen to have been incorporated into the host regenerative process. The collagen fibres were intact and a number of axons were seen to be in close proximity to the fibres (Figure 2.10 and Figure 2.12). Similarly, a number of vascular bundles were seen throughout the tissue and in close approximation to the implanted fibres. The total axonal area was quantified after subtracting the area occupied by the implanted collagen fibres (Figure 2.11) at 100 x magnification using Image J image analysis software. The autograft group showed a 40 % higher axonal area versus all implanted conduit groups ($p < 0.05$) at mid-graft region of the nerve. The nerve tissue area was quantified by subtracting the scaffold area from the overall area of nerve tissue.
The Effect of Intraluminal Guidance

A

Diamidino Yellow Labelled Neurons
(Regenerating axons from the same motor neuron attempt to synapse with peroneal nerve targets)

Fast Blue Labelled Neurons
(Regenerating axons from the same motor neuron attempt to synapse with tibial nerve targets)

Double Labelled Neurons
(Regenerating axons attempting to synapse with both sets of nerve targets)

B

Number of Labelled Neurons

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of labelled neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
<td>*</td>
</tr>
<tr>
<td>Hollow Conduit</td>
<td>*</td>
</tr>
<tr>
<td>Structured Fibres</td>
<td>*</td>
</tr>
<tr>
<td>Unstructured Fibres</td>
<td>*</td>
</tr>
</tbody>
</table>

C

Axonal Dispersion

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>% Dual labelled neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
<td>*</td>
</tr>
<tr>
<td>Hollow Conduit</td>
<td>*</td>
</tr>
<tr>
<td>Structured Fibres</td>
<td>*</td>
</tr>
<tr>
<td>Unstructured Fibres</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 2.9 Retrograde tracing results. (A) Schematic drawing of the concept of simultaneous retrograde tracing. A diamidino yellow dye is applied to the peroneal branch of the sciatic nerve. The dye is applied to the nerve for a period of 30 minutes to allow adequate absorption of the dye. The dye is subsequently transported retrograde to the spinal column over a period of 1 week. The absorption and transport of the dye will label the nuclei of the associated neurons yellow. This confirms regeneration of axons down the peroneal branch of the nerve and that the same motor neurons are attempting to reinnervate peroneal nerve targets. The fast blue dye is simultaneously applied at the same initial time period to the tibial nerve and allowed to be transported retrograde in the same manner as the aforementioned dye. Fast blue dye absorption will label the cytoplasm of the neurons with a blue colour. This confirms regeneration down the tibial nerve branch and that the same neurons are correctly attempting to reinnervate tibial nerve targets. Finally if both dyes are present in the same neuron (yellow nucleus, blue cytoplasm) then it can be concluded that one neuron is incorrectly attempting to synapse with both peroneal and tibial nerve targets. (n=6 per group)
Further quantification was carried out at 1000 x magnification using a systematic stereological approach. The number of myelinated fibres, axon density, mean myelinated fibre diameter, mean axonal fibre diameter, g-ratio and myelin thickness were evaluated and compared. Axonal density was seen to be similar across all conduit groups, with no statistical differences observed between groups \( (p < 0.05) \) (Figure 2.11). Similarly, the autograft group displayed a 42% higher number of regenerated axons at the mid-graft level versus all experimental groups \( (10906.6 \pm 195.11 \text{ axons}) \) \( (p < 0.05) \). The scaffold area to nerve tissue area was similarly evaluated. No statistical differences were seen in terms of mean axonal and fibre diameters \( (p < 0.05) \). Myelin thickness and g-ratio were statistically improved for the autograft group (66% increase and 8.7% reduction respectively) versus all experimental groups \( (p < 0.05) \) (Figure 2.14). The ideal G ratio is between 0.6 and 0.7 [30].

**2.3.8 Ultra-structural Appearance of the Implanted Fibre Groups**

High resolution images of the nerve ultrastructure of both the unstructured and structured collagen fibres were taken using a TEM and compared in terms of histological appearance. The structured fibre group showed distinct formation of fascicular bundles in the fields analysed compared to that the unstructured collagen fibre group where this was not as apparent (Figure 2.16).

**2.4 Discussion**

The use of intraluminal guidance structures is an important strategy for improving nerve regeneration in hollow nerve guidance conduits. These intraluminal fillers act as a replacement for or act as an anchor for the fibrin cable which forms during conduit mediated repair (18). Strategies include the use of various types of scaffolds including gels, fibres, sponges and films. These fillers are made from a number of biological (collagen, fibrin, keratin) and artificial substrates (polylactic acid, poly(lactic-co-glycolic acid)) (40).
The Effect of Intraluminal Guidance

(A) Autograft

(B) Hollow Conduit

(C) Unstructured Fibres

(D) Structured Fibres
Figure 2.10 Histological overview of the regenerated nerve cables seen present at the mid-graft level of each of the experimental groups as follows: (A) Autograft (B) Hollow Conduit (C) Structured Fibre Conduit and (D) Unstructured Fibre Conduit. In both of the collagen fibre groups, fibres can be seen to have successfully been incorporated into the regenerative process with little to no apparent foreign body response. Fibres appear to have been reduced in area from the original circumferential area it is likely that the fibres at this stage have begun to partially degrade. All scale bars: 100 µm.
The Effect of Intraluminal Guidance

![Graphs and diagrams illustrating axonal area and distribution of myelinated axons.]

Legend: Autograft, Hollow Conduit, Structured Fibres, Unstructured Fibres
Figure 2.11. Morphological analysis of axonal area, density and the axonal fibre distribution from the midgraft of the harvested neuronal tissue. (A) Autograft shows a significantly higher nerve regeneration area than all of the experimental conduit groups ($p<0.05$). (B) Graph details the distribution of axons within the regenerated tissue across all experimental groups. Autograft and fibre groups show a higher proportion of myelinated axons with diameters in the range of 3-4 µm than that of control hollow conduits. (C) Graph shows no significant differences seen between all experimental group for axonal density. (D) Figure detailing procedure for quantification of axonal area. The axonal area was traced along its extremities (red) using image analysis software (ImageJ) and the overall axonal area (including the collagen fibres) was quantified. Collagen fibres present in the neural tissue was similarly traced (green) and the overall fibre area was quantified. The final axonal area used in (A) was calculated by subtracting the fibre area from the overall axonal area. (n=8 per group for all measurements)
The addition of such intraluminal guidance structures or fillers results in changes in the response of regenerating axons and Schwann cells during repair. Localised effects occur on the growth cone of the regenerating axons in response to contact mediated intraluminal guidance cues. The effects of this localised signalling on nerve repair are still being realised (21). These localised changes in signalling result in overall changes in the axonal regenerative response. In this investigation, collagen fibres were used to assess their feasibility for nerve repair and to analyse the influence of these fibres on axonal dispersion within a hollow conduit. This study uses collagen as the major component for nerve repair. Collagen which comprises 30% of all proteins in the body is an essential component of nerve tissue (41). Collagen is found in both the inner endoneurium (collagen type I and type II) and outer perineurium of peripheral nerves arranged in bundles of collagen fibres (42). Collagen has a number of benefits for nerve repair. Advantages include controlled degradability (via crosslinking) and stability with minimal foreign body response which are very attractive properties for nerve repair.

Controlled cross-linking of the collagen allows degradation and tensile strength to be tailored for a specific application. As can be seen the EDC:NHS cross-linked intraluminal collagen fibres were still visible within the lumen of the conduit up to 16 weeks after implantation (showing initial signs of degradation) and that axons were seen to have grown in close proximity with the implanted fibres (Figure 2.10). Similarly the outer nerve guidance conduit was structurally intact after a period of 16 weeks with no instance of conduit collapse. Despite having not fully degraded, the intraluminal collagen fibres were shown not to significantly impede nerve regeneration. For nerve area calculations the scaffold area was subtracted from the total area to give a more representative quantification of nerve repair. It can be seen from the results that despite occupying space within the regenerated nerve cable there was no significant difference in nerve area between the fibre groups and the hollow nerve guidance conduit (Figure 2.11) (p < 0.05).
The Effect of Intraluminal Guidance

(A) Unstructured Fibres (400x)  (B) Unstructured Fibres (1000x)

(C) Structured Fibres (400x)  (D) Structured Fibres (1000x)
Figure 2.12. Microscope images (toluidine blue stained) of unstructured (A and B) and structured (C and D) shown to have been successfully incorporated into the host regenerative process. Vascular cells can be seen to be growing in close proximity to the intraluminal fibres as well as a number of regenerated myelinated axons. Scale bars, 25µm and 10 µm for 400x and 1000x images respectively.
The Effect of Intraluminal Guidance
Figure 2.13 Microscopic images (1000x magnification) of regenerated nerve at the midgraft level stained with 1 % toluidine blue. Images shown represent the following groups: (A) Unstructured fibres (B) structured fibres, (C) autograft and (D) hollow conduit. All scale bars, 10 µm. (n=8 per group).
Instead regeneration occurred in close proximity to the fibres. For future studies higher levels of nerve regeneration may be achieved using collagen fibres which degrade faster. The space which is currently still occupied by the intraluminal fibres can potentially be replaced by additional regenerating axons. This study also investigates the incorporation of longitudinal micro-channels on the fibre surface. To date these micro-structured fibres have only been used in short-term nerve regeneration studies (maximum of six weeks) (43). The aim of this study was to characterise such a system in a long-term nerve regeneration study (16 weeks) and compare this directly to their unstructured counterparts. The incorporation of additional topographical guidance features on the surface of the conduits was considered as a potential avenue to further improve nerve repair. It has been shown on flat PLGA films that micro-grooved surfaces as small as 5 µm and 10 µm in width increased aligned neurite outgrowth from PC12 cells (24).

Studies by Ribeiro et al. and later by Nichterwitz et al. have described the use of similar micro-structured intraluminal fibres both in vitro and in vivo (43, 44). In initial in vitro studies, micro-structured poly-e-caprolactone (PCL) fibres displayed not only the ability both to support growth but also to facilitate increased alignment of Schwann cells and increased alignment of neurites extending from seeded rat dorsal root ganglion (DRG). The aligned Schwann cells also displayed increased L1 expression, known to be a marker for the switching of Schwann cells from a mature to a regenerative phenotype. These Schwann cells were said to form artificial glial Bands of Bungner, illustrating another benefit for repair. In a subsequent in vivo study, the PCL fibres were replaced with faster degrading poly-p-dioxanone (PDO) fibres and implanted in a rat sciatic nerve model using an epineurial sheath technique. Six weeks post implantation; Schwann cells showed parallel and aligned migration along the fibres. Axons were seen to subsequently navigate through these Schwann cell bands. These studies further support the use of intraluminal fillers or their micro-structured counterparts.

Despite showing promising results, the aforementioned study had a number of limitations: the studies were carried out over a relatively short-term (6 weeks vs 16 weeks in this current study), the study was carried out with no
outer conduit, no comparisons of the micro-structured PDO fibres were made with control groups (such as autograft and hollow conduit used here or similarly no comparisons between structured and unstructured fibres) and finally no quantification of nerve regeneration were made. Despite this, the proof of concept study elucidates benefits for the current investigation.

Herein, this study evaluates and compares such microstructured fibres in vivo with their unstructured counterparts. Structuring of the fibres was carried out using an excimer laser system. The use of the excimer laser system allows precise control of a number of parameters (including groove width, groove depth and shape of the groove) when structuring the surface of the fibres (Table 2.2 and Table 2.3). Using the manufactured rotary stage in tandem with this system allowed the manufacture of collagen fibres with multiple longitudinal channels along their surface. Structuring of the surface however can become time-intensive especially if higher depths on the surfaces of the materials are required. For use as a tool outside these studies, optimisation of each parameter would be required. The user needs to maintain a sufficient balance between time of production and meeting the required structural requirements.

For this study fibres with a diameter of approximately 50 µm were produced with a groove width of approximately 10 µm running longitudinally across its surface. The choice of this groove width was based on in vitro studies which demonstrated increased axonal alignment and growth, as well as guided Schwann cell migration and alignment (24). Similar studies have shown that fibres of approximately 10 to 80 µm from a broad range of materials have been used successfully for peripheral nerve repair (45-50). These fibres act as a platform for guided Schwann cell migration during the early stages of peripheral nerve repair while providing a means of topographical guidance to regenerating axons. After 16 weeks implantation and despite the incorporation of additional structural guidance cues, there was no significant difference present between the structured and unstructured fibre over a 16 week period ($p<0.05$).
The Effect of Intraluminal Guidance

A

Diameter of Myelinated Axons

B

Axon Counts

Number of Myelinated Axons

C

D

G-Ratio

E

Axon Diameter

F

Myelin Thickness

Legend: Autograft Hollow Conduit Structured Fibres Unstructured Fibres

112
Figure 2.14 Morphological analysis of regenerated nerve. (A) An unbiased counting frame was applied in a systematic random manner to multiple fields over the entire nerve regeneration area. Inclusion lines are represented in blue and exclusion lines are represented in red. Scale bar, 5 µm. (B) Axon counts show significantly more axonal profiles in autograft group versus all experimental groups ($p<0.05$). (C + E) No significant difference was seen in the mean myelinated/unmyelinated diameter between each experimental group ($p<0.05$). (D) G ratio was significantly lower in the autograft group versus all conduit groups. (F) Autograft displayed a significant higher myelin thickness versus all the conduit groups ($p<0.05$). (n=8 per group)
The Effect of Intraluminal Guidance

A

Axonal density

B

Axonal area (um²)

C

Diameter of myelinated axons

D

Structured fibres
Unstructured Fibres

Classical tapered profile
Figure 2.15 Morphological distribution of regenerating axons across the length of the nerve guidance conduit. (A) Axon densities were seen to be significantly lower at the proximal and distal components of the nerve in comparison to the midsection ($p<0.05$). Axons were more diffuse and wide spread and showed a tendency to be become more compact as they approached mid-graft. (B) Axonal area was significantly higher proximal to the midsection for both groups ($p<0.05$). This was due to the lower axonal densities seen in the proximal and distal components. (C) Myelinated axon diameters showed no significant differences from the proximal to distal nerve sections. (D) Diagrammatic representation of the regenerating nerve components showing the classical tapered profile associated with regeneration in a nerve guidance conduit.
The Effect of Intraluminal Guidance

A: Structured Fibre

B: Unstructured Fibre
Figure 2.16 TEM micrographs of the structured and unstructured collagen fibres. (A) Structured fibres result in the formation of defined fascicular bundles with an outer boundary (white arrows) with native collagen being deposited within the bundles and the inter-fascicular space (black arrows). (B) Unstructured collagen fibres can be clearly seen in close proximity to regenerating axons (black arrow), however fascicular bundles are less organised with no apparent boundaries of the bundles. The deposition of native collagen filaments is less dense and scattered compared to that of the structured group. All scale bars, 4 µm.
TEM results also suggested the formation of fascicular bundles with the incorporation of structured fibres (Figure 2.16). Overall however, the beneficial effect of such micro-structured fibres may be masked at such a late time point and may only have proven beneficial at the early stages of repair. Studies by Madduri et al. assessed the effect of aligned silk matrices versus their unaligned counterparts in vitro. This study showed that the introduction of an aligned matrix significantly increased Schwann cell migration and the rate of axonal growth after five days in culture (4). Studies by Kim et al. and later by Clements et al. similarly allude to more efficient migration of Schwann cells and axonal regeneration through the addition of topographical guidance cues (18, 21, 51). Similarly the introduction of structured fibres may have increased rates of migration and growth during the initial stages of repair.

However; addition of intraluminal fibres (either structured or unstructured) had distinct advantages for repair. In simultaneous retrograde tracing studies, intraluminal fibre groups displayed a 13 % reduction in axonal dispersion versus the autograft group (p<0.05). Hence intraluminal fillers in this study were shown to consequently reduce axonal misdirection. Additionally the number of axons which were shown to be growing down their respective branches was statistically increased (2.5 to 3 fold) in comparison to using a hollow conduit alone. Ultimately, this response is likely to result in more defined muscle control for the end patient in the clinic. A further benefit for repair using either of the intraluminal fibre groups, was that the number of axons reaching their distal targets in both fibre groups was significantly greater than that of the hollow conduit group (p<0.05). The combined results from retrograde tracing studies suggest a distinct advantage for repair through the use of intraluminal collagen fibres. Of note the use of autograft for peripheral nerve repair showed the highest amount of nerve regeneration. However, the rate of misdirection using autograft repair was significantly higher than the conduit groups. This high percentage of misdirected axons in autograft repair highlights another limitation of this treatment regime and the requirement for an alternative therapy. Ideally the replacement should match
nerve regeneration levels seen in autograft while maintaining levels of axonal dispersion to a minimum.

To this end, intraluminal fibres have shown the ability to act as a supportive platform for growth and repair. They have shown the ability to guide axonal regeneration and to significantly reduce the levels of axonal mismatch during repair. The true benefits of such a system may only be realised over a critical nerve gap study (>15mm in a rat). Future studies will aim towards targeting such a system. Despite showing distinct benefits for regeneration, levels of nerve regeneration remain lower than those of autograft. Future studies will focus on the creation of a more supportive environment for repair. This may be achieved through the addition of number of extrinsic factors (52). These may be cellular (Schwann cells, stem cells) or molecular (neurotrophic factors, growth factors, ECM molecules) in nature and could aid in increasing overall levels of nerve regeneration.

### 2.5 Conclusion

Intraluminal collagen fibres provide beneficial effects for use in conduit mediated microsurgical repair of peripheral nerve injuries. Both structured and unstructured fibres become fully incorporated into the regenerative process and significantly reduce the level of axonal dispersion. Despite showing some benefits for nerve regeneration, overall the differences between structured and unstructured collagen fibres was not deemed significant.

### 2.6 References


The Effect of Intraluminal Guidance


Chapter Three

Increased Packing Density and Degradation Rate for Regeneration across a Non-Critical and a Critical Nerve Gap

Contents of this chapter are under preparation for manuscript submission:

3.1 Introduction

Intraluminal guidance strategies hold promise for peripheral nerve repair and have shown efficacy in the treatment of both non-critical and critical nerve gap injuries (1-4). Specifically, these strategies seek to overcome the limitations of current hollow nerve guidance conduits by adding additional topographical guidance cues to the guide. These limitations include poor functional recovery in comparison to autograft and a critical gap length of 3 cm in humans (1 cm in a rat). Autograft has its own critical gap length and, despite being the gold standard for repair, shows success in only 40% of patients treated. Additionally autografts are limited in supply, and complications such as donor site morbidity and pain, and mismatches in axonal size, have been reported at the site of implantation. The critical gap distance described herein can be defined as the distance between the proximal and distal stumps of the nerve injury where regeneration is limited, if not absent. This gap distance can vary with the treatment used but still stands as a key hurdle for repair (5).

Strategies to overcome the critical nerve gap have focused on adding structure to the conduit, altering the conduit design or adding molecular/cellular components to the conduit (as reviewed in Chapter 1). This study focuses on how different aspects of intraluminal guidance affect repair, and builds upon the work of Chapter 2. Specifically, the study increases the packing density of the intraluminal fibre conduit (Chapter 2), in an attempt to further improve axonal regeneration. The goal of this study was to accelerate the degradation rate of the intraluminal fibres within the construct to more closely resemble the natural regenerative process. The optimal packing densities for intraluminal fibre structures had previously been shown by Ngo et al. to be approximately 7.8% when using 50 µm poly(l-lactic acid) PLLA fibres for critical gap repair (6). Similarly, Huang et al. compared 0, 100, 200, and 300 silk fibroin (10 µm × 20 µm) fibres in their construct, and found that 200 fibres was the optimal packing density (approximately 4% of the total area) (7). Ngo et al. argued that the higher percentage of intraluminal fibres reduced settling of the fibres to the bottom of the conduit during regeneration and
maintained their distribution in the conduit. Both studies showed statistically higher axonal regeneration at the higher packing densities versus the lower packing densities assessed. This study therefore sought to increase the packing density of the collagen intraluminal fibre conduit in an attempt to increase levels of overall axonal regeneration, as seen in the abovementioned studies. Furthermore, it can be argued that the fibres within the conduit should begin to show early signs of degradation to allow additional regeneration and expansion beyond the 12 week timeline presented herein. This matches the natural regenerative process which takes place during repair of short nerve gaps (<5 mm), where a fibrin cable initially forms between the proximal and distal stumps, providing a platform for repair. Resident cells (Schwann cells and fibroblasts) use this platform to form a natural cellular bridge, after the fibrin cable degrades and is replaced by a natural extracellular matrix (ECM) cable (8, 9). To replicate this, albeit for a longer gap injury, we attempted to use a fibre-filled construct which had a faster rate of degradation for repair.

The current study, therefore, has two main hypotheses:

1) An increased packing density of intraluminal collagen fibres, enclosed within a hollow collagen conduit, will result in increased nerve regeneration and functional recovery across both a non-critical (10 mm) and critical nerve gap (15 mm) in a rat sciatic model versus that of an empty collagen nerve guidance conduit.

2) Intraluminal collagen fibres with an increased rate of degradation will result in increased regeneration and functional recovery due to the enzymatic removal of the implanted fibres from the regenerating nerve once a natural cellular bridge has been established.

In order to test these hypotheses, the following were specific objectives:

a) To evaluate subcutaneously a series of cross-linked and non-cross-linked intraluminal fibres to determine the optimal in vivo degradation rate.

127
Figure 3.1 The transition from a low packing density (Chapter 2) to the high packing density (Chapter 3). Packing density is calculated by multiplying the total number of fibres by the cross-sectional area of an individual collagen fibre and dividing by the total cross-sectional area of the conduit.
b) To implant the intraluminal fibres at an increased packing density in a 10 mm and 15 mm sciatic nerve injury model for a period of 12 weeks.

c) To assess the influence of increased packing density and optimised regeneration using nerve histomorphometry and the assessment of force recovery in distal muscles.

3.2 Materials and Methods

3.2.1 Extrusion and Cross-linking of Collagen Fibres

Collagen fibres were extruded and cross-linked as per Zeugolis et al. (5, 13). Briefly, type I atellocollagen solution (5 mg/ml) was loaded in a 5 ml syringe (BD Scientific, UK) and extruded through 0.03 mm inner diameter silicone tubing (Polymer Technologies Ltd, Warwickshire, UK) at a rate of 0.3 mL/min by a syringe pump (KD-Scientific 200, KD-Scientific Inc., Holliston, MA) into a fibre formation buffer (118mM phosphate buffer and 20% of polyethylene glycol, Mw 8000 (pH 7.50 and 37°C). The fibres remained in the buffer for 5 min and were transferred into a fibre incubation buffer (6.0 mM phosphate buffer and 75 mM sodium chloride; pH 7.10 and 37°C) for a further 5 min.

The extruded collagen fibres were subsequently cross-linked with a number of different cross-linking solutions. N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were used at a number of different cross-linking ratios including 30:10, 5:5, 1:1 (mM–mM) in a 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5), for 24 h. After this period, the fibres were rinsed three times with sterile distilled water and allowed to air-dry, under the tension of their own weight. These fibres were compared to 0.625% glutaraldehyde (GTA) cross-linked and non-cross-linked fibres. GTA fibres were similarly cross-linked for 24 h. For non-cross-linked collagen fibres, fibres were briefly dipped in distilled water for approximately 1 min and then allowed to dry under the tension of their own weight for 24 h. The diameter of the collagen fibres was measured using an IX81 inverted microscope (Mason Technology, Dublin, Ireland). The degree of cross-linking was confirmed and compared by the ninhydrin assay.
3.2.2 **Subcutaneous Implantation of Cross-Linked Collagen Fibres**

Bundles of collagen fibres were assessed in a subcutaneous implant model to characterise *in vivo* degradation rates and to tailor the fibres for implantation in the rat sciatic nerve model. Bundles of fibres were stretched longitudinally and sutured at both ends to hold the fibres together and also to serve as a biological marker for their later retrieval *in vivo*. The fibres were either cross-linked with 30:10 EDC–NHS or non-cross-linked. This would give the ultimate range of *in vivo* degradation time. Prior to implantation, samples were sterilised with 70% ethanol for 2 h and washed thoroughly to remove any residual ethanol from the surface of the fibres.

Samples were randomly assigned to four subcutaneous pockets on the back of four Sprague Dawley rats (*n* = 4 per group per timepoint). Rats were anaesthetised with 4% isoflurane solution. The midback was shaved and the site was sterilised using 10% povidone-iodine solution (Bethidine™). The underlying fascia was exposed by making a longitudinal incision through the outer dermal layers using a no.15 surgical scalpel. The site was widened using a surgical tissue-spreader and the fibre bundles were sutured to the underlying fascia to reduce migration of the scaffold subcutaneously. After implantation, the surgical site was closed using 5-0 surgical sutures, and buprenorphine hydrochloride (0.15 mg/kg) was given to the animal for the alleviation of pain.

