Nerve regeneration through autologous grafts: exploring the ‘Bottleneck’ theory further.

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A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy of University College London.

Division of Surgery and Interventional Science

30th September 2018
I, Jonathan Ian Leckenby, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

Background: Two-stage free functional muscle transfer (FFMT) is the gold-standard treatment modality for unilateral congenital facial paralysis. At the first stage a cross facial nerve graft (CFNG) is harvested and coapted to an isolated buccal branch of the functioning facial nerve. The second stage involves harvesting a suitable muscle with its neurovascular pedicle and transplanting this to the face after a delay of six to nine months to permit nerve regeneration; the nerve supplying the muscle is coapted to the CFNG.

Aims: The purpose of this thesis was to investigate the impact that an autologous nerve grafts has on nerve regeneration. The hypothesis being tested was that nerve grafts with a higher axonal count would yield better outcomes than those with lower axonal counts.

Methods: To test this two parallel experiments were designed. Firstly, a new animal outcome measure was developed selecting the posterior auricular nerve as the subject for intervention; this is an isolated branch of the facial nerve with a distinct measureable outcome through the posterior movement of the ears and recovery of the nerve could be measured accordingly. Secondly, automated axon counts were calculated with a modified technique of serial section electron microscopy.

Results: The results demonstrated that a graft with a higher axon count significantly yielded better outcomes than a graft with a lower axon count: larger grafts produced better clinical outcomes of ear movement and a higher number of axons regenerated across the graft.
Discussion: It was proposed that larger grafts contained higher nerve growth factors, neurotrophins and provided better structural properties than small grafts, however larger grafts are at risk of increased central scarring through ischaemia. In conclusion, this study supports the test hypothesis that a graft with a higher axonal count will out-perform a graft with a lower axon count.
Impact Statement:

The data presented in this thesis has demonstrated for the first time that autologous grafts with higher native myelinated axon counts are preferable for nerve regeneration. A secondary finding confirmed that the likely cause for inferior results obtained with autologous grafts in comparison to direct nerve repairs is as a sequelae of scar formation within the graft.

Within the academic community this offers a target for peripheral nerve manipulation. Firstly, further investigations are required to determine the mechanism of scar formation within the autologous graft. Two proposed mechanisms would warrant initial attention. It is likely that Schwann cells may mimic the glial cells of the central nervous system and switch from a myelin producing phenotype to a scar producing phenotype through fibrosis and increased collagen fibril deposition. Substances that target inhibition of myelination, such as Fingolimod, would have potential therapeutic applications. Alternatively, chondroitin sulphate proteoglycans may be the causative mechanism and inhibition with chondroitinase may produce improved nerve regeneration.

The methodology developed within this work offers an inexpensive reliable animal model for facial nerve surgery. Previous attempts at developing animal models have failed due to cross innervation between facial branches, necessitating complex head restraints of the animals incurring significant expense. The animal model developed in this study provides the academic community with an effective model to study facial nerve surgery and outcomes.
The adaptation of serial section electron microscopy to the extra-cranial nervous system opens the door to other research areas. The potential to uncover cellular interactions in tumour-cell biology or infection could provide key elements to the communication or abnormal communication between normal and pathological tissue types; this is an area that has not yet been explored.

Outside the academic community the primary goal of this research is to improve the outcomes for the facial palsy community. To improve the ability of a nerve to regenerate through an autologous nerve graft will help provide these patients with better outcomes and therefore, a better quality of life. The applications are not only limited to facial paralysis but can also be applied to any peripheral nerve injury, including spinal cord injuries.

The first step of disseminating these results has already begun; the results have been presented to national and international clinical and scientific communities. The aim is that the results will prompt interest from the pharmaceutical industry and that this will lead to successful drug development. Ultimately, the core findings of this thesis will provide a basis for continued research endeavours and provide an excellent foundation to secure institutional and governmental funding to continue to uncover the complexities of nerve regeneration offer new strategies in improving the outcome.
Acknowledgments

This thesis would not have been possible without the support and generosity of several individuals to whom I am deeply in gratitude towards.

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- JL

25th September 2018
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Chapter 1

Background of facial paralysis and nerve regeneration

1.1 Facial paralysis: The scope of the problem

A spontaneous facial palsy can present at any age but is most commonly seen at age 20-50 years, affecting both sexes equally. Incidence is approximately 30 cases per 100,000 per year, and is slightly higher in pregnant women (45 per 100,000) and 1.8 per 100,000 of children are born with facial palsy per year [1-4].

Facial paralysis presents a complex challenge to the reconstructive surgeon. The fundamental problem when considering facial palsy is that there is currently no conceivable way to restore the 17 muscles of facial expression successfully. The second challenge facing the surgeon is that each patient is unique with individualised pathology and concerns; thus not only the medical history and aetiology behind the facial paralysis dictates which operations are available, their age group, the anatomy available and most importantly, what the patient wants all contribute to the decision making process.

The impact facial paralysis has on an individual can be devastating and can be broken down into functional and psychological factors [5, 6]. If a patient is unable to close their eye and the cornea is insensate, the globe will desiccate, potentially leading to ulceration and ultimately blindness. Diminished oral continence, poor speech and obstructive nasal breathing are other functional problems
encountered. A child who is unable to smile or convey emotion effectively may be subject to bullying and psychological trauma, while an adult with profound facial asymmetry at rest may be erroneously perceived as being angry by another individual, resulting in avoidance behaviour [7, 8].
1.2 Current treatment principals in facial reanimation surgery

Functional muscle transfers are the gold standard for both congenital cases of facial paralysis and longstanding acquired facial palsy [9-11]. This concept was pioneered less than 50 years ago by Thompson [12], and the first free functional muscle transfer (FFMT) for facial paralysis was performed by Harii et al. in 1976 [13]. Some surgeons prefer a two-stage approach, using a cross facial nerve graft (CFNG) at the first stage, followed by a functional muscle transfer at the second stage, typically 6-9 months later. Single-stage options exist using the contralateral facial nerve if available, otherwise an alternative donor nerve is used [14-16]. More recently, utilisation of alternative motor nerves has become popular, for example the masseteric nerve, not only for bilateral facial palsy but also for unilateral cases [17-19], however the utilisation of these alternative nerves relies on cortical plasticity to produce a spontaneous smile [20, 21], as only the contralateral facial nerve can produce this in response to emotion. The main restraint with any type of FFMT appears to be the lack of predictability of obtaining an excellent result, as some patients suffer from lack of excursion and symmetry, and others may show signs of tightness of the muscle transfer — classically starting after 6 months since the initial onset of movement [22-24].

Outcomes of FFMT currently suggest that 60% of patients undergoing the procedure will obtain an excellent result; to the layperson, there would be no noticeable evidence of the facial palsy. 20% of the patients will have a poor result; a result of exaggerated asymmetry due to the bulk of the FFMT or due to the muscle becoming ‘over-tight’, both aesthetically displeasing. The remaining 20% have no change in their facial symmetry [24].
1.2.1 Single-stage reanimation procedures: direct nerve repair

Direct nerve repair is used in FFMT for single-stage procedures that do not require a CFNG, and typically an alternative nerve to the facial nerve is selected. The main aim of a neurorrhaphy is to guide regenerating axons towards their target organs thus allowing for successful reinnervation with the minimal loss of axons as they cross the site of repair [25]. Direct nerve repair is optimal when the gap is small and the two ends can be approximated with minimal tension. The most common technique of direct nerve repair is to perform an epineural end-to-end repair. Ideally the corresponding ends of fascicles should be precisely arranged and secured using between 4 – 8 sutures depending on the size of the nerve [26]. Alternatively a fascicular repair can be attempted where multiple sutures are used to coapt matching groups of fascicles; this is a useful technique when repairing mixed nerves however, there is no evidence that a fascicular repair yields better outcomes possibly due to the increased number of sutures required and the resulting scar formation [27]. The suture material acts as a foreign body resulting in local granuloma formation thus inhibiting the growth of axons [28-34].

