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Development and *in vivo* evaluation of a novel nerve
guide conduit containing dorsal root ganglion-derived
extracellular matrix for peripheral nerve repair

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DECLARATION

I declare that the work presented in this thesis is my own.

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Finalmente, esta tesis va dedicada a mi familia.

Abstract

Peripheral nerve injuries can lead to loss of function of injured nerves, significant long-term disabilities and the potential for the development of neuropathic pain, which can cause a reduction in the quality of life of those affected.

The current gold-standard treatment to repair nerve gap injuries is the use of an autograft to bridge the nerve injury, however this is associated with the sacrifice of a healthy donor nerve. While nerve guide conduits (NGCs) can offer an alternative solution for the repair of nerve injuries, current NGCs are limited in their efficacy. Studies suggest that the incorporation of extracellular matrix (ECM) components can be used to improve the function of NGCs by providing a pro-regenerative environment in conduits.

It was hypothesised in this thesis that a nerve-specific ECM would provide an optimal pro-regenerative environment. Therefore, this thesis investigated the use of dorsal root ganglia (DRG) as a source of nerve-specific ECM to improve nerve regeneration.

DRG were isolated from chick embryos and cultured to produce a nerve-specific ECM *in vitro*, which was found to contain relevant ECM components for nerve regeneration by immunofluorescence techniques. The DRG-derived ECM was decellularised and evaluated in a neurite outgrowth assay *in vitro*, showing a positive effect on nerve regeneration.

Subsequent studies evaluated the DRG-derived ECM in an *in vivo* model of nerve repair, where the DRG-derived ECM was deposited onto PCL fibres that were then used as a scaffold in the lumen of a nerve guide conduit.

The level of regeneration obtained by the novel nerve guide conduit containing DRG-derived ECM was similar to that obtained by a nerve autograft in the repair of a 3 mm nerve gap of the Thy-1 YFP mice common fibular nerve, highlighting the potential of DRG-derived ECM as a nerve-specific substrate to support nerve regeneration.

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Abbreviations

ANOVA	Analysis of Variance
ATP	Adenoside triphosphate
BDNF	Brain-derived neurotrophic factor
CAMP	Compound Action Muscle Potentials
CAP	Compound Action Potential
Cy3	Cyanine 3
Dapi	4',6-diamidino-2-phenylindole
dECM	Decellularised ECM (Extracellular matrix)
DRG	Dorsal root ganglion
DRGs	Dorsal root ganglia
ECM	Extracellular matrix
EDD	Embryonic Developmental Day
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAP-43	Growth Associated Protein 43
GDNF	Glial cell-line derived neurotrophic factor
GFAP	Glial Fibrillary
IL1- α	Interleukin 1 alpha
IL1- β	Interleukin 1 Beta
IL6	Interleukin 6
Krox-20	Early growth response protein 2 or Krox20
Laser-SL	Laser microstereolithography
LED	Light emitting diode
LED-SL	LED microstereolithography
LIF	Leukemia inhibitory factor
LPC	Lysophosphatidylcholine
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein
MCP-1	Monocyte chemoattractant protein 1
mRNA	Messenger RNA
NDS	Normal donkey serum
NGF	Nerve growth factor
NSAID	Non-steroidal anti-inflammatory drugs
NT3	Neurotrophin 3
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with triton
PC-12	Pheochromatocytoma cell line
PCL	Polycaprolactone
PCL-MA	Polycaprolactone methacrylate
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PLGA	Poly(lactic co-glycolic acid)
PLLA	Poly L-lactide

PNI	Peripheral nerve injury
RNA	Ribonucleic acid
SB-10	Sulfobetaine-10
SB-16	Sulfobetaine-16
SFE	Surface Free Energy
TCP	Tissue Culture Plastic
TGF- β	Transforming growth factor-Beta
Thy-1	Cluster of Differentiation 90 (CD90 or Thy1)
TNF- α	Tumour necrosis factor alpha
UBS	Ubiquitin Proteasome system
YFP	Yellow Fluorescent Protein

Chapter 1. Literature Review

1.1 The problem: peripheral nerve injuries

Peripheral nerve injuries (PNIs) are a worldwide problem that currently lack suitable treatment options. Inadequate treatment of peripheral nerve injuries can lead to significant, long-term disabilities, as well as a reduced quality of life. The major cause of PNI has shown to be trauma, although iatrogenic events and injuries resulting from nerve tumour dissection or congenital anomalies are also common (Scholz et al., 2009).

Several epidemiological studies estimate a global prevalence of PNIs to range between 1 – 3 % of all patients admitted to trauma centres, based on studies from Canada (Noble et al., 1998), the United States (Taylor et al., 2008), Middle East (Saadat, Eslami and Rahimi-Movaghar, 2011) and Europe (Huckhagel et al., 2018). Despite innate differences in the studies (e.g. retrospective years, patient population), certain trends of PNIs can be highlighted. For instance, the studies have suggested that the nerves in the upper limbs are generally more likely to be injured statistically compared to nerves in the lower limbs, that men often have a higher incidence of PNIs compared to women, and that a major cause of trauma-induced PNIs are due to motor-vehicle accidents.

Murphy et al (2023) recently reported on the prevalence of PNIs in England between 2005 and 2020 and found a mean national incidence of 11.2 PNIs per 100,000 population per year, where males had twice as high incidence compared to women, and the most common site of nerve injury was in the upper limbs (wrist and distal to wrist) (Murphy et al., 2023).

Studies report patients affected by PNIs have been younger than 50 years old, with average ages ranging between 28.6 to 40.6 depending on the study (Saadat et al., 2011, Huckhagel et al., 2018). In England, the average age where nerve injuries are sustained was found to be between 15 – 45 years old (Murphy et al., 2023). It has been estimated that only 40 % of patients recover normal function, where this was found to be highly dependent on the type of the injury and those with most severe injuries saw no functional recovery (Noble et al., 1998). Therefore, with the affected age range representing a large portion of the working-force age group, inadequate treatment of PNIs can not only affect employment and well-being, but the impact of peripheral nerve injuries can be life-long.

Lastly, the aetiology of PNIs seems unpredictable, where studies describe various modes of injury including lacerations and knife injuries (Saadat et al., 2011), motor-vehicle accidents (Noble et al., 1998) or gunshot wounds (Taylor et al., 2008) as the leading cause in trauma-induced injuries. There are also other mentionable causes, for instance, injury to the lingual nerve a common complication

associated with third molar removals due to the proximity of the nerve to the third molar and sensory deficits can include altered taste, impaired speech and neuropathic pain (Atkins & Kyriakidou, 2021).

Given the wide range of mechanisms of injury and subsequent symptoms, as well as the large age group affected, constant research and medical advancements are required to satisfy the clinical demands of ever-evolving global peripheral nerve injuries over time.

1.2 Peripheral Nerve Anatomy

The anatomical defects seen in peripheral nerves following injury are used in their classification, initially described by Seddon in 1943 (Seddon, 1943), and then closely followed with a more comprehensive listing by Sunderland (Sunderland, 1947). Before introducing the types of injury, it is important to describe the anatomy of peripheral nerves (Figure 1.1).

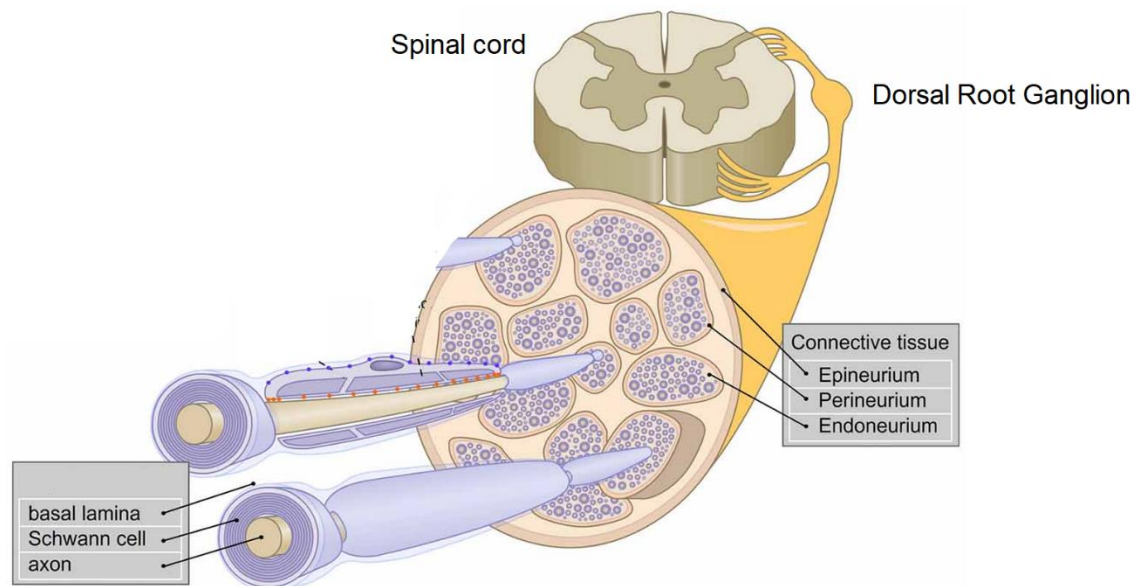


Figure 1.1. Anatomy of a peripheral nerve, adapted from (Belin et al., 2017) with permissions. Copyright © 2017

Peripheral nerves are composed of a number of components and cell types, but in the most basic form, peripheral nerves consist of bundles of axons of neuronal cells. Neuronal cells of the peripheral system are specialised somatic cells with a cell body located either in the dorsal root ganglion (for sensory nerves) or in the grey matter of the spinal cord (for motor nerves) with long, specialised extensions known as the axon. The axons are often, but not always, surrounded by a myelin sheath, which refers to the membrane of a Schwann cell wrapping around the axon repeatedly, forming an insulating, lipid-rich sheath. The junctions between each ensheathing Schwann cell are known as Nodes of Ranvier, and this accelerates the propagation and conduction of signals along nerves. The Schwann cells

produce a basement membrane (or basal lamina), which is a layer and network of extracellular matrix (ECM) components that uninterruptedly ensheathes the whole myelinated axon (Uzman & Villegas, 1960). The myelinated axon is then sequentially surrounded by a number of layers of connective tissue.

The endoneurium is the first layer of connective tissue, which solely surrounds an individual nerve fibre. It is primarily composed of a thin network of collagen, often derived by the underlying Schwann cells, but also by fibroblasts present in the tissue. Other cell types described in the endoneurium include endothelial cells of capillaries and microvessels, pericytes, mast cells and macrophages (Gamble & Eames, 1964). Several nerve axons surrounded by the endoneurium can be grouped together, where each isolated group or bundle of axons is known as a fascicle.

Each fascicle is then covered by a perineurium, a layer of connective tissue shown to vary in thickness as a factor of fascicle size (Sunderland & Bradley, 1952). Perineurial cells, the main components of the perineurium, are held together tightly by gap junctions, thus the perineurium acts as a protective blood-nerve barrier, preventing the diffusion of large molecules into the axonal environment (Jessen & Mirsky, 1999). As well, the perineurium is covered by a basement membrane that not only provides support through structural ECM components (e.g. collagen, laminin) but also contains negatively charged oligosaccharide groups that function as a molecular filter (Bush & Allt, 1990). In the space between fascicles, termed interfascicular inner epineurium, vasculature can be found, where blood vessels supply nerve fascicles through the perineurial septa and thereby reaching individual nerve fibres (Sunderland & Bradley, 1949).

Lastly, fascicles are bound and held together by the epineurium, the outermost layer of a peripheral nerve. The epineurium is composed of large amounts of collagen, as well as some fatty tissue, thus providing mechanical protection to the peripheral nerve from external stimuli. The thickness of the epineurium is also related to the number of fascicles in the nerve (Sunderland & Bradley, 1949), which will differ depending on the nerve location and its target. As branching of a nerve leads to less fascicles in each nerve, there is thinner epineurium, and therefore nerves can become more susceptible to injury.

1.3 Types of Injury

Seddon described three types of nerve injury, from least to most severe: neuropraxia, axontmesis and neurotmesis (Seddon, 1943). Sunderland used a similar categorical severity grading where Type 1 referred to the least severe type of nerve injury while the most severe was categorised as Type 5 (Sunderland, 1947) (Table 1).

In Seddon's classification, neuropraxia refers to a transient lesion of the nerve, where the myelin sheath is affected but the axon is intact, although compressed. Thus, recovery is expected within weeks, making this the least severe type of nerve injury. This type of injury is equivalent to Sunderland's Type I classification. In the case of an injured axon, Seddon uses the term axontmesis (i.e. divided axon). However, the extent of axonal damage can be variable and related to the damage of the surrounding layers, which not only leads to variable recovery, but also can make diagnosis and prognosis difficult to estimate. Therefore, in Sunderland's classification of PNIs, axonal injuries are further classified and distinguished by the affected surrounding tissue. In Sunderland's Type 2 injuries, only the axon is affected, while Type 3 and Type 4 refer to a damaged endoneurium and damaged perineurium, respectively. In these cases (axontmesis or Sunderland's Types 2 – 4), it is worthy to clarify that the epineurium of the nerve is still present, and therefore functional recovery is possible. However, in the case of an injured nerve with no likelihood of recovery, the term neurotmesis was coined by Seddon. This involves a complete injury of the axon, endoneurium and perineurium and the epineurium. This is equivalent to Sunderland's type 5 classification of a PNI where surgical intervention is required for subsequent recovery.

Table 1. Classification of peripheral nerve injuries based on Seddon and Sunderland's classification. Where + refers to anatomical structure being present, while - refers to damage. Table adapted from (Pfister et al., 2011; Robinson, 2000)

Classification		Features					
Seddon	Sunderland	Axon	Endoneurium	Perineurium	Epineurium	Surgical Intervention required?	Recovery rate
Neuropraxia	Type 1	+	+	+	+	No	Fast (days-weeks)
Axontmesis	Type 2	-	+	+	+	No	Weeks
	Type 3	-	-	+	+	Recommended	Weeks - Months
	Type 4	-	-	-	+	Yes	Little/None
Neurotmesis	Type 5	-	-	-	-	Yes	None

1.4 Injury and Regeneration

Injury to peripheral nerves leads to a number of morphological and metabolic changes, which can be loosely described to occur in two phases: during the injury and during regeneration. As mentioned, a number of factors determine the extent of regeneration and subsequent functional recovery, such as the type and cause of injury (as covered in 1.1 - 1.3). To understand the distinct regeneration outcomes, it is important to consider what immediate molecular changes occur following nerve injury.

1.4.1 Wallerian Degeneration

The cells in the peripheral nerve respond to injury by undergoing a process called Wallerian degeneration, a phenomenon that primarily describes the degeneration of injured axons and the surrounding myelin, as well as describing the role of glial (Schwann cells) and immune cells in the injury site (Waller, 1850). The ultimate goal of Wallerian degeneration is to provide an environment supportive of regeneration, as it has been demonstrated that disturbing Wallerian degeneration results in delayed regeneration and thus less functional recovery (Benavides & Alvarez, 1998).

1.4.1.1 Axonal degeneration

Axonal degeneration occurs distally to the injury, while retrograde breakdown of the proximal stump is limited and typically only progressing as far back as a few nodes of Ranvier, unless the site of the injury is close to the neuronal body, in which case whole nerve cell death may occur (Figure 1.2).

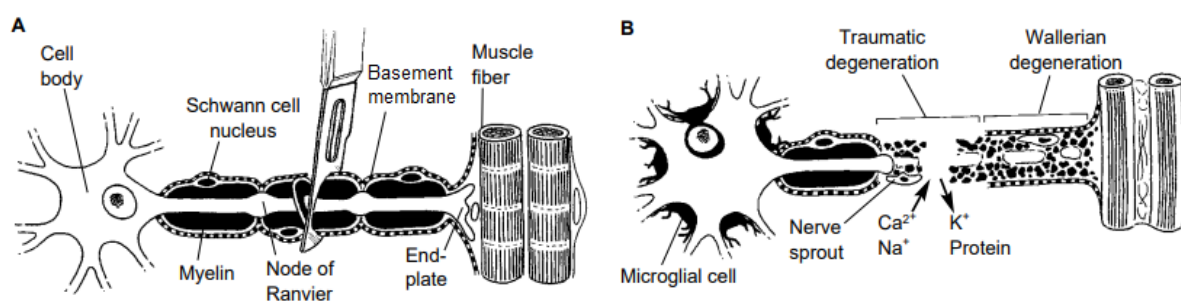


Figure 1.2 Wallerian Degeneration. Injury to a nerve (A) results in a number of cellular changes termed Wallerian Degeneration, that ultimately lead to the degeneration and demyelination of the axon distal from the injury site (B). Image adapted from (S. K. Lee & Wolfe, 2000)

It is worthy to note that studying axonal degeneration immediately after injury in humans is difficult and limited to a small number of tests, therefore a variety of *in vitro* and *in vivo* models have been developed to study the mediators and underlying mechanisms of axonal degeneration. However, axonal degeneration occurs within 2 - 3 days in rodents and rabbits (Gutmann & Holubář, 1950) and within 5 days in the non-human primate (Gilliat & Hjorth, 1972), highlighting species-differences as axonal degeneration is mostly completed within 7 days of injury in humans (Chaudhry & Cornblath, 1992; Wilbourn, 1977). Nonetheless, certain mediators seem conserved in the majority of organisms as a common feature in axonal degeneration, such as the role of calcium ions.

One of the earliest observations following injury is a rapid increase in intracellular axonal calcium (Ca^{2+}) levels, likely due to an influx from the extracellular calcium at the lesion site (George et al., 1995). Calcium ions can activate Ca^{2+} -dependent enzymes such as calpains or phospholipidases, both of which can degrade structural proteins and cell membranes, including myelin. For instance, activation

of calpain I and II has lead to the disruption of axonal neurofilament and microtubule-associated components (Billger et al., 1988). The experimental reduction of the extracellular Ca^{2+} levels has resulted in a slower rate of axonal degeneration (Schlaepfer & Bunge, 1973). Also, recent studies have suggested that phospholipidases can also increase the membrane permeability of myelin, thereby allowing a further influx of calcium ions and activating calcium-mediated cell death (Plemel et al., 2018).

It is worthy to note that distinct mechanisms of neuronal cell-death have been described in the cell body compared to the axon. For instance, caspase inhibitors have shown to prevent necrosis in the cell body of injured neurones, but they could not inhibit degeneration of the axotomised axon (Finn et al., 2000). Another well-described mechanism is the role of local energy levels, where the interruption of axonal transport of organelles, such as mitochondria, can lead to energy depletion and disrupt the maintenance of the axon cytoskeleton (e.g. via depolymerisation of microtubules), leading to axonal fragmentation (Park et al., 2013). The existence of selective and distinct degeneration process in the axon compared to the cell body is advantageous as it allows the elimination of injured segments of the axons, but not the whole nerve. Recent studies have also demonstrated that proteasome inhibitors were able to delay axonal degeneration, thus involving the ubiquitin proteasome system (UPS) in axonal degeneration. The UPS system has also been implicated in the regulation of Schwann cells, which have a distinct function during Wallerian degeneration (Lee et al., 2009)

1.4.1.2 Demyelination

Distal Schwann cells begin to de-differentiate in response to the loss of axonal contact, a process associated with downregulation of myelination-associated genes, such as those for structural proteins in myelin (e.g. Protein zero (P0) or myelin basic protein (MBP)) or cholesterol-related enzymes (Jessen & Mirsky, 2008), overall leading to demyelination. As previously mentioned, Ca^{2+} influx is a mediator in the degradation of structural proteins such as in myelin, where Ca^{2+} activated-phospholipase A hydrolyses phosphatidylcholine (a phospholipid found in myelin) into lysophosphatidylcholine (LPC), which has potent myelin-dissolving properties (Hall, 1972). This switch from myelination to myelin breakdown results in the formation of characteristic myelin ovoids and small myelin fragments (Thomas & Sheldon, 1964) that contain a number of proteins, such as myelin-associated glycoprotein (MAG), that are inhibitory to axonal growth (Li et al., 1996). Therefore, it is imperative the myelin debris is removed during Wallerian degeneration to allow for subsequent axonal regeneration.

Studies have shown that Schwann cells are able to digest most of their own myelin (i.e. myelinophagy) during the early phases of injury (within a week in mice), with little influence of macrophages (Perry et al., 1995). It is in a “later” phase, when Schwann cells stimulate the immune response, that macrophages are recruited to aid in myelin clearance (Martini et al., 2008).

1.4.1.3 Immune response

Macrophage recruitment can be stimulated by a number of cytokine signals released by distally denervated Schwann cells, such as tumour necrosis factor α (TNF- α), interleukin 1 α (IL- α) and β (IL- β) and leukaemia inhibitory factor (LIF) or via chemoattractants such as monocyte chemoattractant protein 1 (MCP-1) that may be released by resident macrophages themselves (Toews et al., 1998). In a healthy nerve fascicle, around 2 - 9% of the endoneurial cellular population are resident macrophages, which can rapidly respond to injury (Arvidson, 1977). Once the blood-nerve barrier becomes compromised after injury, a larger number of blood-borne macrophages can also be recruited to the site via the aforementioned Schwann cell tropism mechanisms. Cytokines released during the immune response can also have paracrine effects on the Schwann cells and promote their survival and proliferation (Rao et al., 1993). Similarly, during myelin clearance, mitogens e.g. platelet-derived growth factor (PDGF), Fibroblast growth factor (FGF), Transforming growth factor- β (TGF- β) can be released to promote direct or indirect Schwann cell proliferation (Salzer & Bunge, 1980). The recruited macrophages assume the main role in debris removal during the “later” myelin clearance phase, while proliferating Schwann cells become more involved in promoting and guiding axonal regeneration.

1.4.2 Nerve Regeneration

After injury, severed axons have the capability to regenerate towards their original target, a function highly dependent on Schwann cells. In fact, a well-defined histological feature of regeneration is the transformation of distal Schwann cells into a “repair” phenotype. Such repair Schwann cells are characterised by proliferation and a morphological elongation where they become longitudinally aligned, resembling a band of cells within the basement membrane of nerves (Thomas, 1963). The aligned elongated Schwann cells are referred to as bands of Büngner, and act as a microskeleton capable of guiding regenerating axons back towards the original target (Thomas, 1964) (Figure 1.3). On the other hand, Schwann cells are also seen migrating from the proximal stump to form a cable across the injury site. Such Schwann cell migration from the proximal stump is delayed and thought to accompany axonal growth, while the distal Schwann cells/Büngner bands contribute to regeneration through the release of axon guidance cues (Williams et al., 1983).

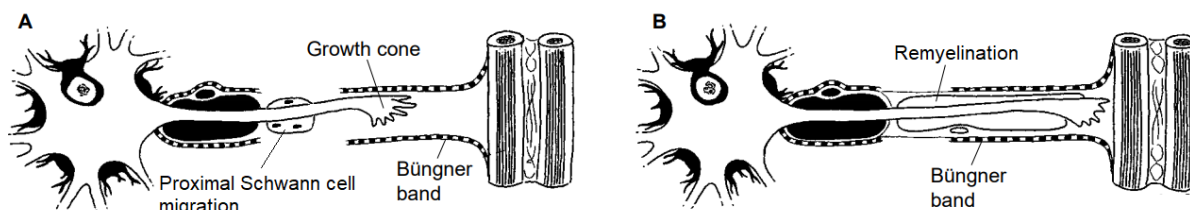


Figure 1.3. Severed axons extend and grow through Büngner bands back towards their original targets (A). Once axons regain contact with Schwann cells, re-myelination occurs (B). Image adapted from (S. K. Lee & Wolfe, 2000)

The induction of the repair phenotype in Schwann cells has been demonstrated to involve the transcription factor c-Jun, which becomes up-regulated rapidly in Schwann cells after injury (De Felipe & Hunt, 1994). The knock-out of c-Jun in mice (c-Jun KO) has resulted in failed regeneration, that is, a failed attempt of axonal growth back to the target, with signs of delayed myelinophagy during degeneration, and disorganised Büngner bands/abnormal Schwann cell morphology and function during the regeneration phase (Arthur-Farraj et al., 2012). As well, the inhibition of Schwann cell activity following nerve injury, such as with an anti-mitotic agents, severely limits regeneration, as demonstrated in a number of animal models (Hall, 1986).

Furthermore, the protein expression of Schwann cells is related to a wide range of cellular functions involved in regeneration. In fact, there are differences in the gene expression of Schwann cells found in sensory nerves compared to motor nerves, giving rise to the notion of “modality-specific” axonal growth, as described by Brushart (1993) who demonstrated motor neurones preferentially elongated through environments containing motor nerve-derived Schwann cells compared to environments of sensory nerve-derived Schwann cells (Brushart, 1993). Thereby, it follows that Schwann cells can produce highly specific neurotrophic factors to guide regenerating axons back to the original targets. In addition, Schwann cells are involved in the release of neurotrophic factors and cytokines, the regulation of cytoskeleton molecules and the production of extracellular matrix components (Brushart, 1993; Bunge, 1993, 1994; Frostick et al., 1998; Nagarajan et al., 2002).

1.4.2.1 Neurotrophic factors

Many neurotrophic factors have been detected following injury and regeneration of neurones, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF) or neurotrophin-3 (NT3) among others, all of which may influence regenerating neurones.

In a healthy nerve, levels of NGF mRNA are low, however, following rat sciatic nerve injury, there is a 10-fold upregulation in NGF mRNA within 12 hours, followed by a decline at 2 days post-injury and

subsequent increase at day 3 that persists for at least three weeks (Meyer et al., 1992). The second increase in NGF mRNA levels has been associated with IL-1 β , which is secreted by macrophages during the immune response, where it is suggested IL-1 β acts upstream of NGF. There is evidence to suggest NGF is associated with nerve regeneration indirectly by increasing migration of Schwann cells towards the injury site (Anton et al., 1994), thereby allowing a larger number of repair Schwann cells to guide regenerating neurones. Another indirect mechanism of NGF in regeneration is thought to be through NGF's angiogenesis effects, through which blood flow is increased to the injury site, thereby allowing an influx of blood-borne immune molecules (e.g. macrophages, cytokines) to release further regeneration-associated factors (Santos et al., 1991).

Another neurotrophic factor found in regenerating axons is BDNF, where its mRNA levels are also low in the intact nerve but become upregulated rapidly following facial nerve injury (within 8 hours) with levels peaking at 2 days post-injury (Kobayashi et al., 1996). Neuronal cell lines treated with BDNF show stimulated neurite outgrowth *in vitro* (Atwal et al., 2000; Dolcet et al., 1999), and *in vivo* administration of antibodies against BDNF after sciatic nerve injury significantly reduced the length of regenerating neurones as well as the proportion of myelinated axons (J. Zhang et al., 2000). Yet, studies have shown that exogenous BDNF administration does not stimulate axonal regeneration unless it is administered to chronically axotomised nerves, as opposed to freshly transected nerves (Boyd & Gordon, 2002). Similar findings have been observed for GDNF on chronically axotomised neurones (Boyd & Gordon, 2003), suggesting a contrasting role for BDNF and GDNF in freshly injured neurones compared to neurones degenerated chronically, highlighting a clinical potential for BDNF and GDNF in delayed repairs.

1.4.2.2 Cytokines

A number of studies have described roles for cytokines in regenerating axons. Levels of IL-6 and LIF mRNA are low in the healthy nerve but are up-regulated following sciatic nerve injury, with a 10-fold increase in LIF mRNA levels by day 1 post injury followed by a gradual decrease lasting one month (Ito et al., 1998). On the other hand, the expression of IL-6 was shown to be more transient, yet still prominent, where levels were increased by 35-fold after 1 day but quickly returned to baseline by day 2 after injury (Ito et al., 1998). Following rat sciatic nerve injury, an increased concentration of IL-6 mRNA has also been detected in the DRG, suggesting a possible intraneural transport mechanism for IL-6 from cell body to regenerating axon (Murphy et al., 1995). The administration of LIF in a rat sciatic nerve injury resulted in enhanced regeneration (Tham et al., 1997), while overexpression of IL-6 in a transgenic line similarly resulted in accelerated nerve regeneration (Hirota et al., 1996). In addition,

knockout of LIF and IL-6 in mice produces impaired regeneration (Zhong et al., 1999), overall suggesting roles for the cytokines IL-6 and LIF in axonal regeneration.

1.4.2.3 Cytoskeleton proteins

A number of the identified regeneration-associated genes following injury encode for proteins involved with the axon cytoskeleton, which is composed of dynamic structures such as microtubules and actin filaments that can assemble and disassemble, thereby allowing remodelling during axonal growth (Figure 1.4).

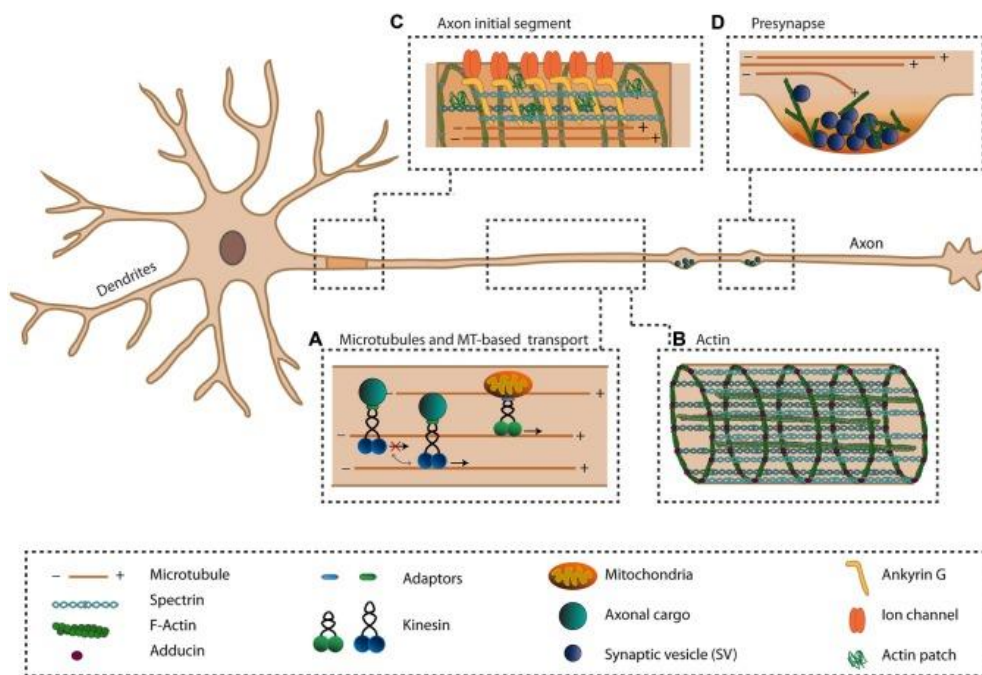


Figure 1.4. The axon cytoskeleton. The axon cytoskeleton is composed of microtubules (MTs), actin filaments and neurofilament. Image adapted from (Kevenaar & Hoogenraad, 2015)

Growth-Associated Protein 43 (GAP-43) or neuromodulin was one of the first neuronal growth-associated proteins identified, initially found to be up-regulated following axotomy of the rabbit hypoglossal nerve, but not expressed after optic tract crush (Skene & Willard, 1981). This finding suggested a role for GAP-43 in nerve regeneration as the hypoglossal nerve is capable of regeneration while the optic nerve is not. GAP-43 was localised to pre-synaptic terminals and growth cones during neurite extension in cultured neurones (K F Meiri et al., 1988) and to regenerating axons *in vivo* (Woolf et al., 1990). In adult rat peripheral nerves, GAP-43 mRNA was rapidly expressed in the DRGs of axotomised sciatic nerves followed by an upregulation of protein synthesis and transportation to newly formed axonal sprouts (Gispén et al., 1990), suggesting a strong association between GAP-43 and axonal growth following injury. Translocated GAP-43 in the neuron may interact with filamentous actin (F-actin) to promote enhanced neurite growth (Tsai et al., 2007). The depletion of GAP-43 mRNA

with small interference RNA (siRNA) in rat dorsal root ganglia resulted in shorter lengths of axons, also suggesting GAP-43 is related to elongation of axons (Donnelly et al., 2013).

GAP-43 can be phosphorylated by casein kinase II (CKII) at serine 191/192 or by protein kinase C (PKC) at serine 42 (He et al., 1997)(Oestreicher et al., 1997), where phosphorylation can be stimulated by a number of cell-derived neurotrophic factors, or ECM-derived molecules (Karina F Meiri & Burdick, 1991). For instance, in the *in vitro* culture of neuronal cell line pheochromatocytoma (PC-12), levels of GAP-43 are elevated when PC-12 cells are induced to phenotypically extend neurites, which happens upon exposure to NGF, suggesting a possible association between the growth factor NGF, GAP-43 and neurite growth (Costello et al., 1990). Alternatively, administration of the cytokine IL-6 in dissociated rat DRG neurones is accompanied by an increased expression of GAP-43, suggesting differential signals can influence GAP-43 and stimulate axonal growth (Yang et al., 2012)

NGF-induced neurite growth in PC-12 cells also leads to the up-regulation of another growth-associated protein called Superior Cervical Ganglion 10 protein (SCG10) (Di Paolo et al., 1996), where the over-expression of SCG10 in PC-12 leads to enhanced neurite outgrowth (Riederer et al., 1997). Studies have shown that SCG10 is involved in the dynamic instability (assembly and disassembly) of the microtubule, another component of the nerve cytoskeleton which is also required for growth cone extension and neurite growth (Belmont & Mitchison, 1996; Mitchison & Kirschner, 1984). Additionally, following rat sciatic nerve transection, levels of SCG10 mRNA levels become upregulated at the proximal stump by 20-fold and the administration of thyroid hormone has resulted in stimulated up-regulation of SCG10 mRNA levels while also promoting axonal growth (Voria et al., 2006).

Small Proline-Rich Repeat Protein 1A (SPRRP1) is another regeneration associated protein that becomes upregulated following rodent nerve injury (Bonilla et al., 2002). Bonilla and colleagues showed the knockdown of the SPRR1A gene resulted in inhibited axon regeneration and the overexpression of SPRR1A lead to increased neurite extension, suggesting a role for SPRR1A in axonal growth. The localisation of SPRR1A is on certain F-actin rich structures such as membrane ruffles found in growth cones, suggesting SPRR1A may regulate axon elongation in growth cones.

1.4.2.4 Remyelination

As axonal regeneration progresses into the distal stump, the re-establishment of contact between regenerated axons and Schwann cells results in the re-induction of the myelination phenotype in Schwann cells (Figure 1.3B). As a result, an up-regulation in genes involved in myelination has been described, including myelination-associated transcription factors (e.g. SCIP, Krox-20), myelin-associated proteins (e.g. P0, myelin basic protein) and enzymes involved in the synthesis of myelin constituents, such as cholesterol, galactocerebroside or sphingomyelin (Mirsky & Jessen, 1996;

Nagarajan et al., 2002). As well, proteins associated with Schwann cell cytoskeleton organisation (e.g. myosin I, myosin VIIa) are upregulated, as these proteins are involved in the migration and elongation of Schwann cells to wrap around axons for myelination (Jessen & Mirsky, 2008).

1.5 Failure of Regeneration

Nerve regeneration is dependent on the re-establishment of injured axons, which have to extend across the injury site towards the distal stump, through Büngner bands, to ultimately reinnervate the original targets. The average rate of axonal regeneration is estimated to be between 1 – 3 mm per day, as approximated by Tinel's sign, yet, as mentioned previously, regeneration can vary as a function of many factors, such as the nerve type, the extent of the injury, presence of scarring, the patient's age or the delay between injury and repair, among other factors (Buchthal & Kühl, 1979; Lundborg, 1987; Pestronk et al., 1980; Seddon et al., 1943). For instance, in some proximal nerve injuries (i.e. injuries close to cell bodies), such as the brachial plexus, the distances to the target can be up to a metre, therefore, considering an average rate of regeneration of 1 mm/day, it could take 2 – 3 years for re-innervation of the target. In such extreme cases of chronic denervation, it is generally accepted there will be little restoration of function (Grinsell & Keating, 2014).

Studies initially suggested that the reason for failed regeneration is due to changes at the target, such as fibrosis of soft tissue or muscle atrophy, which make reinnervation unlikely, leading to loss of functional recovery (Hoffer et al., 1979; Romanul & Hogan, 1965). However, a number of elegant studies by Fu and Gordon (1995) provided evidence that the failure of recovery following long periods post-injury is not due to changes at the target as much as previously thought, but rather due to the failure of chronically denervated nerves and Schwann cells in supporting regeneration for long term.

In a first set of experiments by Fu and Gordon, a rat tibial nerve was axotomised and left without repair for up to 12 months before it was sutured to a freshly axotomised peroneal nerve, thus investigating the effect of chronic axotomy on nerve repair (Fu & Gordon, 1995a). The authors showed that the axons in the proximal tibial nerve, while disconnected from the target for months, were still able to grow and regenerate through the newly attached peroneal nerve to innervate the muscle, where even the force measured in the re-innervated muscle was similar to the force measured after an immediate surgical repair. However, the authors soon found that the number of regenerated axons were reduced after chronic axotomy compared to immediate anastomosis, suggesting that the muscle force measured after chronic axotomy had to be compensated by other factors. It was then reported that there was more collateral sprouting at the muscle and larger motor units, thereby compensating for the reduced axons reaching the target.

In a subsequent second set of experiments by Fu and Gordon, the authors conducted a reversed cross-anastomosis, where the rat tibial nerve was axotomised and the distal stump was left to chronically degenerate for up to 12 months, thus resulting in chronic denervation of the distal stump and the target muscle (Fu & Gordon, 1995b). Then, the distal degenerated tibial nerve was sutured proximally to a freshly axotomised peroneal nerve. The studies found a reduction in the number of peroneal axons capable of regenerating through the chronically degenerated tibial nerve, and similarly, a reduced muscle force was measured. Interestingly, the tibial axons that did reach the target were able to reinnervate more muscle fibres than normal, again, a likely attempt to compensate for the loss of regenerated axons. This study suggested that the poor recovery after long-term denervation is likely due to the failure of the distal stump to accommodate regenerating nerves, rather than the failure of the target muscle to become re-innervated.

As well, the same group has shown that despite the severely compromised axonal regeneration in chronically denervated nerves, where about less than 10% of neurones are able to regenerate into the distal stump, the size of the regenerated axons and extent of myelination was not overly affected (Sulaiman & Gordon, 2000). This suggests that chronically denervated stumps and their “atrophied” Schwann cells may effectively switch back to a remyelinating phenotype upon the return of axonal contact, despite chronic denervation. However, most experiments in rodents suggest the transected distal nerve stump only retains the capacity to support regeneration for about 1 – 6 months, where the regenerative capacity is decreased with increasing time (Li et al., 1997; Sulaiman & Gordon, 2000), with differences in studies likely due to the pre-clinical model and nerve studied.

It is worthy to note that this post-injury regenerative capacity period is estimated to be between 12 – 18 months in humans, with authors and clinicians having reported on the successful regeneration on patients as late as 25 to 36 months after injury (Grinsell & Keating, 2014). For instance, studies report that the timing of surgical repair of injured lingual nerves does not affect the outcome, where nerve regeneration and sensory improvements are seen between early repairs and delayed repairs equally (Atkins & Kyriakidou, 2021). However, this may be due to differences in regards to the nature of the nerve and target, with studies suggesting muscles remain viable for at least two years, while sensory targets (e.g. Meissner corpuscles, Ruffini corpuscles, Merkel cells,) remain viable for longer (Lee & Wolfe, 2000)

Nevertheless, given the time-dependent loss of support from chronically denervated nerves, studies have identified certain components to be associated with impaired regeneration, one of which is scar tissue.

1.5.1 Scar tissue

In agreement with natural wound healing and tissue repair, the immune response to nerve injury is also completed by scar formation, however, the creation of this barrier is well known to impede axonal regeneration (Lane et al., 1978; Ngeow, 2010). Early studies demonstrated a negative correlation between the level of scarring and functional recovery (Bora & William, 1967) where nerve scarring is accompanied by shrunken endoneurial sheaths and a reduction in axonal diameter (Sunderland & Bradley, 1950)

More recently, a study by Atkins and colleagues compared two strains of transgenic mice with different propensities for scarring (increased vs decreased propensity), and showed that the regenerated nerves of mice with increased scar formation had smaller compound action potentials (CAMP) and reduced conduction velocity, findings associated with worse regeneration (Atkins et al., 2006a). As a result, the same group used interleukin-10 as an anti-scarring agent in normal mice and showed improved regeneration, highlighting a therapeutic potential for anti-inflammatory cytokines in peripheral nerve repair (Atkins et al., 2007). Other anti-scarring agents used for peripheral nerve repair have included hyaluronic acid (Özgenel, 2003) and citicoline, a form of vitamin B (Özay et al., 2007). Some studies have reported positive nerve regeneration using antibodies against transforming growth factor such as TGF- β 1 (Davison et al., 1999), but not others (Atkins, et al., 2006b), with differences possibly due to the protocols used.

Scar tissue has shown to be a site for release of inhibitory proteoglycans. Proteoglycans are a component of the ECM in nerves where have shown to be inhibitory for nerve regeneration, such as the chondroitin sulphate proteoglycans (CSPGs). CSPGs have been found to inhibit axonal regeneration *in vitro* (Muir et al., 1989) and *in vivo* (Zuo et al., 1998) and the enzyme-mediated degradation of CSPGs *in vivo* after nerve transection has resulted in accelerated regeneration (Zuo et al., 2002). Studies in rat have also shown that inhibitory proteoglycans, such as NG2 (Morgenstern et al., 2003), may be released by the endoneurial fibroblasts present in scar tissue and play a role in impeding regeneration.

Indeed, scarring can also be associated with the formation of certain neuromas that can lead to the development of abnormal nerve function and neuropathic pain, an often debilitating condition (M Devor, 2001).

1.5.2 Neuropathic pain

Following a nerve injury, there is the possibility for the development of neuropathic pain which may persist after regeneration is complete. As well, axons that fail to reach the distal end of an injured

nerve may form a neuroma, a mass of disorganised and tangled axons, that may also be involved in pain particularly as a site for spontaneous neuronal firing (Devor & Wall, 1976).

It is not entirely clear why some patients with nerve damage develop pain while others do not, but a number of mechanisms have been described to influence injury-induced neuropathic pain. Indeed, cellular and molecular changes can extend beyond the boundaries of the peripheral nerve and are known to be present in the spinal cord and higher regions of the central nervous system, where some of these changes have been implicated in the development and modulation of neuropathic pain.

Early studies demonstrated that peripheral nerve injury resulted in sprouting of nerve terminals into more superficial layers of the dorsal horn of the spinal cord, a re-organisation event associated with the development of pain (Woolf et al., 1992). Furthermore, studies highlighted that peripheral injury could lead to neuropathic pain through loss of inhibition in the transmission of noxious signals in the spinal cord, a key factor involved in the gate control theory of pain (Melzack & Wall, 1965) (Wall & Devor, 1981). In addition, following nerve injury and de-innervation of sensory and motor targets, the cortical representations of the targets are affected, for instance adjacent regions can become enlarged to compensate for the loss of sensory input and this can result in sensory deficits such as hypersensitivity (Li et al., 2011; Wall et al., 1986).

1.5.3 Aberrant regeneration

Lastly, it is worthy to mention that besides neuropathic pain symptoms, another clinical consequence of failed regeneration or incorrect re-innervation is synkinesis, a condition characterised by involuntary movement of a muscle in response to the voluntary movement of a different muscle (Brach et al., 1997). As an example, after injury and repair of the facial nerve, the voluntary action of smiling can trigger contraction of other facial muscles (e.g. eye closure) if there has been abnormal regeneration (Çelik et al., 2000). Other types of aberrant regeneration may be less obvious, for instance, nerves in the arm formerly innervating extensors may incorrectly innervate flexors, leading to pseudomyotonia, an impairment in muscle relaxation (Sumner, 1990). Alternatively, autonomic nerves originally innervating salivary glands may incorrectly innervate sweat glands resulting in sweating while eating, known as Frey's syndrome (Drummond, 2002)

Thus, the failure of regeneration can result in a variety of pathologies, both sensory and motor, that can be highly distressing to patients, thereby highlighting the need for appropriate treatments.

1.6 Current Treatments in Peripheral Nerve Repair

The goal in nerve repair is to effectively re-establish the function of the injured nerve, which can be done by providing a regenerative environment for axonal regeneration towards the target for successful reinnervation and functional recovery. Historically, the vast number of reports on peripheral nerve injuries studies and repairs were based on World War II patients, who often presented with concomitant contaminations and/or infections, requiring a 2 – 3 week delay before nerve exploration/repair, leading to the notion that this waiting period was recommended prior repair (Ducker et al., 1969). Some concepts now support primary repair should be done immediately or within a few days of injury, as it associated with better nerve regeneration (Lundborg, 2000)(Mackinnon, 1989). Nevertheless, this may not always be possible, and reports exist of patients treated up to 25 – 30 months after the initial injury, describing good levels of regeneration (Bertleff et al., 2005; Grinsell & Keating, 2014). Indeed, depending on the type of nerve and injury there can be different treatments, for instance in cases of trigeminal nerve injury related to wisdom tooth removal, crush or compression injury can occur to the branches of the trigeminal nerve such as the lingual or inferior alveolar nerve, and these injuries may not be immediately visible, where in fact many compression injuries will show spontaneous recovery and not require surgery.

However, in the case of transection injuries where spontaneous recovery is unlikely, there are a number of available standard techniques which will be described next.

1.6.1 Gold standard techniques

1.6.1.1 *End-to-end repair*

End-to-end repair is suitable for transection injuries or where minimal retraction of the nerve has occurred, therefore allowing the proximal and distal ends to be reconnected tension-free. It is accepted that suturing nerves under tension can lead to a number of complications (e.g. scarring, neuromas-in-continuity, fibrosis) (Wang et al., 1996) and unsatisfactory regeneration (Scherman et al., 2004; Terzis et al., 1975), therefore end-to-end anastomosis/repair is typically reserved for small defects.

Prior to anastomosis, the nerve may have to be prepared, usually by removal of any necrotic/scarred tissue. This facilitates examination of the injury site and allows for better mobilisation of the nerve ends to replicate the original alignment as accurately as possible, often with the use of epineurial vessels as landmarks (Millesi, 1979) (Figure 1.5). However, outer epineurial alignment does not guarantee internal fascicular alignment, where some studies suggest maintaining fascicular alignment is advantageous for nerve repair. For some nerve injuries, the identification of fascicle groups may be

possible due to a clear internal topographical arrangement, which can be used to suture individual fascicles back together (Millesi, 1979). However, the epineurium may need to be resected over a short distance to allow fascicular repair where there is a risk of scar or oedema formation as a result from excessive nerve handling, which can deter regeneration.

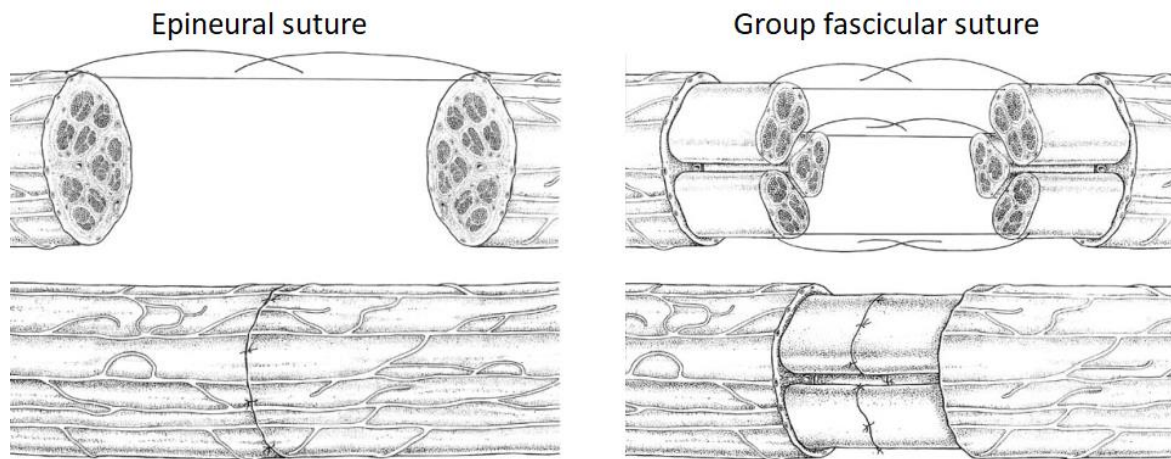


Figure 1.5 End-to-end repair techniques of a peripheral nerve. The transected nerve can be repaired by epineurial sutures (left) or by connecting fascicular groups (right). Adapted from (Lundborg C: Nerve Injury and Repair. Edinburgh, Churchill Livingstone, 1988.)

Depending on the size of the nerve, various sutures may be required for the complete neurorrhaphy e.g. a small digital nerve can be successfully sutured with 2 – 3 sutures, while a larger ulnar nerve requires several more sutures. Alternatively, fibrin glue is a common tool used for coadaptation of nerve, which circumvents any additional trauma by the sutures to the nerves (Narakas, 1988). Both techniques can be combined, i.e. secure sutures with fibrin glue, although there are not many clinical efficacy studies comparing the techniques when used combined or alone (Prasad et al., 2017).

As mentioned, repairs under tension can be detrimental to regeneration, therefore, injuries larger than a few centimetres (>1-2cm) require alternative repair methods.

1.6.1.2 Autografts

In the case where end-to-end neurorrhaphy cannot be done without tension, the “gold standard” technique in repairing a nerve injury involves the use of a nerve autograft, where a healthy donor nerve is taken from the same patient and transplanted to bridge the nerve defect. The most commonly used donor nerve is the sensory sural nerve, but other nerve graft sources include the superficial cutaneous nerve, the medial and lateral antebrachial cutaneous nerves and the superficial radial sensory nerve, where the selection of each donor nerve is based on availability and size matching (Poppler et al., 2015).

Various types of nerve grafting exist, such as single, cable, trunk and vascularised (Lee & Wolfe, 2000). A single nerve graft can be used when the donor nerve matches the diameter of the injured nerve accurately. Otherwise, cable grafting refers to using multiple smaller grafts aligned in parallel to bridge a larger gap, for example, the sural nerve can be sectioned into gap-sized segments, usually allowing for a 10-20% contraction in length, and multiple graft segments (or cables) can be used for transplantation at one site (Millesi et al., 1972). The grafts require correct positioning without adherence to each other to allow diffusion of oxygen and nutrients as well as re-vascularisation. The importance of vascularisation is highlighted in the use of trunk grafts, which refers to the use of a large whole nerve to bridge equally large nerves or otherwise irreparable nerves, for instance, an ulnar nerve can be used to repair a brachial plexus injury. However, trunk grafts have shown little success, as the poor vascularity leads to necrosis at the middle of such grafts (Tarlov & Epstein, 1945). On the other hand, a vascularised nerve graft, i.e. a nerve transposed with its arterial and venous supply, may be used in sites where vascularisation is poor, e.g. a scarred vascular bed. Vascularised grafting has shown good results for long defects, for instance, Doi et al (1992) reported an advanced Tinel sign rate on the vascularised sural grafts (1.8mm/day) compared to vascular-free sural grafts (0.5mm/day), suggesting a faster nerve regeneration rate with vascularised grafts (Doi et al., 1992). Some studies have also reported that the maintenance of vascularisation on medium-sized nerve trunks that would otherwise undergo necrosis show survival due to the presence of vascularisation (Karabekmez et al., 2009). As such, the clinical indication for vascularised grafts is primarily in non-vascularised beds, long and/or proximal injuries, cases with long denervation times and in older patients (D'Arpa et al., 2015).

An obvious limitation of the autograft repair technique is the morbidity at the donor site, however, there is often a risk-benefit consideration, where a given injured nerve may be considered worth salvaging over the loss of an “expendable” nerve. Nevertheless, repair by autograft is not always an option, meaning alternative strategies have to be employed to bridge nerve gaps.

1.6.2 Biological Scaffolds

Given the challenges associated with nerve autografting (i.e. limited donor nerve supply, surgery complications, risk of neuropathic pain, etc.), an alternative strategy used for nerve repair is the use of biological scaffolds to bridge nerve gaps and support regeneration.

Early studies considered the use of biological tube-like structures, such as veins, to repair peripheral nerve injuries. In 1909, Wrede reported on a successful human median nerve repair using a 4.5cm vein graft (Wrede, 1909). Since, it has been shown that regenerating nerves are able to effectively reach the distal stump through vein tube grafts, with return of functional target reinnervation, as demonstrated by histological and electrophysiological findings in rodents (Chiu et al., 1982). Clinically,

Chiu and Strauch (1990) implanted vein grafts in patients with distal nerve defects and reported on the recovery of two-point discrimination, demonstrating sensory recovery (Chiu & Strauch, 1990). However, in comparison to nerve autografting, the level of regeneration obtained by vein-repair was demonstrated as inferior to nerve grafting.

Another downside of vein tubes is that they tend to fail in repairing nerve gaps of more than 1.5cm in length, where the veins are seen collapsing at their midsection (Brunelli et al., 1993), therefore a variety of luminal fillers have been used with the purpose of preventing vein collapse during regeneration, such as muscle or tendon. An interesting filler is nerve, which has been used clinically in combination with a vein tube (i.e. packing the sural nerve inside a large saphenous vein) to repair larger gap injuries and shown recovery of sensory and motor function (Aly & Azab, 2011; Tang, 1995). A small trial also compared the efficacy of a combined nerve-vein conduit to that of a nerve graft alone and showed improved two-point discrimination and muscle strength in the combined nerve-vein group in the two-year follow up (Ahmad & Akhtar, 2014). The studies attribute the efficacy of vein grafts in nerve repair to trophic factors derived from the cells in the vein. However, another important factor is the physical confinement of the regeneration axons in an enclosed environment, thereby preventing any excessive sprouting, misdirection and neuroma formation (Lundborg et al., 1997a). However, the autologous origin of vein tubes is a clear limitation, therefore, an alternative approach to enclose injured nerves in a similar tubular fashion is with the use of nerve guide conduits.

1.6.3 Nerve guide conduits

Nerve guide conduits (NGCs) are hollow tubular devices placed between the proximal and distal ends of a nerve gap with the aim to bridge the gap and provide an enclosed environment to guide regenerating axons. NGCs can be broadly categorised by their properties, e.g. naturally-derived/synthetic material, absorbable/non-absorbable, etc. A number of NGCs have been developed and gained US FDA approval and EU CE approval. Table 2 shows a summary of the current FDA-approved nerve guide conduits that will be discussed in this section.

Table 2. Commercially available, FDA-approved nerve guide conduits used clinically.

Product name	Company	Date of FDA approval	Conduit length	Material /structure	Degradation	K510
Neurotube®	Synovis Micro Companies (Neuroregen)	Mar 22, 1999	2 - 4 cm	Poly (glycolic acid)	3 mo	K983007
SaluMedica™ SaluTunnel™	Salumedica LLC, Atlanta, GA, USA	Nov 24, 2000, Aug 05, 2010	6 cm	Polyvinyl Alcohol (Salubria™ biomaterial), hydrogel. Wrap version available	No degradation	K002098 K100382
NeuraGen® NeuroWrap™	Integra Lifescience Corporation, Plainsboro, NJ, USA	Jun 22, 2001 July 16, 2004, Apr 24, 2014	2 - 4 cm	Collagen Type 1 (bovine) with inner matrix of collagen and glycosaminoglycan (chondroitin-6-sulfate).	36-48 mo	K011168 K041620 K130557
NeuroMatrix® NeuroMend™ Neuroflex™ R-NeuroFlex®	Collagen Matrix Inc., Oakland, NJ, USA	Sep 21, 2001, April 05, 2006, Apr 03, 2014, June 01, 2017	2.5 - 5cm	Collagen Type 1 (bovine). Wrap version available Reinforced version available	4-8 mo	K012814 K060752 K131541 K170656
Surgisis® Nerve Cuff AxoGuard™	CookBiotech, USA	Mar 15, 2003, Oct 01, 2014, Oct 31, 2016	1 - 5 cm	Porcine Small Intestinal Submucosa (SIS), extracellular matrix.	3 mo	K031069 K132660 K162741
Neurolac®	Polyganics BV, Rozenburglan, Netherlands	Oct 10, 2003, May 04, 2005, Oct 20, 2011	2 cm	Poly(DL-lactide-ε-caprolactone)..	16 mo	K032115 K050573 K112267
Reaxon Plus®	Medovent GmbH, Mainz, Germany	Dec 02, 2015	1 - 3 cm	Chitosan	11-17 mo	K143711
Nerbridge™	Toyobo Co., Ltd, Japan	June 22, 2016	3 - 5cm	Poly (glycolic acid) with collagen I and III (porcine) filling, nerve wrap	3 mo	K152967

1.6.3.1 Synthetic conduits

1.6.3.1.1 Non-absorbable nerve guide conduits

Some of the first synthetic nerve guide conduits were made from inert materials like silicone (Lundborg et al., 1982) and polytetrafluoroethylene (PTFE), given that they had been previously used in a number of medical applications and shown to be physiologically well-accepted (Dang et al., 1990) (Hurlbert et al., 1998).

However, silicone-based nerve guide conduits were soon discredited due to being non-degradable, where they were shown to cause compression on the regenerating nerves of patients often leading to a revision surgery for removal of the conduit (Braga-Silva, 1999; Merle et al., 1989). Besides the discomfort and pain consistent with symptoms of nerve compression, further exploration of the silicone NGC regenerated nerves showed severe narrowing and poor vascularisation, likely due to the excessive formation of scar tissue around the conduit, overall discouraging the use of silicone-based conduits.

Similarly, a non-degradable PTFE nerve guide conduit was used to bridge a 3cm ulnar nerve defect in a patient who subsequently reported discomfort at the three-year follow up (Stanec & Stanec, 1998). Other small studies have also reported limited nerve regeneration with PTFE conduits (Pogrel et al., 1998).

A larger randomised trial compared silicone conduits to end-to-end repair of small median and ulnar defects (0.4 cm), and while they reported similar levels of recovery for both groups at the one-year follow up, 7 out of 11 patients had to have the conduits removed by the three-year follow-up due to signs of compression (Dahlin et al., 2001; Lundborg et al., 1997b). Interestingly, in the 5 year follow-up, the patients who had been in the NGC conduit group demonstrated less sensitivity to cold-intolerance, compared to those patients treated by end-to-end-repair (Lunborg et al., 2004), thereby supporting the use of nerve guide conduits during the early phases of regeneration, which can be achieved by the use of biodegradable/re-absorbable NGCs.

1.6.3.1.2 Absorbable nerve guide conduits

The synthetic, re-absorbable materials currently used in FDA-approved nerve guide conduits include polyglycolic acid and poly (dl-lactide-e-caprolactone).

1.6.3.1.2.1 Polyglycolic Acid (PGA)

Polyglycolic acid (PGA) is the synthetic material used in the first ever FDA-approved nerve guide conduit, NeuroTube® (Synovis, USA). The PGA-based Neurotube® conduit is reported to break down

within 3 months, likely by means of hydrolysis, although earlier reports in vivo have shown almost complete resorption of PGA conduits after 1 month post-implantation in the rat (Rosen et al., 1983).

The first trial involving PGA conduits included 15 patients with defects ranging from 0.5 – 3.0cm, where 13 out of 15 patients demonstrated sensory recovery after 1 – 3 years from implantation (Mackinnon & Dellon, 1990b), thereby establishing the efficacy of PGA conduits. A subsequent larger randomised trial in the US (98 patients, 136 defects) compared PGA (Neurotube®) conduits to the standard repair techniques of end-to-end anastomosis for small defects, and to autograft repairs in larger defects (Weber et al., 2000). The sensory recovery was evaluated by 2-point discrimination (PD), which measures the threshold ability of distinguishing two sharp points in the skin where a smaller value reflects improved discrimination as a result of fine innervation. It was found that sensory recovery in of smaller injuries (>0.4 cm) was shown to be better in the patients treated with the PGA conduit group compared to the standard end-to-end (2 PD= 3.7mm vs 2PD= 6.1mm, respectively.) Similarly, for the larger injuries (>0.8cm), nerves repaired by the PGA conduits also demonstrated better regeneration compared to nerves repaired using an autograft. However, the conduit had to be removed in three patients due to conduit extrusion, although this was identified to be due to the poor quality of the sutured skin tissue across the wound.

Soon after the FDA approval of PGA nerve guide conduits, experimental studies started investigating modifications to improve the efficacy of the synthetic conduit. Studies showed the potential for PGA tubes filled a collagen sponge in repairing large nerve gaps (8 cm) in the canine peroneal nerve (Toba et al., 2001) (Figure 1.6).

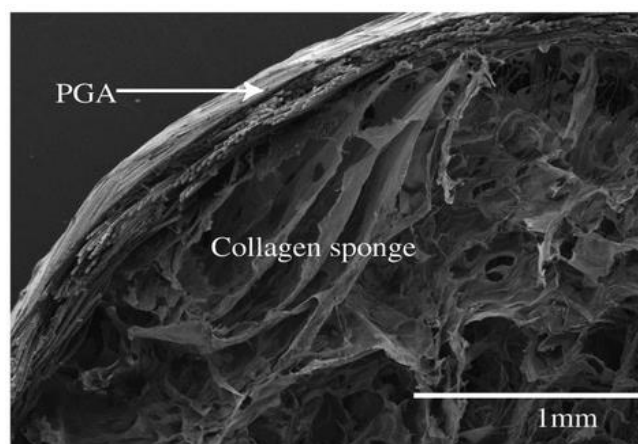


Figure 1.6 PGA-collagen sponge conduit developed by Toba et al. study (2001). Image taken from same paper (Toba et al., 2001)

This and other studies by the group lead to a number of small clinical studies where patients were successfully treated with PGA conduits filled with collagen in the repair of various nerve defects (2 -

6.5cm) (Hagiwara et al., 2002; Nakamura, 2003; Inada et al. 2004, 2005, 2007). As a result, PGA-collagen conduits are commercially available (Nerbridge®, Japan) and gained US FDA approval in 2013. However, there are no randomised trials comparing the Nerbridge® conduit to autografts.

1.6.3.1.2.2 Poly (DL-lactide- ϵ -caprolactone) (PDLC)

Poly (DL-lactide ϵ -caprolactone) (PDLC) is the synthetic material used in the fabrication of the FDA-approved nerve guide conduit Neurolac® (Polyganics, Netherlands). While PDLC is biodegradable, two long-term regeneration studies in rats have found remnants of the PDLC biomaterial on the edge of the regenerated nerves after 16 months (Jansen et al., 2004) and after 24 months (Meek & Jansen, 2009), however this did not seem to influence regeneration.

Early pre-clinical studies in rodents demonstrated the effectiveness of PDLC conduits in repairing nerve gaps of up to 1 cm (den Dunnen et al. 1996a; 1996b), where a large number of myelinated axons have been observed by 3 months (den Dunnen et al., 1998).

A multicentre, randomised trial in the Netherlands compared regeneration using the PDLC nerve conduit (Neurolac®) to end-to-end repair of defects of up to 2cm in the hand of 30 patients (Bertleff et al., 2005). Overall, the trial demonstrated no significant difference in terms of sensory recovery between the groups after 1-year post-repair, suggesting repair by PDLC conduit was as good as the “standard” end-to-end repair. However, it is worthy to note that two patients included in the end-to-end control group has defect sizes of 0.8 – 2.0 cm, which could be considered as repair under-tension (Sunderland et al. 2004) therefore this may have affected the results.

On the other hand, a clinical trial by Chiriac et al (2011) used PDLC conduits (Neurolac®) to repair a number of mixed nerves (n=28 lesions) in various locations, with defect sizes ranging between 0.3 – 2.5 cm (Chiriac et al., 2011). The trial reported modest regeneration using PDLC conduits, with the only satisfactory results noted in defects at the level of the hand (n=6). The nature of the mixed nerves (i.e. nerves with both motor and sensory components) in the trial hinders direct comparison with previous trials that solely reported on sensory defects (Bertleff et al., 2005), however, overall, Chiriac et al. discouraged the use Neurolac™ conduits. Their conclusion was based primarily on the large occurrence of complications, of which five complications were thought to be due to the PDLC conduit, such as conduit extrusion in two patients. Again, conduit protrusion may be due to the location of the conduit, which is not unlikely when the defect is close to a joint, as previously reported (Mackinnon & Dellon, 1990b). Nevertheless, Chiriac et al. also criticised the hardness of the PDLC conduit, and other authors agree this is to be a disadvantage (Meek and Coert 2012), as it leads to the breaking of thin needles and potentially longer surgery times. However, a thin-walled version of the Neurolac™ conduit was developed to aid with ease of suturing.