Animals were euthanised at two weeks and four weeks post-implantation by CO₂ asphyxiation. Expiration of the animal was confirmed via cervical dislocation in all cases. The implanted fibres and surrounding tissue were subsequently removed and fixed in 4% neutral buffered formalin. Samples were allowed to fix for approximately 24 h and were subsequently cryoprotected in 10% sucrose for a further 24 h. 8 μm thick cross-sections were cut throughout the tissue using a cryostat. Cross-sections were then stained with Masson’s trichrome staining as per Appendix O.
3.2.3 Fabrication of Nerve Guidance Conduits

Collagen conduits were fabricated as described previously in Yao et al. (10-12). Bovine atellocollagen solution (12 mg/ml) was wrapped around a 1.5 mm stainless steel rod which was held in place using two silicone moulds. Collagen was allowed to self-assemble on the surface of the stainless steel bar and the bar was constantly rotated, while coating, to create a uniform coating on the surface. The collagen solution was allowed to dry onto the surface at room temperature and then transferred to a cross-linking solution of 30:10 mM EDC–NHS crosslinking solution for 24 h. The conduits were then rinsed in 0.1 M PBS solution to remove excess cross-linker from the surface and subsequently lyophilised for 24 h. The conduits were manually removed from the stainless steel bar. Conduits produced had an inner diameter of 1.5 mm, an outer diameter of 1.7 mm and a length of 12 mm (for non-critical nerve defects) and 15 mm (for critical nerve defects).

Intraluminal fibre nerve guidance conduits were constructed similarly to (13). Conduits were filled with 65 non-cross-linked collagen fibres (the choice of non-cross-linked fibres was based on degradation results of the subcutaneous study). These 65 fibres (50 µm diameter) accounted for 7.22% of the total cross-sectional area.

3.2.4 Surgical Procedure

The fully assembled constructs were sterilised by immersion in 70% ethanol for 2 h, followed by multiple rinses in sterile 0.1 M phosphate buffer to remove excess ethanol, and stored until use. Forty-eight (220–250 g) adult female Lewis rats were randomly assigned to one of six experimental groups (Table 3.1) (This study may be considered as part of the larger 105 animal study that spans Chapters 3 and 4). All experimental procedures were carried out in accordance with National and Institutional guidelines set out by the Cruelty to Animals Act 1876 as amended by the European Communities’ (Amendment of Cruelty to Animals Act, 1876) Regulations 2002 & 2005. Rats were anaesthetised by inhalation of 4% isoflurane. An incision was made at mid-thigh level on the left leg to expose the underlying musculature.
Table 3.1 Design of Animal Study for Investigating the Effect of a Densely Packed Intraluminal Fibre Conduit on Nerve Regeneration across a Non-Critical and a Critical Nerve Gap.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Non-critical (10 mm)</th>
<th>Critical (15 mm)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Hollow collagen conduit</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Intraluminal filled conduit</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>24</strong></td>
<td><strong>24</strong></td>
<td><strong>48</strong></td>
</tr>
</tbody>
</table>
The gluteal muscle was then split to expose the left sciatic nerve. For the 10 mm non-critical nerve gap, the nerve was transected 5 mm proximal to the distal bifurcation of the tibial and peroneal branches and a 5 mm nerve segment was removed. The proximal and distal ends of the transected nerve were inserted 1 mm into 12 mm long collagen tubes (with/without intraluminal fillers), creating a 10 mm gap and secured to the epineurium using 10-0 monofilament nylon sutures (Johnson & Johnson, Dublin, Ireland). For autograft repair, a 10 mm segment of the nerve was explanted, rotated 180° and implanted between the transected nerve stumps, using 10-0 nylon sutures. For the 15 mm critical gap group, the nerve was transected 2 mm proximal to the distal bifurcation and a 10 mm segment was removed.

The wound was finally closed in layers and the animals received buprenorphine hydrochloride for the alleviation of pain. Animals were continuously monitored for signs of distress or pain.

### 3.2.5 Force Measurement Analysis

Functional recovery of the regenerated peripheral nerve cable was carried out 12 weeks post injury. Muscle force measurements have been established as a valid means to establish the level of recovery of motor function (14, 15).

Twelve weeks post-surgery animals underwent a non-survival surgery for electromyography (EMG) and force measurement evaluation. Animals were anaesthetised by inhalation of isoflorane gas (5% for induction and 3% for maintenance). The sciatic nerve was re-exposed using a dorsal–gluteal muscle splitting approach and the regenerated nerve was isolated from the surrounding tissue to allow application of the stimulating electrodes. Mineral oil was applied to the sciatic nerve to prevent current leakage into the surrounding muscle beds and to prevent dehydration of the nervous tissue. The extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were isolated via a skin incision extending from the dorsum of the foot, along the anterior portion of the lower leg, to the knee. The distal tendons of the EDL and TA muscles was separated from the leg. Upon separation, the muscles were attached to stainless steel S-hooks, by securing the tendon to the hook with a 5-0 suture.
Once secure, the animal was placed in the automated functional assessment station (FAS system, Red Rock Laboratories, St Louis, MO). Animals were placed on an adjustable support and the injured left leg was secured in position by clamping of the femoral condyles. For electrical stimulation of the sciatic nerve, bipolar microwire electrodes were attached to the proximal portion of the sciatic nerve. Electrodes were placed 2–3 mm proximal to the implanted conduit to allow sufficient stimulation without damage to the implanted conduit. For force measurements, EDL and TA muscles were connected to 5 N and 10 N load cells, respectively. Stimulation of the sciatic nerve resulted in an evoked active muscle force which was recorded using custom data acquisition software (RRL V. 1.3, Red Rock Laboratories).

Once the above experimental setup was in place, the optimal stimulus amplitude and muscle length were required to be determined per animal. This normalised the resulting force data between animals. Single electrical pulses (duration 0.2 ms) of increasing amplitude (0–1000 µA) were applied to the nerve and the active isometric twitch force of both the EDL and TA muscles was determined. From these measurements, the amplitude ($I_0$) at which the maximal active isometric twitch force occurred was determined. Optimal muscle length was then determined by applying similar electrical impulses (duration 0.2 ms) at the optimal amplitude ($I_0$) and varying the length ($L_0$) of the EDL and TA muscles until the maximum active twitch force was generated.

For measurement of the maximum isometric tetanic force of the EDL and TA muscles, $I_0$ and $L_0$ were kept constant and trains of electrical impulses (pulse duration = 0.2 ms, burst width = 300 ms) of varying frequency (0–150 Hz) were applied to the nerve. Two minute resting periods were applied between stimuli to reduce muscle fatigue and data contamination. The resulting muscle force was recorded for both the TA and EDL muscles. The maximal force generated was deemed to be the maximal isometric tetanic force.

### 3.2.6 Recovery from Muscular Atrophy

Following force measurement recordings of the TA and EDL muscles, the animals were euthanised by CO$_2$ asphyxiation. The TA, EDL and
gastrocnemius (GC) muscles were isolated from the surrounding tissue on both the operated and the uninjured side. The wet weight of both the uninjured and the injured side was recorded. The percentage recovery from muscular atrophy was determined by dividing the mass of the muscle from the injured side of the animal by the mass of the muscle from the uninjured side.

3.2.7 Analysis and Quantification of Nerve Morphometry

Force measurement and muscle isolation having been completed, the sciatic nerve was fixed in situ using Trump’s fixative solution (4% formaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer saline solution) for 30 min (16). The fixative solution was removed from the injury site, and the sciatic nerve was resected and divided into three 1 mm sections: 1 mm proximal to the graft, at mid-graft and 1 mm distal to the graft. The sections were placed in the Trump’s fixative solution and stored for sectioning. Sections from the midsection of the nerve were post-fixed in 1% osmium tetroxide solution and passed through a series of graded alcohol solutions for serial dehydration of the samples. Sections were embedded in spur resin (EMS, Fort Washington, PA) and 1 µm semi-thin sections were cut using a glass knife on a Leica UltraCut E® Microtome (Rankin Biomedical Corp., Holly, MI), stained with 1% toluidine blue for assessment of the cut section. Once a suitable section was identified at mid-graft, sections were stained with 1% phenylenediamine. Phenylenediamine solution provides a more distinct stain of the myelin sheath of the regenerated axons and provides suitable contrast for analysis of the regenerated nerve morphometry.

Nerve morphometrical analysis was carried out using a semi-automated system for assessment of peripheral nerve regeneration and recently validated in the Mayo Clinic (unpublished data). 100× images of the entire nerve image were systematically and randomly sampled from the total nerve area taken at (10×) and an average of 800 axonal profiles were evaluated (100×). The use of this software allowed rapid evaluation of the sampled profiles and the following parameters were extrapolated and compared: axonal area, number of regenerated axons, axonal density, average axon diameter, average fibre
diameter, myelin thickness, and g-ratio (average fibre diameter/axonal diameter).

### 3.2.8 Ranking of Nerve Regeneration Parameters

Nerve regeneration parameters were analysed together as a group, using a mean-variance ranking system as per Daly, Knight et al. (17, 18). Individual nerve parameters were analysed simultaneously, taking into account the variance between the groups and eliminating bias in one particular parameter. Animals were assigned a score from 1 to 48 based on their performance in that particular parameter. Parameters for nerve morphometry (fascicular area, number of myelinated fibres, myelin thickness and axon diameter), force measurement (tetanic and twitch forces for EDL and TA muscles) and muscle mass recovery (percent recovery of EDL, TA and GC muscles) were grouped together and calculated accordingly. Overall, functional nerve regeneration was calculated by grouping these parameters together and analysing accordingly.

### 3.3 Results

#### 3.3.1 Cross-Linking of Fibres and Subcutaneous Implantation

Fibres were cross-linked at a number of different ratios of EDC–NHS to provide a range of fibres that degrade at different rates once implanted in vivo. Cross-linking of the fibres at the different ratios was confirmed by the ninhydrin assay (Figure 3.1). To get the ultimate range of their in vivo degradation, fibres cross-linked at a maximal rate of 30 mM–10 mM (based on previous studies) were implanted subcutaneously into the dorsum of four female Lewis rats and compared to a non-cross-linked control (two cross-linked and two non-cross-linked bundles per animal at two timepoints). The aim of the study was to have the fibres degrade within the first month of implantation, after which the natural ECM cable would take over the role of structural support for the regenerative process. It can be seen that the fibres in both the cross-linked and control groups were present in the subcutaneous tissue of all animals.
Bridging a Critical Nerve Gap

(A) Two Week Control

(C) Two Week EDC:NHS

(B) Four Week Control

(D) Four Week EDC:NHS

(E) Ninhydrin Assay

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GTA:</th>
<th>30:10 EDC</th>
<th>5:5 EDC</th>
<th>1:1 EDC</th>
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<td>Mole of Free NH₂ Groups</td>
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<td></td>
<td>0.08</td>
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<td>0.08</td>
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<tr>
<td></td>
<td>±0.02</td>
<td>±0.02</td>
<td>±0.02</td>
<td>±0.02</td>
<td>±0.02</td>
</tr>
</tbody>
</table>

* Significant difference
Figure 3.2 Masson’s trichrome image of intraluminal collagen fibres implanted in a rat subcutaneous model. (A+B) Two week and four week images of control non-cross-linked fibres show intraluminal collagen fibres (black arrows) present two weeks post implantation. Dark purple zone at centre of the four week control represents the implanted suture \((n = 3)\). (C+D) Two week and four week images of cross-linked fibres show collagen fibres present at both time points (black arrows) \((n = 3)\). (E) Ninhydrin assay showed significant reduction in number of amines in the 30:10 mM EDC–NHS collagen fibres and 0.625% GTA fibres in comparison to control non-cross-linked collagen fibres \((p < 0.05, n = 4\) for all treatment groups, mean ± SD).
However, after four weeks the fibres in the control group were no longer present in the tissue and were assumed to have been degraded (Figure 3.2 (B)). Meanwhile, the cross-linked fibres were still abundantly present in the subcutaneous tissue (Figure 3.2). From this study, it was concluded that the control fibres, with no additional cross-linking, will be sufficient to provide a faster degrading platform \textit{in vivo}, and these were chosen for investigation in the subsequent nerve regeneration study.

### 3.3.2 Nerve Morphometry 12 Weeks Post-Implantation

Two forms of the collagen conduit (hollow and filled) were compared to the gold standard autograft across two different gap lengths (10 and 15 mm respectively) to analyse the effects of increasing gap distance. A hollow collagen conduit was compared to a filled conduit to confirm that the addition of intraluminal structure allows regeneration across a longer gap length. At 12 weeks, implanted groups were analysed using histomorphometrical analysis (Figure 3.3).

No significant differences in axonal density were seen at 10 mm; however, at 15 mm autograft showed significantly higher density than both conduit materials. At 10 mm, autograft showed significantly higher fascicular area than the hollow conduit and no significant difference with the filled conduit group. At the 15 mm gap length, both the filled conduit and autograft were significantly better than the hollow conduit group (where little to no regeneration was seen). The 10 mm autograft demonstrated significantly higher fascicular area than its 15 mm counterpart.

For the 15 mm gap length, no significant differences were seen for unmyelinated axon diameter and myelinated fibre diameter. However, autograft showed a significantly higher myelinated fibre diameter than the hollow conduit at 10 mm and no significant difference with the fibre filled group. Myelin thickness was significantly higher in autograft than both the conduits at 10 mm; no significant differences were seen at 15 mm.
Bridging a Critical Nerve Gap

A. Axonal Density
B. Fascicular Area
C. Myelinated Fibre Diameter
D. Axon Diameter
E. Myelin Thickness
F. G-Ratio
G. Number of Myelinated Axons

Legend:  
- Autograft  
- Hollow Conduit  
- Filled Conduit
Figure 3.3 Assessment of nerve morphometric parameters at 12 weeks. (A) The 15 mm autograft was seen to be significantly greater, in terms of axonal density, versus the conduit groups. No significant differences in axonal density were seen at the 15 mm gap length. (B) The 10 mm autograft showed significantly more fascicular area than the hollow conduit. No significant difference was seen versus the fibre group. At 15 mm, both the autograft and the fibre conduit showed significantly higher fascicular area than the hollow conduit group. The 10 mm autograft was significantly greater than the 15 mm autograft. (C+D) No significant differences were seen across the two gap lengths in terms of myelinated fibre and axon diameters, except in terms of the 10 mm hollow conduit which was significantly less than autograft in terms of myelinated fibre diameter. (E+F) At 10 mm autograft showed a significantly thicker myelin sheath and higher g-ratio than both the conduit groups. At 15 mm no significant differences were seen. (G) Autograft showed a significantly greater number of axons than both the fibre groups at both gap lengths. Scale bar, 5 µm (* p < 0.05, one-way ANOVA followed by Newman-Keuls *post-hoc* test; # p < 0.05, unpaired Student’s *t*-test; mean ± SEM).
Bridging a Critical Nerve Gap

A

B

C

D

E

F

G

Tibialis Anterior

Extensor Digitorum Longus

Gastrocnemius

Max Tensile Force (N)

Max Tensile Force (N)

Max Tensile Force (N)

10 mm 15 mm

10 mm 15 mm

10 mm 15 mm

Autograft

Fiber Conduit

Hollow Conduit
Figure 3.4 Measurement of regenerated muscle force and recovery from muscular atrophy. (A) Photograph of rat placed in the automated functional assessment station. (B) Assessment of force recovery in the tibialis anterior (TA) muscle. The 10 mm autograft showed a significantly greater max tetanic force than that of the 10 mm hollow conduit (left). No significant differences were seen across the remaining groups. In terms of max twitch force (right), no significant differences were seen across groups. (C) Assessment of extensor digitorium longus (EDL) showed no significant differences in terms of max twitch and force across all experimental groups. (D) Photograph shows the isolated TA, EDL and gastrocnemius (GC) muscles isolated at 12 weeks from both the injured and uninjured sides of the animal. (E) The TA showed significantly more recovery from muscular atrophy in the 10 mm autograft than the 10 mm hollow conduit group. Autograft significantly outperformed both the conduit groups at the 15 mm gap length. (F) The 10 mm autograft showed significantly greater recovery than the 10 mm hollow conduit and both the 15 mm conduit groups respectively. (G) In terms of GC muscle recovery, the autograft was significantly greater than the conduit groups at both gap lengths. The 10 mm autograft showed a significantly higher muscle mass ratio than that of the 15 mm graft (* p-value < 0.05, one-way ANOVA followed by Newman-Keuls post-hoc test; # p-value < 0.05, unpaired Student’s t-test).
G-ratio was significantly higher in autograft versus the conduit groups at 10 mm; however, no significant differences were seen at 15 mm. At both gap lengths, autograft showed significantly more axons than the conduit groups.

### 3.3.3 Force Measurement

Force measurements were carried out on the EDL and TA muscles at 12 weeks (Figure 3.4). Despite differences in nerve morphometry, few significant differences were seen between experimental groups. Autograft demonstrated a significantly greater maximum tetanic force in the TA muscle versus the hollow conduit. No significant differences were seen in the maximum twitch forces of the TA and EDL muscles. Muscle mass recovery demonstrated a number of significant differences between the treatment groups. Muscle mass ratios in the TA and EDL were significantly greater in autograft versus the hollow conduit at 10 mm. No significant differences were seen between autograft and the filled conduit in terms of muscle mass recovery in the TA and EDL muscles. At 15 mm, autograft was significantly greater than both the filled and hollow conduit groups. In the GC muscle, autograft was significantly greater, in terms of muscle recovery, than the two conduits at both gap lengths. The 10 mm autograft demonstrated a significantly higher muscle mass recovery than the 15 mm autograft.

### 3.3.4 Grouped Ranking of Multiple Regenerative Parameters

Ranking of individual components of functional nerve recovery allowed the comparison of multiple regenerative parameters simultaneously and was carried out as per Daly *et al.* (18) (Figure 3.5). Individual parameters were grouped under the headings of nerve morphometry, force measurement, and muscle mass rankings and then considered globally under the heading of overall ranking. Grouped parameters of nerve morphometry were significantly higher in the autograft group at both lengths. At 10 mm, ranking of force components was significantly higher in autograft versus the hollow conduit. The 10 mm autograft was also significantly higher than the 15 mm conduits. In terms of overall muscle mass recovery, the 10 and 15 mm autografts were significantly higher than the conduit groups.
Bridging a Critical Nerve Gap

A  Nerve Morphometry Ranking

B  Force Measurement Ranking

C  Muscle Mass Ranking

D  Overall Ranking

Legend: Autograft  Fibre Conduit  Hollow Conduit
Figure 3.5 Ranking of nerve regeneration parameters. (A) Ranking of grouped nerve morphometric components. Parameters of myelin thickness, number of myelinated fibres, and fascicular area were scored from 1 to 48 based on their performance in that particular category and grouped together to give an overall indication of performance across those parameters. The 10 and 15 mm autograft can be seen to significantly outperform both the conduit groups. No significant differences across gap lengths were seen. (B) For functional recovery, the 10 mm autograft significantly outperformed the 10 mm hollow conduit. Across gap lengths, the 10 mm autograft significantly outperformed the 15 mm autograft. No significant differences were seen across the remaining groups. (C) In terms of muscle mass recovery ranking, the 10 mm and 15 autografts significantly outperformed the conduit groups. The 10 mm autograft performed significantly better than its 15 mm counterpart. (D) Overall, the 10 mm and 15 mm autografts outperformed the conduit groups. And the 10 mm autograft significantly outperformed the 15 mm autograft (* p-value < 0.05, one-way ANOVA followed by Newman-Keuls post-hoc test; # p-value < 0.05, unpaired Student’s t-test).
The 10 mm graft was seen to be significantly higher than the 15 mm autograft. This trend was continued when the regenerative parameters were considered as a whole. Overall, the 10 and 15 mm autografts significantly outperformed the 10 and 15 mm filled and hollow conduits. Similarly, the 10 mm autograft significantly outperformed the 15 mm graft.

3.5 Discussion

This study expands on the previous work carried out in Chapter 2 and involved the use of intraluminal collagen fibres as a platform for repair. The overall experiment described herein formed the first part of a two-part study which consisted of the initial nerve morphometric and force measurement analysis of an “improved” intraluminal fibre conduit (current Chapter) and secondly, understanding the early molecular changes which take place as a result of their implantation during the first two weeks of repair. Intraluminal structures have been presented in a number of forms (gels, fibres, sponges, etc.) over the years with varying degrees of success. From these studies a number of benefits and guidelines for their use have been established in the area of peripheral nerve repair. Namely, the use of intraluminal guidance structures has shown the ability to extend regeneration beyond that of a critical nerve gap and that the packing density of these constructs is of key importance for regeneration and repair.

The incorporation of intraluminal fibres to the conduit enhanced regeneration across critical and non-critical nerve gaps. In particular, in terms of axonal area, the intraluminal fibre conduit was not statistically different to that of the autograft. At the critical gap distance the intraluminal fibre conduit showed a significantly higher axonal area than that of the hollow nerve guidance conduit. Similarly, measurement of the tetanic muscle force showed no significant differences between autograft and the intraluminal fibre conduit. However, it should be noted that the 15 mm autograft significantly outperformed both conduits at that gap length. Studies by Yoshii et al. demonstrated that bundles of collagen fibres (without an outer biomaterial conduit) were able to bridge a critical gap 20–30 mm in length (19, 20). Despite their inclusion, functional recovery remained poor. Similarly, Cai et
al. demonstrated that a permeable poly(lactic acid) PLA conduit filled with 16 poly(D,L-lactide, co-glycolide acid) (PLGA) fibres was able to bridge both a 14 mm and an 18 mm gap in a rat sciatic nerve injury 10 weeks after repair. Samples, however, were not compared to autograft and no functional recovery was conducted (21). A chitosan conduit filled with poly(glycolic acid) (PGA) fibres has similarly shown efficacy in a human median injury model, bridging a critical 3.5 cm nerve injury, and showing increasing signs of functional recovery over a three year period (22).

The packing density in this study was increased from 2.2% to 7.7% to more closely resemble that of studies done by Ngo et al. (6). As stated earlier, the addition of intraluminal fibres increased regeneration to levels similar to autograft in a number of parameters. However, overall, in terms of ranking of morphometrical and functional measurements, autograft remained superior to the intraluminal fibre group. This may partially be due to the lack of degradation of the intraluminal fibres, despite subcutaneous results suggesting their in vivo degradation after two weeks in the non-cross-linked state. Additionally, this may be due to the tight packing of the collagen fibres, which in some cases showed fibres juxtapositioned to each other creating a site of inhibition for regeneration. Ngo et al. similarly demonstrated that juxtapositioning fibres inhibited repair and Stang et al. showed that a collagen sponge encompassing the entire conduit lumen inhibited nerve regeneration (6, 23). This suggests a packing density that is mid-range between the 2.2% and 7.7% from the previous and current studies respectively. Prospectively, a packing density of approximately 4% as suggested by Huang et al. may prove beneficial for repair. However, the packing density appears to vary with the materials and configuration used. Clements et al. and Kim et al. showed that polysulphonate tubes filled with various configurations of poly(acrylonitrile-co-methylacrylate) (PAN-MA) thin films were able to bridge a critical gap of 17 mm in a rat sciatic nerve model. The high surface area-to-volume ratio of these constructs allowed them to achieve regeneration across a critical nerve gap at a low packing density (0.6%) (24, 25). Koh et al. showed the opposite extreme with the use of electrospun PLGA filaments in an outer PLGA conduit by bridging a 15 mm nerve gap with packing density of
approximately 10% of the total cross-sectional area (26). These extremes highlight how slight changes in the material and configuration can completely alter the regenerative response during peripheral nerve repair.

The reasons for the difference in the regenerative response seen between these materials are not understood. Mukhatyar et al. attempted to define this response, albeit in a limited capacity, with the use of aligned films (27). It was found that the use of aligned film constructs in a nerve guidance conduit significantly increased aligned fibronectin deposition from Schwann cells in the native environment, and this may be one of the reasons for the regeneration seen at the low packing densities of Kim et al. and Clements et al. However, this was carried out in limited fashion and considered only a small portion of the overall molecules that may have effected repair. A more global study of the molecular response from the incorporation of such intraluminal guidance structures may hold promise for the design of future constructs.

3.6 Conclusion

Non-cross-linked fibres showed a faster degradation rate when implanted subcutaneously versus a 30 mM–10 mM cross-linked fibre. However, when implanted within a rat sciatic nerve model the fibres displayed little to no degradation, suggesting different mechanisms of regeneration within the nerve guidance conduit. A more densely packed intraluminal fibre conduit, implanted within the rat sciatic nerve, showed similar levels of regeneration and functional recovery to autograft in a number of regenerative parameters in both non-critical and critical nerve gaps. Despite this, autograft remained superior across both gap lengths in overall ranking scores. Interestingly, grouped ranking of the parameters assessed showed a significant reduction in regeneration from the 10 mm to the 15 mm autograft. This confirms that regeneration decreases over increasing gap distances, even within the gold standard for repair. The effect of increased packing density highlights the significance of the material used and its configuration in order to match regeneration as seen in autograft, and that confirms the importance of gap distance in influencing peripheral nerve repair. In an attempt to understand
the molecular mechanisms that are responsible for the changes in peripheral nerve repair, due to the materials used, the addition of intraluminal fillers and increasing gap length, future studies will focus on the early regenerative changes (first two weeks of repair) that occur at the level of the proteome.