End-to-side neurorrhaphies are growing in popularity, particularly by supplementing a CFNG with a sensory branch, to promote and sustain nerve regeneration [35-37]. In this method, the donor nerve is not transected, instead, an epineural window is made at the ‘side’ of the donor nerve at an appropriate point along its length and the recipient is coapted to the epineural opening. The precise mechanism is not completely understood but two main theories exist as to how regeneration occurs. Firstly, it is thought that some axons are damaged
in the process of making the side incision; this results in axonal regeneration into the recipient nerve from the point of injury [38]. Secondly, other studies have demonstrated spontaneous axonal sprouting from the nodes of Ranvier of undamaged axons at the coaptation site [39]. Irrespectively the results appear to vary and the main conclusion is that sensory nerves appear to regenerate better than motor nerves using this technique [40, 41].

1.2.2 Two-stage reanimation procedures: Nerve grafting

Cross facial nerve grafting from the functioning contralateral side is still the treatment of choice in children [42, 43]. Nerve grafting is traditionally used when a gap in a nerve resulting from injury cannot be easily directly repaired in a tension-free fashion, as studies have shown that a direct nerve repair performed under tension yields a poor outcome [44]. Tissue ischaemia as a consequence of the increased tension causing the occlusion of blood vessels within the nerve; this leads to central necrosis and scar formation that prohibits axonal regeneration [45, 46]. Nerve grafting helps overcome this at the expense of introducing a second neurorrhaphy that the regenerating axons must traverse [34]. The results from studies examining direct repairs performed under moderate tension with nerve grafting showed comparable results, however no reported values for tensions were given which makes results hard to interpret [34, 46, 47].

Various materials have been used to bridge a nerve gap, but autologous nerve grafts remain the gold standard for a defect greater than 3 cm [48]. Since the first introduction of autologous nerve grafts in 1885 [49], the understanding of nerve
anatomy and ultrastructure, combined with the advent of microsurgery, has established that autologous nerve grafts yield the most favourable outcomes when treating large nerve gaps [50-52]. An autologous graft offers the ideal environment for axonal regeneration, as a scaffold consisting of Schwann cell basal laminae, neurotrophic factors and adhesion molecules is provided, even though the graft undergoes Wallerian degeneration [53, 54]. Both Schwann cells and the neurotrophic factors released into the axoplasmic fluids are crucial for axonal regeneration and in their absence, regeneration cannot occur [55].

Two important factors affect axonal growth through a graft: the diameter of a graft and the vascularity of the surrounding tissue [54]. The survival of any transplanted graft is dependent on diffusion from the surrounding tissue in the acute phase and then neo-vascularisation by capillary ingrowth in order to remain viable. Large diameter grafts have been shown to undergo central areas of necrosis that is replaced with scar tissue over time [56, 57]. Another factor affecting the outcome of regeneration in autologous grafts is the type of nerve used. Motor nerves have been shown to yield better outcomes than sensory ones [58] and in a rat model, sensory grafts have been shown to inhibit axonal regeneration [59]. In a clinical context the use of a motor nerve as a donor graft is avoided due to the resulting associated functional loss, and therefore sensory nerves are preferentially selected, ideally ones that result in the smallest sensory deficit. In facial palsy surgery, the graft is required to extend from the mid-cheek on one side of the face to just anterior to the tragus on the opposite side, therefore the minimum length required is 20cm. The most common donor nerve selected for autologous grafting is the sural nerve as this provides an abundant length,
between 20 – 50cm in length [60], and the resulting sensory defect is only to a small area over the lateral aspect of the foot.

Several other materials have been suggested as substitutes for autologous grafts. Biological conduits have been extensively studied with veins and arteries being most popular, particularly in digital nerve injuries [61], along with variations of skeletal muscle [62], mesothelial tissue [63] and epineural sheaths [64]; these seem only effective in bridging nerve gaps and are often inferior when morphological studies are performed [64]. Synthetic conduits have been developed and should ideally be permeable enough to allow for sufficient oxygen and metabolite diffusion to support Schwann cell proliferation whilst prohibiting the entry of fibroblast cells. Currently only a handful of synthetic conduits are licensed for human implantation and the indications are specifically for gaps less than 3 cm.

The final group of grafts to be considered is allografts. Hand transplantation has driven our understanding of how allogenic grafts behave as nerve regeneration occurs. Studies have shown that immunosuppression plays an important role not only in the prevention of rejection [65] but also the stimulation of axonal growth when tacrolimus (FK506) is used as the immunosuppressant [66]. Of note, it appears that the requirement for immunosuppression is not life-long [67] and once there is evidence that axons have regenerated beyond the graft, treatment can be stopped [68], however, in the complete absence of immunosuppression a graft is rejected and becomes non-functional [69]. Whilst immunosuppression with FK506 is a beneficial coincidence for hand transplant patients, the indication for using allografts in facial palsy patients is not yet warranted.
There is no question of the benefits of using alternative materials to autologous grafts; there are significant donor site morbidities from graft harvesting and there is a limited length of graft that can be used [70-73]. It is important that research continues to push forward to find a viable alternative to autologous grafts, particularly when considering brachial plexus injuries where multiple roots or cords may be injured requiring multiple grafts of considerable length. In the context of facial palsy surgery, patients seldom require more than two grafts, which can easily be accommodated by the harvesting of either their two sural nerves or other cutaneous sensory nerves.
1.3 Variability of outcomes obtained following facial reanimation

It is well documented that functional muscle transfers only achieve between 40-60% of the original muscle strength [74-76]. The main problem affecting FFMT is the inability to predict the exact result a patient will achieve. There are factors that affect the ability of a nerve to regenerate that can be taken into account, such as age [77, 78], which have a direct impact on the outcome following FFMT. The difficulty arises when these factors are controlled yet the outcome still remains unpredictable. The goal for facial reanimation surgery is to restore symmetry, function and spontaneity to the face; in order for this to be achieved a surgeon must be able to perform a wide range of operations, as no single technique is sufficient.

1.3.1 Surgical variability in FFMT and facial reanimation

A surgeon must make several choices when embarking on a facial reanimation procedure, all of which have the potential to impact on the outcome. If a FFMT procedure for the restoration of smiling is considered, the following variables must also be accounted for: 1) donor muscle, 2) type of graft, and 3) donor nerve used. The most common muscles used for FFMT in facial paralysis are *m. pectoralis minor, m. gracilis* and *m. latissimus dorsi*. These muscles differ in their physical shape and therefore, the direction their force is exerted, their number of motor units and the phenotype of muscle fibre sub-group ratios. Typically the sural nerve is used as a CFNG however other sensory branches can be used. If the sural nerve is used, there are variations in the diameter and axonal loads that may contribute to variations in outcomes. Selection of a suitable donor nerve can
be dependent on available anatomy in bilateral congenital cases, dependent on the surgeon’s preference and can be a cause for the variability seen.