1.6.3.2 *Naturally-derived nerve guide conduits*

The currently used naturally-derived materials in FDA-approved NGCs are collagen, chitosan and small intestinal submucosa.

1.6.3.2.1 *Collagen*

Collagen has been widely used as a biocompatible material in a variety of medical applications, such as a suture material (e.g. Catgut) (Snyder, 1976), or as an injectable filler in cosmetic surgery (Charriere et al., 1989). Animal-derived collagen provides an abundant source of the material, and despite its xenogeneic origin that can raise a concern for the immune response (Cooperman & Michaeli, 1984b, 1984a), the processing and purification of collagen results in a weakly-antigenic, biocompatible material (Hardin-Young, Carr, Downing, Condon, & Termin, 1996).

The efficacy of collagen-based nerve guide conduits has been evaluated in a number of pre-clinical studies. In an early primate model, the efficacy of a collagen conduits was compared to a synthetic bio-absorbable conduit (glycolide thermethylene carbonate, Maxon®) in repairing 2 – 5 cm defects (Mackinnon & Dellon, 1990a). At 14 months, regeneration was considered equally good for both conduits for the smaller (2 cm) defect. However, regeneration was very limited in the 5 cm defect for both conduits, with no significant differences between them, suggesting hollow nerve guide conduits may not support regeneration in longer defects but are effective in small injuries. Another study evaluated collagen conduits in smaller defects (0.4cm) in non-human primates for up to 24 months (Archibald et al. 1991). The authors showed no significant difference in the sensory recovery obtained between nerves repaired by conduit or by autograft at 24 months, suggesting collagen NGCs can provide similar levels of regeneration to repair by autograft. In a similar study by the same group with a longer follow-up of 3.5 years, the authors reported collagen NGCs provided the same level of regeneration as direct end-to-end repair or autograft repair of 0.5cm defects in non-human primates (Archibald et al. 1995).

Such pre-clinical studies lead to a number of small trials evaluating the use of collagen-based conduits in nerve repair in a clinical setting. Collagen-based NGCs gained approval by the US FDA in the early 2000s, with some examples being NeuraGen® (Integra Lifescience Corporation) and Neuromatrix (Collagen Matrix Inc., Oakland, NJ, USA).

A small clinical study (n=9 patients) used collagen conduits (Neuragen®) to repair digital nerve defects between 1 – 2 cm (Bushnell et al., 2008). At the 1-year follow-up, 8 out of 9 patients demonstrated functional sensory recovery with 4 patients showing excellent two-point discrimination (2PD) less than 6mm and the other four showing good 2PD of less than 15mm. Similar sensory recovery has been demonstrated by Lohmeyer (2009), who also used collagen conduits (NeuraGen®) to repair a small

number of nerve defects in the hand (0.6 – 1.8 cm). The group showed sensory recovery in 9 out of 12 patients at the 1 year follow-up, where the 2-PD was less than 7mm in 4 patients, and less than 15mm in 5 patients (Lohmeyer et al., 2009).

A retrospective study reviewed patients treated with NeuraGen® collagen conduits between 2002 – 2007 at a level I trauma centre in the US (Wangenstein & Kallinen, 2009). Out of 64 patients with 89 nerve defects between 0.2 – 2cm, the authors reported sensory recovery in 35 – 45% of the treated nerves. This rate of recovery is less than previously described in past smaller trials (which ranged between 75-89%), and might be due to innate limitations of retrospective studies (e.g. inconsistent follow-up evaluation times/measures, loss of patients, etc.). Nevertheless, the study reported only 11 complications that required a second surgery, where only of the one patients showed poor recovery after the revision, suggesting NeuroGen® conduits are safe to use.

1.6.3.2.1.1 Collagen Nerve Wraps

An interesting use of conduits is as “nerve wraps” to protect end-to-end sutured nerves. In a small study by Farole (2008), collagen conduits (NeuraGen®) were split longitudinally and placed around lingual and inferior alveolar nerves (n=9 injuries in 8 patients) that had been repaired with end-to-end neurorrhaphy (Farole & Jamal, 2008). Post-surgical outcome at 3 – 7 months showed improved symptoms in eight cases on evaluation of touch, pinprick and thermal sensation, while one case showed no improvement. No control group was included in the study, therefore no conclusions can be made on the effectiveness of a combined approach (wrap plus end-to-end anastomosis) to no wrap. As well, it is worthy to note that the study applied hyaluronic acid (Healon®, Advanced Medical Optics) after the nerve was encased to minimise adhesion, and hyaluronic acid has been previously reported to enhance nerve regeneration *in vitro* (Wang et al. 1998, Özgenel 2003), therefore this may have affected the results. Nevertheless, nerve wraps can be advantageous by preventing axonal escape at the suture lines thereby preventing the chances of neuroma-formation, as well as providing nerve entrapment to concentrate growth factors (Lundborg et al., 1997b). Nerve wraps (NeuraWrap™, Integra) are also commercially available and have gained FDA approval.

1.6.3.2.2 Chitosan

Chitosan is a derivative of the natural polysaccharide chitin that can be found in the exoskeleton of arthropods and crustaceans thus making it a naturally derived material, however, there can be environmental concerns requiring ethical sourcing of the material. Chitosan is derived by N-deacetylation of chitin, where the degree of acetylation can be tailored for its application (Figure 1.7).

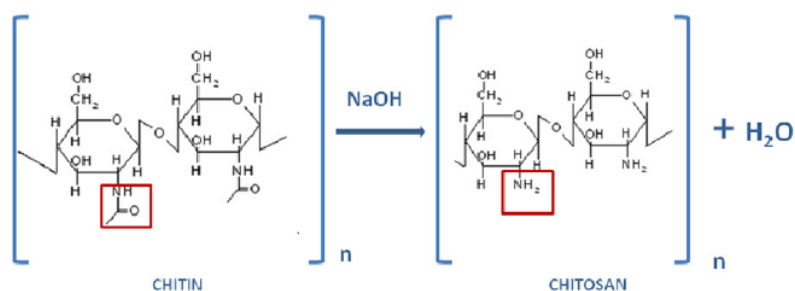


Figure 1.7 Chitosan structure taken from (De Carvalho et al., 2011)

Chitosan has been used in a number of clinical studies, including in wound-healing, making it a biocompatible material (Khor & Lim, 2003). In relation to nerve repair, studies have demonstrated the biocompatibility of chitosan *in vitro* with cultured Schwann cells (Yuan et al., 2004), and with embryonic chick-derived dorsal root ganglia (Freier et al., 2005).

Most pre-clinical studies have used chitosan in combination with other materials due to the mechanical weakness of chitosan alone and the lack of bioactive signals required to guide cell adhesion, therefore composites with collagen or silk (Wei et al., 2003) or synthetic materials like PGA and PCL (Cooper et al., 2011) have been evaluated in nerve repair. A recent study reported on the efficacy of chitosan conduits for small nerve defects to bridge 1 cm defects in the rat and reported similar, although still lower, levels of regeneration compared to an autograft (Haastert-Talini et al., 2013). In addition, the study evaluated the level of acetylation (low vs medium vs high) and found that higher level of acetylation lead to an increased immune response in macrophage activation. Furthermore, tubes made from high acetylation of chitin resulted in faster degradation and reduced structural integrity over time. The structural integrity is an important consideration to prevent the collapse of implanted conduits. However, it is thought that the potential of chitosan in nerve repair can influenced by the metabolites produced, where the degradation of chitosan by hydrolysis produces chito-oligosaccharides, which have been shown to promote cell proliferation and neuronal differentiation *in vitro* with PC-12 cells (Yang et al., 2009). Chito-oligosaccharides have also been used in models of nerve crush injury in the rat and shown enhanced nerve regeneration (Jiang et al., 2009). Moreover, anti-apoptotic effects of chito-oligosaccharides have been found, which may influence the survival of Schwann cells following injury (He et al., 2014). However, the reduced structural integrity in highly acetylated chitin conduits is an important consideration in order to prevent the collapse of implanted conduits, therefore care must be taken to select appropriate composites.

A commercially available chitosan-based nerve guide conduit is Reaxon® (Medovent GmbH, Germany) which launched in 2014 and gained US FDA approval in 2015. A randomised trial by Neubrech et al (2016) recruited 72 patients and compared chitosan (Reaxon®) conduit (n=37) to end-to-end neurorrhaphy (n=37) in the repair of small defects (Neubrech et al., 2018). The study did not specify the

average defect size or range, but it can be assumed it was less than 0.5 cm as there should be no tension for the standard end-to-end repair. At 6 months post-repair, the two-point discrimination (2PD) in the end-to-end repair (2PD = 6.3 mm) was better than that of the chitosan conduit group (2PD=8mm), although still not similar to the contralateral, uninjured 2PD (2PD= 2.68mm). A small proportion of the participants were re-evaluated at 12 months post-repair, where some patients in the chitosan conduit group demonstrated better two point discrimination (2PD = 5.5 mm) than those in the end-to-end repair group (2PD= 8mm) but not all.

1.6.3.2.3 *Small intestinal submucosa (SIS)*

The small intestinal submucosa (SIS) is a layer of the intestinal wall that has been well-established as a suitable biomaterial in a variety of medical applications including tendon (Badylak et al., 1995), bone (Suckow et al., 1999), blood vessels (Badylak et al., 1989) or wound-closure (Brown-Etris et al., 2002). Porcine-derived SIS is the major source of material, which is obtained by mechanical delineation of superficial tissues of the small intestine followed by decellularisation with a mild acid, to produce solely the extracellular matrix structure (SIS-ECM) (Gilbert et al., 2008) (Figure 1.8) .

Clinically, it is reported that more than one million patients have been implanted with SIS-ECM across a variety of applications (Misseri et al., 2005; Jones et al., 2005; Mostow, Haraway, Dalsing, Hodde, & King, 2005). SIS-ECM is commercially available in a variety of medical products with FDA approval (e.g. Biodesign® (Cook Biotech, USA), OASIS® (Smith & Nephew, USA) and Restore™ (DePu, USA)). Importantly, SIS-ECM is used in the production of FDA-approved nerve guide conduits/wraps, Axoguard™ (AxoGen, USA, previously Cook Biotech, USA) and Surgisis® (Cook Biotech, USA).

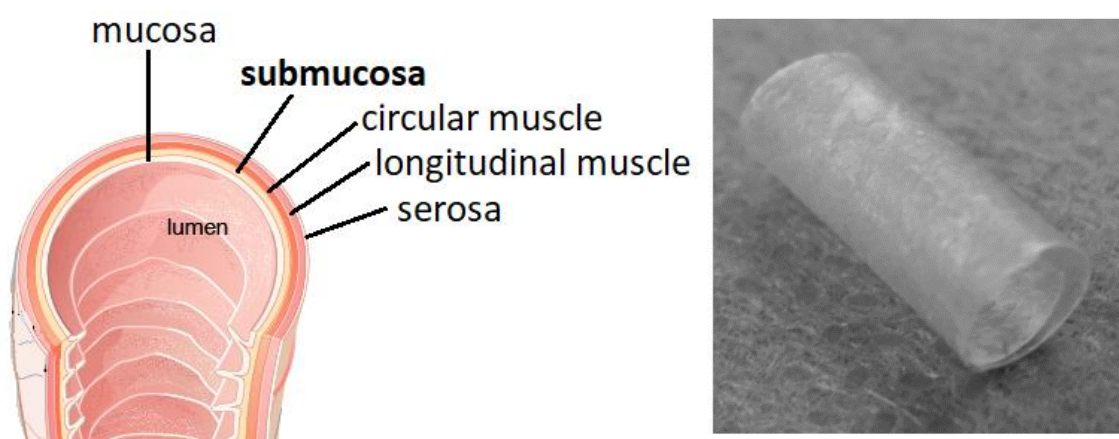


Figure 1.8 Submucosa layer location in the small intestine (left). Porcine-derived small intestine submucosa (SIS) is used to make AxoGuard™ (right). Images adapted from (Britannica, 2018)(Kokkalis et al., 2011).

One of the earliest pre-clinical studies using SIS in nerve repair was by Hadlock et al (2001), who used rat-derived SIS to make a SIS nerve guide conduit by rolling the material into a tubular shape. The authors showed successful repair of 0.7 cm sciatic nerve defect in the rat, as demonstrated by functional measurements and histology (Hadlock et al., 2001). Since, a number of pre-clinical studies have established the efficacy of SIS nerve guide conduits for nerve repair (Smith et al., 2004) (Su, Zeng, Zhang, Zhang, & Xie, 2007; Xei, Zhang, & Su, 2007) and shown similar rates of regeneration for SIS conduits compared to autograft repair, with a high morphological resemblance in regenerated fibres and nerve conduction velocities.

Despite being developed (and advertised) as a nerve guide conduit, AxoGuard™ is mainly used in non-gap injuries, such as for compression injuries (Halsey et al., 2015) or in patients with recurrent carpal tunnel syndrome (Papatheodorou et al., 2015), or used to wrap nerves after anastomosis/nerve grafting (Martin et al., 2018). However, one study compared collagen (NeuraGen®) conduits to the conduit version of AxoGuard® in the repair of injured lingual nerves (Wilson et al., 2017) and reported no significant differences in functional sensory recovery between patients of the two groups.

1.6.4 Neuronal allografts

The final category of current therapies used in the repair of nerve injuries is the use of an allograft. A nerve allograft, i.e. of cadaveric origin, is an attractive option as there can be an abundant supply of donor material and no donor-site morbidity in patients. However, the immunological response created by foreign antigenicity requires immunosuppression, which has been associated with a number of complications such as increased risk of infections and nephrotoxicity, among others (Brenner et al., 2002; Min & Monaco, 1991). However, the rationale behind nerve allografting is that the graft acts as a temporary scaffold, therefore only a predetermined period of immunosuppression would be required until the patient's nerve regenerated through the graft and reached the target, whereupon immunosuppression would be discontinued. Thereafter, the allograft would not be required and the immune response would break down the foreign tissue (Myckatyn & MacKinnon, 2004).

Immunosuppression would be needed during the initial stages after allograft transplantation as studies in rats have shown that a non-immunosuppressed nerve allograft results in scarring and fibrosis of the donor graft, which leads to a mechanical barrier for nerve regeneration (MacKinnon et al., 2001). To reduce the immune response, two options are available: patients can be immunosuppressed with drug therapies or the allograft nerves can be pre-treated prior to implantation to reduce the immunogenicity (via decellularisation).

1.6.4.1 *Immunosuppression*

A series of studies in rat by Mackinnon et al. found that pre-treated allografts resulted in poorer levels of regeneration in comparison to non-treated allografts in animals under immunosuppression (Mackinnon et al., 1984). In fact, the minimum effective doses of the immunosuppressive agents during the regeneration period resulted in enhanced nerve regeneration through the non-treated allograft, with results akin to nerve autografting (Mackinnon et al., 1984). Such pre-clinical studies have helped establish dose-responses for a number of immunosuppressive agents (e.g Cyclosporin A, tacrolimus, azathioprine) that have been subsequently used clinically for nerve allograft repairs of brachial plexus injuries (Mackinnon & Hudson, 1992) and upper- and lower- extremity injuries (Elkwood et al., 2011; Mackinnon et al., 2001) where the donor nerves were from cadaveric origin.

1.6.4.2 *Decellularisation*

Treatment of nerve allografts to reduce the immunogenicity of the nerves involves the removal of any cellular components that would contain foreign major histocompatibility complex (MHC) molecules. This decellularisation process thereby prevents an immune response in the patient following transplantation (Myckatyn & MacKinnon, 2004).

While some decellularisation techniques can offer maximal removal of cellular/immunogenic components, the structural integrity of the nerve allograft may become compromised, thus limiting its use in regeneration. Therefore, combined techniques have been used to find an optimal nerve decellularisation method with effective removal of cells but preservation of connective tissue and extracellular matrix components.

An early chemical decellularisation protocol was developed by Johnson et al. (1982), who used a combination of ionic (sodium deoxycholate) and non-ionic (Triton X-100) detergents, hypertonic and hypotonic solutions, and a nuclease (DNase) to effectively decellularise human cadaveric nerves, although originally developed for the purpose of ultrastructural and biochemical investigations (Johnson et al., 1982). However, this protocol was later modified for the purpose of creating an acellular nerve graft for transplantation, where Sondell et al. (1998) described a refined version with effective decellularisation and less damage to extracellular matrix components (Sondell et al., 1998).

Ultimately, it was Hudson (2004), who developed a milder chemical decellularisation protocol, based on the previous studies, where he showed improved preservation of the native extracellular matrix as well as effective cellular removal (Hudson et al., 2004) (Hudson et al., 2004b). This protocol was used to develop the only currently commercially available acellular human allograft, the Avance® allograft (AxoGen, Inc.).

1.6.4.3 *Avance® nerve allograft*

The Avance® allograft is also the only FDA-approved human allograft currently in use for the treatment of nerve repair. Briefly, the decellularisation protocol involves the use of the zwitterionic detergents sulfobetaine-10 (SB-10) and sulfobetaine-16 (SB-16) and the ionic detergent Triton X-100, with a number of hypertonic and hypotonic washes (phosphate and sodium solutions) between steps. As well, the decellularised allografts undergo a chondroitinase treatment to remove inhibitory CSPGs (chondroitin sulphate proteoglycans) and are sterilised with gamma-irradiation (Krekoski et al., 2001).

The efficacy of Avance® allografts was established in a few pre-clinical studies prior to its clinical use (Hudson et al., 2004). In pre-clinical studies, authors have reported Avance® allografts can provide similar levels of nerve regeneration to autografts in injuries of 1.4cm and 2.8cm injuries (Whitlock et al., 2009), making the acellular allografts a highly promising alternative.

One of the earliest clinical studies reported on 7 patients treated with the Avance® graft to repair sensory defects (n=10) of lengths between 0.5 – 3cm, from 2007 – 2008 (Karabekmez et al., 2009). The functional recovery, determined with two-point discrimination (2PD), showed excellent sensory recovery in 5 patients (PD<6mm) and good recovery (2PD<10mm) in the remaining patients, however, the report is limited by the short post-op evaluation time. Another trial used Avance® allografts to repair 18 digital nerve defects ranging between 0.5 – 3cm, where successful functional recovery was reported for 15 out of 18 defects at 15 months post-op (Taras et al., 2013). In addition, a study used Avance® allografts and AxoGuard™ wraps to repair lingual and inferior alveolar nerve injuries (0.8 – 7 cm) and showed improvement in 87% of the cases (n=23), including 5 cases (out of the 7) with longer defects (3 – 7 cm) (Zuniga, 2015). Although no control group was used, the study used the neurosensory test (NTS) scale definition of “improvement”, where this referred to recovery of sensory perception and pain thresholds to normative limits.

A larger multi-centre study, sponsored by the manufacturing company (Axogen), investigated the efficacy of Avance® grafts in a number of mixed, motor and sensory nerves of various lengths (0.5 – 5cm) (Brooks et al., 2012). Of interest, the authors reported positive nerve recovery in 91.8% of sensory nerves (n=49), 88.9% of motor nerves (n=9) and 83.3% of mixed nerves (n=18), showing a good range of recovery across nerve types. In terms of length, the acellular allografts provided better regeneration in shorter nerve defects, but still reported good functional recovery in 76.2% of intermediate-sized gaps (1.5 - 2.9cm, n=21) and 90.9% of the larger gaps (3 - 5cm, n=22). Overall, the study concluded the Avance® grafts have been used widely and safely, with less than a 5% revision rate observed among the clinics. Other retrospective studies have reported similar findings (Cho et al., 2012).

A recent randomised prospective study compared the Avance® allograft to commercially available collagen-based conduits (NeuraGen®/NeuroMatrix®) in the repair of digital nerves (n=31) of 0.5 – 2.0 cm defects and showed improved recovery of sensory function in the Avance® group patients (PD= 5mm) compared to the collagen conduit (PD= 8mm) at the 12 month follow-up (Means et al., 2016). Therefore, these studies highlight the potential of acellular nerve grafts in nerve repair in short gap injuries of up to 2-3cm.

1.6.5 Current Treatments Summary

The current treatment options for nerve repair include end-to-end anastomosis, repair by nerve autograft, acellular nerve grafts or a number of nerve guide conduits made from a variety of materials (natural, synthetic or acellular xenogeneic tissue). In the largest randomised trial evaluating nerve guide conduits to autografts, Weber et al (2000) reported that (PGA) conduits were able to support regeneration as effectively as autografts in small defects (0.4 – 0.8cm) (Weber et al., 2000), but not for larger defects. Many retrospective reports have highlighted the efficacy of acellular allografts (Avance®, AxoGen) in nerve repair, with some small trials reporting repair in injuries of up to 5cm (Brooks et al., 2012). However, there have been no controlled, randomised, prospective clinical studies comparing the Avance® allografts to the gold standard of an autograft. But, a randomised study showed improved functional recovery with the Avance® allografts compared to collagen-based nerve guide conduits, again in small defects (2cm) (Means et al., 2016).

The clear failure of these therapies in larger nerve injuries stresses the requirement for further advancements in peripheral nerve repair. While a number of studies have investigated alternative decellularised nerves, such as in the form of xenografts (Zilic et al., 2016), the vast number of experimental studies are addressed at enhancing the efficacy of nerve guide conduits.

1.7 Experimental Strategies for Peripheral Nerve Repair

One of the reasons attributed to the failure of nerve guide conduits (NGCs) in large defects is the inability of Schwann cells to migrate across long distances and support effective axonal regeneration, which is required for re-innervation of targets to re-establish functional recovery. Therefore, a number of experimental therapies have been directed at improving Schwann cell support and increasing the rate of axonal regeneration in nerve guide conduits in a variety of ways (Vallejo et al., 2022). These strategies can be loosely categorised as chemical and physical modifications to the conduit designs, the addition of support cells and the use of extracellular matrix components (Figure 1.9).

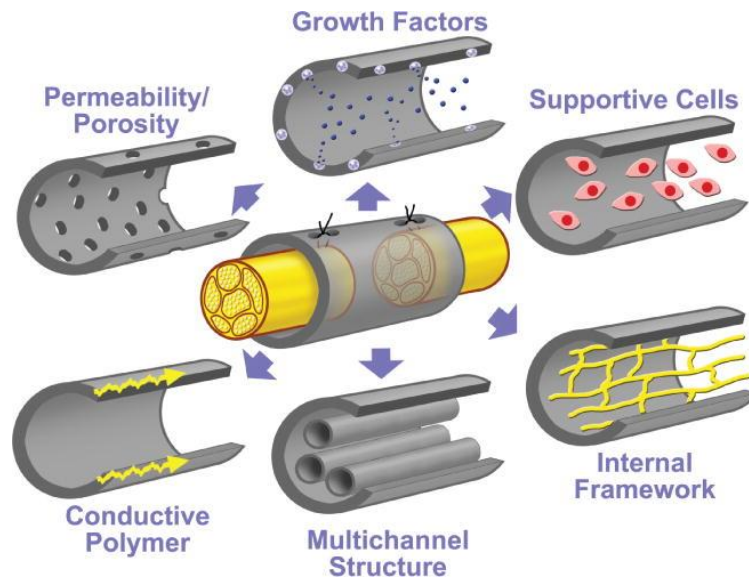


Figure 1.9. Strategies to improve nerve guide conduits. Taken from (Ruiter et al., 2009) with permissions.

1.7.1 Physical and Chemical Modifications

In an attempt to replicate the morphology of peripheral nerves, studies have developed multichannel-conduits which replicate the fascicular anatomy of the peripheral nerve. An early study in 1998 by Tessa Hadlock evaluated nerve guide conduits with 2, 4 or 5 multi-channels (or sub-luminae as referred to in their paper) in an *in vivo* rat model of nerve repair showing limited regeneration in the multi-channelled conduits (2, 4 and 5 channels) in comparison to repair with a conduit with a single lumen and in comparison to the autograft repair (Hadlock et al., 1998). Specifically, the authors reported that in the single lumen conduit repair, axons occupied 15 - 30% of a cross section taken at the mid-point of the repair, while in the autograft repair the cross-sectional space occupied by regenerating axons was higher at 40 - 50%. In contrast, there were only a few regenerating axons in the mid-point of the repair when using the multi-channel conduits which was not deemed quantifiable, suggesting poor regeneration as a result of the reduction in available cross-sectional area for axons to grow. However, a number of the conduits failed in their study where the authors suggested that there might have been high residual solvent in the multi-channel conduits, rather than due to the effect of the multi-channels.

Nevertheless, the study was based on the concept that increased surface area as a result of the channels would provide improved cellular adherence during regeneration. And indeed, subsequent studies since have supported this notion. For instance, Yao et al (2010) compared 2-, 4- and 7- multi-channelled conduits (Figure 1.10 top row) to a single lumen conduit and autograft and showed reduced axonal sprouting, which can be associated with axonal misdirection and incorrect re-innervation, in the 2- and 4- channel conduits compared to the single-lumen conduit. Overall, the

study reported that the reduction in cross-section did not impose a “barrier” or “blocking” effect on regeneration, as all groups showed the formation of regenerating axons, which were deemed functional based on electrophysiological recordings, however the functional regeneration was still higher in the autograft group. In addition, in a different way to increase surface area, studies have also used conduits with spiral internal structures (Figure 1.10 bottom row) and showed nerve regeneration could be supported in rat models of nerve repair (Shah et al., 2019). Furthermore, the modifications are not only limited to the luminal space of the conduits, as the porosity and roughness of nerve guide conduits can be modified, such as with the addition of grooves or micro-patterns on the inner surface of the conduits to increase cell adhesion (Aebischer et al., 1990) and such conduits have shown to positively influence nerve regeneration *in vivo* too (Hsu & Ni, 2008).

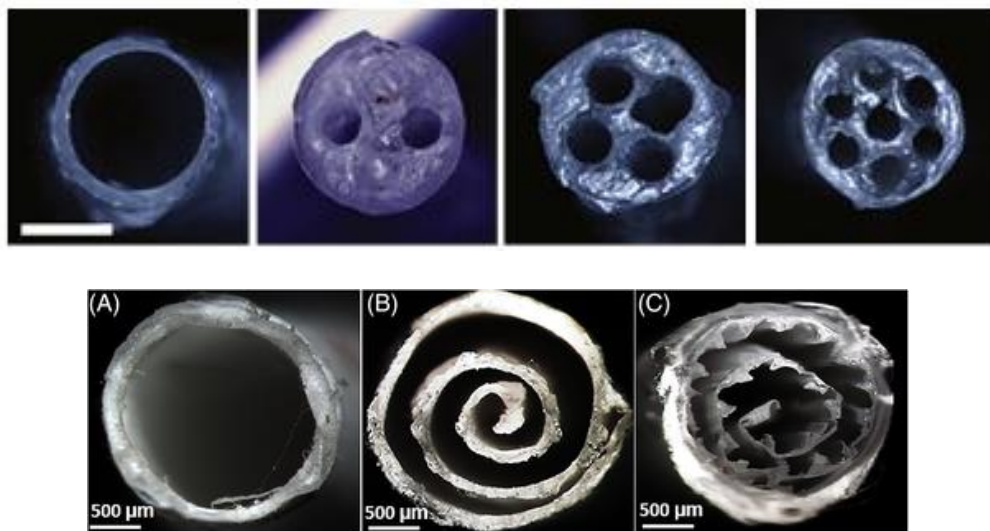


Figure 1.10 Top row: Multichannel conduits showing a single lumen, and 2-, 4-, 7- multi-channels in the nerve guide conduit. Adapted from (Yao et al., 2010). Bottom row: nerve guide conduits with a single lumen, a spiral lumen, and a mixed spiral-multichannel lumen. Adapted from Shah et al. (2018).

1.7.1.1 Intraluminal fibres

Another method to increase surface area in conduits is using thin filaments or fibres in the micron or nano-range as these provide a large surface-area-to-lumen-volume ratio, that can be used to increase cell adherence. It is hypothesised that such fibres can be used to mimic the structure of longitudinally-aligned bands of Bungers formed by Schwann cells following nerve injury, and therefore help to recapitulate the regenerating environment to improve axonal extension across the nerve gap.

The fabrication of micro- or nano- fibres for tissue engineering is commonly achieved using electrospinning, a manufacturing technique that uses an electric field to produce fibres from a polymer solution. The use of electrospun fibres and their potential use in nerve regeneration is discussed further in Chapter 2, however, briefly, filaments and fibres in the nano- and micron- range

have shown a positive effect in improving axonal outgrowth of neurones in a number of *in vitro* models, with an emphasis on the effect of the fibre diameter size. For instance, a study by Daud et al. (2012) investigated the outgrowth of axons from neuronal NG108-15 cells on PCL fibres and showed significantly increased outgrowth of neurites on 8 μm PCL fibres ($142.36 \pm 13.69 \mu\text{m}$) compared to 1 μm fibres ($61.83 \pm 6.89 \mu\text{m}$).

Studies using more complex systems of nerve outgrowth evaluation, however, such as using dorsal root ganglia explants, show a variable effect of fibre diameter. For instance, Wen and Tresco (2006) showed improved outgrowth of dorsal root ganglia (DRG) axons on fibres in the 5 – 30 μm range in comparison to fibres in the 100 to 500 μm range (Wen & Tresco, 2006). Furthermore, Wang et al (2010) compared three fibre diameters of 0.2, 0.7 and 1.3 μm using embryonic chick DRG, and showed similar levels of neurite outgrowth from DRG on the 0.7 and 1.3 μm , which in turn only showed a slight increase in axon outgrowth in comparison to the smaller fibre diameter group (Wang et al., 2010). Whilst these two studies used DRG, the former used post-natal rat DRG and the latter used embryonic chick DRG, suggesting differences in the effect of fibres depending on the model and stage of the tissue, which suggests that differences found *in vitro* and *ex vivo* may not necessarily translate *in vivo*.

In fact, studies evaluating intraluminal fibres in nerve guide conduits *in vivo* show a range of fibre diameters have been used successfully to support regeneration with studies reporting fibres between the ranges of 200nm to above 250 μm (G Lundborg et al., 1997; Matsumoto et al., 2000; Ngo et al., 2003; Koh et al., 2010).

Clinically, Fan et al. (2008) reported on using chitosan nerve guide conduits containing longitudinally aligned PGA filaments in the repair of median nerve injuries in two patients (3.0 – 3.5cm defects) and shown good functional recovery in the 3 year follow-up (Fan et al., 2008; Gu et al., 2012). The conduits were based on previous pre-clinical studies where it was reported that 2000 PGA filaments of 14 μm were used to fill the conduits at a fibre packing density of 1.9% (2000 fibres of 14 μm in a 4.5mm internal diameter conduit) (Wang et al., 2005).

1.7.1.2 Surface modifications

Chemical surface modifications have also been conducted on the surface of nerve guide conduits by using a number of small peptide molecules known to enhance adhesion and proliferation *in vitro*, such as IKVAV (Ile-Lys-Val-Ala-Val) and RGD (Arg-Gly-Asp) (de Luca et al., 2016; Hosseinkhani et al., 2013). The surface modification of nerve guide conduits with RGD sequences has shown superior regeneration in 1.5cm defects in the rat compared to no surface modification (Xiao et al., 2013), however, the level of regeneration was still inferior to that of an autograft.

It has been previously reported that electrical stimulation enhances *in vitro* neurite outgrowth (Kotwal & Schmidt, 2001; Sharifi et al., 2023; Valentini et al., 1992), although the underlying mechanisms are still inconclusive. Nerve guide conduits have been modified with electroactive molecules, for instance, Wang et al (2010) immobilised B-tricalcium phosphate (B-TCP) particles on chitosan conduits, where B-TCP was either polarised to store an electric charge or was left unpolarised before implantation in a rat sciatic nerve defect model (Wang et al., 2010). Histologically, polarised B-TCP chitosan conduits showed increased blood vessels along the regenerated axons compared to non-polarised chitosan conduits as well as increased myelinated axons in the polarised chitosan conduit at 12 weeks post-transplantation. PTFE conduits have also been electrically charged and shown more myelinated neurones after repair of the mice sciatic nerve in comparison to un-charged PTFE conduits alone (Valentini et al., 1989).

1.7.2 Cellular strategies

As previously mentioned (section 1.5), there is a time-dependent loss of support from Schwann cells in chronically denervated nerves (Fu & Gordon, 1995a). This is associated with a loss of neurotrophic factors, growth-associated components and a down-regulation of mechanisms maintaining the “repair” phenotype for long-term (Jessen & Mirsky, 2008; Jessen & Mirsky, 2016). In fact, Schwann cell senescence markers (e.g. p16) have been identified in higher levels in grafts used to repair longer defects compared to p16 levels in shorter grafts used for shorter defects, suggesting there is an enhanced loss of Schwann cell survival in chronically denervated grafts (Poppler et al., 2016).

Therefore, a number of experimental studies have evaluated Schwann cells for nerve repair, with generally positive outcomes. For instance, an early study supplemented nerve guide conduits with two concentrations of Schwann cells (low and high) and compared their efficacy to cell-free NGCs in the repair of 1.8 cm defects in the rat sciatic nerve (Ansselin et al., 1997). Cell-free conduits did not support regeneration across the critical gap when examined 3 months post repair, while Schwann cell-supplemented NGCs demonstrated regeneration, with an increased number of myelinated axons, faster conduction velocity and improved functionality (toe-spread reflex) in the “high concentration” Schwann-cell NGC group, showing a positive effect of Schwann cells on regeneration.

However, in comparison to an autograft repair, Stang et al (2004) showed that regeneration across a 2 cm rat sciatic nerve with a Schwann cell-supplemented NGC resulted in only about 100 regenerated axons in the distal stump compared to 5000 axons following autograft repair, suggesting autograft repairs are still superior (Stang et al., 2005).

Nevertheless, in smaller injuries, the use of Schwann cells seems to improve the level of regeneration, as shown by studies using Schwann cell-NGCs in 1 cm defects, although again not to the same extent as an autograft (Wei et al., 2011). Schwann cells have also been used in acellular small intestine submucosa grafts and shown improved regeneration to cell-free grafts in 0.7 cm defects (Hadlock et al., 2001).

The delivery system of the Schwann cells may also likely affect regeneration. For instance, Georgiou et al (2013) grew Schwann cells on an aligned sheet of collagen hydrogel that was rolled and inserted into nerve guide conduits (SC-NGC) and used these to bridge 1.5 cm rat sciatic nerve defects (Georgiou et al., 2013). The authors reported on a significantly larger number of axons in the distal stump of the regenerated nerve with the SC-NGC compared to the nerve repaired with a hollow NGC, where it possible that the combined effect of the aligned collagen hydrogen and the Schwann cells improved the rate of regeneration. However, the study had a short evaluation period where there may have been more sprouting leading to an increased axonal count.

Indeed, while there are positive reports for the use of Schwann cells in nerve repair, particularly in smaller defects, the clinical use of Schwann cells in patients may be limited, for instance by the requirement of an autologous source of the cells, where the isolation may be invasive to the patient. This would also involve specialised cell culture facilities for culturing adequate number of cells, meaning such a therapy may not be a likely option for acute repairs.

As a result, therapies targeted at increasing host Schwann cell proliferation and migration shown great promise, which can be achieved by the manipulation of the local environment, including the extracellular matrix and related components.

1.7.3 Extracellular matrix

The extracellular matrix (ECM) is a three-dimensional network of macromolecules whose precise composition and structure are dependent not only on the tissue, but also on the developmental and patho-physiological stage of the tissue including during development, injury and regeneration. The ECM components of the peripheral nerve are found in two main locations, the connective tissue layers of the nerve and the basal lamina surrounding Schwann cells.

A pioneering study by Ide et al. (1983) elucidated a role for the ECM in nerve regeneration, where they showed regenerating axons were able to grow through nerve stumps previously processed to remove living cells (i.e. decellularised) but still conserving the basal lamina and ECM components (Ide et al., 1983). The authors showed regenerating axons growing and migrating along the Schwann-cell derived basal laminae, suggesting the components involved played a key role in guiding axonal regeneration.

As a result, a large number of studies have investigated the individual roles of specific extracellular matrix components for nerve repair. More details are provided in Chapter 2, but briefly, Schwann cells and neuronal cells are able to attach and migrate on a number of ECM-coated surfaces, such as collagen (Koopmans et al., 2009), laminin (Carbonetto et al., 1983; Sandrock & Matthew, 1987), fibronectin (Baron-Van Evercooren et al. 1982) and proteoglycans, like heparan sulphate (Lander et al., 1982). Some ECM-coated surfaces also show high levels of Schwann cell proliferation, such as laminin (Armstrong et al., 2007), while other ECMs components are mitogenic to Schwann cells when administered exogenously (Baron-Van Evercooren et al., 1982).

ECM components can bind on integrin and non-integrin (e.g. dystroglycans) receptors expressed on Schwann cells to mediate cell behaviour (Fernandez-Valle et al. 1994) (Yu, Chen, North, & Strickland, 2009; Morrissey, Levi, Nuijens, Sliwkowski, & Bunge, 1995). For instance, studies implicate collagen V signalling with myelination, where the siRNA-mediated suppression of the type V collagen receptor on Schwann cells has resulted in inhibited myelination in co-cultures of Schwann cells and neurones *in vitro* (Chernousov et al., 2006). As well, studies have associated laminin with the phosphorylation and activation of certain Schwann cell transcription factors, such as transcription factor nuclear factor kappa B (NF-kappaB), where NF-kappaB is thought to modulate axonal outgrowth (Armstrong et al., 2008).

A number of *in vivo* studies have evaluated ECM components as fillers in nerve guide conduits, such as in aqueous form or hydrogels, and shown various regeneration outcomes. Early studies and the majority of studies have used collagen-based fillers and gels, showing improved regeneration in comparison to hollow conduits (Satou et al., 1986), but not in comparison to autograft repairs (Rosen et al., 1989). Combined fillers of laminin and fibronectin have also been used in conduits and shown improved regeneration in comparison to hollow conduits (Woolley et al., 1990), and nerve guide conduits filled with a collagen, laminin and fibronectin together (2:1:1 concentration) have also shown enhanced regeneration across a 1 cm gap in the rat compared to empty conduits (Chen et al., 2000).

Indeed, cells grown on a combination of ECM components, such as laminin-coated surfaces and treated with heparan sulphate, a proteoglycan, also show enhanced neurite outgrowth compared to the growth on laminin surfaces alone (Hantaz-Ambroise et al., 1987), suggesting the interaction between the several ECM components (and/or other local components) can provide a more favourable regeneration outcome. In agreement, in the presence of neurotrophic factors (such as NT3 or BDNF), studies have shown increased neurite outgrowth on surfaces containing both laminin and fibronectin rather than either surface alone (Gómez Sazo et al., 2012). The effective use of various combined ECM components, in comparison to each component alone, is not surprising, considering

the native *in vivo* environment in regenerating nerves comprises a variety of ECM molecules, cell-protein interactions, and various signalling mechanisms between them.

1.8 Summary and project rationale

Current treatments for peripheral nerve injuries are associated with a number of limitations, particularly related to the size of the defects. End-to-end suturing is only suitable for tension-free defects, otherwise, the gold standard intervention of an autograft can be used, but donor site morbidity associated with the sacrifice of the healthy nerve is a clear limitation. Decellularised allografts have shown potential in the repair of various sized nerve defects but have not demonstrated clinical superiority to the autograft and can be costly which limits their widespread use.

While nerve guide conduits may be useful in the repair of smaller defects, they are limited in the repair of larger defects due to the failure of maintaining a favourable environment for regeneration. This environment is highly dependent on the presence and migration of Schwann cells, which are able to guide regenerating axons through the release of trophic factors, cell adhesion signals and extracellular matrix (ECM) interactions.

As mentioned, a number of strategies can be used to improve the function of nerve guide conduits, such as physical and chemical modifications to the design of the conduit, the use cellular therapies such as Schwann cells or luminal fillers, where the use of extracellular matrix components seems to hold potential (Vallejo et al., 2022; Zennifer et al., 2023).

Overall, replicating the peripheral nerve environment in nerve guide conduits is key in providing a permissive environment to regenerating axons. However, individual ECM components in NGCs inherently fail to recreate the native ECM architecture and composition seen in peripheral nerves. Therefore, this represents a gap in the literature and inspires the development of novel nerve guide conduits that can replicate and mimic a native-like ECM environment.

A recent study by Gu et al (2014) used Schwann cells to produce and deposit extracellular matrix on silk fibres which were then decellularised to obtain solely the Schwann cell-derived ECM components coating the fibres (Gu et al., 2014). The ECM-coated fibres (8 μ m) were inserted into the lumen of nerve guide conduits (SC ECM-conduit) and used for the repair of a 1 cm nerve gap injury in the rat. In comparison to an empty conduit, the SC ECM-conduit showed an increased number of regenerated axons in the distal end compared to plain conduits. While there was no comparison with an autograft group, the study included an acellular nerve graft group, where the levels of regeneration were similar between the ECM-conduit and the acellular graft, highlighting the potential of a NGC with SC-derived ECM components. However, the study used primary rat Schwann cells to deposit the SC-derived ECM

in vitro, and whilst this is an effective way of producing nerve-specific ECM, other cells in the peripheral nerve also contribute and synthesise components relevant to the extracellular matrix that may not be fully recapitulated using only Schwann cells (Cornbrooks et al., 1983).

Therefore, other sources to deposit a more native-like nerve specific ECM can also be investigated. For instance, co-cultures of various cell types can be used to produce a better representation of the native ECM of a nerve, which can then be used to provide a more appropriate and tissue-specific ECM substrate for regeneration. Indeed, a study compared the co-culture of primary Schwann cells and fibroblasts on chitosan-collagen sponges with neuronal cells (primary motor neurons) (Gingras et al., 2003) and showed that in comparison to either surface alone, the co-culture of Schwann cells and fibroblasts lead to increased myelination of the motor neurones, increased neurites and increased axonal diameter, demonstrating a positive effect of combined cell types involved in the native peripheral nerve environment.

A recent study by Wang et al. (2021) compared Schwann cell-derived ECM to a variety of other cell-derived ECMs (such as from fibroblasts, bone marrow mesenchymal cells, and precursor Schwann cells) and showed a differential response of DRG primary neurones when grown on each. Specifically, whilst all cell-derived ECMs showed enhanced neuronal lengths in comparison to DRGs grown on poly-L-lysine, the Schwann cell-derived ECM and the BMSC-derived ECM supported enhanced DRG outgrowth in comparison to fibroblast-derived ECM and precursor Schwann cell-derived ECM. The study also characterised the ECMs derived from the different cell sources and showed clear proteomic differences in the level of ECM relevant components, again suggesting that individual cells may not recapitulate nerve-specific ECM.

Therefore, the selection for the source of ECM for nerve regeneration represents an area for optimisation where an alternative way to mimic the peripheral nerve environment is with the use of a dorsal root ganglion (DRG). The DRG is the location of the cell bodies of pseudo-unipolar sensory neurones, whose axons bifurcate into a peripheral and central branch (Figure 1.11). DRG have been widely used as a model of the peripheral nerve to conduct a variety of studies investigating mechanisms underlying peripheral nerve functions, such as development, axonal degeneration and outgrowth, myelination or targeted innervation (Schmeichel et al., 2003; Werth & Thayer, 1994; W. Zhang & Li, 2013). But importantly, DRG demonstrate the upregulation of a large number of regeneration-associated genes and proteins following injury, making it a suitable model to mimic processes associated with regeneration (Nunez & Fischer, 1997; Riederer & Barakat-Walter, 1992; Sundaram et al., 2021).

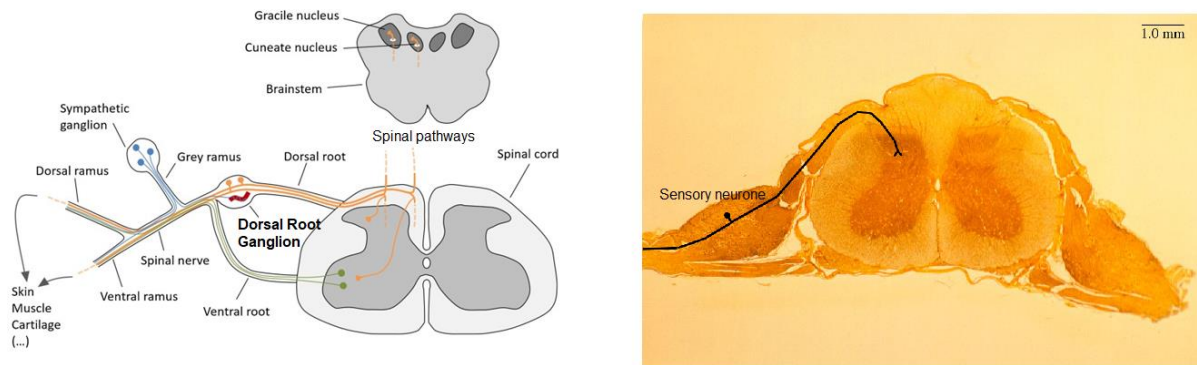


Figure 1.11 Spinal cord and dorsal root ganglion schematic illustration (left) and histological visualisation (right). Images adapted from (Nascimento et al., 2018; Todt, 2017)

DRGs can be isolated from various sources and developmental stages, such as embryonic or adult sources. A distinct advantage of the DRG as a model of the nerve is that the DRG-derived neurones are capable of retaining a similar *in vivo* architecture due to the preserved relationship of neurones with other non-neuronal cell types found in the DRG (Melli & Höke, 2009). In fact, DRG are composed of not only various subtypes of sensory neurones, but also non-neuronal cells like non-myelinating Schwann cells, satellite glial cells and cells of blood vessels or immune cells, all capable of interacting with the nerve environment during normal physiology and regeneration (Haberberger et al., 2019). Importantly, following axotomy of DRG neurones, which models a peripheral nerve injury, there is an upregulation of genes associated with survival and regeneration, similar to *in vivo* injuries, such as c-jun (Koistinaho et al., 1993) and fibroblast growth factor 2 (FGF-2), which may be involved in proliferation of the resident glial cells, thereby also modelling aspects of regeneration.

Therefore, given that DRGs are used to model peripheral nerves, it follows that the DRG can also be used to mimic the native extracellular matrix seen in nerves. Meaning, DRG could be cultured to produce DRG-derived cells (neurones and non-neuronal cells), which would deposit a “native-like” and nerve-specific extracellular matrix. This DRG cell culture can then be decellularised to obtain solely the ECM components, which can then be tested for nerve regeneration.

It is hypothesised that the DRG-derived ECM may recreate the peripheral nerve environment more efficiently than individual ECM components, thereby offering a more optimal environment for nerve regeneration. Therefore, this thesis aims to investigate the potential use of DRG-derived ECM in nerve regeneration, both *in vitro* and *in vivo*. One method to evaluate DRG-derived ECM *in vivo* is by coating fibres with DRG-derived decellularised matrix and using these as intraluminal fillers in a nerve guide conduit (Figure 1.12).

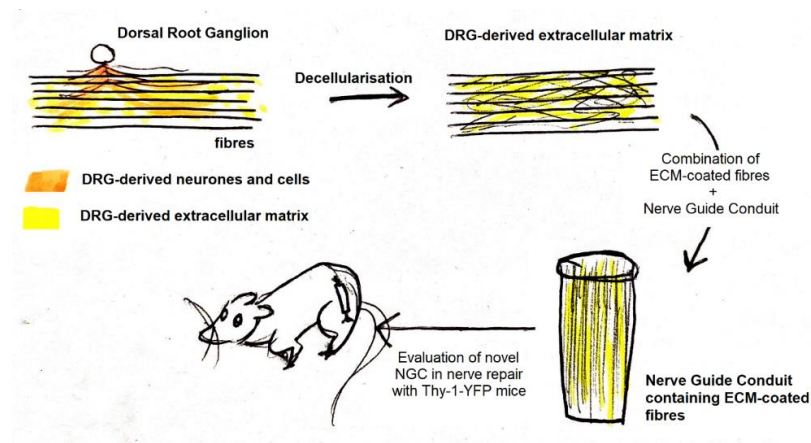


Figure 1.12 Graphical summary of project. DRG- dorsal root ganglion, ECM- extracellular matrix. NGC- nerve guide conduit.

1.8.1 Project Hypothesis and Aims

It is hypothesised that dorsal root ganglia, a source of neuronal and non-neuronal cells in the peripheral nerve, may be used to model the peripheral nerve environment to produce a native-like extracellular matrix, which can be obtained by decellularisation.

The DRG-derived ECM can be evaluated for nerve regeneration by depositing the matrix on PCL fibres and using these as intraluminal fillers in nerve guide conduits to develop a novel nerve guide conduit for peripheral nerve repair.

The aims of this thesis are described below:

1. Obtaining a DRG-derived ECM capable of improving nerve regeneration *in vitro*
2. The development of a novel nerve guide conduit with intraluminal fibres coated with DRG-derived ECM (NGC+dECM)
3. The evaluation of the NGC+dECM in an *in vivo* model of nerve repair using YFP-Thy-1 transgenic mice

In order to achieve these aims, this thesis reports on the following experimental chapters:

Chapter 2: The fabrication of a nerve guide conduit containing an optimal internal framework composed of intraluminal electrospun microfibers.

Chapter 3 Optimisation, characterisation, decellularisation, and evaluation of DRG-derived ECM *in vitro*

Chapter 4: The *in vivo* evaluation of the NGC containing a DRG-derived ECM

Chapter 2. The fabrication of a nerve guide conduit containing an optimal internal framework composed of intraluminal electrospun microfibers.

Abstract

A variety of synthetic and natural materials have been used to fabricate nerve guide conduits with clinical success, where properties regarding biocompatibility are key. Various physical and chemical modifications have been shown to possess great potential in improving the function of nerve guide conduits, such as incorporating intraluminal fibres to provide increased adherence and contact guidance to regenerating axons and Schwann cells.

This chapter involved the development of a nerve guide conduit containing aligned fibres in the lumen to improve contact guidance in regenerating axons and cells following injury.

The conduit was fabricated via stereolithography and injection moulding from a photo-curable version of polycaprolactone. The tensile strength of the material was tested and evaluated for cytocompatibility with a neuronal cell line. The fibres were fabricated via electrospinning of polycaprolactone and treated with air plasma. The incorporation of the fibres within the lumen was also investigated by pycnometry.

2.1 Background

2.1.1 Introduction

A review of nerve guide conduits (NGCs) has been provided in Chapter 1 (Literature Review), however, to summarise, the ideal nerve guide conduit can satisfy a number of desired requirements to optimise the potential of conduits for improving nerve repair outcomes. Properties such as biodegradability and bioresorbability are particularly relevant due to the requirement for long-term exposure of the conduit until nerve regeneration is complete. Moreover, chemical and physical modifications of nerve guide conduits can be undertaken to help the regenerative process of regenerating axons and support cells across injury sites. In addition, the conduit should be clinically-compatible, meaning it should be sterilisable, but also easily handled and implantable, features related to the mechanical and physical properties of the material. Importantly, the conduit should provide a functional level of regeneration that is at least on par or better than the gold-standard of an autograft repair, as ultimately the aim is to provide improved regeneration and quality of life for patients affected by nerve injuries. Therefore, the selection of the material and subsequent modifications is of relevance when developing novel nerve guide conduits for peripheral nerve repair.

2.1.2 Polycaprolactone

Polycaprolactone (PCL) is a synthetic polymer that has been widely used in a variety of clinical and biomedical applications, such as for drug delivery (Capronor, (Darney et al., 1989)), or as a suture material (Monacryl; co-blend of caprolactone and glycolide (Bezwada et al., 1995)). The use of PCL in such devices that have FDA approval or CE mark registration suggest that subsequent technologies using PCL may have a faster route to commercialisation and approval.

Polycaprolactone is a polyester which can be synthesised by ring-opening of the monomer ϵ -caprolactone or by polycondensation of 6-hydroxyhexanoic acid (Figure 2.1), where the ring-opening polymerisation is generally preferred (Bartnikowski et al., 2019).

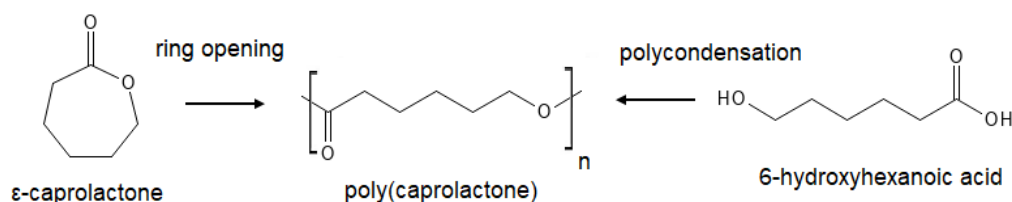


Figure 2.1 Structural formula and reaction scheme of two methods of polymerisation of poly(caprolactone), by ring opening of ϵ -caprolactone or by polycondensation from 6-hydroxyhexanoic acid.

PCL is semi-crystalline polymer with a molecular weight ranging between 3000 – 80000g/mol and is soluble in a wide range of solvents such as chloroform and dichloromethane as well as being miscible with a variety of other polymers. PCL's melting point is between 59 -64 °C. The biodegradability rate of PCL polymer structures has been shown to range between several months to several years depending on the physiological environment and specific structure (Bartnikowski et al., 2019; Ikada & Tsuji, 2000). Long term degradation studies *in vivo* using radioactive tracers bound to PCL have shown that the material did not accumulate in body tissue or organs (Sun et al., 2006). PCL degradation is typically by non-enzymatic chain scission and hydrolysis of the ester links in the polymer chain backbone, resulting in a range of intermediate products (e.g. 6-hydroxylcaproic acid, acetyl coenzyme A) that can ultimately enter the citric acid cycle and be eliminated from the body (Albertsson & Karlsson, 1996; Woodruff & Hutmacher, 2010), therefore making PCL bioresorbable.

These properties make PCL attractive for some applications, for instance drug delivery or as a slowly-degrading suture material. However, depending on the application, functionalisation or copolymerisation of PCL can be conducted to modify its structural properties, and tailor the use of PCL for a wide range of tissue types as well as with different fabrication methods, such as for the fabrication of nerve guide conduits.

2.1.3 Manufacture methods of NGCs

Due to the simplicity of the shape of a nerve guide conduit, that is, a hollow tube, most fabrication methods can be easily employed to create conduits. However, there are a few techniques more commonly used to fabricate conduits.

2.1.3.1 Dip coating

Dip coating involves submerging or dipping a cylindrical mould (e.g. a rod) into a liquid polymer solution, allowing the solution to coat the mould thinly and evenly. The polymer solution is left to dry allowing the solvent to evaporate prior to polymerisation of the material. The dip-coating process can be repeated until a desired coating thickness is achieved and then it is removed from the mould. Early pre-clinical studies using Neurolac, an FDA approved and commercially available nerve guide conduit made from a copolymer of caprolactone and lactide, poly(DL-lactide- ϵ -caprolactone) (PDLC), describe dip-coating for the fabrication of the conduit (Hoppen et al., 1990; Jansen et al., 2004).

2.1.3.2 Solvent casting and injection moulding

In solvent casting, a polymer solution is poured into a mould and left to dry allowing the solvent to evaporate resulting in a mould-shape made from the solidified polymer. For nerve guide conduits, a mould in the shape of a tube can be used directly, or alternatively, a sheet of polymer of given

dimensions can then be further processed, such as by extrusion or compression, into a tubular structure (Widmer et al., 1998).

Alternatively, polymer solutions can also be injected into moulds (injection moulding) which can allow for filling of more complex-shaped moulds where pouring would not be possible. For instance, Sundback et al. (2003) used a tubular mould containing several filaments that were held in place by attaching the ends of the filaments to supports at each side of the mould. Then the polymer solution was injected to fill the space between the filaments and the outside of the tubular mould creating a number of channels for axonal regeneration. Alternatively, other groups have employed 3D printing to create different shaped moulds to also be used in casting/injection moulding (Moore et al., 2006)

2.1.3.3 Additive manufacturing

Additive manufacturing, or 3D printing, is an umbrella term used to describe fabrication techniques that can build a 3D structure in a layer-by-layer fashion. The main technologies used in 3D printing are based on fused-deposition modelling (FDM), stereolithography or selective laser sintering.

2.1.3.3.1 Fused Deposition Modelling (FDM)

Fused-deposition modelling (FDM) is a technology based on melt-extrusion of a polymer through a heated nozzle which extrudes and deposits the polymer in a layer-by-layer fashion onto the platform guided by computer-aided design (CAD) software. Due to the high-temperature deposition, each layer is able to adhere and bond with the next layer until it is constructed and then left to cool and solidify in the desired structure. The technology was developed and patented by Scott Crump in 1989 (Stratasys Inc,(Crump, 1992)) using acrylonitrile butadiene styrene as the polymer, however, since then a variety of polymers have been used such as polylactic acid and polycaprolactone. The scaffold dimensions are limited by the size of the nozzle where the fibres extruded by the construct are typically in the 100-700 μm range. For this reason, a large number of studies using FDM scaffolds have been used mainly for hard tissue like as bone which much success (Schantz et al., 2006)

2.1.3.3.2 Selective Laser Sintering (SLS)

Selective Laser Sintering is based on fusing powder materials with a laser, causing the powder to melt, fuse and polymerise into a 3D construct, again based on a CAD program. A fresh layer of powder is provided through a roller for the sintering of each layer, and the remaining powder (kept at a lower temperature) is used for structural support of the construct. Due to the high resolution that can be obtained with laser beams, SLS allows the printing of more complex and detailed structures. A case study in 2016 used laser sintering of a polyamide material (PA2200) to print a 3D model of a patient's cranium (based on data from computer tomography scans) which helped the clinicians in designing an appropriate and customized implant for the patient based on the model (Fernandes et al., 2016).

2.1.3.3.3 Stereolithography

Stereolithography is based on the use of light to cure a liquid photosensitive polymer via photopolymerisation. A typical stereolithography set-up involves a liquid photosensitive polymer (or resin) contained in a reservoir containing a stage where a light-source is directed on the surface of the resin, either through a top-down or bottom-up approach, allowing polymerisation of the first layer (Figure 2.2). After which, the stage is moved allowing fresh resin to flow over the previously-formed layer, and again a new layer is polymerised and formed. In the top-down approach, light is projected from underneath through a transparent window plate, and the structure is built by the stage moving upwards. In the bottom-up approach, the light is project from above on the stage as it moves down, where a thin layer of resin is illuminated at each time.

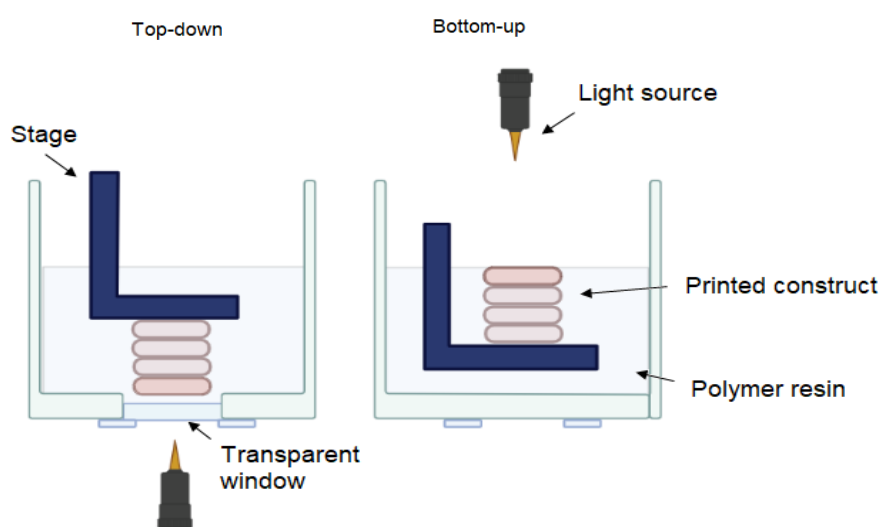


Figure 2.2 Schematic representation of two stereolithography approaches: top-down (left) and bottom-up (right). Made with Biorender.com

The light source is able to photopolymerise the resin guided by a pattern provided in a computer-aided design file, where various designs can be used based on data from magnetic resonance imaging, computed tomography or simple 3D drawing software.

The image can be projected onto the resin either by a computer-controlled beam or by using a digital mirror device (DMD). With the computer-controlled beam, the beam is guided by the CAD file to draw on the resin, often referred to as scanning-stereolithography as the beam scans along the resin. Alternatively, the DMD contains an array of mirrors that can be rotated to an “on” or “off” state where each mirror can represent a pixel on the image and reflect the light beam accordingly depending on the state of the mirrors to produce photopolymerisation at once on all parts of the pattern. Therefore, DMD-based light illumination is significantly faster and can also be employed to build simultaneous structures at one time.

A number of parameters can be modified in stereolithography to control the dimensions of the cured structure, where typically the curing depth is determined by the energy of the light, therefore modifying the intensity of the light source as well as the exposure times can be used to control the thickness of the layers cured. In context, most additive manufacturing techniques produce structures in the 50 – 200 μm range, however, with stereolithography the accuracy can reach 10 μm , with some SLA set-ups using single-photon polymerisation report resolution of up to 0.43 μm . For this reason, additive manufacturing methods are highly appropriate for the fabrication of experimental nerve guide conduits.

The main consideration in SLA is the requirement of a photo-sensitive material. Many materials have been successfully developed for this purpose by functionalisation with acrylate, methacrylate and fumarate groups. In addition, initiation of photopolymerisation of such materials is typically dependent on the generation of a reactive species which is provided by a photoinitiator that is capable of absorbing and converting photolytic energy into a reactive species (e.g. free radical formation). Depending on the photoinitiator system, different wavelengths of light can be used (i.e. UV, visible and IR) and thus different sources for light irradiation can be used, such as xenon, mercury, light emitting diode (LED) lamps or lasers. It is important to note that any residual photoinitiator or uncured resin in the 3D printed structures have to be thoroughly removed as these chemicals can be cytotoxic.

2.1.3.4 Electrospinning

Electrospinning involves the formation of fibres from a polymer solution. Typically, most electrospinning set-ups are made up of a syringe containing the polymer solution, which is topped with a needle and connected to a syringe pump to control the rate at which the solution flows, as well as a high voltage power supply and a grounded collector (Figure 2.3). An electric field is established between the needle and the collector by applying a high voltage, which in turn charges the polymer solution. At a sufficient critical voltage, the electrostatic repulsion created in the electric field will overcome the surface tension of the polymer droplet, thus deforming the polymer ejected by the needle into a conical shape called a Taylor cone before a polymer jet is ejected. The electric field accelerates and elongates the ejected charged polymer jet towards the direction of the collector, during which the solvent in the polymer jet evaporates resulting in the fabrication of solidified polymer fibres which are deposited onto the grounded collector. The fibres can be collected in various ways such as with stationary collectors to produce randomly orientated fibres, or alternatively onto a rotating mandrel set a specific speed to collect aligned fibres.