3.7 References


Chapter Four

The Proteomic Response to Intraluminal Guidance, Type of Biomaterial Conduit and Gap Distance Treated in Peripheral Nerve Repair

Contents of this chapter are under preparation for manuscript submission:

Table 4.1 List of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>A1BG</td>
<td>Alpha-1-B glycoprotein</td>
</tr>
<tr>
<td>ACTB</td>
<td>Actin, beta</td>
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<tr>
<td>AFM</td>
<td>Afamin</td>
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<td>AHNAK nucleoprotein</td>
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<td>ATP5A1</td>
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<tr>
<td>C4A/C4</td>
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<td>CADM4</td>
<td>Cell adhesion molecule 4</td>
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<td>CKB</td>
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<td>CKM</td>
<td>Creatine kinase, muscle</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>DBI</td>
<td>Diazepam binding inhibitor</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GC</td>
<td>Group-specific component (vitamin D binding protein)</td>
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<tr>
<td>KCTD12</td>
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<td>Mitogen activated kinase-like protein</td>
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<td>MARCKS</td>
<td>Myristoylated alanine-rich protein kinase C substrate</td>
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<td>MYLPF</td>
<td>Myosin light chain, phosphorylatable, fast skeletal muscle</td>
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<td>NEFH</td>
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<td>Neurofilament, medium polypeptide</td>
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<td>NME2</td>
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<tr>
<td>YWHAE</td>
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4.1 Introduction

Peripheral nerve injuries affect more than one million people and accounts for approximately 2.8% of multiple trauma cases worldwide (1). The peripheral nervous system is known for its ability to regenerate itself across relatively short distances, and recovery is quite robust with the use of end-to-end repair (i.e. direct suturing). For treatment of larger gaps, the primary treatment methods are auto/allograft or the use of hollow biomaterial conduits. Although these treatments have been available for the past thirty years, success has been limited and results unsatisfactory. Autograft, despite being the gold standard for repair, shows limited success with a number of limitations. The use of clinically approved hollow conduits, made from either biological or synthetic materials, fails to match this limited success and often meets with failure after long-term implantation (2). The use of some synthetic materials such as polylactic acid (PLA) has shown numerous complications after clinical trials (3, 4). Attempts to improve these conduits have focused on the use of a variety of materials and designs, either alone or in combination with cellular or molecular additives. A number of these strategies have achieved success in animal trials, but these strategies often fail to translate to the clinic. This is possibly due to a limited understanding of the biological response.

This study uses high-throughput gel-free multi-dimensional protein identification technology (MudPIT) to understand the molecular changes that occur within nerves treated with either autograft or biomaterial conduits. Gel-free mass spectroscopy overcomes the limitations of gel-based systems, which are often biased towards housekeeping enzymes, and high-abundance proteins (5). The use of tandem isobaric tagging (ITRAQ™) with MudPIT allows groups of materials to be assessed on the same run of the mass spectrometry system, reducing instrument use as well as system and equipment error and increasing reproducibility, sensitivity and accuracy of quantitation (6, 7). Initial attempts to define the biological changes that occur on a broader scope have focused on regeneration within silicone and elastomer tubes (8-12). These efforts have documented the genomic and proteomic changes (often using gel-based systems) over crush (possible
different mechanisms of repairs) (13-19) or across short transection injuries, or considered a limited range of proteins using proteomic microarrays in biomaterial conduits (15 proteins analysed) (20). The complex orchestrated series of events needs to be further characterised in order for an optimal treatment strategy to be achieved.

In an attempt to define this complex series of events, with the respect to the biomaterial and tissue engineering field, this study uses a proteomics approach to characterise the changes that occur based on a number of criteria: First, differences in protein regulation were characterised and compared based on the specific conduit material that was used for repair. It is well known that various biomaterials have a differential effect on nerve regeneration – with some materials showing a greater affinity towards repair (21). In this study two materials – a natural (collagen) and synthetic material (poly (lactic-co-glycolic) acid (PLGA)) are compared to the gold standard (autograft). These materials have previously shown efficacy in nerve repair (2, 22, 23). It was hypothesised that the use of different biomaterial conduits will produce different proteomic profiles in comparison to the gold standard treatment for peripheral nerve repair. Second, proteomic changes were characterised as a function of the gap length treated. The use of autografts and biomaterial conduits are limited to defined distances of nerve injury (3 cm in a human and 1 cm in a rat, i.e. a non-critical gap length) and beyond this critical gap, regeneration becomes minimal or absent (critical gap length) (21). The molecular mechanisms behind the reduced regeneration across these distances are not well understood. It can be hypothesised that differences in levels of axonal regeneration across a non-critical and critical gap were related to differences in the initial proteomic changes that occur during repair. Finally, recent reports have shown that the inclusion of intraluminal structures/fillers enhances regeneration in hollow conduits across a critical gap distance (24-26). Therefore, the final comparison focuses on how the addition of intraluminal structure affects nerve repair at the base proteomic level, at both non-critical nerve gaps in comparison to a hollow biomaterial conduit and the gold standard treatment for repair. Using 8-plex iTRAQ™ mass spectrometry this study attempts to analyse the proteomic
changes which occur according to these criteria and contribute to the design of the next generation of biomaterial nerve conduits (summarised in Figure 4.1).

4.2 Materials and Methods

4.2.1 Preparation of Conduit Groups for In Vivo Implantation

PLGA conduits were prepared as described in (27). The conduit was produced using an immersion based method, wherein a 20% solution of 85:15 monomer ratio was prepared in insert solvent. A glass mandrel was dipped three times in a PLGA solution and was subsequently immersed in 95% isopropyl alcohol for two h. The PLGA conduit was allowed to air-dry and then removed from the glass mandrel. After removal, the conduit was lyophilized to produce a porous conduit. This yielded a conduit with an inner diameter of 1.6 mm (based on the diameter of the glass rod) and a length of 12 mm. This conduit served as a comparative control to document a response for synthetic conduits.

Collagen conduits were prepared as described previously in Yao et al. (28-30). Bovine atellocollagen solution (12mg/ml) was wrapped around a 1.5 mm stainless steel rod which was held in place using two silicone moulds. Collagen was allowed to self-assemble on the surface and the stainless steel rod was constantly rotated to create a uniform coating on the surface. The collagen solution was allowed to dry onto the surface at room temperature and then transferred to a 30:10 mM EDC: NHS crosslinking solution for 24 h. The conduits were rinsed in 0.1 M PBS solution to remove excess cross linker from the surface and subsequently lyophilised for 24 h.

The conduits were then manually removed from the stainless steel bar. The resulting conduits produced had an inner diameter of 1.5 mm (diameter of stainless steel bar) and a length of either 12 mm (for material based comparisons) or 15 mm (for comparison of non-critical and critical nerve defects). The collagen conduits served as a comparative control for biological/natural conduits.
Figure 4.1 Schematic diagram of the factors assessed which have an effect on peripheral nerve repair. The material used, gap distance treated, and the incorporation of intraluminal structure have been shown extensively to influence nerve regeneration. This study aims to understand this influence at the base proteomic level in the acute phase (first two weeks) of nerve repair as a function of time and their spatial expression. This analysis allows for the identification of key regulators which are important for successfully nerve repair, throughout the conduit, as a function of both the material and strategy used for repair.
For the study on the effect of gap distance on nerve repair, collagen fibres were extruded as per Zeugolis et al. (31, 32). Briefly, type I atellocollagen solution (5 mg/ml) was loaded in a 5 ml syringe (BD Scientific, UK) and extruded through 0.03 mm inner diameter silicone tubing (Polymer Technologies Ltd, Warwickshire, UK) at a rate of 0.3 mL/min by a syringe pump (KD-Scientific 200, KD-Scientific Inc., Massachusetts, USA) into a fibre formation buffer (118mM phosphate buffer and 20% of polyethylene glycol, Mw 8000 (pH 7.50 and 37°C)). The fibres remained in the buffer for five mins and were transferred into a fibre incubation buffer (6.0 mM phosphate buffer and 75 mM sodium chloride; pH 7.10 and 37°C) for a further five mins.

Intraluminal fibre nerve guidance conduits were constructed similarly to (33). Conduits were filled with 65 non-cross-linked collagen fibres. 65 fibres accounted for 7.22 % of the total cross-sectional area and matched the optimal density as laid out by Ngo et al. (34).

4.2.2 Surgical Procedure
The fully assembled constructs were sterilised by immersion in 70 % ethanol for 2 h, followed by multiple rinses in sterile 0.1 M phosphate buffer to remove excess ethanol and stored until use. 105 adult female Lewis rats (220 – 250 g) were randomly assigned to one of seven experimental groups (Figure 4.2). All experimental procedures were carried out in accordance with National and Institutional guidelines set out by the Cruelty to Animals Act 1876 as amended by the European Communities’ (Amendment of Cruelty to Animals Act, 1876) Regulations 2002 & 2005. Rats were anesthetised by inhalation of 4% isoflorane. An incision was made at mid-thigh level on the left leg to exposure the underlying musculature. The gluteal muscle was then split to expose the left sciatic nerve. For the 10 mm non-critical nerve gap, the nerve was transected 5 mm proximal to the distal bifurcation of the tibial and peroneal branches and a 5 mm nerve segment was removed. The proximal and distal ends of the transected nerve were inserted 1 mm into 12 mm long collagen tubes (with/without intraluminal fillers) creating a 10 mm gap and secured to the epineurium using 10-0 Ethilon® monofilament nylon sutures (Ethicon, Dublin, Ireland).
Figure 4.2 Schematic diagram of the study layout (A) and workflow of ITRAQ tagging and MudPIT mass spectrometry analysis (B).
For autograft repair, a 10 mm segment of the nerve was explanted, rotated 180° and implanted between the transected nerve stumps, using 10 – 0 nylon sutures. For the 15 mm critical gap group, the nerve was transected 2 mm proximal to the distal bifurcation and a 10 mm segment was removed. The wound was finally closed in layers and the animals received buprenorphine hydrochloride to alleviate pain. Animals were continuously monitored for signs of distress or pain.

4.2.3 Isolation of the Sciatic Nerve

Two weeks post implantation, animals were sacrificed by overdose of pentobarbital and transcardially perfused using ice cold phosphate buffered saline. Perfusion was required to reduce the albumin levels within the nerve, which would otherwise mask the expression of low abundance proteins (35). The sciatic nerve was subsequently re-exposed under a dissecting microscope using a gluteal muscle splitting approach. The sciatic nerve was exposed proximally close to the spinal cord and distally near the muscles. This procedure allowed for a complete exposure of the sciatic nerve and its branches. The nerve was transected 5 mm proximal and distal to the nerve conduit and isolated for further sectioning as per (19). Under the microscope, sutures were carefully removed using a fine forceps and microscissors and the proximal and distal stumps were carefully removed from the conduit. After removal, approximately 6 cm nerve segments were left behind and were subsequently weighed, snap frozen and transferred to the -80 ºC for storage until further processing was required. The remaining conduit was split and a solid mass of tissue (or if no tissue present, the tissue lysate) was removed, weighed, snap frozen and stored as per the nerve segments.

4.2.4 Protein Isolation, Precipitation and Quantification

For extraction of the protein from the tissue samples, samples were removed from the -80 ºC storage, weighed and suspended in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) (Sigma® R0278, Dublin, Ireland) mixed with a protease inhibitor cocktail for mammalian tissues (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin) (Sigma® P8340, Dublin, Ireland) and
transferred to a prechilled magnetic bead homogenizer (Qiagen® TissueLyser LT™). Samples were homogenized and subsequently centrifuged at 12,000 x g at 4 ºC for 5 mins to pellet the tissue debris from the sample. The supernatant was removed and transferred to a pre-chilled test tube. Acetone was pre-chilled to –20 ºC and transferred to protein-containing tube (six volumes of acetone per one volume of protein solution). The tube was inverted three times and incubated overnight at -20 ºC until a flocculent had formed. Samples were centrifuged at 6,000xg for 10 mins and the acetone was decanted from the tube. Samples were then lyophilized and stored for later analysis.

Prior to 8-plex iTRAQ isobaric label tagging and MudPIT analysis of the extracted protein. The total amount of protein was determined using the BCA assay. Samples were resuspended in dissolution buffer (10 mM triethylammonium bicarbonate (TEAB), pH 8.5). The Pierce® BCA micro assay was carried out as per the manufacturer’s instructions (Thermo Scientific™, Dublin, Ireland) to determine the protein concentration in each sample. BSA was used to generate a standard curve.

4.2.5 8-plex Protein Tagging

Samples from proximal, middle and distal nerves were run separately in three individual protein 8-plex iTRAQ® experiments (Figure 4.2). Normalisation was kept constant for all groups by running the same amount of protein (100µg) for all the samples and across all iTRAQ® groups. Each individual iTRAQ® experiment was carried out according to the manufacturer’s instructions (Applied Biosystems®, Foster City, CA). Eight tubes were each filled with an appropriate volume of the pooled protein solution to give 100 µg of protein per tube. Samples were vacuum dried and resuspended in 20 µl of the dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5) and 1 µl of the denaturant (2% SDS) buffer to denature the sample. This process buffers the reaction and partially denatures the protein. 2 µl of a reducing agent (50 mM tris-(2-carboxyethyl) phosphine (TCEP)) was added to each sample tube. This addition reduces the disulphide bonds of the proteins and increases accessibility of trypsin sites for digestion into peptides. The samples were incubated in this solution for 1 h at 37ºC while gently rocking. After
incubation, samples were cooled to room temperature. 1 µl of cysteine blocking agent (200 mM methyl methanethiosulfonate (MMTS) in isopropanol) was added to each sample tube to block cysteine groups and to prevent reformation of disulphide bonds within the protein. Samples were allowed to remain in this solution for 10 mins at room temperature.

After this step, 10 µl of trypsin solution (1 µg of trypsin per 10 µg of protein) was added to the sample tubes and was incubated overnight at 37 °C for digestion of the protein (trypsin is bovine trypsin with CaCl₂ (AB Sciex, Framingham, MA). The digested protein sample was subsequently centrifuged at 1,200 rpm for 30 seconds bringing the digested protein to the bottom of the sample tube. Individual 8-plex reagents were prepared in 50 µl of isopropanol and each pooled treatment group was assigned an isobaric tag (113-121) as per Figure 4.2. 50 µl of the assigned isobaric reagent was added to the treated protein sample and the pH was adjusted to a slightly basic value that was between 7.5 and 8.5 (by adding 5 µl more of the 500 mM TEAB (8.5) buffer), for optimal labelling efficiency, and incubated at room temperature for 2 h. This isobaric tag, when added, complexed to the pooled protein sample and confirmation of tagging was carried out using mass spectroscopy analysis. This is a primary amine-modifying reagent as it will covalently reacts with all amino group (N-terminus and lysine residues). Upon confirmation of successful tagging, the individual tagged protein samples were pooled together for LC/MS/MS analysis (8-plex).

4.2.6 Multidimensional Protein Identification Technology (MudPIT) Analysis

Two-dimensional peptide fractionation was used to maximize the number of identified peptides before using mass spectrometry. This was carried out as per (36). The 8-plex pooled protein samples were concentrated through the use of vacuum centrifugation. This removed residual organic solvents from the solution. 1 ml of buffer A (10mM KH₂PO₄, 25% acetonitrile, pH2.8) was added to the pooled protein solution prior to strong cation exchange (SCX) chromatography. This was performed by separating the peptides over a 4.6 x 100 mm POROS® HS/20 column (Applied Biosystems®, Washington DC, USA) using a salt (KCl) gradient to elute bound peptides. A 1100/1200 HPLC
system (Agilent Technologies®, Santa Clara, CA) was used to carry out liquid chromatography on the samples. 96 fractions were collected that spanned the entire range of the gradient including all unbound and bound peptides.

A two-step gradient was applied at a flow rate of 0.5 ml/min for 50 mins. The 96 fractions were then pooled back into 45 consecutive fractions spanning the entire unbound and eluted peptide peaks based on analysis of the chromatogram at a UV absorbance of 214 nm. Vacuum centrifugation was used to dry the samples and each fraction was resuspended in 100 µl of reverse phase buffer A (2% acetonitrile, 0.1% trifluoroacetic). Reverse phase chromatography was carried out by injection of the pooled fractions into a Ultimate® Plus NanoLC (Dionex®, Sunnyvale, CA) system equipped with an Acclaim® C18 PepMap™ 100 µ-Precolumn (300 µm x 5 mm, 5 µm beads, 100 Å pores) in conjunction with an analytical nanoflow C18 PepMap™ 100 column (75 µm x 15 cm, 3 µm, 100 Å pores) (Dionex®, Sunnyvale, CA). The samples were eluted over a period of 60 mins with a 5 – 50% gradient of acetonitrile. Fractions were directly spotted onto an ABI 4800 Opti-TOF™ MALDI (matrix-assisted laser desorption ionization) target plates (GE Healthcare Bio-Sciences, Pittsburgh, PA) and mixed with a α-cyano-4-hydroxycinnamic acid (CHCA) ionization matrix (Sigma Aldrich) at a ratio of 1:2 using a Probot printing robot (Dionex, Sunnyvale, CA). Each SCX fraction results in approximated 500 MALDI spots on the plate and five SCX fractions are printed per 4800 target plate.

Mass spectrometry (MS) was carried out on the isolated pool of fractions using an ABI 4800Plus MALDI-TOF/TOF™ (time-of-flight/time-of-flight) tandem MS system (GE Healthcare Bio-Sciences, Pittsburgh, PA). Collision-induced disassociation (CID) was used under medium gas pressure in ambient air to fragment the 15 most abundant precursors of each spot by MS-MS. A 200 ppm mass tolerance was set for the precursor ions. For filtering of individual spectra, a minimum signal to noise ratio was set to 50. This threshold maximised sensitivity and the number of spectra considered for identification of proteins.
4.2.7 Peptide and Protein Identification and Statistical Analysis

ProteinPilot™ software (version 3.0, Applied Biosystems, Software Revision 50861) was used to calculate the relative abundance, and to identify the peptide and protein IDs obtained from MudPIT analysis. Database matching and identification was carried out using the Paragon algorithm (36-39). The search incorporates more than 150 biological modifications simultaneously including those that may be induced by iTRAQ® sample preparation and Cys alkylation. This allows identification of unusual protein modifications, increase the number of proteins detected (higher sequence coverage and an increase in multi-hit proteins) and increases the resolution of the protein being detected. The database searched against was a combined and a redundant one of all known protein sequences from rat (*Rattus norvegicus*) and mouse (*Mus musculus*) downloaded from publicly available databases. These include the UniProtKB / Swiss-Prot and the UniProtKB / TrEMBL databases ([http://www.uniprot.org/taxonomy/complete-proteomes](http://www.uniprot.org/taxonomy/complete-proteomes)) and NCBI/RefSeq ([http://www.ncbi.nlm.nih.gov/refseq/](http://www.ncbi.nlm.nih.gov/refseq/)). The combined database contained 152422 protein sequences from these two species. Loading error was accounted for by normalising data using the bias and background correction features in ProteinPilot™. The bias correction reduces error introduced by unequal mixing of the tagged protein solutions, and assumes that the unpooled samples prior to mixing had the same amount of total protein. After identification and bias correction, each peptide was assigned a confidence value which was based on the level of agreement between the theoretical and experimental fragmentation patterns. A confidence score was also assigned to each protein based on the confidence scores of its constituent peptides.

For comparability among individual iTRAQ® labels, the weighted average of the iTRAQ® log ratio was calculated as per (38). Further processing was carried out using the Pro Group™ Algorithm within the Protein Pilot™ software. This yielded the final average ratio for each protein after bias correction, an unused protein score, a total protein score and a *p* value. Total protein score incorporates all peptide evidence for a particular protein sequence. However, these peptides may be better explained in a higher ranking protein. The unused protein score, therefore, is all peptide evidence
which does not have a better explanation as being part of a higher ranking protein (39). Therefore, the unused protein score does not allow the same peptide to be used in a different protein once detected. The \( p \) value outputted by the Paragon™ algorithm allowed the results to be evaluated based on the certainty of changes in expression, not just the magnitude of change and increases confidence if any differential expression has occurred.

For the purpose of this study, high confidence proteins were selected by applying the following threshold: (a) an unused protein score \( \geq 1 \) (i.e. protscore \( \geq 1 \) = proteins have > 90 % confidence), (b) at least two peptides (of two distinct spectra) of 95% confidence identifying the protein and (c) a \( p \) value < 0.05. Differentially expressed proteins were compared for each iTRAQ® set against the normal uninjured control (iTRAQ® label: 121) yielding an iTRAQ® ratio for each treatment group and a fold change cut-off was applied similar to all groups. A fold change of 2.0 or higher (either upregulated/downregulated) was deemed to be differentially expressed.

4.2.8 Functional Enrichment Analysis Based on the Conduit Material
For all treatment groups, samples were divided into proximal, middle and distal components to account for the variation in biological processes which occur throughout the length of the conduit (2, 20, 40). For definition of the biological response based on the material used, autograft, the hollow PLGA and the hollow collagen conduit groups were compared using Ingenuity® Pathway Analysis (IPA) software (build version 220217). This analysis identified the most significant biological functions, networks and diseases related to the input protein data set. IPA takes the inputted focus molecules and assigns them to a node. The software then forms connections between the focus molecules based on protein-protein and physical interactions as well as on their biological function. These focus molecules are then related to other molecules in the literature from the curated Ingenuity® Knowledge Base and assigned a limit of 35 molecules to reduce the introduction of irrelevant material in the analysis. From the network, a score is generated based on the number of focus molecules and their connectivity with respect to the entire network. The score is generated from the log ratio of the \( p \) value generated by Fisher’s exact test. The score is representative of how likely the differential
proteins in the network are likely to be expressed together by chance i.e. a score > 2 indicates a 99% confidence that the network is not being generated by chance alone. A large number of interactions between the focus molecules suggests that these molecules are working together as part of a larger biological system. The score also takes into account the size of the network and the relevance of this network to the focus molecules (41, 42). From this analysis, differences in biological function and networks could be elucidated, based on the material used and the underlying mechanism at the site of analysis.

4.2.9 Functional Enrichment Analysis Based on the Gap Length
For the material groups relating to the gap distance, similar functional enrichment analysis was carried out using IPA. The groups considered were: the 10 mm (non-critical nerve gap) autograft, collagen conduit and filled/fibre collagen conduit groups; and the 15 mm (critical nerve gap) autograft, collagen conduit and filled/fibre group. PLGA was not considered as part of this analysis because of the limited availability of spots on 8-plex iTRAQ and also as the hypothesis was no longer material based. Differences in protein expression resulting from increasing gap length were analysed for the gold standard, a hollow collagen nerve guidance conduit and a collagen conduit filled with intraluminal structure during the acute phase of nerve regeneration. Differences in protein expression and top functional networks were compared as per the abovementioned material response.

4.2.10 Validation of Protein Expression by ELISA
Multiple enzyme-linked immunosorbent assays (ELISAs) were used to validate the change in expression of significantly regulated proteins from the proteomic mechanistic study. Proteins were selected for validation based on their presence throughout each material and differences in expression. ELISAs for DCN, VIM, SOD, APOE, MARCKS and MBP were used to validate a number of the identified targets. All ELISAs were carried out as per the manufacturer’s instructions (Antibodies-Online.com, Atlanta, USA) and compared to the normal uninjured nerve.
4.2.11 Statistics
A one-way ANOVA followed by Newman-Keuls post-hoc analysis was used for all statistical analysis unless otherwise mentioned. Data was deemed statistically significant if a $p$ value less than 0.05 was demonstrated. All data analysis was carried out using Graphpad Prism® statistical analysis software (v5.01, La Jolla, CA).