There is no standardised outcome measure that is universally used to assess outcomes of facial reanimation surgery. Up to 25 different systems are used in clinical practice and although they are similar, each takes into account different outcomes and has a different scoring system [79]. This lack of standardization makes pooling of data from different surgical units difficult and as a result, makes comparison between groups almost impossible.

Large series of clinical data from individual units are becoming available where the same muscle, CFNG and donor nerve have been used and all outcomes assessed using the same scoring system [80, 81]. The results of over 658 pectoralis minor FFMT revealed 60% of patients obtained an excellent outcome. Overall the majority of patients obtain good or excellent results (60 – 80%) [24] and the operative procedures for restoring facial animation are successful. What is unclear is why outcomes are unpredictable within individual series, performed by the same surgeon, using the same muscle and the same donor nerves. Another concern is the situation where a patient initially obtains an excellent result but develops over-tightness with the muscle in a state of continual contraction [82, 83]; this leads to significant disfigurement and the patient is reclassified as having a poor outcome.
### 1.3.2 Axonal load of the donor nerve

There are several options available to a surgeon when it comes to selecting a donor nerve to supply the FFMT. The decision is made on what anatomy is available, such as in the case of bilateral congenital paralysis where the facial nerve is absent on both sides. In these cases, the surgeon can choose from another functioning cranial nerve such as a branch of the masseteric nerve (Vth), the hypoglossal (XII) or the accessory nerve (XI); axon counts for these donor nerves have shown to be significantly different in humans [84]. The axonal load of a donor nerve has also been shown to influence the outcome of FFMT in experimental models in rabbits leading to the proposal of the ‘Bottleneck Theory’ [23, 85, 86]. The findings of these studies demonstrate that the rectus abdominus muscle had stronger contractions if the donor nerve axonal count exceeded the axonal count of the muscle; if the balance was converse, the contractions were weaker. The authors concluded that there was a bottleneck between the axonal supply of donor nerve and the nerve of the muscle, and in the situation where the donor nerve had the lower axonal count, there was increased axonal sprouting to compensate, thus each motor unit was consequently under-powered and force production was limited by nerve fibre number [86] (Fig. 1.1). More recently Snyder-Warwick has demonstrated similar results [84] when examining outcomes in paediatric patients being treated for unilateral facial paralysis. Their study compared the axonal counts of two groups, the first using a buccal branch of the facial nerve with a CFNG and the second with the masseteric nerve directly coapted to the FFM - in both cases the gracilis muscle was used. Although the buccal branch (6757 per mm²) had a higher axonal count compared to the masseteric branch (5289 per mm²), once regeneration had occurred through the
Figure 1.1. The ‘Bottleneck Theory’.

An illustration showing the effect of axon number on motor unit size following muscle reinnervation (Reproduced with permission from Urso-Baiarda et al [23]).
CFNG the axonal count reaching the distal CFNG was 1647mm² suggesting only 75% of axons regenerated through the CFNG. Unsurprisingly this resulted in more movement of the muscle in the masseteric group at the cost of spontaneous movement. To date, there are no reports of the ‘Bottleneck Theory’ being applied to the CFNG component in FFMT. If one controls the axonal load of the donor nerve, does the axonal load of the autologous graft have a similar effect on functional outcome? If the theory is applied, the assumption would be that grafts with higher axonal counts should lead to better outcomes as there is less of a bottleneck between the donor nerve and the nerve graft in comparison to grafts with lower axonal counts; this is a key concept which this thesis seeks to explore.

The decision to select one donor nerve over another is not without clinical consequence. By selecting a contra-lateral functioning buccal branch and combining this with a CFNG the patient will spontaneously smile once the muscle is re-innervated whereas if other donor nerves are chosen, the patient must re-learn how to use the muscle and is reliant on cortical plasticity to obtain a spontaneous result and thus a challenge in older patients.

1.3.3 Selection of free functional muscle:

A second variable that has the potential to affect outcome is the muscle chosen for FFMT. Whilst many different muscles have been chosen historically, the main muscles used are *m. gracilis, m. latissimus dorsi* and *m. pectoralis minor* [87]. Despite the structural differences in these three muscles most commonly used, the clinical data reported in the literature cannot be adequately compared due to the lack of standardized reporting. There are extensive case-series published
from individual units, but there are multiple technical variables used within these groups that prohibit statistical evaluation. There is little in the scientific literature investigating this variable in the context of FFMT. No experiments have been reported examining the effect changing muscle size, and therefore motor units, has on contractility when the donor nerve is controlled.

In general, the transplanted muscle is expected to atrophy within the time it takes for re-innervation; the degree of atrophy is unpredictable and will depend on the time the axons take to re-establish the neuromuscular junctions and prevent irreversible end-plate degeneration. The impact the axonal load of the donor nerve has on functional muscle outcomes is established, and the results demonstrate that the lower the axon count available for reanimation the weaker the outcome; if the assumption is that the number of muscle motor units will always far outweigh the number of axons available for re-innervation, then muscle selection may not have an impact on the outcome.

1.3.4 Autologous nerve grafts

The final element of FFMT that can be changed is the selection of the nerve chosen as a cross facial nerve graft in two-stage facial reanimation procedures. Traditionally, autologous sensory nerve grafts are used with the sural nerve being the most popular as the associated donor-site morbidity is low, and the length of graft harvested is sufficient to reach from the non-paralyzed side of the face subcutaneously across to the pretragal region of the paralyzed side [88].
The choice of sensory nerve grafts or motor nerve grafts affect the outcome of motor nerve regeneration [58]. Animal experiments have demonstrated a better restoration of motor function when motor nerve grafts were used to reconstruct tibial nerve defects in comparison to sensory nerve grafts [58, 59]. In clinical practice the use of motor nerve grafts is not recommended as the morbidity associated with the motor function loss is too great and therefore is currently not of practical use.

Altering the length or the diameter of a nerve graft influences the outcome of nerve regeneration. A multitude of studies have shown that increasing the length axons need to regenerate over is detrimental to the outcome obtained [89-91]. Similar evidence has been reported for grafts with large calibres. Evidence presented by Best et al. [56] demonstrated that in large calibre grafts the outcomes were poorer in comparison to smaller grafts. On histological analysis, the central portions of the grafts appeared to be poorly revascularised and developed scarring which limited axonal regeneration. However, there is a sparsity of information regarding whether the axonal count of a nerve graft has the potential to impact nerve regeneration. A graft with increased axonal counts would contain a higher level of beneficial nerve growth factors and Schwann cells that would promote a greater degree of axonal regeneration. Not only is the abundance of growth and neurotrophic factors beneficial but the physical architecture allows for multiple channels where axons may regenerate through.
1.4 **Hypothesis and Specific Aims**

Having established that the axonal count of the donor nerve impacts the outcome of FFMTs the next step is to explore the role an autologous graft plays in FFMT; there is a gap in the published literature addressing this directly. Using similar concepts established by MacQuillan and Grobbelaar [85, 86], the goal is to determine whether the ‘Bottleneck Theory’ can, in some way, be applied to the nerve graft. The hypothesis this work looks to prove is:

“Autologous nerve grafts with higher native myelinated axonal counts will yield better outcomes, both clinically and histologically, than autologous nerve grafts with lower native myelinated axonal nerve counts”

1.4.1 **Specific Aim 1a: Establish a rodent model for clinical outcomes following facial nerve surgery**

Multiple animal models exist designed to monitor recovery following peripheral nerve surgery. The most popular tools are typically based around sciatic nerve injury in rats and assess the animals gait or ability to walk along ladder rungs. As the purpose of this work is based on improving outcomes for facial paralysis, these models may not be appropriate. The most important distinction is that the facial nerve is a cranial nerve and thus may behave differently to peripheral nerves [92-94]; the cell body is located centrally and there is evidence that there are key difference in how cranial nerves recovery from injury. Consequently, it would be more appropriate to investigate the recovery of facial nerve manipulation by establishing an animal model using the facial nerve.
1.4.2 Specific Aim 1b: **Assess the impact the native myelinated axon count of autologous nerve grafts has on clinical outcomes of facial nerve surgery in a rodent model**

Having established and validated an outcome model for facial nerve surgery in rodents, two different types of autologous graft will be used. Two groups of grafts will be examined which will have significantly different myelinated axonal count populations. The results from these experiments will seek to prove the hypothesis and examine the impact the native axonal count of an autologous nerve graft has on clinical outcomes of facial nerve surgery.