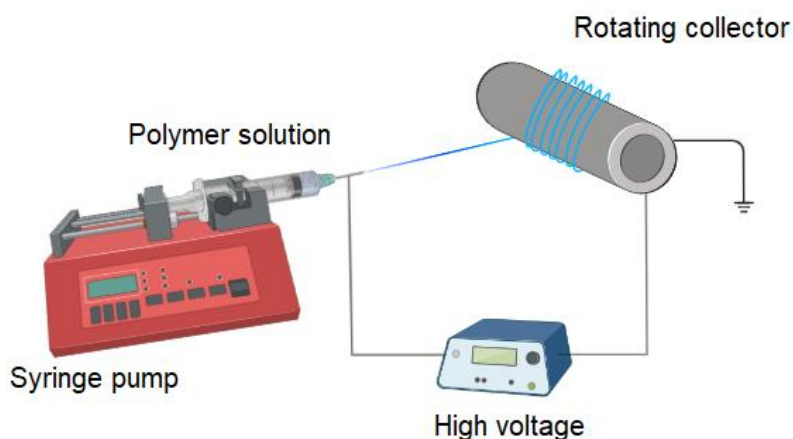


Figure 2.3 Schematic representation of an electrospinning set-up including the syringe pump, the polymer solution contained in the syringe and ejected from the needle towards the grounded collector set to rotate. Made with Biorender.com

2.1.3.4.1 Factors influencing electrospinning

These electrospinning parameters (voltage, flow rate, distance) can be varied to control the formation of the jet and resulting features of the fibres (Cui et al., 2007; Xue et al., 2019; Xue et al., 2017). For instance, the voltage needed to charge a polymer and overcome the surface tension will be dependent on the viscosity of the polymer solution, where higher electrical forces and therefore higher voltages are required to electrospun more viscous solutions (Hu et al., 2011) (Yuan et al., 2017). Moreover, higher voltages will typically result in the formation of thinner fibres, however, with lower viscosity solutions the high voltage can result in the formation of several jets which in turn repel each other and influence the distribution of the fibres on the collector (Demir et al., 2002). The flow rate at which the polymer is ejected can also influence the fibre diameter, typically with increased flow rates resulting in increased volumes of polymer being pumped and thus in the formation of larger fibres (Tang et al., 2014). The distance between the needle and the collector can also influence fibre formation, where a minimum working distance is needed to ensure the jet can elongate and the solidify before reaching the collector, otherwise, the jet will evaporate after reaching the collector and this can lead to morphological changes in the fibres. Typically, longer distances between the needle and the collector result in increased elongation of the fibre jet and thus thinner fibres (Doshi & Reneker, 1993). Lastly, when collecting aligned fibres, increasing the rotation speed of the collector can help with improving fibre alignment (Yuan et al., 2017), although excessive collector rotation can in itself create a turbulent airflow that may affect fibre alignment (Beachley et al., 2012).

Clearly, there is an intricate relationship between all the aforementioned parameters influencing the electrospinning process and the morphology of the resulting fibres, which calls for careful optimisation depending on the polymer. In addition, the selection and preparation of the polymer also require

consideration, as the viscosity of the solution (dependent on the polymer's molecular weight, concentration of the solution and solvent) largely affect the electrospinning process. In fact, in low concentration solutions, the polymer chains are not sufficiently strong enough to maintain the formation of a continuous polymer jet and instead result in “broken” droplets of polymer beads, a process which is known as electrospraying (Doshi & Reneker, 1995; Shin et al., 2001). Alternatively, if the concentration or viscosity of the polymer solution is too high, then difficulties may arise in pumping the polymer solution and in forming a jet (due to not overcoming the increased surface tension of the polymer). The inability to form a jet is also a feature in polymers that are not conductive, however, as mentioned, increasing the voltage may help with fibre formation in high viscosity polymer solutions, and certain additives can be used to increase the conductivity of polymer solutions. In addition, different polymers will have different solubilities depending on the solvent, and the various volatilities of the solvent will also influence the evaporation rate and solidification rate of the polymer jet, affecting fibre morphology. Lastly, the environment and ambient conditions in which the electrospinning set-up is conducted can also affect the electrospinning process, where changes temperature can affect the viscosity of the polymer, and the relative humidity in the air can influence the evaporation and solidification rate of the polymer jet.

2.1.3.4.2 Electrospinning in tissue engineering

As reviewed, by controlling the materials and parameters of electrospinning, the morphology of the resulting fibres can be tailored for specific applications. As different tissues have distinct regeneration mechanisms and challenges, the properties and requirements for any tissue-engineered scaffold must be carefully tuned and tested for each application, and electrospinning has been used extensively in the fabrication of such scaffolds in a large number of fields, including for bone (Lin et al., 2020), skin (Keirouz et al., 2020), cartilage (Chen et al., 2016) and nerve-tissue engineering. This is partly because the high-surface-area-to-volume ratio that can be achieved by electrospun scaffolds is an attractive feature for clinical applications, such as drug delivery (Silva et al., 2007), but also the porosity and structural similarity of fibres to the extracellular matrix allow close matching of the scaffold infrastructure to the morphology of the tissue (Rahmati et al., 2021). In addition, biodegradable materials are often selected to match the regeneration period of the tissue with the resorption of the scaffold.

Electrospun-devices have also been tested in a smaller number clinical trials with promising results, such as in the treatment of oral lichen planus with the Rivelin[®] patch (Afyx), a mucoadhesive patch composed of an electrospun membrane loaded with clobetasol and an electrospun impermeable backing layer, where the results of the Phase 2 study have been recently released demonstrating

safety of the patch as well as ease of use and comfort as reported by the participants (Brennan et al., 2022; Colley et al., 2018).

2.1.3.4.3 *Electrospinning in nerve tissue engineering*

Electrospinning for peripheral nerve tissue applications has been used in two main methods. Firstly, electrospinning can be used as the fabrication method of nerve guide conduit. For example, Panseri et al. electrospun a blend of Poly(DL-lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) onto a copper wire (diameter 1.29 mm) held onto a grounded collector, where the wire was rotated allowing the fibres to coat the wire evenly, resulting in the surface of the conduit (Panseri et al., 2008). Alternatively, fibres can be electrospun into a sheet and rolled into the shape of a tube. For instance, Chew et al. electrospun a blend of poly(ϵ -caprolactone-co-ethyl ethylene phosphate (PCLEEP) fibres onto a flat film of the same material, and rolled the film in two different directions to create conduits with an inner surface of fibres that were either aligned longitudinally or perpendicularly (also referred to as circumferentially) (Figure 2.4) (Chew et al., 2007).

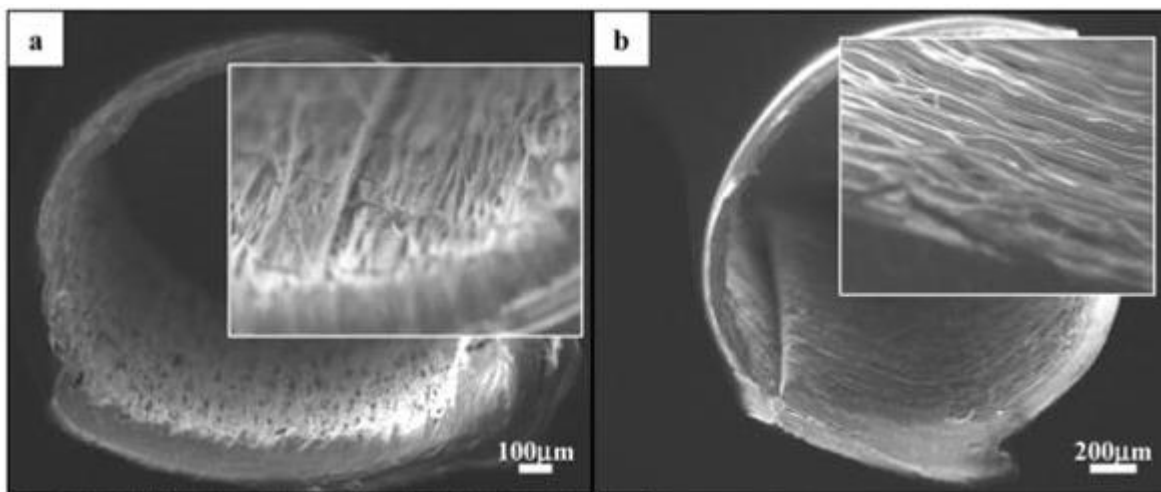


Figure 2.4 Nerve guide conduit with inner surface made of electrospun fibres orientated longitudinally (a) or perpendicularly/circumferentially (b) along the axis of the conduit. Reprinted from Chew et al. Copyright permissions 2023 John Wiley & Sons, Inc.

Similar to the Panseri study, the conduit with the fibres running in a perpendicular orientation to the axis of the conduit can be beneficial as they can provide increased flexibility, as well as high porosity and permeability for nutrient exchange. However, in the Panseri study, the authors reported regular lumen shrinkage at the mid-point of the conduits and in 40% of the implantations into their rat model the conduit had collapsed or been displaced despite being sutured. It is possible that the perpendicular orientation of the fibres limited the elongation or the tensile strength of the conduit, whilst in the Chew study the addition of the extra layer on the outside surface improved the mechanical strength of the conduit, nevertheless mechanical properties were not studied in either of the papers.

The conduits in the Chew study were used repair a nerve gap injury in rats and compared to a hollow, smooth conduit (made by rolling a smooth sheet of PCLEEP), where they found an increased number of regenerated axons had grown in both the longitudinally and randomly orientated fibre-conduit groups compared to the smooth conduit. However, there were no significant differences between the fibre-conduit groups, that is, between the fibres aligned longitudinally vs perpendicularly, therefore the authors suggested that the positive effect of the fibres was mainly due to the increased surface area provided for more cell adhesion. However, it is worthy to mention that the electrophysiological data reported in the study showed very limited functional regeneration with all the conduit groups, therefore while the fibre-conduits may have increased axonal sprouting and cell elongation, it is difficult to conclude that the increased surface area as a result of wall topography had a big effect on regeneration in this study.

Moreover, it is important to note that changing the surface of the wall of the conduit is not the only way to modify surface area to increase cell attachment. An alternative method is by modifying the lumen of the conduit, with early studies dating back to the mid-1990s. In a 1996 study by Goran Lundborg and Martin Kanje reported on a silicone conduit containing 13 nylon filaments in the lumen (filaments of 250 microns in thickness), which was used to repair a 10 mm sciatic nerve gap in a rat model (Lundborg & Kanje, 1996). The authors used a “pinch-reflex” test as a measure of sensory regeneration and found a positive response in only four of the eight animals in the group, which was inferior to repair with a hollow conduit (100% response).

Nevertheless, the study served as a successful proof-of-concept, simply requiring further optimisation of the conduit design. In fact, the same authors repeated the study and addressed some of the limitations, such as reducing the number of filaments in the conduit from 13 to 8 (reducing the space occupied by the filaments from 25% to 15%) and subsequently reported on improved regeneration. In more detail, the second study design was slightly different as it used a critical gap model of 15 mm in the sciatic nerve of the rat (as opposed to 10 mm), which meant that regeneration with a hollow silicone conduit would be unlikely, where regeneration was only seen in 2 out of 8 of the animals treated with the hollow conduit (Lundborg et al., 1997). However, all animals (12 out of 12) treated with the filament-filled conduits showed signs of regeneration, and histological and immunohistochemical studies confirmed the presence of axons along the filament-filled lumen from the proximal to the distal site, suggesting that the presence of the filaments was key in supporting regeneration in their model. Moreover, this paper suggests that there was a specific range at which the inclusion of the filaments was beneficial, whereas using too many filaments would create a physical barrier of “dead space” that would obstruct regeneration.

As such, it makes sense that with the influence of this proof-of-concept, many studies employed electrospinning as the approach to increase surface area within conduits, not only due to the high surface-area-to-volume ratio that can be obtained with micro or nano fibres, but also because of the ease of producing and modifying the properties of the fibres, which can help elucidate on specific mechanisms of surface topography.

In a paper by Kim et al, poly(acrylonitrile-co-methylacrylate) (PAN-MA) was electrospun to create sheets of either aligned fibres or randomly-orientated fibres (400-600 nm), which were then inserted into the lumen of a conduit (Kim et al., 2008). The study compared the effect of the alignment vs random intraluminal fibres on the repair of a nerve gap injury in a rat model and found improved regeneration with the aligned surface morphology. Specifically, a higher number of axons were counted at the distal stump (aligned fibres: ca. 6000 vs randomly orientated: less than 100 axons counted) with comparable results of the aligned group to the autograft control group. Moreover, the compound action potential (CAP) recordings of mixed, sensory and motor nerves were similar between animals in the autograft group and the aligned-sheet group suggesting that the axons that regenerated in the groups were also functional, meanwhile, no CAP measurements were detected for the nerves repaired with the randomly-orientated sheets. The authors reported that, given the surface area was maintained by using fibre sheets of the same material and thickness, enhanced regeneration was due to the improved guidance, extension, and migration of axons and schwann cells on the aligned fibre surface compared to that of the randomly orientated fibres, suggesting a positive effect of longitudinally aligned fibres in nerve guide conduits.

Therefore, it seems that the interplay between surface area and the contact guidance of fibres used within conduits must be carefully selected to provide the best design for regeneration, where increased contact guidance is beneficial as long as the presence of the fibres do not significantly occlude the lumen and obstruct the path of regenerating axons and migrating cells (G Lundborg et al., 1997; Ngo et al., 2003).

2.1.4 Biocompatibility

Biocompatibility is a concept that refers to a variety of interactions between a biomaterial and the host tissue that are dependent not only on the material, but also on the specific application (Williams, 2008). For instance, early implantable medical devices were often designed to be inert and unreactive, in order to reduce adverse effects as a result of the long-term implantation of a foreign object in the human body. In fact, a lot of the requirements of such devices surrounded the idea of safety in terms of the materials being non-toxic, non-carcinogenic, non-irritant, etc. These features, although appropriate and still relevant, limit the meaning of biocompatibility to be defined solely by the

biomaterial, which is not a complete picture, especially in regards to tissue engineering. This is because typically the aim of tissue engineering is to help the regeneration process of a specific tissue, which will differ depending on the tissue type and the application of the scaffold, ergo, various interactions exist between the biomaterial/scaffold and the host tissue that define biocompatibility. Biocompatibility therefore can be evaluated by the chemical, physical, and biological properties of a material and subsequently by the specific response of various host tissues, including on the immune response and the repair and regeneration processes.

A starting point when evaluating biomaterials is typically assessing the cytocompatibility of the material, as well as that of its degradation products or leachable components, with the host tissue. Leachable components are of particular importance when evaluating 3D polymer scaffolds used in tissue engineering, as they can refer to unreacted monomers, uncured resin, photoinitiator residues, or other processing additives that can often negatively impact cells in vitro and in vivo. Cytotoxicity and viability of cells can be assessed qualitatively (e.g. microscopic examinations of cell morphology) or quantitatively (e.g. measuring cell growth, cell death, metabolic activity or protein quantification). Additionally, the physical and chemical properties of a material and scaffold can also be investigated to evaluate biocompatibility. For instance, bulk properties such as the molecular and structural composition of the material can influence the permeability and degradability of the scaffold, as well as mechanical properties such as strength and stiffness. These properties in turn can influence local and systemic immune responses that may be favourable or detrimental to the regenerative process. The surface properties of a material are also key as most tissue-engineering approaches rely on cells to physically come into contact with the scaffold, and at the surface of the scaffold, or the cell-material interface, the activity of cells regarding adhesion, proliferation or differentiation can be modulated by surface modifications. Surface properties can be assessed by looking at surface chemistry, such as wettability properties, surface charge as well as surface topography (roughness, patterning), all of which can influence cell absorption, morphology and migration of cells on scaffolds (Amani et al., 2019).

2.1.5 Summary and Chapter Aims

Polycaprolactone is an FDA-approved material that benefits from appropriate properties such as biodegradability, and can be easily functionalised to use in photo-polymerisation systems for the fabrication of conduits. Various physical and chemical modifications have been shown to possess great potential in improving the function of nerve guide conduits, such as incorporating intraluminal fibres to provide increased adherence and contact guidance to regenerating axons and Schwann cells.

Therefore, this chapter aims to develop a nerve guide conduit made from a photocurable version of PCL, containing intraluminal aligned fibres made by electrospinning.

Aims:

1. Fabricate a nerve guide conduit made from methacrylated-PCL (PCL-MA) using stereolithography and injection moulding.
2. Assess the cytocompatibility of PCL-MA *in vitro* with NG108-15 neuronal cells and evaluate mechanical properties of PCL-MA bulk material
3. Produce aligned PCL fibres using electrospinning to use as intraluminal fillers in conduits and treat with air plasma to increase wettability to improve cell attachment
4. Combine PCL fibres and conduits and validate the method for fibre packing density using gas pycnometry

2.2 Methods

2.2.1 Nerve guide conduits fabrication

Nerve guide conduits were fabricated via a home-made microstereolithography set-up, using a photocurable methacrylated-polycaprolactone as the polymer.

2.2.1.1 Polymer preparation

Photocurable polycaprolactone (PCL) was obtained by methacrylation of the hydroxyl terminal groups of PCL triol (Mn: 900 g/mol, Sigma) (Fig. 2.2). The methacrylation protocol was based on the study by Field et al. (2022) which was based on the procedures by Elomaa et al. (2011) (Elomaa et al., 2011) (Field et al., 2021). For purposes of this project due to time constraints, methacrylated polycaprolactone was provided by a colleague in the lab, Mr Boyang Liu.

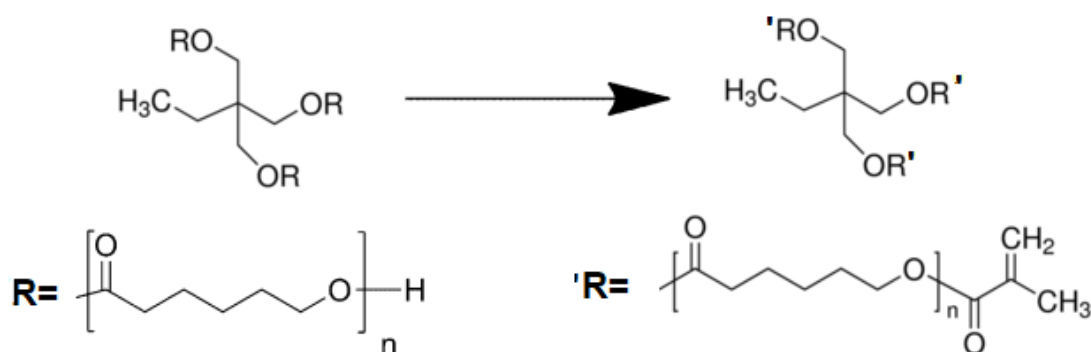


Figure 2.5 PCL triol (left) was dissolved in DCM and reacted with MAA and TEA to produce methacrylated PCL (right). Image adapted from (Elomaa et al., 2011)

2.2.1.1.1 Methacrylation

PCL triol (Mn=900g/mol, Sigma) was dissolved in dichloromethane (DCM, Fisher Scientific, Loughborough, UK) and reacted with methacrylic anhydride (MAA, Sigma), a methacrylating reactant, in the presence of trimethylamine (TEA, Sigma) under nitrogen purging. The solution was continuously stirred at 0°C (using an ice-bath) to cool down the synthesis reaction. The reaction was left stirring for 20 hours in the dark and a two-fold molar excess ratio was used to produce a methacrylation degree of 50% of polycaprolactone.

The methacrylated-PCL was washed three times with 1M hydrochloric acid (Fisher Scientific), thus removing a substantial amount of DCM from the polymer and two times with deionised water to remove any remaining hydrochloric acid. Next, the remaining solvent was further removed from the polymer through rotatory evaporation. Subsequently, methanol washes were conducted to purify the methacrylated polymer, which consisted of washing the polymer solution with methanol by mixing

the solution together, leaving the solution mixture to separate into two layers at -80°C overnight and discarding the supernatant layer containing contaminants (mainly the solvents). This was repeated a total of five – seven times, each time with new methanol. Finally, the solution was brought back to room temperature, resulting in a clear liquid, before rotatory evaporation was used to remove any final remnants of methanol, resulting in a viscous methacrylated-PCL (PCL-MA) polymer mixture.

2.2.1.1.2 Addition of photoinitiator

In order to use methacrylated-PCL in microstereolithography, a photoinitiator was added to produce photocurable methacrylated-polycaprolactone (PCL-MA). The photoinitiator used was a commercially available blend of diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) and 2-hydroxy-2-methyl propiophenone (Sigma), which was used as 2%. The solution was mixed at 200 rpm for 20 minutes followed by 20 minutes of sonication in a water bath.

2.2.1.2 Conduit fabrication methods

Throughout the progress of the PhD project, a number of conduit fabrication methods were evaluated: microstereolithography-based printing of conduits and injection-moulding casting of conduits.

2.2.1.2.1 Microstereolithography 3D printing

The home-made microstereolithography set-up consisted of a Digital Micromirror Device (DMD, Texas Instruments Incorporated), a 405 nm tuneable Mounted LED light source (ThorLabs, M405LP1) or a 405 nm violet laser (Vortran Stradus, Vortran Laser Technology Inc), and a motorised z-axis stage (Thorlabs Ltd) (Figure 2.6)

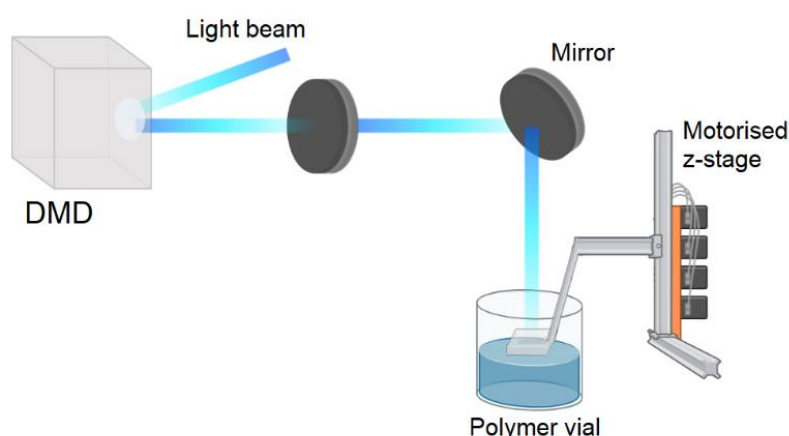


Figure 2.6 Graphical illustration of the micro-stereolithography setup used to fabricate nerve guide conduits. The set-up consists of a light-source derived from either a laser or LED lamp, a digital micromirror device (DMD), a motorised z-stage (C) and a glass vial containing a liquid photocurable polymer. Image created with BioRender.com

A cross-sectional image (1024 x 786 pixels) of a conduit was uploaded to the DMD. The light beam was shone on to the surface of the DMD and reflected on to a series of lenses and mirrors which in turn allowed the cross-sectional conduit image to be reflected onto the surface of the polymer solution contained in a glass vial. The motorised z-stage was lowered in the polymer-containing glass vial, allowing photo-polymerisation of the solution to be carried along layer by layer, until the 3D conduit structure was fully produced.

2.2.1.2.2 *Injection moulding of conduits*

Injection moulding was investigated as a method for conduit fabrication as described in Figure 2.7. Briefly, PCL-MA was injected through a small opening in the silicone tubing, filling the space between the tubing and the hypodermic needle. The hypodermic needle (20G, Microlance) was chosen to match the internal diameter of the conduit (0.9mm), while the space filled by the polymer between the silicone tubing (ID: 1.2mm) and the needle would give rise to the wall thickness of the conduit. The whole construct was cured under UV light (OmniCure S1000, Exfo) for 5 minutes at 100% power and disassembled to retrieve the hollow conduit.

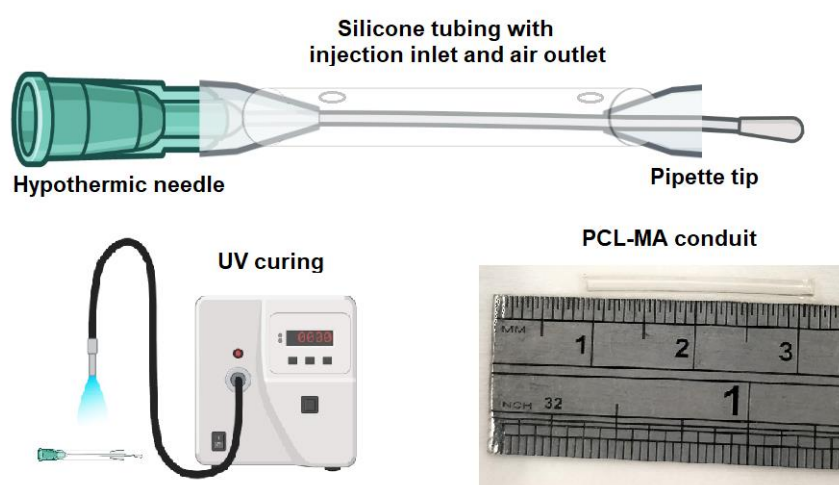


Figure 2.7 Schematic representation of the injection moulding printing method for conduits. Image created with BioRender.com.

2.2.1.3 *Conduit washing and sterilisation*

Conduits were placed into a solution of 100% isopropyl alcohol for 1 - 2 weeks to remove any excess photoinitiator and uncured polymer from the conduit. The solution was changed daily. For sterilisation, the conduit was sterilised with 70% ethanol for at least 1 hour and then washed in deionised water three times and overnight before use. The conduits were kept in sterile deionised water until used in *in vivo* studies.

2.2.1.4 Imaging of conduits

Conduits were imaged using an optical microscope (Stemi 305, Zeiss, equipped with Axiocam 105 camera) or scanning electron microscopy (SEM) (Inspect F50, FEI). Prior to SEM imaging, tubes were sputter coated with gold (SC500, Emscope). Image analysis was carried out in ImageJ (NIH)

2.2.2 Characterisation of PCL-MA constructs

2.2.2.1 Dog bones preparation

PCL-MA “dog bones” were prepared for tensile testing according to specifications from ASTM D638, (referred to as dumbbell-shaped test specimens in the ASTM D638 (Matrix & Specimens, 2006).

In order to produce the dog bones, negative moulds made using silicone were used. Firstly, dog bone test pieces were printed with a commercially available 3D printer (Form 1, Formlabs) and glued to the bottom of a petri dish. Then, a 1:10 ratio of silicon elastomer curing agent to silicon elastomer (Sylgard) was prepared by mixing vigorously and centrifuged at 1000 rpm for 5 minutes to remove air bubbles from the mixture. The silicone mixture was slowly poured into the petri dish to surround and cover the dog bone pieces. The petri dish was then cured at 60°C for 2 hours. The cured silicone constructs were removed from the petri dish to reveal a negative silicone-mould for dog bone preparation. The PCL-MA polymer was poured into the negative silicone mould and the construct was cured with a UV lamp (OmniCure, S2000) at 100% power for 5 minutes on both sides. The cured PCL-MA dog-bone constructs were removed from the mould and briefly washed in methanol and left to dry prior to mechanical testing (Figure 2.8).

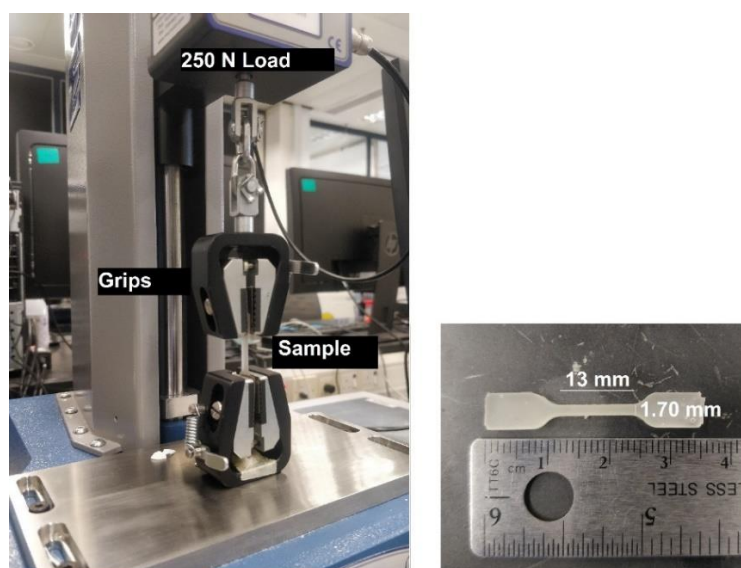


Figure 2.8. Mechanical testing equipment showing the PCL-MA “dog bone” sample being held in between the grips of the mechanical tester.

2.2.2.2 Tensile testing

The mechanical properties of PCL-MA dog bones were measured using a MultiTest-dV tester (Mecmesin, Slinfold, UK) (Figure 2.8).

The PCL-MA dog bone pieces were attached to the grips of the mechanical tester and clamped in place, and the tensile strength of the samples was tested using a 250 N load cell with an applied strain rate of 1 mm/s. The dimensions (thickness, gauge length and width) of each individual sample were input into the operating software (Vector Pro software, Mecmesin) and used to obtain load/displacement data, and stress-strain data. The Young's Modulus was calculated for each sample as the linear (or elastic) region of the stress-strain curve. The maximum elongation, expressed as a percentage of the original length, was also measured.

2.2.3 Preparation of PCL-MA discs for cytocompatibility testing

PCL-MA flat surfaces were prepared by spin-coating the polymer onto glass coverslips using a commercial spin-coater (Laurell Technologies Corporation). The glass coverslips were first functionalised with air plasma to allow binding of the polymer with the glass. Then, the polymer was spun at 3000 rpm for 30 seconds to produce an even coat of polymer on the glass coverslip (ID = 1.3mm). The polymer was then cured under a UV lamp for 5 minutes (100%). The PCL-MA substrates were washed in IPA to remove uncured photoinitiator for 1 week prior to cell culture studies. PCL-MA discs were sterilised with 70% ethanol for 1 hour and washed 3 times with deionised sterile water (dH₂O). PCL-MA discs were kept in sterile dH₂O until further use in cell culture studies to evaluate cytocompatibility with a neuronal cell line.

2.2.4 Cell culture

For cell culture studies, aseptic techniques were adopted in a class II biological safety cabinet equipped with laminar airflow.

2.2.4.1 General cell culture

Cells were expanded in T75 flasks, where they were cultured with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 10% foetal bovine serum (FBS, v/v), 5% amphotericin B (v/v), 5% L-glutamine (v/v) and 1.25% penicillin and streptomycin (v/v) (complete media), unless otherwise specified.

2.2.4.2 Cell passaging and seeding

Cells were cultured in T75 flasks until 80 - 90% confluent, thereafter ready for passaging. A volume of 5 ml of trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA, T3924, Merck) was added per flask for a 5-minute incubation period at 37°C. Cells were detached from the surface of the T75 flask with the aid of gentle tapping and the Trypsin-EDTA was deactivated by adding 10 mL of growth medium per flask. The cells were transferred to a universal tube and centrifuged at 1000 rpm for 5 minutes or until the formation of a pellet. The supernatant was removed, and the pellet was re-suspended in 1 ml of growth medium.

For expansion and cell passaging purposes, 10 µL of the cells were directly counted using a hemocytometer (Hawksley, UK) by counting the number of visible cells in the four corner squares and averaging. For cell seeding purposes, such as in experiments, 10 µL of the cells were first diluted with 10 µL of Trypan blue (1:1 dilution) before counting only viable cells, which were distinct from dead cells due to the preferential staining of Trypan blue in dead cells.

2.2.4.3 Cell freezing and resurrection

For cell freezing, cells were detached from confluent flasks and centrifuged as described before, but re-suspended in “freezing media”. The freezing media consisted of 10% dimethyl sulfoxide (DMSO) and 90% FBS. Cells were pipetted into cryovials at 1 ml per vial and transferred into a freezing container (Nalgene® Mr Frosty, Sigma) for 24 hours in -80°C. Following, cells were either kept in -80°C for short-term storage (less than 3 months) or stored in liquid nitrogen for long-term storage (more than 3 months). Generally, one confluent T75 was used for three cryovials.

For resurrection of cells, cryo-vials were transferred to a warm water bath (37°C) until the contents of the cryo-vial had semi-thawed. Then, the cells were transferred to a universal tube containing 25mL of media and centrifuged to remove the DMSO. Cells were then transferred to T75 flasks for cell culture and expansion.

2.2.4.4 Cell fixing

Depending on the experiments, cells (with or without scaffolds) were washed with PBS. Then, cells were fixed with 3.7% paraformaldehyde (v/v) for 15-20 minutes and washed 2 – 3 times with PBS. All steps were done on a rocker and at room temperature.

2.2.4.5 Immunolabelling

Cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS (PBS-T) for 20 minutes (2 washes, 10 mins each) and incubated with Dapi (0.2mg/ml, D8417, Sigma) and Phalloidin-FITC prepared in PBS (10ug/ml, P5282, Sigma) to label cell nuclei and F-actin in the cytoskeleton, respectively, for 1 hour.

2.2.4.6 NG108-15 cell culture

NG108-15 neuronal cells, a mouse neuroblastoma x rat glioma hybrid, were obtained from the European Collection of Authenticated Cell Cultures (ECACC, UK). Cells were cultured until 80% confluent in complete media (DMEM + FBS + P/S + G + F). Cells were detached with trypsin, centrifuged and counted using a haemocytometer.

For experiments on PCL-MA discs for cytocompatibility, 5000 cells were seeded in 150µL of complete media overnight to allow cell attachment to the surfaces. Cultures were then topped up with complete media to 500 µL before changing the culture to serum-free growth media (after 48 hours from initial seeding) to encourage the formation of neurites.

2.2.4.7 Metabolic assay

Metabolic activity was measured using the Resazurin (BioReagent) fluorometric reduction assay. The resazurin salt was diluted to a concentration of 0.025mg/ml in media. Cell culture media was removed from cells and replaced with media containing resazurin salt. As a non-toxic, permeable compound, the non-fluorescent resazurin would be reduced by living cells to a highly detectable fluorescent dye, resorufin. After 4 hours of incubation, the media was pipetted into 96-well plates in triplicate for every sample, and the fluorescent signal of resorufin was measured at 530-570 nm (excitation) and 585-595 nm (emission), giving a measurement of metabolic activity.

2.2.5 Fluorescence microscopy

For fluorescence microscopy, a Leica DMI8 inverted microscope at the School of Clinical Dentistry (University of Sheffield) was used. The microscope was equipped with a LED 3 fluorescence light source, offering white light in the 390 – 680 nm range, and three filter cubes for epifluorescence illumination of FITC, Cy3 and LED 405. Images were captured using the microscope's FlexaCam C1 Camera system and obtained and processed using the Leica Application Suite X (LAS X) software.

Further image processing was done in ImageJ (NIH). For cell culture studies involving cell counting analysis, images were taken from the whole wells and 3 separate sections per image were analysed manually using the cell counter plug-in. This was repeated for all samples and in 3 replicates at least (N=3, n=3)

2.2.6 Electrospinning

The fabrication of aligned polycaprolactone (PCL) fibres was achieved through electrospinning. The electrospinning set-up consisted of a syringe pump (WPI-Europe), a voltage power supply (Genvolt UK) and a motorised rotating cylindrical collector (IKA works) (Figure 2.9).

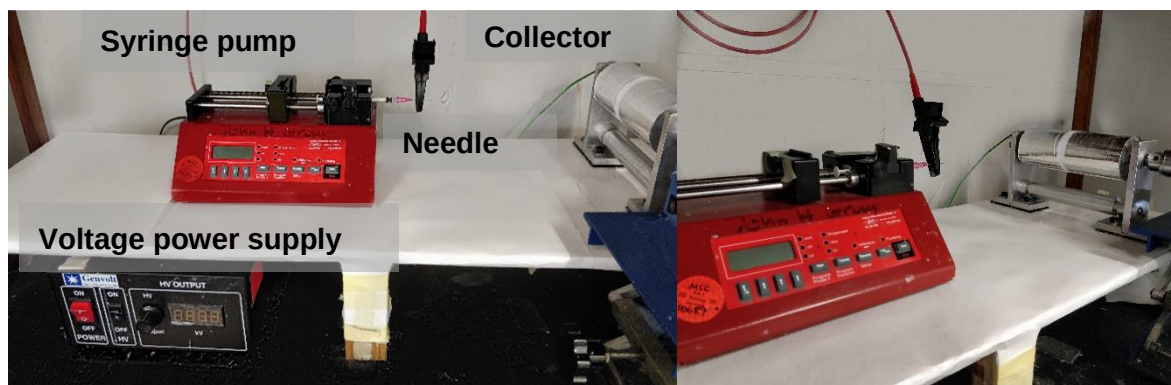


Figure 2.9 Electrospinning set-up for the fabrication of PCL fibres.

Polycaprolactone (Mn 80,000 g/mol, Sigma-Aldrich) was dissolved at a concentration of 20% (w/w) in dichloromethane (DCM). A plastic syringe (Becton Dickinson, 1ml) was used to draw the polymer solution before being topped with a hypothermic blunt needle (20G). The PCL-loaded syringe was connected to the syringe pump and supplied with voltage in order to produce PCL fibres. Fibres were collected on a sheet of aluminium foil wrapped around the earthed rotating collector set at a speed of 2000 rpm. The specific conditions used to fabricate PCL fibres can be found in Table 3.

Table 3 Summary of electrospinning parameters used to fabricate PCL fibres.

Needle-to-collector distance (cm)	PCL concentration (in DCM)	Solvent	Duration (min)	Flow-rate (ml/h)	Voltage (kV)	Collector speed (rpm)	Diameter (μm)
20	20%	DCM	4	6	18-20	2000	8-10

2.2.7 Air plasma treatment

Samples were treated with air plasma in a commercial Zepto plasma system (Diener Electronic) equipped with a 40 KHz radio frequency generator. The sample was placed in the chamber, where the pressure was evacuated to 0.2 mbar to remove any contaminants and subsequently flooded with ambient air. The inner pressure was maintained at 0.4 mbar, and air plasma was generated at a power of 50 W for a treatment duration of 60 seconds.

2.2.7.1 Water droplet contact angle

A Drop Shape Analyser (DSA 100, Kruss, Germany) was used to analyse the wettability property of surfaces. Briefly, a 1 ml syringe was used to deposit a 5 μ L droplet of deionised water on a given surface. The position of the water droplet was captured using the attached high-resolution camera, allowing the measurement of the contact angle between the water droplet and the solid surface which was indicative of a hydrophobic (angle > 90°) or hydrophilic (angle < 90°) surface (Figure 2.10). Three to five contact angle measurements were taken per sample, in triplicate.

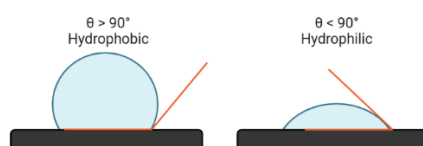


Figure 2.10. Schematic representation of the water contact angle to indicate a hydrophobic or hydrophilic surface.

2.2.8 Insertion of PCL fibres into nerve guide conduits

PCL fibres were inserted into conduits through a manual threading procedure. A strip of the fibres was attached to the end of a needle and then threaded through the conduits one by one. A blade was used to cut the fibres between each conduit, giving individual nerve guide conduits containing the same packing density of PCL fibres (Figure 2.11).

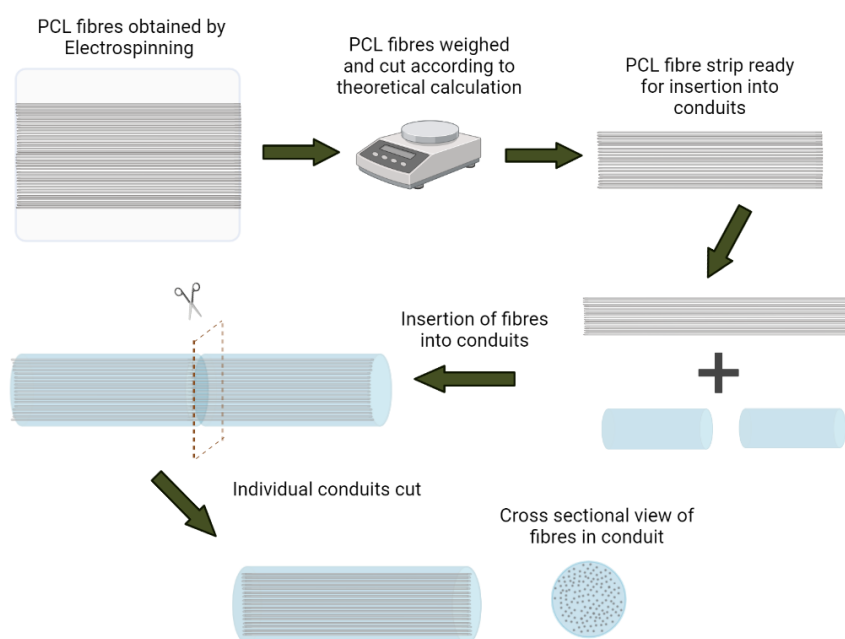


Figure 2.11 Schematic representation of manual combination of electrospun PCL fibres with nerve guide conduits via threading. A blade was used to cut individual nerve guide conduits (red dotted lines).

2.2.8.1 Fibre packing density calculation

The fibre packing density refers to the volume of space in the lumen of a nerve guide conduit taken up by the fibres. This relationship can be described as:

$$\text{Fibre packing density} = \frac{\text{Volume of PCL fibres } (V_s)}{\text{Volume of conduit } (V_c)} \times 100$$

In order to calculate the packing density, two volumes are needed: the luminal volume of the conduit and the volume of the fibres, which were calculated as follows.

For a conduit of a given inner diameter and length, we can calculate the luminal volume using the following equation:

$$(\text{volume of lumen}) V_c = \pi r^2 \times l$$

For a packing density of x , it follows that the volume of fibres within a conduit have to be $x\%$ of the volume of the conduit:

$$(\text{volume of fibres}) V_f = \frac{x}{100} \times V_c$$

Using the theoretical volume of the fibres, an equivalent mass can be calculated using the following equation:

$$m = \rho \times V_f,$$

where ρ is the density of polycaprolactone

Thus, for a chosen fibre packing density of $x\%$, a theoretical mass can be calculated as described above and used to obtain a fibre bundle to insert into the conduit.

This method was used by selecting a given packing density e.g. 10% and using the conduit dimensions to calculate the relevant mass of the fibres to be used as a filler in the conduit. The fibres were then analysed by gas displacement pycnometry for validation of the method.

2.2.8.2 Pycnometry

A gas displacement pycnometer (AccuPyc II 1340, Rodwell) was used to measure the packing density of the electrospun PCL fibres within the conduits. The PCL fibres to be inserted into a given conduit were placed in the sample chamber of the pycnometer system where helium gas was pumped, allowing the inert gas to fill any pores in the sample. Any gas unable to fill the chamber fully, due to the presence of the sample, was displaced into a second chamber where volume measurement was conducted, thus giving the gas displacement volume. The system was then capable of using the

volume of the displaced gas, along the pressure equilibrations of the chambers, to calculate the equivalent volume of the sample using the following equation:

$$V_s = V_1 - \frac{V_2}{\frac{P_1}{P_2} - 1}$$

where V_s is the sample volume, V_1 and V_2 are volumes of the first and second chamber, respectively and P_1 and P_2 are gas pressures of the first and second chamber, respectively.

Thus, it follows that the measured volume of the sample (V_s) i.e. the fibres, should be equivalent to volume calculated using the theoretical method described above.

2.2.9 Statistical analyses

Statistical analyses were made using GraphPad Prism (GraphPad Software v9, San Diego, CA). Data was tested for normality and analyses of variance (ANOVA) was used to test for differences between groups for parametric data. For data with one dependent variable a one-way ANOVA was used and for two or more dependent variables a two-way ANOVA was used. Experiments were replicated independently three times ($N = 3$), and within each replicate, three separate samples were collected ($n = 3$) unless reported otherwise in the text. Post-hoc multiple comparisons were tested with Tukey's.

2.3 Results

2.3.1 Nerve guide conduit fabrication

2.3.1.1 Polymer preparation

Polycaprolactone methacrylate (PCL-MA) was kindly synthesised in-house and provided by Mr Boyang Liu, from Prof. Fred Claeysens' Research Group in the Biomaterials Department of the Kroto Research Institute (Material Science and Engineering).

2.3.1.2 Mechanical testing of cured PCL-MA

Polycaprolactone methacrylate (PCL-MA) was used to make dog bones as detailed in Methods 2.2.2.1 and tensile strength of the samples was tested. Specifically, different batches of PCL-MA were provided throughout the project (denoted as batches 1, 2 and 3) and used to make dog-bones to compare the mechanical properties of each batch of material.

The Young's Modulus was calculated for each sample as the linear region of the stress-strain curve. The Young's Modulus of the tested batches 1, 2 and 3 were 3.719 ± 0.113 MPa, 7.94 ± 0.7173 MPa and 13.94 ± 0.416 MPa, respectively. There was low variation in the Young's Modulus calculated from samples within each batch, as evident in the small standard deviations (Figure 2.12). However, there was a significant difference in the Young's Modulus between different batches of PCLMA produced (1 vs 2, 2 vs 3, 1 vs 3, $p < 0.0001$).

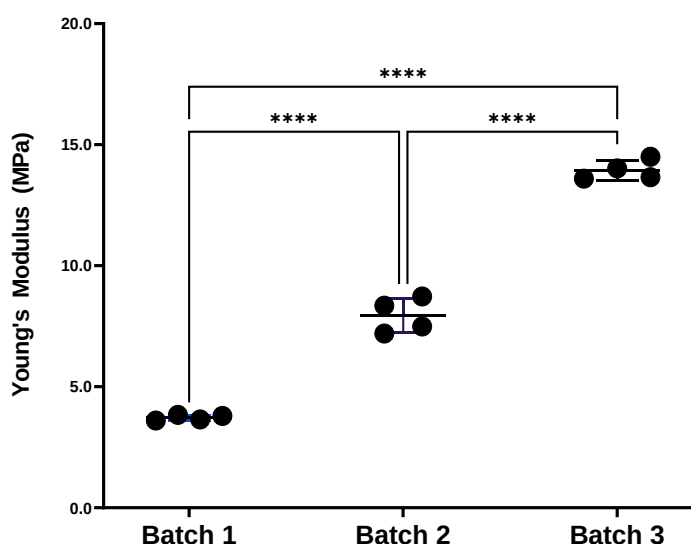


Figure 2.12 . Mechanical tensile testing of cured dog-bones structures made by different batches of PCL-MA (Batch 1, 2 and 3) was performed to calculate the Young's Modulus of each batch. Values displayed mean $\pm$ SD. **** denotes a significant difference ($P < 0.0001$) assessed by 2-way ANOVA with Bonferonni multiple comparisons. (N=1, n=4)

2.3.2 Optimisation of conduit printing methods

Photocurable polycaprolactone methacrylate (PCL-MA) was used with 2% photoinitiator to fabricate nerve guide conduits. Three nerve guide conduit fabrication methods were trialled throughout the project: microstereolithography-based printing (LED and laser light sources) and injection moulding.

2.3.2.1 *Microstereolithography: LED lamp*

In LED-based microstereolithography (LED-SL) an LED lamp was used as the light source in the microstereolithography set up described in Methods 2.2.1.2.1. The LED lamp was tested at powers ranging from 20 to 90% alongside various stage speeds (0.01 – 0.07 mm/s) for the initial optimisation runs (Figure 2.13). At lower LED powers (20 - 50%) and high stage speeds (0.03 – 0.07mm/sec) there was either no formation of a 3D construct, or there were holes present in the walls of the conduit. At low stage speeds (0.01 – 0.03 mm/s) but high lamp powers (60% - 90%) the constructs also failed, and some conduits were not hollow throughout due to over-curing of the walls. It was found that lower stage speeds (0.01 – 0.03mm/s) and lamp powers at 30 – 40% resulted in more promising conduits. However, upon closer inspection, many conduits still printed with holes in the walls (although not always) and there was a difference in the thickness of the walls at one end of the conduit (Figure 2.14). In an attempt to address these issues, PCL-MA was pre-heated to 50°C prior to printing, however, this resulted in overall thinner conduits and more variable conduits compared to printing with room temperature PCL-MA (Figure 2.15, Figure 2.16). It was found that PCL-MA used at room temperature and using a stage speed of 0.01 mm/s and a lower power of 20 - 30% produced the best conduits without any holes.

Power Stage	20	30	40	50	60	70	80	90
0.01								
0.02								
0.03								
0.04								
0.05								
0.06								
0.07								

Figure 2.13. The effect of LED power (%) and stage speed (mm/s) on the quality of PCL-MA nerve guide conduits produced with microstereolithography

PCL-MA printing at room temperature

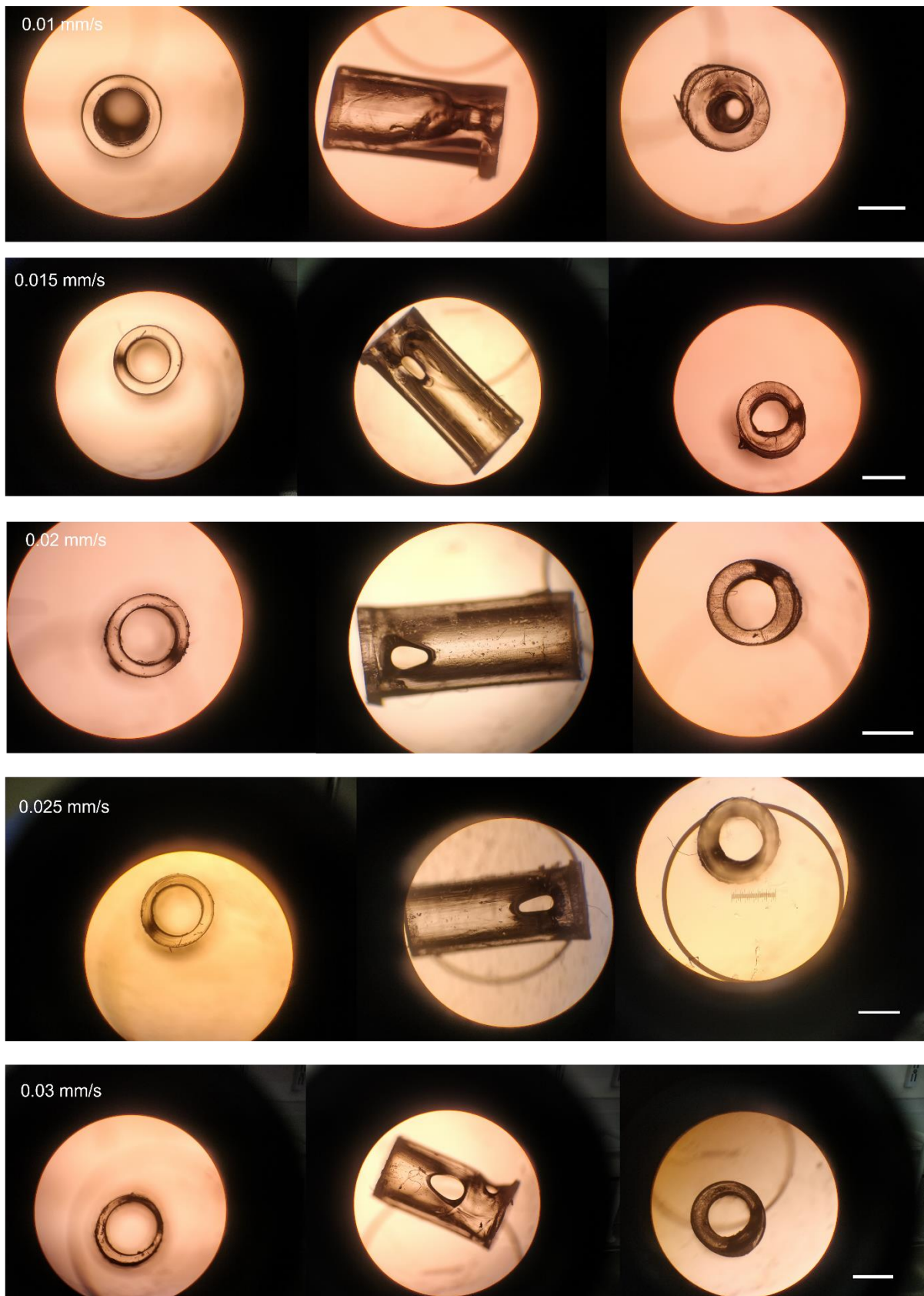


Figure 2.14 The effect of stage speed (mm/s) on the quality of nerve guide conduits produced with microstereolithography using PCL-MA at room temperature. Scale bar 1 mm.

Printing with pre-heated PCL-MA

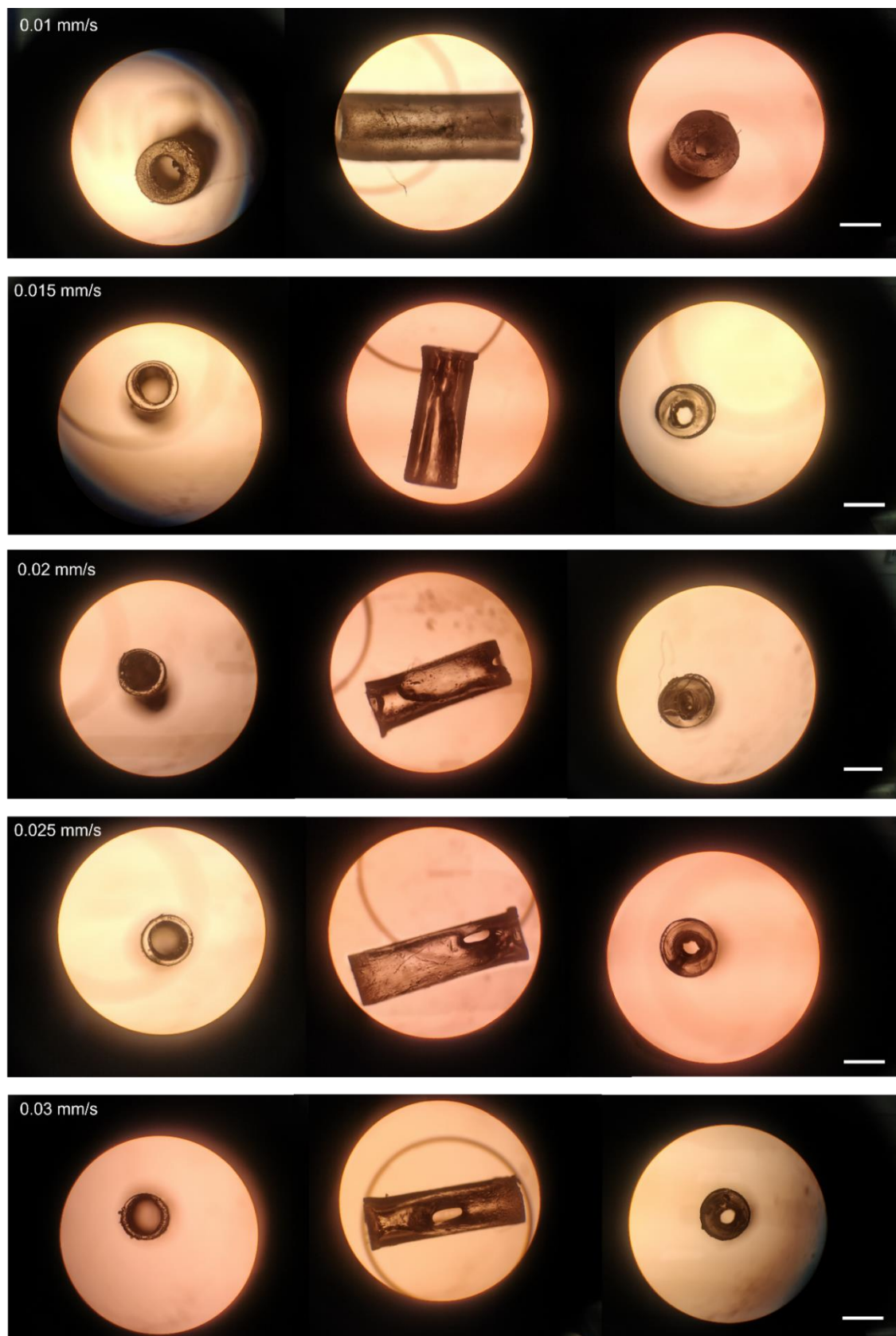


Figure 2.15 The effect of stage speed (mm/s) on the quality of nerve guide conduits produced with microstereolithography using PCL-MA pre-heated to 50°C. Scale bar 1 mm.

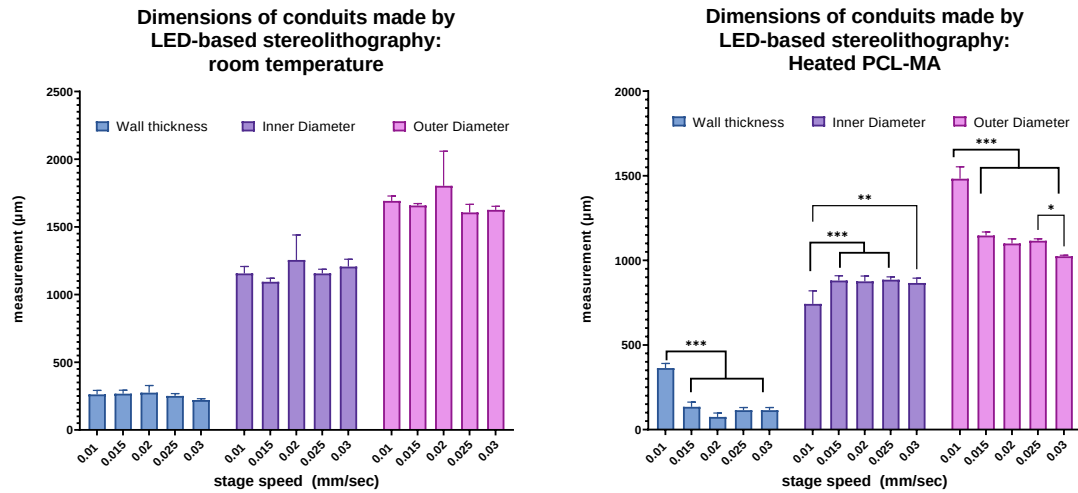


Figure 2.16 Comparison of dimensions of nerve guide conduits made by microstereolithography using PCL-MA at room temperature or pre-heated PCL-MA. Values displayed as mean $\pm$ SD. * denotes a significant difference assessed by 2-way ANOVA with Tukey's multiple comparisons, where $p < 0.05$ for *, $p < 0.005$ for **, $p < 0.0001$ for ***.

2.3.2.2 Laser-based microstereolithography

Laser-based microstereolithography (Laser-SL) was the original method selected for nerve guide conduit fabrication as this set-up had been previously optimised and used extensively in our own group over several years to successfully produce nerve guide conduits (Field et al., 2021; Pateman et al., 2015). Laser-SL offers high reproducibility and low variation in the conduits fabricated, as demonstrated in the SEM image (Figure 2.17) and the low standard deviation of the inner and outer diameters of the conduit and the wall thickness measurements (Figure 2.18).

However, this method was only used to provide a small number of conduits suitable for characterisation because the use of 3B lasers, and therefore the use of this technique, became restricted in the department during this PhD project and therefore by the time conduits were required for the final *in vivo* chapter an alternative technique had to be employed.

2.3.2.3 Injection moulding

An alternative conduit fabrication method investigated was injection moulding using silicone tubing and a hypodermic needle as described in Methods 2.2.1.2.2. As visible in the SEM image in Figure 2.17, there was a variability in the wall thickness of conduits produced by injection moulding, with one side of the conduit having a thicker wall. This is also demonstrated in the larger standard deviation of the wall thickness measurement of injection moulding conduits ($334 \mu\text{m} \pm 159$) compared to conduits made via microstereolithography, both by Laser ($267 \mu\text{m} \pm 17$) or LED lamp ($267 \mu\text{m} \pm 71$) (Figure 2.18). However, there were no significant differences in the dimensions of the conduits fabricated by the three methods.

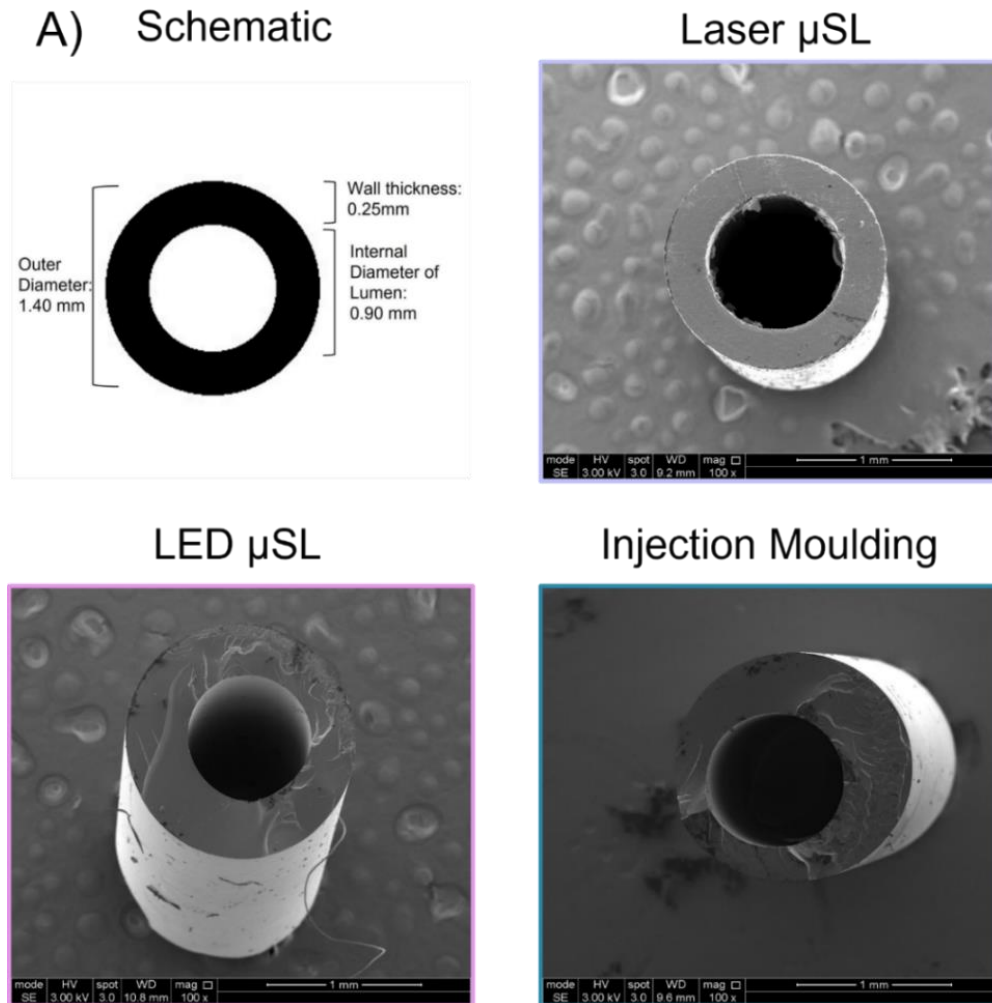


Figure 2.17 Scanning electron microscopy (SEM) images of PCL-MA conduits fabricated by three methods: Laser-based microstereolithography (Laser μ SL), LED-based stereolithography (LED μ S) and Injection Moulding. The conduits were printed following the dimensions shown in the schematic (A).

Comparison in dimensions of conduits fabricated via stereolithography and inject moulding

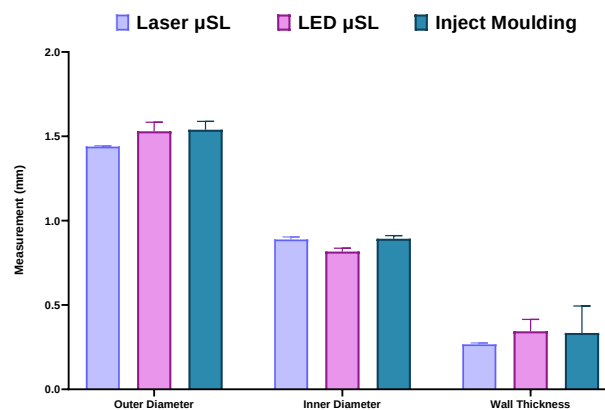


Figure 2.18 Dimensions of nerve guide conduits made from PCL-MA produced by three methods: : Laser-based microstereolithography (Laser μ SL), LED-based stereolithography (LED μ S) and Injection Moulding. Values displayed mean $\pm$ SD. (N=3, n=3)

2.3.3 Cytocompatibility of PCL-MA with a neuronal cell line.

In order to assess the potential use of methacrylated PCL-MA for the fabrication of nerve guide conduits in nerve repair, the cytocompatibility of the polymer was firstly checked *in vitro*. Briefly, PCL-MA was spin coated to create thin films of the polymer (Methods 2.2.3) and cultured with a neuronal line, NG108-15 neuronal cell line, and assessed for cytocompatibility. Specifically, cells were seeded and grown on PCL-MA films and the metabolic activity of the cell line was measured after 48 hours and compared to TCP and glass. There was no significant difference in the metabolic activity of cells cultured on different PCL batches compared to TCP or glass (Figure 2.19).