4.3 Results

4.3.1 Mass Spectrometry and Functional Enrichment Analysis
Two weeks post-implantation, nerve segments from the proximal, middle and distal nerve segments were subjected to 8-plex ITRAQ™ protein tagging and subsequent MudPIT MS/MS analysis (Figure 4.2). Proteomic analysis yielded 1934, 2404, and 1338 identified protein candidates before initial filtering of the protein data at the proximal, middle, and distal segments of the isolated groups respectively. After filtering, a total of 161, 157, and 116 high-confidence proteins were detected for the proximal, middle, and distal components respectively. A protein was considered to be significantly altered if a fold change threshold of two or higher was reached and the protein was identified with a $p$-value less than 0.05. This resulted in the identification of 41, 60, and 36 differentially regulated high-confidence proteins in the proximal, middle, and distal nerve environments. These molecules were deemed “the focus molecules” for functional enrichment analysis in the IPA software platform. Focus molecules were related to associate proteins in the literature based on their expression profile and biological function creating a functional biological network. From this, the focus molecules were subdivided into groups of biological functions. Focus molecules appeared across the multiple functions to represent the diversity of their function. The produced biological network was assigned a score based on connectivity to the focus molecules. The higher the score, the less likely it was generated by random chance and the more likely it is part of the biological system in question. For all groups, network analysis of the significantly identified and regulated focus molecules showed a relation to neurological disease.
### Proximal Top Biological Functions and Networks

<table>
<thead>
<tr>
<th>Function Affected</th>
<th>-log(p-value)</th>
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<tbody>
<tr>
<td>Cell Assembly and Organisation</td>
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<td>Cellular Function and Maintenance</td>
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<td>Small Molecule Biochemistry</td>
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<td>Cell Morphology</td>
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<tr>
<td>Molecular Transport</td>
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</tr>
<tr>
<td>Nucleic Acid Metabolism</td>
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</tr>
<tr>
<td>Cellular Development</td>
<td>3.0</td>
</tr>
<tr>
<td>Cell-to-Cell Signalling and Interaction</td>
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<tr>
<td>Free Radical Scavenging</td>
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<tr>
<td>Cellular Movement</td>
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<tr>
<td>PLGA Conduit</td>
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<tr>
<td>Collagen Conduit</td>
<td>5.5</td>
</tr>
<tr>
<td>Autograft</td>
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</table>

#### Protein Expression Heatmap

- **Vimentin**
- **Transferrin**
- **Creatine Kinase M-Type**
- **Myelin Protein PO**
- **Hemopexin**
- **Ceruloplasmin**
- **Myelin Basic Protein**
- **Hemoglobin Subunit Alpha-1/2**
- **Decorin**
- **Superoxide Dismutase**
- **Annexin A2**
- **Myosin Light Chain 1/3**
- **Nucleoside Diphosphate Kinase**
- **Elongation Factor 1-Alpha**
- **Myristoylated Alanine-Rich C-Kinase Substrate**
- **Tropomyosin 2 Beta**
- **Creatine Kinase B-Type**
- **ATP Synthase Subunit Alpha**
- **Glyceraldehyde-3-Phosphate Dehydrogenase**
- **Myosin-4**
- **Alpha-Enolase**
- **Heat Shock Cognate 71 kDa Protein**
- **Maltate Dehydrogenase**
- **L-lactate Dehydrogenase A Chain**

#### Overlap of Molecules in Top Networks

- **Autograft**
- **Collagen Conduit**
- **PLGA Conduit**

#### Proximal Environment
Figure 4.3 Biological network analysis of differentially regulated proteins as a function of the material used in the proximal component of the graft. (A) Top biological networks corresponding to each material and the most significant biological functions regulated in each. (B) Overview of the significantly identified and regulated proteins in the proximal graft. (C) Overlap of molecules in the top biological networks. (D) Snapshot of proximal regenerative environment – proteins that are synthesised locally in axons and Schwann cells, macrophages and blood borne components infiltrate the injured nervous area and are responsible for the changes in protein expression demonstrated herein.
Figure 4.4 Differences in biological function, networks, and focus molecules as a function of the material used. (A) Top biological functions and the associated biological networks and comparative differences in their regulation. (B) Differences in regulation of focus molecules, red (upregulated) and green (downregulated). (C) Overlap of focus molecules in top networks. (D) Schematic of the contribution of cellular, neuronal, and debris components at midgraft.
### Distal Top Ten Biological Functions and Networks at Distal End

**Legend:**
- Autograft
- Collagen Conduit
- PLGA Conduit

<table>
<thead>
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<th>Function Affected</th>
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<td>Cell Morphology</td>
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<td>Cellular Compromise</td>
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<td>Cellular Function and Maintenance</td>
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<td>Cellular Development</td>
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<tr>
<td>Cellular Movement</td>
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<tr>
<td>Cell Death and Survival</td>
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<tr>
<td>Small Molecule Biochemistry</td>
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<tr>
<td>Cell Cycle</td>
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</table>

### Protein Expression Heatmap

<table>
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<tr>
<th>Protein Name</th>
<th>Autograft</th>
<th>Collagen</th>
<th>PLGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein E</td>
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<tr>
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<tr>
<td>Myelin Protein P0</td>
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<td></td>
</tr>
<tr>
<td>Myelin Basic Protein</td>
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<td></td>
</tr>
<tr>
<td>Tropomyosin Beta Chain</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-1B-Glycoprotein</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Prelamin-A/C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified Protein (Fragment)</td>
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<td>Transgelin-2</td>
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<td>Lamican</td>
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<td>Ah2-162</td>
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<td>Fructose-Bisphosphate Adolase</td>
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<td>Transgelin-2</td>
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<tr>
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<td>14-3-3 Protein Epsilon</td>
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**Color Scale Bar:**

-4.5 to 4.46

### Overlap of Molecules in Top Networks

- Autograft
- PLGA Conduit
- Collagen Conduit

### Distal Micro-Environment
Figure 4.5 Material-specific differences at the distal end of the regenerating nerve. (A) Biological networks and top tier biological functions as a function of the material used. (B) Focus molecules and their regulation at midgraft. (C) Overlap of molecules in top biological networks generated by IPA software.
4.3.2 The Material-Specific Proteomic Response at Two Weeks
To define the material-specific protein changes associated with conduit-mediated nerve repair, nerve conduits made from either natural (collagen) or synthetic materials (PLGA) were implanted within a rat sciatic nerve model and the protein expression patterns of each group were analysed after two weeks after implantation and compared to the gold standard autograft.

4.3.2.1 Autograft – Gold Standard Protein Expression
Top tier biological functions were initially analysed. This strategy allowed for focus molecules to be considered as a whole and allowed the materials to be compared in terms of overall functional regulation. Autograft showed superiority in a number of biological functions in the proximal graft. These biological functions include: cellular assembly and organisation, cellular function and maintenance, molecular transport, nucleic acid metabolism, cell movement, free radical scavenging and lipid metabolism (Figure 4.3). The second tier functions break-down the top tier functions into their specific biological roles. Proximally, the most significant second tier functions are related to organisation of filament components (intermediate filaments, neurofilaments, etc.), regulation of extracellular ions (iron, calcium, heme, etc.), myelination of axons, outgrowth of neurites, organisation of extracellular matrix components, neuroinflammation, and the influx of phagocytic cells.

The molecules involved in these biological functions that form the top biological network are: ANXA2, DCN, SOD1, HBA1/HBA2, MARCKS, MPZ, MBP, HPX, CKM, NME2, VIM, TF, and TPM. Molecules in this network (including focus and implicated molecules) have 8 out of 35 molecules in common with the top network of PLGA and 5 out of 35 in common with collagen proximally. Focus molecules that show differential expression to the conduits and are responsible for the differences in biological networks generated include upregulation of NME2, SOD1, and TF and downregulation of ANXA2, HBA1/2, and VIM. Molecules and pathways that have a close relationship to focus molecules include: ERK1/2, AKT, MAPK, P38 MAPK, PKC, APLN, VEGF, and actin.
At midgraft, top tier functions relate to cellular compromise, cell morphology, cell-to-cell signalling and interaction, cellular function and maintenance, and cellular assembly and organisation (Figure 4.4). By and large, the autograft undergoes less significant regulation than its hollow conduit counterparts. At midgraft, axonal and myelin components seem to be undergoing a process of secondary Wallerian degeneration. Significant second tier functions include axon and myelin degeneration, the influx and binding of macrophages, reorganisation of filament and extracellular matrix components, activation of neuritogenesis, regulation of neuroinflammation and repair. Focus molecules responsible for these functions include: APOE, NEFM, TF, KCTD12, DCN, MBP, Kng1/Kng111, MSN, ALB, C4A/C4, and RYR3. Associated molecules are related to angiogenesis, neuroinflammation, and regeneration and repair. These include TGFβ, VEGF, JUN, RHOA, MAPK, p38 MAPK, ERK 1/2, AKT and IL12. The top network has 17 out of 35 molecules in common with the collagen conduit and 15 out of 35 molecules in common with PLGA. In comparison to PLGA, unique/differentially expressed molecules in the autograft include upregulation of APOE, C4A/C4B, HPX, KCTD12, KNG1/KNG, MSN and RYR3 and downregulation of ALB, DCN and TF. Compared to the collagen conduit, the upregulation of HPX, KNG1/KNG111, and MSN and downregulation of AFM were responsible for the differences in the protein response midgraft.

Distally, significance levels of top tier functions are maintained similar to that of the central graft (Figure 4.5). These include cellular compromise and cellular morphology, cell-to-cell signalling, cellular function and maintenance and cellular assembly and organisation. The conduit groups show more significant regulation of distal functions than that of autograft. The second tier of biological functions remains similar to that of the middle. Functions are primarily related to breaking down and restructuring of the native environment. Specific functions relate to changes in the morphology of native axons and their myelin sheath, in particular atrophy of axons and degeneration or changes in the native neuroglia. Molecules are largely associated with macrophage influx and their interactions, interactions of vascular tissue, and the promotion of neuritogenesis. Molecules also appear
to reduce cell death in the distal graft. The top network consists of: NEFM, LUM, HPX, MSN, HMGN2, SPTAN1, A1BG, APOE, MBP, LMNA, KHSRP, DCN, and TF. These molecules are associated with HDL, P38 MAPK, ERK1/2, Mapk, Akt, and VEGF and IL1. Autograft has 19 out of 35 molecules in common with both the collagen and PLGA conduits. Focus molecules not common with PLGA include the upregulation of APOD, LMNA, and MSN and the downregulation of SPTAN1 and HMGN2. In comparison to the collagen conduit, unique molecules in the autograft include upregulation of A1BG, APOD, KHSRP, MSN, and TF.

4.3.2.2 The Influence of a Natural ECM Material on Nerve Regeneration

Proximally, the collagen conduit has a differential response to that of autograft (Figure 4.3). Top tier functions relate to amino acid metabolism, small molecule biochemistry, and cell morphology, assembly, and organisation. Cellular assembly and organisation and cell function and maintenance show less significant regulation than that of autograft and show similar levels of significance between both the collagen and PLGA conduit. Molecules related to small molecule biochemistry are similarly regulated to that of autograft and are two-fold higher than PLGA. Amino acid metabolism is highly affected in the collagen conduit versus that of PLGA, where little to no regulation is being detected. Second tier biological functions are related to myelination of axons and nervous tissue, hypomyelination of axons, and cellular infiltration. The downregulation of CKB and upregulation of CKM are responsible for the significant regulation of amino acid metabolism. Their expression also implicates the activation of Schwann cells, binding of microtubules, proliferation of neuroepithelial cells and regeneration of motor neurons. Compared to autograft, there is less regulation of blood-borne components such as extracellular heme and iron and superoxide radicals. Focus molecules responsible for the most significant biological functions were MBP, CKB, MPZ, GAPDH, CKM, DCN, and MYL, ATP5A1, MYH4. Unique to collagen’s top network was the upregulation of ATP4A1, GAPDH, MYH4 and MYL1, and downregulation of CKB and UBC.

Mid-conduit, functions show similar regulation to the proximal autograft, and both treatments show higher regulation in a number of molecules versus that
of PLGA. The collagen conduit showed more significant regulation of cell signalling, free radical scavenging, cell movement, amino acid metabolism, vitamin and mineral metabolism, lipid metabolism, cellular growth and proliferation, and small molecule biochemistry (Figure 4.4). Differences in second tier functions relate to regulation of reactive oxygen species and homeostasis of extracellular ions. In particular, data suggests increased synthesis of hydrogen peroxide and reactive oxygen species. Homeostasis of cholesterol and lipid components became implicitly similar to the middle and distal autograft. Amino acid metabolism, i.e. metabolism of phosphocreatine, remains strongly implicated and this pathway is one of the main hallmarks with the use of a collagen conduit. Other functions relate to a phagocytic response, with increased cellular infiltration and restructuring of the environment. Focus molecules responsible for the top network include: CKM, SOD1, DBI, TF, NEFM, RYR3, ALB, CKM, CRP, APOE, DCN, C4A/C4B, and KCTD. At the midpoint, the collagen conduit has 19 out of 35 molecules expressed similarly with autograft in the top network. Differential proteins unique to the collagen network include: downregulation of CKB, CKM, DBI, and SOD1 and upregulation of CRP. At midgraft, ERK1/2, HDL and LDL, IL1, and TGFβ are implicated similarly to autograft.

At the distal end, top biological functions are related to cell morphology, cellular assembly and organisation, cellular compromise, cell movement, cellular function and maintenance, cell death and survival, and lipid metabolism (Figure 4.5). Across these biological functions the collagen conduit shows more significant regulation autograft or PLGA. Second tier functions include axonal and myelin degeneration and cellular movement, similar to autograft, suggesting that Wallerian degeneration is also occurring in the distal conduit. Distally, focus molecules responsible for significant changes in functional expression include: MPZ, HPX, HMGN2, MBP, MPZ, APOE, NEFM, SNCG, CKB, LUM, HBA1/HBA2, LMNB1, SPTAN1, and DCN. These molecules are closely related to those of the ERK1/2, Akt, MapK, IL1, and the Ap1 (CJUN) pathways as well as being related to collagen synthesis and the LDL and HDLs. Unique molecules include
upregulation of C3 and HBA1/HBA2 and downregulation of CKB, MPZ, LMNB1, SNCG, and SOD1.

4.3.2.3 PLGA: A Synthetic Alternative to the Native ECM

Proximally, PLGA showed less regulation of top tier functions than autograft, similar to collagen. Top tier functions in cell morphology, cellular assembly and organisation, cell development, and cellular function and maintenance remained similar to collagen (Figure 4.3). Second tier functions relate to a large cellular influx, in particular fibroblast infiltration. This is in contrast to collagen, where activation of Schwann cells and regeneration of motor neurons are highly implicated. The PLGA conduit, proximally, has a biological network with 8 molecules out of 35 in common with autograft. Focus molecules in the network include: LDHA, ENO1, MPZ, DCN, TPM2, DCN, MDH2, HPX, MYL1, HSPA8, MBP, MARCKS, and TPM1. Focus molecules responsible for the differences include upregulation of ENO1, HSPA8, MDH2, MYL1, and TPM1 and downregulation of LDHA. Common regenerative pathways to the autograft include MAPK and ERK1/2 pathways, compared to collagen which had only the AKT pathway in common.

At midgraft, PLGA showed higher regulation of cell death and survival, as well as functions related to cell-to-cell signalling, cellular assembly and organisation versus the other groups (Figure 4.4). Second tier functions suggest a high cellular influx, axon and myelin degeneration components, and regulation of cholesterol, lipid, and free metal components. Second tier functions include the formation of intermediate filaments and neurofilaments, and neurite outgrowth. Two high scoring biological networks are generated at the midpoint. The top biological network related to neurological disease and organismal injury has 17 out of 35 molecules. Focus molecules at the core of this network include A1BG, AHSG, APOE, CE, DBI, DPYSL2, HPX, KCTD12, MDH2, MPZ, NEFM, PPIB, and TF. Molecules with a differential expression in comparison to autograft include: upregulation of A1BG, AHSG, C3, ITIH4 and downregulation of DPYSL2, DBI, MDH2, MPZ, and PPIB. The biological network implicates the ERK1/2 pathways, both HDL and LDL, p38 MAPK but also JNK.
Distally, top tier functions remain at a similar level to the other groups (Figure 4.5). Second tier functions relate to degeneration of axons, myelin sheaths, and their associated neuroglia. Similarly, there is upregulation of molecules related to extracellular iron and heme clearance. The highest scoring network had 18 molecules in common with autograft and 19 in common with collagen conduit. Top tier and second tier functions suggest similar processes occur despite the change in material. Differential expression in the distal network includes upregulation of CKM, HBA1/2, and LMNB1 and downregulation of MPZ, PRX, VIM, and YWHAE. Focus molecules are strongly associated with regenerative pathways and molecules including ERK1/2, HDL and LDL, IL1, NFκβ, MAPK, P38 MAPK, AKT, and TCR.

4.3.2.4 Validation of Material Specific Protein Expression (ELISA)

ELISA was used to confirm the identification, magnitude and direction of the fold change in the protein samples. Proteins which generated the most significant biological networks were considered for analysis and included: DCN, MARCKS, APOE, SOD, VIM, and MBP. These proteins were validated for all groups, for the material specific and the distance specific protein responses. With respect to material specific changes (Fig 4.9), DCN was shown to be detected in all sample groups and downregulated across all groups. At the midpoint, both the collagen and PLGA conduits were significantly more downregulated than that of the autograft. This matched both the magnitude and direction of the fold change that was seen mass spectroscopy analysis. This also highlighted the extent of downregulation in the conduit groups versus that of the autograft group. APOE was shown to be upregulated across all groups with no significant differences seen in proximal and distal components of the graft. However, midgraft APOE expression was significantly higher in the hollow collagen conduit versus that of the PLGA and autograft groups. This was contrary to the mass spectroscopy data, however the ELISA kit may not have considered all isoforms (of which there are three primary (43)) of the APOE protein which may account for differences in its expression. VIM identification was confirmed throughout the graft and was shown to be downregulated across all groups in the proximal component of the treatment groups. At midgraft, VIM was significantly
downregulated in both the conduit groups versus that of the autograft. Distally no significant differences were seen between the groups. SOD (Cu-Zn) expression was differentially expressed (downregulated) in the hollow collagen conduit only as both the collagen and autograft groups were below the fold change cut-off. At midgraft, the autograft showed upregulation of SOD (Cu-Zn) whereas the conduit groups showed little to no change, although this difference was not significant. In the distal component of the graft, SOD (Cu-Zn) was downregulated for all groups. MARCKS showed upregulation in the autograft group proximally, similar to that of the mass spectrometry results, and the two conduit materials were downregulated. Midgraft upregulation of the autograft was maintained and similar downregulation of the conduits was seen. No significant differences however were seen across all groups, and in the distal end there was no bias towards up/downregulation in the autograft group. MBP showed significant differences proximally in the conduit groups when compared to that of autograft, however no differences in expression were seen in the middle and distal components in the ELISA data. MBP was downregulated across all groups, as expected, due to the breakdown of myelin components. Autograft in the middle and distal components fell below the fold change cut-off and was not considered to be differentially regulated.

4.3.3 The Effect of Gap Distance on the Proteomic Response
To understand the initial proteomic response responsible for the differences in regeneration seen in different configurations of intraluminal collagen conduits at critical and non-critical gap lengths which occurred at 12 weeks post implantation (Chapter 3), proteomic analysis was carried out at two weeks as per the material response group.

The comparison similarly included two lengths of autograft, hollow collagen conduits and intraluminal collagen fibre conduits.

4.3.3.1 The Effect of Gap Distance on Autograft
The proximal component was first analysed which was now geometrically further away from the distal components of the injured nerve.
The Biomaterial-Induced Proteomic Response
Figure 4.6 Material Specific ELISA data validating core proteins in the proximal, middle and distal environments of the injured nerve two weeks post implantation. Data was compared using one-way ANOVA followed by the Newman-keuls post-hoc test (* p-value < 0.05 was deemed significant).
### Protein Expression Heatmap

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<th>Autograft 15 mm</th>
<th>Hollow Conduit 10 mm</th>
<th>Hollow Conduit 15 mm</th>
<th>Filled Conduit 10 mm</th>
<th>Filled Conduit 15 mm</th>
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### Top Differentially Regulated Functions

#### Autograft

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Figure 4.7 Pathway analysis of distance-specific proteomic changes at the proximal end of the graft/conduit. Differential networks are produced depending on the distance of the proximal graft from the distal end. Across all treatment groups, top biological functions show different degrees of biological significance depending on the gap distance treated.
### Protein Expression Heatmap

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### Top Differentially Regulated Functions

#### Autograft
- **Biological Function:** Cellular Compromise
  - Log(p-value): 7.0

#### Hollow Conduit
- **Biological Function:** Cell Function & Maintenance
  - Log(p-value): 7.0

#### Filled Conduit
- **Biological Function:** Cell Assembly & Organization
  - Log(p-value): 8.5
Figure 4.8 Pathway analysis of distance-specific proteomic changes at the midpoint of the graft. The figure shows the networks generated and significant biological networks of each treatment group and how they vary across increasing gap lengths.
Figure 4.9 Changes in protein expression and associated changes in biological networks and top tier functions at the distal end of the graft. The figure shows changes in the autograft, hollow, and filled collagen conduits as a function of the gap length treated.
As expected, there was less significant regulation of top tier biological functions including cellular assembly, cellular function and maintenance, small molecule biochemistry, nucleic acid metabolism, free radical scavenging, and cellular movement. Interestingly, a number of functions were more significantly regulated in the longer graft including vitamin and mineral metabolism, cell morphology, and molecular transport (Figure 4.9). Pathway analysis of the two gap distances yielded distinct biological networks, with only 4 out of 35 molecules in common between the two autografts. Focus molecules responsible for the differential regulation in the longer graft include: upregulation of AHNAK, CADM4, MDH2, MYL1, NEFH, NEFL, PRPH, PRX, and SPTBN1 and downregulation of CALR and ATP5B. Second tier functions relate to clustering of neurofilament structures, dissociation of microtubules, Ca2+ sequestration, and axonal transport. At midpoint, there seems to more significant regulation of top tier functions in the 15 mm graft (Figure 4.10). In particular, cellular compromise, immune cell trafficking, inflammatory response, cellular assembly and organisation, cell function and maintenance, and cell movement are being significantly regulated. In the 15 mm graft, second tier biological functions show significant regulation of macrophage binding and quantity, and a high inflammatory response.

This is associated with the dissociation and atrophy of local axons within the graft. Also implicated is the invasion of blood components and cells, as well as angiogenesis and glial cell activation. In the 10 mm autograft group, the most significant functions are related to axonal atrophy and degeneration of neural glia. There is also an increase in the number of molecules related to organ inflammation and immunological influx. In the 15 mm graft, there is an increase in molecules related to lipid metabolism and cholesterol uptake. The two lengths have a total of 19 molecules in common in their most prominent networks. Main focus molecules specific to the 15 mm autograft include upregulation of C3, H2AFX, and TLR7 and downregulation of MPZ and S100A9. In the 10 mm graft, specific molecules include downregulation of ALB, AFM, and TF and upregulation of HPX and RYR3. In the longer
graft, VEGF and TGFβ beta are no longer implicated. Instead the network suggests regulation of the complement system and interferon alpha.

Distally, 10 and 15 mm grafts show similar regulation of top tier functions (Figure 4.11). The most notable differences in the 15 mm graft are more significant regulation of cell movement, molecular transport, carbohydrate metabolism, and lipid metabolism. Second tier functions relate to the regulation of cell movement and migration, as well as macrophage binding, axon and myelin degeneration. The distal end grafts have 20 out of 35 molecules in common in their most prominent networks. Focus molecules responsible for the changes in the 15 mm graft include downregulation of DPYSL3, LMNB1, MPZ, and YWHAE. In the 10 mm graft, upregulated LMNA and downregulated HMGN2, SPTAN1, and NEFM are responsible for the differences in functional networks.

4.3.3.2 The Effect of Gap Distance in a Collagen Conduit

Proximally the top networks of two conduit lengths have 7 out of 35 molecules in common. Top tier functions are differentially regulated across gap lengths (Figure 4.9). Amino acid metabolism and small molecule biochemistry are more significantly regulated in the 10 mm conduit. In the 15 mm conduit, molecules related to cell morphology, cellular compromise, and carbohydrate and lipid metabolism are more significantly expressed. Second tier functions relate to morphology and degeneration of the myelin sheath and neuroglia; organisation of filament structures and the expression pattern of focus molecules suggest an increase quantity of phagocytes in the proximal conduit. Interestingly, in the 15 mm graft focus molecules suggest an increased quantity of blood cells and cell death. Molecules giving rise to the differential network in the 15 mm group include downregulation of APOE and HBA1/A2 and upregulation of C3, HSPA8, PKM, TF, and TPM2. In the 10 mm conduit, molecules include upregulation of ATP5A1, MYH4, and MYL1 and downregulation of CKB and UBC.

Midgraft, the two conduit lengths behave similarly in top and second tier functions (Figure 4.10). Second tier functions in the 10 mm group include synthesis of reactive oxygen species, leukocyte migration, ion homeostasis,
the activation of neuroglia degeneration of axons and blood cell adhesion. Comparatively, second tier functions in the 15 mm group relate to neuroglia activation, ion homeostasis, reactive oxygen species synthesis, organ inflammation, and increased activation of blood cell components. In both the 15 mm autograft and hollow conduits, the pattern of expression suggests a hypersensitive immunological response. Molecules significantly expressed at 15 mm include upregulation of C3 and LOC299282 and downregulation of ENO2. In the 10 mm group, differences include upregulation of KCTD12 and RYR3 and downregulation of DBI, NEFM, and SOD1.

Distally, increasing distance results in differences in top tier functions, including cellular assembly and organisation, cell movement, lipid metabolism, molecular transport, cell signalling, and vitamin and mineral metabolism (Figure 4.11). Interestingly, there is an increase in amino acid metabolism at the distal end which is seen throughout the shorter conduit. At 15 mm, second tier functions are related to axonal morphology and degeneration, metabolism of phosphocreatine, and cellular movement and necrosis. Molecules responsible for the changes include upregulation of A1BG, CKM, HSP90AB1, ITIH4, and KHSRP and downregulation of TMSB10/TMSB4X. In the shorter graft, downregulation of LUM, MPZ, SNCG, and HMGN2 and upregulation of C3, HBA1/HBA2, and HPX are responsible for differences in function.

4.3.3.3 The Addition of Intraluminal Structure to a Hollow Conduit and the Effect of Gap Distance

The addition of intraluminal structure to a hollow conduit was assessed at a 10 mm gap length in comparison to the hollow conduit group. Proximally, the addition of fibres reduced regulation of two top tier functions – amino acid metabolism and small molecule biochemistry. The remaining functions remained similar. The two networks had 11 out of 35 molecules in common. Molecules responsible for changes in the filled conduit included the upregulation of HPX, MARCKS, TF, and TPM2. This differential suggested more regulation of blood-borne components and homeostasis of extracellular molecules with the addition of structure. The hollow conduit shows downregulation of CKB and upregulation of GAPDH.
At midgraft, the addition of structure increases significance of second tier functions. The filled conduit’s prominent functions are related to neuroglia activation, ion homeostasis, and homeostasis of reactive oxygen species. Top tier functions more significantly regulated by the filled conduit include cell-to-cell signalling and interaction, cellular function and maintenance, cellular assembly and organisation, and vitamin, mineral, and molecular transport. Top biological networks of hollow and filled conduits have 22 molecules in common midgraft. The addition of structure results in a network where the upregulation of C3, ACTB, LOC299282, and TLR7 and downregulation of MPZ are responsible for the changes. Molecules showing differences in the hollow conduit include downregulation of CKB, CK, DBI, NEFM, and SOD1, and upregulation of CRP.