1.4.3 Specific Aim 2a: **Utilise principals of serial section electron microscopy to establish a semi-automated tool to calculate myelinated axonal counts**

Serial section electron microscopy (SSEM) has recently been used to analyse large volumes of cortical tissue. Using this technique, it is possible to uncover every connection a cell makes with another in the quest for a better understanding of neural connections. By modifying this technique, myelinated axonal counts will be automated reducing the work-load associated with traditional methods of nerve histomorphological analysis.

1.4.4 Specific Aim 2b: **Assess the impact the native myelinated axonal count of autologous nerve grafts has on facial nerve regeneration histomorphologically using serial section electron microscopy (SSEM)**
By modifying the technique of SSEM and applying the principals to nerve regeneration, the process of nerve regeneration through grafts of differing myelinated axonal counts will be examined histomorphologically. This aim seeks to develop a rapid, automated technique of accurately producing myelinated axonal counts and observing the regenerative process in unprecedented detail.

1.4.5 Specific Aim 3: Determine the relationship between the clinical outcomes and cellular outcomes following facial nerve surgery using autologous nerve grafts with differing native myelinated axon counts

By combining the results from Specific Aims 1 and 2, the relationship between events occurring at a cellular level and at a clinically appreciable level will be determined. The results from combining the two methods of nerve regeneration analysis will seek to prove the hypothesis of this thesis.
1.5 Bibliography:


Chapter Two

Development of an animal model to use as an outcome measure for facial nerve surgery

2.1 Introduction

Outcome measures are crucial to reporting results of interventions used in facial paralysis. This allows for critical evaluation of results obtained and should be unbiased, reproducible and objective in nature. Currently more than 25 clinical outcome measuring systems exist for facial paralysis [1-3] since the original proposed by House [4] leading to non-standardized reporting of results [5].

Animal models for the assessment of nerve regeneration are well established. Most models are centred on peripheral nerve injury to the hind limb and assessment of walking patterns, ladder rung climbing and swimming [6]. Whilst these systems have been well studied and offer good inter-user reliability, there are fundamental differences between the regenerative properties of the central nervous system (CNS) axons and peripheral nervous system (PNS) axons [7]. Although the facial nerve exits the cranium and has many similar physiological and histological properties to a peripheral nerve, its cell bodies remain in the facial motor nucleus, located in the brainstem. Studies demonstrate that the CNS environment is inhibitory to axon growth, and that the PNS environment is stimulatory [8-11]; therefore the supposition that the facial nerve behaves exactly the same as a peripheral nerve may be inaccurate.
Animal models specifically addressing facial nerve assessment exist for this reason. In rodents, studies have utilised two main outcomes as a marker for facial nerve function and regeneration: eye closure and whisker/vibrissae movement [12-15]. These outcome measures require restraint of the animal or the application of head fixators which can only be used in rats, not mice, increasing discomfort to the animal and cost of the experiments, whilst excluding the many transgenic mice breeds that facilitate our understanding of nerve regeneration. More recently, alternative facial nerve independent mechanisms have been identified that can also achieve eye closure and whisking, making these outcome measures sub-optimal [12, 16].
2.2 Anatomical study of the extra-cranial course of the facial nerve in the laboratory mouse

In order to achieve Specific Aim 1, an anatomical study to determine a suitable branch of the facial nerve was undertaken. Five, three-month old YFP-16 transgenic mice were selected for the study as these animals have high level expression of yellow fluorescent protein (YFP) throughout their nervous system. The animals were euthanized in a CO$_2$ chamber and death was confirmed via thoracotomies. The animals were perfused with 50mls 4% paraformaldehyde (PFA) in phosphate buffered saline via the left cardiac ventricle. The skin and superficial soft tissue of the head and neck area was removed and dissection of the facial nerve was performed under the guidance of a fluorescent macro-zoom microscope (Axio Zoom, Zeiss).

The facial nerve was seen to emerge from the cranium via the stylomastoid foramen, approximately 12mm inferior and 5mm posterior to the most inferior aspect of the bony external auditory meatus. The branching pattern was consistent in all animals. The first branch, the posterior auricular branch, arose as the facial nerve emerged from the stylomastoid foramen, and coursed immediately behind the auricles to innervate the interscutularis muscle. The facial nerve trunk proceeded towards the face and separated into three divisions: the temporal, the zygomatic/buccal and the mandibular/cervical divisions. Continuing distally, these divisions separated into their terminal branches - the temporal, zygomatic, buccal, mandibular and cervical branches. However, it was noticeable that there were several interconnections between these terminal branches. Henstrom et al. [17] have previously demonstrated this in the Wister rats and
coined the term ‘distal pes’ to describe the convergence just proximal to the whisker pad, however, due to the assistance of the fluorescence microscope and YFP-16 transgenic animals, the cross-innervation in all areas was clearly demonstrated (Fig. 2.1).

The posterior auricular branch, or the posterior auricular nerve (PAN) as it will be referred to henceforth, had no identifiable cross-innervation with any other facial nerve branch. The nerve passed deep to the greater auricular nerve (GAN) and was noted to give off three consistent branches to the posterior surface of the auricle, thus forming discrete neuromuscular junctions (NMJs) in a banded fashion at the distal third of the auricle. These findings were confirmed immunohistochemically with α-bungarotoxin staining which innervates type IIB muscle fibres (Fig. 2.2). The PAN continued distally as a terminal branch to supply the interscutularis muscle.

To confirm the function of the PAN a second group of experiments was undertaken. Six YFP-16 mice were selected and anaesthetized by an intraperitoneal injection of ketamine and xylazine (a solution containing 87 mg/kg ketamine + 13 mg/kg xylazine). Under fluorescent microscope guidance (Axio Zoom, Zeiss), a curved incision was made around the posterior aspect of the auricle, extending towards the angle of the mandible. The greater auricular nerve was identified as the most superficial structure encountered. By retraction of the trapezius muscle dorsally and then sternocleidomastoid ventrally, the extra-temporal emergence of the facial nerve trunk could be identified. The PAN was isolated from the surrounding tissues and insulated with a latex cuff. To confirm
**Figure 2.1** Anatomical dissection of the facial nerve of a mouse

A) The superficial anatomy of the face of a YFP-16 mouse. The skin has been removed to expose the underlying facial nerve and musculature. The main branches of the facial nerve can be appreciated.

B) The same animal under fluorescent imaging. Under these conditions the cross-innervation patterns may be fully appreciated. Cross-innervation can be seen between the temporal and zygomatic branches, between the zygomatic and buccal branches and extensively between the buccal and mandibular branches; no demonstrable cross-innervation was seen with the posterior auricular nerve (PAN).