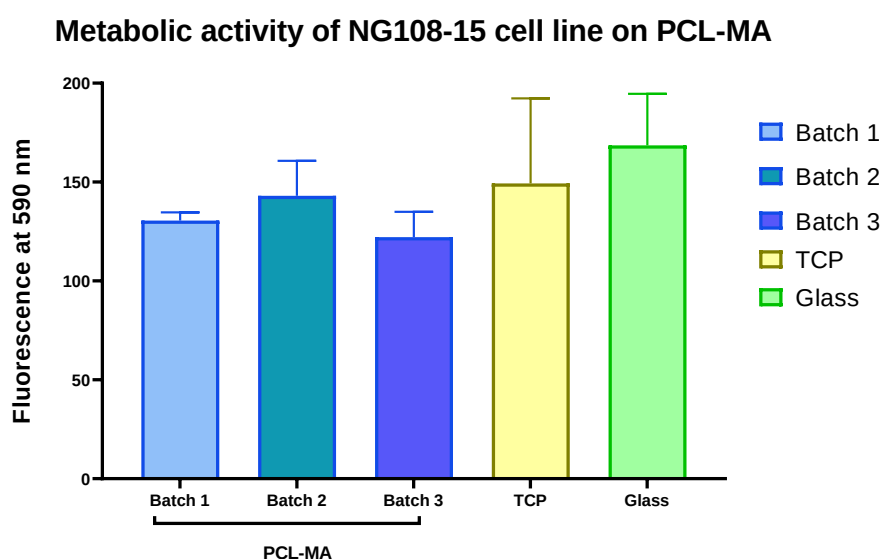


Figure 2.19 Metabolic activity of NG108-15. Neuronal cells were cultured on different batches (Batch 1, 2,3) of PCL-MA and tissue culture plastic (TCP) and glass and the metabolic activity was measured after 48 hours using a resazurin assay. Values displayed mean ± SD. N=3, n=3

In addition, cells were fixed and stained for cell nuclei and the cell attachment was assessed by counting the Dapi-stained nuclei after 5 days of cell culture on different PCL batches and compared to TCP (relative attachment) (Figure 2.20). There was a significant difference in the attachment of NG108-15 cells on TCP compared to the PCL batches ($P < 0.05$), but there was no significant difference between the attachment of cells on the different PCL batches (1 vs 2, 2 vs 3, nor 1 vs 3).

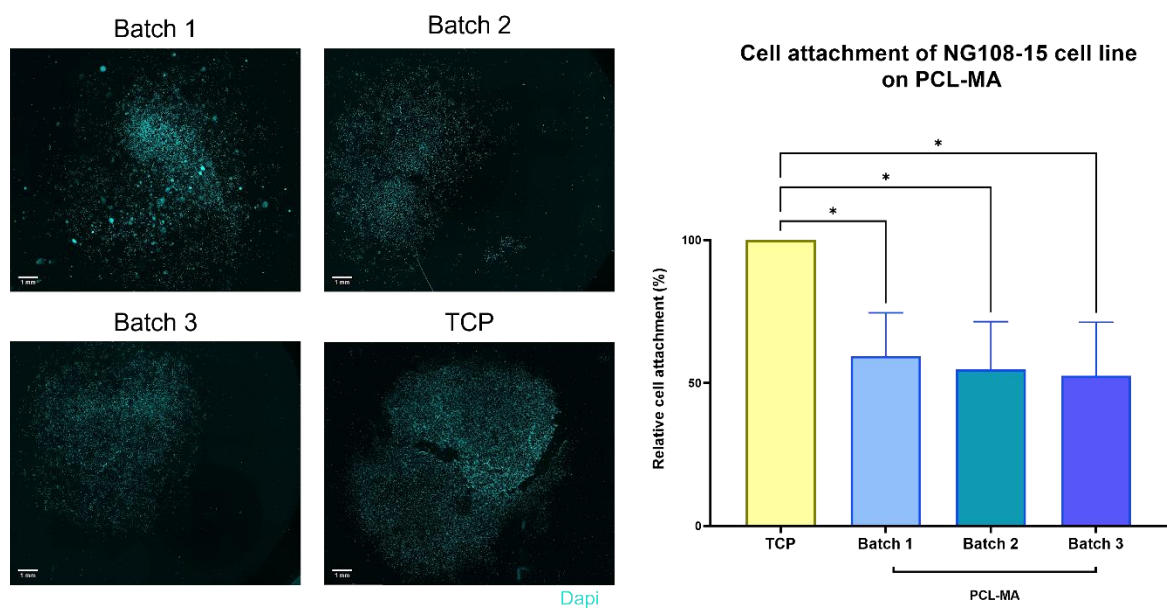


Figure 2.20 Cell attachment of NG108-15 Neuronal cells on different PCL-MA batches and on TCP. Values were normalised in reference to TCP attachment where TCP is 100%. Values are mean % $\pm$ SD. * ($p < 0.05$) denotes a significant difference assessed by 2-way ANOVA with Tukey's multiple comparisons. Scale bar 1 mm. (N=3, n=3)

2.3.4 Electrospinning of PCL fibres

Aligned polycaprolactone (PCL) fibres were fabricated by electrospinning using a 20% PCL solution in dichloromethane (DCM). Briefly, a 1 mL syringe filled with the PCL polymer solution was pumped with flow rates of 5.0 - 6.0 ml/hr and a voltage of 18 – 20 kV onto a rotating collector set at 2000rpm. The distance between the syringe needle and the rotating collector (needle-to-collector distance) was varied between 9, 11 and 14cm to investigate any effects on the resulting diameter of the fibres. The SEM images of the electrospun fibres (Figure 2.21) show high alignment in all groups, albeit within some groups there was higher variability in fibre sizes as demonstrated in the larger standard deviations in Figure 2.22. Despite the trend of increased fibre size with increased flow-rate, there were no significant differences in the average fibre diameter produced under the selected conditions with various needle-to-collector distances ($P > 0.5$), with all measured fibres ranging between $6.79 \pm 0.71 \mu\text{m}$ and $7.75 \pm 0.39 \mu\text{m}$ (Figure 2.22).

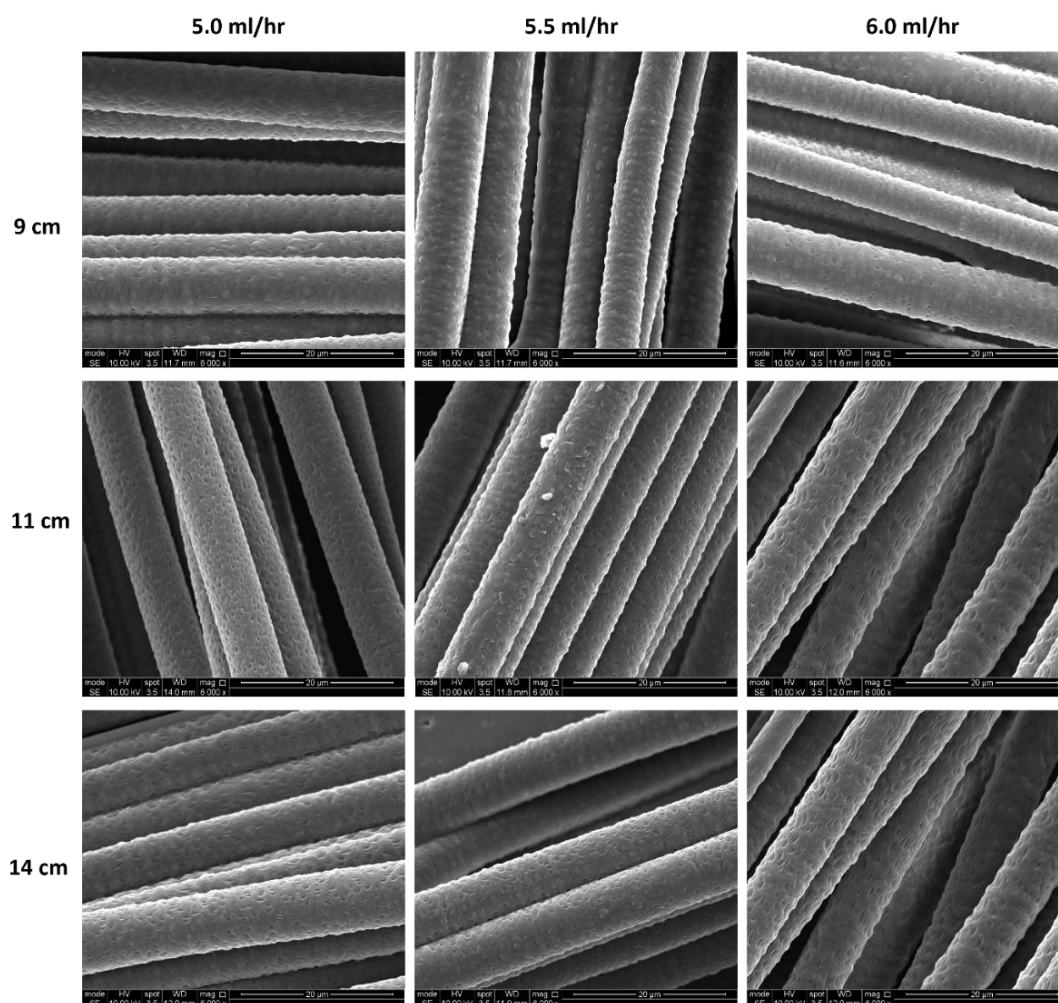


Figure 2.21. SEM images of PCL fibres fabricated by electrospinning technique under varying conditions of flow rate (5.0, 5.5 and 6.0 ml/hr) and needle-to-collector distance (9 cm, 11 cm, 14 cm). Scale bars 20 microns.

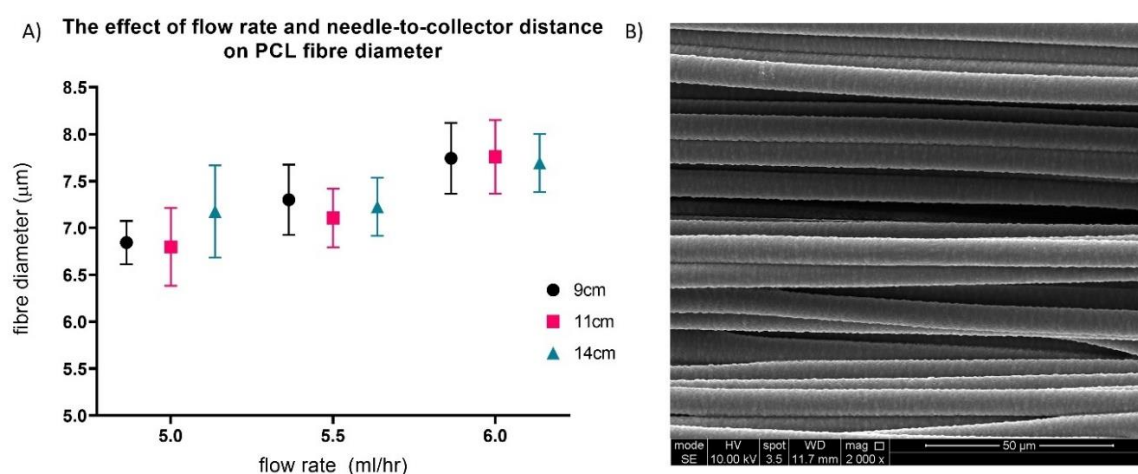


Figure 2.22 A) The effect of flow rate and needle-to-collector distance on electrospun PCL fibre diameter. No significant differences between groups (2-way ANOVA with Tukey's post-hoc multiple comparisons). Data presented as mean and standard deviation. B) Low magnification SEM image of electrospun fibres showing high alignment in fibres (scale bar 50 µm).

2.3.5 Air plasma treatment of PCL fibres

Air plasma treatment was used to modify the surface hydrophilicity of polycaprolactone. To evaluate the hydrophilicity of PCL, water contact angle measurements were performed on flat surfaces of PCL substrates treated with plasma and compared to control, non-treated PCL films. Methacrylated PCL (PCL-MA) and glass were also used as controls. The water contact angle on non-treated PCL was $110.2 \pm 8.06^\circ$ (Figure 2.23). After plasma treatment there was no droplet formation on plasma-treated PCL, measured as a contact angle of 0° , showing a significant increase in hydrophilicity as a result of plasma treatment. Glass and PCL-MA had a contact angle of $61.90 \pm 1.49^\circ$ and $88.99 \pm 6.65^\circ$, respectively.

In order to assess the stability of the air plasma treatment, the contact angle of plasma-treated PCL was measured after 2 weeks, where the water contact angle was measured as $91.47 \pm 4.77^\circ$ demonstrating a significant loss in hydrophilicity. However, this value was still significantly lower than non-treated PCL ($P < 0.05$).

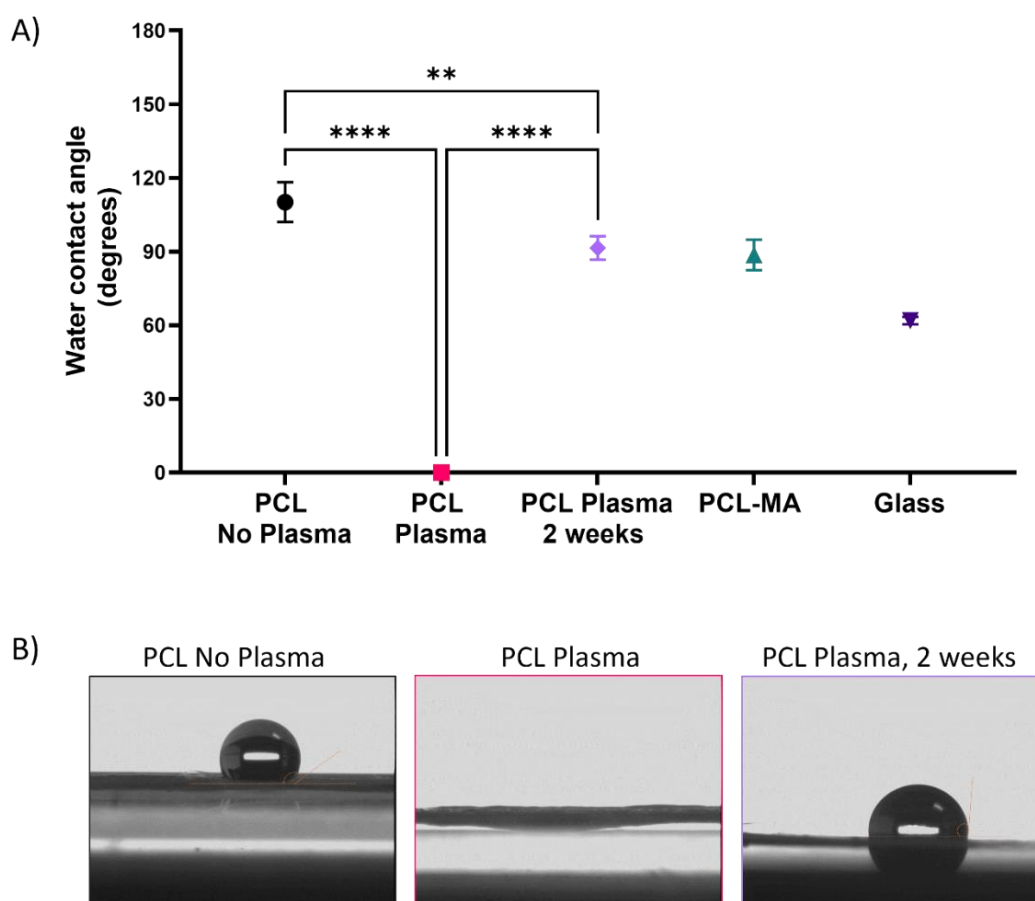


Figure 2.23 (A) Water contact angle measurements for PCL surfaces, specifically untreated PCL (PCL no plasma), PCL treated by air plasma (PCL Plasma), PCL treated by air plasma and stored for 2 weeks (PCL Plasma 2 weeks), methacrylated PCL (PCL-MA) and Glass. Values are mean $\pm$ standard deviation (SD). ** ($p < 0.005$) and **** ($p < 0.0001$) denotes a significant difference assessed by 2-way ANOVA with Tukey's multiple comparisons. ($N=3$, $n=5$)

2.3.6 Combination of PCL fibres with NGC

PCL fibres were inserted into PCL-MA nerve guide conduits, where the relationship between the fibres and the conduit can be described as the volume of the fibres contained in the volume of the conduit lumen, also called fibre packing density. Briefly, using the density of the material of PCL, a corresponding mass for the fibres was calculated in order to satisfy a given packing density within the lumen of a nerve guide conduit. PCL fibres were inserted into conduits using the methods described in 2.6.1 and two examples are shown in Figure 2.24. Figure 2.24 shows two conduits with different wall thicknesses, but the same internal diameter (800 μ m) containing a high (left) and low (right) packing density.

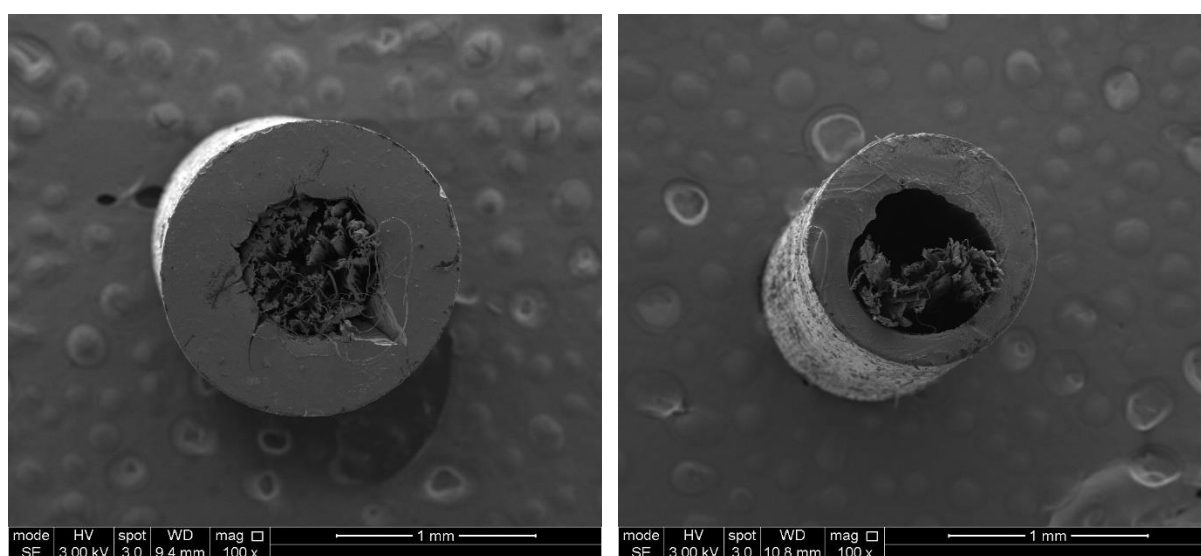


Figure 2.24. PCL fibres inserted into the lumen of two types of conduits with the same internal diameter containing a high packing density (left image) and low packing density (right image).

In order to validate the theoretical method of packing fibres into the conduits, gas pycnometry was used to measure the density and volume of the fibre bundles. Three fibre bundles equivalent to three packing densities were tested by pycnometry: 30%, 15% and 5%. The fibre bundles were measured to obtain density and volume data by gas pycnometry to calculate a “measured” packing density.

Firstly, the “measured” density was significantly different in each group, suggesting the method is appropriate for the development of different packing densities. Then, the “measured” packing density was compared to the “theoretical” fibre packing density in each group, where it was hypothesised that there would be no significant difference between the theoretical packing density and the measured packing density (Figure 2.25). However, there was a significant difference between the “theoretical” packing density and measured packing density for all three groups ($P < 0.005$). For the high packing density selected to be 30%, the pycnometry data showed the packing density was actually

30.6 ± 0.12 %. For the medium packing density of 15%, the measured packing density was 15.5 ± 0.09% and for the low packing density (5%), the measured packing density was 5.5 ± 0.05%. Therefore, the measured values were consistently significantly larger than the theoretical values, although by less than 1%.

Validation of packing density method

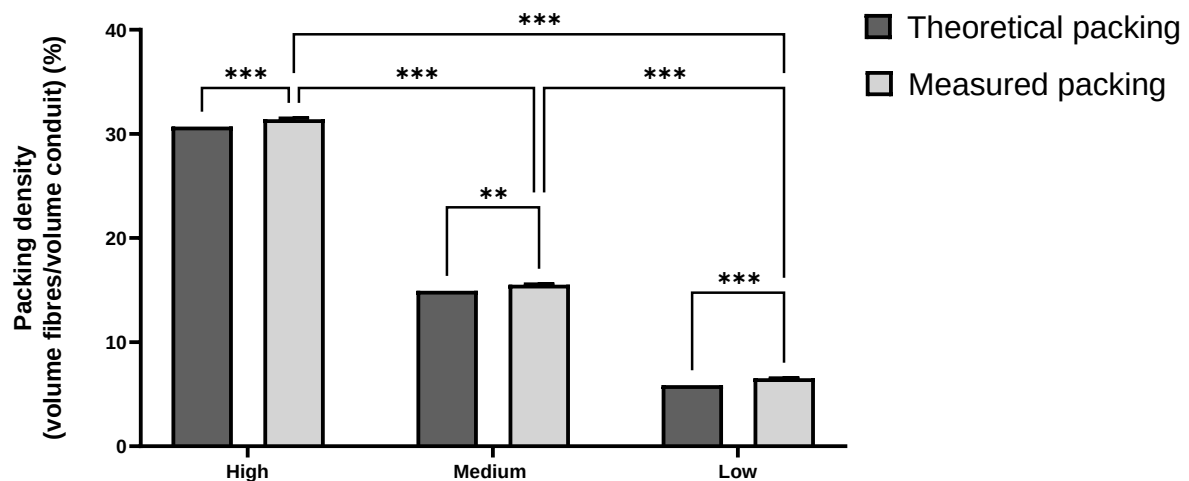


Figure 2.25 Comparison of the fibre packing density. Values are mean $\pm$ standard deviation (SD). ** ($p < 0.005$) and *** ($p < 0.001$) denotes a significant difference assessed by 2-way ANOVA with Tukey's multiple comparisons.

2.4 Discussion

2.4.1 PCL-MA mechanical properties

Throughout the studies involving fabrication of nerve guide conduits, various batches of methacrylated PCL (PCL-MA) polymer were required and kindly provided by an external source. Characterisation was conducted on all batches to evaluate batch-to-batch reproducibility prior to cell culture studies. To do this, a small amount of each batch of PCL-MA was used to make dog-bones in order to evaluate the tensile strength of the constructs.

The Young's Modulus of the tested batches 1, 2 and 3 were 3.719 ± 0.113 MPa, 7.94 ± 0.7173 MPa and 13.94 ± 0.416 MPa, respectively, which were shown to be significantly different from each other. This difference could be due to a number of reasons.

Firstly, the number of methacrylate groups in each polymer batch may have been different, meaning that upon addition of the photoinitiator and subsequent exposure to the UV light, the polymerisation that occurred between the functional groups (i.e. methacrylate groups) was affected, thereby resulting in differences in the crosslinking density of the polymer which in turn affects the Young's modulus. A higher crosslinking would result in a stiffer material (higher Young's Modulus), therefore this would suggest that Batch 3 had the highest methacrylate groups and Batch 1 had the lowest methacrylation level.

The relationship between methacrylation and mechanical properties was recently studied by Field et al. (2021) where they showed that changing the methacrylation content of PCL from 17% to 77% resulted in a significant increase in the Young's moduli of the cured samples (from 0.12 MPa to 3.51 MPa). This was explained by the increase in cross-linking as a result of the increase in methacrylate groups. The study also reported the Young's moduli for PCL-Ma samples with a degree of methacrylation of 39% and 49% was less than 1 MPa. The PCL-MA used in our study was synthesised with a similar process to the Field et al. study in order to fabricate a PCL polymer with approximately 50% methacrylation degree, where we report a Young's moduli between 3 and 13 MPa, much higher than that reported by Field et al. Their study revealed that remnant of solvent in their scaffolds which may have affected the mechanical properties, which may account for the differences found between their study and ours.

However, the level of functionalisation of a polymer is not the only factor that can affect photopolymerisation and cross-linking; molecular weight can also influence this process and therefore influence mechanical properties. Elomaa et al synthesised photocurable PCL through methacrylation of polycaprolactone-triol, resulting in samples with Young's Modulus of 6.7MPa to 15.4MPa, which

are closer in line with the results reported in our study. The change in Young's modulus in the Elomaa study was due to changing the molecular weight of the polycaprolactone polymer but keeping the functionalisation parameters (degree of functionalisation) the same. In such PCL samples, the polymer with the smaller molecular weight resulted in higher young's modulus and a decrease in the elongation at break (Elomaa et al., 2011). This is expected, as typically if the level of functionality is kept the same, decreasing the molecular weight leads to an increase in cross-linking density, which in turn limits the ability of the polymer to stretch upon an applied stress, hence a lower elongation at break. In addition, polymers with an increased cross-linking density are able to resist and distribute the stress through a larger number of polymer chains and therefore a stiffer material with a higher young's modulus is produced.

Similar findings were reported by Green et al. who produced photocurable PCL through acrylation (addition of acrylate groups) of PCL-triol and PCL-diol of different molecular weights, resulting in PCL-triacrylate or PCL-diacrylate, respectively (Green et al., 2018). The PCL-triacrylate pre-polymer samples showed higher polymerisation rates than the PCL-diacrylates samples upon UV exposure, again likely due to the increased number of functional acrylate groups in PCL-triacrylate in comparison to PCL-diacrylate, which suggests an increased cross-linking density.

In addition, Green et al also compared the PCL pre-polymers (both diacrylate and triacrylate) properties by their molecular weight, where the shorter molecular weight polymers had a faster polymerisation rate than their higher molecular counterparts, again suggesting increased cross-linking in shorter molecular weight samples of the same functionalisation. The mechanical properties of the different PCL-acrylate samples were also evaluated, and the study reported an increased Young's modulus in the lower molecular weight PCL-triacrylate (6.9 MPa) in comparison to its higher molecular counterpart (4.0 MPa), and similarly with PCL-diacrylate. Other studies with acrylated PCL have also reported similar findings (Tzeng et al., 2018), demonstrating that the influence of both functionalisation and molecular weight can affect the polymerisation and mechanical properties of cured samples.

However, as the PCL-MA was provided by a reliable external source, we can assume the methacrylate functionalisation level of the batches of PCL-MA polymer used in this study was the same, therefore, the batches found to have a higher Young's modulus (Batch 3) may have been due to the polymer having smaller molecular weight molecules. However, the molecular structure of the PCL-MA batches in this study were not investigated therefore we cannot confirm this theory.

Alternatively, assuming the methacrylate content and the molecular weight of the samples were the same, then a difference in the photoinitiator concentration used may have caused a change during

the photo-polymerisation and cross-linking of the polymer. Specifically, an increased concentration of photoinitiator under the same light exposure conditions would result in increased free radical generation and an increased degree of polymerisation (Alonso et al., 2014). However, a high concentration of photoinitiator can also result in a quick and large generation of free radicals, which could not only increase initiation and propagation of polymerisation but also termination (as free radicals may react with each other and annihilate their activity) leading to polymers with lower molecular weights.

As far as the author is aware, all efforts were made to keep the synthesis process of the polymer constant among batches (therefore, the expected level of methacrylation and molecular weight should be constant), and the photoinitiator concentration was also kept the same throughout the studies. Therefore, it is unknown if during the time between polymer synthesis and the use of the polymer any changes could have occurred in the batches.

For instance, considering that the methacrylate groups added to the polymer are reactive and can often interact with each other, it is possible that some batches had increased intra-cross linking of methacrylate groups which can influence the mechanical properties of the cured structure. In addition, it's possible that some batches may have "aged", meaning the polymer may have degraded more due to a longer time between synthesis and polymer curing, and this degradation may have resulted in polymers with smaller molecular weight chains, which again can lead to increased cross-linking density and a higher Young's modulus.

However, in this study, the "oldest" batch that aged longest before mechanical testing was Batch 1, which had the lowest Young's modulus. Nevertheless, as this study did not include any characterisation of the molecular structure of the polymer, nor polymer's reaction kinetics or functionalisation data, it is difficult to pinpoint exactly what may have caused the difference in the batches.

Nevertheless, the mechanical properties of PCL-MA polymer can be considered to be within a suitable range for the use in the fabrication of nerve guide conduits. Studies investigating conduits pre-clinically report materials with Young moduli between 0.28MPa and 470MPa, using synthetic materials like polyglycerol sebacate (0.28MPa), polylactic-co-glycolic acid (7 – 140MPa), polycaprolactone (0.12MPa – 15MPa) or polyethylene glycol (470MPa) (Elomaa et al., 2011; Kehoe et al., 2012; Pateman et al., 2015; Sundback et al., 2005). The wide range of mechanical properties of materials used pre-clinically may be due to the fact that the pre-clinical conduits are tailored for *in vivo* purposes, where the mechanical requirements will differ depending on the model.

Previous studies have reported on differences in the Young's moduli of selected nerves of different animals such as rat (0.576 MPa, (Borschel et al., 2003)), mice (7 MPa, (Beel et al., 1984)), rabbit (11.7 MPa, (Rydevik et al., 1990)) and porcine nerves (7.75 MPa, (Zilic et al., 2016)). In addition, a small number of studies have also measured the mechanical properties of human nerves (from patients who underwent limb amputations or from cadaveric sources) and report a range of Young's moduli of 9.50 - 15.87MPa for human tibial nerves or 10.41MPa for peroneal nerves (Dumont & Born, 2005; Kerns et al., 2019). Indeed, these values are closer to the Young's moduli for commercially available conduits such as Neurotube® (polyglycolic acid) with a Young's modulus of 4.0 MPa, Neuragen® (collagen) with 0.8 MPa and Neurolac® (Poly(DL-lactide-ε-caprolactone)) with 0.136 MPa (Hsu et al., 2017; Madduri et al., 2010). However, the current study did not measure the Young's modulus of our PCL conduits therefore it is unknown where our nerve guide conduits would fall within the range of clinically available conduits. Therefore, future studies should investigate the properties of PCL tubes by tensile testing. Nevertheless, the mechanical properties of the samples obtained by the various PCL-MA batches in this study can be considered suitable for nerve applications and for use in our *in vivo* model in Chapter 4.

2.4.2 Cytocompatibility of PCL-MA with a neuronal cell line

To evaluate the use of methacrylated PCL (PCL-MA) for the fabrication of nerve guide conduits, the cytocompatibility of the polymer was firstly investigated *in vitro*. Specifically, the metabolic activity of a neuronal cell line, NG108-15, was measured in response to different batches of PCL-MA. Briefly, PCL-MA was spin coated to create thin films of the polymer and cultured with the NG108-15 neuronal cell line. There was no significant difference in the metabolic activity of cells cultured on PCL-MA in comparison to cells cultured on tissue culture plastic or glass, suggesting that PCL-MA was not cytotoxic to NG108-15 neuronal cells *in vitro*. In addition, the attachment of NG108-15s on PCL-MA substrates was also evaluated and compared to TPC showing no significant difference in attachment of NG108-15 neuronal cells on the tested surfaces.

Field et al. also reported on the *in vitro* cytocompatibility of PCL-MA with a Schwann cell line (RN22) and with primary rat Schwann cells, where cells cultured on PCL-MA showed no significant difference in their metabolic activity in comparison with the cells grown on tissue culture plastic or glass, suggesting cytocompatibility of PCL-MA with RN22 and primary Schwann cells (Field et al., 2021) .

In addition, other studies have also evaluated the cytocompatibility of PCL-MA with human dermal fibroblasts and a human osteosarcoma cell line by investigating the cell proliferation rate of the cells grown on PCL-MA in comparison to TCP and showed no significant difference between the substrates, suggesting PCL-MA was not cytotoxic to these cells either (Aldemir Dikici et al., 2022).

2.4.3 Nerve guide conduit fabrication and characterisation

Photocurable polycaprolactone methacrylate (PCL-MA) was used to fabricate nerve guide conduits by two methods: microstereolithography, using either a LED lamp or laser as the light source, and injection moulding.

Conduits produced by laser-based microstereolithography were highly reproducible and uniform. This was demonstrated in particular by the uniform dimension of the internal and external diameter and the resulting wall thickness of the conduits throughout the structure.

The stereolithography set-up in our department was built in-house and has been used extensively over the last decade to produce a variety of scaffolds for tissue engineering, including nerve guide conduits that have demonstrated a high preclinical potential in mouse models of nerve injury and repair. The process involved expanding and reflecting a laser beam through a series of lenses and mirrors selected to maximise the irradiation intensity of the laser beam onto a DMD. The DMD reflected the light beam through another series of collimating and focal lenses to generate the light pattern, according to the cross-sectional image uploaded on to the device's proprietary software, onto the surface of the liquid polymer. The set-up uses a continuous motion of the stage moving in the z-dimension (into the polymer resin) which enables a fast photocuring of the layers and production of a conduit. At the optimised parameters (laser power and z-stage speed), a single conduit of 5 mm can be produced in 4 minutes. Unfortunately, the use of class 3B lasers and thus laser-based stereolithography equipment became restricted in the department and the work could not be conducted elsewhere in the remaining time, therefore, while this method was used to provide a small number of conduits suitable for characterisation, this technique became restricted by the time conduits were required for the final *in vivo* chapter.

In an attempt to address the class 3B laser restrictions, the laser beam was replaced by a light emitting diode (LED) of similar wavelength. However, initial attempts at printing with the LED lamp were unsuitable, especially in regards to the quality of the conduits and also the curing time. As mentioned earlier, the optimised stereolithography set-up with the laser enabled the fabrication of a conduit in under 5 minutes. In contrast, the LED-based set-up required a significantly longer exposure per layer of the conduit, increasing the printing time per conduit to 60 minutes, a 12-fold increase.

This can be due to the inherent differences between an LED and a laser, where the latter emits a narrow mono-chromatic wavelength whilst an LED emits a wider, Gaussian-like distribution of mono-chromatic wavelength. Meaning, the light of the laser does not diverse as much as an LED so the laser retains its dimensions, and the intensity of the centre of the light is higher in a laser compared to an

LED. Given that different photo-initiators absorb energy at different wavelength ranges, to produce free radicals to initiate photocuring, the relationship between a selected photoinitiator and the light source may affect the curing of a photocurable material. Typically, at shorter emission wavelengths, the resolution of the curing system is increased but the penetration depth is lower, leading to increased curing times (Raman & Bashir, 2015). Therefore, it is possible that the LED light emitting a less intense light source resulted in poor penetration depth hence the increased curing time in comparison to the laser.

Numerous attempts of optimising the stereolithography set-up, such as modifying the objective lenses and mirrors to improve the irradiation of the LED lamp onto the DMD as well as optimising the LED power and stage speeds, resulted in shorter curing times for conduit fabrication. However, the conduits were again not suitable due to uneven curing, for instance, in some conduits there were holes in the walls whilst with other printing parameters there was over-curing and no lumen could be formed. Due to time constraints, as a result of the Covid-19 pandemic, further optimisation was not possible. Therefore, a new method to produce conduits was explored: injection moulding.

Injection moulding was selected due to the simplicity of the method and the ease of producing a large number of conduits rapidly. However, the dimensions of the conduits were not consistently uniform. For instance, the wall thickness of conduits produced by injection moulding showed high variability within the same construct ($334\ \mu\text{m} \pm 159$), meaning that the wall thickness ranged from roughly $170\ \mu\text{m}$ to $500\ \mu\text{m}$, almost a 3-fold difference between the wall thickness at its largest and smallest measurement (Figure 2.17). This variation in wall thickness can be attributed to the imperfect placement of the needle in the middle axis of the tubing.

Again, due to time constraints, alternative methods could not be explored and the method of injection moulding was selected as the fabrication method for nerve guide conduits used for in vivo evaluation.

2.4.4 Electrospinning of PCL fibres

2.4.4.1 *Fabrication of fibres*

Electrospinning was employed to produce aligned PCL fibres. Briefly, a 20% solution of polycaprolactone in dichloromethane was dispensed at selected flow rates (5.0, 5.5, 6.0 ml/hr) from a syringe pump onto a rotating mandrel collector set at three distances from the needle (needle-to-collector distance) of 9, 11, and 14 cm. The voltage applied to the needle was kept at 20 kV for all electrospinning conditions. The PCL fibres fabricated with the selected parameters all produced highly aligned fibres, with fibres ranging between $6.79 \pm 0.71\ \mu\text{m}$ and $7.75 \pm 0.39\ \mu\text{m}$ with no significant differences between any of the fibre diameters produced by the selected electrospinning conditions.

Factors like flow rate and needle-to-collector distance are known to affect the formation of the polymer jet and therefore the features of collected fibres, including fibre diameter (Cui et al., 2007; Xue et al., 2019; Xue et al., 2017). Typically, increasing the flow rate to increase the amount of polymer solution ejected leads to larger fibre diameter, while increasing the needle-to-collector distance results in increased elongation of the fibre jet and thinner fibres (Doshi & Reneker, 1993, Tang et al., 2014). In our study, smaller fibres were formed at lower flow-rates and shorter needle-to-collector distances, where a small trend was seen with increased fibre diameter at higher flow-rates and longer distances, suggesting flow-rate may have a bigger influence on fibre diameter.

This is an important consideration to ensure reproducible results, particularly when using in-house built systems where other environmental factors surrounding the fabrication of fibres cannot be entirely controlled. In our experience, during colder months, attempts at electrospinning with previously optimised parameters failed. This is not surprising as changes in the environment, such as temperature and humidity, can affect the electrospinning process by altering the viscosity of the polymer and/or solvent evaporation rate during fibre jet formation which may impede fibre formation or affect fibre morphology including diameter or alignment which in turn may affect the applicability of the fibres (De Vrieze et al., 2009) (Doshi & Reneker, 1993).

For example, in a study by Daud et al (2012), neuronal NG108-15 cells cultured on PCL fibres showed increased outgrowth of neurites in terms of neurite length on 8µm PCL fibres ($142.36\mu\text{m} \pm 13.69\mu\text{m}$) compared to 1µm fibres ($61.83 \pm 6.89\mu\text{m}$). Similarly, previous research in our group investigating different PCL fibre diameters (1, 5, 8, 10 and 13 µm) showed increased NG108-15 neurite extension with PCL fibres of 8 µm ($151.7 \pm 30.3\mu\text{m}$) and 10 µm ($155.5 \pm 21.6\mu\text{m}$) compared to the rest of the fibre diameter groups (M. Behbehani thesis, unpublished).

Nevertheless, PCL fibres in this study were fabricated with two aims in mind, firstly and importantly to support nerve growth *in vivo*, which involves a more complex system than one cell line such as the NG108-15 cells. In addition, the fibres in this study were fabricated also to support the outgrowth of dorsal root ganglia, as this will be used to deposit and coat the fibres with DRG-derived extracellular matrix (Chapter 2). Studies evaluating PCL fibres with DRG have used fibres between 0.2 µm to 10 µm and have shown good outgrowth of DRG cells. Specifically, marginal differences with no significant effect of fibres diameters were reported when DRG were grown on fibres between 1µm to 8µm (Daud et al. 2012). Similarly, Wang et al (2010) compared three fibre diameters of 0.2, 0.7 and 1.3 µm using embryonic chick DRG, and showed similar levels of neurite outgrowth from DRG on the 0.7 and 1.3 µm, which in turn only showed a slight increase in axon outgrowth in comparison to the smaller fibre

suggesting that while 0.2 μm fibres may not support regeneration, larger fibres in the range of 0.7 to 8 μm are appropriate.

Therefore, in our study we were able to consistently produce aligned PCL fibres between 6 – 8 μm in diameter in a reproducible manner with our electrospinning set-up, where this range of diameters is consistent with that previously reported to optimise neurite outgrowth in *ex vivo* models and *in-vivo*.

2.4.4.2 Air plasma treatment

The effect of air plasma treatment on polycaprolactone films was studied by measurement of the water contact angle on treated and untreated surfaces. It was demonstrated that air plasma treatment reduced the water contact angle on PCL surfaces from 110.2° down to 0°, highlighting a change in surface wettability following air plasma treatment. In terms of surface composition, air plasma treatment has been shown to result in an increase of hydroxyl (-OH) polar groups, which are responsible for the increase in wettability and reduced contact angle (Cui & Brown, 2002).

While surface composition was not evaluated in this study, Zander et al (2012) analysed PCL nanofibers after air plasma-treatment by X-ray photoelectron spectroscopy (XPS) and showed an increase in the atomic ratio of oxygen to carbon after plasma treatment. Specifically, the atomic spectra of untreated PCL consisted of mainly carbonyl oxygen bonds (O-C=O) and ester oxygen bonds (O-C=O) at 50% each, while after plasma treatment the spectra showed a reduction in carbonyl oxygen and ester oxygen bonds to 41 and 23.5% respectively, and an increase in a third component for hydroxyl bonds (C-OH) at 35.5% (Figure 2.26). Other groups have also confirmed the increase in the oxygen to carbon content ratio after plasma treatment and have attributed this to the increase in wettability and hydrophilicity of the plasma-treated surfaces (Jeon et al., 2014; Nandakumar et al., 2012).

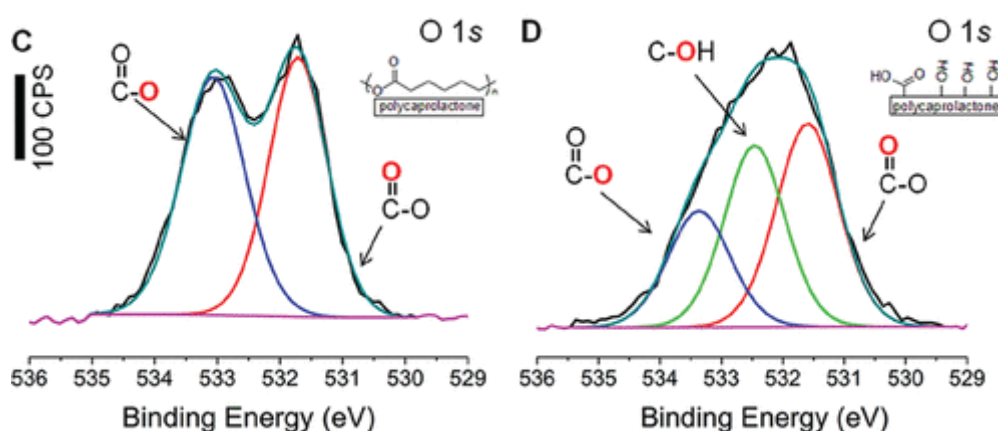


Figure 2.26 Reprinted with permission from (Zander et al., 2012) Copyright 2012 American Chemical Society.

PCL plasma-treated samples showed a reduction in wettability after 2 weeks (contact angle increased to 91.47°). This can be explained by the instability of the polar groups on the samples, where the orientation of polar groups on surfaces is not permanent and will rearrange depending on the environment, a phenomenon referred to as hydrophobic recovery (Bormashenko et al., 2013). This phenomenon is dependent on the surface free energies of the material and the surrounding environment. Whilst the surface free energy (SFE) of the samples was not tested directly, it can be predicted by the water contact angle where typically surfaces with high SFE will express high wettability and thus show a low water contact angle.

Therefore, samples treated by air plasma showed high wettability and hence it can be predicted that they had high SFE. These samples were then stored for 2 weeks at room temperature in a cupboard, in contact with air, and showed a decrease in their wettability and therefore the SFE of the sample was decreased over time.

At an air-solid interface, the molecules on the surface of a solid material will have excess energy compared to the bulk of the material, where the magnitude of the energy is dependent on the interaction between the molecules of the material. The difference in the SFE between such a solid and the medium (i.e. PCL with air in this case) influences the reorganisation of the functional groups. When the surface free energy of the plasma-treated material (i.e. PCL) is larger than the surface energy of the surrounding environment (i.e. air), the functional groups on the surface of the material migrate and orientate towards the inner bulk of the material, as this is done to reduce the difference in free energy between the materials (principle of minimum energy), which is measured as a loss in wettability of the material.

Alternatively, it follows that if a sample is kept in an environment with a higher surface free energy, for instance PBS or water, then the functional groups would orientate towards the surface of the material, again to reduce the difference in the free energy between the materials, and therefore the wettability in this case would not be lost and in some cases would be increased. This can be of relevance for storing samples in the long term to retain wettability after air plasma treatment.

However, it is difficult to estimate what storage medium could be used in our sample to conserve the wettability over time as we did not measure the surface free energy on the plasma-treated PCL sample in our study. Therefore, for our studies, PCL samples were kept in deionised sterile water and used within 2 days after plasma-treatment.

2.4.5 Combination of fibres with nerve guide conduits

The packing of PCL fibres into conduits (packing density), described as the volume of the fibres contained in the volume of the conduit lumen, was validated by means of gas pycnometry. Briefly, using the density of the material of PCL, a corresponding mass for the fibres was calculated in order to satisfy a given packing density within the lumen of a nerve guide conduit. Then, the same fibre bundles were measured to obtain density and volume data by gas pycnometry to calculate a “measured” packing density. The “measured” packing density was statistically larger than the “theoretical” fibre packing density, however the difference was less than 1% in each fibre packing density studied.

Therefore, while the method of packing the fibres into nerve guide conduits can be considered appropriate, gas pycnometry only offers data on the skeletal density and volume of the fibre bundles, and therefore it may be appropriate to investigate other methods to evaluate the packing densities. For instance, micro computed tomography can provide a visualisation of the fibres *in situ* within the conduits. Alternatively, fibres within conduits can be embedded or frozen and sections can be taken at different lengths of the conduit, and cross-section images can be analysed by imaging software, again to provide further information on the overall position of fibres within conduits.

It is important to validate the method in which fibres are inserted into conduits, as it has been demonstrated that while increased contact guidance in the form of intraluminal fibres within nerve guide conduits can be beneficial, this is only as long as the presence of the fibres does not significantly occlude the lumen and obstruct the path of regenerating axons and migrating cells (Ngo et al., 2003).

For instance, Ngo et al (2003) used poly-L-lactide (PLLA) microfibres of 40 - 100 μm in diameter as intraluminal fillers in conduits inserted at different densities (3.75, 7.5, 15 and 30%) to bridge a 1cm nerve gap in the rat sciatic nerve. While improved regeneration was shown in the fibre-filled conduits in comparison to a hollow conduit, the study showed that the repairs using conduits with less fibres (i.e. the lower packing densities) showed an increased number of axons at the distal stumps compared to repairs with the higher density groups (3.75% vs 30%). In addition, a study by Koh et al. (2010) used a 10% fibre packing density to fill nerve guide conduits that were evaluated *in vivo* with a model of nerve repair in rat where the conduits were able to support regeneration to a similar level as that obtained by a hollow conduit and an autograft repair (Koh et al., 2010).

Studies in our group have also shown that a 10 – 12% packing density of PCL fibres was appropriate in supporting nerve regeneration in an *in vivo* mouse model of nerve repair (Field, thesis, unpublished).

This packing density was based on a previous study that evaluated the same PCL fibres in an *ex vivo* model, where 6000 to 7000 PCL fibres of 5µm in thickness were used to fill a nerve guide conduit (equivalent to roughly 10% packing density) and cultured with a rat DRG showing good outgrowth of neurones and Schwann cells (Behbehani et al, 2018).

2.5 Conclusions

In this chapter, methacrylated polycaprolactone (PCL-MA) was investigated as a material for the fabrication of nerve guide conduits for peripheral nerve repair. The material demonstrated appropriate cytocompatibility using a neuronal cell line and the tensile strength of the material was also tested and found to fall within a suitable range of materials previously used for the fabrication of conduits.

Nerve guide conduits were fabricated by two methods, stereolithography and injection moulding. Conduits fabricated by stereolithography were more reproducible and showed even dimensions throughout the construct, compared to conduits fabricated by injection moulding. However, due to time constraints injection moulding was selected for subsequent studies.

Electrospinning was used to successfully fabricate highly aligned fibres from polycaprolactone and treated with air plasma to improve the hydrophilicity and wettability of the fibres. The method for inserting PCL fibres into the lumen of the nerve guide conduits was done following a theoretical calculation using the density of PCL fibres to measure the corresponding volume occupied by the fibres, which was then validated by pycnometry.

2.5.1 Upcoming chapters

As a reminder, this thesis aims to develop a nerve guide conduit containing PCL fibres which will be coated with decellularised-extracellular matrix derived from DRG, which will then be evaluated in an *in vivo* model of nerve repair.

This chapter involved the first part of the project, which was the development of the conduit and PCL fibres. Therefore, the following chapter focuses on the optimisation and decellularisation of DRG-derived ECM and coating the fibres with the DRG-derived dECM.

Chapter 3. Optimisation, characterisation, decellularisation, and evaluation of DRG-derived ECM *in vitro*

3.1 Abstract

A large number of extracellular matrix (ECM) components have been used in various tissue-engineering approaches to enhance regeneration following nerve injury, however, individual ECM components do not replicate the native ECM in peripheral nerves. This chapter hypothesised that replicating a nerve-specific ECM would provide an optimal environment to improve nerve regeneration.

In order to do this, this chapter evaluated the use of dorsal root ganglia as a model to produce a nerve-specific ECM.

The optimisation and characterisation of DRG culture *in vitro* was conducted both on tissue culture plastic and PCL fibres, using immunocytochemical methods to reveal the presence of neuronal and glial markers in the cultures, as well as important ECM components specific to the basal lamina of Schwann cells, which were maintained following decellularisation. Therefore, this provided proof and validation of the use of DRG as a model for nerve-specific ECM.

Next, DRG-derived ECM was evaluated for nerve regeneration using a nerve outgrowth assay, indicating a positive effect on nerve regeneration *in vitro*. Overall, this chapter provided valuable insights into the potential use of DRG-derived ECM as a nerve-specific environment for nerve regeneration.

3.2 Background

Nerve injury results in Wallerian degeneration of affected axons, where regeneration is then dependent on axons extending across the injury site towards the distal stump and back to the original targets (reviewed in 1.5). A large number of molecular events are involved in providing a pro-regenerative environment to the regenerating axons, including the induction of the repair phenotype in Schwann cells, recruitment of immune cells and the secretion of different neurotrophic factors, all of which involve various types of complex signalling occurring between the cells involving the extracellular environment or matrix (ECM) (Gomez-Sanchez et al., 2017; Jessen & Mirsky, 2019; Stoll & Müller, 1999).

3.2.1 The extracellular matrix of the peripheral nerve

The extracellular matrix (ECM) is a three-dimensional network of macromolecules whose precise composition and structure are dependent not only on the tissue, but also on the developmental and pathophysiological stage of the tissue including during injury and regeneration. The ECM components of the peripheral nerve are found in two main locations, the connective tissue layers of the nerve and the basal lamina. The basal laminae (or sometimes called basement membranes) refers to the dense network of ECM components found in close proximity to cells, for instance surrounding Schwann cells and axons (Uzman & Villegas, 1960). Figure 1.1 shows an electron micrograph cross-sectional image of a peripheral nerve with both myelinated and unmyelinated axons surrounded by basal laminae (black arrows) and the endoneurium, found between the axon-Schwann cell units.

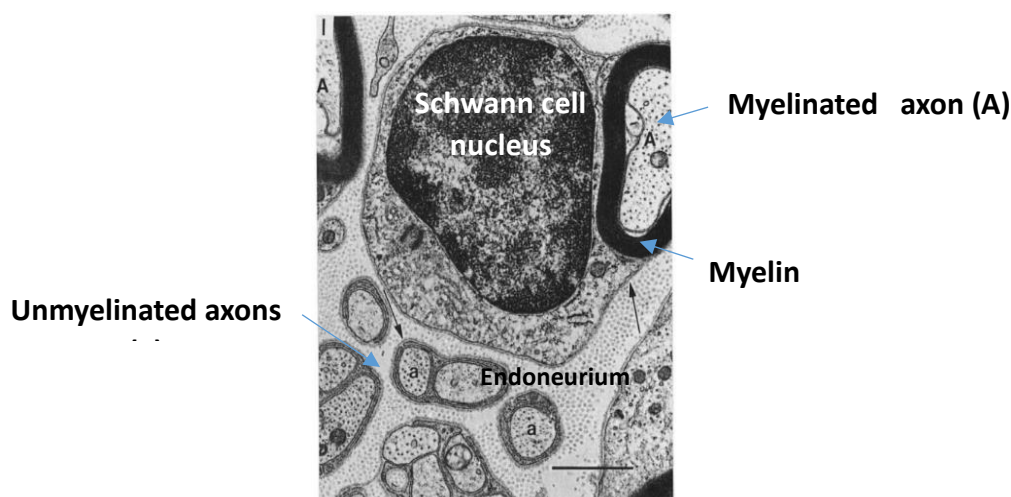


Figure 3.1 Electron micrograph of a peripheral nerve (mouse sciatic nerve) showing myelinated (A) and unmyelinated nerves (a) are surrounded by basal laminae (black arrows). Magnification 23,000 X, Scale bar = 1 micron. Image taken from (Ide et al., 1990) with permission, Copyright © 1990 Published by Elsevier Ltd.

A number of relevant ECM components for nerve regeneration are discussed below, where the major components of the basal lamina ECM include collagen type IV, which assembles into a network binding to other ECM components such as glycoproteins like laminin and fibronectin, and proteoglycans (PGs). Outside of the basal laminae, ECM components in the connective tissues are found mainly in fibrillar form, such as collagen types I, III and V.

3.2.1.1 Collagens

Collagens are a large family of triple-helical proteins, accounting for more than 30% of the total protein in the human body, with up to 28 different types described according to the assembly of the corresponding polypeptide chains. These polypeptide chains, called alpha chains, are composed of repeating amino acid sequences of Glycine-X-Y, where X and Y are usually proline and hydroxyproline and are held in a triple helix through hydrogen bonding and electrostatic forces, resulting in a rod-like structure (Figure 3.2). At each end of a collagen molecule are non-collagenous domains (NC domains) that are involved in the self-assembly of collagens into specific supramolecular structures. Collagens can also be categorised by their distinct supramolecular structures, such as fibrillar or non-fibrillar structures.

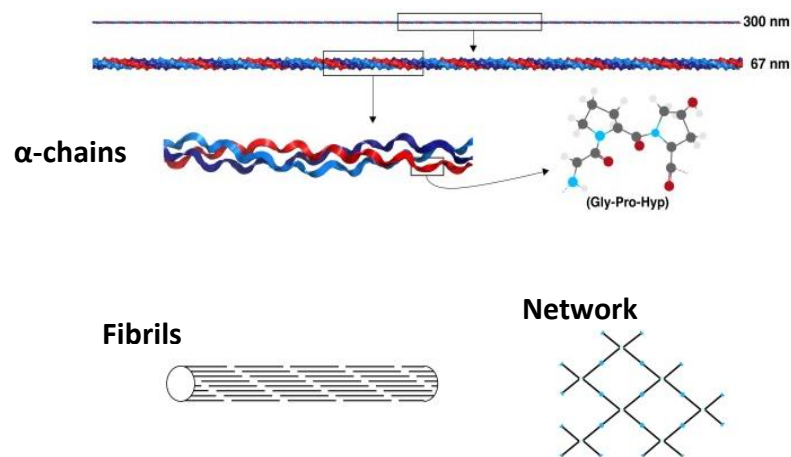


Figure 3.2. Schematic representation of collagen's triple helix structure and Glycine-Proline-Hydroxyline (Gly-Pro-Hyp) amino acid sequence (top), with two supramolecular structures of collagen, fibrils and network. Images used with permission from (Kirkness et al., 2019; Ricard-Blum, 2011). Copyright Cold Spring Harbor Perspectives in Biology. © 2019 Elsevier Ltd.

In the peripheral nerve, fibrillar collagens type I, III and V are found in all three connective layers (the epineurium, perineurium and endoneurium), often running longitudinally in the direction of the nerve which, along elastin fibres, provide mechanical strength to the nerve (Gamble & Eames, 1964). In the perineurium, some collagen fibrils are also found obliquely-orientated, separating the layers of the perineurial cells surrounding each nerve fascicle. In the epineurium, collagen fibrils bundles are

arranged in an undulated or zig-zag pattern that allow for an increase in elongation without tensioning the fibres (Stolinski, 1995).

Cell culture studies have shown that collagens can be produced *in vitro* by immature Schwann cells (M. B. Bunge et al., 1980), and indeed, during development, collagen fibrils can be seen in the endoneurium of embryonic mouse sciatic nerves, a stage where no fibroblasts could be detected, suggesting immature Schwann cells are a source of collagen *in vivo* too. Collagen type IV, on the other hand, is found in a non-fibrillar form, resembling a network, and is found exclusively in the basal laminae, where it modulates cell adherence and regulates matrix stability and structural organization.

Besides a role in mechanical support, collagens have also been implicated in other aspects of regeneration after nerve injury. For instance, *in vitro*, collagen IV has been shown to increase axonal lengths of mouse DRG neurones (Tonge et al., 1997) and recent studies have described a role for collagen IV in guiding regenerating axons back to the correct targets following nerve injury in zebra fish (Isaacman-Beck et al., 2015). Studies also implicate collagen signalling with myelination, for instance, the siRNA-mediated suppression of Glypican-1, a collagen V receptor on Schwann cells, resulted in inhibited myelination in co-cultures of Schwann cells and neurones *in vitro* (Chernousov et al., 2006). In addition, collagen VI-null mice show impaired regeneration and reduced migration and polarization of macrophages (Chen et al., 2015), which is an essential step required following nerve injury to clear myelin debris during Wallerian degeneration.

3.2.1.2 Laminin

Laminin is a heterotrimeric glycoprotein consisting of three polypeptide chains, alpha, beta and omega which assembles into a T-shaped molecule, with one long arm and three short arms (Figure 3.3). In the adult nerve, the main isoform of laminin found in the basal lamina of Schwann cells is laminin-211, composed of one $\alpha 2$ chain, one $\beta 1$ chain and one $\gamma 1$ chain (2-1-1).

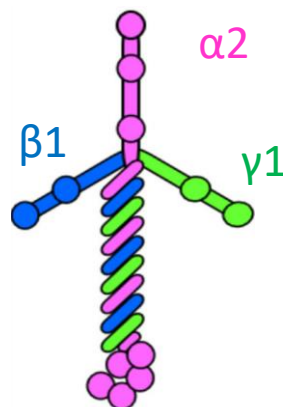


Figure 3.3. Schematic representation of laminin 211. Image used with permission from (Durbeej, 2010).

Laminins are important ECM factors known to influence Schwann cell migration and myelination, primarily acting via the integrin receptors $\alpha 1\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$, but also via non-integrin receptor dystroglycan (Saito et al., 2003).

Early studies showed that the specific disruption of laminin's $\beta 1$ integrin receptor on Schwann cells *in vitro* resulted in inhibition of myelination and abnormal radial sorting (Fernandez-Valle et al. 1994). Furthermore, mouse models where the expression of laminin $\alpha 2$ and consequently of laminin-211 is reduced have also shown abnormal myelination with a larger number of unmyelinated axons, resulting in motor dysfunctions in the animals (Bradley & Jenkison, 1973). In addition, some studies show that lack of laminin $\gamma 1$ leads to reduced proliferation of Schwann cells, which in turn can impact the effect of repair Schwann cells following injury. In fact, other studies have shown a reduced number of axons are regenerated following nerve injury in animals lacking laminin $\gamma 1$ chain compared to wildtype, suggesting a role for laminin in axonal regeneration (Chen & Strickland 2003; Yu et al., 2005).

Indeed, laminins are also known to increase neuronal outgrowth of various cell lines *in vitro* (Engvall et al., 1992) and after axotomy of DRG primary neurones, expression for laminin and laminin-integrin receptors are upregulated on neurones too (Wallquist et al., 2004). In mice, the expression levels for laminin-211 and 411 are significantly upregulated following nerve injury in the injured stump, with a higher expression in the proximal stump compared to the distal stump, suggesting a role for laminin on the regenerating axons originating from the proximal stump (Wallquist et al., 2002).

3.2.1.3 Fibronectin

Fibronectin is another glycoprotein found in the basal lamina, composed as a heterodimer fibrillar molecule with multiple binding sites for other ECM components such as collagens, laminins and proteoglycans, as well as cell-binding sites. In addition, fibronectin can also bind to cell surface receptors such as the $\alpha 5\beta 1$ integrin receptor found on Schwann cells and axons to modulate regeneration (Lefcort et al., 1992; Vogelezang et al., 2001).

While levels of fibronectin are low in adult nerves, compared to during development, after nerve injury there is an increase in the expression of fibronectin in various *in vivo* models of nerve injury (Lefcort et al., 1992). *In vitro*, fibronectin has been shown to act as chemoattractant and mitogen for Schwann cells (Baron-Van Evercooren et al., 1982), and fibronectin substrates can increase neurite extension of DRG neurones (Humphries et al., 1988; Plantman et al., 2008).

3.2.1.4 Proteoglycans and glycosaminoglycans

Proteoglycans are glycosylated proteins, meaning they consist of a core protein with a varying number of glycosaminoglycans (GAGs) chains. GAGs are linear polysaccharides that may be sulphated, such as

chondroitin sulphate and heparan sulphate, or not, such as hyaluronan/hyaluronic acid. Proteoglycans are typically named according to the attached GAG chains (Gandhi & Mancera, 2008). The polysaccharide chains, made of repeating disaccharide units, typically give GAG chains a negative charge, allowing binding to various positively-charged molecules such as cytokines and growth factors.

Proteoglycans and their respective GAGs are involved in a large variety of functions. For instance, Syndecan-3 is a transmembrane heparan sulphate proteoglycan found on Schwann cells thought to mediate binding to Collagen V, likely via the $\alpha 4$ chain (Erdman et al., 2002).

Of relevance to the adult peripheral nervous system, proteoglycans of chondroitin sulphate (CSPGs) are present in the extracellular matrix and have been related to inhibiting nerve regeneration (Gause II et al., 2014; Zuo et al., 1998). Following transection of the mouse sciatic nerve, CSPGs are upregulated in the distal stump, localised primarily to the Schwann cell basal lamina (Braunewell et al., 1995), and degradation of CSPGs with chondroitinase has been shown to increase the number of axons that are able to enter distal stumps during regeneration in a number of models (Groves et al., 2005; Neubauer et al., 2007; Zuo et al., 2002). In fact, the clinically-used Avance® allografts provided by Axogen undergo a chondroitinase treatment to remove CSPGs (Krekoski et al., 2001).

3.2.1.5 Fibrinogen and fibrin cable

Finally, another component present in the extracellular matrix for peripheral nerve regeneration is fibrinogen, a glycoprotein found in vasculature which is released and leaked into the injury site and coagulates into fibrin. As a reminder, following nerve injury, the formation of a fibrin cable within the first week of repair is critical as it acts as a structural guidance in supporting the elongation of axons and Schwann cells across injury site (Daly et al., 2012; Williams & Varon, 1985).

The fibrin cable is thought to degrade within 2 weeks in rodents and therefore if axonal elongation and cellular migration is not complete between the proximal and distal stump by this time, nerve regeneration fails (Williams et al., 1987). It is suggested that the fibrin cable can influence Schwann cells by inducing and maintaining Schwann cells in a non-myelinating phenotype, which in turn results in longer lasting support for axons following injury (Akassoglou et al., 2002).

3.2.2 Modelling a nerve-specific ECM

Therefore, as reviewed above and in 1.7.3, the extracellular matrix in the peripheral nerve is capable of influencing neuronal and Schwann cell behaviour, such as migration, elongation, phenotype differentiation and proliferation, through a variety of signalling mechanisms during normal development and physiology but importantly following injury and regeneration, that ultimately provide an optimal, pro-regenerative environment.

As such, ECM components have been used in a number of tissue-engineering approaches and evaluated in a number of studies to replicate the pro-regenerative environment and to enhance regeneration following injury. For instance, ECM components have been used as fillers in nerve guide conduits, either as single purified ECM proteins or used in various combinations (Chen et al., 2000), but ultimately, individual ECM components do not fully recapitulate the true ECM environment in a peripheral nerve.