Distally, molecules related to cell morphology, cellular assembly and organisation, cellular compromise, and cell movement are less significantly regulated in the filled conduit. Second tier functions in the filled conduit relate to axonal and myelin morphology, influx of phagocyte and blood cell components, uptake of cholesterol and homeostasis of extracellular ions. The hollow and filled conduits have 21 molecules in common in their top scoring networks. The addition of fibres to the conduit results in upregulation of A1BG, APOD, KHSRP, and TF. In the hollow conduit, differences are due to downregulation of CKB, HMGN2, LUM, NEFM, SNCG, SOD1, and SPTAN1 and upregulation of LMNA.

The effect of increasing gap length was similarly analysed in the filled conduit group (Figure 4.9). Proximally, increased gap distance results in significant regulation of extracellular iron and blood-borne components. In 15 mm graft, similarly to autograft and the hollow conduit, molecules relate to regulation of organ inflammation and cell death. With increasing gap distance there appears to be more significant regulation of cellular assembly and organisation, carbohydrate and nucleic acid metabolism, and amino acid and lipid metabolism. Top networks have 11 out of 35 molecules in common. At 15 mm, unique expression includes downregulation of ANXA2, HBA1/2, LMNA, MYH9, and VIM and upregulation of ENO1, ENO3, GC, MDH2,
MYLPF, NME2, and PGAM1. At 10 mm, differences include upregulation of ATP5A1 and downregulation of UBC.

Mid-conduit, the 15 mm filled group maintains activation of molecules related to neuroglia activation and blood cell adhesion which are not maintained in the 15 mm hollow conduit (Figure 4.10). However, molecules related to ion homeostasis, free radical metabolism and cell death and survival are reduced across the gap length. The longer graft also shows a number of molecules related to cell recruitment being significantly regulated. Biological networks have 27 molecules in common midgraft across both gap lengths. Upregulation of FN, Mug1/Mug2, and downregulation of NEFM and VIM are responsible for changes at midgraft in the longer conduit. At the shorter gap length, upregulation of ACTB and LOC299282 and downregulation of ALB and TF are responsible for the differential networks.

Distally, increased gap distance in the filled conduit results in a number of changes in top tier functions (Figure 4.11). At 15 mm, molecules related to molecular transport, cell movement, and cell signalling are more significantly regulated. Interestingly, lipid metabolism and cell death and survival are less regulated than in the 10 mm conduit. In the 15 mm conduit, the most significant second tier functions are related to the homeostasis of metal ions, inflammation, axonal and myelin degeneration, macrophage recruitment, cell death, and cellular movement. Top biological networks at both distances have 28 out of 35 molecules in common. Differences in the 15 mm conduit include upregulation of CALR and downregulation of DPYSL2 and S100A4. In the 10 mm filled conduit, upregulation of C3 and downregulation of MPZ are responsible for the difference in the biological network.

4.3.3.4 Validation of Expression at Different Gap Lengths
Core proteins were similarly validated for all treatment groups at both gap lengths and compared for differences in protein expression. DCN was downregulated across all groups proximal to distal. The 10 mm and 15 mm hollow and filled conduits showed more significant downregulation of the protein at midgraft than the respective 10 and 15 mm autograft groups. No significant differences were seen within groups at different gap lengths (e.g.
10 mm autograft V 15 mm autograft). APOE was upregulated for all groups across all gap lengths. No significant differences were seen between groups. VIM was shown to be downregulated proximally and upregulated distally for all groups. The 15 mm hollow conduit was significantly more downregulated versus that of the 10 and 15 mm autograft groups. SOD was detected for all groups showing downregulation for the majority of samples proximally and distally. MARCKS was similarly detected for all groups. Despite showing differences in the mass spectroscopy data no significant differences were seen across all groups, however, the direction of the fold change matched that seen in the autograft group. MBP showed significant downregulation at both gap lengths for both the filled and hollow conduits versus that of the 10 mm and 15 mm autograft groups. Similarly distal expression of MBP in the 10 mm filled conduit was significantly more downregulated than that of the 10 and 15 mm autograft groups. Overall ELISA results in this instance confirm the presence of core proteins and in some cases show significant differences in regulation of core proteins within the group.

4.4 Discussion
A large number of studies have reported on strategies to improve regeneration and functional recovery in the injured peripheral nervous system using a broad range of materials and combination therapies, but there has been little investigation of the global changes at the molecular level that occur as a result of these interventions. This study attempts to elucidate the mechanisms of repair that occur with the use of the primary surgical techniques for treatment of large gap injuries, i.e. autograft and biomaterial conduits. In the first part of the study the use of autograft is compared to biological or synthetic conduits. Material-specific changes occurring two weeks after repair were analysed.

To elucidate the differential spatial response that occurs in response to the conduit/autograft, the materials were divided into the proximal, middle and distal regenerate environments. At the tissue and cellular level these changes have been well characterised (44, 45). Proximally, axon regeneration is beginning to occur, with concurrent interaction of local Schwann cells and fibroblasts, as well as blood and endothelial cell components. The axon itself
The Biomaterial-Induced Proteomic Response

is responsible for the synthesis of a number of proteins. Recent studies have focused on their synthesis and transport to and from the site of injury, i.e. local protein synthesis, and anterograde and retrograde transport (reviewed in (46-49)).

The middle of the grafts/conduits undergoing a milieu of biological processes simultaneously, receiving proteins synthesised in the proximal and distal environments, as well as synthesis from cells in the local environment. In the conduits a fibrin cable forms between the proximal and distal stumps creating a natural ECM bridge. This forms a platform for cellular migration and regrowth of regenerating axons (2, 40, 45). In the distal segment of the graft, Wallerian degeneration should be under way for all materials. Changes in these environments have been analysed to a certain extent by Bryan et al., who used a reverse phase proteomic array of a small subset of proteins (15 proteins analysed) and established their expression pattern over 28 days at the proximal, middle, and distal environments in a polyethylene guide (20). The current study expands on this, looking at numerous proteins across the length of the nerve. The study focuses on the changes that occur in these processes at the protein level, in response to both the material used and the gap distance that was treated.

In the autograft, the main regenerative pathways associated with peripheral nerve regeneration are maintained throughout the graft, e.g. MAPK, ERK ½, and the AKT pathway (50), and, unlike the two conduit materials, VEGF expression seems to be implicated throughout the graft, highlighting the importance of angiogenesis (51-53). The middle of the graft appears to be undergoing secondary Wallerian degeneration and resembles closely that of the distal graft. The generation of this microenvironment and the molecules involved may be the reason for the increased regeneration seen in such a graft. Distally, data suggests Wallerian degeneration may be accelerated in autograft and the upregulation of APOD may be one of the contributing factors. APOD speeds up the initial macrophage recruitment and subsequent clearance of macrophages after phagocytosis (54).
Figure 4.10 ELISA data for treatment groups based on the gap distance treated. All data was analysed by one-way ANOVA followed by a Newman Keuls post-hoc test for comparison of groups at different gap lengths and compared to the 10 mm ($) and 15 mm (*) autograft respectively (a $p$-value < 0.05 was deemed significant).
APOE shows increased expression throughout the graft and is an important antioxidant and anti-inflammatory molecule, as well as promotor of neurite outgrowth and regeneration. The apolipoproteins are essential during regeneration for uptake of cholesterol and lipid components from the degenerating axons which later provide the raw products for subsequent remyelination and regeneration. APOE and its mimetics have received much attention as possible therapeutics for the cardiovascular, pulmonary, and central nervous systems (55, 56).

Molecules known for the clearance of toxic by products such as extracellular iron and heme (TF and HPX respectively) are similarly expressed throughout the autograft (57-59). The expression of these factors varies in the conduit materials. TF levels appear to be at lower midgraft and possibly creates a natural gradient from the proximal to middle nerve environments. TF is an antioxidant and its primary role relates to the removal of extracellular iron and its utilisation for repair. TF receptors have previously been shown to be upregulated after sciatic nerve injury and are associated with the dedifferentiation of local Schwann cells and activation of local macrophages (60-63).

Other molecules that create a favourable environment in the autograft include upregulation of SOD1 proximally and MSN at midgraft. SOD1 has a primary role in removal of free oxygen radicals and its upregulation is not seen in the conduit materials throughout their lengths. Toxicity from these free oxygen radicals may be a reason for the poor performance in the biomaterial conduits. MSN is important for growth cone formation and its guidance through the regenerated environment (64). The combination of these factors may be essential for successful nerve regeneration. MARCKS shows upregulation in the autograft group which is implicated in the breakdown of the polysialic acid–NCAM complex, which has been shown to have beneficial effects for peripheral nerve repair (65-67). Interestingly, DCN is consistently downregulated in all materials, and appears to have levels of dysregulation in the conduit materials. DCN, a large glycoprotein, has recently been implicated in the promotion of regeneration in the injured central nervous system. Upon application of DCN to the inhibitory components of the glial
scar, axons were shown to overcome this inhibition and extend neurites beyond the site of injury (68).

The use of a biological conduit creates a differential response to that of autograft. Proteins specific to collagen include upregulation of a number of components related to local energy synthesis. In particular, CKB is downregulated throughout the conduit and molecules related to ATP synthesis are upregulated proximally (ATP4A1). Knockouts of CKB in neurons have shown increased quantity of motile mitochondria to compensate for the loss of the enzyme (69). Similarly DBI, associated with lipid metabolism and energy metabolism, is downregulated at the mid-conduit (70). SOD1 is downregulated at the middle and distal conduit, suggesting an increase in oxygen free radicals, and this accounts for reduction in the number of regenerated axons seen versus the autograft group. Increases in C3, a component of the complement system, in the distal component are associated with more efficient clearance of axon and myelin components during Wallerian degeneration (71, 72). Downregulation of LMNB1 (associated with demyelination in the CNS) and MPZ is associated with variations in Wallerian degeneration as the molecules are associated with disruption of myelin components (73).

Alternatively the use of a synthetic PLGA conduit results in differential changes to that of the collagen conduit. MARCKS is upregulated proximally similarly to autograft and leads to the upregulation of MDH2 seen (proteins have previously been shown to peak concurrently (74). C3 is upregulated at midgraft instead of at the distal graft in the PLGA conduit, and DBI is similarly downregulated midgraft. PRX, which is downregulated distally, is associated with degeneration of the myelin sheath and is a protein that is exclusively found in Schwann cells, suggesting a variation in Wallerian degeneration in the distal axons (75). Interestingly, A1BG is upregulated in the PLGA group at midgraft, which also becomes upregulated with the addition of fibres to the hollow conduit. This molecule similarly sees upregulation in the distal autograft and would be an interesting target for further analysis.
The effect of gap distance was similarly characterised. The results of the study show that gap distance has a direct effect on the regeneration in both the autograft and conduit materials. Within the autograft, alterations in a large number of proteins can be seen throughout the graft. These changes are due to an increased abundance of macrophages and phagocytic cells within the longer graft and result in prolonged secondary degeneration. These changes result in a decrease in performance. Unexpectedly, CADM4 is upregulated in the longer graft and is known for initiating myelination between Schwann cells and axons (76, 77). At this stage of repair it may be serving another function. Increased expression of NFH and NFL proximally suggests increased axonal transport and restructuring of the proximal environment, possibly attempting to compensate for increased distance from the distal environment. Increasing gap distance in the hollow conduit results in altered expression in a number of proteins but not to the same extent as that of autograft. Data suggests a heightened immune response within the larger graft which may result in decrease in recovery. Increased gap distance resulted in the downregulation of APOE, which may result in the build-up of cholesterol and lipid components or adversely affect their reutilisation in the regenerative process. Surprisingly, in the longer grafts there is increased synthesis of TF, which may beneficial effects for repair. Also increased is the expression of C3, which is now seen throughout the graft. The addition in structure to the conduit suggested increased regulation of extracellular toxicants with the upregulation of proteins such as TF and HPX, which may help to protect axons during regeneration and repair. MARCKS is similarly upregulated to match that of the autograft and increased expression of APOD is seen distally, which may result in more efficient degeneration in the distal graft. Multiple proteins are similarly alternatively regulated at higher gap lengths, suggesting an adaptive response within the material conduit.

The results presented herein support the hypothesis that regeneration in large peripheral nerve injuries is altered at the proteome level as a function of the material used and the distance treated. In particular it highlights the need to generate material-specific strategies to counteract deficiencies specific to that material. However, due to limitations of proteomic techniques, growth factor
and transcription factors could not be directly identified. Analysis of these factors with respect to the materials used would further support these results. Importantly, molecules and their expression profiles have been identified that are specific to materials used and the distance treated, with potential for targeting of future therapeutics.

4.5 Conclusions
This phase of the study analysed the molecular changes that occur as a function of different biomaterial treatments and their altered expression at different gap lengths. The study has identified proteins whose expression is altered based on the material used and the gap distance treated. Similarly, the study has identified proteins which become differentially regulated as a result of adding intraluminal structure to a hollow biomaterial conduit. These changes can be correlated with the beneficial effects of intraluminal fibre conduits seen in the previous chapters and can be used to identify deficiencies in the repair strategy for future development (outlined in Chapter 5).

4.7 References


Chapter Five

Summary and Future Studies
5.1 Introduction

The overall aim of this project was to develop a suitable biomaterial platform to promote regeneration and functional recovery of the injured peripheral nerve. It was hypothesised that intraluminal collagen fibre conduit can synergistically act as a platform for cellular migration and topographical guidance of axons, while creating a functional microenvironment for nerve regrowth, with the ultimate aim of treating both non-critical and critical nerve injuries. The intraluminal collagen fibre conduit was developed and evaluated over four main phases: first a tissue-engineered platform for peripheral nerve repair was created and evaluated both in vitro and in vivo for its ability to promote peripheral nerve repair across a non-critical nerve gap.

Having identified the benefits and limitations of the intraluminal fibre system, the second phase of the project attempted to improve regeneration seen in phase I and overcome some of its limitations. The optimised system was analysed in vivo in a non-critical nerve gap (10 mm) and a critical nerve gap (15 mm) and assessed for both nerve histomorphometry outputs and functional outputs.

Based on beneficial results from the initial two phases of the project, the third and fourth phases of the project were designed to understand the mechanistic effects of the incorporation of intraluminal fibres into a hollow conduit system. This was carried out with the aim to further improve the intraluminal fibre conduits at the molecular level which is specific to the material used. In particular, phase III of the project sought to analyse the initial proteomic changes that occur from the use of different hollow conduit materials and how they compare to the gold standard for repair. This provided a basal level for the proteomic changes which occur from the use of different hollow conduit materials and how these materials compared to autograft. In this way the missing molecular components of hollow conduit structures could be elucidated. Phase IV expanded upon this and was used to identify the effect of intraluminal guidance on the proteomic profile of conduit-mediated nerve repair by comparing the proteomic results directly to an empty collagen conduit. In addition, phase IV focused on the alterations in protein expression of specific biomaterials and of autograft as nerve injuries transitioned from a
non-critical to a critical nerve gap length. From this, the effect of increasing gap length was elucidated.

5.2 Summary

5.2.1 Phase I: The Development of a Biomaterial Platform for Repair
The aim of phase I (Chapter 2) of the project was to develop, characterise and assess a natural ECM bridge for its ability to promote repair in the injured peripheral nerve. This was achieved by assessing the use of structured or unstructured collagen fibres, enclosed within a hollow collagen nerve guidance conduit and evaluating their ability to promote regeneration across a non-critical nerve gap. Specifically, this involved developing and characterising the process for producing both the structured and unstructured fibres, characterising them in vitro and assessing the effect of the fibres, enclosed within a conduit, in vivo within a rat sciatic nerve model. Both sets of fibre groups were successfully fabricated using an extrusion based system and cross-linked sufficiently to allow them to be retained throughout the regenerative process (fibres were present 16 weeks post-implantation).

The extrusion based system was used with an excimer laser system to create structured fibres with a large degree of control over the structural parameters. Both fibre groups showed the ability to guide re-growing neurites from PC12 cells in vitro. In PC12 studies, the structured fibres showed significantly more neurite outgrowth than the unstructured fibre group, however, in vivo the structured and unstructured fibres showed no significant differences sixteen weeks post-implantation. When implanted within a rat sciatic nerve model, the inclusion of fibres within the collagen conduits, showed a number of benefits for repair. The fibres were seen to be successfully incorporated within the regenerative process with minimal prolonged tissue response (no fibroblast or macrophage layer in close proximity to the fibres). The addition of fibres to the conduit showed the ability to reduce axonal dispersion within the collagen nerve conduit, due to the reduction in available area for axons to migrate and disperse within the conduit.

At midgraft, the fibre groups showed no significant differences in the number of regenerating axons. However, retrograde tracing revealed significantly
more axons regenerating down the distal branches of the sciatic nerve, than that of the hollow collagen conduit group. The benefits of reduced axonal dispersion and increased number of distal axons suggested that the intraluminal fibre conduit will provide a suitable platform for repair.

5.2.2 Phase II: Optimising Packing Density, Collagen Fibre Degradation and Assessing Functional Recovery

Despite intraluminal fibre conduits showing benefits for nerve repair, levels of nerve regeneration failed to match that of the autograft group. In an attempt to further optimise the use of an intraluminal fibre conduit, phase II (Chapter 3) of the project sought to optimise the packing density and degradation properties of the implanted conduits and assess their ability to promote functional nerve regeneration across a critical and non-critical nerve gap. Packing densities, which in Phase I were 2.2 % of the total cross-sectional area, were increased to 7.22% to match the packing density reported by Ngo et al., (PLLA filaments) (1). Increased packing densities, raised concerns over excess scaffold material being present in the conduit and possibly limiting regeneration in the long term. To counteract this, degradation properties of the collagen were adjusted to more closely match the natural fibrin cable which forms during regeneration and degrades rapidly within the first month of regeneration. Subcutaneous studies demonstrated that by using non-crosslinked fibres, degradation times appeared to be dramatically increased versus their cross-linked counterparts. The non-crosslinked fibres were demonstrated to have disappeared after two weeks subcutaneously, versus their cross-linked counterparts where they remained four weeks after implantation. Using the optimised fibres, the higher density intraluminal fibre conduit was implanted within a rat sciatic nerve model. After 12 weeks nerve histomorphometry and additionally muscle force recovery were analysed. The fibre conduit group was shown to be comparable to the autograft group across a number of comparisons. Whereas in the hollow conduit group, autograft remained significantly superior. The fibre conduit and hollow conduit groups showed the ability to regrow across a 15 mm gap. Although the hollow conduit only had successful regeneration in two out of eight cases, the fibre conduit showed regeneration in five out of eight cases. Overall
autograft was superior at both gap lengths. This highlighted the need to further understand the processes which occur as a result of autograft repair and where the materials used are failing to match the overall regenerative response.

5.2.3 Phase Three: A Biomaterials-Induced Proteomic Response to Conduit-Mediated Nerve Repair

Phase III (Chapter 4) of the project focused on understanding how the proteomic response changes during peripheral nerve regeneration as a function of both the treatment method and the biomaterial used, two weeks after injury. These changes were primarily assessed at a non-critical nerve gap (10 mm) and involved the comparison of two different hollow nerve guidance conduits, namely one natural conduit (collagen) used throughout the prior studies, a synthetic (PLGA) conduit and the gold standard autograft. The spatial proteomics expression for each of these materials was analysed and compared at two weeks and a further understanding of the molecular mechanisms of repair was achieved. Particularly, a spatial pattern of expression was established for each of the material used. The spatial expression of proteins was similarly established for the gold standard autograft and used as the main point of comparison for the materials used. Deficiencies in the materials used included less regulation of superoxide free radicals, extracellular cholesterol, and mineral ion components. Autograft also demonstrated what appeared to be an extended version of the distal nerve graft, in terms of protein expression, into the middle component of the graft which was not seen with the use of hollow conduit materials. This may be one of the principal reasons, at the molecular level, for the increased regeneration seen in autograft.

5.2.4 Phase IV: The Effect of Intraluminal-Collagen Fibres on the Biomaterial-Induced Proteomics Changes during Peripheral Nerve Repair across both Non-Critical and Critical Nerve Gaps

The final phase (Chapter 4) of the project assessed the addition of intraluminal collagen fibres to the hollow nerve guidance conduit. In addition, this phase of the study assessed the effects of increasing gap length on the proteomic response in the materials used.
**Summary and Future Studies**

**Figure 5.17** Schematic diagram summarising the conclusions of the four main phases of the project.

<table>
<thead>
<tr>
<th>Phase I: Development of a Suitable Platform for Repair</th>
<th>Phase II: Optimising Packing Density and Bridging a Critical Nerve Gap</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Structured and unstructured collagen fibres fabricated as a platform for repair</td>
<td>• Subcutaneously non-crosslinked fibres degraded two weeks after repair.</td>
</tr>
<tr>
<td>• Guided axons <em>in vitro</em> and <em>in vivo</em>.</td>
<td>• Intraluminal fibre conduits showed the ability to bridge a critical nerve gap.</td>
</tr>
<tr>
<td>• Successfully incorporated into regenerative process.</td>
<td>• 12 weeks after repair, the intraluminal fibre conduit showed no significant differences versus autograft at a 10 mm gap in a number of parameters.</td>
</tr>
<tr>
<td>• Reduced axonal dispersion.</td>
<td>• Overall ranking of multiple parameter revealed that autograft remained superior in terms of overall regeneration.</td>
</tr>
<tr>
<td>• Increased number of distally labelled axons.</td>
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<tr>
<td>• Regeneration remained lower than autograft.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Phase III: Proteomic Analysis of Material Specific Changes</th>
<th>Phase IV: The Proteomic Effect of Intraluminal Guidance and Gap Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Identified the spatial protein expression pattern of the gold standard for repair.</td>
<td>• Elucidated the effect on intraluminal guidance on the spatial proteomic response in hollow nerve guidance conduits.</td>
</tr>
<tr>
<td>• Identified the spatial proteomic profile of a natural ECM based conduit.</td>
<td>• Identified the alterations in gold standard protein expression as a function of gap length.</td>
</tr>
<tr>
<td>• Identified the spatial proteomic profile of a synthetic material conduit.</td>
<td>• Identified the spatial proteomic changes that occur in hollow and filled nerve guidance conduits as a function of gap length.</td>
</tr>
<tr>
<td>• Identified the missing proteomic components of natural and synthetic conduits during the early stages of repair.</td>
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Hollow and filled collagen conduits were compared to autograft at two gap lengths, essentially a 10 mm and 15 mm gap length, non-critical and critical respectively. The objective of this phase was to investigate the proteomic changes that occur using an intraluminal fibre/hollow collagen conduit across a non-critical and critical nerve gap and compared it to the gold standard for repair. The addition of intraluminal fibres to the conduit had a similar proteomic profile to that of the collagen conduit, but also had an increased number of molecules in common to that of the autograft group. The identification of these molecules gives some indication to how the addition of intraluminal fibres to the lumen of a hollow biomaterial conduit may alter the regenerative response.

The most important findings of the project are illustrated in Figure 5.1. Overall, the use of intraluminal fibre conduits showed the ability to regenerate across both non-critical and critical nerve gap injuries with functional recovery, in some instances, showing similarities to that of the current gold standard for repair. The proteomics analysis of the material-based regenerative response, the effect of intraluminal guidance and the gap distance, allowed the identification of proteins and/or pathways which will form the basis for the generation of the next phase of intraluminal guidance conduits. One could envision the incorporation of a protein of interest (such as apolipoprotein E) within the fibre based conduit and evaluating its effect on repair. If the protein is delivered in a correct dosage with a stable conformation this may lead to superior functional recovery in the nerve guidance conduit. Based on these results, a next generation nerve guidance conduit with the advantages of the reduction in axonal misdirection, similar if not superior nerve regeneration and functional recovery comparable to that of autograft could be achieved. For the end patient, this would provide a suitable alternative to autograft repair with reduced rehabilitation time due to the more accurate regeneration of the injured nerves.
5.3 Limitations

5.3.1 Phase I

The use of intraluminal collagen fibres as a filler material for nerve guidance conduits although showing benefits for nerve repair had a few caveats in their implementation. The use of the fibres suspended within the conduit limited spatial control of the construct (fibres were freely suspended in solution and may juxtaposition to each other creating areas of inhibition) and as such introduced variability into the conduit design. Saline was used to fill the void in this study to avoid the introduction of additional variables to the study. As such, all fibres introduced into the conduit were not uptaken into the regenerative process. Presumably the excess fibres would sink to the bottom of the conduit and not take part during regeneration. Therefore, a suitable gel material such as collagen or fibrin may provide a suitable spacer material to reduce the fibre-to-fibre contact and maintain their spatial location once implanted. This gel could be injected at a low concentration and at low cross-linking ratio to ensure adequate cellular migration and allow infiltration of regrowing axons. Additionally the introduction of such a gel material will allow the introduction of cellular/molecular components for potential future studies. Another potential limitation was the exclusion of laminin from the surface of the collagen fibres. Studies have shown that the addition of laminin to a collagen or even a synthetic conduit material have beneficial effects \textit{in vivo} and in previous studies using collagen constructs may have contributed to their success (2-8). Laminin or other neurotactic peptides were not added in this study, as the initial concept of this study alone was that topography alone will be sufficient to enhance regeneration and that collagen (a major component of normal uninjured nerve) will provide the necessary binding ligands for repair. Contrary to this, it can be argued that native cells (Schwann cells, fibroblasts etc.) may have aligned along the fibres, and will have produced their own ECM on their surface and thus reducing the need for the introduction of exogenous ECM molecules.