[Key: p – posterior auricular nerve, t – temporal branch, z – zygomatic branch, b – buccal branch, m – mandibular branch, dp – distal pes]
Figure 2.2  Anatomical and immuno-histological study of the mouse ear.

A) and B) The posterior surface of the mouse ear following de-epithelialization. Three discreet branches of the posterior auricular nerve (PAN) [white arrowheads] were consistently observed to form a band of neuromuscular junctions (NMJs).

C) α-bungarotoxin was used to confirm the presence of NMJs [white arrowheads].

D) Cross-section of mouse ear tissue. Staining with anti-MyHC2B (Life Technologies, Carlsbad, CA, USA) demonstrated the presence of type IIB muscle fibers [white chevron markers] associated with auricular cartilage (white arrowheads). These findings support the discovery of a previously unreported muscle of the mouse ear.
the action of the PAN a nerve stimulator (Vari-Stim® III, Medtronic, Xomed Inc.) was used. Stimulation of the PAN at the origin resulted in retraction of the auricles posteriorly and flattening towards the skull; when stimulated distally to the three branches given off to the auricle, only retraction of the ear could be demonstrated, suggesting the three previously undefined branches supplying the posterior auricular region are responsible for the curling of the auricle (Fig. 2.3 A - D). To understand the consequence of injuring the PAN, each animal had a 5 mm segment of the right nerve segmentally excised. The skin incision was closed with absorbable sutures and the animals were allowed to recover from surgery. Analgesia and post-operative care was provided according to local protocols. Assessment of the animals post-operatively showed the right ear was held in a forward/ventral posture but was not retracting posteriorly in comparison to the contra-lateral side (Fig. 2.3 E&F)
Figure 2.3  Function of the posterior auricular nerve in a mouse.

A) The anterior position of the right ear prior to stimulation of the posterior auricular nerve.

B) During stimulation of the posterior auricular nerve the ear can be seen to retract towards the mid-line.

C) The lateral position of the ear at rest.

D) During stimulation of the posterior auricular nerve, the ear is retracted posteriorly.

E) Baseline video still of a mouse recorded whilst they drank from the water feeder in the fabricated video-suite.

F) Post-operative video still following excision of the right posterior auricular nerve demonstrating the inability to retract the ear.
2.3 Rationale for selecting the posterior auricular nerve (PAN) in experimental design

The anatomical study of the branching patterns demonstrated extensive cross-innervation between most of the facial branches. Although initial studies promoted the implementation of either the assessment of whisking or eye-closure [13-15, 18], these have largely been retracted [12, 16, 17], citing alternative mechanisms which enable these functions to occur independent of the facial nerve; the anatomical dissection of YFP-16 mice demonstrates an abundance of cross-linking between the main branches of the facial nerve. Contrastingly, the PAN is an isolated branch of the facial nerve that has an identifiable function and furthermore, all the branches of the nerve have been mapped in a ‘connectome’ by Lu et al. [19] confirming that the interscutularis is not innervated by any other motor nerve (Fig. 2.4).

An ideal animal model for facial nerve injury would not be invasive or require animal restraint. Animals convey emotion primitively in contrast to humans, however there are key features which disclose basic emotional states. In canines and felines, vocal cues such as barking or purring, offer some understanding of emotional response, however even in higher animals, non-vocal signs are difficult to interpret, particularly when concentrating on the facial nerve. Animal behavioural research has demonstrated that the ear position can convey the animal’s situational behaviour. Behavioural studies in sheep investigating non-invasive methods of assessing pain have reported ear-position as being one component of a reliable indicator of suffering [20, 21].
Figure 2.4  The *m. interscutularis* connectome.

The detailed report from Lu *et al.* demonstrate that the *m. intercutularis* has no innervation from any other nerve other than the posterior auricular branch of the facial nerve. [Reproduced with permission from Lu *et al.*]
If animals were distressed or in pain the ear posture was more likely to be retracted and furthermore, has been shown to convey positive and negative emotion in sheep [22-24]. These studies noted that ear-movement is the most powerful indicator of emotive characteristics. When an animal is threatened, or prior to retaliation, the ears are retracted and flattened against the skull; these auricular muscles are largely vestigial in humans.

The PAN would appear to be an excellent nerve to use for the facial reanimation surgery: there is no cross-innervation, there is a potentially measurable outcome by monitoring ear movement and finally, it is linked to an emotional and behavioural response in animals.
2.4 Development of a mouse model for facial nerve surgery using the restoration of ear movement as an outcome measure

2.4.1 Animal groups

Having identified the PAN as being a nerve that was potentially suitable for studying the outcomes of facial nerve surgery, it was important to determine whether the restoration of ear movement could be used to assess this. Most reports of outcomes following surgery use three distinct animal groups; a sham group, a nerve crush group and a nerve repair group. The understanding is that this best simulates clinical injuries in the form of neuropraxia and neurotmesis [25-30]. In order to evaluate this proposed ear-movement model, it was important to design a pilot study to have comparable groups to published data.

Female YFP-16 mice were chosen at six-weeks of age and were assigned to three groups, each group containing ten animals with the exception of the sham and excision groups which contained five animals:

1. Sham group (Sh, n=5)
2. Nerve crush group (Cr, n=10)
3. Nerve excision group (Ex, n=10)

All interventions were performed on the right PAN and thus the left ear served as the control for each animal.
2.4.2 Surgical technique

Before and after surgery, all animals were allowed access to food and water *ad libitum*. All animals were anaesthetized with an intra-peritoneal injection of ketamine and xylazine (87mg/kg of ketamine and 13mg/kg of xylazine, dose = 0.1ml/20g). Once adequate anaesthesia was confirmed the area surrounding the right auricle was prepared for surgery by removing the fur around the surgical field with Nair™ (Church & Dwight Co., USA) after 3 minutes of application. The surgical site was prepared using 70% ethanol solution. A 15mm curvilinear incision was made extending from the posterior auricular margin to the angle of the mandible. Surgical dissection proceeded as described in Section 2.2 under a fluorescent operating microscope to expose and define the PAN. In all groups the nerve was dissected freely and identification of the nerve was confirmed using a nerve stimulator (Vari-Stim® III, Medtronic, Xomed Inc.). The following differences for each group occurred:

**Sham Group (Sh, n=5)**  
After identifying the nerve the surgical field was irrigated with 10mls of normal saline solution (0.9% NaCl). No further manipulation of the PAN was performed.

**Crush Group (Cr, n=10)**  
After identifying the nerve the PAN was crushed 3mm from the origin of the facial nerve trunk. Size 5 jeweller’s forceps were used to squeeze the nerve between the tips of the forceps for two periods of twenty seconds. Confirmation of an effective crush injury was verified using the fluorescent microscope to demonstrate a non-fluorescent gap in the nerve (Fig. 2.5). The surgical field was irrigated with 10mls of normal saline solution (0.9% NaCl).
Figure 2.5  Surgical procedures on the posterior auricular nerve.
A) Curvilinear incision around the base of the auricle exposes the posterior auricular nerve. In the sham group, the nerve was skeletonized with no added intervention.
B) The posterior auricular nerve was crushed until no fluorescence was visualized (between the red arrowheads).
[Key: fn – facial nerve trunk, p – posterior auricular nerve].
**Excision Group (Ex, n=10)** After identifying the PAN a 3mm section of the nerve was sharply excised and preserved in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) solution (for histological analysis as described in Chapter 3). Confirmation of a nerve gap was verified using the fluorescent microscope to demonstrate a non-fluorescent gap in the nerve. A 2 mm epineural incision was made in the facial nerve trunk and the proximal stump of the PAN was sutured in an end-to-side fashion to the facial nerve trunk using four 11-0 Ethilon® interrupted sutures (Ethicon, USA) at the 12, 3, 6 and 9 o’clock positions; this was to prevent the PAN from potentially neurotising the interscutularis muscle as it regenerated from the proximal stump. The surgical field was irrigated with 10mls of normal saline solution (0.9% NaCl).