Therefore, alternative methods can be used to replicate tissue-specific ECM, where the most obvious is the decellularisation of nerves, such as the Avance® allograft used clinically (reviewed in 1.6.4.2), however, as mentioned these are not acceptable to some patients, can be costly and are not offered routinely. Many studies have also investigated decellularisation of nerves from various xenogeneic sources (Zilic et al., 2016) which can be used either as acellular grafts or further processed and modified to be delivered in formats such as hydrogels with much potential (Meder et al., 2021). But, xenogeneic acellular grafts may be limited by inherent differences between species, such as improper fascicular matching of nerves, and hydrogels can be limited by their mechanical properties. Therefore, an alternative method to obtain tissue-specific ECM is by using cells in culture, to obtain cell-derived ECM.

In order to replicate nerve-specific ECM, studies have used Schwann cells. For example, Gu et al (2014) deposited Schwann cell-derived ECM on silk fibres, which were decellularised (dECM) and inserted into nerve guide conduits and evaluated in the repair of a 1 cm nerve gap in rat (Y. Gu et al., 2014). The Schwann cell-derived dECM conduit showed an increased number of regenerated axons in the distal stump compared to plain conduits, and while there was no comparison with an autograft group, the study included an acellular nerve graft group, where the levels of regeneration were similar between the dECM-conduit and the acellular graft, highlighting the potential of cell-derived ECM in nerve regeneration.

However, other cells in the peripheral nerve contribute to the extracellular matrix that may not be fully recapitulated using only Schwann cells. An alternative method to produce cell-derived ECM that is specific to the peripheral nerve is by using dorsal root ganglia.

3.2.2.1 The Dorsal Root Ganglia

The dorsal root ganglia (DRG), a type of sensory ganglia found adjacent to the spinal cord but not part of the CNS, consist of the cell bodies of peripheral sensory neurones, which are pseudo-unipolar neurones whose axons bifurcate into two branches: one extending into the periphery and forming sensory endings with skin, muscle, and other target organs, while the other shorter branch enters the spinal cord.

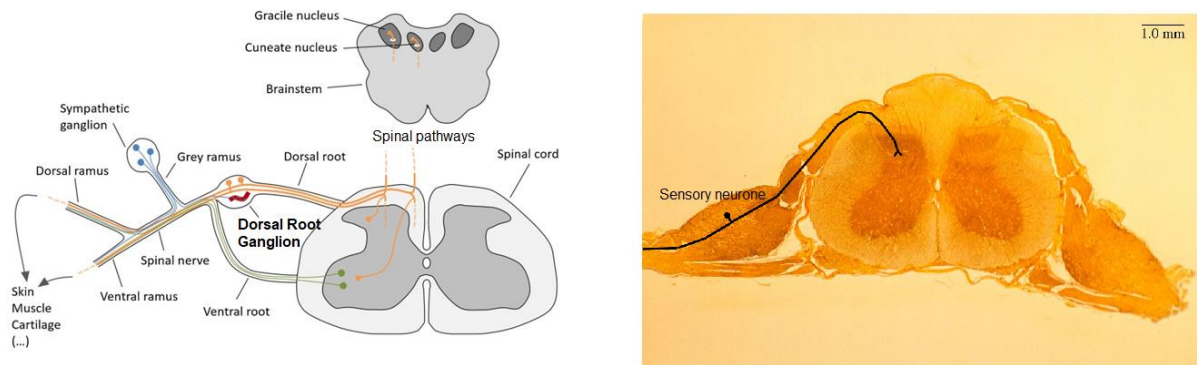


Figure 3.4 Spinal cord and dorsal root ganglion schematic illustration (left) and histological visualisation (right). Images adapted from (Nascimento et al., 2018; Todt, 2017) with permission.

Various subtypes of sensory neurones, including myelinated A α and A β fibres, but also smaller unmyelinated A δ and C fibres, are found in the DRG. Therefore, myelinating and non-myelinating Schwann cells are also present in the DRG. Surrounding and enveloping the cell bodies are also specialised glial cells called satellite glial cells, which are involved in maintaining the microenvironment between neurones in the ganglia, and interestingly can modulate actions that promote regeneration following injury to the peripheral axon processes (Hanani, 2010)(Bella et al., 2006). The ganglia are also covered in a connective tissue capsule made of endothelial and smooth muscle cells, and small blood vessels and immune cells are also present (Haberberger et al., 2019).

DRG have been widely used as a model of the nervous system in various field to conduct studies investigating mechanisms underlying a variety of peripheral nervous system functions, during development, normal physiology also injury and regeneration (Schmeichel et al., 2003; Werth & Thayer, 1994; W. Zhang & Li, 2013).

Furthermore, DRGs can be isolated from various vertebrate animals (e.g. rodent, chicken) at different developmental stages (embryonic or adult), but also from different patho-physiological states which can be used to model different diseased conditions in culture. For example, DRG taken from diabetic rats show differential metabolic profiles compared to normal rat DRG and differential responses to pharmacological interventions (Zherebitskaya et al., 2009). Alternatively, healthy DRG neurones can be isolated and co-cultured with diseased Schwann cells instead, for example Schwann cells from animal with Charcot Marie Tooth disease, and the co-culture can be used to model the pathological de-myelination of neurones *in vitro* and a number of molecular assays can be used to reveal potential underlying mechanisms of de-myelination, and other pathologies in a wide variety of diseases related to neuropathies (Päiväläinen et al., 2008).

Whilst DRG are commonly cultured following dissociation and isolation to obtain a mono-culture of primary cells, they can also be cultured whole as explants (also referred to as *ex vivo* cultures), where each culture type or model offers unique features dependent on the experimental design and aims in question.

Although, a distinct and relevant advantage of using whole explant DRGs as a model is that the DRG are capable of retaining qualities of their *in vivo* architecture, due to the preservation of the relationship of neurones with other non-neuronal cell types (glia, fibroblasts) as well as the extracellular environment found in the DRG capsule that would undeniably become disturbed when dissociated (Melli & Höke, 2009).

Furthermore, harvested DRG undergo axotomy as a result of being removed from their native location, and therefore the culture of DRG *in vitro* is a rather useful model to study nerve injury. DRG explants (like most tissues and cells when studied *in vitro*) will also undergo a large number of cellular and molecular changes, for instance most DRG neurones in culture lose their unipolar morphology and instead become multi-polar, showing a large number of neurite extensions and branching. Interestingly, this is similar to the response seen *in vivo* where following injury, regenerating axons branch into several processes.

As reviewed in 1.4, axotomy of peripheral nerves *in vivo* results in extensive altered gene expression, both at the axons and site of injury but also in the cell bodies of the injured neurones, where these changes contribute to different aspects associated with survival and regeneration (Martin et al., 2019). Similarly, DRG neurones in culture also show upregulation of genes associated with regeneration, such as GAP-43, microtubule-associated protein 5 (MAP5), or ATF-3, which are involved in neurite and axonal outgrowth (Nunez & Fischer, 1997; Riederer & Barakat-Walter, 1992). Satellite glial cells also respond to injury by increasing the production of certain cytokines involved in the immune response which help with debris clearance to promote survival of axons, and have also been shown to contribute to axonal regeneration and sprouting through upregulation of regeneration associated genes (Avraham et al., 2020). Similarly, transcription factors associated with neuronal survival and Schwann cell proliferation, such as c-Jun (Lindwall & Kanje, 2005) or fibroblast growth factor 2 (FGF-2), are also upregulated in DRG cultures (Koistinaho et al., 1993), likely also influenced by signals retrogradely transported from the lesion site to the cell bodies (De Felipe & Hunt, 1994) (Carlin et al., 2019). Therefore, a large portion of critical molecular aspects relevant to *in vivo* regeneration are also modelled in DRG cultures (Sundaram et al., 2021).

Importantly, DRG also show upregulation of a large number of genes associated with the ECM composition (laminin, fibronectin, collagens), as well as cell-adhesion molecules and integrin receptors

required for ECM and cell interactions (integrin B1) (Fudge & Mearow, 2013; Szpara et al., 2007) suggesting that relevant ECM secretion and remodelling processes critical for axonal outgrowth and extension are also appropriately modelled in DRG cultures.

Therefore, given that DRG can recapitulate relevant molecular and cellular events related to nerve injury and regeneration, including components and signalling molecules relevant to the extracellular matrix, it follows that the DRG can be used to model a nerve-specific ECM.

As far as the author is aware, no studies have evaluated DRG as a source of ECM for nerve regeneration, which represents a gap in the literature that holds potential for further investigation, particularly as DRG demonstrate the upregulation of a large number of regeneration-associated genes and proteins that may provide an ECM that is not only nerve-specific but also pro-regenerative.

Studies have shown that adult DRG explants require longer times to establish neurite networks compared to embryonic DRGs (Neto et al., 2017), therefore the use of embryonic DRGs may be more favourable.

3.2.3 Summary and Chapter Aims

It is hypothesised that the DRG-derived ECM can be used to model a nerve-specific ECM that may offer an optimal environment for nerve regeneration. This chapter focuses on the development of DRG derived ECM, where DRG are taken from embryonic chick and cultured as whole explants in an *ex vivo* model, firstly on tissue culture plastic for initial optimisation and characterisation and subsequently on PCL fibres with the ultimate aim of evaluating the DRG-ECM coated PCL fibres in an *in vivo* study in the next Chapter.

Aims:

1. Optimise the deposition and decellularisation of chick DRG-derived ECM on tissue culture plastic.
2. Evaluate chick DRG-derived ECM using a neuronal cell line (NG108-15 cell line) *in vitro*.
3. Optimise the eposition and decellularisation of chick DRG-derived ECM on PCL fibres.

3.3 Methods

3.3.1 Dorsal Root Ganglion culture

Dorsal root ganglia (DRG) were dissected from embryonic chicks and cultured as whole explants on tissue culture plastic (TCP) or electrospun fibre scaffolds made from PCL. Throughout the chapter the culture is also referred to as an *ex vivo* model.

3.3.1.1 DRG dissection

Fertilised chicken eggs (Henry Steward Ltd, UK) were incubated for 12 days in a specialised egg incubator kept at 37.5°C, which featured a gradual rotation device (45 degrees rotation per hour). The chick embryos used in these studies did not require a home office license as they are not considered protected animals under the Animals Scientific Procedures Act (1986) as long as the embryonic developmental stage of the chick is below the last third of the incubation period; Considering that chicken eggs hatch at 21 days, the last third of their incubation period is considered at day 14. Three embryonic developmental days prior to this limit were evaluated in our study: EDD 13, 12 and 11, referring to the day on which DRG were dissected and cultured.

On the dissection day (EDD 11, 12 or 13), the chicken eggs were cracked in sterile Petri dishes, revealing 12-day-old embryonic chicks (Figure 3.9). In some cases, chicks developed incorrectly or seemed deformed, thus these were not included in the experiments. Otherwise, in healthy embryonic chicks, dorsal root ganglia were dissected following the procedure described by Powell et al. (2014) (Powell, Vinod, & Lemons, 2014). Briefly, immediately after cracking the eggs, the chicks were culled by decapitation and the internal organs were removed using blunt, rounded tweezers. Then, chicks were washed in sterile PBS and transferred to a dissecting microscope. Fine tweezers (Dumont, number 5) were used to remove individual whole DRGs from the chick embryo and transferred into sterile PBS where any attached nerve roots could be then removed by trimming, using a scalpel blade.

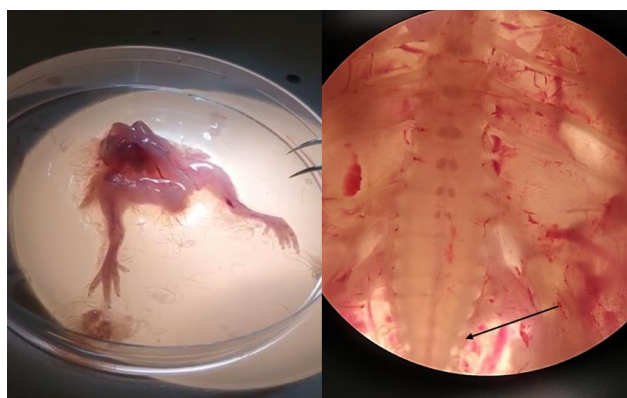


Figure 3.5 Dissection of spinal dorsal root ganglia from embryonic chick embryos. Arrow points at location of DRG.

3.3.1.2 DRG models

DRG were placed either on the surface of wells of a tissue culture plate (TCP) directly or on electrospun PCL fibres. PCL fibres were fabricated as detailed in the previous experimental chapter, meaning, by electrospinning as detailed in 2.2.6 and treated by air plasma as detailed in 2.2.7. Fibres were cut and placed into 6-well plates with the aid of a silicone-based aquarium sealant, prior to sterilisation with 70% ethanol for 30 minutes, followed by 3 PBS washes (1 hour each) and stored in PBS overnight for a maximum of 2 days (in order to conserve the plasma treatment effect).

3.3.2 Decellularisation methods

3.3.2.1 0.5% Triton X-100 in ammonium hydroxide

A solution of 0.5% Triton X-100 (v/v) was made in a buffer of 20mM ammonium hydroxide (NH_4OH) prepared in deionised water and stored at 4°C for up to 1 month. The solution was filtered through a 0.22 μm filter before use.

DRG cultures were washed twice with PBS and then subjected to decellularisation with the Triton X-ammonia solution for 10 mins at room temperature. The decellularisation solution was removed and the surface was washed gently twice with PBS.

For DRG cultures on PCL fibres, a modified decellularisation solution was also trialled consisting of 2.5% Triton X-100 in 20mM ammonium hydroxide buffer for 10 minutes at 37°C, followed by treatment with DNase I (D5025, Sigma) at a concentration of 10mg/ml, and incubated for 30 minutes.

3.3.2.2 Freeze-thaw

Another method trialled for DRG cultures on PCL fibres was freeze-thaw decellularisation, where cultures and scaffolds were washed twice with PBS and covered with the 0.5% Triton X-100 in ammonium hydroxide solution (prepared as described above) and frozen by placing in a -80 freezer for 15 minutes. Then cultures were removed and allowed to thaw for 20 minutes by placing in a water bath set to 37°C. This constituted one freeze-thaw cycle. Three cycles were used in total.

3.3.3 DNA quantification

3.3.3.1 PicoGreen assay

In order to quantify DNA in DRG and DRG cultures before and after decellularisation, Quant-iT™ PicoGreen™ dsDNA Assay (P11495, Thermo Fisher) was used.

Firstly, the cell cultures were lysed to obtain the DNA as follows: Cell cultures were washed with PBS and treated for 30 minutes with a lysis solution consisting of 1% Proteinase K and 1% Triton X-100 in

0.15M NaCl, 50mM Tris-HCL buffer (pH 8.0) (TE buffer). The cell lysates were scraped and detached from the surfaces and then transferred to columns for DNA purification (NucleoSpin® DNA, Takara, Macherey-Nagel) following the manufacturer's instructions. Briefly, the lysates were loaded on to a series of silica-membrane columns with a selective binding for DNA and washed with respective buffers, before eluting from the columns to obtain purified DNA.

The PicoGreen™ dsDNA (200X) reagent supplied in the kit was diluted 1:200 in TE buffer (200mM Tris-HCL 20mM EDTA, pH 7.5) to prepare a working solution of 1X PicoGreen™. The working solution was incubated with eluted sample lysates at a 1:1 dilution for 5 minutes, protected from light. Then, samples were transferred in triplicate to a 96-well plate for reading. A DNA standard curve was included in every 96-well plate ranging from 25 pg/mL to 500 ng/mL. Sample fluorescence was read at excitation = 480 nm and emission = 520 nm using a spectrophotometer plate reader (FLx800, Bio-Tek Instruments Inc.) and KC4 software (version 3.3, Bio-Tek Inc.).

3.3.3.2 Dapi (4',6-diamidino-2- phenylindole) staining and cell counting

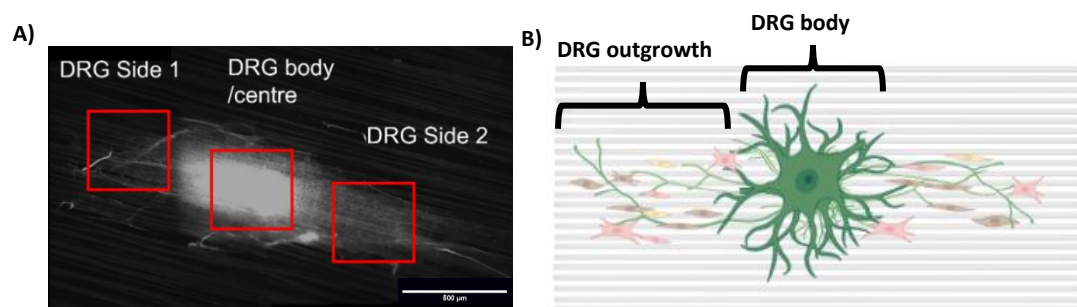
DNA quantification was also conducted on DRG cultures by means of image cell counting analysis based on 4',6-diamidino-2- phenylindole (Dapi) staining of dsDNA in cell nuclei. Briefly, cell cultures were fixed with 3.7% paraformaldehyde for 20 minutes and stained with Dapi (0.2mg/ml, D8417, Sigma) prepared in PBS for 1 hour. Then, images were taken with a fluorescent microscope (2.2.5) and Dapi-stained nuclei were counted using the CellCounter plugin in Fiji for each condition: control (no decellularisation) and post-decellularisation, where the data was normalised to pre-decellularised DRG cultures (100%).

3.3.4 Live/Dead cell staining assay

Cell viability was determined with a Live/Dead® Viability Kit (Thermofisher) based on the permeability of two DNA stains into cells.

Syto-9 (0.02%) and propidium iodide (0.03%) were added to serum-free media and incubated with cells for 1 hour. Syto-9 (green-fluorescent tagged) is a cell-membrane permeable stain capable of entering all and binding to DNA, while propidium iodide (red-fluorescent tagged) is cell-membrane impermeable and can only enter dead cells with compromised membranes. Therefore, all cells stain green with Syto-9 but only dead cells stain red, suggesting the green-stained cells not stained red are alive with intact membranes, serving as a cell viability assay.

Each sample was imaged at three locations: at the DRG body and at either side of the body (Figure 3.6). The fluorescence area for each stain was quantified on ImageJ and expressed using the formula below.



$$\text{Dead cells (\%)} = \frac{\text{Cells stained Red}}{\text{Cells stained Green}} \times 100$$

Figure 3.6 Representation of DRG imaging sites for Live/Dead analysis. A) Microscopy image of DRG grown on PCL fibres. Scale bar 500 microns. B) Schematic representation of the DRG on PCL fibres, showing the different areas: DRG body in the centre where the highest density of cells are, and the outgrowth at each side. Formula used to calculate the number of Alive or Dead cells based on each area.

3.3.5 Immunohistochemistry staining

Cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS (PBS-T) for 20 minutes (2 washes, 10 mins each, and protein-binding sites were blocked with 10% Normal Donkey Serum (NDS) in PBS-T (v/v) for 1 hour. The primary antibodies were prepared in 5% NDS (Table 4) and incubated with the cells overnight at 4°C. The following day, the primary antibodies were washed off (PBS, 2 washes, 10 min each) and the secondary antibodies were prepared in 2.5% NDS and incubated for 3 hours at room temperature. Nuclei were stained with Dapi.

The table below describes the antibodies used along the secondary antibodies, where the dilutions were selected and optimised based on previous studies in the laboratory.

Table 4. List of primary and secondary antibodies used for immunohistochemistry.

	Product code	Dilution in NDS	Stock concentration (mg/ml)
Primary antibodies			
Mouse, anti-Beta III Tubulin	Biologend,801202	1:1000 to 1:500	1.0
Rabbit, anti-S100B protein	Dako, Z0311 Proteintech, 15146-1-AP	1:100	1
Rabbit, anti-Collagen IV	Abcam, Ab6586	1:200	1
Rabbit, anti-Laminin	Abcam Ab11575	1:50	0.7
Secondary antibodies			
Donkey, anti-mouse FITC	715-095-150	1:400	1.25
Donkey, anti-rabbit Cy3	711-165-152	1:400	1.25

3.3.5.1 Antibody specificity and controls

The specificity of the secondary antibodies to the primary antibodies was tested by omitting the primary antibodies from the protocol and using only secondary antibodies.

For immunocytochemical studies of dorsal root ganglia cultures, it was found that the body of the DRG showed fluorescence in the absence of the primary antibody (Figure 3.7). However, this fluorescence was only in the DRG body (top row), as opposed to the cells or neurites extended from the DRG (bottom row).

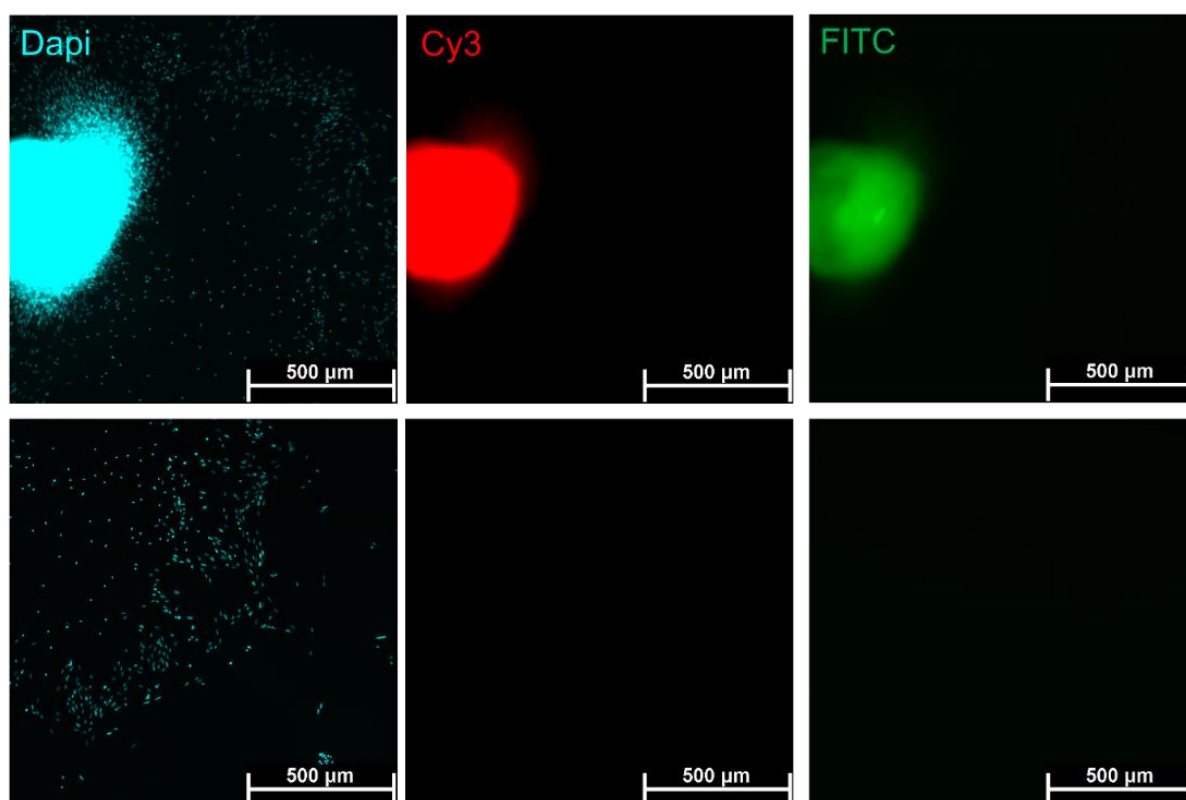


Figure 3.7 Immunocytochemistry controls for DRG cultures on TCP. DRG explants were cultured on TCP for 1 week and stained using the immunocytochemistry protocol but omitting the primary and using only the secondary antibody, showing obvious autofluorescence of the DRG body (top row). However, the cells derived from the DRG did not show any autofluorescence in the absence of the primary antibody as shown in the bottom row where Dapi-stained cell nuclei are seen, but no autofluorescence staining is seen under the excitation for the secondary antibodies (bottom row). Scale bars 500μm. Representative images are shown from three independently performed experiments (N=3, n=3).

A similar observation was noted with DRG on PCL fibres in the absence of the primary antibody, particularly in the green and blue channel where some autofluorescence of the fibres could be seen as well in the absence of staining (Figure 3.8). However, under the same excitation parameters, a clear difference can be seen in samples stained as positive controls (i.e., primary and secondary incubations). Therefore, images were captured and processed identically using the same excitation

parameters and any contrast enhancements or background removals were also applied consistently to allow direct comparisons.

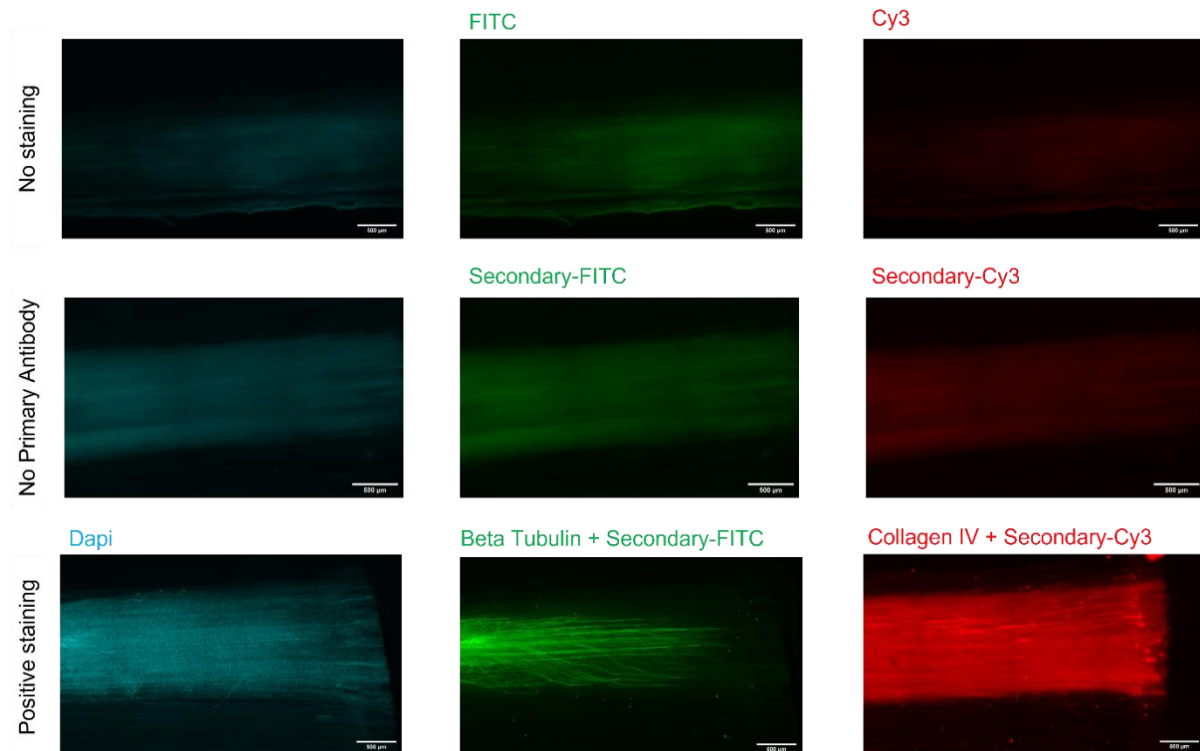


Figure 3.8. Immunocytochemistry controls for DRG cultures on PCL fibres. The top row shows DRG explants cultured for 1 week on PCL fibres and stained using the immunocytochemistry protocol but omitting the primary and secondary antibody, showing some background autofluorescence of DRG. The middle row similarly shows 1 week DRG cultures on PCL fibres stained following the immunocytochemistry staining protocol omitting the primary antibody and using only the secondary antibodies, showing also background staining and autofluorescence from DRG. Lastly, the bottom row shows DRG cultures on PCL fibres after 1 week stained with the standard immunocytochemistry protocol including both primary and secondary antibodies, demonstrating positive staining, particularly where DRG axons are clearly stained with Beta Tubulin (green), as well as positive staining for collagen (red). Scale bars 500µm. Representative images are shown from three independently performed experiments (N=3, n=3).

3.3.6 Cell culture of NG108-15 cells on dECM surfaces

General cell culture aseptic techniques were adopted as described in 2.2.42.2.4.6 and based on previous studies by the group (Daud et al., 2012)

Neuronal NG108-15 cells (ECACC, UK) were cultured at a seeding density of 5000 cells in 150µL of complete media overnight to allow cell attachment to the surfaces. Cultures were then topped up with complete media up to 300 µL, before changing the culture to serum-free growth media (after 48 hours from initial seeding) to encourage the formation of neurites from the cell line.

NG108-15 cells were cultured on decellularised DRG-matrix (dECM) or TCP as control for 1 week to evaluate cell attachment and neurite elongation. The metabolic activity of cells was also measured using the resazurin assay as detailed in 2.2.4.7

3.3.7 Statistical analyses

Statistical analyses were made using GraphPad Prism (GraphPad Software v9, San Diego, CA). Data was tested for normality and analyses of variance (ANOVA) was used to test for differences between groups for parametric data. For comparison between only two groups a Student's T-test was used. For data with one dependent variable, a one-way ANOVA was used and for two or more dependent variables a two-way ANOVA was used. Post-hoc multiple comparisons were tested with Tukey's. Experiments were replicated independently three times ($N = 3$), and within each replicate, three separate samples were collected ($n = 3$) unless reported otherwise in the text.

Results

This chapter investigated the hypothesis that dorsal root ganglia (DRG) can be used to deposit an extracellular matrix (ECM) that is nerve-specific and may offer an optimal environment for nerve regeneration. In order to investigate the hypothesis, the results are split into two parts where DRG are cultured, decellularised and evaluated based on the surface used for the model, namely tissue culture plastic (Part 1) or PCL scaffolds (Part 2)

3.4 Part 1: Tissue culture plastic (TCP)

3.4.1 Optimisation of chick-derived DRG model on tissue culture plastic (TCP)

Dorsal root ganglia were dissected from embryonic chick embryos and cultured on tissue culture plastic (TCP) for 1 week. On average, 20-30 DRG could be dissected within a reasonable time from one egg (Figure 3.9).

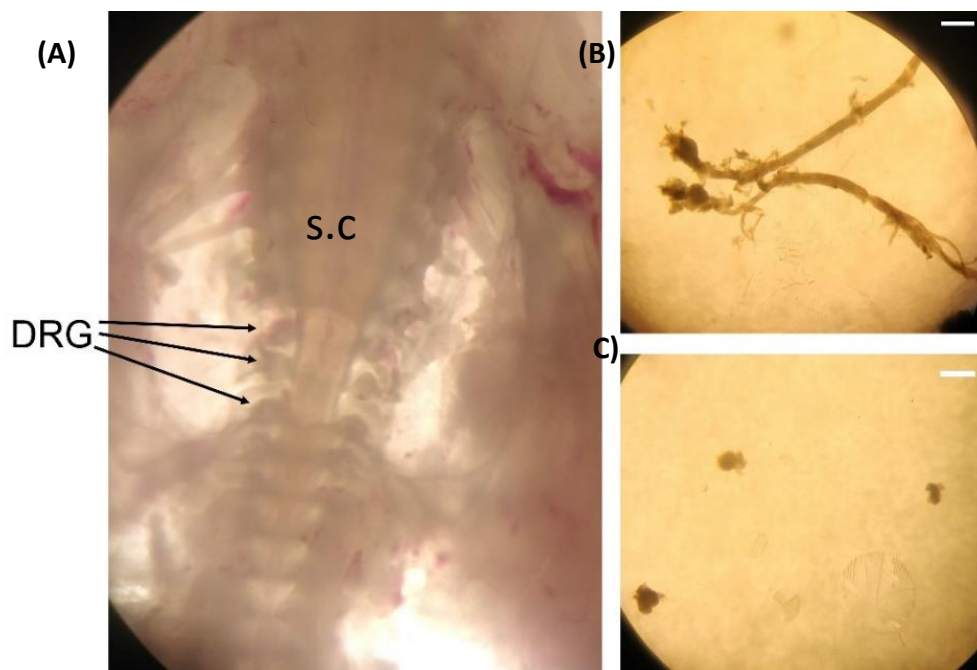


Figure 3.9 Dissection of spinal dorsal root ganglia (DRG) from embryonic chick embryos. (A) Arrows point at location of three DRG adjacent to the spinal cord (s.c). (B) Dissected DRG (two ganglia) with peripheral nerves still attached. (C) DRG without nerve roots. Scale bars 1mm

The outgrowth of cells from the DRG explants seeded on TCP was found to be highly variable, with some DRG showing minimal outgrowth while other ganglia showed extensive cell migration and neurites (Figure 3.10). Specifically, the outgrowth and migration of cells from the ganglia ranged from between a few cells that migrated 20 μm away from the DRG body, to extensive migration where Dapi-stained cell nuclei were found up to 5000 μm away from the body), although the average migration was 2213. In addition, the migration of cells and the extension of neurites from the ganglia

often showed bias towards one side, rather than extending radially equally. Furthermore, the attachment rate of the DRG explants to the tissue culture plastic was also highly variable.

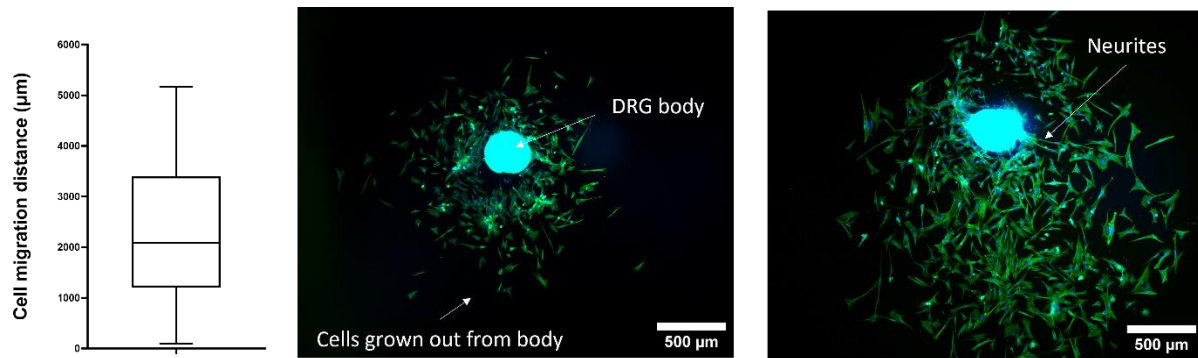


Figure 3.10 DRG outgrowth was highly variable on TCP. The box plot represents the spread of the cell migration data from different DRG showing a large variability (data from all DRG samples included i.e. $N=5$, $n=20$). Two images of DRG cultures are shown with varying levels of outgrowth from the DRG body (large blue mass of cells in the middle). Blue: Dapi staining for cell nuclei and Green: Phalloidin staining of F-actin in cytoskeleton of cells. Scale bars 500µm.

Therefore, in order to optimise DRG outgrowth to develop a reproducible model, several parameters were investigated. Firstly, the type and size of the tissue culture plates used to culture DRG was investigated as preliminary experiments suggested DRG were prone to floating and detaching depending on the plate used.

Specifically, DRG were seeded individually in the wells of tissue culture plates of various sizes (6 well plate to 96 well plate) and the attachment rate and outgrowth was calculated after 1 week of culture by counting the number of DRG that remained attached and grew cells out of the number of DRG seeded, reported as percentage growth, as shown below.

$$Growth (\%) = \frac{\text{number of DRG attached with cell outgrowth}}{\text{number of DRG seeded on TCP}} \times 100$$

Overall, DRG attachment and outgrowth was generally low on TCP, ranging from $11.6 \pm 16\%$ outgrowth for DRG seeded on 6 well plates, to $31.2 \pm 10\%$ for DRG seeded on 48-well plates (Figure 3.11). Whilst the percentage growth was highest when DRG were seeded on a 96 or 48 well plate compared to 24-, 12- and 6- well plate, this was not significantly different ($p=0.07$). However, because the DRG outgrowth on 48-well plates showed the smallest variance, 48-well plates were selected for subsequent studies.

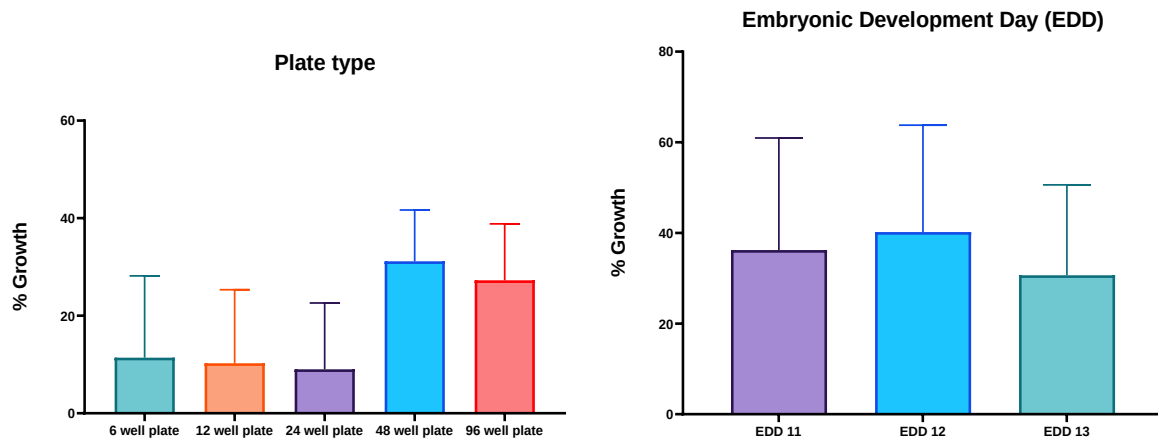


Figure 3.11 DRG culture on TCP optimisation. The percentage growth rate of DRG on different well plates was investigated showing no significant difference in the plate type used ($p>0.05$). In addition, DRG from chicks of different embryonic developmental days were investigated and no significant effect was found on the percentage growth ($p>0.05$). Data was tested using a one-way Anova. ($N=3$, $n=3$).

Next, the embryonic developmental day (EDD) of the chick at the day of the dissection of DRG was also studied to evaluate if there were any effects of DRG “age” on cell attachment and outgrowth on TCP. There was no significant difference in the percentage of DRG that attached and grew between DRG taken from EDD 11, 12 or 13 ($p>0.05$) (Figure 3.11), suggesting no effect of the EDD on DRG attachment and growth. However, DRG outgrowth was slightly less when DRG were dissected from EDD 13 chick embryos, therefore subsequent studies used DRG from chick embryos of EDD 11 and 12.

Furthermore, it was noticed that the DRG explants were morphologically different in size, which upon closer inspection of the DRG *in situ*, a difference in size could be seen depending on the location of the DRG in relation to the spinal cord. Specifically, the thoracic DRG (found at the spinal level between the ribs of the chick embryo) and the lumbar DRG (found adjacent to the lumbar enlargement of the spinal cord) were visually larger than the DRG at the lower sacral spinal cord. Therefore, we wanted to investigate if the spinal level and size of the DRG had an effect on attachment and outgrowth of cells.

Dorsal root ganglia were dissected from different spinal cord levels (thoracic, lumbar, and sacral) from EDD 12 chick embryos and cultured for 1 week on a 48-well plate and showed no significant difference in terms of the percentage growth. In addition, the DRG outgrowth was then quantified in terms of the average maximum migration distance travelled by the cells derived from the ganglia of the various spinal levels, showing a small but non-significant trend with increased maximum migration in ganglia taken from thoracic DRG compared to lumbar and sacral DRG (Figure 3.12).

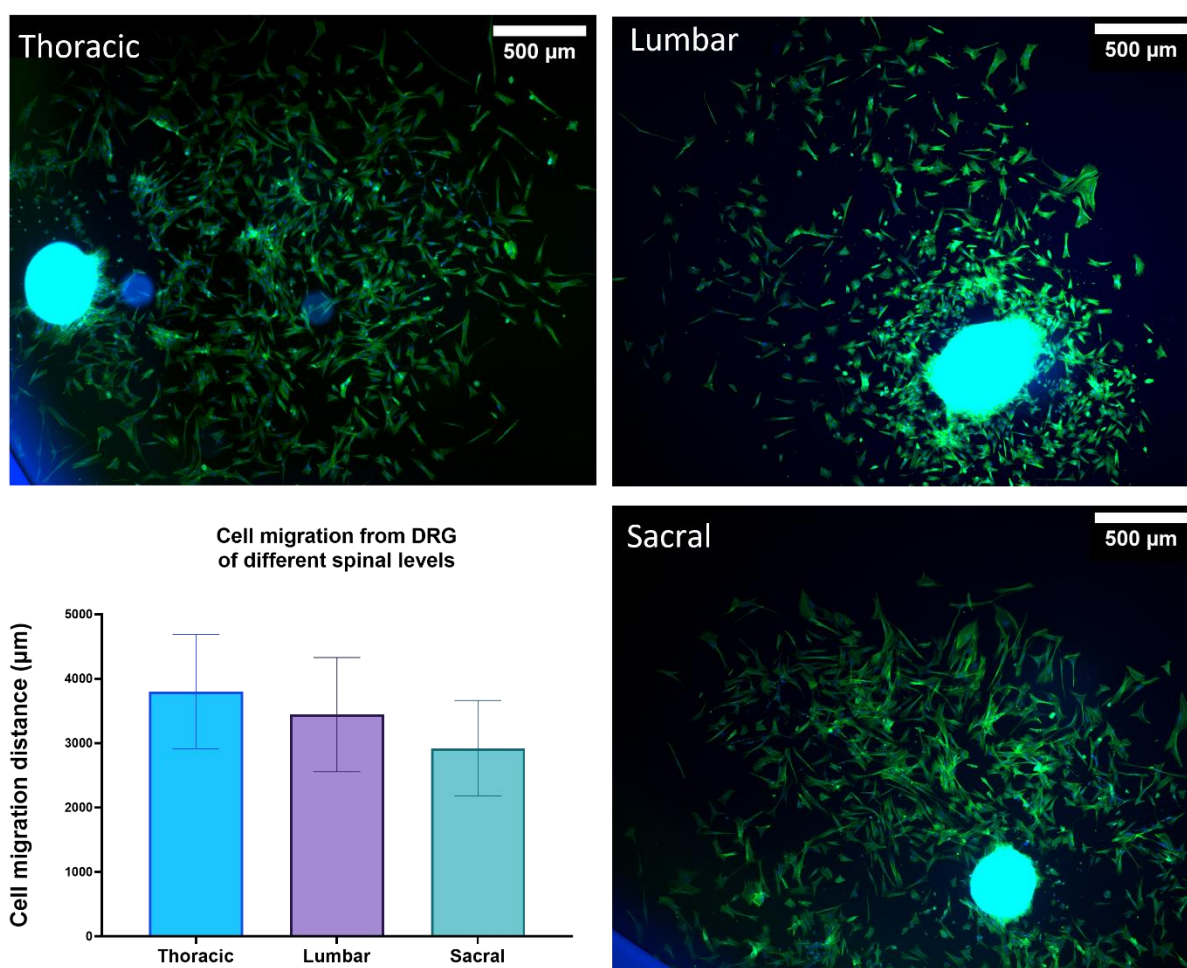


Figure 3.12 Effect of spinal level origin of DRG on DRG outgrowth and cell migration. DRG were dissected from different spinal levels (thoracic, lumbar and sacral spinal cord level) and the explants were cultured on 48-well plates individually for 1 week. DRG showed outgrowth of cells where the maximum migration distance was measured for each condition. Cells were stained by Dapi stain for cell nuclei and Phalloidin stain for F-actin in the cytoskeleton of cells. Data is represented as mean + standard deviation and tested by a one-way Anova. No significant differences were found ($p>0.05$). Scale bars 500μm. Representative images are shown from three independently performed experiments ($N=3$, $n=3$).

Overall, no significant effects of plate type, EDD day or spinal level were found on the DRG attachment rate and outgrowth of cells from the explants on TCP. Therefore, in order to maximise the number of available DRG for experiments, DRG were dissected from all spinal levels and from chick embryos of embryonic developmental (EDD) day 11 and 12, and cultured on 48-well plates as these showed the highest rates of percentage growth.

3.4.2 DRG culture characterisation and decellularisation

The next set of experiments aimed to investigate the optimised DRG *ex vivo* models by characterisation of the cells in the DRG cultures as well as the ECM components. In addition, the effect of decellularisation of DRG cultures was also evaluated.

DRG from chick embryos (EDD 11 and 12) were cultured as whole explants on TCP (48 well-plate) for 1 week. Then, the samples were either subjected to decellularisation with a detergent-based solution consisting of 0.5% Triton X-100 in an ammonium hydroxide buffer for 10 minutes at room temperature, or with PBS as control. The solutions were removed, and the DRG cultures were washed gently twice with PBS prior to fixing with 3.7% PFA and immunocytochemical staining as described in methods 3.3.5 against a number of relevant antibodies to characterise the cultures.

DRG cultures contained neuronal cells and axons stained by neuronal-specific antibody Beta Tubulin as well as containing Schwann cells, evident by glial-specific S100B staining, as shown in the top row of Figure 3.13, referred to as “control”. In contrast, DRG cultures that were subjected to the decellularisation protocol did not show any positive staining for neuronal cells or axons, nor Schwann cells, (Figure 3.13) suggesting the decellularisation protocol was successful in removing neurones and Schwann cells from the DRG cultures.

DRG cultures were also positive for extracellular matrix components of collagen IV (Figure 3.14) and laminin (Figure 3.15), shown in the top row of the figures (DRG + ECM). DRG cultures subjected to decellularisation resulted in a sheet-like film being left behind that was positive for collagen IV and to a lesser extent for laminin, suggesting the decellularisation protocol was capable of retaining ECM relevant components.

Any remaining Dapi-staining evident in DRG cultures after decellularisation showed abnormal morphology with fragmented nuclei found clumped, and cell nuclei stained by Dapi were quantified and showed a $98.1 \pm 1.98\%$ reduction after decellularisation (Figure 3.16). In addition, double-stranded DNA content was quantified using Quant-iT™ PicoGreen™ dsDNA Assay also showing a relative reduction of $97.5 \pm 3.5\%$ in dsDNA after decellularisation (Figure 3.16).

DRG EX VIVO CULTURE ON TCP

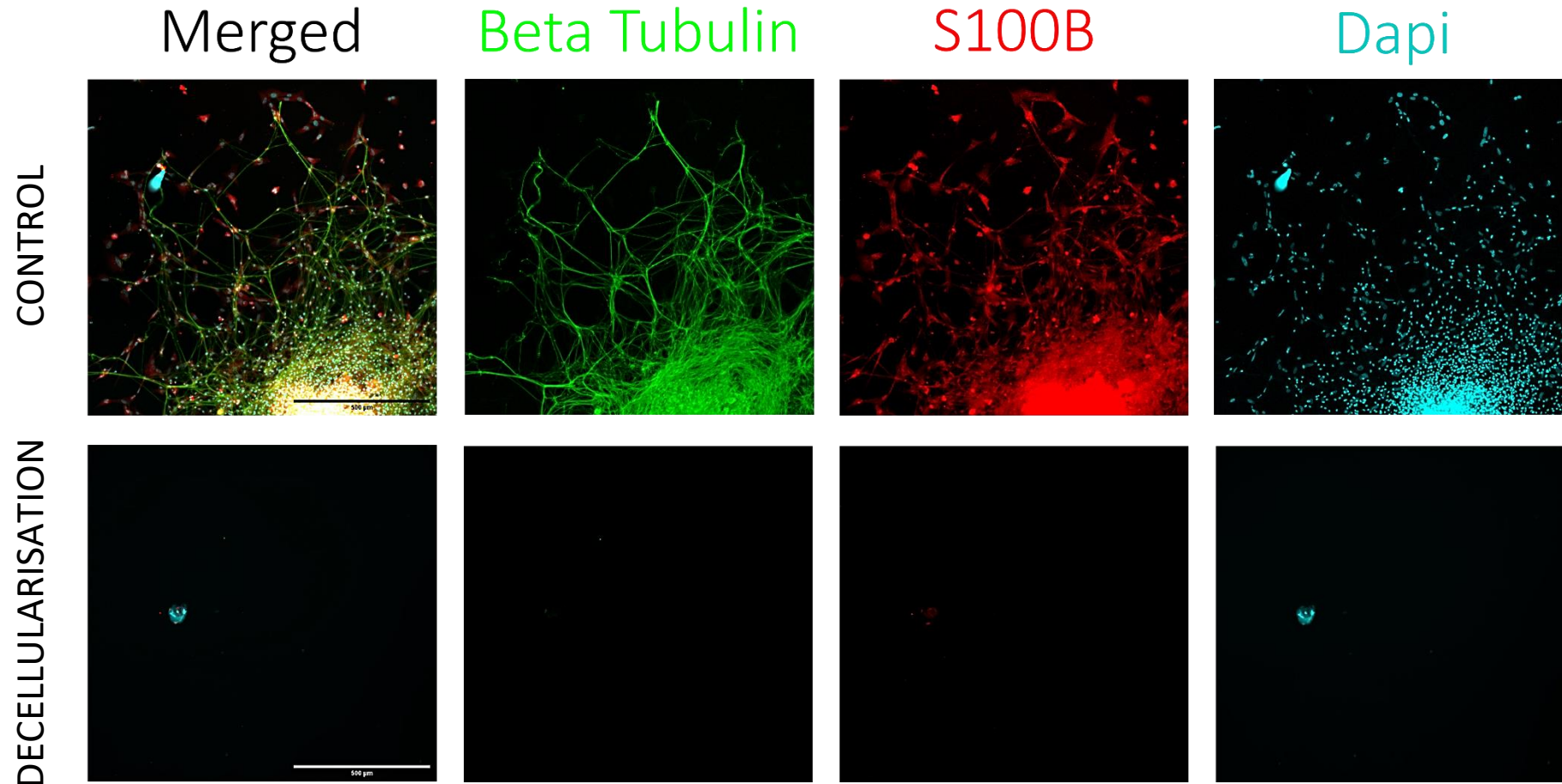


Figure 3.13. Representative immunofluorescence images of DRG cultures. The top row shows DRG cultures with labelling of neuronal marker B-tubulin III and Schwann cell marker S100B, along with nuclei marker DAPI after 1 week of culture (Control). The bottom row shows cultures following decellularisation, with no positive neuronal or Schwann cell staining. Scale bars 500 μ m. Representative images (N=3, n=3).

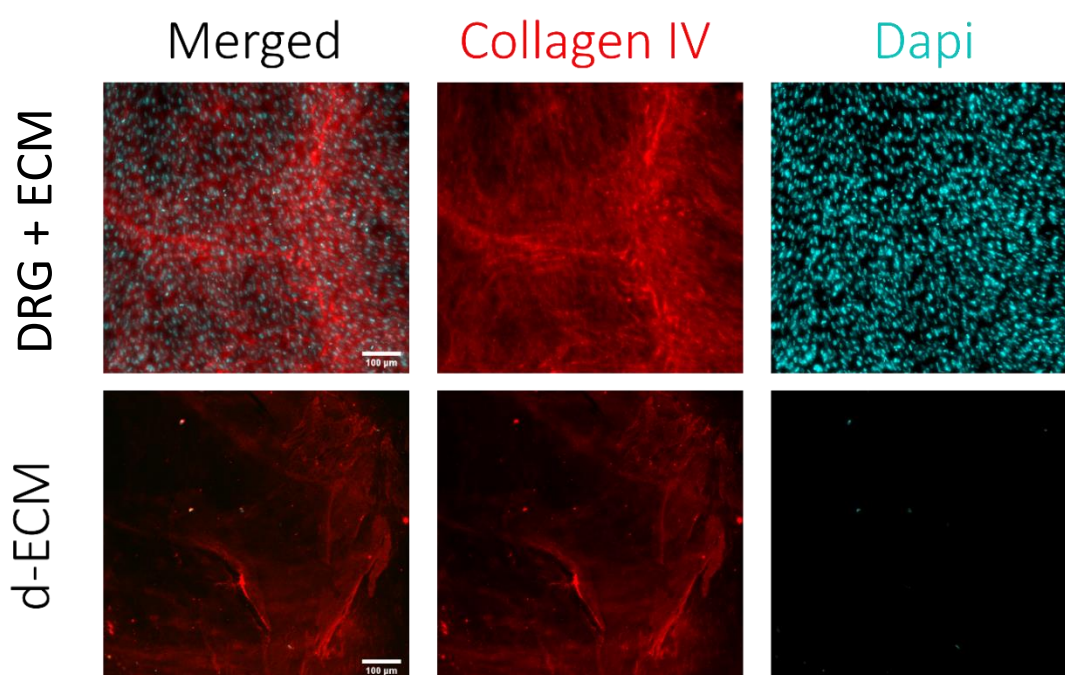


Figure 3.14 Representative immunofluorescence images of DRG cultures before (DRG+ECM) and after (d-ECM) decellularisation. The top row shows DRG cultures with labelling of collagen IV, an ECM component found in the basal lamina of nerves, along with nucleic marker DAPI after 1 week of culture and the bottom row shows decellularised cultures where collagen IV is positive, suggesting the ECM is maintained, but no Dapi-staining confirming decellularisation. Scale bars 100 μ m. (N=3, n=3).

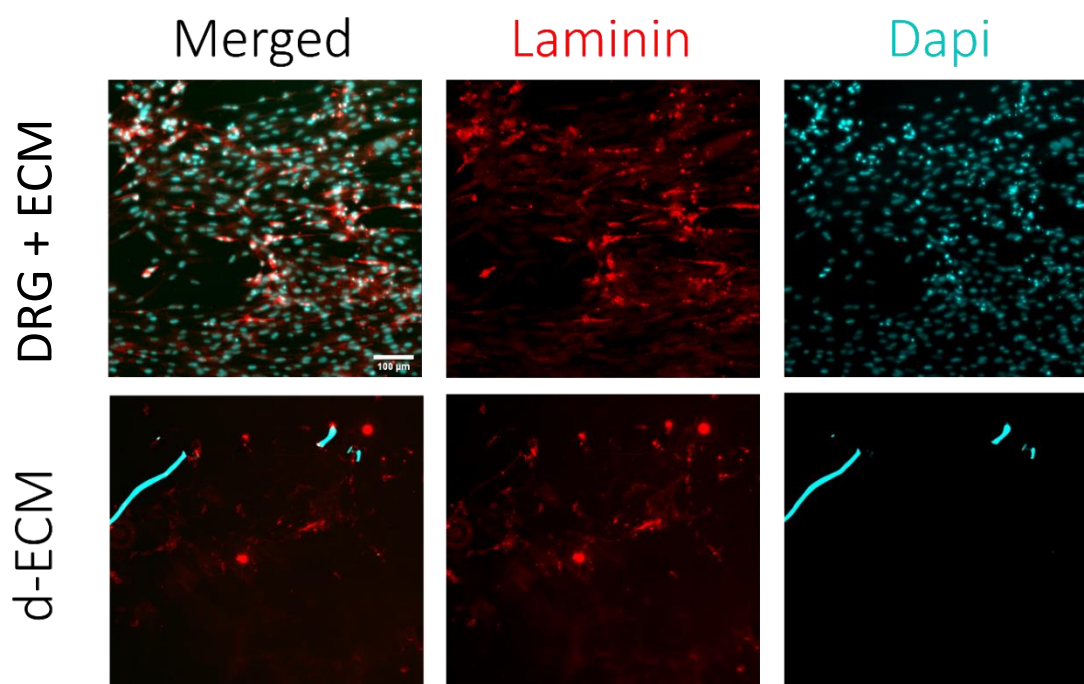


Figure 3.15 Representative immunofluorescence images of DRG cultures before (DRG+ECM) and after (d-ECM) decellularisation. The top row shows DRG cultures with labelling of collagen IV, an ECM component found in the basal lamina of nerves, along with nucleic marker DAPI after 1 week of culture and the bottom row shows decellularised cultures where collagen IV is positive, suggesting the ECM is maintained, but no Dapi-staining confirming decellularisation. Scale bars 100 μ m (N=3, n=3).

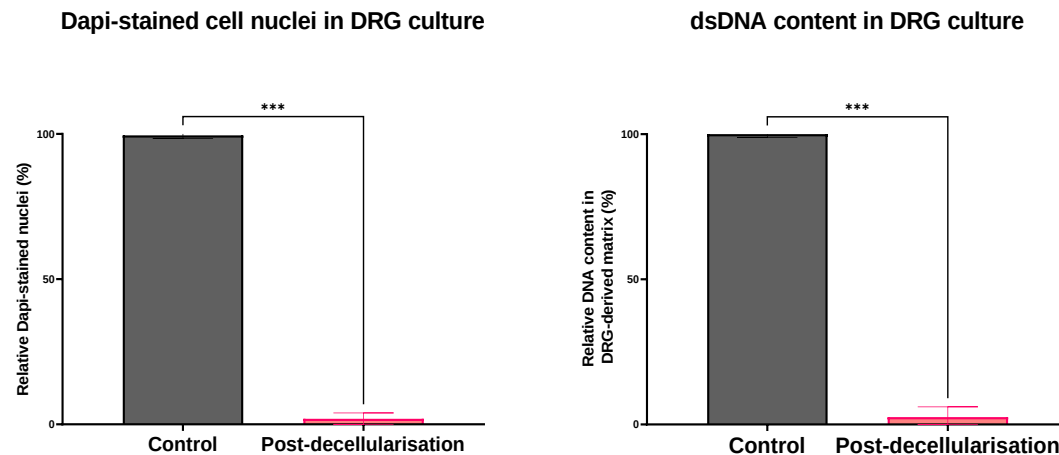


Figure 3.16. DNA quantification was assessed after 1 week of culture of DRG on TCP (control) and on DRG cultures subjected to decellularisation (post-decellularisation) by two methods: presence of dapi-stained nuclei and dsDNA quantification using a commercially available kit (PicoGreen™ Assay). Data was analysed using the unpaired Student's T-test where *** signifies $p < 0.001$. ($N=3$, $n=3$).

3.4.3 Evaluation of DRG derived dECM as a surface for nerve regeneration

Finally, the DRG-derived decellularised extracellular matrix (dECM) was evaluated as a novel surface for nerve regeneration potential by using a neurite outgrowth assay using NG108-15 cells. Briefly, NG108-15 cells were seeded on the dECM surfaces or on tissue culture plastic (TCP), which served as a control, and the media of the cells was changed after 48 hours to serum-free media to induce the extension of neurites from the cell line.

The cell morphology of NG108-15 cells cultured on the dECM was compared to TCP by counting cells remaining attached at the end of the culture period and neurite counts, as well as measuring neurite lengths (Figure 3.17). There was an increased number of cells counted on dECM surfaces compared to TCP at the end of the 5-day culture period ($p < 0.01$). In addition, a larger number of neurites were significantly extended from cells cultured on the dECM compared to TCP ($p < 0.01$). However, the length of the neurites was not significantly different between cells grown on TCP and dECM.

The metabolic activity of NG108-15 cells was also measured at three time points during the culture of neuronal NG108-15 cells on the two surfaces, where there was no significant difference in the metabolic activity measured between cells grown on TCP or dECM on each day ($p > 0.05$) (Figure 3.18). On day 7, there was slightly increased metabolic activity measured on cells grown on dECM compared to TCP, although not significantly ($p = 0.06$). There was also a small decline in the metabolic activity of cells as a function of time (Day 1 to Day 7) related to the detachment of cells as a result of repeated changes of cell culture media.

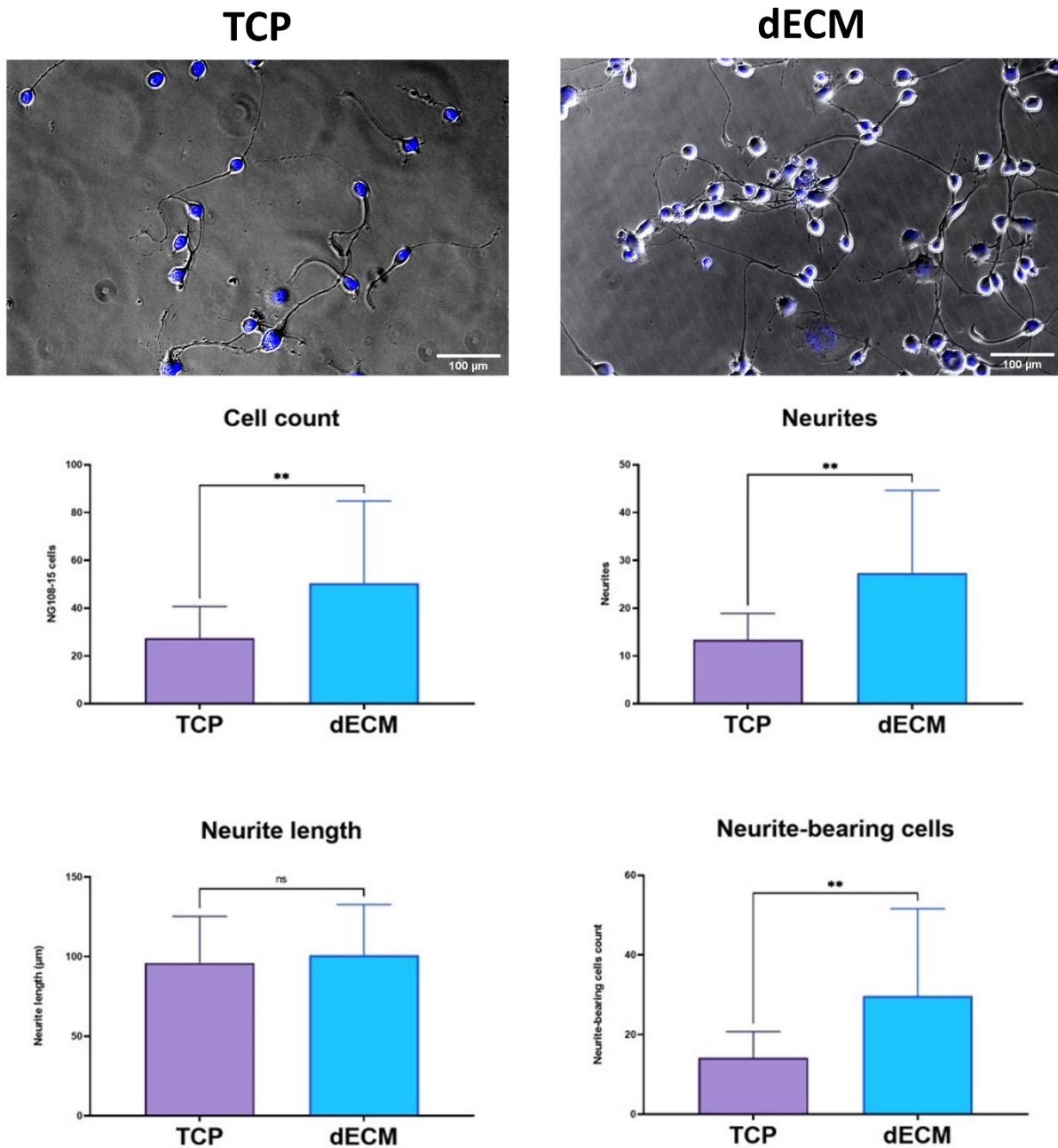


Figure 3.17 NG108-15 cells were seeded and cultured for 1 week on tissue culture plastic (TCP, top left) and on the decellularised extracellular matrix (dECM, top right). Images are phase-contrast with Dapi stain for nuclei (blue). Cells and neurites were analysed by cell count, neurite count and neurite length. Scale bar 100μm. Data was analysed with an unpaired Student's T-test for each graph, where ** signifies $p < 0.01$. Representative images ($N=3$, $n=3$).