5.3.2 Phase II

The introduction of increased packing density and increased degradation rates had some unforeseen effects. In some cases fibres juxtapositioned to each
other creating a site of inhibition for regenerating axons. This was potentially limiting regenerating, despite being higher than that of the hollow collagen conduit. This indicates that the packing density may have been two high and that a medium packing density (between 2.2 % and 7.7%) would be optimal for nerve regeneration. Surprisingly, the non-crosslinked collagen fibres did not degrade over the regenerative period, as suggested by the subcutaneous results and were still present in abundance in the regenerated nerve tissue 12 weeks after regeneration. Force measurement data in the EDL and TA muscles similar showed recovery even in instances where little to no regeneration (15 mm hollow conduit group). This is reflected in the muscle mass recovery, where there is a similar degree of recovery in the conduit groups at the 15 mm gap length. This indicates that the muscles may still be receiving some compensatory means of recovery, possibly from an afferent nerve that is not part of the sciatic nerve trifurcation. The gastrocnemius muscle which shows differences in recovery of the 10 mm and 15 mm autograft groups should be included in force recovery measurements in the future. The gastrocnemius muscle was not evaluated in this instance, due to the time required for the evaluation of each muscle and necessity to harvest the sciatic within a reasonably time frame (to prevent atrophy of the nerve before fixation). Additionally, the strain gauge required to measure force outputs in the GC muscle was not available at the time of the analysis.

5.3.3 Phase III

The proteomics analysis due to the pooling of the initial samples resulted in all data being technical replicates and as such biological variation was not fully considered in the analysis. Instead the effect of biological averaging in the pooled samples was assumed throughout the study. Ideally, samples would have been carried out at least in triplicate for all samples and subjected to principal component analysis or further hierarchical clustering analysis to identify the most significant proteins detected during the experiment. This would have provided a quantitative aspect to the proteomics analysis. Instead the mass spectroscopy analysis was used primarily as a discovery tool and the direction and not the overall magnitude were considered for analysis. Differences between materials and the core proteins of the top networks were
validated via ELISA to confirm the direction of the change and if the proteins were indeed being significantly regulated (>1.5 fold change). Additionally the spatial component of the graft was considered as important, due to the various processes which occur throughout the graft. However, the expression of proteins are temporal in nature and as such additional time-points would have been beneficial to track the changes in protein expression over time. The study carried out during phase III, could be used as benchmark for future studies. A group of ten to fifteen proteins can potentially be identified from the study and their spatial and temporal expression tracked, to increase the validity of this study. This study can be carried out similar to Bryan et al. who used protein arrays to track the expression of a select number of proteins as a function of time and position (i.e. the spatiotemporal expression of selected proteins)(9).

5.3.4 Phase IV
Phase IV of the project had similar limitations to that of phase III. Also the inclusion of PLGA for both gap lengths was not possible due to the limitations of the isobaric tagging system. A maximum of eight samples can be run at the same time and the limit had already been reached. Thus, a 15 mm PLGA sample was not included in the current experimental setup without introducing additional variation to the experimental setup. If the sample was introduced on a separate run of the equipment, the sample will introduce a degree of variability caused by differences between the equipment runs (10, 11). When all eight samples were run at the same time, this variability was kept to a minimal, as all samples were analysed in the system at the same time. As such, a 15 mm PLGA conduit could not be included in the analyses. Additionally, results from the 10 mm PLGA samples would not be applicable in this suggestion as this aspect of the study is more focused on the influence of gap length on the proteomic response in axonal regeneration and also on how the inclusion of intraluminal fibres to a hollow collagen conduit influences repair. For the 10 mm PLGA results to be relevant, an additional group which included a PLGA conduit with intraluminal fibres would be necessary to be incorporated to the study. However, this was outside the scope of this study.
Additionally, proteomics analysis of the injury based response can be limited when looking at individual proteins. Proteins which are highly upregulated or downregulated in individual groups may not have a large effect on the overall regenerative response. In order to characterise how these proteins may be affecting nerve repair it would be appropriate to analyse how the repair mechanisms would occur if the protein was underexpressed or knocked out and similarly what the effect of overexpression of the protein may be. This would highlight the importance of the molecule of interest and its benefits as a molecular additive for future repair strategies. It must also be considered that the proteomics analysis carried out herein was on the whole neural tissue and as such any responses considered must be interpreted at this level and not on individual cellular responses. It is hard to elucidate from the results the cell type which is the origin of the proteins of interest but it may be correlated based on the relevant abundance of cells at the site analysis. For example, in the distal portion of the analysed samples it is well characterised that there are a high proportion of Schwann cells compared to other resident cell types and any proteins expressed could be associated with this cell type. However, in order to be certain of this origin the isolated tissue would have to be depleted of any additional cell types without altering the expression of the extracted protein. This would be worthwhile to consider in future investigations.

5.4 Conclusions

5.4.1 Phase I

**Overall aim:** To develop, characterise and assess a natural ECM bridge for its ability to promote repair in the injured peripheral nerve.

**Conclusions**

- Intraluminal fibers provide a suitable platform for guiding regrowing axons and neurites *in vitro* and *in vivo*.

- The structured fibre conduit showed no significant differences in repair versus the unstructured fibre group.
Summary and Future Studies

- The incorporation of intraluminal fibres in a hollow collagen conduit resulted in a significant decrease in axonal dispersion versus that of autograft.
- Intraluminal fibres were successfully incorporated into the regenerative process.
- Overall the gold standard autograft remained superior to the autograft and hollow conduit groups.

5.4.2 Phase II

Aim: To optimise the packing density and degradation properties of the implanted intraluminal fibre conduits and assess their ability to promote functional nerve regeneration across a critical and non-critical nerve gap.

Conclusions

- Non-cross linked collagen fibres degraded over a two-week time period when implanted subcutaneously in the dorsum of a rat.
- The short degradation time was not translated to the sciatic nerve of the rat and fibres were still present 12 weeks after implantation.
- An increased packing density of intraluminal fibres showed no significant differences versus autograft at 12 weeks in a number of parameters including: fascicular area, myelinated fibre diameter and axonal density.
- At a critical nerve gap, the 15 mm fibre filled group showed no significant differences versus autograft. Both groups were significantly greater than the hollow conduit group.
- In terms of grouped ranked parameters autograft significantly outperformed both conduit groups at both gap lengths.

5.4.3 Phase III

Overall Aim: To assess the proteomics changes that occur during peripheral nerve regeneration, as a function of the treatment method and biomaterial used.

Conclusions
Proteomic analysis of the three different materials assessed (autograft, a collagen conduit and a PLGA conduit) showed a material specific proteomic response that was spatial in nature at two weeks.

The middle and distal components of the autograft showed evidence of extended Wallerian degeneration, extending the regenerative environment to that of the proximal environment.

PLGA and collagen conduits showed differences in proteomic expression, but displayed evidence for reduced regulation of free oxygen radicals, lipids, blood and free metal components which may be toxic for repair.

The use of a collagen conduit displayed significant regulation of amino acid components in comparison to both the autograft and PLGA groups.

PLGA showed alterations in a number of molecules related to lipid metabolism (e.g. APOD)

### 5.4.4 Phase IV

**Aim:** To investigate the proteomic changes that occur using an intraluminal fibre conduit across a non-critical and critical nerve gap and compare it to the gold standard for repair.

**Conclusions**

- The addition of intraluminal fibres to a hollow biomaterial conduit resulted in increased expression of molecules related to the regulation of extracellular iron components, lipids, and free oxygen molecules.
- Similarly the addition of fibres increased expression of a number of glycoproteins and surface proteins responsible for their regulation (e.g. A1BG and MARCKS).
- In the hollow conduit group, increasing gap distance results in loss of regulation of amino acid metabolism and increased expression of components related to the complement system and heat shock proteins suggesting increased cellular stress.
- In the autograft group, increasing gap distance resulted in increased expression of complement components, extracellular matrix
Figure 5.18 Schematic showing concepts of future studies and their sites of application.
organisation and a decrease in regulation of extracellular heme and other blood borne components.

- In the 15 mm fibre filled group, there is no upregulation of complement components seen in the hollow and autograft groups, however, molecules related to extracellular ion and lipid metabolism are downregulated across the gap length.

5.5 Future Studies

The overall project sought to address the limitations of current hollow biomaterial conduits for the treatment of peripheral nerve injuries and to attempt to match/improve the regeneration seen with the use of autograft repair. The main focus being on the use of intraluminal filler materials in a hollow collagen conduit and how it influences peripheral nerve regeneration. Later, the spatial proteomic response was characterised for all materials used throughout the study and compare. Future directions of the project could seek to address the limitations of the four main phases and expand on the initial work carried out at each phase. Further investigation of this work could therefore expand down a number of avenues. These are highlighted and summarised as per Figure 5.2.

5.5.1 An Intraluminal Fibre Conduit as a Controlled Drug/Cell Delivery System

5.5.1.1 Optimising Packing Density and Fibre Distribution

Chapter 3 sought to identify the optimal packing density and degradation rate for the incorporation of intraluminal fibres into a collagen conduit. The higher packing density (7.22%) used in Phase II showed regeneration similar to that of autograft across a number of regenerative parameters. There were, however, instances where fibres abutted each other and inhibited regeneration. This may possibly be due to the high density of fibres in the conduit or the lack of spatial control between each fibre. One potential study can seek to optimise this density in a single controlled experiment, replicating similar experiments done by Ngo et al. and Huang et al. (1, 12) using PLLA fibres and silk fibres respectively.
Ngo found that a packing density of 7.8% was ideal for their studies at both a non-critical and critical nerve gap, similarly Huang et al. showed that approximately 200 fibres were ideal for repair. Simultaneously, to reduce fibre to fibre contact a low concentration/minimally cross-linked natural/synthetic hydrogel can be introduced to the system. This would provide a number of benefits: 1) an additional carrier for cellular/molecular components which can be used to provide additional release (if the fibres themselves are used as a delivery vehicle) creating a dual release system 2) if an aligned hydrogel system was used with appropriate nanoscale topographies such as (13, 14) in a softer/low stiffness hydrogel, axon regeneration would benefit from both the microscale topographical features provided by the stiffer intraluminal collagen fibres (young’s modulus of nerve being in the range of 0.5 to 70 MPa (15-17)) and nanoscale guidance provided by the softer nanofibres within the hydrogel.

5.5.1.2 Controlled Release and Local Gradients

The intraluminal fibre conduit, can be further optimised by the incorporation of a number of molecular or cellular components as discussed in chapter one or supplementation of proteins identified in the proteomic phases of the study (Phase III and Phase IV). These factors will need to be released in a controlled and sustained manner for their benefits to be realised. This can be achieved using a number of controlled release systems e.g. incorporation of molecules into and diffusion from the fibre materials, diffusion from the conduit wall, suspension within a suitable hydrogel etc. (reviewed in Chapter 1). New release systems will be of particular interest e.g. the use of a dual release system to deliver more than one molecule, or to use a spatially controlled system to create local gradients in vivo.

This can be achieved using a fibrin-in-fibrin systems as per Kulkarni et al. (18, 19), collagen microspheres in a larger hydrogel (20), PLG microspheres in a PEG hydrogel system (21) or a similar release system. Gradients can be created by creating a high density of spheres at one end of the conduit (high concentration of the molecule of interest) and reducing the density of spheres at the opposite end (low concentration of the molecule of interest) – creating a localised gradient in the conduit. Gradients have similarly been created
using photo-immobilisation and micropatterning of molecules within the material (22), however these are largely in vitro. Sufficient spatial control will be required for the system to be successful and will likely need to be pre-gelled before implantation within the conduit. Alternatively an injectable system which will control the concentration of injected spheres will provide an alternative. Gradients of molecules have been shown to be beneficial for nerve repair – DRGs were shown to grow towards high concentrations of laminin or IKVAV or to low concentrations of chondroitin sulphate proteoglycans (CSPGs) (22-25). Local gradients of NGF within a biomaterial conduit have similarly been shown to increase axonal regeneration versus that of immobilised NGF alone. NGF was differentially absorbed along the length of poly (ε-caprolactone)-block-poly (l-lactic acid-co-ε-caprolactone) (PLCA) to create a diffusional gradient from the proximal to distal component. When used in vivo, in a 14 mm rat sciatic nerve model, regeneration was significantly greater than a PLCA conduit releasing NGF in a uncontrolled manner (26).

5.5.1.3 Triggered Release of Temporal Components for Early and Late Stage Nerve Regeneration

The regenerative process of peripheral nerve repair is a multiple stage process and this has been highlighted concisely in Chapter 1. Essentially the five main phases (from the fluid phase to myelination phases) can be simplified into two main components – one the regenerative phases (the regeneration of axons and their growth toward their distal targets) and two the myelinating phase (once their target has been reached myelination is restored and mature axons are formed). Therefore, molecular therapies which target the initial pro-regenerative phase and the later pro-myelination phase can prove beneficial for repair. This can be achieved using sequential injections of molecules at defined timepoint or can similarly be achieved using a triggered release system. Triggered release systems would allow temporal control of delivery throughout the regenerative process, as they can be initiated at any time with the introduction of an external stimuli (magnetic field, ultrasound etc.). These triggered release systems usually consist of a polymeric, liposomal or natural molecule which responds to external stimuli such as light, electrical or
magnetic fields, or ultrasound to the patient to trigger the release (27-29). These can be controlled by the clinician or patient for controlled release. In this case variations in stimuli can allow an initial release of a pro-regenerative molecule for the first stage of repair and at a later stage for the promotion of myelination within the construct. This may provide an ideal mechanism for promoting regeneration at both phases of repair and lead to enhanced functional recovery. The application of ultrasound as an external stimuli will provide additional benefits for repair. Previous studies have shown that the application of ultrasound during nerve regeneration leads to an increase in the rate of nerve regeneration as well as the formation of a thicker myelin sheath (30-32). As such the combination of triggered release and ultrasound may have dual benefits for repair. Furthermore, for the distal component of the regenerating nerve, triggered release at a later stage can be used to encourage neuromuscular junction formation, as detailed in Section 4.5.5.

**5.5.1.4 Cellular Depots Releasing Molecules of Interest**

An alternative methods of functionalising the nerve guidance conduit can be the incorporation of a cellular component. This approach can be combined with the hydrogel system to ensure their survival upon implantation and they can subsequently be tracked using an appropriate molecule such as green fluorescent protein (GFP) (similar to Ding *et al.* (33) ). The cellular component can be combined with the molecular therapy to ensure the implanted cells receive similar benefits to repair as will the native cells. The implanted cells can be engineered *ex vivo* to overexpress a key molecule of interest such as those identified in Chapter 4. For sustained transfection and release, a gene vector can be released in a controlled manner from the conduit wall or a similar delivery system as discussed in Sections 4.5.1.2 and 4.5.1.3. This will provide a means of maintaining the cells in vivo and maintaining the enhanced expression of the molecule of interest throughout the regenerative process. In particular, if combined with a triggered release system the implanted cells can be switched to phenotype which is more conducive for myelination (e.g. switch of an immature regenerating Schwann cell to a mature myelinating Schwann cell). This will provide a further means to enhance functional nerve regeneration in the construct.
5.5.2 The Identification of Novel Protein/Gene Targets

The analysis of changes in protein expression over a variety of different treatment groups yielded a number of molecules specific to the material used and how their expression became altered as a function of the distance treated. From this, molecules which are expressed throughout the autograft and not in the hollow biomaterial conduits can be identified. In particular, analysis of the gap distance, furthered this understanding and identified differentially regulated molecules in the hollow collagen conduit and intraluminal fibre filled conduit groups. However, analysis of these molecules was largely based on the underlying biology and related to the neurological injury and disease. Additional analysis by bioinformatics may yield additional protein targets which were otherwise unidentified. Less strict filtering of molecules and lowering the fold change threshold may yield further molecules which may have remained undetected. For example, the protein prosaposin which was initially detected in the proximal component of the autograft group was upregulated in comparison to the two conduit materials. However, filtering of data, removed this molecule from further analysis. Prosaposin is a protein that is deemed a neurotrophic factor and myelinotrophic factor, which has shown efficacy in attenuating diverse models of neurological pain (34-37). The protein and its peptides has seen limited use to date in the treatment of peripheral nerve injuries and is a target still worthy of consideration. Although filtered from the study, further analysis of the proximal proteins by ELISA may yield sufficient expression for its exploration. Similar molecules could be found by further exploration of the data.

5.5.3 The Potential of Transcriptional Regulators, Glycoprotein and Apolipoprotein Neural Therapeutics

Proteomic analysis of the injured nervous system identified a number of molecules that are intrinsic to repair. Specifically, it highlighted a number of proteins which were at the core of the regenerative network and important for the regenerative process. Molecules which were highly under or overexpressed in the autograft may be key to enhancing regeneration with a specific material. In particular, regulators of these molecules may hold key interest. One such molecule of interest is c-JUN, which is closely related to a
number of proteins seen at the midpoint of the autograft. Previous work by Farraj et al. have shown that c-Jun is one of the key molecules required to reprogram Schwann cells to a more regenerative phenotype and that overexpression of this molecule may hold beneficial properties for repair (38). Exploration of similar regulators of nerve regeneration may hold promise for future repair strategies. Identification of potential novel regulators can be achieved with the use of upstream analysis of the protein data from phase III and IV.

Glycoproteins and their mimetics have shown potential in both the peripheral and central nervous system. Polysialic acid mimetics (PSA), as well as a human natural killer epitope (HNK-1) have shown beneficial effects in peripheral nerve repair. In vitro HNK-1 was shown to be beneficial to motor neuron outgrowth and PSA was shown to be beneficial to both motor and sensory outgrowth. In vivo PSA filled collagen conduits showed an increase in the number of regenerated axons versus the hollow conduit. HNK-1 showed no significant differences. Beneficial effects of PSA require the interaction with neural cell adhesion molecule (NCAM) for their beneficial effects to take place. This complex, however, cannot directly interact with its target cell and needs to be broken down. Myrotinsylated adenine rich c-kinase substrate (MARCKS) has recently been shown to be required for this breakdown. Proteomic analysis revealed that this was highly upregulated in the autograft group and downregulated in the collagen conduit group. This suggest that the application of PSA to site of injury can be further enhanced by upregulating expression of MARCKS in the target cells. Possibly showing additional benefits for repair.

Decorin (DCN) another glycoprotein has shown similar potential in the central nervous system. In vitro application of DCN overcame inhibition of CSPGs from reactive astrocytes and inhibitory myelin components to promote robust neurite outgrowth in the model. Particularly DCN suppresses expression of neurocan, brevican, phosphocan and NG2 (39). DCN similarly has been used in vivo to treat spinal cord injury and shown benefits for repair, allowing growth of axons across an acute spinal injury by inhibiting components of the glial scar (40, 41). DCN was revealed in the proteomics
study to be downregulated across all groups. Despite clearance from infiltrating macrophages and phagocytic Schwann cells, a number of inhibitory components may remain that may hinder regeneration. Therefore the application of DCN as a supplementary molecule for repair may also hold potential for future therapies.

Aside from the glycoproteins, the apolipoproteins play a vital role in the regenerative process (42) and were expressed throughout the treatment groups. Apolipoprotein D and E in particular were shown to be upregulated in the autograft and as such may hold key importance for repair. APOD has shown to be essential for efficient and timely myelin clearance, as well as ensuring rapid remyelination of injured axons. APOD knockouts showed that axon and remyelination were delayed without the addition of APOD and enhanced with the addition of excess APOD (43). APOD which is only upregulated in the autograft group and not in the material groups in the comparative study in phase III may be beneficial as an additive. Ensuring that rapid clearance of inhibitory myelin components and later rapid remyelination occur in the treatment groups. APOE and its peptides similarly may show beneficial effects for repair. Qiu et al. showed that APOE receptors mediates neurite outgrowth through the MAPK pathway in primary neurons (44). In a nerve crush injury model, Li et al., that application of APOE mimetics to the injured nerve significantly increased motor and sensory functional recovery and increased myelin uptake by Schwann cells during the early stages of repair (45). Application of APOE and its mimetics have shown the ability to modulate inflammation and promote neural stem cell survival, in addition their role in cholesterol and lipid regulation, in numerous studies of the peripheral and central nervous systems (46-49). To date, APOE and its mimetics have not been studied in the repair of the peripheral nervous system.

5.5.4 Targeting the Neuromuscular Junction or Dorsal Root Entry Zone

The peripheral nervous system can be considered as the central connection between the musculoskeletal and sensory organs and the central nervous system. As such the transition zones for each of these systems are of key importance for treatment of extensive and multi-modal injuries. These transition zones are known at the neuromuscular junction and dorsal (sensory)
Summary and Future Studies

/ventral (motor) root entry/exit zones respectively. A multimodal scaffold that has an entry or exit point that is geared towards these junctions will be a potential future project. Taking advantage of the gradients or triggered release above, these could be used to potentially enhance end functional recovery of both peripheral and spinal cord injuries.

The proximal component of these repair strategies as per Figure 5.2 focuses on strategies that encompass injuries to the area of the dorsal and ventral roots. Injuries to the central nervous system are often multifactorial and inhibition at the dorsal/ventral roots can result in limited regeneration towards it peripheral targets. Inhibition at the dorsal root entry zone is a particular problem, resulted in chronic pain and sensory loss. Even in dorsal crush injuries, peripheral nerves will regenerate down the tracts but will fail at the dorsal root entry zone of the spinal column. This may be due to the change in the somewhat proregenerative environment of the periphery (primary cells being Schwann cells) and the transition to the more central dorsal root (astrocytes and oligodendrocytes) (50). Similarly, patients who not only have spinal cord injury but damage to these roots, may experience little regeneration down these tracts even if the cord itself is repaired. A multi-modal scaffold, similar to those used for bone-tendon junction could provide a valuable solution (51-53). This could consist of growth promoting and anti-inhibitory component, potentially having a proximal side suited to that of the central nervous system, a central component for removal of inhibition at the neuromuscular junction and distal component for repair of the injured peripheral components. These could be used in addition to spinal and peripheral strategies mentioned herein.

On the distal side of peripheral nerve injuries (as per Figure 5.2), once regeneration is achieved, correct re-innervation and synapse formation with the regenerated nerve to the underlying muscle is of key importance. Studies focused on biomaterial strategies for peripheral nerve repair are now beginning to look at the newly formed junctions (i.e. the neuromuscular junction) as important for functional repair (54-56). A repair strategy for injuries in close proximity to these junctions could be implemented that will allow the identification of key factors necessary for their repair. In particular,
injuries where there is limited distal nerve to suture into will require such a strategy. Molecular studies (proteomic or genomic) which are focused on the formation of the neuromuscular junction with respect in combination with a biomaterial nerve guidance conduit, will allow identification of key components needed for more successful synapse formation. Similarly, the use of a multimodal conduit, this time with a distal muscular component could provide an ideal solution. Successful strategies for repair in muscular regeneration studies could be combined with those of the peripheral nervous system to provide an ideal transitionary zone for this area for repair. This will essentially combine two environments where axons entering the transitionary zone will be exposed to mixed environment that contains components from both regions, and enter one that is primarily musculoinductive and potentially a promotor of synapse formation.

5.6 References
Summary and Future Studies

Summary and Future Studies


Summary and Future Studies


APPENDICES
## A. Materials and Reagents

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>8plex iTRAQ® protein tagging kit</td>
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<tr>
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<td>Ethicon™, Somerville, NJ</td>
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<tr>
<td>6-0 Vicryl Rapide™ sutures</td>
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<td>10-0 Ethicon™ sutures</td>
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<td>16- G needle</td>
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<td>Material</td>
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</tr>
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<td>18-G needle</td>
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<tr>
<td>23-G needle</td>
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</tr>
<tr>
<td>Amphotecerin B</td>
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<tr>
<td>Anti-S100 antibody, rabbit</td>
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<tr>
<td>Anti-thy1.1 antibody</td>
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<td>Ascorbic acid</td>
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<td>Citric acid</td>
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<td>Dodeca-molybdosphoric acid</td>
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<td>Eosin</td>
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<tr>
<td>Fast blue</td>
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<tr>
<td>Fast green</td>
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<td>Formaldehyde</td>
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<td>Forskolin</td>
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<td>Fuchsin</td>
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<td>Hematocycin</td>
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<td>Horse serum</td>
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<td>2-(N-morpholino)ethanesulfonic acid (MES)</td>
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<td>N-Hydroxysuccinimide (NHS)</td>
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</tr>
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<td>Phosphate buffered saline</td>
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<td>Pituitary extract</td>
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</tr>
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<td>Polyethylene glycol diglycdyl ether</td>
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</tr>
<tr>
<td>Poly-L-lysine (PLL)</td>
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<tr>
<td>Potassium permanganate</td>
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</tr>
<tr>
<td>Potassium phosphate monobasic</td>
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</tr>
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<td>Propylene oxide</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td></td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
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</tr>
<tr>
<td>Sodium metabisulphite</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate dibasic</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate monobasic</td>
<td></td>
</tr>
<tr>
<td>Sulphuric acid</td>
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<td>Tin II chloride</td>
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<tr>
<td>Toluidine blue</td>
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</tr>
<tr>
<td>Tris hydrochloride</td>
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<td>Triton™ X-100</td>
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<td>Trypsin-EDTA</td>
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<tr>
<td>Tween 20</td>
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<td>Uranyl acetate</td>
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<td>Xylene</td>
<td></td>
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<td>Xylazine hydrochloride</td>
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<tr>
<td>Histoclear™</td>
<td>Thermoscientific, Fitchburg, WI</td>
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<tr>
<td>Pierce® BCA Assay</td>
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</tr>
</tbody>
</table>

**B. Extraction of Atellocollagen Solution from Bovine Achilles’ Tendon**

1. Mince the tendon and wash in 0.1 M phosphate buffered saline solution.
2. Weigh the mass of the minced tissue.
3. Resuspend the minced tendon solution in 0.5 M acetic acid solution and stir at 4 °C for two to three days.
4. Add pepsin solution at a ratio of 1:100 (w/w) to the wet weight of the suspended tissue, and stir overnight at 4 ºC to allow digestion of the N and C terminals of the collagen molecules.