In all animals, the surgical incision was closed using 5-0 Vicryl Rapide™ (Ethicon, USA) interrupted sutures. All animals received a subcutaneous injection of 0.5ml of warmed 0.9% NaCl for rehydration, and a subcutaneous injection of buprenorphine (0.05mg/kg) for post-operative pain control. Animals were recovered from anaesthesia in a heated chamber (32-36°C) with 95% oxygen and observed continuously until they become sternally recumbent, in control of their airways and could eat and drink on their own. Experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) PHS Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act (7 U.S.C. *et seq.*), and an animal use protocol approved by the Standing Committee on the Use of Animals in Research and Training of Harvard University (Protocol 24-08). At the end of the data collection period, all animals were euthanized in a CO₂ chamber.
2.4.3 Development of an automated video capture of ear movement

To capture ear retraction, the study design was to startle the animals as they were drinking from a metal spout. This concept was devised based on behavioural studies of animals in response to being startled, and in combination with established methods assessing eye closure as an outcome method. To record changes in ear movements a custom video-capture suite was fabricated. A colourless round Perspex cage was fabricated measuring 30cm in diameter with 30cm high walls. At one end a rectangular extension was added measuring 10cm long, 4cm wide and 30cm high. At the far end of this extension a hole was made so that a bottle water feeder with a metal spout could be mounted and allow the animal free access to water. A copper-conductive plate was mounted on the floor of the extension which was joined by conductive wiring to a copper collar which was mounted around the drinking spout. A second 3 mm diameter hole was made adjacent to the water feeder to permit the passage of a silicon tube connected to a valve that would open to supply a puff of laboratory oxygen, regulated at 90 mmHg, directed at the face of the animal as described by Heaton et al. [14, 31]. The air-puff system was connected to an Apple iMac desktop computer with Adobe Photoshop CS6® (Adobe Systems Inc., USA), MATLAB® (MathWorks, USA), LabVIEW® (National Instruments, USA), QuickTime© Player (Apple Inc., USA) and iMovie© (Apple Inc., USA) installed. A USB 2.0-powered high-definition monochrome digital camera with an in-built Progressive Scan CMOS CCD image sensor (ImagingSource DMK 72BUC02) fitted with a macro lens (Pentax™ TV lens 16mm F1.4) was mounted to a fixed height of 45cm above the floor of the extended section of the cage to record the animal as it drank from the water feeder. The camera was set to record 1024x768 (Y800) at 45 frames per second. The system was set up so that when the animal was drinking from
the water feeder, an electrical circuit was established with the animal providing conduction between the copper floor pad and the copper collar around the water feeder. The purpose of this was two-fold: firstly, to open the motorised-valve so that a puff of air could be discharged and secondly, prompt the computer to save the video recording ten seconds prior to the connection being made and continue saving the recording for a total of thirty seconds. To prevent learned avoidance, a random generator was applied to the valve system so that an air puff was generated unpredictably as it drank from the feeder. The MATLAB code was written to permit the investigator to leave the animal unattended for several hours at a time and to ensure only 30 seconds of buffered video were captured around a drinking event, avoiding the need to manually screen through hours of empty footage. An overview of this system can be seen in (Fig. 2.6).

The trial run of the video suite was a failure. The system operated as designed, however the animal was startled to a degree where the captured footage was unusable. The air-puff would cause the animal to quickly recoil in an unpredictable manner making analysis of the captured video uninterpretable. Whilst reviewing the footage the investigator began to notice that on the occasions where the air-puff was deliberately not initiated, the behaviour of the animals was universal; every animal retracted their ears of their own accord. This prompted the investigator to abandon the air-puff system altogether but retain the buffered video-capture technology. Anecdotally, a senior investigator working in the laboratory suggested that a better drinking response could be obtained by filling the water feeders with chocolate milk (Nesquik®, Nestle, Switzerland). This practice was adopted in all future video capture sessions as an increased drinking behaviour was observed however, this was not critically evaluated.
Figure 2.6  Overview of the video-suite used to assess ear movement.

A) The equipment utilized to record mice whilst they took a drink from the water feeder. The data was set to use buffered video recordings, 10 seconds prior to a drink being taken and then for the next 30 seconds.

[Key:  a – regulator to control the pressure of 'air-puffs' delivered,  b – USB 2.0 controlled valve programmed to release an air-puff for every 1/5 episodes from the water feeder that were randomized.  c) The cooper floor implemented to ensure conductivity between the animal and the water feeder.  d) Water feeder.  e) USB link to computer software system (LabView).]

B) Overview of an occurrence of a mouse drinking from the water feeder.
2.4.4 Automated video-capture of pilot study and data analysis.

For all animals, a pre-operative baseline recording of their ear movement during drinking was captured. A data-captured session was complete once ten episodes of the animal drinking were recorded which, typically accomplished in a one-hour time period; this also served to allow the animals to acclimatise to the video-suite.

Subsequent video recording sessions were planned for all groups post-operatively. On post-operative days (POD) 1, 10, 20 and 30, animals in all groups were recorded in the video suite drinking for a minimum of ten occasions. For each animal, a freeze frame was obtained from the buffered recording when the animal drank from the feeder; these were exported as .TIF files to Adobe Photoshop. To determine the change in ear movement, the right ear (interventional side) was compared to the left side (control side). Using the imaging software, a line (the ‘inter-canthal line’) was drawn linking the medial canthus of the right and left eyes. Two further lines (the ‘posterior auricular lines’) were drawn parallel to the inter-canthal line, one was placed at the most posterior point of the left auricle and one placed at the most posterior point of the right auricle. The distance was measured, in numbers of pixels, between the two posterior auricular lines and the inter-canthal line (Fig. 2.7). To calculate the ratio difference between the movements of the two ears the following equation was used:

\[
\text{ratio of recovery} = \left\{ \frac{(x-(x-y))}{x} \right\}
\]

Where:  
\(x = \text{distance left ear moved (control)}\)  
\(y = \text{distance right ear moved (intervention)}\)
The results were analysed using GraphPad Prism5 (GraphPad Software Inc., USA). Student’s paired t-test was used to compare the results between the groups and a 2-way analysis of variance (ANOVA) test was used to analyse each individual group.

Figure 2.7 Calculation of ear movement ratio.

A) Pre-operative measurement of both ears using the left ear as the control. The yellow line represents the normal position of the control ear during ear retraction, x is the distance (measured in pixels) from the inter-canthal line. The blue line represents the amount of retraction of the right ear from the inter-canthal line (y).

B) POD01 demonstrates the right ear in a fixed forward position following crush injury of the PAN nerve; the improvement in retraction was expressed as the ratio of recovery with respect to the left ear.
2.5 Results of pilot data

All 25 animals survived for the duration of the study and there were no complications from the surgical procedures. The animals tolerated the video capture sessions without any negative behavioural outcomes and all animals achieved recordings pre-operatively and at POD01, 10, 20 and 30 that captured ten occasions of drinking from the feeder.