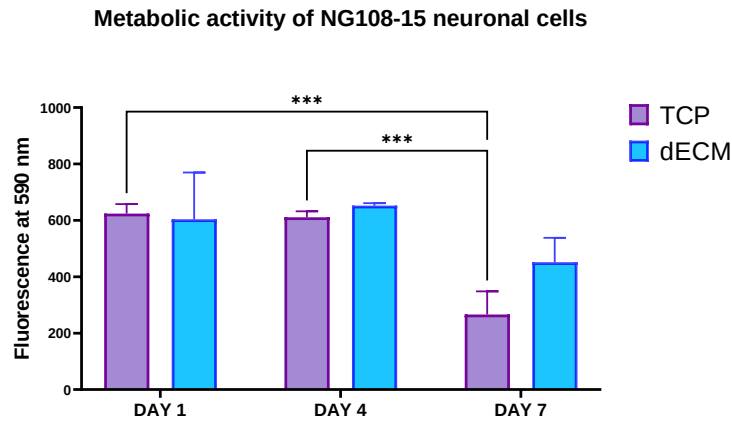


Figure 3.18 Metabolic activity of neuronal NG108-15 cells was measured on day 1, day 4 and day 7 of the cell culture period when cells were grown on TCP (purple bars) or on the decellularised chick-derived extracellular matrix (dECM) (blue bars). Statistical analyses were performed using a two-way repeated measured ANOVA, $p < 0.01$. Representative images ($N=3$, $n=3$).

3.5 Part 2: Fibres

As mentioned, the results were split into two parts depending on the surface used for the culture of the DRG explants. In the second part of the results, dorsal root ganglia were dissected from embryonic chick embryos and cultured on polycaprolactone (PCL) fibres previously fabricated by electrospinning as described and optimised in Chapter 2. On average, 20-30 DRG could be dissected within a reasonable time from one egg.

3.5.1 DRG culture on PCL fibres

Dorsal root ganglia (DRG) were seeded on PCL fibres (6 - 8 microns in diameter) and cultured for 2 weeks. Figure 3.19 shows a schematic of a DRG explant seeded in the middle of PCL fibre scaffolds which were cut to 1 cm and inserted in 24 well plates, showing the extension of axons from neuronal cells in the DRG body as well as the migration of other cells in the ganglia like Schwann cells.

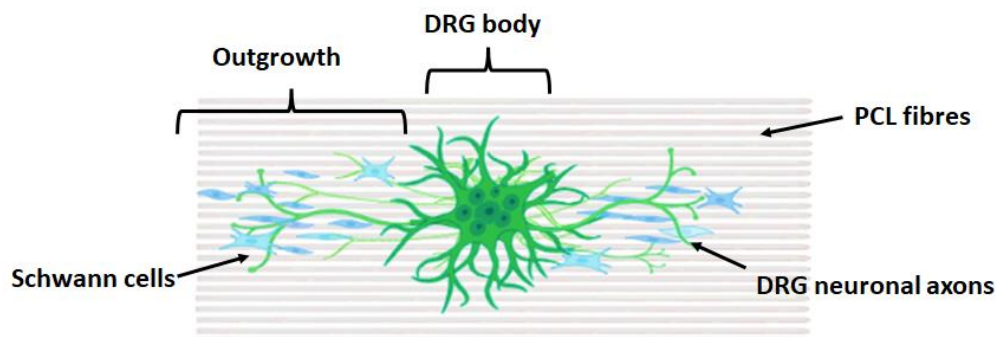


Figure 3.19. Schematic of DRG culture on PCL fibres. DRG explants were seeded in the middle of PCL scaffolds and cultured for 2 weeks. Neurones with cell bodies in the DRG explant (DRG body) extended axons and neurites along the PCL fibres. DRG-derived glial cells (Schwann cells) also migrated and extended along the fibres.

DRG cultures on PCL fibres were fixed and stained for neuronal specific anti-beta tubulin which stains axons but also the cell bodies of neurones, but does not stain other cells derived from the ganglia. Figure 3.21 shows a DRG explant where positive beta-tubulin staining (green) shows characteristic neuronal cell bodies as well as the axons that are extended longitudinally (pointed by arrows, middle row). In addition, Dapi-staining of cell nuclei is also evident all along the fibres, showing the presence of cells derived from the ganglia that are not neurones.

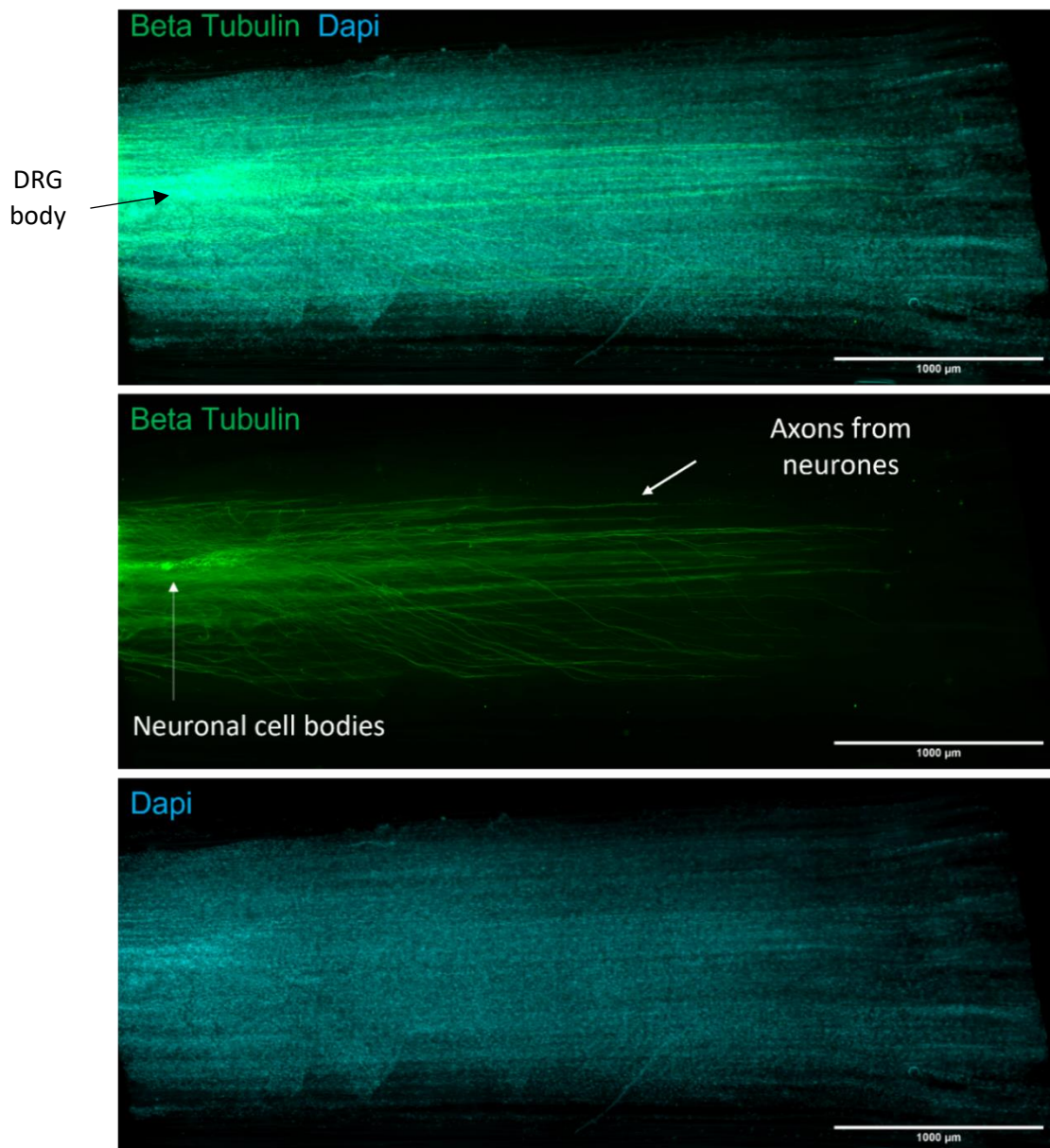


Figure 3.20 DRG grown on PCL fibre scaffolds. (A) Immunofluorescence images of DRG explant cultured on PCL fibre following 2 week of culture and stained for neuronal-specific anti-Beta Tubulin, showing axons (green) and also neuronal cell bodies (arrow). Dapi staining for cell nuclei stains all the cells derived and grown out of the ganglia (blue) covering the scaffold. Scale bar 1000 µm.

The average maximum length of the axons extended by DRG neurones along the PCL fibres was $3691 \pm 658 \mu\text{m}$. The average migration of DRG-derived non-neuronal cells was $3947 \pm 531 \mu\text{m}$ after 2 weeks (Figure 3.21). The maximum migration of cells was limited by the size of the PCL scaffolds, which were cut to 1 cm in length in total, and DRG were seeded in the middle allowing for roughly 0.5cm ($5000 \mu\text{m}$) of scaffold space for cell outgrowth on each side. In the majority of samples, the migration of the non-neuronal cells stained by Dapi surpassed the maximum axonal lengths, suggesting axons followed closely behind the non-neuronal cells.

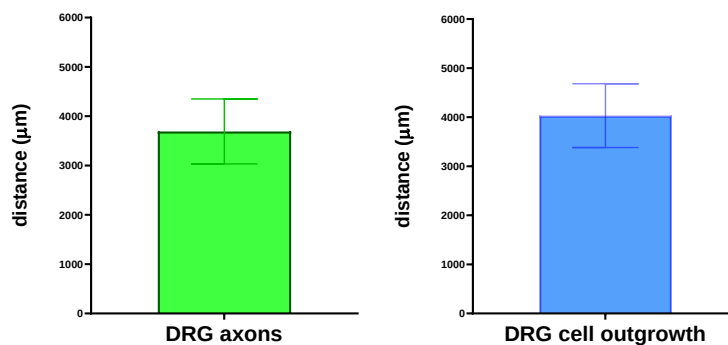
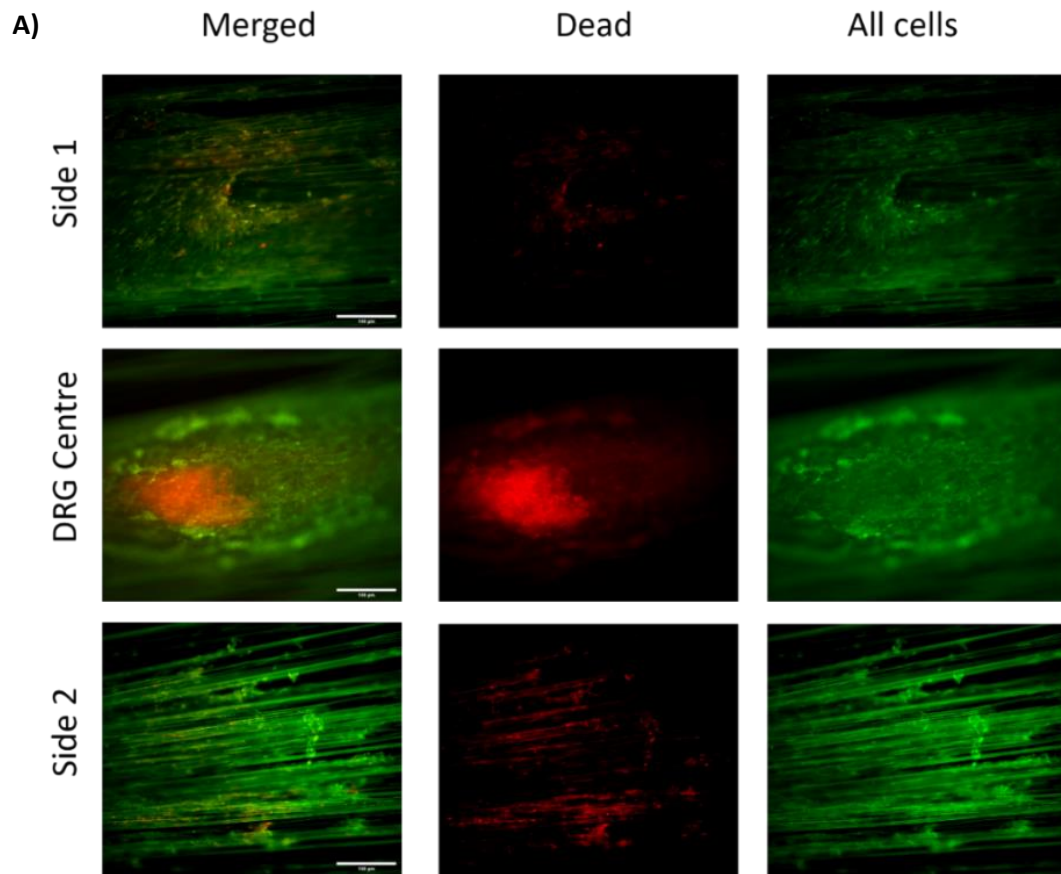


Figure 3.21 DRG grown on PCL fibre scaffolds. The extension of neurites (green) and dapi-stained cell nuclei (blue) derived from the DRG was quantified by measuring the average maximum axon outgrowth from DRG neurones, and the maximum distance travelled by cells based on the Dapi-stained nuclei. (N=3, n=3).

3.5.1.1 Live/Dead

Cell viability of DRG explants on PCL fibres was evaluated using a Live/Dead assay as described in 3.3.4, where the percentage of living and dead cells were measured at three locations on the ex vivo models: the DRG body and at either side of the body where cell outgrowth and axons extension had occurred, referred to as side 1 and side 2 (Figure 3.22).

Within the DRG body, there was a similar ratio between the percentage of live and dead cells, where $31.5 \pm 24 \%$ of cells were stained red, signifying dead cells, with no significant difference compared to the percentage of live cells ($68.5 \pm 31 \%$) ($P > 0.05$). However, on either side of the DRG, where axons and cells had grown, the majority of the cells were alive ($97.2 - 98.0\%$) with only $1.9 - 2.7\%$ of cells being stained red.



B) Cell viability of DRG explants on PCL fibres

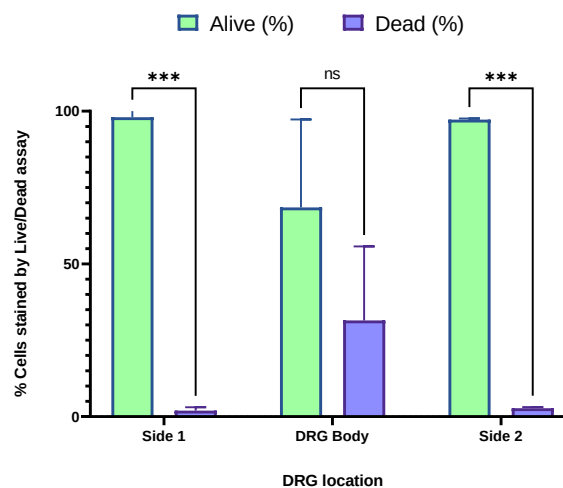


Figure 3.22 Cell viability of DRG cells on PCL fibres quantified by Live/Dead assay. (A) Representative images of DRG explants cultured for 2 weeks on PCL fibres and stained with Live/Dead kit containing two DNA stains, syto-9 which stains all living cells (green) and propidium iodide, which stains dead cells only (red). The images show three regions of the culture, the DRG body and the two outgrowth sides from the body (side 1 and side 2). Scale bars 100µm. (B) The percentage of live and dead cells were quantified by region, where there were significantly more alive cells in the cell outgrowth sites side 1 and side 2 compared to dead cells(***, $p < 0.001$), but there was no significant difference in the DRG body where an equal ratio of living and dead cells were found ($p > 0.05$). (N=3, n=3).

3.5.2 Optimisation of decellularisation protocol

The next set of experiments aimed to optimise the decellularisation protocol for DRG on PCL fibres.

DRG from chick embryos (EDD 11 and 12) were cultured as whole explants on PCL fibres for 2 weeks. Then, the samples were either subjected to decellularisation, or washed with PBS as a control. Three methods were evaluated as a decellularisation protocol. Firstly, the standard protocol used for DRG on TCP which was 0.5% Triton X-100 in an ammonium hydroxide buffer (Method 1). Secondly, a similar decellularisation protocol with a stronger concentration of 2.5% Triton X-100 alongside DNase I was also trialled (Method 2). Finally, a third method was evaluated where the DRG and fibres were decellularised by repeated freeze-thaw cycles.

The solutions were removed, and the DRG cultures were washed gently twice with PBS prior to fixing with 3.7% PFA and staining with Dapi for cell nuclei and Phalloidin for F-actin present in the cytoskeleton (Figure 3.23). This staining was selected as it provided a simple assay to visualise decellularisation.

Visually, Methods 1 and 2 were able to remove the DRG body from the fibres, which is also evident microscopically by the reduction in Dapi-staining of cell nuclei in the middle of the explants seeded on PCL fibres. In contrast, DRG bodies were still visibly attached to the fibres when decellularised by Method 3, which is also evident by the Dapi-fluorescence in the middle of the explants (shown by asterisk).

Nevertheless, as shown in Figure 3.23, Dapi-staining was still evident along the length of the fibres after all three decellularisation protocols. Upon closer inspection (Figure 3.24), the Dapi-stained nuclei after decellularisation showed an abnormal and fragmented morphology, therefore showing the decellularisation techniques were able to disrupt the cell membranes of DRG cells successfully, but not completely remove the residual cells and DNA from the fibres

The cell viability of the DRG cultures on PCL fibres after decellularisation was also evaluated using the Live/Dead assay, confirming that the majority of cells remaining after each decellularisation protocol were in fact dead cells. Method 1 resulted in cell death of $96.3 \pm 5.3\%$ of all cells, Method 2 in $95.1 \pm 1.1\%$, and Method 3 in $91.4 \pm 6.7\%$ of cells. Whilst Method 3 showed the lowest percentage of dead cells following decellularisation, there were no significant differences in the percentage of live or dead cells between the three decellularisation protocols, suggesting an equal cell-lysing ability by the three methods ($P > 0.05$). (Figure 3.25).

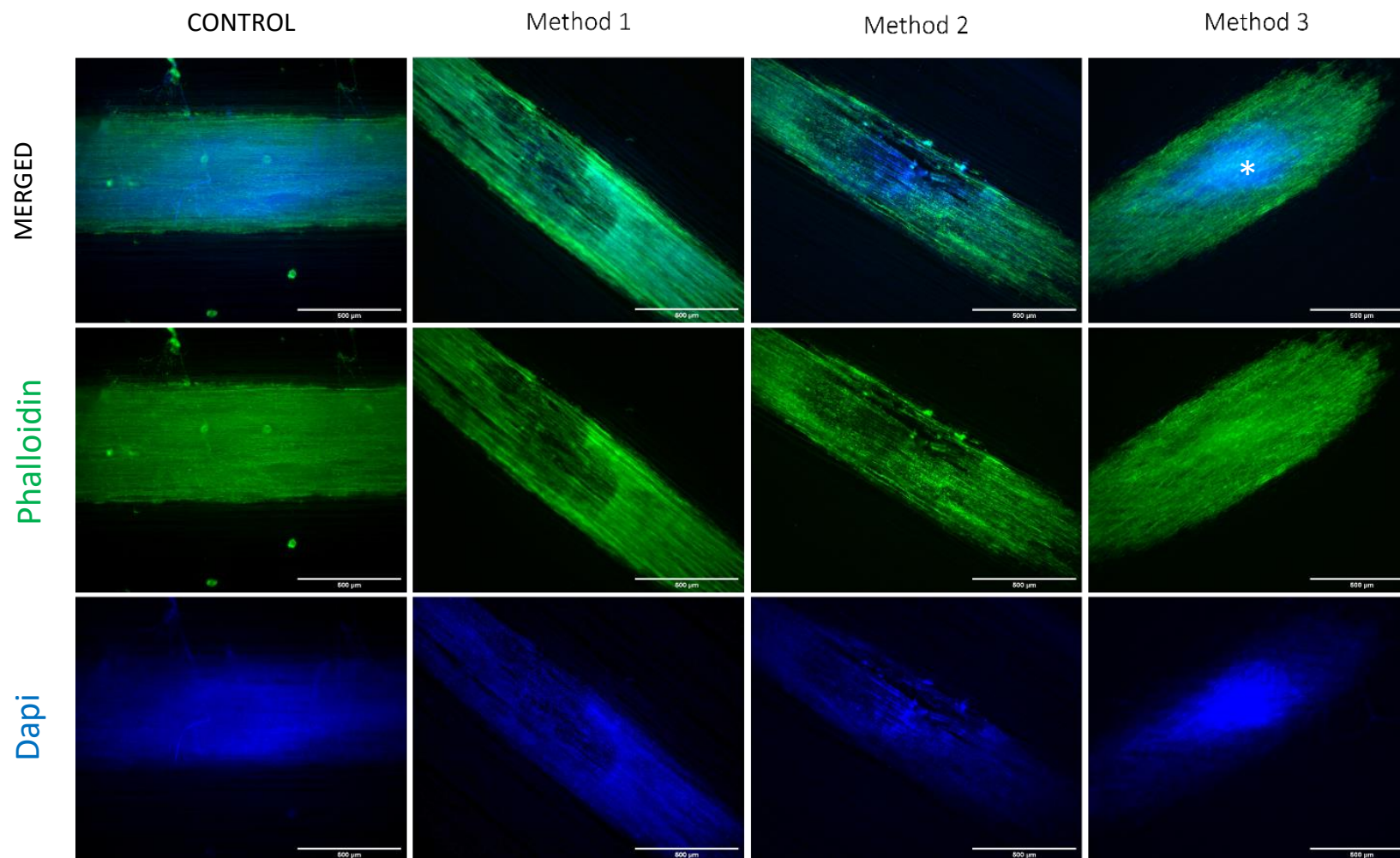


Figure 3.23. Decellularisation of DRG model on PCL fibres. DRG cultures on PCL fibres were decellularised using three methods, Method 1: 0.5% Triton X-100 in an ammonium hydroxide buffer, Method 2: 2.5% Triton X-100 in an ammonium hydroxide buffer with DNase I, and Method 3: Three cycles of freeze-thawing. Control samples were not decellularised. Cultures were fixed and stained with DNA-binding Dapi to visualise cell nuclei, and Phalloidin-FITC, an F-actin stain to visualise cell cytoskeletons. (N=3, n=3).

Control

Post-decellularisation

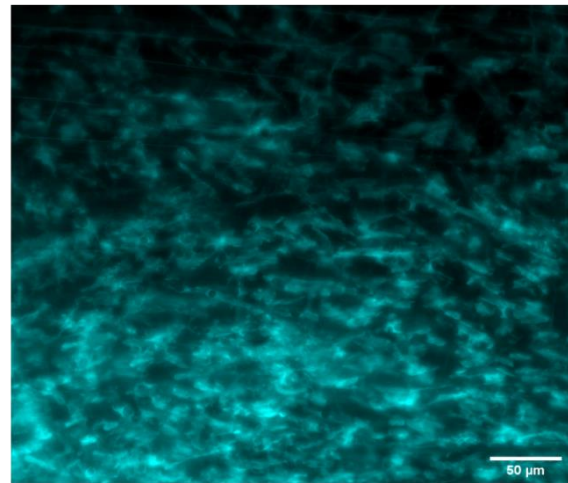
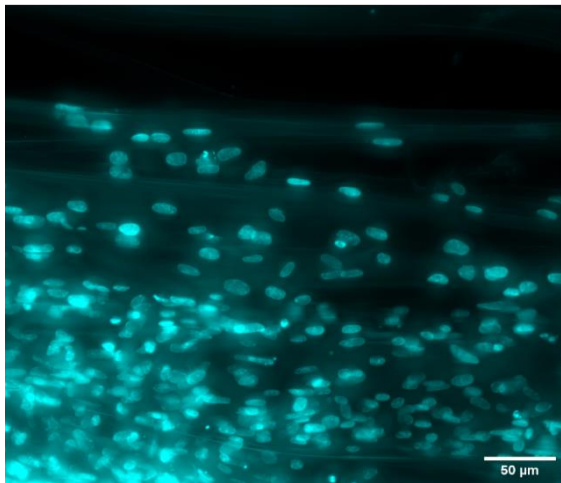


Figure 3.24. High magnification image of cell nuclei of cells derived from DRG explants cultured on PCL fibres after 2 weeks and stained with Dapi. The left image shows a control culture with healthy, round cell nuclei found along the PCL fibres and the right image shows a similar DRG culture following decellularisation. The morphology of the nuclei show fragmentation and successful lysis of cell membranes after decellularisation, however the lysed cells and DNA fragments remain attached to the fibres resulting in Dapi-staining. (N=3, n=3).

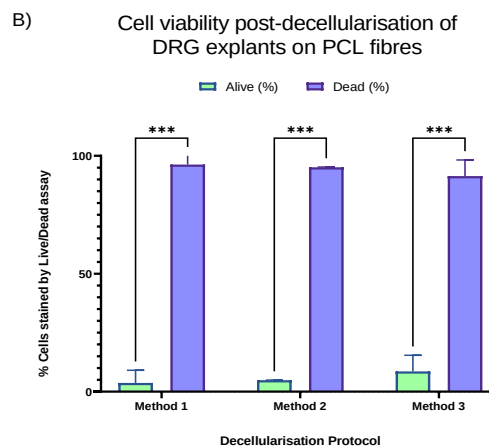
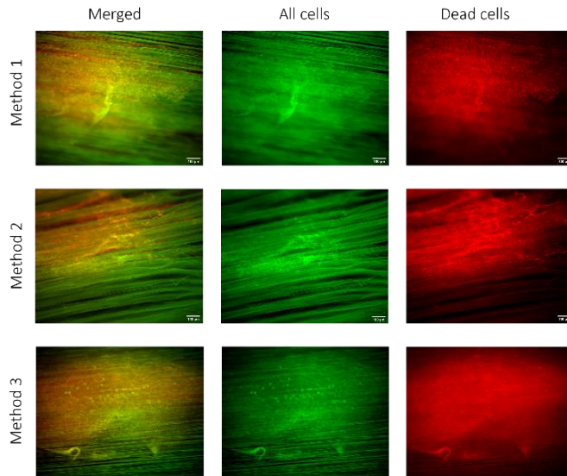


Figure 3.25 Cell viability of DRG cultures on PCL fibres after decellularisation was quantified by Live/Dead assay. (A) Representative images of DRG explants cultured for 2 weeks on PCL fibres and decellularised using three different methods and stained by Live/Dead kit where syto-9 (green) stains all living cells and propidium iodide (red) stains dead cells only. Scale bars 100μm. (B) The percentage of live and dead cells were quantified, where there were significantly more dead cells compared to alive cells after each decellularisation protocol (One-way Anova, *** $p < 0.001$). There was no significant difference in the number of dead or alive cells between the decellularisation protocol methods ($p > 0.05$). (N=3, n=3).

Overall, Method 1 and 2 provided the highest level of decellularisation, based on visual examination of the cultures where Dapi-staining at the DRG body was reduced in comparison to Method 3. In addition, the live/dead assay suggested a larger number of alive cells remained in the cultures following Method 3. Therefore, based on this data and due to time constraints where further optimisation was not possible, Method 1 was selected as this was also based on the protocol used for DRG cultures on TCP.

3.5.3 Characterisation of DRG and decellularised DRG models on PCL fibres

The dorsal root ganglia (DRG) cultures and extracellular matrix (ECM) deposition onto PCL fibres was characterised by immunofluorescence. DRG from chick embryos (EDD 11 and 12) were cultured as whole explants on PCL fibres for 2 weeks. Then, the samples were either subjected to decellularisation using the standard protocol of 0.5% Triton X-100 in an ammonium hydroxide buffer, or washed with PBS as a control. The solutions were removed, and the DRG cultures were washed gently twice with PBS prior to fixing with 3.7% PFA and immunocytochemical staining as described in methods 3.3.5 against a number of relevant antibodies to characterise the cultures.

DRG cultures showed neurones (stained by Beta III Tubulin) and glial cells (stained by S100B) at both the DRG body and adjacently where cell outgrowth had occurred successfully onto PCL fibres (Figure 3.26). Due to the high density of the cells in explant, only a subset of cell bodies of neurones at the surface of the scaffold were able to be visualised at the location of the DRG body, as the majority of the DRG cells had likely migrated in the z-direction (i.e. within the deeper layers of the scaffold) and imaging limitations did not allow visualisation of the more deeper cell bodies.

After decellularisation, some neurones were still present at the site coinciding with the DRG body, although to a lesser extent, evident by the presence of characteristic neuronal cell bodies (Figure 3.26). Whilst no individual glial cells could be seen morphologically, positive staining for S100B in comparison to the “no primary control” suggests the remnants of Schwann cells were still present on the fibres after decellularisation at the DRG body.

In contrast, the axons of neuronal cells were more easily visualised throughout the outgrowth sites, showing axons extending along the PCL fibres, accompanied by S100B positive Schwann cells (Figure 3.27). After decellularisation, there was no staining for neurones and a reduction in Schwann cell staining.

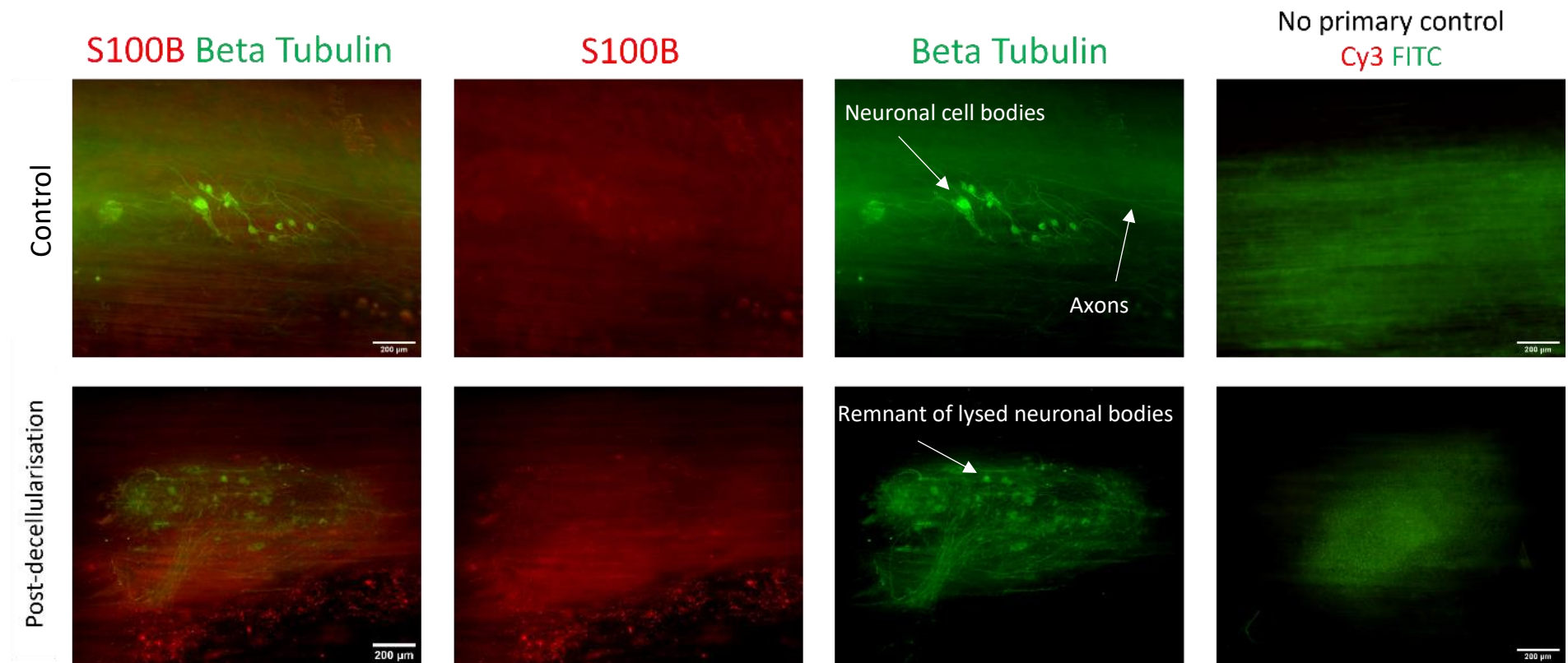


Figure 3.26 Representative immunofluorescence images of the site where DRG were seeded on PCL fibres, i.e. the DRG body. The top row shows DRG cultures with labelling of neuronal marker Beta tubulin III and Schwann cell marker S100B, as well as a control culture stained with the omission of the primary antibodies to serve as a comparison (no primary control column). The bottom row shows DRG cultures on PCL fibres after decellularisation, with some positive neuronal cell bodies still present as well as positive S100B staining suggesting remaning Schwann cells. Scale bars 200 µm. (N=3, n=3).

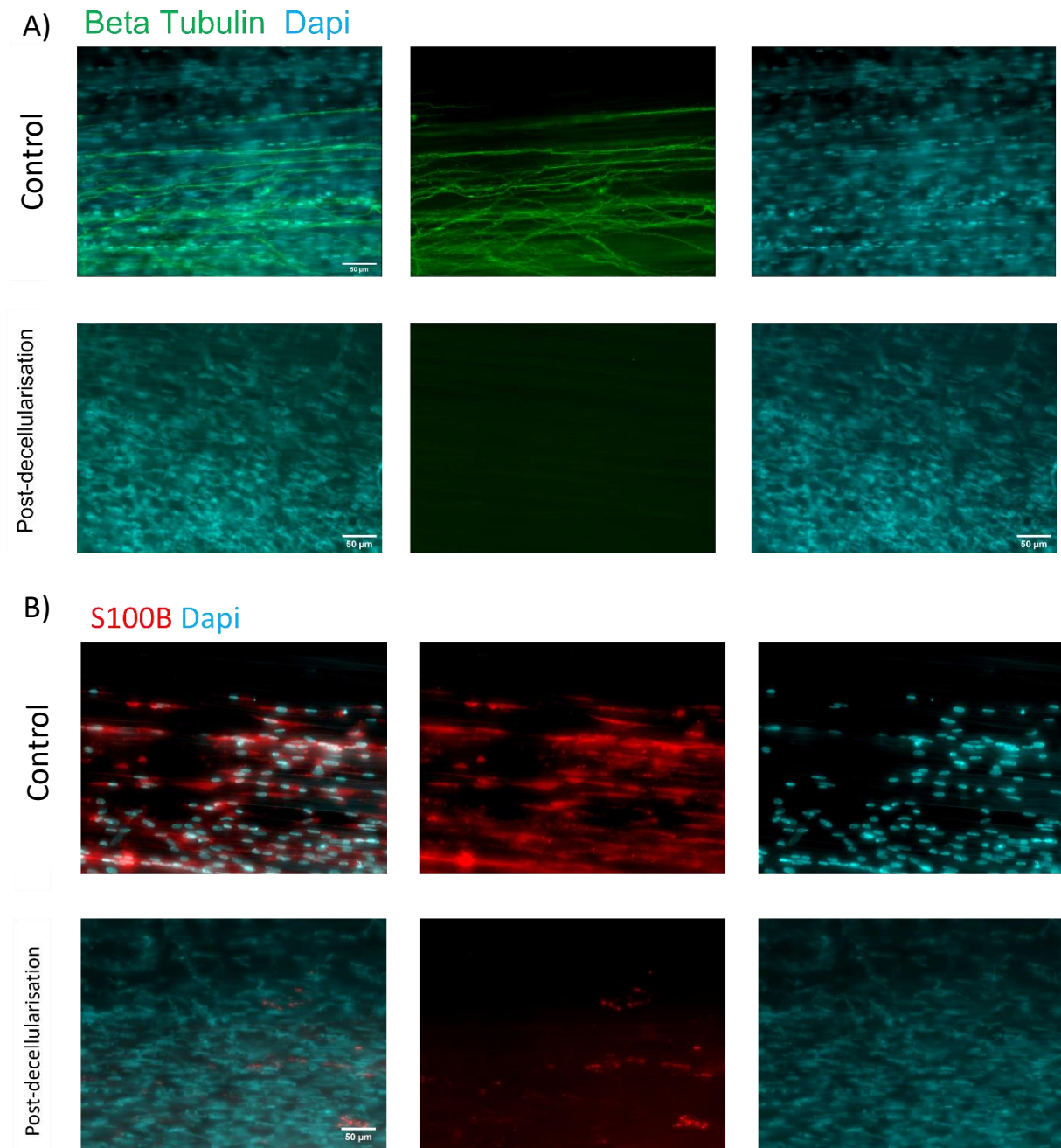


Figure 3.27. Representative immunofluorescence images of the outgrowth sites of DRG cultures on PCL fibres stained for (A) Beta Tubulin for neuronal cells and (B) S100B for Schwann cells. (A) The top shows neuronal axons (green) running in parallel to PCL fibres. Dapi-staining reveals cell nuclei of cells derived from the ganglia that are not neuronal as they do not show co-labelling with beta tubulin. The bottom row shows cultures following decellularisation where no beta-tubulin labelled axons remain and the fragmented morphology of the lysed cells is still stained with Dapi. (B) The top shows Schwann cells (red) extending along PCL fibres where the cell nuclei of Schwann cells are also labelled by Dapi. The bottom row shows cultures following decellularisation with significantly reduced Schwann cell staining, and the fragmented morphology of the lysed cells is still stained with Dapi. Scale bars 50 μm. (N=3, n=3).

DRG cultures on PCL fibres were also positive for extracellular matrix components of laminin (Figure 3.28) and collagen IV (Figure 3.29), as shown in the top figure of each image (Control row). DRG cultures subjected to decellularisation were also positive for laminin and collagen, suggesting the decellularisation protocol was capable of retaining ECM relevant components. The bottom row in the images shows samples stained with the omission of the primary antibody, where there is minimal background staining under Cy3 excitation confirming the positive staining are due to the antibodies.

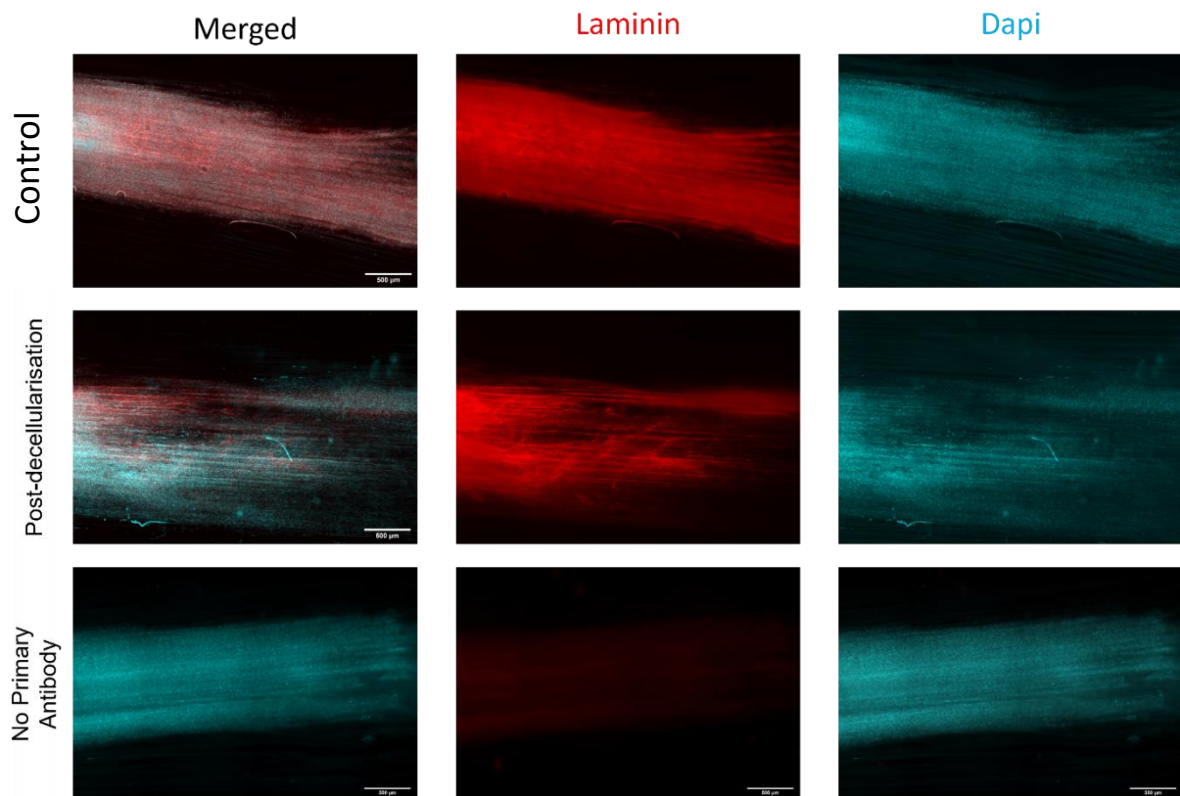


Figure 3.28 Representative immunofluorescence images of the DRG cultures on PCL fibres stained for anti-laminin, an ECM component. The top row shows laminin staining (red) present all along the fibres as well as Dapi-staining (blue). The middle row shows DRG on PCL fibres following decellularisation with positive staining for laminin (red). The bottom row shows DRG cultures stained with the omission of the primary antibodies to serve as a comparison (no primary antibody). Scale bars 500 μ m. (N=3, n=3).

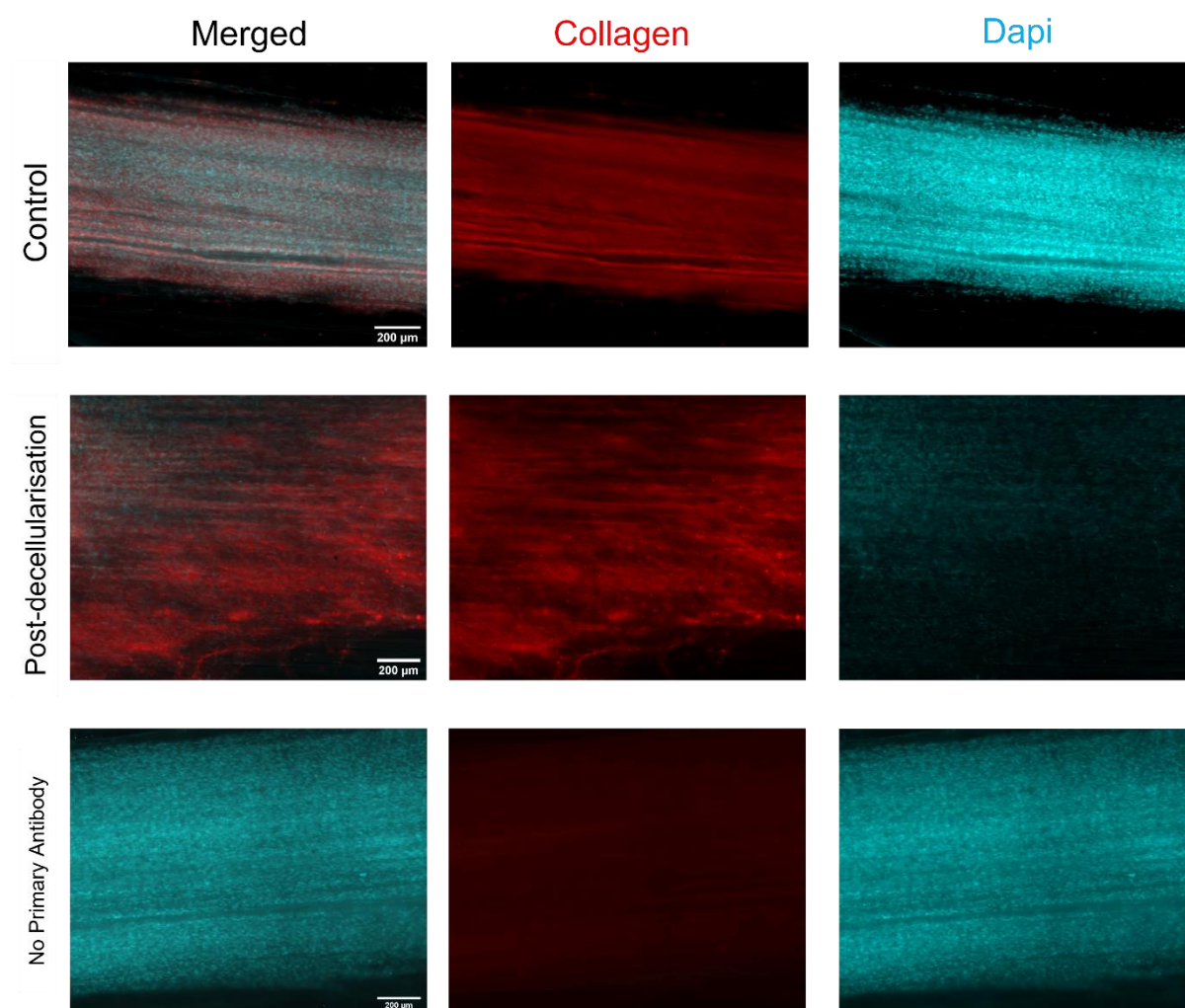


Figure 3.29. Representative immunofluorescence images of the DRG cultures on PCL fibres stained for anti-collagen IV, an ECM component. The top row shows collagen staining (red) present all along the fibres as well as Dapi-staining (blue). The middle row shows DRG on PCL fibres following decellularisation with positive staining for collagen (red). The bottom row shows DRG cultures stained with the omission of the primary antibodies to serve as a comparison (no primary antibody). Scale bars 200 μm . (N=3, n=3).

3.6 Discussion

This chapter set to investigate the hypothesis that dorsal root ganglia (DRG) can be used to deposit an extracellular matrix (ECM) that is nerve-specific and therefore may offer an optimal environment for nerve regeneration. In order to investigate this hypothesis, the first step was to optimise the culture of DRG, where whole DRG explants were seeded onto tissue culture plastic resulting in an *ex vivo* model.

3.6.1 Optimisation of DRG model on TCP

The average attachment and outgrowth of DRG on standard tissue culture plastic (TCP) was found to be highly variable, ranging between little or minimal growth to some DRG explants expressing extensive cell and neurite outgrowth. Additionally, some DRG cells showed bias towards one direction whereas others showed more even migration from all sides of the ganglion body. Due to this variability, various parameters were investigated in an attempt to optimise a reproducible model.

3.6.1.1 Cell culture conditions

Firstly, the size of the well was investigated on the outgrowth of the DRG to maximise the number of models/cultures available. The size/type of the well plate would affect not only the surface area provided for cells but also the volume of cell culture medium provided per cm² (Table 5).

Table 5. Relationships between surface area and volume of media per each well type. The max volume of media is specific to the current model and not the well plate.

	SURFACE AREA (CM²)	MAX* VOLUME OF MEDIA (ML)	VOLUME OF MEDIA PER CM² (ML/CM²)
6-WELL	9.6	1	0.104
12-WELL	3.5	0.4	0.114
24-WELL	1.9	0.25	0.132
48-WELL	1.1	0.15	0.136
96-WELL	0.32	0.1	0.313

Whilst not significantly different, the outgrowth of DRG cells was higher on 48- and 96-well plates compared to the other wells (6-, 12-, 24), which coincides with increased DRG outgrowth with increased volume of media per cm² (i.e. or per DRG).

The volume of media per cm², or the amount of media over the cells, can influence the diffusion rate of oxygen, where a large volume of media may act as a diffusion barrier, restricting oxygen supply to the cells. Alternatively, elevated levels of oxygen in the media can be toxic to the cells due to increased free radicals. Smaller volumes of media can also cause uneven distribution of not only oxygen supply but also nutrients in the media due to the formation and effect of the meniscus (which is more

noticeable in smaller wells). Therefore, the volume of media per well has to be optimised to provide correct oxygen diffusion and this will vary depending on the cell type and culture.

The recommended volume of media for monolayer cells is suggested as 0.2ml/cm² (McAteer, 1994), however, the volume per cm² provided in this study differed for each well plate, ranging between 0.1 to 0.3 ml/cm². This is because there was a maximum volume of media that could be provided per well safely to prevent the detachment of the DRG from the bottom surface of the well. If too large of a total volume was provided, then the DRG would detach and float (due to differences in the density of the DRG and the liquid media), which would prevent the outgrowth and attachment of cells on the surface.

It is worthy to mention that whilst there are reports of other studies using larger media volumes of 0.2ml/cm² for the culture of DRG explants (Bryce & Ribchester, 1993) successfully, these studies used coatings in the wells to increase the adherence and attachment of the DRG. However, in contrast to such studies that use DRG as models to evaluate the effect of external factors on DRG neurite outgrowth, the aim of our study was to evaluate the native extracellular matrix derived by DRG cells and the decellularised matrix which includes components such as laminin and collagen, which are often used as coating agents. Therefore, in our study we decided to not use such coatings as this would have added another variability in our model which would have complicated the analysis of DRG-derived matrix and decellularised matrices.

Another consideration when selecting the well type for the culture of DRG was providing sufficient surface area to allow optimal outgrowth of the cells from the DRG. Typical DRG cultures in the literature report on radial migration and extension of cells and neurites (Pettigrew et al., 2019), however, in our studies the majority of cells and neurites showed bias towards one direction (Figure 1 B vs C). This could have been due to the fact that despite seeding DRG explants in the middle of wells, the explants would still move due to routine handling of the cultures, often drifting towards the edges of the wells which inherently limited DRG outgrowth on one side. This movement was more noticeable in larger wells compared to smaller wells. Therefore, considering the above effects of surface area and volume, DRG were cultured primarily on 48 well plates for subsequent studies on TCP.

3.6.1.2 DRG origin

Once the plate type and cell culture conditions were selected, the origin of the DRG was evaluated based on the embryonic developmental age of the chick as well as the spinal level where DRG were dissected.

The chick embryos used in this study did not require a home office license as they are not considered protected animals under the Animals Scientific Procedures Act (1986) as long as the embryonic developmental stage of the chick is below the last third of the incubation period; Considering that chicken eggs hatch at 21 days, the last third of their incubation period is considered at day 14. Three embryonic developmental days prior to this limit were evaluated in our study: EDD 13, 12 and 11, referring to the day on which DRG were dissected and cultured. Younger chick embryos were not considered due to the small size of the embryo which prevented comfortable DRG dissection in a timely manner. There was no significant difference in the percentage of DRG that attached and grew on TCP between DRG from EDD 11, 12 or 13. In addition, dorsal root ganglia dissected from different spinal cord levels (thoracic, lumbar, and sacral) also showed no significant difference in the distance travelled by the DRG cells (cell migration) on TCP.

There are molecular differences in dorsal root ganglia at different developmental stages that may affect migration, for instance the level of $\alpha 1$ integrin, laminin and collagen in dorsal root ganglia of avian embryos is elevated between EDD 4 to 11 but declines after EDD 11 to EDD 16 (Duband et al., 1992), which may suggest that younger DRG (<EDD11) could result in increased adherence to surfaces due to increased content of adherence-related molecules.

In addition, there were visual differences in the size between DRG from different spinal levels in our study, which may suggest that larger DRGs, such as those from the thoracic and lumbar spinal level, would contain a larger amount of cells than smaller sacral DRGs, and therefore increased DRG-derived ECM could be provided.

DRG cells at different spinal levels are also suggested to be molecularly distinct; indeed, they are a reflection of the targeted migration of neural crest cells during development, where specific signalling pathways modulate specific differentiation of the populations of neurones in DRG and relevant glia (Rubin & LaMantia, 1999; Teillet et al., 1987). As far as the author is aware, no reports could be found on molecular characterisation of embryonic chick DRG at different spinal levels. However, studies in rodents have shown differences in the molecular expression of lumbar vs sacral DRG cells based on specific expression of subpopulations of neurones (Kozłowska et al., 2017) as with differential responses to neurotrophic factors (Vetter et al., 2010). Furthermore, in response to injury, upregulation of regeneration associated genes (RAG) occurs in the DRG and it may be possible that certain ganglia with a higher content of cells or higher content of RAG genes can improve the rate of survival of DRG neurones and therefore improved attachment and outgrowth on TCP. Therefore, future studies may benefit from evaluating the molecular characterisation of chick DRG in order to identify and optimise the selection of DRG.

3.6.2 DRG-derived ECM for nerve regeneration

3.6.2.1 Characterisation of dECM on TCP

Dorsal root ganglia (DRG) from all spinal levels of embryonic chicks (EDD 11-12) were cultured on tissue culture plastic (TCP) showing the extension of axons/neurites from neuronal cells located in the ganglia (beta tubulin-positive) as well as the migration of Schwann cells (S100B-positive cells) from the DRG body after 1 week of culture.

In addition, cultures stained positively for extracellular matrix components of laminin and collagen IV, which are associated to Schwann cells basal lamina *in vivo* (M B Bunge et al., 1980; Jessen & Mirsky, 2016). This suggests that the DRG-derived cultures developed in this chapter can be used as a model of peripheral nerve with appropriate cellular and ECM markers relevant to regeneration. Following decellularisation, there was successful removal of the cellular components (Dapi-stained cells and neurites) whilst laminin and collagen IV were still present in the decellularised matrix (dECM).

Collagen IV and laminin have been implicated in a variety of processes following nerve injury and regeneration, including promoting axonal growth (Isaacman-Beck et al., 2015; Lein et al., 1991; Tonge et al., 1997) and influencing Schwann cell migration (Saito et al., 2003) and myelination (Fernandez-Valle et al. 1994), therefore the ability to maintain these components in the dECM is considered a success.

However, there are a large number of other ECM components involved in regeneration, as reviewed previously (1.7.3, 3.2.1), but due to time constraints only two components were evaluated in this study as these were deemed most relevant due to previous literature. Nevertheless, it may be of particular interest in the context of regeneration for future studies to evaluate more ECM components, such as fibrinogen as this component is not typically found in the native ECM of the peripheral nerve until the occurrence of injury, where it becomes released from blood vessels and capillaries (which are present in the capsule surrounding the DRG) and is involved in the formation of the fibrin cable that helps guide regenerating axons and Schwann cells (Daly et al., 2012; Williams & Varon, 1985). Therefore, the presence of this specific ECM component may be useful in further validating the use of DRG in culture as a model for peripheral nerve injury and regeneration.

Furthermore, while we were able to demonstrate the deposition of ECM based on immunocytochemical methods, future studies may also benefit from quantification of the components. As mentioned already, there was a large variability in the outgrowth of the cells and neurites from ganglia on TCP, and this variability was also mirrored in the deposition of ECM components. Specifically, DRG with less outgrowth and minimal cell migration on TCP showed smaller

sections of positive staining compared to DRG cultures that had more cell migration where staining for ECM components was more widespread in the well of the tissue culture plate. In turn, this would suggest that following decellularisation, there would be an uneven content of ECM in the decellularised matrices of different samples. In order to address this shortcoming for subsequent studies, DRG cultures were selected for decellularisation based on showing a minimum level of outgrowth and migration, as a way to standardise the content of the dECM that was then evaluated for nerve regeneration.

3.6.2.2 Regenerative potential of dECM using a neurite outgrowth assay

In order to evaluate the regenerative potential of DRG-derived ECM (dECM) on nerve regeneration, a neuronal outgrowth assay was employed based on measuring the effect of the dECM surface on cell adherence and axon elongation of a neuronal cell line, NG108-15s. The outgrowth assay is based on the initial starvation of the cells whereby removing serum from the media induces the formation of the neurites from the cell bodies, a feature that can then be analysed to evaluate the effect of different surfaces on the neurite phenotype of the cells, including the formation and elongation of the neurites but also cell adherence (Seidman et al., 1996).

NG108-15 cells grown on the DRG-derived dECM showed increased cell adherence at the end of the assay, as well as increased neurite formation compared to cells grown on control tissue-culture plastic (TCP), showing a positive effect of the DRG-derived dECM on cell attachment and neurite extension from cells. Indeed, this effect can be attributed to the presence of the deposited ECM, such as laminin and collagen that were characterised in the cultures and have shown to increase axonal outgrowth and formation of neurites in previous studies (Tonge et al., 1997) (Armstrong et al., 2007).

Interestingly, the lengths of the neurites of NG108-15 cells were not significantly different between cells grown on dECM compared to TCP in our study. Indeed, some studies have reported that the effect of laminin is more pronounced on neurite formation and branching as opposed to neurite elongation on NG108-15 cells (Weeks et al., 1991), which may explain the similar findings in our study.

In addition, differences in protocols may account for the findings in our study in terms of the neurite length of NG108-15 cells on dECM, such as due to differences in the duration of the assay. Previous studies have evaluated neurite outgrowth on ECM components within a few hours to days (48 hours) of cell seeding, and shown that the ECM components stimulated the extension of neurites from NG108-15 cells while those grown on TCP still had a round shape (Armstrong et al., 2007). In contrast, in this study we evaluated neurite outgrowth from NG108-15 cells for a week, a longer time point than typically reported, where neurites were clearly present in both surfaces, the dECM and on TCP. It is possible then, that as a result of the increased cell density (as more cells remained attached by the

end of the assay on dECM compared to TCP) and the increased number of neurites present on the dECM which result in more connections between neurites, that there was less space available per individual neurite for elongation on the dECM compared to the cells cultured on TCP, which may inherently limit the neurite length measurement. Therefore, future studies may benefit from shorter evaluation periods when using NG108-15 cells for outgrowth assays.

Moreover, it is known that neuronal cells in culture can very easily become disturbed, and indeed during early preliminary experiments optimising the cell line for our study, any small currents created by pipetting of media (e.g., for media changes) resulted in cells detaching from the wells. Therefore, the fact that a larger number of cells remained attached on the dECM compared to TCP, even after multiple media changes, certainly highlights the positive effect of the dECM properties. However, in order to gain a deeper understanding of the composition of the ECM, certain controls could have been included in our neurite outgrowth assay, such as a laminin-only or collagen-only surface. However, since the exact content of the dECM components were not known, as we did not quantify the dECM, it was difficult to select appropriate control groups that were suitable for the study, and therefore we selected tissue culture plastic due to the widespread use. However, future studies may benefit from including a selection of various concentrations of different individual ECM components.

Indeed, the use of neuronal cell lines as assays can sometimes present with such limitations in the interpretation of the results, and furthermore in the translation capability. Therefore, a variety of different assessments and methods can be used together to address the shortcomings of individual assays and provide a fuller understanding of the effect of novel therapies on nerve regeneration.

For example, plans were made to evaluate the DRG-derived ECM using another dorsal root ganglia, to evaluate the effect of the dECM on DRG neurones, where we hypothesised improved axonal outgrowth would occur. However, due to time constraints, it was not possible to schedule these experiments in a timely manner. Plans were also made to evaluate different ECMs from longer culture periods, where the involvement of myelination may have been more significant and influenced the composition of the ECM. However again, due to time constraint this was not possible.

Nevertheless, considering that our results *in vitro* provided evidence of a positive effect of DRG-derived ECM on nerve regeneration, we wanted to evaluate the dECM surface further, specifically in an *in vivo* model of nerve repair. In order to do this, we employed the use of PCL fibres as a scaffold for the deposition of the DRG-derived matrix, to ultimately insert into nerve guide conduits.

3.6.3 Culture of DRG on PCL fibres

Dorsal root ganglia (DRG) from all spinal levels of embryonic chicks (EDD 11-12) were cultured on PCL fibres showing the extension of axons from DRG neuronal cells located in the ganglia (beta tubulin-positive) as well as the migration of Schwann cells (S100B-positive cells) from the DRG body after 2 weeks of culture. In addition, cultures were positively stained for extracellular matrix components of laminin and collagen IV, suggesting that the DRG-derived cultures on PCL fibres can be used as a model of peripheral nerve with appropriate cellular and ECM markers.

It is worthy to explain that DRG cultures on PCL fibres were kept for twice as long as the explants on TCP. This was because fibres in this thesis were fabricated with the ultimate goal of using in a nerve guide conduit as intraluminal fillers to evaluate in an *in vivo* model of nerve repair, where the minimum requirement for the fibres was a length of 3 mm to match the nerve gap. Preliminary experiments of DRG on PCL fibres showed that the maximum migration of DRG axons and cells was around 1500µm, which was considered insufficient as this would not provide enough coverage of the DRG-derived cell outgrowth onto the PCL fibres. Indeed, this DRG neuronal axon length has also been reported by previous studies in our group using chick DRG cultured on PCL fibres (1200 to 1400 µm) (Sandoval-Castellanos et al., 2021; Taylor et al., 2023). Therefore, cultures were left in culture for longer resulting in an increase in the axon lengths up to 3691 ± 658 µm and DRG migration up to 3947 ± 531 µm, which satisfied our minimum requirements for DRG outgrowth on fibres. In addition, in comparison with previous studies from our group, it can be concluded that DRG outgrowth doubled within the extra week of regeneration.

We evaluated the cell viability of DRG cultures on PCL after 2 weeks of culture with a commercial Live/Dead assay kit that uses the differential permeability of two DNA binding dyes; Syto-9 a DNA stain that is cell membrane permeable and therefore capable of staining all living cells, and propidium iodide which is impermeable to cell membranes and can only enter and bind DNA in the nucleus when the cell membrane is compromised, such as in the case of dead and apoptotic cells.

We found differential cell viability based on Live/Dead staining depending on the region of the DRG explant on the PCL fibres, specifically, 30 – 60 % of cells were classified as dead cells in the DRG body of the explants, while the rest of the regions on either side of the ganglion, where the outgrowth and migration of Schwann cells had occurred, showed primarily live cells (over 97.2% of cells were classified as alive). Indeed, the body of DRG are highly and densely populated by neuronal cells, where one study counted the cells of a lumbosacral DRG in a chick embryos (EDD 10) to be around 9173 ± 966 neurones (Bhattacharyya et al., 1992). However, it is important to note that their study harvested the DRG for immediate fixation and analysis as opposed to culturing the DRG, where cell death is likely

to occur, so their value may not be an accurate representation of the number of neurones in DRG cultures. Few studies have described the patterns of cell programmed death in chick embryos, and neuronal cell death is a naturally occurring process during development while neurones attain appropriate connections, so it is difficult to estimate the number of neuronal cells in our culture. Future studies may benefit from characterising the number of cells, both neuronal and non-neuronal, in culture.

As well, interestingly, one study by Ciutat et al (1996) found that in chick embryos Schwann cell death peaked twice during development, once by day 5 and again by day 9 (although smaller) and gradually declined from EDD 10 until hatching (Ciutat et al., 1996). These peaks in Schwann cell death were associated with the loss of axonal-derived signals as a result of lost connections during development, where neuroregulin-1 has been found to play a critical role. There are various isoforms of Neuregulin-1, some membrane-bound and other as soluble proteins, where it has been suggested that soluble neuregulin is released from axons in response to BDNF signalling to promote survival of Schwann cells (Ma et al., 2011). This provides further supports to the notion of using whole DRG explants in order to maintain the natural signalling occurring between resident cells.

Nevertheless, it is interesting that in comparison to other studies in the literature that commonly report on using neurotrophic-supplemented media to maintain embryonic chick DRG (either as whole explants or dissociated) in culture (Mrówczyńska & Mazur, 2021; Wiens et al., 2016), studies from our group have shown that chick DRG can survive in culture for 1 week (Sandoval-Castellanos et al., 2021) and now we have shown this to be true for 2 weeks in the results of this chapter.

However, it is worthy to note that this is an area for improvement and optimisation of the DRG model used in this study. Previous studies from our group have evaluated the effect of a wide range of concentrations and combinations of neurotrophic factors (such as NGF and BDNF) on embryonic chick DRG explants, primarily evaluating the effect on neurite outgrowth (Sandoval-Castellanos et al., 2020). Sandoval-Castellanos et al. found that the supplementation of NGF and BDNF at a concentration of 1µg/mL in the cell culture medium significantly quadrupled the neurite outgrowth of DRG cells when grown on TCP after 1 week. Similarly, in a subsequent study it was found that the same concentration of the growth factors significantly increased the average DRG axon length by a factor of almost 2 for DRG cultures on PCL fibres (Sandoval-Castellanos et al., 2021).

These findings may be useful for future studies in order to optimise and maximise the extension of DRG neurones. However, it should be noted that the previous studies evaluated DRG cultures for only a period of 1 week, therefore it may be worth evaluating the effect of neurotrophic factors in long-term DRG cultures as well. And as mentioned, due to the differential effects of neurotrophic factors

on different DRG of different spinal levels as previously reported in rat studies(Vetter et al., 2010), it may be worth to investigate this effect on embryonic chick DRG of various levels too.

Finally, the effects of decellularisation of DRG on PCL fibres were also investigated in this chapter, specifically after 2 weeks of culture, decellularisation of DRG on PCL fibres was carried out using Triton X-100, a non-ionic detergent capable of solubilising and disrupting cell membranes.

Live/dead cell viability assays demonstrated that over $95.1 \pm 1.1\%$ of the DRG cells has been successfully lysed, confirming the efficacy of the decellularisation protocol. In addition, staining with dapi, a DNA binding dye, demonstrated that the morphology of dapi-stained nuclei, although remaining after the decellularisation protocol, were highly fragmented and characteristic of apoptotic cells (Eidet et al., 2014).