5. Add 0.9 M NaCl solution to the solution and leave stirring overnight at 4 ºC to precipitate the collagen out of the solution.

6. Centrifuge the solution at 8,000 rpm for 20 minutes at 4 ºC.

7. Decant the supernatant and collect the remaining solid mass.

8. Record the wet weight of the solid collagen.

9. Resuspend in 0.5 M acetic acid and stir overnight at 4º C.

10. Precipitate the collagen a second time from solution by adding 0.9 M NaCl to the solution and leave to stir overnight at 4 ºC.

11. Collect the precipitated collagen flocculent by centrifuging at 8,000 rpm for 20 minutes at 4°C.

12. Reweigh the collected collagen precipitate and suspend in a lower volume of 0.5 M acetic acid to concentrate the solution.

13. Dialyse the atellocollagen solution against 0.1 mM acetic acid for a period of five to six days.

14. Confirm concentration and purity by Sircol assay and SDS page respectively.

C. Extrusion and Cross-linking of Collagen Fibres

1. Prepare fibre formation buffer (FFB) and fibre incubation buffer as below:
   a. FFB: 20% PEG, 94 mM sodium phosphate dibasic, 24 mM sodium phosphate monobasic, pH 7.8
   b. FIB: 5.5 mM sodium phosphate dibasic, 1.5 mM potassium phosphate monobasic, 75 mM sodium chloride, pH 7.1

2. Heat fibre formation buffer (FFB) and fibre incubation buffer (FIB) to 37 ºC in incubator.

3. While water bath is heating, fill 5cc syringe with required amount of collagen (3 ml of collagen = approximately ten 20 cm fibres).

4. Place the filled syringe onto the syringe pump and secure in place with the attached clamp.
5. Place an ice pack in close proximity to the filled syringe to prevent denaturation of collagen solution.

6. Turn on the pump and input the following parameters (using chart command):
   a. Syringe make and size (as used)
   b. Volume = 0 (continuous)
   c. Rate = 0.3 ml/min

7. Connect the silicone tubing to the FFB container, via the silicone tubing, to the free end of the syringe.

8. Confirm that tubing is secured firmly to the syringe tip.

9. Place the other free end of the tubing just underneath the surface of the FFB in its container and secure in place with an appropriate weight.

10. Begin extrusion of collagen solution by pressing the ‘start’ button on the front of the syringe pump.

11. Allow air bubbles to clear from the submerged tubing and wait for the collagen to reach the end of the silicone tubing.

12. Upon emergence of the collagen solution into the fibre formation buffer apply a steady air flow to the collagen solution to guide extrusion of collagen fibres.

13. Stop the pump once the end of the container has been reached or a collagen fibre of desired length is formed.

14. Leave for five minutes in fibre formation buffer to ensure collagen has attained sufficient mechanical strength to be transferred to the fibre incubation buffer.

15. Transfer the collagen fibre by securing the fibre to the free end of stainless steel rod to the fibre incubation buffer and leave for an additional five minutes in fibre incubation buffer (this step further stabilises the extruded fibre and removes any residual PEG the surface of the fibre.

16. After incubation, transfer to either to distilled water, cross-linker (as per Table 0-1) or PBS, as desired, and leave for 24 hours (two five minutes rinses in the case of distilled water).
17. After overnight incubation in either the cross-linker or PBS, rinse three times in distilled water and dry the fibres under the tension of their own weight.

**Table 0-1: Range of Cross-linkers Used**

<table>
<thead>
<tr>
<th>Cross-linker</th>
<th>Concentration Used</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC : NHS</td>
<td>30 mM :10 mM (and dilutions)</td>
<td>50 mM MES buffer</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.625 %</td>
<td>PBS / 70 % ethanol</td>
</tr>
<tr>
<td>Ethylene glycol diglycdl ether</td>
<td>4%</td>
<td>PBS / 70 % ethanol</td>
</tr>
<tr>
<td>Polyethylene glycol diglycdyl ether</td>
<td>4 %</td>
<td>PBS / 70 % ethanol</td>
</tr>
<tr>
<td>Diisocyanathohexane</td>
<td>5 %</td>
<td>PBS / 70 % ethanol</td>
</tr>
</tbody>
</table>

**D. Ninhydrin Assay - Confirmation of Collagen Crosslinking**

1. Weigh samples.
2. Place in a 1.5 ml microtube.
3. Add 200 µl of distilled water to the samples.
4. To each tube, add one ml of the Ninhydrin solution:
   - Ninhydrin solution is made in two parts (not mixed until used):
     a) 4% Ninhydrin solution in 2-ethoxy ethanol (light sensitive store in aluminum foil).
     b) 200 mM citric acid solution in 0.16% w/v tin II chloride pH 5.0.
4. Place tubes in heating block at 95 – 100 °C for 30 to 35 minutes and cover in foil to prevent exposure to light.
5. Allow tubes to cool to room temperature.
6. In new 1.5 ml microtubes add one ml 50% isopropanol.
7. Add 250 µl of sample solution to each tube and mix well.
8. Transfer 200 µl of solution to a 96 well plate.
9. Read on nanoplate reader at 570 nm.
10. Normalize sample values i.e. divide by the original mass.
11. Compare results to glycine standard curve.

**E. Sircol Assay – Measuring Collagen Quantity and Concentration**

1. Prepare 100 µl of reagent blanks and collagen standards in 1.5 ml microtubes according to the manufacturer’s protocol (Biocolor, Carrickfergus, United Kingdom).
2. Prepare 100 µl of desired test substrate in another set of 1.5 ml tubes.
3. Add one ml of Sircol Dye Reagent to each tube.
4. Mix tube by inversion and shake for 30 minutes on a mechanical shaker (this will allow precipitation of an insoluble collagen-dye complex from the soluble unbound dye).
5. Centrifuge the tubes at 12,000 rpm for ten minutes, and then carefully invert and drain.
6. Add 750 µl of acid-salt wash reagent to the collagen-dye pellet for removal of unbound dye in the tube.
7. Centrifuge the tubes at 12,000 rpm for ten minutes and decant solution from the tubes.
8. Remove any residual liquid around the lip of the tube using a cotton bud.
9. Add 250 µl of the alkali reagent to the reagent blanks, standards and samples and mix for five minutes.
10. Transfer 200 µl of each sample to individual wells of a 96 micro well plate.
11. Read absorbance of the plate at 555 nm and calculate the resulting concentration.

**F. Scanning Electron Microscope – Focused Ion Beam (SEM – FIB) Analysis of the Structured Collagen Fibers**

1. Mount samples on SEM pedestal stubs using a sticky carbon tape.
2. Sputter-coat samples with 15 nm thick Palladium over-layer.
3. Mount sample in FIB sampling chamber and take preliminary SEM images.
4. Tilt the sample from 0º to 54º to determine a suitable region for FIB cross-sectioning.
5. Once identified, cross-section the sections using high beam currents and polish the cross-section at low beam currents.
6. Capture images of the resulting cross-section at the desired magnifications.

G. Fabrication of Collagen Conduit
1. Place one stainless steel bar (1.5 mm diameter) on a suitable stand.
2. Wrap the collagen solution (12 mg/ml) around the surface of the bars and dry under vacuum.
3. Insert the dried conduit into the crosslinking solution (30 mM: 10 mM EDC: NHS in 50 mM MES Buffer, pH 5.5) and allow the conduit to crosslink for eight hours at ambient temperature (15 – 25 °C).
4. Remove the conduit from the cross-linker, rinse clear all cross-linker residues.
5. Neutralise the conduit using a sodium carbonate buffer.
6. Freeze dry for 24 hours and remove the stainless steel bar.
7. Sterilise the conduit using 70% ethanol for a period of two hours.
8. Rinse the conduit with sterile saline solution to ensure removal of excess ethanol solution.

H. PLGA Conduit Production for Proteomics Study
1. Make up 10% (w/w) PLGA solution in 2, 2, 2-trifluoroethanol and leave rotating overnight.
2. Dip a glass rod into the PLGA solution briefly and slowly remove from the polymer solution (taking to avoid spillage of excess solution).
3. Immediately immerse coated conduit in 95 % isopropyl alcohol for approximately five minutes.
4. Allow the conduit to air-dry to remove access alcohol for the surface of the conduit and manually de-mould from the glass rod.
5. Lyophilize overnight if required.

I. Sciatic Nerve Surgery and Isolation of Nerve Tissue
1. Weigh the rat prior to surgery to ensure it is of sufficient weight and for monitoring weight loss post-surgery.
2. Anaesthetize the animal using preferred method:
   a. Intraperitoneal injection of ketamine (80 – 100 mg /Kg) and
      xylazine (10 mg / kg).
   b. Inhalation of two – five percent isoflorane.
3. Shave the animal leg of the animal and sterilize the surgical site with
   povidine iodine solution.
4. Maintain hydration of the rat’s eyes throughout the surgery by the
   application of eye drops.
5. Expose the sciatic nerve by first making a lateral incision on the left
   thigh above the sciatic notch using a scalpel.
6. Spread the underlying muscle using a blunt forceps to separate the
   muscle fibers and reduce damage to the tissue.
7. Identify the sciatic nerve and open the surgical site using a tissue
   spreader.
8. Clear the sciatic nerve of surrounding and underlying fascia.
9. Snip the afferent nerve found proximal to the spinal column.
10. Identify the trifurcation of the sciatic nerve.
11. For conduit groups remove a 5 mm section of the nerve to create a 10
    mm nerve gap, and a 12 mm section to create a 15 mm nerve gap.
12. For autograft remove a 10 or 15 mm section of the nerve and rotate
    180° prior to suturing.
13. Suture the conduit or graft to the proximal and distal nerve stumps
    using 10 – 0 Ethicon™ sutures.
14. Once secure, close the wound in layers (i.e. muscle followed by skin)
    using 6-0 Vicryl Rapide™ sutures.
15. Inject animal with buprenorphine hydrochloride (0.1-0.25 mg/kg)
    subcutaneously for management of post-surgical pain and saline for
    restoration of fluids lost during surgery.
Figure 0-19 Isolation of sciatic nerve using a gluteal muscle splitting approach.

Figure 0-20 Removal of desired nerve segment using microscissors

Figure 0-21 Suturing of the collagen nerve guidance conduit using 10 – 0 Ethicon™ sutures.
19. Monitor animals until fully recovered from surgery and continue daily monitoring of animals health and application of analgesia as required post-surgery.
20. At a defined endpoint, euthanize animal by CO₂ asphyxiation and cervical dislocation.
21. For nerve morphometry analysis, fix in situ using trumps fixative solution (4 % formaldehyde, 1 % glutaraldehyde in 0.1 M PBS).
22. For protein analysis or immunohistochemistry, transcardially perfuse the animal with ice cold saline solution, transect the nerve and flash freeze.

J. Preparation of Spurr Resin
1. Using balance and tripour beaker, weigh out the first three components of the resin (ERL4221, DER 736, NSA) as per Table 0-2.
2. Gently mix first three components together using bulb end of a plastic pipette for a couple minutes.
3. Place the mixture in the 60 °C oven for five minutes.
4. Remove from oven and finish thoroughly mixing for another five minutes.
5. Add catalyst and mix gently for several minutes.
6. Transfer to 50 ml centrifuge tubes.
7. Degas resin in vacuum desiccator for five minutes.
8. Seal up tubes and store at 4 °C until used, warm up prior to use.

Table 0-2 Ratio of Components for Spurr Resin

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<tr>
<th></th>
<th>~40 ml total</th>
<th>~80 ml total</th>
<th>~160 ml total</th>
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<td>ERL 4221</td>
<td>10 g</td>
<td>20 g</td>
<td>40 g</td>
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<tr>
<td>DER 736</td>
<td>8 g</td>
<td>16 g</td>
<td>32 g</td>
</tr>
<tr>
<td>NSA</td>
<td>25 g</td>
<td>50 g</td>
<td>100 g</td>
</tr>
<tr>
<td>DMAE</td>
<td>(0.35 ml)</td>
<td>(0.75 ml)</td>
<td>(1.4 ml)</td>
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K. Embedding Nerve Samples in Spurr Resin

Process samples as follows:

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<th>Temperature</th>
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<td>1</td>
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<td>2</td>
<td>PBS</td>
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</tr>
<tr>
<td>3</td>
<td>1% OsO₄</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>DH₂O</td>
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<tr>
<td>6</td>
<td>DH₂O</td>
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<tr>
<td>7</td>
<td>2% uranyl acetate</td>
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<td>14</td>
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<td>15</td>
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<td>16</td>
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<td>18</td>
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<td>19</td>
<td>Pure spurr 4 hours Room Temp Yes</td>
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<tr>
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<td>Embed samples in moulds</td>
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<tr>
<td>21</td>
<td>Place in oven 50 -55 C 3-4 days 50 – 55 ºC Yes</td>
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</table>

**L. Manual Stereological Analysis of Nerve Sections**

1. Observe toluidine blue / pheneylendiamine stained sections under an optical microscope.
2. Capture nerve sections at 4 / 10 x (as appropriate) magnification to capture the entire nerve section. (Figure L-1)
3. Using ImageJ analysis software, trace the outline of the nerve section (excluding the conduit if present) and measure the total area. If intraluminal fibres are present in the tissue, trace the fibres, measure the total area and subtract from the total area.
4. Under 100 x magnification, count the approximate number of fields needed to cover the entire nerve section.
5. If the total number of fields is twenty, and the number of fields to be sampled is five, count every fourth section to achieve systematic random sampling of the entire nerve section.
6. Using an unbiased counting frame (Figure L-2), count the number of axons within the frame. Exclude axons which touch the left and bottom borders of the counting frame (red) and include axons which touch the top and right lines of the counting frame (blue).
7. Using Image analysis software, measure axon diameter, myelin thickness and the myelinated fibre diameter.
8. Calculate the total number of axons by multiplying the average axonal density of the sample fields by the total axonal area.
Figure L-1 Toluidine blue stained nerve section shown traced, for evaluation of the axonal area. Total axonal area is represented in red and fibre area is represented in green. Scale bar, 30 um.
Figure L-2 Unbiased counting frame for evaluation of axon number, density and diameter of fibers. Exclusion lines are represented in red and inclusion lines are represented in blue. Scale bar, 5 µm.
M. Retrograde Tracing (Fast Blue & Diamidino Yellow)

1. One week prior to the terminal end-point, re-expose the sciatic nerve using a gluteal muscle spreading approach (outlined in sciatic nerve surgery protocol).

2. Identify the tibial and peroneal branches of the sciatic nerve.

3. Transect the tibial nerve distal to the sciatic nerve.

4. Place the transected nerve in 5% fast blue solution for a period of 30 minutes.

5. After 30 minutes, rinse the nerve with sterile saline solution and suture into the surrounding fascia to prevent dye leakage.

6. Transect the peroneal nerve distal to the sciatic nerve.

7. Place the transected peroneal branch in 5% diamidino yellow solution for 30 minutes.

8. Rinse the nerve with sterile solution and suture into the surrounding fascia as above.

9. Re-close the injury site in layers using 6-0 Vicyrl® Rapide sutures.

10. Seven days after application of the tracer solution, transcardially perfuse the animal with 4% paraformaldehyde/10% sucrose on 0.1 M PBS solution.

11. Once perfused, isolate the L1 to L6 vertebrae of the spinal cord from the animal and post-fix overnight.

12. Transfer the cord to 10% sucrose solution for a further 24 hours for cyroprotection.

13. Once the cord has sank to the bottom of the tube, embed the sample longitudinally in Cyro-gel™ solution.

14. Section 30 µm sections of the cord using a suitable cyrostat.

15. Immediately evaluate sections under a fluorescent microscope.

16. Count the number of fast blue labelled cytoplasms (tibial nerve axons) diamidino yellow nuclei (peroneal axons) and dual labelled profiles (polyinnervated axons) under the microscope.

N. Evaluation of Force Measurement

1. At the terminal end-point, anaesthetize the animal using 2 – 5% isoflorane.
Figure 0-22 Rat placed within the automated functional assessment station. In the picture the tibialis anterior muscle is attached to the strain gauge (black arrow) and the sciatic nerve has been attached to the stimulating electrodes (blue arrow).
1. Shave the back and both legs of the animal, and sterilize the surgery site using povidine iodine solution.

2. Expose the muscles of the leg by creating an incision which extends from the dorsum of the foot along the anterior portion of the lower leg to the knee.

3. Severe the distal tendons of the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles from the extensor reticula.

4. Attach the free ends of the tendons to stainless steel S-hooks and secure in place using 5-0 Ethicon™ sutures.

5. Expose the sciatic nerve as per sciatic nerve surgery protocol and extend the incision laterally to ensure sufficient exposure of the nerve.

6. Isolate the nerve from the surrounding fascia and transfer animal to the automated functional assessment station (Red Rock Laboratories, St. Louis, Missouri, USA).

7. Place the animal in an adjustable support and secure the knee using the provided clamps.

8. Ensure sufficient lubrication of the exposed muscles and nerve throughout the procedure by the addition of mineral oil (mineral oil will also prevent current leakage into the surrounding muscle beds).

9. Attach bipolar electrodes to the proximal portion of the sciatic nerve for stimulation.

10. Attach the EDL and TA muscles to the 5 N and 20 N load cells respectively.

11. For normalization of measurements between animals determine the optimal stimulus amplitude and optimal muscle length.

12. For determination of the optimal stimulus amplitude, apply single electrical pulses (pulse duration = 0.2 msec) of increasing amplitude (0 – 1000 µA) to the nerve and record the isometric twitch force in the EDL and TA muscles. The maximal twitch force corresponds to the optimal stimulus amplitude.

13. For calculating optimal muscle length, apply optimal stimulus amplitude and a single electrical pulse (pulse duration = 0.2 msec) to the nerve and adjust the length of the nerve until the maximal twitch force is generated.
14. For determination of the maximal tetanic muscle force, apply a train of electrical pulses (pulse duration = 0.2 msec, burst width = 300 msec) of varying frequency to the nerve at the optimal stimulus amplitude and length. Allow two minutes of rest between each stimulus to reduce muscle fatigue and data contamination.

15. Repeat measurements for the opposite muscle if required.

16. Once all data acquired, euthanize animal by CO₂ asphyxiation and cervical dislocation.

17. Isolated TA, EDL and gastrocnemius (GC) muscles and record the wet weight of both the injured and uninjured muscles to determine the relative degree of recovery from muscular atrophy.

O. Subcutaneous Implantation of Collagen Fibres

1. Bundle the desired number of fibres together longitudinally and suture at both ends to hold samples together.

2. Sterilise the samples in 70 % ethanol for two hours.

3. Wash thoroughly with sterile 0.1 M PBS solution to remove excess alcohol.

4. Randomly assign the experimental groups to four subcutaneous pockets on the back of the rat.

5. Anaesthetize the animal using 5 % isoflorane for induction and 2 % for maintenance of anaesthesia.

6. Shave the back of the animal, and sterilize the surgery site using povidine iodine solution.

7. Using a sterile no. 15 scalpel blade make a small incision in the back of the animal and spread the wounds using a tissue spreader or surgical forceps.

8. Suture the bundled fibers in place to prevent migration, subcutaneously, using non-degradable sutures.

9. Close the wound using 5-0 Vicryl® Rapide sutures.

10. Inject the animal with buprenorphine hydrochloride (0.15mg/kg) for the alleviation of pain.

11. At the terminal endpoint, euthanize the animal by CO₂ asphyxiation and harvest skin of the dorsal region of the rat.
12. Isolate the implanted fibers and surrounding fascia, using the embedded non-absorbable suture as a marker.

13. Fix in 4 % neutral buffered formalin and store for 24 hours for further processing.

14. For cyro-sectioning, transfer to 10 % sucrose for a further 24 hours or until the tissue sinks to the bottom of the solution.

**P Protein Isolation, Quantification, Isobaric Tagging**

**P.1 Isolation of Precipitation of Protein from the Sciatic Nerve**

1. Thaw flash frozen nerve sample and weigh mass of tissue.
2. Place tissue in pre-chilled microtube designed for the magnetic bead homogenizer with RIPA buffer (1 g of tissue to 12 ml reagent) and protease inhibitor cocktail (1 ml per 20 g of tissue).
3. Homogenize the tissue as per the manufacturer’s instructions.
4. Centrifuge the lysed sample for ten minutes at 12,000 x g to remove excess tissue debris.
5. Transfer the supernatant to a pre-chilled test tube and filled with six volumes of chilled acetone (pre-chilled to –20 ºC).
6. Invert tube three times.
7. Incubate tube at -20 ºC overnight (flocculent will form in solution).
8. Spin at 6,000 x g for ten minutes.
9. Decant acetone from the flocculent.
10. Use precipitated pellet for Pierce® BCA protein assay and isobaric tagging experiments.

**P.2 Quantification of Precipitated Protein**

1. Carry out protein quantification as per the manufacturer’s instructions (Pierce® BCA Protein Assay Kit) with small modifications for subsequent isobaric tagging.
2. Resuspend the precipitated protein pellet in dissolution buffer (10 mM triethylammonium bicarbonate (TEAB), pH 8.5).
3. Calculate the required working reagent amount (WR) using the following equation:
(# standards + # unknowns) x # replicates x (volume of WR per sample) = total volume of working reagent required.

4. Prepare WR by mixing reagent A with reagent B at 50:1 ratio.
5. Transfer 25 µl of standards and unknowns into a 96 well plate.
6. Add 200 µl of WR to each well and shake for 30 seconds.
7. Cover plate and incubate at 37 ºC for 30 minutes.
8. Allow plate to return to room temperature and read at 562 nm on a plate reader.
9. Subtract the absorbance value from the blank well from all standards and unknowns.
10. Use the generated BSA standard curve to calculate the quantity of precipitated protein.

P.3 Isobaric Tagging of Experimental Groups

1. Before all tagging experiments, draw out plan for tagging assignment to avoid confusion during the pooling phase of the experiment.
2. 8plex ITRAQ® is carried out as per the manufacturer’s instructions.
3. To all eight sample tubes add 20 µl of dissolution buffer and 1 µl of denaturant.
4. Vortex samples and add two µl of reducing agent.
5. Vortex samples again and incubate for one hour at 60 ºC.
6. Spin to bring samples to bottom of the tube and add one µl of cysteine blocking reagent.
7. Mix samples and incubate at room temperature for ten minutes.
8. Reconstitute four vials of trypsin and add two µl of trypsin per tube.
9. Mix and incubate tubes overnight at 37 ºC.
10. Centrifuge at 1,500 rpm for five minutes to allow protein to settle to the bottom of the tube.
11. Prepare 8plex ITRAQ® reagent:
   a. Allow ITRAQ® vial to reach room temperature.
   b. Centrifuge at 1,500 rpm for five minutes.
   c. Add 50 µl of isopropanol to each vial.
   d. Mix well and centrifuge at 1,500 rpm for five minutes.
12. To each sample tube add the contents of one ITRAQ® vial as laid out in the experimental plan (i.e. tag 113 for sample one, tag 114 for sample two etc.)
13. Mix well and adjust pH of combined tube to 8.5 using provided dissolution buffer.
14. Incubate tubes at room temperature for two hours.
15. Confirm successful tagging of sample tubes on the mass-spectrometry system before pooling.
16. Pool the contents of the eight tubes, mix thoroughly and run on the mass-spectrometry system.

Q Colorimetric Histology Staining

Q.1 Haematoxylin and Eosin Staining
1. De-wax sections in xylene for ten minutes and repeat.
2. Remove xylene and pass through two changes of absolute alcohol (two minutes each).
3. Bring to water through 95%, 70% and 50% alcohols (two minutes per bath).
4. Remove alcohol in running tap water for two minutes.
5. Stain in Mayer’s Haematoxylin for six minutes.
6. Blue nuclei in running tap water for four minutes.
7. Examine under microscope and differentiate in acid alcohol if necessary.
8. If acid alcohol is used, rinse in running tap water for four minutes.
9. Stain in Eosin for two minutes.
10. Rinse in tap water quickly.
11. Dehydrate through graded alcohols, two minutes each.
12. Clear in xylene, two changes of approximately 15 minutes.
13. Cover sections with D.P.X. mounting medium and apply coverslip.
14. Place slide in oven to allow mounting medium to solidify.