Sham Group (Sh, n=5) Animals exhibited a statistically significant reduction in right ear movement at POD01 in comparison to all other POD recordings (P < 0.05). From POD05 onwards, there was no difference between ear movements on either side and there was no significant change in right ear movement during the study period (p = 0.77).

Crush Group (Cr, n=10) There was a significant reduction in right ear movement in comparison to the left ear at POD01, POD10 and POD20 (p < 0.05). At POD30, there was no significant difference between the ear movement on either side (p = 0.35). Two-way ANOVA analysis demonstrated a significant change in right ear movement during the data collection period (p < 0.05).

Excision Group (Ex, n=10) All animals had a significant reduction in right ear movement at all PODs in comparison to the left ear (p < 0.05). Further analysis failed to demonstrate any change in right ear movement during the study period (p = 0.94).

A summary of the pilot data can be seen in Figure 2.8.
The recovery of right ear movement displayed as a ratio in comparison to the left side (control).

**Figure 2.8** Pilot study results of ear recovery following posterior auricular nerve manipulation.

During the study period, the crush group demonstrated a significant recovery in ear movement in comparison to the sham and excision groups. The results suggest that ear movement is a reliable assessment measure of functional outcome following facial nerve surgery.
2.6 Pilot study discussion

The results from the pilot study demonstrated that assessment of ear movement can successfully differentiate between the three experimental groups over the study period. The sham group was selected to demonstrate any unforeseen consequences of surgery in the region of the PAN, such as damage to musculo-skeletal structures which would affect ear movement. Indeed, at POD01 there was a significantly reduced level of movement between the right ear and the contra-lateral control side. Movement returned to normal and no difference was demonstrated in the remaining study period. The fact that ear movement recovery to normal by POD10, suggests that no inadvertent damage has taken place to compromise ear movement. One proposed rationale for this difference is that at POD01 the animal is still recovering from the immediate local sequelae of surgery, such as pain, and that the reduced movement may be unrelated to the nerve. A second explanation is that a slight neuropraxia has developed as a consequence of intra-operative handling of the PAN during surgical dissection. Given this data, future experiments excluded video analysis at POD01 as benefits of analysing this data were minimal.

The excision group served as a negative control to ensure that the PAN was responsible for the assessed ear movement. To ensure there was no inadvertent neurotisation of the muscle, a segmental excision was completed and an end-to-side neurorrhaphy was performed to redirect the PAN to grow along the facial nerve trunk. There was a significantly reduced level of ear movement at all PODs and a failure to demonstrate any level of recovery. The results from this group confirmed that the PAN was responsible for the ear movement being evaluated.
The crush group was included as the outcome of crush injuries have been extensively documented and are predictable. Previous rodent studies have reported that full recovery from crush injuries of the sciatic, tibial, phrenic, and facial nerves can be expected within the range of 21 – 28 days [6, 25, 26, 30], justifying the length of the data collection period for this pilot. This pilot study successfully demonstrated that the crush group recovered to normal function by POD30, with no statistical difference both between the movement of each of the animal’s ears and the movement observed between the crush and sham groups. This result agrees with and confirms previous reports concerning motor recovery following crush injuries. A second important result was that a significant change in movement was observed during the study period. There was a graduated change in the recovery of ear movement at each POD that was statistically significant. This suggests that using this methodology is sensitive enough to track changes in the recovery of movement and not only differences between positive and negative controls.
2.7 Conclusions from the pilot study

The aim of the pilot study was to determine whether ear movement could be analysed to produce a viable outcome measure in facial nerve surgery. The results show that significant differences were demonstrated between the experimental groups and that significant changes within the crush group were identifiable making this an acceptable model to develop as an outcome measure following rodent facial nerve surgery.
2.8 Bibliography:


Chapter 3

Development of an automated technique for establishing axonal counts using serial section electron microscopy

3.1 Introduction

The nervous system has been extensively studied since the advent of microscopy. Great progress was made by Ramón y Cajal in the late 19th century when he described the organisation of the nervous system with remarkable accuracy [1]. Traditional techniques are still frequently used to study the peripheral nervous system as the high-lipid content of myelin is particularly suitable for visualization with lipophilic stains such as toluidine blue. Morphological analysis of the peripheral nervous system (PNS) can supplement clinical assessment and provide important information on various aspects of regeneration [2-4].

Electron microscopy is a particularly useful tool for analysis at an ultra-structure level. Modern electron microscopes are capable of imaging at a resolution of 4nm pixels which allows for vesicles within synapses to be clearly visualised [5] and extensive geometrical parameters can be used to assess nerve fibres [6]. Whilst this is an impressive accomplishment, the resources required to achieve are extensive, restricting this technology to the research environment.

The advent of transgenic species has greatly enhanced our analysis of nerve regeneration in combination with fluorescence microscopy [7]. Since Chalfie
introduced the green fluorescent protein from jelly fish into mice [8], transgenic lines have been created that exclusively express fluorescent protein in axonal tissue (YFP-16 strains) and can be further modified to only express the protein in ~10% of axons (YFP-H) [9]. These transgenic lines have enabled in vivo analysis of axonal regeneration to be visualised with confocal microscopy techniques [10] and has greatly facilitated evaluation of neuromuscular junctions (NMJs) [11-13].

The challenge that the nervous system presents is largely a result of the size and number of cells involved. Although fluorescent imaging of transgenic species can provide a large amount of detail, only descriptive observations regarding the pathways of regeneration can be made. A useful example to consider is a direct repair of a nerve lesion (Fig. 3.1). Several conclusions can be made regarding the observations, whilst taking into consideration that only ~10% of the axons are visible: 1) the axonal behaviour proximal to the neurorrhapy is organised and appears normal, 2) there is a loss of organisation as the axons attempt to regenerate across the neurorrhapy with several axons appearing to escape into the surrounding tissue and 3) distal to the neurorrhapy the axons become more organised. The observer cannot provide any information regarding which axons regenerate successfully or describe any associated behaviour of the successful or unsuccessful axons. If the traditional cross-sectional methods are employed, either using light or electron microscopy, an observer could choose to section the regenerating nerve at a number of areas and be able to provide information at that specific point, however it would be impossible to link the sections together. An analogy to illustrate this is that of a motorcycle racer (Fig. 3.1b-d and Video 3.1). Initially the racer has good form and appears to be in complete control, indeed if a snap-shot is taken at this point, the conclusion would be that he was
**Figure 3.1** A confocal image of a neurorrhaphy with motorcyclist analogy

**A)** A fluorescent confocal image of a YFP-H mouse 6 weeks after nerve repair. The white arrow heads identify the shadow created by the nylon suture used to repair the nerve. The left side of the image is the proximal nerve and the organized structure quickly becomes disrupted at the site of repair; axons appear to spiral out from the nerve into the surrounding tissue. Distal to the repair, order is restored. An analogy can be made with a motorcyclist who almost falls during a race (see Video 3.1).

**B)** If still frames were taken at points **B), C) or D)** and evaluated in isolation, three different conclusions would be drawn illustrating the need to bridge the gap between isolated cross-sectional analysis and confocal fluorescent observations. *(Image reproduced with permission from Woollard, A., UCL PhD Thesis 2017)*
likely to be a good racer. If the video plays for another three seconds, the situation changes completely. The racer has lost complete control and if the observer was to comment, the conclusion would be that the racer would be unsuccessful and crash. Similarly, if the video is allowed to continue, the racer can be seen to avoid falling off the motorbike and get himself back to being upright in his seat, however the motorcycle is now off the track and an observer may conclude that the racer looks fine but is in the wrong location.