Detergents such as Triton X-100 are considered milder detergents that maintain the ECM integrity, and therefore are used for decellularisation of cell-derived ECMs, as opposed to decellularisation of whole tissues(Harris et al., 2018). Although, there are studies who have used Triton X-100 (0.5%) and ammonium hydroxide to decellularise whole sciatic rat nerves successfully with no reports on any immune responses as a result of the acellular nerve being grafted *in vivo* (Kim et al., 2004).

Nevertheless, despite the widespread use of Triton X-100 based decellularisation protocols for cell-derived ECM, some studies have shown that Triton X-100 is not effective in eliminating all components, with specific regards to some actin filaments and DNA still being positively stained following decellularisation. However, the studies emphasise that despite the staining, no obvious morphological cell nuclei are visible (Lu et al., 2012), as was the case in this chapter where the Dapi-stained nuclei were highly fragmented. Therefore, future studies should optimise the washing steps following decellularisation (as well as using molecular techniques to quantify any remaining DNA) as this might have been a limitation in our study as we did not want to disturb the DRG-derived ECM so very gentle washes were used.

Nevertheless, it is also worthy to remember that this thesis has built on a novel concept as the decellularisation of DRG-derived ECM has not been previously reported, and due to time constraints as a result of several lockdowns, further optimisation of the decellularisation protocol could not be conducted.

However, the main aims of the chapter have been successfully completed, chief being modelling the DRG explants, both on TCP and PCL fibres, and demonstrating the suitability in deriving ECM from this novel ganglion source with positive effects on DRG-derived ECM on nerve regeneration *in vitro*.

3.7 Conclusions

This chapter set to investigate the hypothesis that dorsal root ganglia (DRG) could be used to deposit an extracellular matrix (ECM) that is nerve-specific and therefore may offer an optimal environment for nerve regeneration.

We optimised the DRG model by considering factors such as cell culture medium volume as well as surface area to maximise the outgrowth of DRG on TCP, where the origin of the DRG during development and anatomical location was also taken into account.

Next, we characterised the DRG model for relevant neuronal and glial markers associated with DRG cultures as well as critical ECM components specific to the basal lamina of Schwann cells, namely collagen IV and laminin, suggesting DRG can be used to model a peripheral nerve environment. In order to obtain the ECM components, decellularisation was conducted successfully resulting in the removal of cellular material whilst retaining the relevant ECM components. This is a significant and novel finding as the first report of decellularization of DRG-derived ECM.

Furthermore, DRG cultures were successfully modelled on PCL fibres for a culture period of 2 weeks and the decellularisation of DRG-derived ECM on PCL fibres resulted in the maintenance of ECM components and the removal of morphological-cellular material, however some DNA still remained attached to the scaffolds.

DRG-derived ECM on TCP was evaluated for nerve regeneration using a nerve outgrowth assay showing increased cell adherence and neurite formation compared to control, indicating a positive effect of the DRG-derived dECM on features relevant to nerve regeneration.

Overall, this chapter provides valuable insights into the use of DRG-derived ECM as a potential nerve-specific environment for nerve regeneration and serves as a foundation for further research.

3.7.1 Upcoming chapter

As a reminder, this thesis aims to develop a nerve guide conduit containing PCL fibres which will be coated with decellularised-extracellular matrix derived from DRG, to then be evaluated in an *in vivo* model of nerve repair.

The first experimental chapter (Chapter 2) involved the fabrication of the conduit and PCL fibres. This chapter focused on the optimisation of DRG cultures for the derivation of extracellular matrix, with positive reports for the use of DRG-derived ECM in improving nerve regeneration using an *in vitro* assay. Finally, the last experimental chapter will evaluate the DRG-derived ECM within a novel nerve guide conduit in an *in vivo* model of nerve repair.

Chapter 4. *In vivo* evaluation of a novel nerve guide conduit containing PCL fibres coated with DRG-derived ECM

Abstract

This chapter evaluated a novel nerve guide conduit containing intraluminal fibres coated in decellularised extracellular matrix previously derived from dorsal root ganglia (DRG). DRG were used as a source of ECM as they mimic more closely the peripheral nerve environment, and it was hypothesised that a native-like ECM such as the one derived by DRG would improve regeneration outcomes following nerve injury.

The novel conduit containing dECM was evaluated in a common fibular nerve gap model of nerve injury and repair in Thy-1-YFP mice and compared to a hollow conduit, a conduit with PCL fibres and a nerve graft, which served as a control. Regeneration was evaluated by axon analysis along the length of regenerated nerves, showing regeneration was supported in all groups to a similar level, with no significant differences between groups.

Furthermore, the potential for the development of pain as a result of nerve injury was evaluated by monitoring glial activation in the spinal cord, where no significant differences were observed as a result of the repair type suggesting the novel conduit does not exacerbate glial activation.

4.1 Background

4.1.1 Introduction

The current treatments for peripheral nerve injuries are based on end-to-end suturing for smaller defects or the use of an autograft for the repair of larger injuries, where the latter is associated with donor-site morbidity and therefore presents a limitation despite offering the best regeneration outcome. Recent studies and clinical trials have frequently demonstrated the potential of using nerve guide conduits for the repair of nerve injuries, however, the level of regeneration obtained by conduits is still not as effective as an autograft. Nevertheless, the potential of nerve guide conduits is untapped particularly in regard to various experimental therapies than can be investigated using conduits. For instance, studies have shown the potential for using extracellular matrix components to improve nerve regeneration, and indeed a number of ECM components (e.g. collagen) have been used successfully to support regeneration following injury in a number of experimental models and clinically as well.

In this thesis we hypothesised that a native-like nerve specific ECM may provide improved regeneration following nerve injury. In chapter 2, dorsal root ganglia were used as a source of ECM and decellularised to obtain a DRG-derived ECM containing components relevant to the basal lamina of Schwann cells such as laminin and collagen IV. In this chapter, we evaluate a novel nerve guide conduit containing fibres coated in decellularised-ECM in a model of nerve repair *in vivo* and compare the regeneration outcomes with a hollow conduit, a conduit with PCL fibres and an autograft. In order to provide a full understanding of the regeneration outcome, we also study the potential for the development of neuropathic pain by monitoring glial activation in the spinal cord following injury.

4.1.2 Methods for assessing nerve regeneration *in vivo*.

Nerve regeneration is a multifaceted process; therefore, a myriad of methods have been developed and adopted to evaluate regeneration following various nerve repair therapies and treatments.

Generally, the methods to evaluate nerve regeneration involve the use of animals (although *in vitro* investigations can also provide useful information), where regeneration can be assessed by looking at behavioural changes and functional improvements following nerve injury and repair, as well as immunohistochemical and molecular analyses of regenerated axons to dissect the role of specific components involved in the regeneration process.

Typically, three main types of nerve injury are used in *in vivo* models which loosely relate to Seddon's classification for nerve injuries (Table 1 in Chapter 1.3) and therefore provide clinical relevance. In the

first case, compression injuries can be modelled by cuffing a nerve, which can lead to demyelination but not degeneration of axons, hence modelling neuropraxia. Nerve crush injuries can be modelled by pinching nerves, leading to the degeneration of axons distally to the site of injury (axonotmesis) but the basal lamina tubes remain intact, and lastly transection injuries by cutting the nerve where all the anatomical components of the nerve become fully separated at the site of injury (neurotmesis). Transection models of injury can also be further sub-divided depending on the size of the injury, which can reflect clinical observations where larger gap injuries usually do not show spontaneous regeneration unless surgically treated, and this is relevant to the field of tissue engineering as nerve gaps can be modelled *in vivo* and used to identify promising therapies for repair, including novel types of nerve guide conduits.

The general consensus suggests a combination of approaches should be used to provide an overall assessment of nerve regeneration and function as no single method is considered superior (Navarro, 2016), but indeed, certain methods to evaluate regeneration are more suited to specific models of injury and in turn to experimental aims for specific clinical translations. For instance, studies with an interest in maxillofacial clinical translation may benefit from the specific selection of nerve models involving supply to the oro-facial region, with relevant assessment methods, such as lingual nerve injury models and behavioural tests evaluating the function of drinking behaviours (which can be affected by the lingual nerve).

Nevertheless, the vast majority of nerve injury studies in rodents have been conducted on hindlimb nerves (e.g. the sciatic nerve), largely attributed to the ease of access to the nerve, where results can be applied to represent motor function generally, and in addition, such models benefit from a large variety of available behavioural and functional tests allowing comparison of data between studies (Tos et al., 2009).

4.1.2.1 Behavioural observations

Behavioural methods for assessing nerve recovery are selected based on the nerve target and motor or sensory nature of the nerve. For instance, injured sensory nerves can be evaluated by checking responses to sensory stimuli, such as hot, cold, or mechanical stimuli which would elicit different response thresholds depending on the level of regeneration and the recovery of different sensory neurones. Alternatively, gait analyses are often used when assessing motor nerves, which can provide information on the timeframes of reinnervation and recovery of target muscles (Costa et al., 2009).

Whilst behavioural tests may require long habituation and training periods which may discourage their use, the non-invasive nature of behavioural observation is a clear advantage for assessing nerve function, however, behavioural observation may often times be confounded by other factors, and

small differences in different repair therapies may not be as detectable. Therefore, the level of regeneration also needs to be assessed at a cellular level, which can be done by evaluating properties of regenerated nerves, such as conduction.

4.1.2.2 Electrophysiology studies

The excitable nature of nerves provides electrophysiological information, where action potentials can be induced and recorded to provide information based on nerve conduction properties. In fact, nerve conduction studies are performed clinically in a variety of application, such as in the diagnosis of conditions like carpal tunnel syndrome (Committee et al., 1993).

In nerve regeneration studies, electrodes can be placed at given locations on a nerve and stimulated to measure the degree of axonal regeneration at a distal site, for instance electrodes can be placed at either side of a nerve injury and recorded distally where regenerated axons have travelled, and the recorded sum of the potentials (compound action potential) can provide information on the number of axons that have successfully regenerated across the injury site. Different electrodes can be placed at different levels of the nerve and indeed action potentials can also be recorded at motor endplates to provide information on motor re-innervation. In addition, the conduction velocities of nerves can be measured at different stages of regeneration, for instance providing information on nerve maturation as a function of the different myelinated and unmyelinated axons in nerves.

4.1.2.3 Immunohistochemical studies

Counting the number of nerve axons found at specific sites following a nerve injury can be used as an indicator of regeneration and provide useful information when comparing different therapies. Typically, transverse sections of a regenerated nerve are used for counting axons, where staining procedures can also be applied to differentiate myelinated axons from unmyelinated ones, thereby providing information on the later phases of regeneration. Indeed, following injury, regenerated axons often show a higher percentage of unmyelinated nerve fibres and smaller fibres compared to the native nerve, which can correlate with the reduced conduction velocity of regenerated nerves and therefore can be used to suggest changes in functional recovery (Navarro, 2016).

However, technical issues can arise due to different methodologies of morpho-quantitative analysis of axons, often as a result of different processing, staining, and imaging techniques, which may lead to over- or under-estimations of axon count which can limit comparisons.

In addition, it is important to recognise that during regeneration, axons can branch and sprout giving rise to several branches that may not make appropriate connections. This may lead to an over-estimation of axons which may not necessarily reflect improved regeneration, as indeed some of the

branches may be later pruned or may cause inappropriate and/or pathological innervation at the targets. Indeed, axon counting on transverse sections of nerves do not usually take into account the sprouting of axons. For this reason, whole mount imaging of nerves can also be used to follow the path of axons following injury (Dun & Parkinson, 2015).

4.1.2.4 *Thy-1-YFP-H mouse model*

Whilst whole nerves can be stained and imaged without sectioning to provide a longitudinal view of the regeneration pathway, the sheer amount of axons in a nerve complicate the visualisation of individual axons for analysis despite the use of clearing agents.

Alternatively, more groups have taken advantage of genetic modifications using fluorescent proteins to allow the visualisation of axons without the need of post-processing immunostaining techniques. Feng et al (2000) were able to generate a number of transgenic mice lines with specific patterns of expression of fluorescent proteins on neuronal tissues, based on a modified Thy-1 vector. A popular line generated by Feng is the thy-1-YFP-H line where the staining is limited to a subset of peripheral axons and the expression is consistent along the whole length of the axon (Feng et al., 2000). The subset specificity reduces the number of visible axons allowing individual axons to be distinguished from one another, and therefore permitting axons to be traced along the regeneration pathway.

It has been approximated that roughly 2.6% of all peripheral sensory nerves are YFP+ in the Thy-1-YFP-H mouse line, based on observations from the L4 and L5 spinal nerves where YFP+ labelled axons were 122.6 in the dorsal root corresponding to sensory axons (out of an estimated 4265 neurones found in the ganglia) and 87.2 were YFP+ in the ventral root, corresponding to motor axons. Therefore, a larger number of sensory axons seem to be labelled YFP+ compared to motor axons (58% sensory and 42% motor in L4, and 57% sensory and 43% motor in L5) (Groves et al., 2005). Using this ratio, nerves originating from L4 and L5, such as the sciatic nerve and its branches, may also express a similar ratio of YFP labelled sensory and motor axons. For instance, the study suggested that out of the 36.03 YFP+ axons found in the common fibular branch of the sciatic nerve, 58% would be sensory (21) and 42% would be motor axons (15). Whilst studies in our own group have not analysed the proportion of YFP+ axons from motor or sensory origin, we have also found approximately 36 axons in the common fibular are YFP+, suggesting consistency between same strains used by different groups.

While the aforementioned methods can be used in combination to evaluate nerve regeneration and recovery of function, an often-times overlooked component related to nerve injuries is the probability for the development of neuropathic and chronic pain (Haroutiunian et al., 2013). Therefore, in order to provide a fuller overview of the recovery following nerve injury and regeneration, evaluating aspects related to the development of neuropathic pain may also be of interest.

4.1.3 Neuropathic Pain

Following a nerve injury, there is the possibility for the development of neuropathic pain which may persist after regeneration is complete. As well, axons that fail to reach the distal end of an injured nerve may form a neuroma, a mass of disorganised and tangled axons, that may also be involved in pain (Devor & Wall, 1976). Cellular and molecular changes can extend beyond the boundaries of the peripheral nerve and are known to be present in the spinal cord and higher regions of the central nervous system, where some of these changes have been implicated in the development and modulation of neuropathic pain.

Early studies demonstrated that peripheral nerve injury resulted in sprouting of nerve terminals into more superficial layers of the dorsal horn of the spinal cord, a re-organisation event associated with the development of pain (Woolf et al., 1992). Furthermore, studies highlighted that peripheral injury could lead to neuropathic pain through loss of inhibition in the transmission of noxious signals in the spinal cord, a key factor involved in the gate control theory of pain (Melzack & Wall, 1965; P. D. Wall & Devor, 1981). Multiple underlying mechanisms have been described in spinal disinhibition, such as GABA depletion and reduced pre-synaptic GABA release, leading to loss of GABAergic inhibition on superficial dorsal horn neurones (Moore et al., 2002). Alternatively, other studies point at reduced excitatory signalling to GABAergic interneurons as the main culprit in spinal disinhibition, possibly as a result of nerve-injury induced reorganisation (Polgár & Todd, 2008).

It has also been shown that following nerve injury, regenerated sensory neurones can fire spontaneously (ectopic firing), which can contribute to the initiation of abnormal and unpleasant sensations (M Devor, 2001). Such underlying hyperexcitability in neuropathic pain can be attributed to a reorganisation of expression of sodium and calcium channels in injured neurones, resulting in altered excitability and thereby underlying a mechanism in neuropathic pain (Waxman, Cummins, Dib-Hajj, Fjell, & Black, 1999). Neuromas can also be a site for ectopic firing, leading to similar symptoms of pain (Lane et al., 1978). As such, studies have demonstrated that in some cases, agents that block sodium channels (e.g. lidocaine) can be used to block conduction to alleviate neuropathic pain (Marshall Devor et al., 1992; Kostyuk et al., 1981).

Another mechanism implicated in neuropathic pain involves changes in the regulation of injury-induced neuropeptides (e.g. substance P, neuropeptide Y, galanin), either in the cell body, neuroma or site of injury, that can act as modulators of ectopic firing (Fried et al., 2001; Hökfelt et al., 1994). Cytokines and inflammatory factors released during the immune response can also alter the sensitivity of nerves, both directly and indirectly, ultimately leading to reduced activation thresholds and hypersensitivity to stimuli (Woolf et al., 1992).

Alternatively, the loss of sensory afferents from targets can cause the neighbouring neurones to sprout collateral axons in the denervated region, likely to compensate for the loss of innervation, where this can not only lead to spinal changes, but also cortical reorganisation. Studies in non-human primates have shown that both the loss and subsequent regeneration of nerves leads to changes in the organisation in the somatosensory brain cortex (J. T. Wall et al., 1986). For instance, in a transected median nerve, the cortical representations of the denervated skin are lost, while the cortical representations of the adjacent skin zones become expanded. The injury-induced enlargements may persist well after the regeneration/re-innervation period, and thus may prevent the re-establishment of original cortical representations, thereby leading to sensory deficits, such as diminished recognition of tactile stimuli or hypersensitivity.

Therefore, it is important to monitor such injury-induced changes in order to provide a fuller understanding of the effect of nerve repair therapies. One way this can be done is by looking at the role of glial cells in the spinal cord following nerve injury.

4.1.3.1 Glial activation

Peripheral nerve injury has been associated with the activation of glial cells in the spinal cord, such as microglia and astrocytes.

In healthy conditions, microglial cells are present in a “surveillance mode” as they are part of the natural physiological immune response, however, while the exact time course varies with the injury model, species and examined location, it has been shown that within 24 hours from peripheral nerve injury, microglial activation is seen in the spinal dorsal horn of rats, evidenced by morphological changes (Eriksson et al., 1993). A peak in microglial proliferation (microgliosis) is also evident around 2 – 3 days post-injury (Gehrmann & Banati, 1995), with some studies stating a two-to-four fold increase in cell number.

Astrocytes make up 20 to 40% of all glial cells in the CNS and are primarily involved in providing metabolic support to neurones as well as maintaining homeostasis of glutamate, ATP, and potassium (Ji et al., 2019). Under pathological conditions, astrocytes also respond to nerve injury by proliferation and undergoing cellular hypertrophy, particularly increasing in size, and showing thickened and elongated processes. Studies suggest that the peak of astrocyte proliferation starts slower and relatively later than microglial proliferation following injury, with some reports mentioning a steady increase in astrocyte activation from day 1 post injury to a large peak at day 7 post-injury (Hashizume et al., 2000).

Astrocytes are characterised by the presence of glial fibrillary acidic protein (GFAP), which is a key component involved in the assembly of the intermediate filament protein inside their cytoskeleton, therefore increased GFAP expression is consistent with the remodelled morphology of hypertrophic states in astrocytes (Eng et al., 2000). Garrison et al (1991) first reported on an increase in astrocyte activation and GFAP staining in the spinal cord of rats that underwent peripheral nerve injury and developed behavioural symptoms consistent with neuropathic pain. Specifically, increased GFAP staining in the spinal cord was found on the side consistent with nerve injury in the body (ipsilateral to the injury) and localised at the specific spinal levels concerned with projections of the injured nerve (Garrison et al., 1991). Since, a number of studies have consistently demonstrated GFAP staining following peripheral nerve injury in a number of models (Ji et al., 2019; Romero-Sandoval et al., 2008).

Signalling between activated microglia, astrocytes and neurones in the spinal cord has been shown to contribute to a number of neuropathic pain symptoms. For instance, a study by Chen et al (2014) reported on an upregulation of connexin-43 expression in spinal astrocytes following nerve injury in mice, where connexin-43 is a predominant gap-junction protein involved in connecting astrocytes to each other allowing the diffusion of ions and molecules between coupled cells and also the extracellular environment. Three weeks after injury, blocking connexin-43 resulted in reduced symptoms of neuropathic pain in the injured animals, suggesting a role for astrocytic connexin-43 in neuropathic pain maintenance. Furthermore, the study suggested connexin-43 undergoes a functional switch following nerve injury, resulting in the release of neuropathic pain mediators such as ATP and glutamate (Bennett et al., 2012; Chen et al., 2014).

Specific receptors found on microglia are also upregulated following nerve injury and thought to be involved in mechanical allodynia, which is a hypersensitive painful response evoked by otherwise innocuous stimuli. For instance, studies showed that following nerve injury in rodents, mechanical allodynia was accompanied by an increase in the expression of ATP purinergic P2X₄ receptors in activated microglia in the ipsilateral spinal cord, and that the pharmacological inhibition of the receptors reversed mechanical hypersensitivity (Tsuda et al., 2003), suggesting a role for P2X₄-expressing microglia in allodynia and neuropathic pain. Furthermore, the administration of activated P2X₄-expressing microglia in healthy naïve rats induced robust tactile allodynia (Tsuda et al., 2003), while targeting P2X₄ receptors with antisense oligonucleotides or genetically deleting the encoding gene (*P2rx4*) prevented the development of hypersensitivity (Ulmann et al., 2008).

Subsequent studies revealed that activated microglia released BDNF in the spinal dorsal horn, where BDNF could bind to its TrkB receptors in laminae I neurones and result in a loss of inhibition and thus transmission of pain (Thompson et al., 1999; Trang et al., 2009). The loss of inhibition was found to

involve a downregulation of the neuronal K-Cl co-transporter 2 (KCC2), which resulted in an increase of intracellular Cl⁻ ions. Under this pathological accumulation of intracellular Cl⁻ ions, GABAergic stimulation, as a result of touch, could cause laminae I neurones to depolarise (via opening of Cl⁻ channels), rather than hyperpolarise (Coull et al., 2003) (Figure 4.1). As such, innocuous mechanical stimulation could result in aberrant depolarisation and transmission of pain (allodynia).

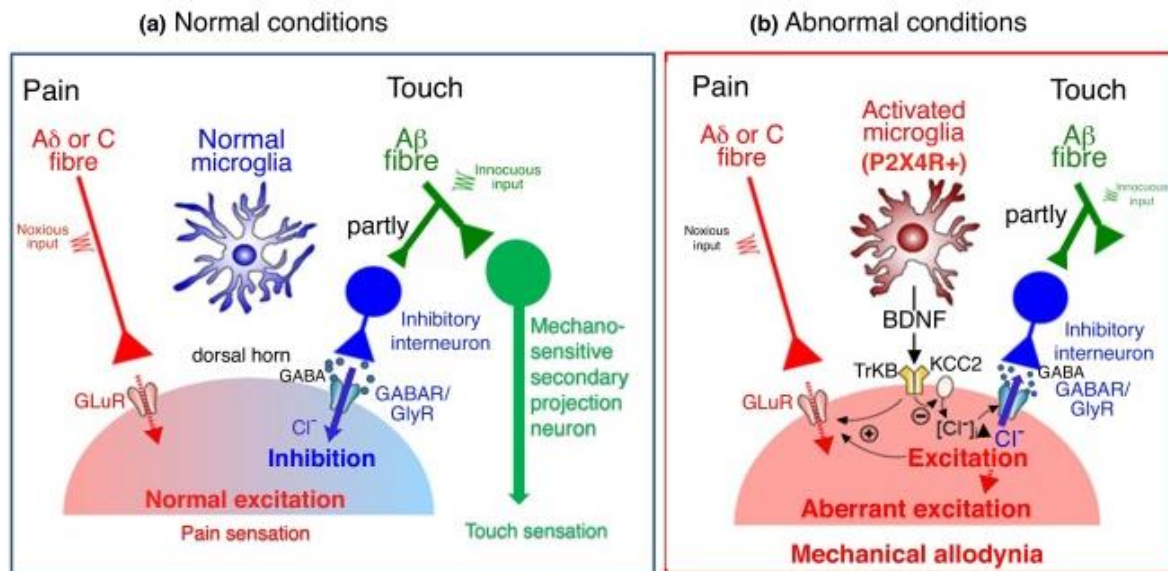


Figure 4.1. (a) Under normal conditions, touch stimuli are transmitted by large Aβ fibres that stimulate GABAergic neurones to inhibit transmission of spinal laminae I neurones. This GABA-mediated inhibition involves hyperpolarisation of the neurone. (b) However, in abnormal conditions, microglial-derived BDNF can bind to TrkB receptors and cause a downregulation in the expression KCC2 transporters, resulting in a pathological intracellular accumulation of Cl⁻ ions. In this abnormal state, mechanical stimulation cannot cause hyperpolarisation, but instead, the opening of Cl⁻ channels results in an outflow of Cl⁻ ions, causing depolarisation (and thus excitation/firing) of spinal laminae I neurones. As such, touch transmission can be converted to allodynia. Image adapted from (Inoue, 2019; Tsuda & Inoue, 2016)

4.1.4 Summary and Aims

A novel nerve guide conduit was fabricated from polycaprolactone methacrylate (PCL-MA) using the methods of injection moulding and electrospinning as described in Chapter 1. The electrospun fibres were coated with decellularised-extracellular matrix (ECM) previously obtained from DRG cultures to model the peripheral nerve environment as described in Chapter 2. Therefore, this final experimental chapter aims to evaluate the novel conduit in the repair of a common fibular injury in Thy-1-YFP mice. In addition, to evaluate the efficacy of the conduit and the potential for development of neuropathic pain, glial activation in the spinal cord is assessed following nerve injury and repair.

Aims

1. Using a common fibular nerve gap model in Thy-1-YFP mice, assess the *in vivo* nerve regeneration following repair with nerve guide conduits: hollow conduit, conduit with intraluminal fibres, and conduit with intraluminal fibres coated with decellularised matrix (as described in Chapter 3) and with a nerve graft.
2. Evaluate the potential for neuropathic pain development following nerve repair of the common fibular nerve with the different repair groups by assessing glial activation in the spinal cord.

4.2 Methods

4.2.1 Common fibular nerve gap injury model

PCL conduits were evaluated in the repair of a common fibular nerve injury as described previously by our group (Harding et al., 2014)(Pateman et al., 2015). Briefly, a 3mm nerve gap was created in the common fibular nerve which was then repaired using either a nerve graft taken from a littermate, or three types of conduits: hollow conduit, conduit with fibres, conduit with fibres coated with decellularised extracellular matrix (Table 6). The nerve graft repair was used as the control as this is the gold-standard method used clinically.

Table 6. Groups and animal numbers in each repair type group.

Repair Type	Number of animals used in group
Nerve graft repair	5 (plus 3 donors)
Hollow PCL Conduit	5
PCL conduit with PCL fibres	5
PCL conduit with PCL fibres coated with decellularised ECM derived from DRG	5

4.2.1.1 Conduit preparation

Nerve guide conduits were produced as described in Method 2.1.2.2 (Chapter 2) by injection moulding from methacrylated PCL. All conduits were 4.0 mm in length with an internal lumen of 0.9 mm and outer diameter of 1.4 mm.

PCL fibres were prepared by electrospinning as described in 2.2.6 and plasma treated as described in 2.2.7. Then, PCL fibres were coated with extracellular matrix as developed in Chapter 3. Briefly, PCL fibres were used as scaffolds for the deposition of extracellular matrix (ECM) derived from embryonic chick dorsal root ganglia (3.3.1.2) and decellularised (3.3.2) to obtain PCL fibres coated with decellularised ECM. PCL coated fibres and control PCL fibres were inserted into conduits based on the methods described in 2.2.8 at a packing density of 6%.

4.2.1.2 Sample size calculations

The sample size for this chapter were calculated in order to obtain a statistical power of 80% using PiFace software [v1.76: homepage.stat.uiowa.edu/~rlenth/Power]. Details of the calculations are shown in Figure 6.1 (Appendix), where standard deviation (SD) data was obtained from previous studies in our laboratory (Harding et al., 2014; Singh et al., 2018). The sample size chosen for the study

was n=5 for each group, which would be sufficient to detect differences between groups of 19.5% for axon counting/sprouting analysis.

Extra animals could not be included to account for failures due to time constraints as a result of Covid-19 and difficulties in the availability of appropriate animals due to the uncertainty and variability of in-house breeding limitations as these were disturbed during the pandemic.

4.2.2 Experimental set-ups

4.2.2.1 Ethics

All experimental procedures were approved by the UK Home Office in accordance with the UK animals (Scientific Procedures) Act 1986 guidelines, under the project license numbers PPL 70/8194 (before 2020) and P34A34A72 (2020 onwards). The experiments were completed by the author under a granted personal license I0CB8FA04. All efforts were made to minimise animal suffering and to reduce the number of animals used in the studies.

4.2.2.2 Animals

Thy-1-YFP-H mice were obtained from a Home Office-approved UK supplier (JAX Mice, Maine USA via Charles River UK Limited) and bred for experimental purposes. Mice were maintained on a standard 12:12 h light/dark cycle and housed in groups of 3-6, with both YFP and wild type mice as littermates. Temperature and humidity were maintained at a constant of $21 \pm 2^\circ\text{C}$ and $50 \pm 2\%$, respectively with water and food pellets access *ad libitum*. The experimental procedures were conducted between 9am and 6pm.

4.2.2.3 Anaesthesia and analgesia

General isoflurane gas anaesthesia (Isoflurane®, Abbot Laboratories) was used for induction of anaesthesia in recovery mice (induction at 4-5% and maintenance at 1.5% - 2% in O₂ carrier gas). Mice that were used as donors for nerve autografts or during the end of the experiments prior to tissue harvest were induced with an injectable anaesthetic (Ketavet, 100mg/kg and Domitor, 1mg/kg, i.p.), as this is more suited for terminal procedures.

Analgesia was provided to recovery animals once the procedure had ended using a long-lasting NSAID (Metacam, 5mg/kg, s.c.) as well as providing local pain relief at the site of surgical incision with Marcaine (0.25%, 1:20 dilution). Animals were checked daily and scored for pain, and analgesia was given accordingly where needed.

4.2.2.4 Fibrin glue

Fibrin glue was used as a sealant to secure nerves to grafts and conduit. Fibrin glue was made by applying equal quantities of fibrinogen (10mg/ml of fibrinogen in saline, Sigma Aldrich) and thrombin (40IU/ml in distilled water, Sigma Aldrich). Hamilton syringes or insulin syringes were used to apply a small droplet (5-10 μ L) of each component separately onto the ends of the injured nerve to secure the graft or conduits in between the proximal and distal stumps.

4.2.2.5 Autograft surgery

Surgical procedures were performed under aseptic condition with all instruments washed and autoclaved. Surgeries were conducted using a surgical dissection microscope (Global Microscope: DP Medical Systems Ltd, Surrey, UK) and kept to under an hour. Surgeries were conducted on a heated mat to conserve body heat, and eye drops were administered to prevent eye irritation and animal breathing was checked regularly to monitor the effects of anaesthesia.

The experimental set-up involved the unilateral repair of the common fibular nerve in YFP mice with a nerve graft provided by a littermate. Two procedures were therefore required within the same experiment: harvest of a donor nerve from a littermate and transfer of the donor nerve to an YFP mouse for the repair of a nerve gap.

A YFP mouse was placed under general anaesthesia on a heated mat and the surgical site (left hindleg) was shaved and cleaned with hibitane before an incision was made first on the skin and then through the bicep femoris muscle to expose the three terminal branches of the left sciatic nerve: the sural, common fibular and tibial nerves (Figure 4.2).

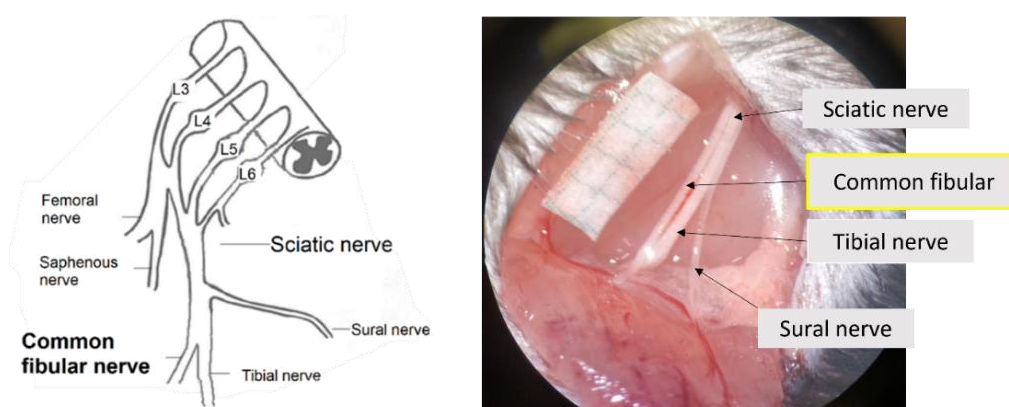


Figure 4.2 Common fibular nerve injury. Schematic representation of the branches of sciatic nerve (left). Sciatic nerve and branches exposed on the left hind leg with a piece of graph paper (4 mm).

The common fibular nerve was freed from surrounding tissue and fascia and an appropriate length of the nerve was measured (approx. 5mm). The nerve was re-covered with the muscle to prevent the

site from drying out while the recipient animal was prepared for transfer, where then the donor nerve would be immediately transferred to the recipient YFP mouse. As soon as the donor nerve was transferred, animals were overdosed with pentobarbital (140mg/kg, diluted 1:1 in saline).

The recipient YFP mouse was placed under general anaesthesia and the common fibular nerve was exposed similarly to the donor animal. The left common fibular was carefully freed from surrounding tissue and a 3 mm gap was subsequently made in the common fibular nerve, making sure to leave surrounding nerves intact. The donor nerve graft from the littermate was placed within the gap and the nerve ends of the graft were trimmed to align between the distal (terminal branch) and proximal (closest to sciatic nerve) ends of the gap. A small piece of parafilm was placed under the graft to help contain the fibrin glue that was applied to each end of the nerve and allowed to set for 3-5 minutes. Once the nerve graft was secured to the recipient nerve, muscle and skin were closed in separate layers using 6-0 Vicryl sutures and analgesia was administered. Animals were then placed in an incubator at 37°C allowing them to recover fully from the effects of anaesthesia, monitoring their behaviour closely prior to returning them to their home cages for a period of 3 weeks. Animals were kept in their original cages and with littermates unless any issues arose with sutures, then animals were caged separately until the wound sites were fully healed in order to prevent wound re-openings. Animals were checked daily and scored for signs of infection, inflammation or pain, and analgesia was given accordingly if needed.

4.2.2.6 Nerve repair with conduit implantations

For nerve repairs of the common fibular nerve, the nerve was exposed as described above, but the nerve was repaired using a nerve guide conduit from the groups described above in Table 6.

Briefly, the common fibular nerve was cut to produce a 3 mm gap. A conduit measuring 4 mm was used to repair the gap by placing the ends of the proximal and distal stump into each end of the conduit (approximately 0.5mm of the nerve stumps were placed in either end). The conduit was secured in place using fibrin glue. Muscle and skin were closed, and mice were returned to home cages for a recovery period of 3 weeks.

4.2.3 Tissue harvest and processing

4.2.3.1 Harvest of repaired nerve

Following a 3-week recovery period, the mice were re-anaesthetised in a terminal procedure using the injectable anaesthetic and the common fibular nerve was re-exposed. If the nerve was found covered in any scar tissue, this was carefully removed to free the repaired nerve and/or conduit underneath. The skin (and muscle tissue if necessary) were sutured to a brass ring held above

the nerve repair site, allowing the formation of a “pool” wherein the repaired nerve was visibly accessible. The repaired nerve site was filled with 3.7% (v/v) paraformaldehyde allowing *in situ* fixing for 15 minutes. Autograft repair nerves were collected directly into Eppendorf tubes for further fixation for 30 minutes. For nerve repairs using conduits, the conduits were carefully cut open using small surgical scissors and the nerve was removed and also fixed for a further 30 minutes. Animals were overdosed by pentobarbital injection (140mg/kg) and culled. The nerves were mounted on a microscope slide using Vectashield® and imaged within a week.

4.2.3.2 Spinal cord harvest

The spinal cord was harvested once the animal had been culled. Briefly, animals were placed facing down and an incision was made along the back to remove the skin as distally as possible. The vertebral column was removed and immersed in a 50 ml falcon tube filled with 3.7% (v/v) paraformaldehyde for overnight fixation at 4°C. A small length of the ribs was kept attached to the vertebral column for identification of spinal segments. The following day, the spinal cord was freed from the vertebral column and the ribs allowed the identification of lumbar segment L1-L6. The contralateral side of the spinal cord on the ventral orientation was marked with a blade along the whole cord. The spinal cord L1-L6 segment was then placed in a 50ml falcon tube and further fixed for 4 hours and then transferred to a solution of 30% sucrose overnight. The following day, the spinal cord was embedded in OCT fluid (Thermo Shandon Ltd) and the tissue was frozen using the fast-freeze function and station of a cryostat (CryoStar NX50). The embedded spinal cord segment was stored in a -80°C freezer until required use for immunofluorescence staining.

4.2.4 Glial staining

The frozen spinal cords were mounted on chucks and attached to the cryostat for cutting. Transverse sections were cut until lumbar segment L4 was reached (as the common fibular nerve projects to L3-L6 in the mouse spinal cord) using a mouse spinal cord atlas for reference and identification of each spinal level. Once L4 had been reached, 30 µm thick spinal cord sections were then collected and placed in separate wells of a 24-well plate containing PBS for free-floating staining.

Tissue sections were stained for astrocytes using rabbit polyclonal anti-GFAP (Abcam, ab7260, 0.5mg/ml) at a 1:2000 dilution and an anti-rabbit Cy3 secondary antibody (1.25mg/ml) at a 1:500 dilution (Jackson Immuno Research, 711-165-152). Briefly, sections were permeabilized with 0.1% (v/v) Triton X-100 in PBS (PBS-T) for 20 minutes (2 washes, 10 mins each), and protein-binding sites were blocked with 10% Normal Donkey Serum (NDS) in PBS-T (v/v) for 1 hour. The primary antibody was prepared in 5% NDS and incubated with the sections overnight at 4°C. The following day, the primary

antibody was washed off (PBS, 2 washes, 10 min each) and the secondary antibody was prepared in 2.5% NDS and incubated for 3 hours at room temperature. The sections were washed and mounted onto glass slides and covered with Vectashield® and covered with a glass coverslip.

The specificity of the secondary antibody to the primary antibody was tested by omitting the primary antibody from the protocol and using the only secondary antibody during the protocol, demonstrating no positive staining under Cy3 excitation (Figure 4.3 top row). The minimal background staining is a result of the transgenic nature of the tissue as the animals are Thy-1 YFP so a small subset of neurones in the spinal cord also express the YFP fluorophore that can be inadvertently viewed under Cy3 excitation, as demonstrated when the red and green channel images are merged, the fluorescence is visualised as yellow completely. On the other hand, when the primary antibody anti-GFAP is used, positive astrocyte staining is visible that does not overlap with the YFP neurones (Figure 4.3 bottom row).

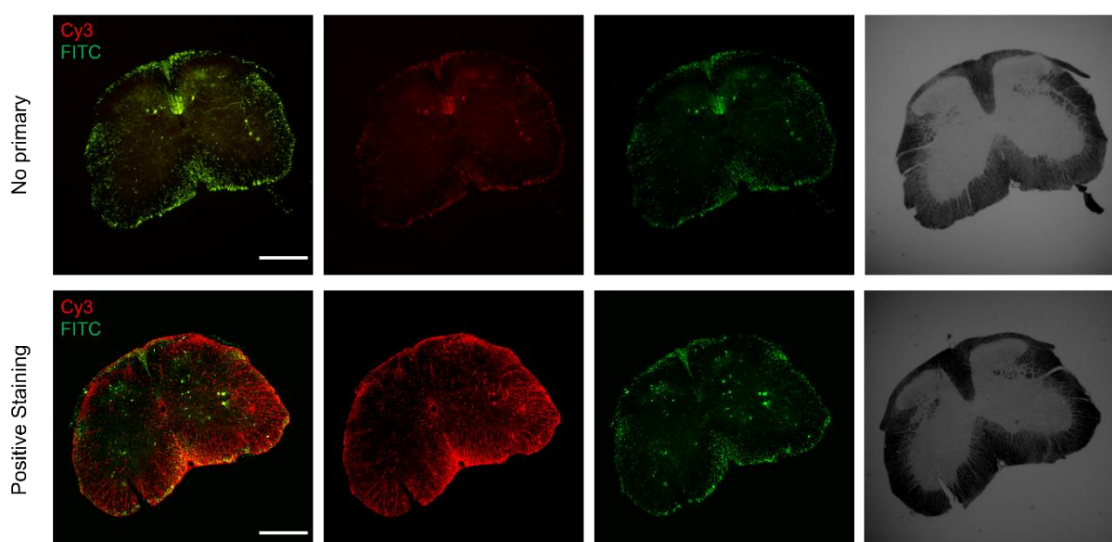


Figure 4.3 Antibody controls for anti-GFAP. Top (no primary): Application of secondary antibody (anti-rabbit Cy3) with omission of primary antibody shows no positive staining for astrocytes. Some background staining is evident due to the YFP nature of the animals. Bottom (Positive staining): Primary antibody anti-GFAP is used following secondary antibody application showing positive astrocyte staining. Scale bars 500µm.

4.2.5 Image acquisition and processing

4.2.5.1 Fluorescence microscopy

For fluorescence microscopy, a Leica DMI8 inverted microscope at the School of Clinical Dentistry (University of Sheffield) was used. The microscope was equipped with a LED 3 fluorescence light source, offering white light in the 390 – 680 range, and three filter cubes for epifluorescence illumination of FITC, Cy3 and LED 405. Images were captured using the microscope's FlexaCam C1

Camera system and obtained and processed using the Leica Application Suite X (LAS X) software. Further image processing was done in ImageJ (NIH).

4.2.5.2 Image analysis for nerve repairs

A 10× objective was used to obtain z-stack images with optimal slice intervals determined by the imaging software through the depth of the tissue, giving an average of 30-60 slices per image. Z-stack images were taken at sequential positions along the nerve stitched together into a composite image with z-stacks. The stitched z-stack images produced a single image for each repaired nerve. Visual inspection of the nerve showed the location where the nerve graft or conduit, as the axons at each end of the graft site expressed irregular morphology and sprouting. The start of regeneration was identified by the visible branching in the proximal stump (closest to the sciatic nerve) and thus labelled with a reference vertical line (0.0 mm). Further vertical lines were drawn along the horizontal axis of the nerve in 0.5mm increments until the end of the nerve graft, where axons entered the distal stump, thus creating intervals across the nerve. Additional interval lines were drawn before the proximal stump (- 1.5mm) to demonstrate the morphology of the nerve in its native state.

Axon count analysis to evaluate nerve regeneration involved counting individual axons at each interval point to determine axon count. Specifically, the axons were counted at each individual z-plane for each sample, taking care to only count unique axons.

4.2.5.3 Image analysis for glial activation

Images of the dorsal horn and ventral horn of the spinal cord were taken for analysis of glial labelling. In Figure 4.4 a whole spinal section is shown with the dorsal horn and ventral horn highlighted (A) and the same section with positive anti-GFAP staining (B). Two high magnification images were then taken at the dorsal horns, localised to the superficial laminae of the dorsal horn (laminae I – III) and at the ventral horns (two images) and used for analysis.

The images for analysis were converted into 8-bit and thresholding was done to measure only the GFAP staining in the images (Figure 4.4C). The percentage area data were reported as the ratio of staining of the ipsilateral side to contralateral side (Ipsilateral/Contralateral). Two high magnification images were taken at each area of interest. Three spinal cord sections were analysed per each animal in each group.

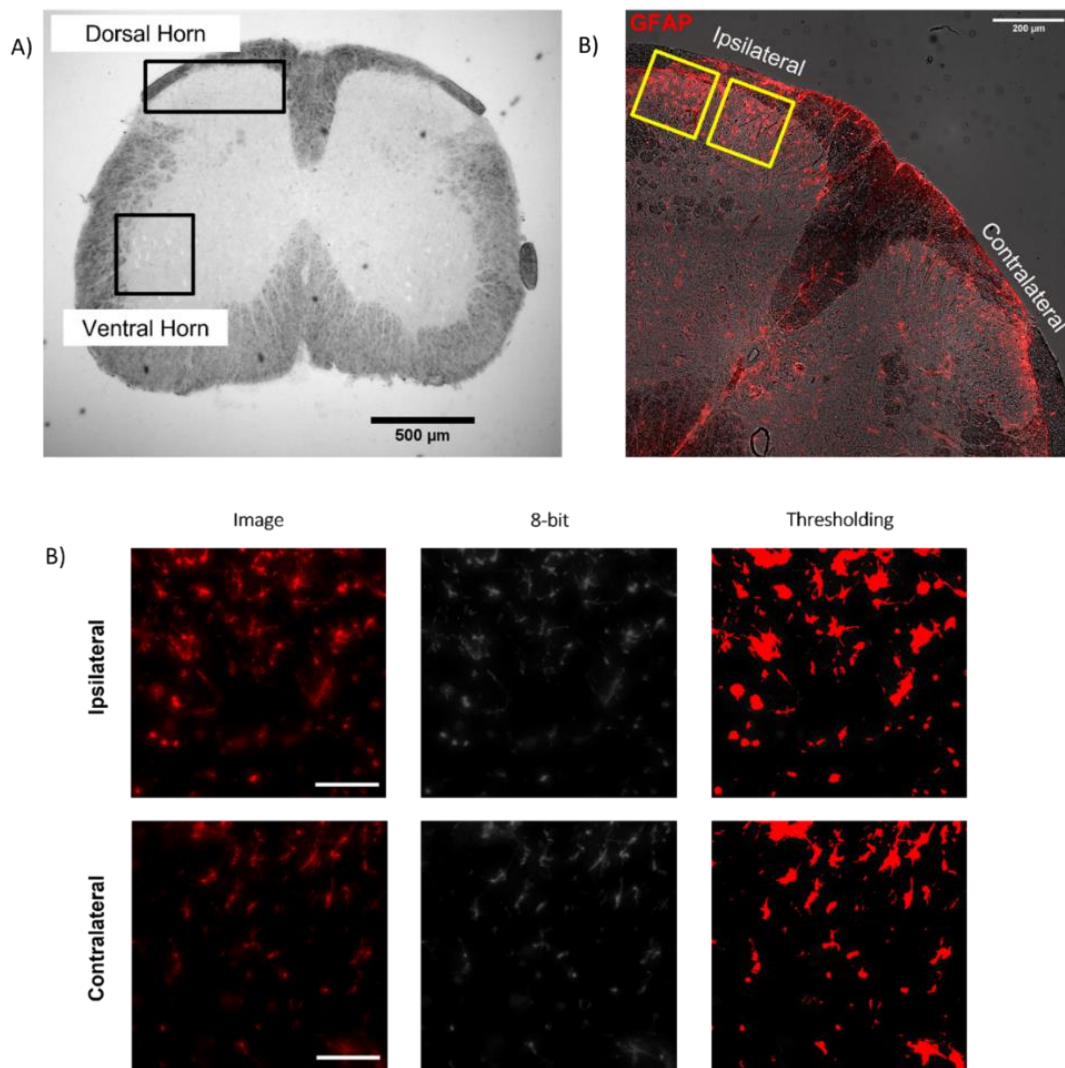


Figure 4.4 Low magnification image of a L4 spinal cord section taken with a x5 objective with two boxes highlighting the dorsal and ventral horn (A). Spinal cord L4 section stained with anti-GFAP for astrocyte staining, with two boxes highlighting the location on the dorsal horn where high-magnification images with a 40x objective are taken and used for analysis of GFAP staining in laminae I-III (B). High-magnification images with a 40x objective taken at the dorsal and ventral horns of the spinal cord for image processing and analysis with ImageJ, where images are converted into 8-bit and thresholding was used to measure only the positive staining (C). Scale bars 500μm, 200μm and 100 μm in A, B and C.

4.2.6 Statistical analyses

Statistical analyses were made using GraphPad Prism (GraphPad Software v9, San Diego, CA). Data was tested for normality and analyses of variance (ANOVA) was used to test for differences between groups for parametric data, followed by relevant multiple comparison test. Axon counting/sprouting index and glial activation were tested using a two-way ANOVA. All data is presented as mean + standard deviation.

4.3 Results

4.3.1 Nerve regeneration with novel nerve guide conduit

In this study, a 3 mm nerve gap in the common fibular nerve of a YFP mouse was repaired with either a graft taken from a littermate to serve as the control, or with a nerve guide conduit from three experimental groups: hollow conduit, conduit with PCL fibres and conduit with PCL fibres containing decellularised ECM derived from DRG.

Five animals were included in each group, however not all groups showed regeneration (Table 7). Specifically, while all animals repaired with an autograft and hollow conduit showed regeneration at the end of the recovery period, regeneration failed in two cases in the other two groups. Regeneration success was defined as axons regenerating and reaching the distal stump. In the failure cases, there were several reasons identified. Firstly, whilst axons from the proximal stump were able to enter the conduits and fibres, axons ended before reaching the distal stump where a large neuroma formed of disorganised axons and scar tissue was found obstructing the path (Figure 4.5). In another failure case, a large clot was found inside the conduit, likely formed from fibrin glue and blood cells due to the colour of the clot, and once again obstructed the path for regenerating axons from entering the conduit. Lastly, in the final failure case, when the surgical site was opened to harvest the conduit, the conduit had been completely dislodged and was found adhered to the underlying muscle. These failure cases are attributed to the inability of the fibrin glue to secure and hold the conduits in place as opposed to the conduit designs failing.

Table 7. Summary of regeneration outcome following repair with repair types

	Nerve graft	Hollow Conduit	Conduit + Fibres + dECM	Conduit + Fibres
Animals in group	5	5	5	5
Regeneration success	5	5	3	3
Success (%)	100%	100%	60%	60%
Reasons for failure:				
Neuroma at distal site			1	1
Clot found inside conduit				1
Dislodged conduit			1	

It is also worth noting in Figure 4.5 in the distal stump that no individual YFP positive nerve fibres can be seen morphologically, and rather only a few “specks” are seen, which confirms that the axons we see following nerve regeneration in the graft and the distal stump originate from the proximal stump.

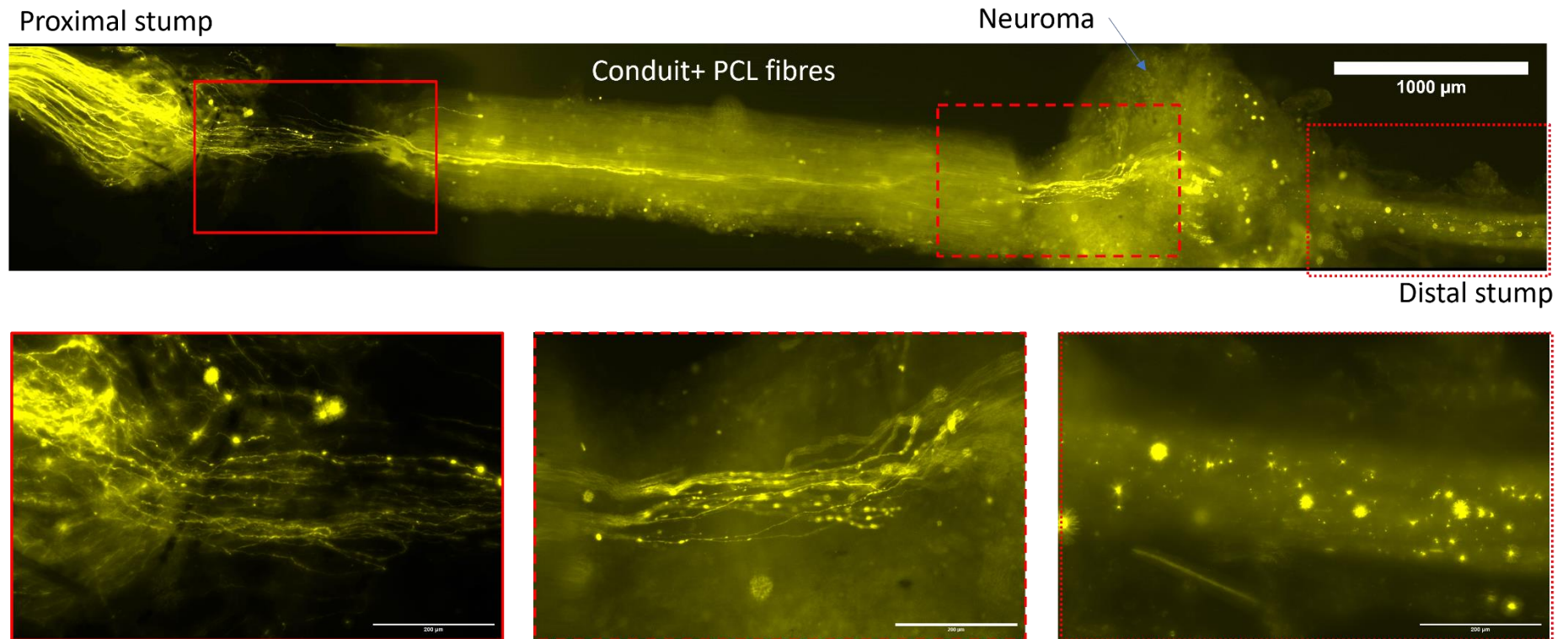


Figure 4.5 Failed regeneration. Images showing common fibular nerve at 3 weeks following repair of 3mm gap with conduit + PCL fibres, showing axons entering the conduit from the proximal stump, crossing the gap injury across the fibres but not entering the distal stump. Axons end at the site of the neuroma found just before the distal stump. Top row shows the whole common fibular gap site, with highlighted areas shown magnified in bottom row. Scale bars 1000µm for top image and 500µm for bottom row images.

Representative images of successful regeneration for each repair group are shown in Figure 4.6 to Figure 4.9, and quantified in Figure 4.10 and Figure 4.11. Quantification was done by counting the number of axons at pre-determined intervals of 0.5mm in the regenerated nerve, including proximal to the injury site (denoted as negative numbers) which reflects the native nerve. The axonal count at each interval for each group was normalised and compared to the number of axons at the most proximal site (-1.5mm).

Due to the thickness of the fibre bundles, the autofluorescence of the material PCL and the variability of the outgrowth of the axons on the fibres (where some axons grew within the fibre bundles while others grew more superficially), it was difficult to image the axons in the conduit + fibre groups. Therefore, Figure 4.10 is missing the axons in the middle portion for the two conduit + fibre groups, as axons could not be reliably quantified for all animals and these intervals were not included in the statistical analyses where all four groups are compared.

Nevertheless, a general trend can be observed in the regenerated nerves, where an increase in disorganised axons and sprouting is seen adjacent to the injury site at the proximal side. This is where axons begin to regenerate and elongate out from the proximal stump through the conduit or graft (from interval - 0.5mm to 0.5mm). Similarly, as axons exit the graft or conduit in search of the distal stump, another increase in axonal count and disorganisation of axons is seen (around interval 3-3.5mm), although smaller than the first peak. The level of sprouting at either of these locations is similar between all repair types as there was no significant difference between groups ($p>0.05$).

Next, in order to reflect the difference in the regenerated axons and sprouts along the regeneration path following nerve repair with the conduits and graft, the number of axons at each interval for each group were compared to the number of axons at their proximal site. There was no significant difference in the axons at the intervals in comparison to the proximal site for either of the groups ($p>0.5$), suggesting the number of axons that regenerated from the proximal site to the distal site stayed equal between repair types.

Figure 4.11 compares the axons at the proximal site (-1.5mm) to the distal site (-6.0mm) and an average reduction in the relative axons can be seen for each group. It's important to note the large variability in the axons counted for each group. In animals repaired with a nerve graft, an average of $58.9 \pm 21.5\%$ of axons regenerated from the proximal site, across the graft and into the distal stump. In comparison, hollow nerve guide conduits supported the regeneration of $42.8 \pm 31.5\%$ of axons, while conduits with PCL fibres supported $43.10 \pm 24\%$ of axons and conduits with PCL fibres coated with decellularised ECM supported the regeneration of $44.8 \pm 23.4\%$ fibres from the proximal stump to the distal stump.

NERVE GRAFT REPAIR

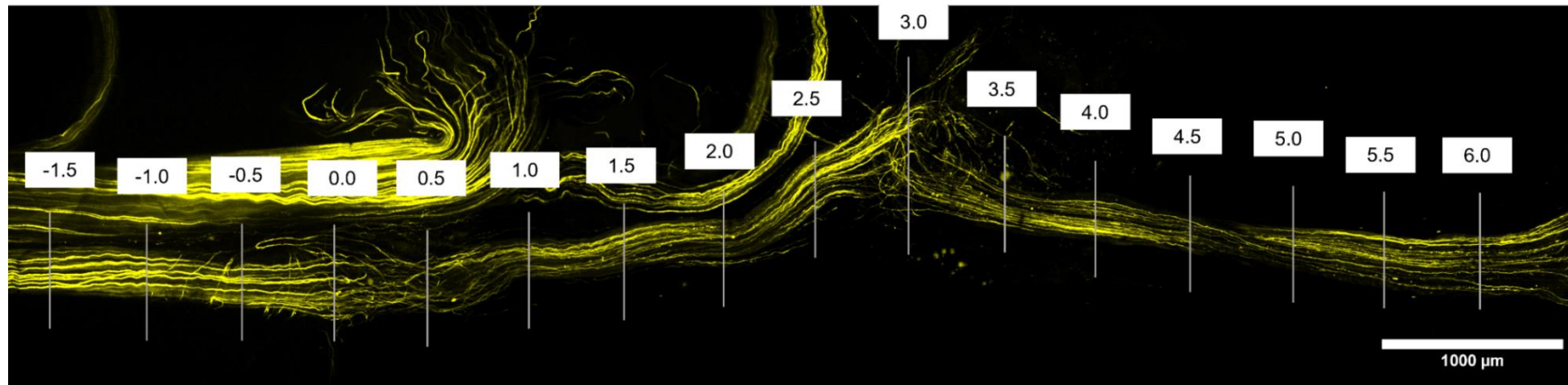


Figure 4.6 Nerve graft repair. Image showing common fibular nerve at 3 weeks following repair of 3 mm gap with nerve graft. Intervals are marked in reference to the site where axons enter the graft at 0.0mm and exit at 3.0mm to enter the distal stump. Scale bar 1000μm

HOLLOW CONDUIT

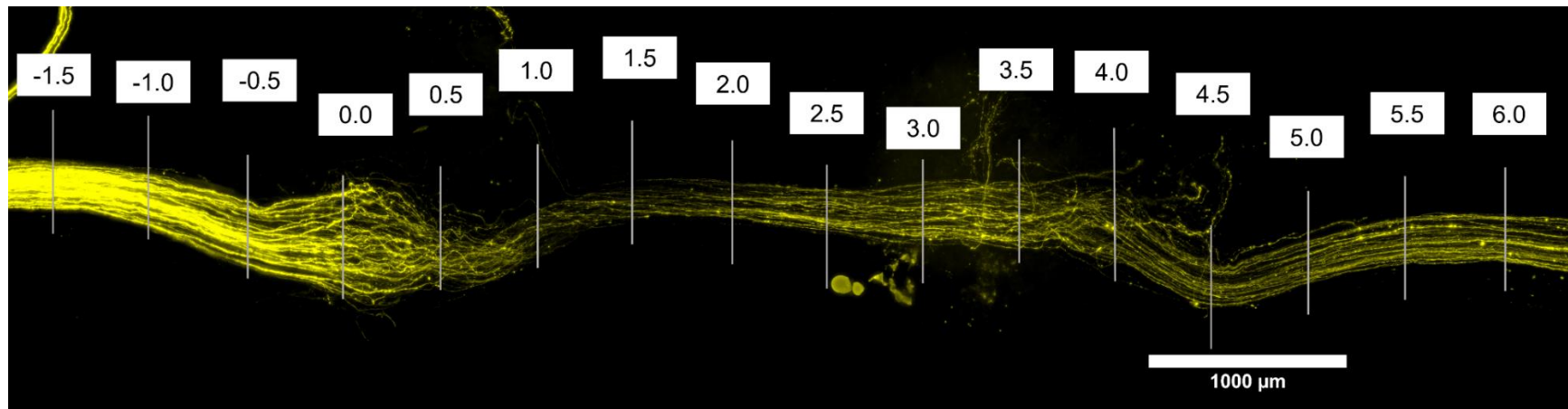


Figure 4.7 Hollow conduit repair. Image showing common fibular nerve at 3 weeks following repair of 3 mm gap with a hollow conduit. Intervals are marked in reference to the site where axons enter the graft at 0.0 mm and exit at 4.0mm to enter the distal stump. Scale bar 1000μm

PCL CONDUIT + FIBRES + dECM

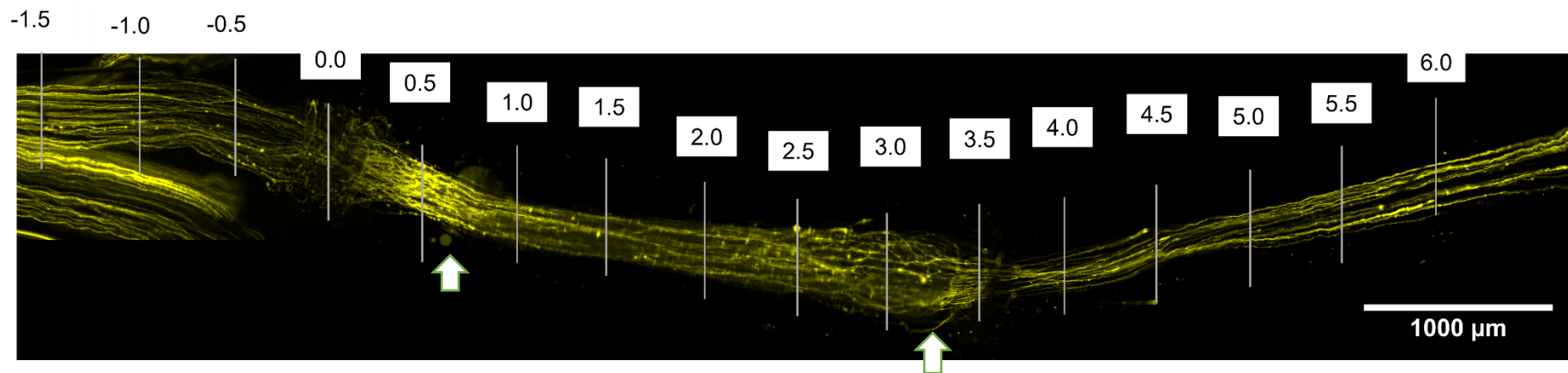


Figure 4.8. Conduit with PCL fibres + dECM. Image showing common fibular nerve at 3 weeks following repair of 3 mm gap with a conduit containing PCL fibres coated with dECM. Intervals are marked in reference to the site where axons enter the conduit at 0.0 mm and exit at 4.0mm to enter the distal stump. Scale bar 1000µm. Arrows show the start and end of the fibres.

PCL CONDUIT + FIBRES

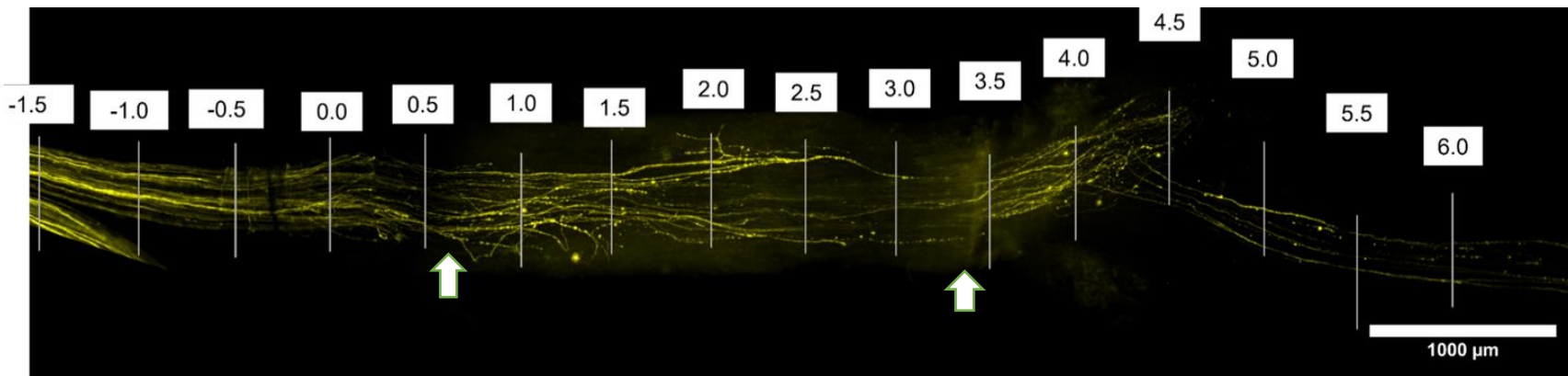


Figure 4.9 Conduit with PCL fibres. Image showing common fibular nerve at 3 weeks following repair of 3 mm gap with a conduit containing PCL fibres. Intervals are marked in reference to the site where axons enter the conduit at 0.0 mm and exit at 4.0mm to enter the distal stump. Scale bar 1000µm. Arrows show the start and end of the fibres.