Q.2 Masson’s Trichrome with Gomori’s Aldehyde Fuchsine.
1. De-wax sections in Histoclear™ twice for ten minutes each.
2. Remove excess Histoclear™ from the slide.
3. Rinse in two changes of absolute alcohol for one minute each.
4. Hydrate the sections by placing in baths of 95%, 70% and 50% alcohols; one minute per bath.
5. Remove residual alcohol from the slides in running tap water for two minutes.
6. Oxidise in 0.5% KMnO₄ / 0.5% H₂SO₄ (equal parts) for two minutes.
7. Rinse in tap water and bleach in 2% sodium metabisulphite (Na₂S₂O₅) for two minutes.
8. Wash in water for 30 seconds, followed by 70% alcohol for one minute.
9. Stain in Gomori’s Aldehyde Fuchsin for one minute.
10. Rinse very quickly in water, then in 95% alcohol for ten seconds and then in water again for ten seconds.
11. Stain in Celestine blue for four minutes.
12. Rinse in water for 30 seconds.
13. Stain in Mayer’s Haemalum for four minutes.
14. Quick rinse in water for 20 seconds.
15. Differentiate in acid alcohol for 20 seconds.
16. Blue nuclei in running tap water for four minutes.
17. Stain in Masson’s cytoplasmic stain for one minute.
18. Rinse very quickly in water and transfer to 1% dodeca-molybdophosphoric acid, for two minutes.
19. Rinse in water and counterstain in Fastgreen™ for one minute.
20. Differentiate in 1% acetic acid for one minute.
21. Dehydrate through 50%, 70%, 95% alcohols and absolute alcohol for one minute per bath.
22. Clear in xylene for ten minutes (twice).
23. Mount in DPX (in the fume hood).

Q.3 Toluidine Blue Staining

1. Cut semi-thin sections (one µm) on ultramicrotome.
2. Mount sections on a slide and dry rapidly on a 90º C hotplate.
3. Remove sections from the hotplate and cover with 1 % toluidine blue solution.
4. Return the slide to the hotplate for approximately ten seconds.
5. Remove from hotplate and rinse under cool running water until all excess dye is removed.
6. Mount and view.

**Q.4 Phenylendiamine Staining**

1. Prepare a 1% solution in distilled water (discard after two weeks. Store in the refrigerator in the darkbottle). Filter solution before use.
2. Place a few drops of freshly filtered stain (use #42 Whatman™ filter) on a 0.75-1.0 micron section for one hour at room temperature. If the stain is made up the same day as the slides are stained, wait one and a half hours.
3. Rinse in the running tap water for ten minutes.
4. Decolorize with 95% alcohol for 10-15 minutes (this step removes crystals that form on sections by phenylendiamine. Check the sections under the microscope; if the crystals remain, leave in the 95% alcohol longer).
5. Rinse in running tap water for ten minutes.
6. Air dry.
7. Coverslip with preserve-a-slide.

**R. Whole Explant Dorsal Root Ganglion (DRG) Primary Culture**

**R.1 Coating Tissue Culture Dishes with Collagen**

1. Flame a couple of pipettes for delivering the collagen and bend two or three more at a 45° angle to spread the collagen.
2. Add one drop of collagen to each dish and spread evenly.
3. Leave the dishes half open under the hood to dry for about one hour.
4. Re-hydrate collagen with MEM for a minimum of four hours. Re-hydration overnight usually works best.
5. Remove the MEM and add exactly eight to ten drops of the culture medium (usually AN2 media with 10% calf bovine serum). If too much media is added, the DRGs will not adhere to the bottom of the dish and will float. If too little media is added, the cultures may dry out.
6. Put dishes into the incubator until ready to plate the DRG.

**R.2 Cell Culture Set Up. (On Morning of Surgery)**

1. Place in the hood the following:
   - **Instruments for micro dissection:**
     i. one x no.4 forceps
     ii. one x scissors
     iii. two x no.5 sharp forceps
     iv. one x microscissors
     v. instrument holder
   - L-15 media
   - Dissecting microscope
   - Beaker with 80% alcohol (gauze in the bottom to keep fine instruments from bending)
   - 1-100 mm glass tissue culture dish with L-15
   - 60 mm plastic tissue culture dishes

**R.3 Gross Dissection**

1. Anesthetize the rat with 100 mg/kg pentobarbital
2. Inject 25 mg pentobarbital into the heart or dissect the diaphragm to euthanize the animal.
3. Soak the belly with alcohol and wipe with sterile gauze.
4. With the scalpel, make and incision from the sternum all the way down towards the urethra. Dissect the top layer of skin. Do not cut into the abdomen.
5. With the homeostatic forceps, pull the skin away on either side to expose the abdomen.
6. Using the forceps and scissors, cut through the abdomen, again from the sternum to the urethra.
7. Grab the base of the uterus with the forceps, cut it and remove the uterus with pups. Be careful not to cut the bowel. This can be a source of bacterial contamination.
8. Place into the sterile, clean glass petri dish.
9. Take the pups to the hood right away to remove them and put them into L-15.
10. To remove pups from the uterus, start by making an incision from the top of one of the horns toward the base of the uterus.

11. The pups will be encased in a sac with the placenta off to its side. With the larger forceps grab between the pup and the placenta and cut away the placenta.

12. Place the pups into the 100 mm dish containing L-15.

13. Place the pups individually into clean 60 mm plastic dishes containing L-15, removing the sac around the pups.

**R.4 Micro Dissection**

1. Flip the pup on its side and remove the head.
2. Turn the pup (facing the side) and hold it still with the micro forceps by spearing the abdomen where the liver is present.
3. Using the micro scissors in the other hand, make an incision from between the tail and hipbone straight up the side of the animal and above the front shoulder.
4. Turn the pup over and do the same on the other side.
5. At this time, it is possible to separate the spinal cord area and tail from the rest of the body.
6. Lay the cord on its dorsal side, so that the ventral side is facing up.
7. Remove any residual organs or large blood vessels.
8. Turn the cord so that it runs horizontal.
9. To remove the vertebral bodies, hold the ribs on either side of the cord with the micro forceps.
10. Make an incision along both sides of the spinal cord and remove the vertebral bodies. (Do not cut downward it is possible to cut the DRG off the cord. Dissect with a slight upward angle.)
11. The spinal cord should be exposed. One should be able to grab the cord at the top and pull it out.
12. Place all of the cords into another clean 60 mm dish containing L-15.

**R.5 Plating the DRG**

1. Retrieve the coated dishes from the incubator.
2. Hold the spinal cord with one micro forceps and remove the DRG with another micro forceps in the other hand. Grab the connective
tissue between the spinal cord and the DRG and pluck the DRG from the cord.

3. Transfer the DRGs to the coated plate using the forceps.
4. Plate 4 DRG per dish and 16 DRG per condition.
5. Return the dishes to the incubator for one hour.
6. Check regularly to ensure the dishes do not dry out. Add 20 more drops of media to cover the DRG.

**R.6 Disassociating DRG**

1. Hold the spinal cord with one micro forceps and remove the DRG with another in the other hand. Grab the connective tissue between the spinal cord and the DRG and pluck the DRG from the cord.
2. Place all of the DRG into another 60 mm dish containing L-15.
3. After all DRG are removed from the cords, pipette them into a plastic centrifuge tube. When using a glass pipette, wash the pipette a couple of times with AN2 medium. The serum in the media will prevent the DRG from sticking to the glass pipette.
4. Spin the tube 800 rpm for ten minutes and remove the supernatant.
5. Add one ml of 0.25 % trypsin in Hank’s balanced salt solution.
6. Incubate for 30 minutes at 37 ºC.
7. Spin the tube at 800 rpm for five minutes and remove supernatant.
8. Add one ml of AN2 media and pipette up and down. The pipette needs to have a small hole to be certain the cells are dissociated. Flame the pipette to reduce the aperture (ideal aperture size should provide some resistance when pipetting medium up and down.)
9. Into the dry dishes, pipette two drops of re-suspended cells and let sit in the incubator for one to two hours.
10. Add ten drops of media to cover the cells so that they won't dry out.
11. Feed cells every two days with fresh AN2 media containing 15% serum and NGF.
S. Neonatal Schwann Cell Isolation (Complement-Mediated Lysis Method)

S.1 Preparation (Day One) – Before Obtaining Nerves

1. Set up dissection board of Styrofoam™ wrapped in aluminum foil and wash with 70% ethanol.
2. Prior to dissection, soak all instruments in 70% ethanol.
3. Coat tissue culture dishes with poly-L-lysine (PLL) as follows:
   a. dilute PLL solution 1:100 with sterile dH₂O for tissue culture dishes
   b. coat dishes (10 mL/dish) 20 min at room temp
   c. wash 2x sterile dH₂O
   d. air dry in tissue culture hood
4. Place one sterile 15-mL centrifuge tube containing 10 mL L-15 medium on ice and another with 10 mL L-15 medium in a 37°C water bath.

S.2 Dissection and Isolation of Schwann Cells (Day One)

1. Pin animal dorsal side up onto dissection board with rear legs pinned in the shape of an inverted “V” (23-G needles) and with the torso pointed away (18-G needle).
2. Spray rat with 70% ethanol.
3. Using a small scissors and dissecting forceps, make a continuous incision around each hind limb and the lower back and remove all skin covering those areas.
4. Using a pair of dissecting forceps to gently tease apart the hamstring muscles to either side, expose the sciatic nerve along its entire course in the hind limb from the pelvis to the knee.
5. Without stretching the nerve, lift it slightly with one forceps and, using micro-dissecting scissors, cut the nerve at each end (approx. one cm long).
6. Place nerve in ice-cold L-15 in 15-mL tube.
7. Harvest the sciatic nerve from the animal’s other hind limb.
8. Repeat sciatic nerve dissection for other animals as quickly as possible.
9. Once all the nerves have been harvested, centrifuge three minutes at 700 rpm.
10. Gently decant supernatant and add eight mL 37°C L-15.
11. Add one mL each of pre-warmed 1% collagenase and 2.5% trypsin, and mix by flicking or inverting the tube several times.
12. Incubate 30 minutes in a 37°C water bath, flicking/inverting the tube every ten minutes.
13. Centrifuge for ten minutes at 2000 rpm and remove supernatant by pipet (do NOT aspirate).
14. Add 10 mL D-medium, re-suspend pellet by flicking/inverting tube, and repeat centrifugation.
15. Wash pellet once more as above.
16. Pre-rinse a two mL pipet in D-medium, then re-suspend pellet in two mL medium by pipetting up and down ten times.
17. Flame-polish a long Pasteur pipet to a 0.5 mm aperture and pipet cell suspension up and down 20 times.
18. When cells are evenly dispersed, remove 12 µL to count using a hemacytometer (do not count red blood cells, which are small, round, yellow phase-bright cells with dark centers).
19. Plate cells at 10^6 cells per 10 cm tissue culture dish in ten mL D-medium.

S.3 Elimination of Proliferating Cells (Day Two)
1. Wash cells gently twice with HBSS.
2. Replace medium with ten mL D-medium containing ten µM Ara-C (1:100 stock solution).

S.4 Schwann Cell Recovery Phase (Day Four)
1. Wash cells gently twice with HBSS.
2. Replace medium with ten mL D-medium.

S.5 Complement Killing of Fibroblasts (Day Six)
1. Wash cells once with HBSS.
2. Wash cells once with D-medium containing 20 mM HEPES.
3. Add two mL of D-medium containing 20 mM HEPES and 40 µL (1:40 dilution in media) anti-Thy1.1 antibody to cells.
4. Incubate for 15 minutes at 37 °C.
5. Directly add 400 µL (1:4 dilution in media) rabbit complement and gently swirl dish to mix evenly.
6. Incubate 40 minutes at 37 °C, checking cells every ten minutes to assess health of Schwann cell and death of fibroblasts.
7. Remove medium and wash cells gently twice with HBSS.
8. Add ten mL D-medium containing two µM forskolin and 20 µg/mL pituitary extract, and return to 37°C incubator.

S.6 Schwann Cell Proliferation (Day Eight)
1. Replace medium with fresh D-medium containing 2 µM forskolin and 20 µg/mL pituitary extracts every two days.
2. Repeat complement-mediated cell killing of residual fibroblasts as necessary.

T. Adult Schwann Cell Isolation (D-Valine Method)

T.1 Preparation (Day One) – Before Obtaining Nerves
1. Set up dissection board of Styrofoam™ wrapped in aluminum foil and wash with 70% ethanol.
2. Prior to dissection, soak all instruments in 70% ethanol.
3. Use: T75 Tissue Culture Superior flasks, 250 ml, Sarstedt™ (T75 with yellow cap) or alternative coating as follows.
4. Coat tissue culture dishes with poly-L-lysine (PLL) as follows:
   e. Add one ml for 35-mm petri dish (0.3 ml for 24 well-plate) PLL solution.
   f. Incubate ten minutes at room temperature.
   g. Discard used solution and dry for at least two hours.
5. Coat plates with laminin on the day of the isolation as follows:
   a. Add one ml for 35-mm petri dish (0.3 ml for 24 wellplate) PLL solution.
   b. Incubate for 30 minutes at 37°C.
   c. Discard used solution.
   d. Wash twice with PBS, keep final PBS on the plate.
6. Place one sterile 15-mL centrifuge tube containing 10 mL L-15 medium on ice and another with 10 mL L-15 medium in a 37 °C water bath.
7. Make stocks as follows:
   a. Laminin solution:
      a. Make aliquots and store at -20°C for up to one month.
   b. Forskolin solution:
      a. Dissolve ten mg forskolin in one ml DMSO store at -20 °C up to one month.
   c. N2 supplement and bovine pituitary extract:
      a. Make aliquots: one ml of N2 supplement and 150 µl bovine for 100 m store at -20°C for up to 1 month.
   d. Collagenase solution:
      a. Dissolve collagenase in serum-free DMEM to make a 0.05% (wt/vol) collagenase solution.
      b. Add: ten ml of medium to 5 mg of collagenase powder and filter-sterilize using a 0.2 µm syringe filter.

8. Prepare Schwann cell culture medium, from stocks, as follows:
   a. To 500 ml of DMEM-D-valine add 10 ml glutamine (20 mM), 50 ml fetal cave serum, 5 ml N2 Supplement, 750 µl bovine pituitary extract, 100 µl forskolin solution, 5 ml P/S and 125 µg amphotericin B.
   b. Store the medium at 4 °C for maximum of one month.

T.2 Dissection and Isolation of Schwann Cells (Day One)
1. Pin animal dorsal side up onto dissection board with rear legs pinned in the shape of an inverted “V” (23-G needles) and with the torso pointed away (18-G needle).
2. Spray rat with 70% ethanol.
3. Using a small scissors and dissecting forceps, make a continuous incision around each hind limb and the lower back and remove all skin covering those areas.
4. Using a pair of dissecting forceps to gently tease apart the hamstring muscles to either side, expose the sciatic nerve along its entire course in the hind limb from the pelvis to the knee.
5. Without stretching the nerve, lift it slightly with one forceps and, using micro-dissecting scissors, cut the nerve at each end (approx. one cm long).

6. Place nerve in ice-cold L-15 in 15-mL tube, centrifuge it for five minutes at 1100-1200 rpm.

7. Harvest the sciatic nerve from the animal’s other hind limb.

8. Repeat sciatic nerve dissection for other animals as quickly as possible.

9. Once all the nerves have been harvested, strip of epineurium using fine pointed forceps to pinch the outermost layer to remove the connective tissue from proximal to distal end.

10. Tease the nerves apart and cut the tissue in two to three mm fragments.

11. Incubate the nerve fragments in ten ml of 0.05% collagenase solution per pair of nerves for 60 minutes in the incubator with agitation.

12. Filter through a 40-µm cell strain.

13. Centrifuge at 400g for six minutes and remove supernatant.

14. Wash the cell pellet with two ml Schwann cell culture medium.

15. Incubate in a 24-well plate add per well: 0.1 ml cell suspension + 0.5 ml Schwann cell culture medium for 7 d. (0.9 ml cells + 1.5ml medium for 35 mm dishes/ 9 ml cells + 15ml media in T75) Do not disturb cells, observe cell growth only after day five under the microscope.

T.3 Schwann Cell Culture (Day Seven)

1. Replenish Schwann cell culture medium.

2. Incubate for three to four days.

T.4 Schwann Cell Culture (Day Ten)

1. Remove 50% of the used medium and replace it with fresh Schwann cell culture medium. Continue to culture and change medium every two to three days until day 19-20 when cells are confluent.
U. Cell Culture

U.1 Thawing of Liquid Nitrogen Frozen Cells
1. Warm Dulbecco’s Modified Eagles Medium (DMEM), Fetal Bovine Serum and Penicillin & Streptomycin (P/S) in a 37 ⁰C water bath for 15 minutes.
2. Make up a stock solution of media
   a. Remove 50 ml from the 500ml DMEM using a 25 ml pipette and pipette boy.
   b. Add 50 ml FBS (10%).
   c. Add 5 ml P/S (1%).
3. Mix carefully, label with name, date and components used.
4. Retrieve cell line from liquid nitrogen storage tank.
5. Place the vial removed from nitrogen storage into a water bath for one to two minutes to quickly defrost.
6. Add the contents of the vial to 7.5 ml media in a 15 ml tube and centrifuge at 1,200 rpm for five minutes.
7. Remove the supernatant from the tube, leaving the pellet of cells at the bottom.
8. Resuspend in 7.5 ml Hanks Balance Salt Solution (37°C).
9. Centrifuge again at 1,200 rpm and re-suspend in 5 ml of media.

U.2 Counting of Cells
1. Pipette 20µl of the cell suspension into a well in a 96 plate and mix with 20 µl of trypan blue (1 in 2 dilution).
2. If necessary repeat the dilution to achieve a 1 in 4 dilution.
3. Assemble the haemocytometer on the table and transfer 10 µl of solution to each side of the haemocytometer.
4. Place the on the light microscope and adjust focus and orientation until the counting grid is in place.
5. Count cells on the haemocytometer using a counter and ignoring cells touching the bottom and lower right boundaries of the grid. Cells touching the top and upper-left boundaries are included. Cells stained dark blue are not counted and considered dead.
6. Repeat this counting procedure for the opposite side of the haemocytometer.

7. Cell number found on each side is multiplied by the dilution factor and multiplied by $10^4$ to give the total number of cells/ml of solution.

**U.3 Seeding Cells on a Culture Plate**

Cells are seeded at an ideal concentration per the total surface area. For example in a T 75 flask (measuring 75 cm$^2$, 20,000 cells/cm$^2$ is the ideal concentration of cells i.e. $20,000 \times 75 = 1,500,000$ per flask. Cells concentrations counted from above will need to be adjusted accordingly.

1. Add a sufficient volume from the cell containing solution to the flask such that the desired cell concentration is reached.
2. Label the flask appropriately with contents, name and date.
3. Place inside the incubator at 37° C and 5% CO$_2$ for time period desired.

**U.4 Cell Maintenance and Feeding of Cells**

1. Transfer the flask from the incubator to the fume hood.
2. Invert the flask such that the cell covered side is now face up in the container and carefully remove media.
3. Using a new pipette, add 10 ml fresh media into the flask.
4. Reclose the lid of the flask and invert the flask again with the cell containing side now face down.
5. Ensure adequate coverage of the underlying cell surface by media.
6. Confirm cell integrity under light microscope and return to incubator.

**V. Collagen Type I Hydrogel Formation and Seeding**

**V.1 Formation of a Type I Collagen Hydrogel**

1. Mix the constituents, on ice, in the following order for a five ml gel solution:
   a. PBS 10 X (500 µl).
   b. Collagen type I in 0.05 M acetic acid (5 ml).
   c. 2 M NaOH (115 µl).
2. Confirm a neutral pH using universal indicator solution (if neutral pH not achieved adjust the amount of NaOH accordingly.

3. Form the gel by placing the gel solution into a 37 °C incubator for 10 to 20 minutes.

V.2 Seeding Cells in a Collagen Hydrogel

1. Sterilise all components of the collagen gel using a sterile filter within the fume hood and keep on ice until time of use.

2. Mix gel components when ready and store under ice as per collagen hydrogel protocol.

3. Count cells as per the counting of cells section.

4. Resuspend cells to desired cell concentration.

5. Centrifuge to form cell pellet.

6. Remove media from pellet inside the hood.

7. Resuspend cells in collagen gel solution.

8. Place in incubator at 37 °C for approximately 30 minutes, along with large sample of the gel solution to confirm gelation if necessary.

9. After gelation, add media to cover samples and maintain in culture for the required period.

V.3 Seeding of Collagen Fibres Using a Collagen Hydrogel System

1. Prepare solution for collagen gel formation (for one ml of collagen solution) (carried out under ice):
   a. Add 115.5 ml of 10X PBS to 1.5 ml microtube.
   b. Add one ml of collagen solution and mix thoroughly and slowly for approximately five minutes.
   c. Add 19.8 ml of 2 M NaOH to mixed solution and mix thoroughly and slowly for approximately five minutes.
   d. Check pH, if pH 7.0 reached proceed, if not adjust levels of NaOH and PBS until pH is neutral.
   e. Keep on ice until ready to use.

2. Sterilise platform under UV or 70 % ethanol if UV not appropriate.

3. Rinse three times with sterile distilled water.

4. Rinse and incubate scaffold and platform with media for 30 minutes.
5. Mix gel solution and cell suspension 1:1 thoroughly for five minutes using a plastic bulb pipette (100 µl of gel + 100 µl of 80,000 cells/ml solution = 4000 cells per gel).

6. Place in incubator for 10-15 minutes to allow gel formation.

7. Leave cells in gel to equilibrate for one to two hours.

8. Top up with DMEM and incubate until desired time point.

9. Fix in 4 % paraformaldehyde and stain accordingly.

**W. Immunocytochemistry and Immunohistochemistry of *In Vitro* and *In Vivo* Samples**

**W.1 Immunohistochemical Staining for Cryosectioned Nerve Samples**

1. Rehydrate sections by immersion in PBS for ten minutes.

2. Incubate for 20 minutes in blocking solution (20% newborn goat serum, 0.2% Triton-X, in PBS).

3. Dilute primary antibody in a 10x dilution of blocking solution (2% newborn goat serum, 0.02% Triton-X, in PBS) and incubate for two hours at room temperature or overnight at 4 °C. Use primary concentrations as follows for tissue sections:
   a. NF-160: 1:100
   b. S100: 1:100
   c. Vimentin: 1:100
   d. βIII-tubulin: 1:100
   e. P0: 1:100

4. Wash sections three times in PBS.

5. Dilute secondary antibody in PBS and incubate for one hour at room temperature or overnight at 4°C in the dark.

6. Wash sections three times in PBS.

7. Counterstain using a DAPI stain for five minutes in the dark.
   a. Alternatively, for a Hoeschst stain, dilute Hoeschst to 1 µg/mL in PBS.

8. Wash sections three times in PBS.

9. Add a drop of mounting media onto each sample and place a coverslip over each.

10. Seal with clear nail polish.
W.2 DRG and PC12 Immunocytochemistry

1. Remove media from slides, add paraformaldehyde for 20 minutes at 4º C to fix cells.
2. Remove paraformaldehyde, rinse three times in PBS.
   a. At this stage, samples can be left overnight at 4 ºC.
3. Add fresh PBS, place on shaker for ten minutes at room temperature.
4. Remove PBS, add 2% Triton-X to permeabilise cells, place on shaker for five minutes at room temperature.
5. Rinse three times in PBS for five minutes each on the shaker at room temperature.
6. Block samples for one hour at room temperature in blocking solution: 20% new-born goat serum, 0.5% BSA, 0.4% Triton-X, and 0.2% Tween 20 in PBS OR 2% BSA and 0.4% Triton-X in PBS.
7. Rinse three times in PBS for five minutes each on the shaker.
8. Add primary antibody diluted in 10% blocking solution in PBS for two hours at room temperature on the shaker.
   a. For βIII-Tubulin, 1:300 is sufficient.
9. Rinse three times in PBS for five minutes each on the shaker.
10. Add secondary antibody diluted in PBS for one hour on the shaker at room temperature in the dark.
    a. For an Alexa Fluor™ 488, use a 1:350 (approx.) dilution.
11. Rinse three times in PBS for five minutes each on the shaker (RT, dark).
12. Add Phalloidin conjugated to TRITC at 1:200 in PBS for 20 minutes on the shaker in the dark.
13. Rinse three times in PBS for five minutes each on the shaker in the dark.
14. Add the Hoechst™ counterstain (or alternatively DAPI solution) at one μg/mL for three minutes on the shaker in the dark.
15. Rinse covered three times in PBS for five minutes each on the shaker.
16. Add either PBS (short term storage and imaging, less than 1 week) or fluorescent mounting media (long term storage and imaging, more than one week) and seal samples with clear nail polish.
X. List of Publications

X.1 Journal Article Publications


X.2 Manuscripts to be Submitted


X.3 Manuscripts in Preparation

1. Angius D., Daly W., Gutierrez-Cotto Y., Charlesworth C., Madden B., Yaszemski M., McCormick D., Spinner R., Windebank A. Adipose derived stem cells inhibit regeneration in rat sciatic nerve: a functional, physiological, morphometric and proteomic study.

**X.4 Conference Proceedings – Podium Presentations**


6. **Daly, W.**, Abu-Rub, M., Zeugolis, D., O’Connell, C., Yao, L. and Pandit, A. ‘Biofunctional collagen fibres: bridging the gap that is peripheral nerve regeneration.’ Podium presentation at the **Neuroscience Research Day**, Galway, Ireland, 2010. **Best Podium Award.**
Appendices

X.5 Conference Proceedings – Poster Presentations