Axon count

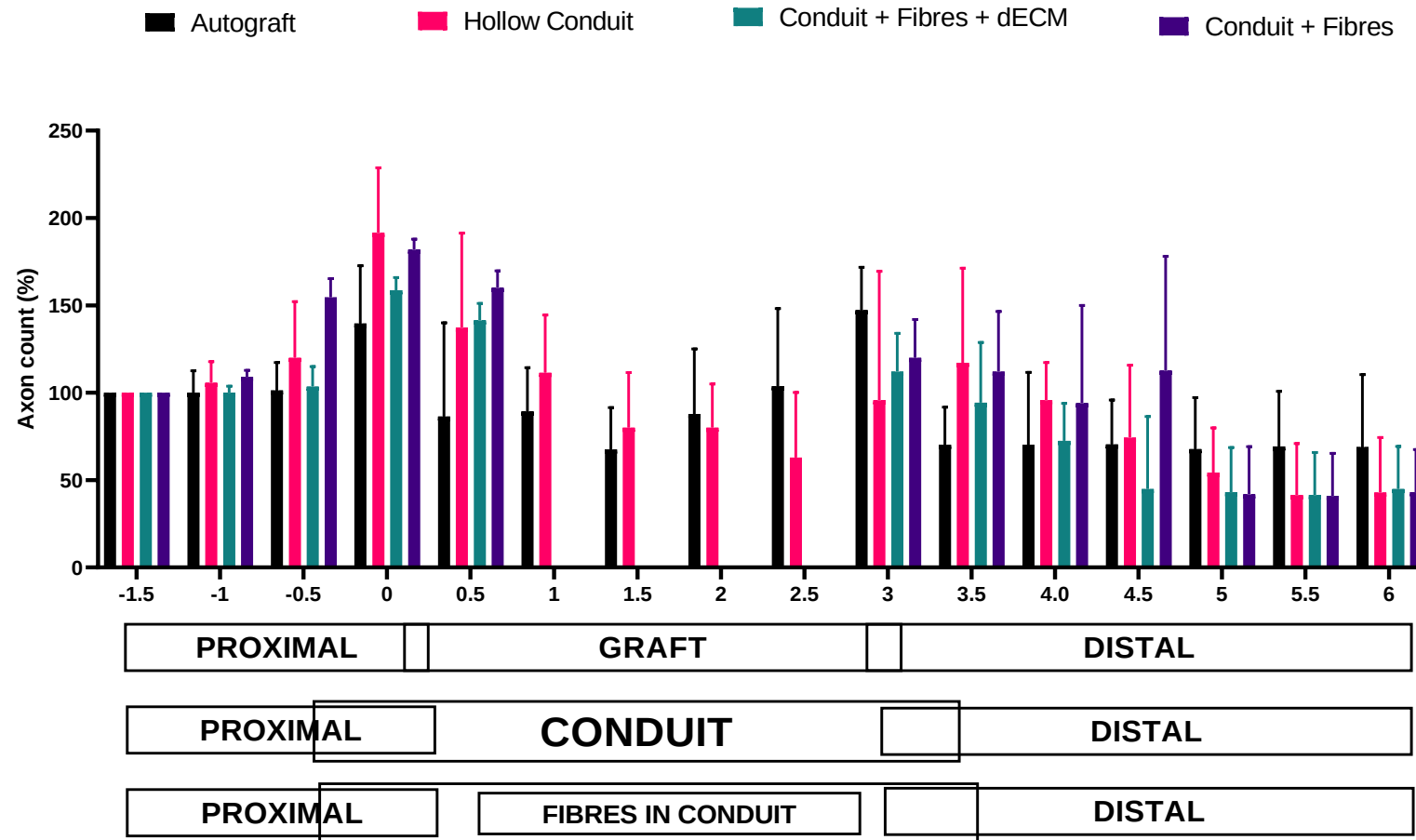


Figure 4.10. Quantification was done by counting the number of axons at pre-determined intervals of 0.5mm in the regenerated nerve. The axonal count at each interval for each group was normalised and compared to the number of axons at the most proximal site (-1.5mm).

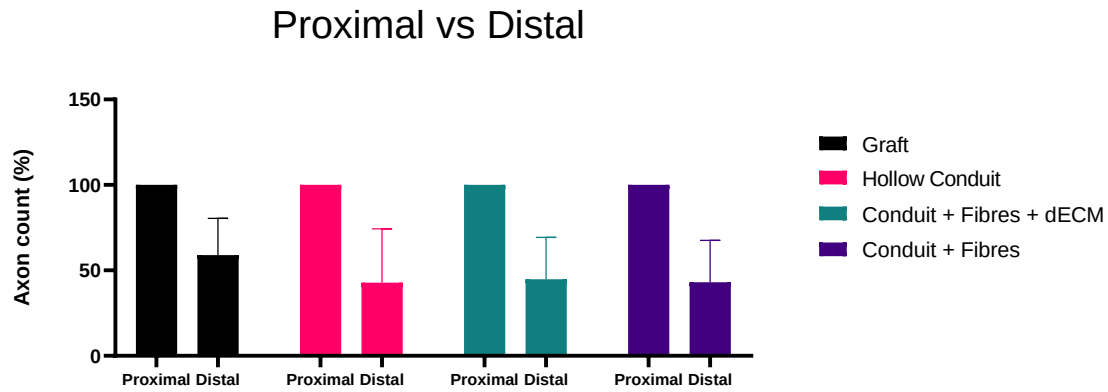


Figure 4.11. Comparison of axon count at the proximal stump prior to conduit/graft entry (-1.5mm interval) with the axon count at the distal stump (6 mm interval).

4.3.2 Glial activation in spinal cord

The spinal cord of animals that underwent common fibular injury and nerve repair with the three nerve guide conduits and the nerve graft were harvested and stained for astrocyte activation with GFAP. Following peripheral nerve injury, astrocyte activation occurs in the ipsilateral spinal cord, as show in Figure 4.12, where in the dorsal horn astrocytes show signs of hypertrophy, such as thicker processes in comparison to the contralateral side, as well as evident proliferation (gliosis).

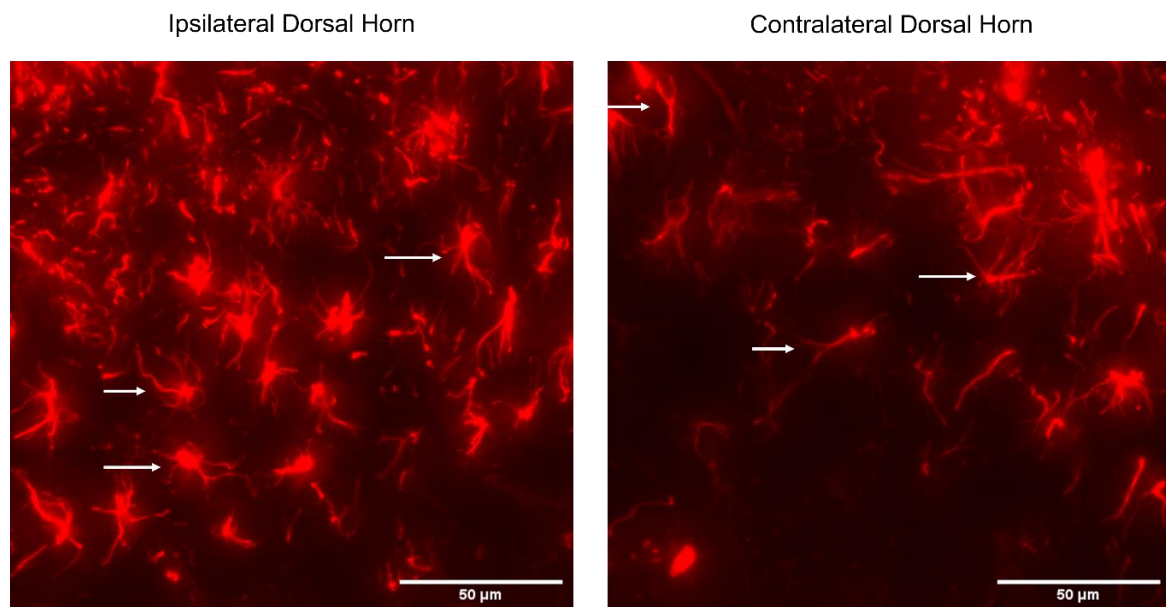


Figure 4.12. High magnification images taken at the dorsal horn of the spinal cord, showing the ipsilateral and contralateral side to injury. In the ipsilateral dorsal horn, activated astrocytes stained by GFAP are shown evident by the hypertrophic morphology of the astrocytes, with increased processes per cell as well as an increase in astrocyte number in comparison to the contralateral side.

Images of GFAP staining in the L4 segment of the spinal cord at the dorsal and ventral horns for animals in each repair type is shown in Figure 4.13 to Figure 4.16. The analysis was only carried out for successful repairs. As shown in the images, the dorsal and ventral horn spinal cords showed increased GFAP staining on the ipsilateral side (to the injury) compared to the contralateral side for all repair groups: nerve graft, hollow conduit, conduit with dECM fibres and conduit with PCL fibres.

GFAP analysis was then conducted where specifically the staining in the ipsilateral and contralateral side were measured using image processing (as described in 4.3.4), and the percentage area of staining in each side was then reported as the ratio of staining of the ipsilateral side to contralateral side (Ipsilateral/Contralateral) (Figure 4.17). As shown in Figure 4.17, the percentage staining was reported as the ratio of the contralateral to ipsilateral side, where all groups showed a ratio of above 1, confirming an increase in GFAP staining in the ipsilateral side. However, there was no significant difference in the GFAP staining ratio between the different repair groups, suggesting the increase in GFAP was consistent for all repair types and that the novel PCL conduit with fibres coated in decellularised matrix did not affect GFAP staining in the spinal cord in comparison to the other groups.

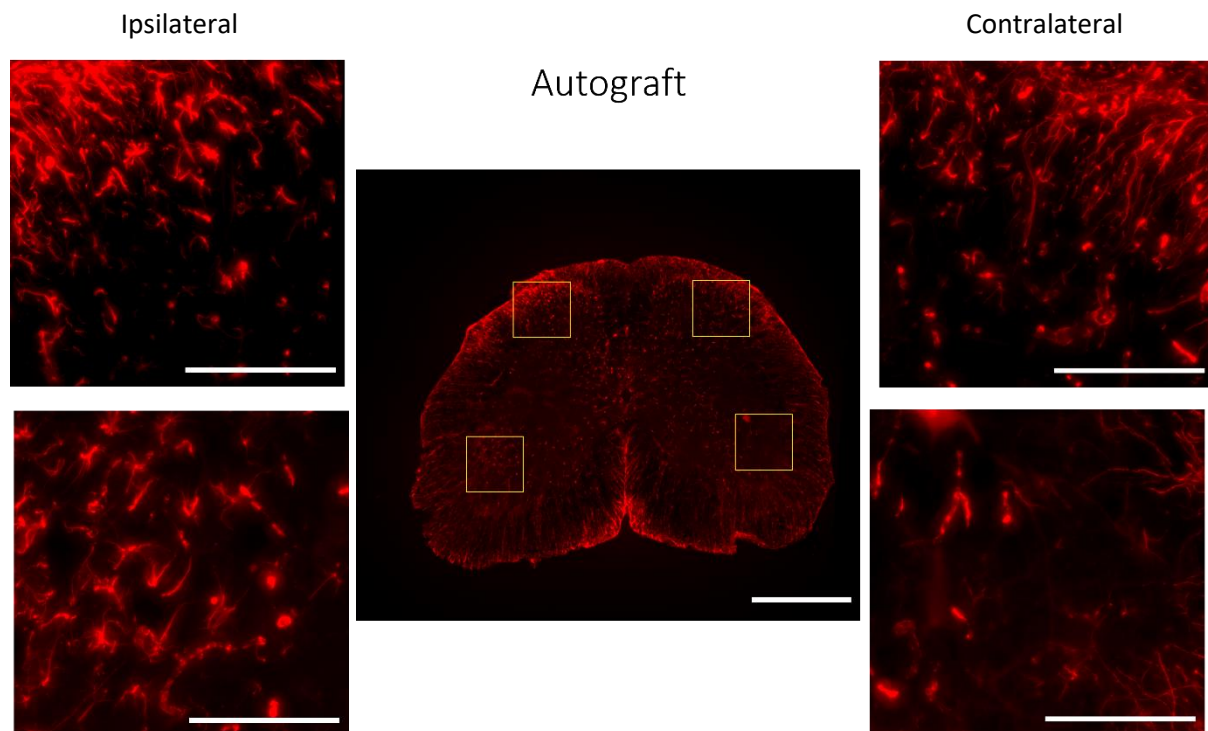


Figure 4.13 Spinal cord sections following nerve repair with a nerve graft. High magnification images are highlighted in the cord section at the ipsilateral and contralateral side of the spinal cord in reference to the injury side. Scale bar 100µm (in 40x images) and 500µm in whole spinal cord view.

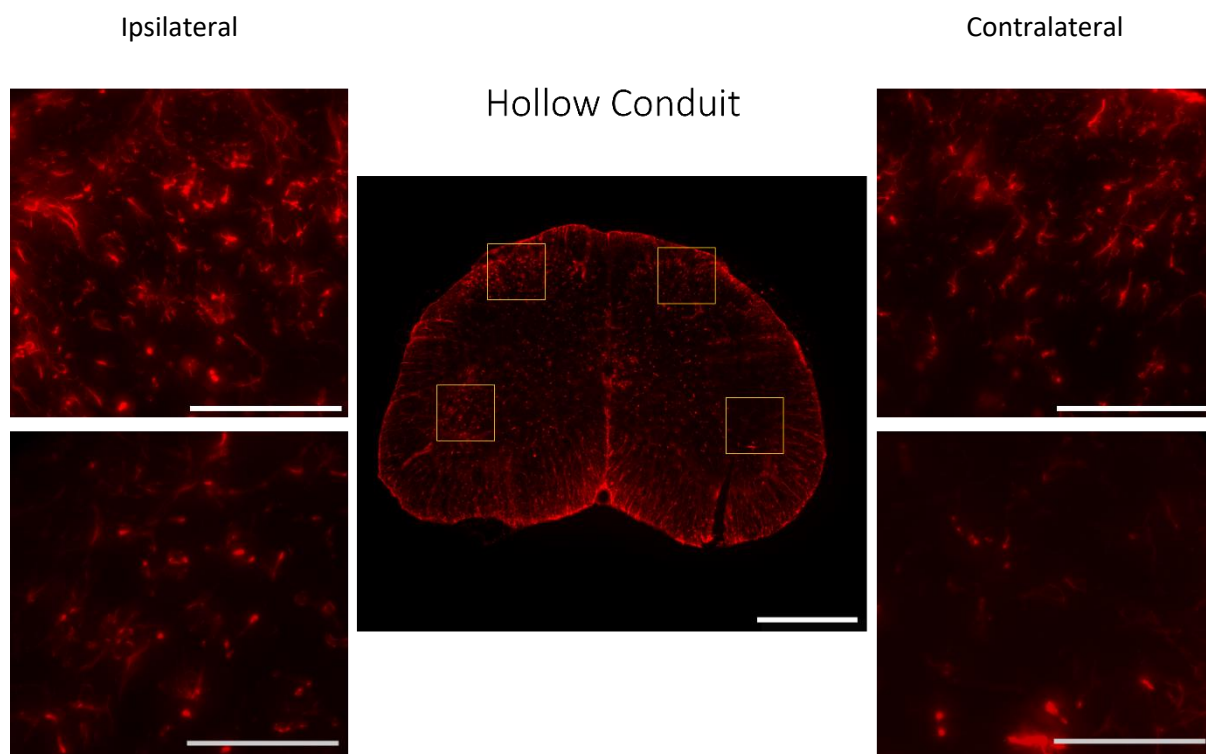


Figure 4.14 Spinal cord sections following nerve repair with a hollow conduit. High magnification images are highlighted in the cord section at the ipsilateral and contralateral side of the spinal cord in reference to the injury side. Scale bar 100 μ m (in 40x images) and 500 μ m in whole spinal cord view.

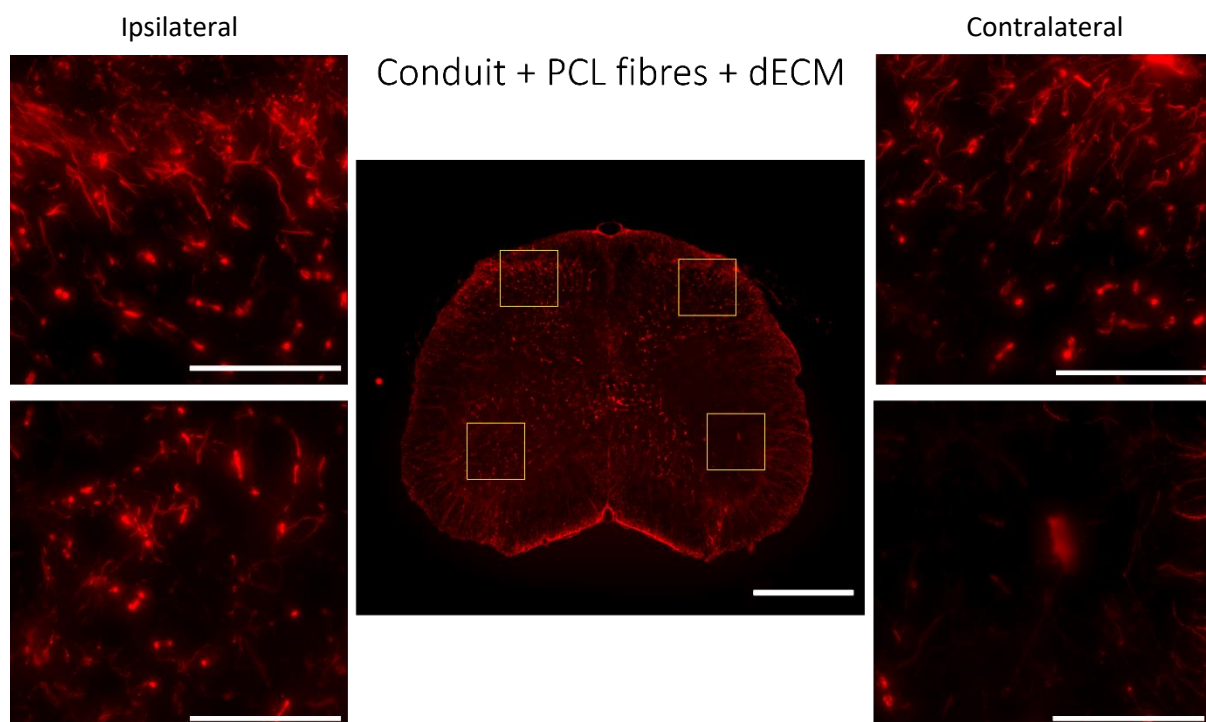


Figure 4.15 Spinal cord sections following nerve repair with a conduit with PCL fibres coated in dECM. High magnification images are highlighted in the cord section at the ipsilateral and contralateral side of the spinal cord in reference to the injury side. Scale bar 100 μ m (in 40x images) and 500 μ m in whole spinal cord view.

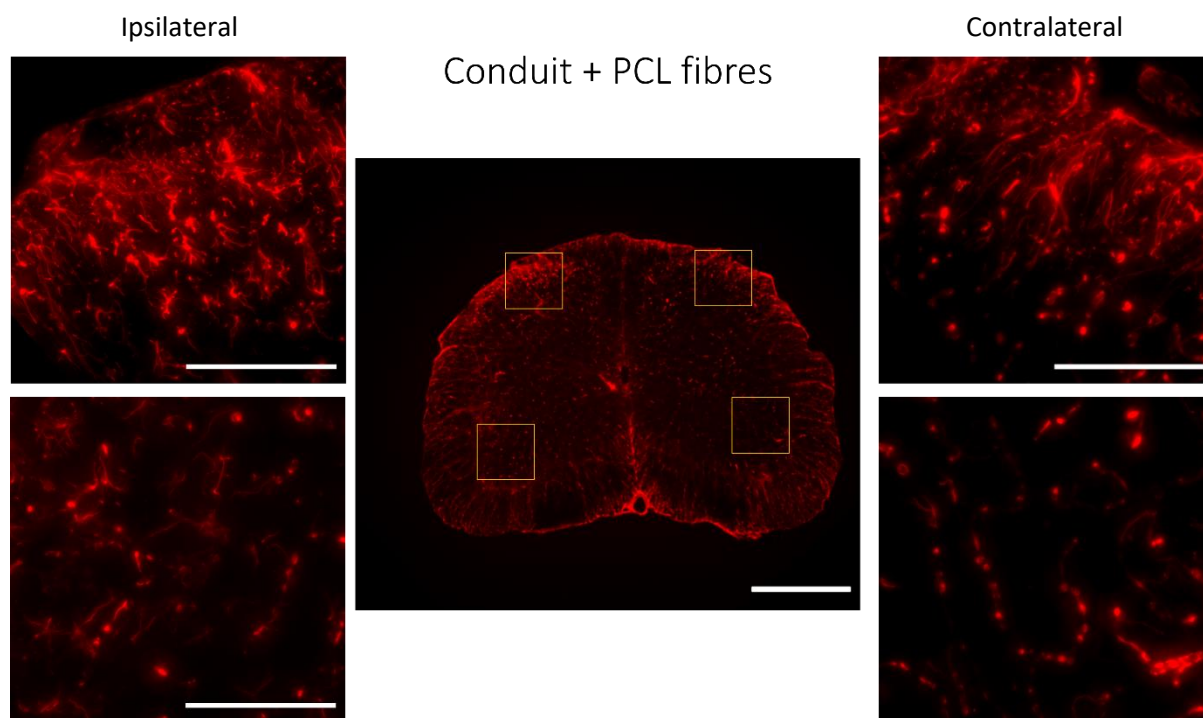


Figure 4.16 Spinal cord sections following nerve repair with conduit + PCL fibres. High magnification images are highlighted in the cord section at the ipsilateral and contralateral side of the spinal cord in reference to the injury side. Scale bar 100 μ m (in 40x images) and 500 μ m in whole spinal cord view.

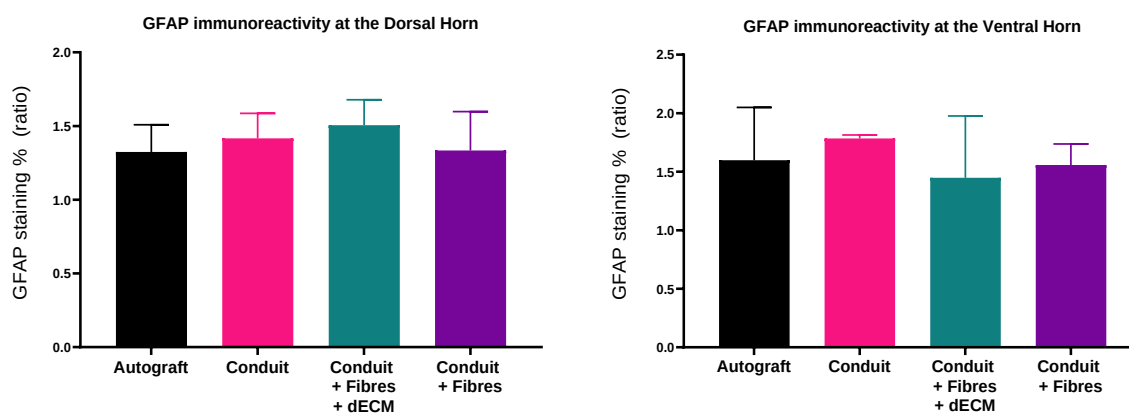


Figure 4.17 Quantification of GFAP staining in the ipsilateral spinal cord (as a ratio of the contralateral uninjured side) at the dorsal horn level and ventral horns following nerve injury and repair with an autograft, conduit, conduit+PCL fibres coated with dECM, conduit + PCL fibres.

4.4 Discussion

4.4.1 Nerve regeneration

Nerve injury results in the degeneration of axons and loss of innervation of original targets which can lead to motor and sensory deficits. Therefore, successful nerve regeneration is dependent on the re-establishment and re-innervation of original targets in order to restore nerve function. The gold standard for treating nerve injuries is the use of an autograft, however, nerve guide conduits are now showing significant potential as a suitable alternative for supporting nerve regeneration.

In this chapter, three experimental nerve guide conduits were investigated for the potential of treating nerve injuries and compared to a nerve graft, which served as a control as this is the gold-standard used clinically. Specifically, a 3 mm nerve injury was created in the common fibular nerve of YFP mice and subsequently repaired using either a nerve graft, or with three experimental nerve guide conduits: a hollow PCL conduit, a conduit with intraluminal fibres and a conduit with intraluminal fibres coated with decellularised extracellular matrix (dECM).

As nerve injury results in the degeneration of axons in the distal stump, the presence of axons in the distal stump can be used as a measure of regeneration and can be reported as the percentage of the axons that regenerate and travel successfully from prior to the injury site (which would reflect the native nerve), across the injury gap, and into the distal stump. This is advantageous when evaluating different repair therapies, as the percentage of axons in the distal stump can be compared between groups to identify conduits that show improved potential for supporting nerve regeneration.

In this chapter, in animals repaired with a nerve graft, an average of $58.9 \pm 21.5\%$ of axons regenerated from the proximal site, across the graft and into the distal stump. In comparison, hollow nerve guide conduits supported the regeneration of $42.8 \pm 31.5\%$ of axons, conduits with PCL fibres supported $43.10 \pm 24\%$ of axons and conduits with PCL fibres coated with decellularised ECM supported the regeneration of $44.8 \pm 23.4\%$ fibres from the proximal stump to the distal stump.

Whilst the nerve graft repairs showed the highest percentage of axons growing across the injury site, which was expected as this is the gold-standard repair therapy, there was no significant difference in between any of the repair groups, suggesting an equal ability in the experimental conduits to support regeneration on par with the nerve graft repair.

These findings support the notion that nerve guide conduits can serve as an appropriate and suitable alternative to nerve autografting, particularly considering the limitations surrounding donor-site morbidity of harvesting suitable nerves. Overall, the results in this chapter add to the constantly-

growing field of nerve guide conduits, therefore the results in this chapter need to be discussed within the context of the field.

4.4.1.1 The need for novel nerve guide conduits

Following nerve injury and repair with hollow conduits, the formation of a fibrin cable within the first week of repair is critical in supporting the elongation of axons and Schwann cells across the conduit to the distal stump (Daly et al., 2012; Williams & Varon, 1985). The fibrin cable is a result of the accumulation of trophic factors and ECM components being synthesised by resident cells following injury, which is thought to degrade within 2 weeks in rodents and therefore if axonal elongation and cellular migration is not complete between the proximal and distal stump by this time, nerve regeneration fails (Williams et al., 1987). It is suggested also that the fibrin cable can influence Schwann cells by inducing and maintaining Schwann cells in a non-myelinating phenotype which in turn results in longer lasting support for axons following injury (Akassoglou et al., 2002). Nevertheless, maintaining the non-myelinating, proliferative status of Schwann cells is also desired in order to sustain the formation of bands of Bungers that support and direct axonal growth, and a strategy to mimic this is using intraluminal fibres.

Various studies have shown a positive effect of intraluminal fibres during nerve regeneration using a range of materials, diameters and packing densities. For instance, Ngo et al (2003) used poly-L-lactide (PLLA) microfibrils of 40 - 100 μm in diameter inserted at different densities (3.75, 7.5, 15 and 30%) in a nerve guide conduit to bridge a 1 cm nerve gap in the rat sciatic nerve and showed improved regeneration in comparison to a hollow conduit. The authors reported that repairs using conduits with less fibres (3.75% packing density) showed an increased number of axons at the distal stumps compared to repairs with the higher density groups (30% packing density). Koh et al. (2010) evaluated polylactic-co-glycolic acid (PLGA) fibre bundles of 25 μm in diameter where each bundle was composed of smaller tightly packed fibres in the range of 200 – 600nm. The bundles were packed at a 10 % packing density into conduits and showed the conduits were able to support regeneration in a rat model of nerve injury to a similar level as that obtained by a hollow conduit and an autograft repair (Koh et al., 2010).

In this chapter, PCL fibres of 6 - 8 μm in diameter were inserted into PCL-MA conduits with a packing density of 6%, based on previous studies from our group demonstrating the suitability of this range in a number of separate studies. Specifically, Daud et al. (2012) cultured DRG explants on PCL fibres of varying ranges (1 μm , 5 μm and 8 μm) and showed all fibre diameters supported DRG neurite outgrowth length equally. Similarly, Behbehani (2018) demonstrated that PCL fibres packed at a low packing density of 10% in nerve guide conduits supported increased DRG outgrowth when compared

to DRG seeded on a high packing density of 40% in an *in vitro* model. Importantly, Field (2018, thesis, unpublished) built on this work by evaluating the same nerve guide conduits with a 10% PCL fibre packing density *in vivo* and demonstrated the fibre-filled conduits were able to support nerve regeneration.

Despite the similarity in the fibre packing density between the study in this chapter and the aforementioned studies, it is worthy to highlight that the packing density may not necessarily reflect how fibres are distributed within conduit, and this can have an influence on regeneration.

For instance, the Ngo et al (2003) study and the Koh et al (2010) study both used larger fibres, which resulted in fibres being more evenly spread along the cross section of the conduits and meaning that larger gaps are present between each fibre. In contrast, in our study we used electrospun fibres in the form of sheets, which may have not evenly distributed across the cross-section of the conduit lumen, and rather may have concentrated together at parts resulting in more densely-packed fibres bundles.

Densely-packed fibre sheets were studied by Kim et al, who electrospun poly(acrylonitrile-co-methylacrylate) (PAN-MA) to create thin sheets (<10 µm) of either aligned fibres or randomly-orientated fibres (400-600 nm), which were then inserted as into the lumen of a conduit (Kim et al., 2008). Their study compared the effect of the alignment vs random intraluminal fibres on the repair of a nerve gap injury in a rat model and found improved regeneration with the aligned surface morphology. The authors reported that, given the surface area was maintained by using fibre sheets of the same material and thickness and the packing density was calculated as 0.6% in both cases, then the enhanced regeneration was due to the improved guidance, extension, and migration of axons and Schwann cells on the aligned fibre surface compared to that of the randomly orientated fibres, suggesting a positive effect of longitudinally aligned fibres in nerve guide conduits. Indeed, subsequent studies from the same group then evaluated the effect of the number and configuration of thin sheets of electrospun fibres in conduits and showed that one sheet of electrospun fibres showed the best results in comparison to 3- sheets placed in the conduit. By changing the number of sheets in the conduits, the fibre packing was minimally affected, only increasing from 0.6 % to 1.8 %, which is still a lower packing density than that reported in literature (Clements et al., 2009). However, by adding more sheets, the conduit lumen became compartmentalised, where one sheet in the middle of the conduit resulted in two compartments – resembling two semicircles, and the use of 3 sheets in a “Z” configuration resulted in four compartments. The authors attributed the failure of the 3 sheets to the fact that the configuration of the sheets in the “Z” formation resulted in decreased open space for regenerating axons as well as asymmetric regeneration and migration of support cells, particularly where cells from the proximal and distal site were found in different “areas” or “compartments”

during the early stages of regeneration suggesting a potential mismatch. Therefore, this study highlights that despite the low packing density of a conduit, the arrangement of fibres within the conduit is also a critical aspect relevant for regeneration.

Therefore, it is possible that the assembly of PCL fibres in the conduits used in this chapter resemble the ones described by Clements et al., meaning that some regenerating axons were limited and restricted by the lack of space in the more densely packed sheet as opposed to a conduit with evenly spread fibres. However, as we were not able to view or image individual axons along the fibres bundles, rather only at the distal stump, it is difficult to estimate whether the majority of the regenerating axons were growing in contact with the fibres or alternatively in between the gaps of the fibres. Therefore, future studies may benefit from looking at cross sections along the fibres to further the understanding of regeneration axons along intraluminal fibres.

4.4.1.2 The case for ECM components

Indeed, the potential of intraluminal fibres in conduits is a highly reported strategy used to provide additional support during regeneration, with considerations regarding diameter, packing density and configuration mentioned above. However, while fibres can provide a structural and physical platform for cell migration, synthetic fibre such as PCL lack the inherent biological cues that are critical for regeneration, where the fibrin cable is an evident example, as well as other signalling cues from ECM components involved in the basal lamina of Schwann cells that are able to drive and induce cell signalling to support nerve regeneration. For this reason, in this chapter we evaluated PCL fibres coated with extracellular matrix.

A number of studies have shown the potential of using individual ECM coatings on intraluminal fibres in nerve guide conduits, such as early studies by Matsumoto et al (2000) who evaluated laminin-coated collagen fibres in a critical 80 mm common fibular gap in a canine model showing regeneration was supported, to recent clinical trials using collagen-fibre filled nerve guide conduits in the repair of 30 mm medial nerves (Saeki et al, 2018). Therefore, while the use of ECM components in nerve guide conduits is an upcoming and suitable therapy, these and other similar nerve guide conduits are still not able to provide a level of regeneration on par with that obtained by using the gold-standard autograft repair.

As explained in Chapter 3, studies suggest that nerve guide conduits lack the inherent cues in a nerve autograft that can support nerve regeneration, where a critical feature is the presence of native, nerve-specific ECM and associated signalling cues. For this reason, in this thesis we hypothesised that replicating a nerve-specific ECM within a nerve guide conduit can serve as an alternative method to improve the function and efficacy of conduits in supporting nerve regeneration. In Chapter 3 we

identified dorsal root ganglia as a source nerve-specific extracellular matrix, due to the relevance of DRG in modelling peripheral nerves *in vitro*, and the capability of DRG in recapitulating relevant molecular and cellular events concurrent with peripheral nerve regeneration. Indeed, our *in vitro* studies in Chapter 3 demonstrated that DRG-derived ECM was capable of supporting increased nerve adhesion and the formation of neurites from a neuronal cell line, a highly promising finding that warranted further investigation. In this chapter, we were able to evaluate the potential of DRG-derived ECM *in vivo* and we have shown similar regeneration outcomes compared to the level of regeneration obtained with a nerve graft.

4.4.1.3 Limitations

It is important to mention a number of limitations in our results. Firstly, at the end of the recovery period it was noted that regeneration had not occurred in a number of animals, partly due to the formation of a neuroma at the site connecting the conduit with the distal stump. Visual analysis of the distance between the ends of the proximal and distal stump and the conduit suggest that the distal ends of the nerve had slipped out of the conduit, resulting in regenerating nerves forming a neuroma. The reason for this is likely due to the fact that the fibrin glue may have not been optimal in securing the conduits in place, and due to the natural movement of the leg and extension of the nerve, the distal site became dislodged. Indeed, in one of the repairs the whole conduit had become dislodged. In the conduits tested in this study, suturing was not an option due to the mechanical properties of the conduit's material. Secondly, in another failure case, a fibrin clot was found inside the conduit obstructing the path for regenerating axons to enter the conduit. It was noted that during the surgery, some bleeding had occurred in this specific animal and whilst the bleeding was deemed manageable at the time, it is possible that the wound re-opened and the excess bleeding may have contributed to the large fibrin clot. It is worthy to mention that future studies should attempt at optimising the fibrin glue or sourcing a commercially available alternative.

Therefore, due to the failure cases, the number of available regenerated nerves for analysis was reduced, and sample size can affect the statistical power of studies. Specifically, our power calculations were based on N=5 animals to detect differences of 19.5% with a statistically appropriate power of 80%. However, at N=3 sample size, the detectable differences would need to be 26%. In addition, there was a large variability in the analysed samples (as evident by the large error bars), where increased sample sizes could counter the effects of the large errors.

On another note, it is important to discuss the limitations of the type of analysis used as a measure of regeneration. The mice used in this study were Thy-1-YFP animals, which are a transgenic mouse line that express a yellow fluorescent protein in a subset of axons in the peripheral nerves along the whole

length of the axon. This pattern of expression is advantageous as the density of axons visible by fluorescent imaging is reduced in a manner that allows individual axons to be distinguished from one another. This feature can be advantageous as it allows for accurate analysis of the regeneration process by visualising individual axons extending across the injury site back towards the targets.

In this study, regenerated nerves were imaged throughout the whole thickness and length of the nerve using z-stacking. Then, at predetermined intervals along the length of the regenerated nerve, axons were counted at each slice of the z-stack, which allowed for a reliable axon count compared to imaging a maximum intensity projection image where overlapping axons could be missed. Nevertheless, it is important to clarify that axonal counting at these intervals may have been confounded by the presence of axonal branching emerging from regenerated nerves, which may lead to an over-estimation of axonal count, as such branching sprouts may have originated from a smaller number of unique axons. Ideally, axons should be traced throughout the whole length from prior to the injury site (i.e. in the proximal stump), along the graft and conduit until the distal stump, as this would then allow the identification of unique axons for quantification.

Indeed, previous studies have suggested that individual axon tracing can align closely with electrophysiological data, which is a common method of assessing functional nerve recovery by measuring the conduction ability of regenerated axons in a nerve. Specifically, in a study from our group, compound action potentials (CAP) were measured at a proximal and distal site of a regenerated nerve repaired with a conduit, and the CAP ratio of the distal to proximal site (25.3%) closely correlated with the percentage of unique axons found in the distal site compared to the proximal site (27.1%)(E. Albadawi, 2018, thesis, unpublished). Therefore, it is possible that axon tracing can be used considered as a pseudo-measure of functional regeneration based on the similarity to electrophysiological data. Therefore, future studies should focus on using axon tracing of unique axons over axonal counting.

4.4.1.4 Estimation of unique axons

Unique axon tracing was not possible in the repair groups of conduits with PCL fibres due to the combination of the fibre thickness and autofluorescence of the material presenting an obstruction during imaging, which prevented the visualisation of individual regenerated axons. Whilst axons could be counted at the distal stump, it is not possible to say how many of these axons are unique. However, an estimate can be made by looking at the relationship between the number of unique axons at the distal stump and the total number of axons at the distal stump (as a result of sprouting) reported in previous studies using the same 3 mm nerve gap model in the common fibular of Thy-1-YFP mice from our group.

Harding et al. (2014) traced unique individual axons following a nerve injury and 3 mm graft repair of Thy-1-YFP mice and showed that approximately 24% of unique axons from the proximal stump were represented in the distal stump, suggesting at least 24% of axons regenerated across the injury site and reached the distal stump. In comparison, when all the axons and sprouted branches were counted at the most distal interval (4.0 mm) and normalised to the proximal stump, the value of axon and branching count (or so-called sprouting index) was 77% at this distal interval, representing an increase by a factor of 3.2 (77 divided by 24). This suggests that each axon originating from the proximal stump therefore sprouted into at least three branches by the time it reached the distal stump, which is in accordance with the literature where it's reported that axons branch more than once during the regenerative process (Navarro, 2016).

Similar findings have been observed by Pateman et al. in the same model with a graft repair, who counted approximately 65% of total axons and branches that were found in the distal stump compared to the proximal stump, whilst unique axon tracing was reported as 20% of axons in the distal stump, again suggesting each unique axon at the proximal stump branched three times (factor of 3.25) by the time it travelled through the graft and reached the distal stump.

Therefore, as the current study in this chapter used the same common fibular nerve gap model as the Harding et al. (2014) and Pateman et al (2015) studies, it is likely that the sprouting relationship with unique axons is maintained, where each axon sprouts three times. However, it is important to compare the axons at the same interval; this study following nerve repair with a graft at the same interval (4.0 mm) we found a sprouting index of 70%, which may reflect an actual unique number of axons of 23% (70 divided by 3). This value is indeed close to those reported by the aforementioned studies with nerve graft repairs.

In order to estimate, using this relationship, the unique axons in the repairs with nerve guide conduits, two considerations need to be taken.

Firstly, the repair length distances were slightly longer in conduit repairs compared to grafts. This may be due to the fact that while the nerve gap distance was cut to 3 mm between the proximal and the distal stump, the stumps may have retracted due to not being held securely enough by the glue. Indeed, the average distance between stumps in the graft repairs was 3 mm, as expected as this is the length of the graft, but in the conduit repairs the distance was 4 mm, which was the length of the conduit, suggesting retraction of the stumps to the very end of the conduit. This means that in order to maintain the same starting point, the 0 mm mark at the proximal stump was selected as the point where axons entered the graft or conduit, and therefore the distal end is different for graft repairs vs conduit repairs.

Secondly, the relationship between the total number of axons and unique axons may be different in nerve guide conduits compared to repairs with nerve grafts, however previous studies from our group suggest that this number is still close to 3, suggesting 3 branches per unique axon. For example, Harding (2014) counted 67.80% of sprouted axons from the proximal stump reached the distal stump following nerve repair with a conduit, of which 21% were considered unique axons (a factor difference of 3.2). In the current study, we found counted 42.80% of axons from the proximal stump reached the distal stump, which may reflect an estimated unique axon count of 13%.

Furthermore, Harding (2014) also reported on some preliminary nerve guide conduits containing fibres, where the relationship factor was found to be similar, albeit slightly smaller at 2.7 (based on 11.0 % of sprouted axons from the proximal nerve were counted at 4.0mm interval of which 4 % were considered unique). This was based on axons counted at a 4.0 mm interval (which is equivalent to 1.0mm into the distal stump).

In the current study we were able to count the number of axons up until a 5.0- and 6.0-mm interval (1.0 and 2.0 mm after the nerves exit the conduit and enter the distal stump), which using the relationship between axons sprouting and unique axons, this may reflect an actual number of 13% of unique axons (43% divided by 3.2). This estimated value of unique axons for our study is significantly larger than the unique axons reported by Harding (4%), and indeed visual comparison of the images also suggests improved regeneration with the conduits used in this study. However, the conduits in the Harding study were preliminary and the material (PEG) may have not been optimal for the conduit.

Another study from our lab more recently reported on fibre-filled nerve guide conduits which were more optimised. Specifically, Field (2019) also evaluated PCL fibre filled conduits and showed that the number of axons declined rapidly from the proximal site as axons entered the conduits with fibres, where approximately 20% of axons and sprouts could be counted by the time axons reached the most distal interval (6.0 mm, equivalent to 1mm after axons entered the distal stump). In contrast, in this study we counted twice as many axons and sprouts at the distal 5.0 mm and 6.0 mm interval, equivalent to axons entering 1 mm and 2 mm in the distal stump (43%). Applying the above relationship to both of these studies, the unique axons would be equivalent to 6.25% for the Field study (20 divided by 3) and 13.4% (43 divided by 3), suggesting improved regeneration of axons in the conduits used in this study.

A reason for the potential improved repair with fibre-filled conduits in our study in comparison to previous studies in our group might be in the difference between the packing density of the fibres, where Field (2019) used a 10% packing density while in this study we used a reduced fibre packing

density of 6%. This warrants that future studies evaluate different configurations and percentages of fibre packing densities in nerve guide conduits to optimise the regenerative capacity.

Finally, it is worthy to highlight visual differences of images reported in between studies. A notable difference is found in the current study between the fibre-filled conduits with dECM (Figure 4.8) in comparison to the repairs with the conduit fibres without the coating (Figure 4.9). Specifically, axons are running more parallel to each other in the distal stump in the PCL fibres + dECM, and this may indeed suggest improved regeneration.

It is likely that the small sample size in our study has limited our comparisons between groups statistically, and due to time constraints as a result of Covid-19 we were not able to include more animals in the current study, however, visual differences between the repair types suggest that the PCL + dECM conduit holds potential for improving nerve repair and future studies should investigate this relationship further.

4.4.2 Potential for neuropathic pain development

As mentioned, peripheral nerve injury can result in the development of neuropathic pain, where a variety of mechanisms have been described, particularly surrounding peripheral nerve sensitisation and hyperexcitability as a result of the re-organisation of ion channels on injured axons.

In addition, neuromas have also been implicated in the development of pain, which can be formed by excessive axonal sprouting. Indeed, following nerve injury and repair, axonal sprouting is particularly evident at two sites in the regeneration pathway, when axons exit the proximal stump to enter the graft/conduits and when axons exist the graft/conduit to enter the distal stump. Therefore, increased sprouting can be considered as a way to increase the chances of an axon entering the graft/conduit/distal stump to regenerate towards the original target and studies show that axons that don't reach their original targets are lost (Navarro, 2016). Therefore, axonal sprouting is a natural response to nerve injury that may seem beneficial. For instance, in patients with ALS (amyotrophic lateral sclerosis) the neuromuscular junctions are pathologically de-innervated resulting in motor neurone death, and increased axonal sprouting as a compensatory mechanism to the loss of motor neurones at the neuromuscular junctions is suggested to ameliorate the progression of the disease (Marshall & Farah, 2021).

However, away from the motor end plates, in the peripheral nerves along the body, axonal sprouting can lead to the formation of neuromas, and neuromas have been known to be involved in neuropathic pain pathways, such as being a potential site for ectopic firing of axons which can lead to symptoms of pain (Lane et al., 1978).

Furthermore, it is known that following nerve injury, there is a potential for the development of pain even following regeneration due to incorrect re-innervation of targets. Therefore, in order to fully evaluate repair therapies such as those evaluating novel and experimental conduits, it is important to assess the possibility for the development of neuropathic pain. Glial activation is known to influence the development of neuropathic pain, therefore, assessing glial activation following nerve repair can be used to evaluate the efficacy of nerve repair therapies and the potential for the development of neuropathic pain.

4.4.2.1 *Glial activation*

As mentioned, peripheral nerve injury can induce the activation of glial cells, such as microglia and astrocytes, in the spinal cord, where glial activation has also been linked with a number of neuropathic pain conditions, such as mechanical allodynia and with the maintenance of neuropathic pain states (Ji et al., 2019; Romero-Sandoval et al., 2008).

Astrocyte activation is characterised by proliferation and changes in cellular morphology including an increase in size and extension of thicker and longer processes (Garrison et al., 1991). These changes can be visualised and reflected by increased labelling of GFAP, a protein involved in the cytoskeletal remodelling of hypertrophic astrocyte cells in the spinal cord. Therefore, activated astrocytes can be quantified by measuring GFAP-labelled cells in the spinal cord following nerve injury.

In this chapter, spinal astrocyte activation was quantified following injury and repair of the common fibular nerve with a nerve graft or three experimental nerve guide conduits. There was no significant difference in the level of astrocyte activation between the groups, suggesting that the experimental conduits did not exacerbate astrocyte activation following injury, and therefore do not increase the potential for development of neuropathic pain.

4.5 Conclusions

In this chapter, a novel nerve guide conduit made containing intraluminal fibres coated with DRG-derived decellularised ECM was evaluated in a model of nerve repair in the common fibular of Thy-1-YFP mouse and compared to repair with a hollow conduit, a conduit with PCL fibres or a nerve autograft. Regeneration was evaluated by axon analysis along the length of regenerated nerves, showing regeneration was supported in all groups to a similar level, with no significant differences between groups. Therefore, this suggests the use of DRG-derived ECM to model a nerve-specific ECM can be used to nerve support regeneration. Furthermore, the potential for the development of pain as a result of nerve injury was evaluated by monitoring glial activation in the spinal cord, where

no significant differences were observed as a result of the repair type suggesting the novel conduit does not exacerbate glial activation.

Chapter 5. Conclusions and Future Work

Peripheral nerve injuries are a worldwide problem that can lead to significant, long-term disabilities, as well as a reduced quality of life. Current treatments for peripheral nerve injuries are associated with a number of limitations, particularly related to the size of the defects. End-to-end suturing is only suitable for tension-free defects, otherwise, the gold standard intervention of an autograft can be used, but donor site morbidity associated with the sacrifice of the healthy nerve is a clear limitation. Decellularised allografts have shown potential in the repair of various sized nerve defects but have not demonstrated clinical superiority to the autograft, are not always suitable for all patients and can be costly which limits their widespread use.

Nerve guide conduits (NGCs) offer an alternative solution to the treatment of nerve repairs, but they are limited in the repair of larger defects due to the failure of maintaining a favourable environment for regeneration. A number of promising strategies have been developed to improve the function of nerve guide conduits, where the use of extracellular matrix (ECM) components seems to hold potential, particularly in mimicking a pro-regenerative environment within conduits. However, individual ECM components in NGCs inherently fail to recreate the native ECM environment and composition seen in peripheral nerves. Therefore, this represents a gap in the literature and inspires the development of novel nerve guide conduits that can replicate and mimic a native-like, nerve-specific ECM environment.

In this thesis, we hypothesised that dorsal root ganglia can be used to model the peripheral nerve environment to produce a nerve-specific ECM, which in turn may offer a more optimal environment for nerve regeneration.

In order to address this hypothesis, DRG were isolated from embryonic chick and cultured *in vitro*, where immunocytochemical methods identified the presence of DRG-derived neuronal and glial cells as well as the secreted extracellular matrix, which was positive for collagen IV and laminin, two ECM components with critical roles during regeneration. Therefore, this validated the use of DRG as a model to obtain nerve-specific ECM. Decellularisation of the DRG cultures revealed the ECM components could be successfully maintained, and evaluation of the DRG-derived ECM with a neuronal cell line demonstrated positive effects on nerve regeneration, chief being increased cell adherence and neurite formation of cells on the DRG-derived ECM, features that can suggest improved regeneration.

The *in vitro* results provided promising evidence for the potential of DRG-derived ECM as a nerve-specific environment to enhance nerve regeneration, which we then aimed to investigate further in

an *in vivo* model. Specifically, we aimed to develop a novel nerve guide conduit that would contain the DRG-derived ECM, where the PCL fibres as an intraluminal scaffold in the lumen of the conduit could be used to deposit the ECM onto, and therefore provide it as a substrate for regenerating axons.

The nerve guide conduit was fabricated from methacrylated polycaprolactone (PCL), a suitable material that we evaluated to have appropriate mechanical properties based on the Young's modulus of the material, as well as cytocompatibility *in vitro* with a neuronal cell line (Chapter 2). The PCL fibres were fabricated by electrospinning, resulting in appropriately sized fibres in the 6 – 8 μm range as previously optimised by our previous studies. The PCL fibres were used as a scaffold for the culture and deposition of DRG-derived ECM *in vitro* for a period of 2 weeks, which following decellularisation were found to maintain relevant markers of the native nerve ECM, namely collagen IV and laminin.

Finally, in Chapter 4, the novel conduit containing DRG-derived ECM-coated fibres was evaluated in a common fibular nerve gap model of nerve injury and repair in Thy-1-YFP mice and compared to a number of controls, including a hollow conduit, a conduit with PCL fibres without the ECM coating and a nerve graft, which is equivalent to the clinical gold-standard of autografting. Regeneration was evaluated by counting the number of axons that regenerated, showing regeneration was supported in all groups to a similar level, with no statistically significant differences between groups. However, it is likely that the small sample size in our limited the statistical comparisons, as qualitative examination of the regenerated nerves suggested that the outgrowth of regenerated axons was improved in repairs using the PCL fibres with dECM conduit in comparison to the conduits with PCL fibres without the coating, particularly due to the fact that axons in the distal stump appeared in a more aligned and directed fashion, suggesting a greater degree of organization and guidance provided by the dECM. It is also possible that the 3-week recovery period was too long to appreciate differences between the repair types, partly due to the high regenerative capacity of mice. Therefore, future studies may also benefit from evaluated shorter time periods. Furthermore, due to potential for the development of neuropathic pain as a result of nerve injury, animals were evaluated by monitoring glial activation in the spinal cord, where no significant differences were observed as a result of the repair type suggesting the novel conduit does not exacerbate glial activation, providing some safety information on the novel conduit.

Overall, this thesis was able to report on the novel use of DRG-derived ECM as a substrate for nerve regeneration showing the potential for modelling nerve-specific ECM in nerve guide conduits to improve regeneration.

Whilst each chapter has identified certain limitations and potential areas for improvement in specific studies, it is also worth discussing possible future avenues for this research.

5.1.1 Future work

Despite optimisation in order to maximise the outgrowth of DRG-derived ECM, particularly by evaluating factors such as the embryonic developmental age of the chick as well as the spinal level at which DRG were dissected, DRG outgrowth was found to be highly variable on TCP. In contrast, PCL fibres supported the growth of DRG explants in a more consistent manner and showed long and extensive DRG axons and Schwann cell migration. Indeed, the immunocytochemical studies also revealed more evident staining of ECM components on PCL fibres compared to TCP. However, the extent of deposition of ECM component was not quantified in the studies which warrants further investigation. In particular, future studies may benefit from evaluating the molecular characterisation of the DRG-derived ECM, and investigate whether any differences between ganglia from different embryonic development days or spinal levels produce a distinct molecular profile that may result in improved ECMs.

For example, studies have shown different cellular events occur in the ganglia of chick embryos during development, such as the peaks in proliferation of cell death of Schwann cells (Ciutat et al., 1996). Molecular characterisation may help standardise the selection of DRG and identify more optimal DRG that may provide an increased number of viable cells in order to maximise the deposition of DRG-ECM.

In relation to cell viability, a large number of studies report on using neurotrophic factors during the culture of embryonic neuronal cells, and indeed previous studies from our own group have also identified optimal growth medium supplementations that can maximise the outgrowth of chick DRG neurites in culture (Sandoval-Castellanos et al., 2020). The success in using growth factors in media is based on recapitulating the neurotrophic environment during development that stimulates neurogenesis, as well as the maturation of neurones and related glia. As a result of harvesting DRG, this neurotrophic environment is disturbed which can cause cell death, and furthermore, studies suggest that neuronal death is significantly increased when axons are injured more proximally to the cell bodies (Ygge, 1989), therefore these two factors suggest that DRG neuronal survival can be quite low in culture. Future studies may benefit from harvesting DRG more distally i.e. by leaving a small section of the peripheral spinal nerves attached or using supplemented-media.

Furthermore, molecular characterisation can be used to identify DRG that express a larger number of regeneration-associated genes, which may result in the deposition of a more relevant, pro-regenerative ECM. And considering that harvesting of a DRG explant clearly mimics an injury to induce this upregulation, there may also be subsequent methods to enhance this molecular response further with whole DRG explants. For instance, dissociation of DRG neurones similarly induces the upregulation of regeneration associated genes (Zou et al., 2009), however certain studies report on

“injuring” the axons of cultured dissociated DRG neurones (Shin & Cho, 2020) to induce a further “second wave” of upregulation of regeneration associated events in culture. This concept could also be applied to whole explants to induce a similar wave of upregulation of regeneration associated genes and evaluate the effect of these on the subsequent secreted ECM (Figure 5.1).

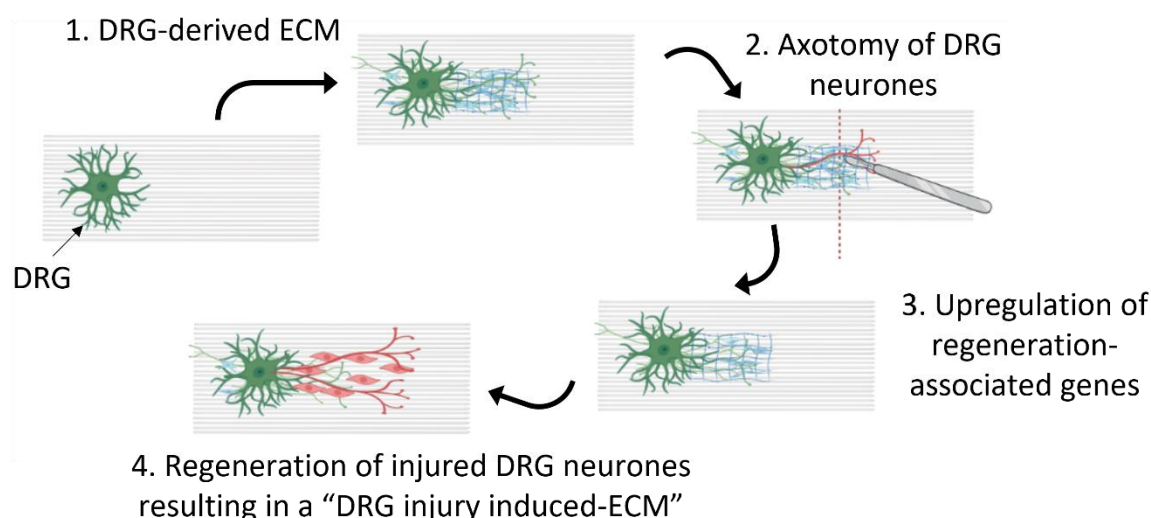


Figure 5.1 Schematic of a potential model to induce further upregulation of regeneration associated genes in DRG cultures. DRG explants can be cultured as described in this thesis on PCL fibres, after which the axons of the DRG neurones can be axotomized/cut with a blade to induce an injury, resulting in an upregulation of regeneration-associated genes in culture in the remaining DRG cells, which may allow subsequent regeneration with marked axonal growth as a result of the upregulated genes, resulting in the deposition of a modified ECM. Image made with Biorender.com

5.1.1.1 Alternative methods to model nerve specific ECM *in vitro*

Whilst we have shown that DRG can be used to deposit nerve-specific ECM, it is worthy to remember that DRG are the location of cell nuclei of sensory neurones, which ultimately suggests that the DRG derived ECM may be a sensory-specific ECM. And indeed, while the DRG-derived ECM was able to support nerve regeneration in the *in vivo* study for the repair of the common fibular nerve, a mixed nerve with both motor and sensory functions, it begs the question on whether the effects on nerve regeneration would have been more apparent using a purely sensory nerve as the injury gap model to evaluate DRG-derived ECM.

In relation to this, it would be interesting to evaluate the development of a nerve specific ECM from a motor neurone population with a motor nerve-specificity. And indeed, studies could compare the effect of motor-neurone derived ECM with DRG-derived ECM and evaluate the differential responses for nerve regeneration, both *in vitro* and *in vivo*. For example, the two different dECM surfaces could be evaluated in a modified Campenot-based compartmentalised chamber (Figure 5.2). This initial

chamber system was developed by Robert Campenot in the late 70s providing a physical separation between the location of cell bodies and axons, allowing axons to grow on separate compartments allowing the study of various manipulations (typically pharmacological) of the compartments on axons (Campenot, 1977). Since, many modified chambers have been developed, therefore it may be worth investigating the use of such chambers for evaluating dECM surfaces, too. Furthermore, the different nerve-specific ECMs can also be evaluated *in vivo* by using a purely motor or sensory nerves on each dECM, which may provide further insights into the development of optimal nerve-specific ECMs depending on the injured nerves.

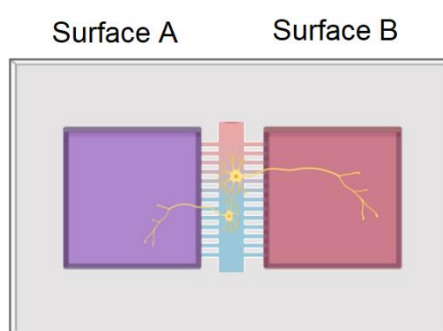


Figure 5.2 Schematic representation of a Campenot chamber where two different surfaces (A and B) can be evaluated on the same neuronal cells. Image made with Biorender.com

Finally, human cell sources can also be investigated for the development of nerve-specific ECM, which can provide a clear clinical translation. Indeed, while human DRG may be unlikely to source, although there are reports on the culture of human DRG from cadaveric origin (Valtcheva et al., 2016), a large number of studies are reporting on the development of human neuronal models *in vitro*, derived from a variety of stem cells, which may be used to model nerve-specific ECM (Lampert et al., 2020).

In fact, previous work in our group has resulted in the development of a human cell line capable of differentiating into a neuronal-sensory phenotype (Solis-Castro et al., 2020). These cells were obtained as a by-product from routine dental treatments and although further validation is needed, future studies could explore co-cultures with human Schwanns to induce myelination of neuronal cells, which in turn may provide a more representative *in vitro* model of the human nerve that can be used to obtain a human cell-derived and nerve specific ECM.

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Appendix

Power calculation

The sample size for this chapter were calculated in order to obtain a statistical power of 80% using PiFace software [v1.76: homepage.stat.uiowa.edu/~rlenth/Power]. Details of the calculations are shown in Figure 6.1, where standard deviation (SD) data was provided from previous studies in our laboratory, (Harding et al., 2014; Singh et al., 2018).

The sample size chosen for the study was $n=5$, which would be sufficient to detect differences between groups of 19.5% for axon counting/sprouting. Differences between 14 – 35% have been detected in previous studies reporting on sprouting/axonal count, suggesting differences of 19.5% is suitable for the current study.

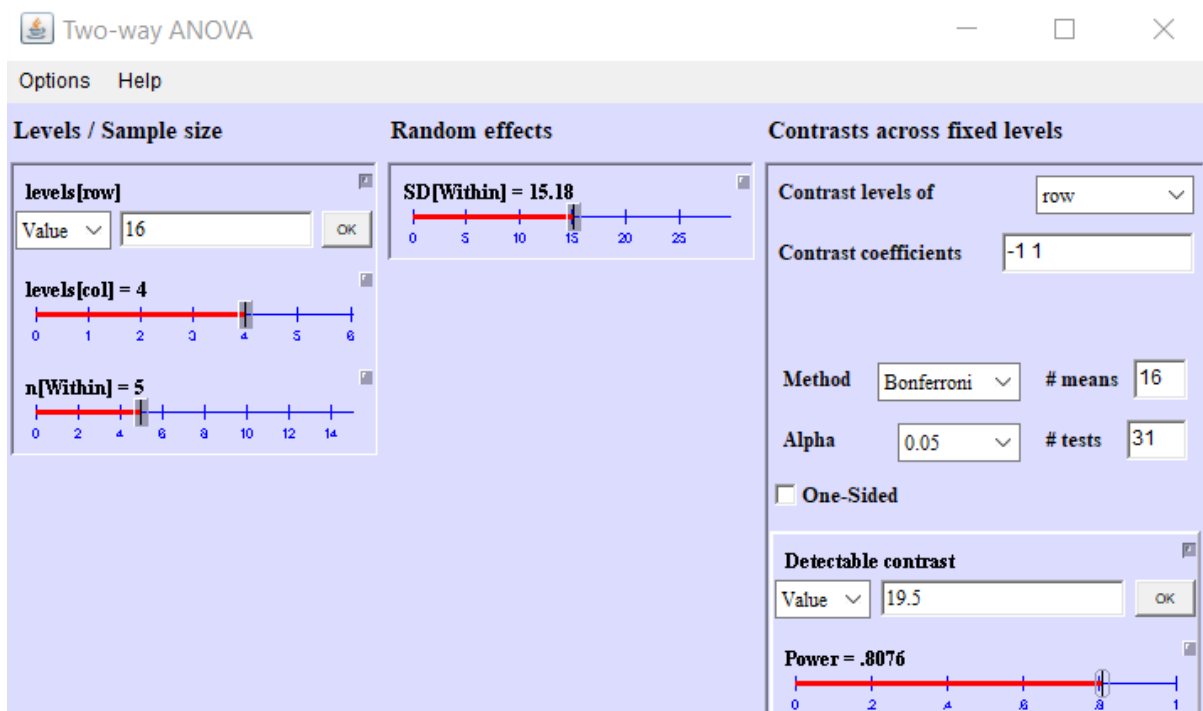


Figure 6.1 The levels[row] refers to the number of intervals; levels[col] refers to the number of groups; n[Within] = sample size; SD[Within] = standard deviation value derived from previous published data. Number of intervals [# means] multiplied by number of repair groups minus one gives the number of t-tests required for the Bonferroni post-tests [# tests].