To solve this observational problem of snap-shot cross-sectional analysis in isolation, Specific Aim 2 seeks to use the principals of serial section electron microscopy and apply this to peripheral nerve regeneration.
3.2 Application of Serial Section Electron Microscopy to the peripheral nervous system.

Serial section electron microscopy (SSEM) is the process of sequentially imaging sections from a block of tissue. Similar techniques are used in everyday healthcare through computed tomography or magnetic resonance imaging. Recently, the principal has been applied to the study of the connective pathways of the brain using electron microscopy in an attempt to map every connection every cell makes within the brain as outlined by the BRAIN initiative [14, 15]; the term ‘connectomics’ has been coined to describe this [16]. Connectomics presented many challenges that needed to be overcome. Firstly, a new method of processing sections was developed; the Automated Tape-collecting Ultra-Microtome (ATUM) was engineered to accomplish this [17]. This technique incorporates a continuous collection of ultra-thin sections onto a reel of Kapton® tape. Each section is imaged in sequential order, to produce a ‘stack’ of images that can be analysed to examine the cellular processes within the volume of tissue processed. In published datasets [5, 18, 19], 25 nm thick sections are cut and imaged at a pixel size of 20-30 nm - requiring more than 33,000 sections to be collected in order to image 1 mm$^3$ volume of data which is a much smaller volume than analysis of a peripheral nerve; thus in order to analyse this area, several parameters were modified.

3.2.1 Specimen fixation and resin embedding

All animals were maintained and underwent anaesthesia as described in Chapter 2.4.2 and according to the Animal Care and Use Committee guidelines. Ten six-
week old female wild type laboratory mice were selected for this study. After anaesthesia had been confirmed, the animals were trans-cardially perfused with 50 mls of 4% PFA diluted in PBS. A posterior auricular incision was made to expose the root of the facial nerve on each side. A 4 mm section of the facial nerve trunk was harvested from each side and placed in individual 1ml vials containing 4% PFA diluted in PBS for twenty-four hours at 4°C. The nerve specimens (n=20) were post-fixed in 1% osmium tetroxide diluted in PBS for 2 hours at room temperature, and then rinsed three times in PBS for 10 minutes. Dehydration was performed at 4°C while the specimens were agitated on a BioRocker™ (labForce, Thomas Scientific, USA) through a graded series of 20%, 50%, 70%, 90% and 100% ethanol, for 5 minutes at each concentration. Following dehydration, the specimens were placed at 4°C in pure acetone for 15 minutes and then washed with pure propylene oxide twice for 10 minute durations.

To determine the correct hardness of embedding mixture to be used, three different formulas were used. Five samples were embedded in a ‘Hard’ mixture and five in a ‘Soft’ mixture, according to Glauert’s formulas [20]. The EMBed-812 embedding kit (Electron Microscopy Science, USA) was adjusted according to the hardness of the epoxy-resin desired. Embedding began at room temperature with a 1:2 mix (epoxy:propylene oxide) for 1 hour, then exchanged for a 1:1 mix for a further hour before being placed in pure epoxy for 12 hours with the specimens being agitated on a rocker constantly. Next, the specimens were transferred to a fresh mixture of epoxy for one hour prior to being transferred to silicon moulds and baked at 60°C for 24 hours.
3.2.2 Sectioning, post-fixation and carbon coating

Prior to ultra-thin sectioning, all embedded blocks were first semi-thin sectioned at 2µm for light microscopy. The semi-thin sections were placed on glass microscope slides and stained with toluidine blue solution (0.25 g of sodium borate and 0.25 g of toluidine blue O, dissolved in 25 ml of distilled water). This confirmed adequate fixation and osmication of the specimens prior to ultra-thin sectioning for electron microscopy (Fig. 3.2).

All specimens were prepared for ultra-thin sectioning by trimming the block faces to minimise the surface area being sectioned with a trim 20® diamond knife (DiATOME, USA). Ultra-thin sectioning was completed using a UC7 Ultra-microtome (Leica, Germany) in combination with ultra 45° diamond knife and water reservoir (DiATOME, USA) at a section thickness of 40nm; five sections from each sample were transferred to Kapton® 1/4” tape (Electron Microscopy Sciences, USA). All sections were mounted on 100 mm diameter silicon wafers (Electron Microscopy Sciences, USA) which are flat, conductive and vacuum safe with double-sided conductive carbon tape. To enhance the signal for electron microscopy, the sections were post-stained with 2% uranyl acetate dissolved in deionised water for two minutes and rinsed with 18.2 MΩ deionised water, and secondarily rinsed with Photo Flo 200 solution (Electron Microscopy Sciences, USA) and then dried with an air gun to prevent the formation of water marks. To improve the contrast, a secondary post-stain was applied using lead citrate (5 mg of lead citrate in 1 ml of sodium hydroxide) for two minutes, rinsing with distilled water and Photo Flo as described above.

To prevent the sections from becoming electrically charge during electron microscopy imaging they must be grounded. To achieve this, the sections must be coated in a thin film of carbon using a thermos-evaporator (Auto 306,
Figure 3.2  Light microscopy assessment of sections using Toluidine blue stain.

A) The overall structure of the facial nerve was sufficiently preserved during fixation and osmication.

B) Higher magnification demonstrates sufficient myelin staining
Edwards, United Kingdom). Carbon fibre cord was placed between the two electrodes, and the wafer-mounted specimens were inserted beneath the cord into the vacuum dome until a vacuum of $8 \times 10^{-5}$ Torr was achieved. Current was applied across the carbon ribbon until the wafer was finely coated.

### 3.2.3 Scanning electron microscopy imaging

Once the specimens had been prepared for imaging they were transferred to a Magellan 400L scanning electron microscope (FEI, USA). Sections were imaged individually utilising the auto-focus and auto-contrast inbuilt functions. The image parameters used were 50nm pixel size, 200ns dwell time, 12,000 x 12,000 pixel field of view (12k x 12k FOV) and a working distance of 6mm (7.0 kV @ 26nA).

### 3.2.4 Analysis of optimal resin mixture

The two different mixtures of epoxy-resin were evaluated using the same (SEM) imaging parameters. The harder resin produced far more wrinkles in the sections and occasionally caused the tissue to shear from the block during sectioning (Fig. 3.3). In contrast, the softer resin consistently produced fewer wrinkles and no difficulties were experienced during sectioning with shearing.

In both groups, there was no noticeable difference between the contrast and resolution of the images acquired in areas unaffected by wrinkling. The decision to use the softer epoxy-resin formula was made.
Figure 3.3  
Sectioning difficulties encountered with the use of ‘hard’ resin formulas.

Once thin sections were cut, distortion with excessive wrinkling was encountered necessitating a change to using ‘soft’ epoxy-resin formulas.

A) Low magnification
B) Higher magnification
3.3 Establishing ideal imaging parameters

SSEM imaging of the PNS has different obstacles in comparison to the CNS. The CNS has far more inter-cellular connections and finer ultra-structure, however published datasets report studied volumes equivalent to 0.13 mm$^3$ and thus, with current imaging modalities using a resolution of 5 nm pixels and a section thickness of 50 nm, one cubic millimetre of tissue equates to 20,000 sections, 40 Gigapixels per image and a data volume of 800 TB [5]. This is an impractical size requiring computational power beyond most research capabilities. Fortunately, one of the attractions of the PNS, and in particular axons, is that this level of detail is not required but conversely, a larger volume of data is required, which presents a new challenge.

To capture a data-set required to study nerve regeneration, a required minimum length of 3 mm of nerve tissue was anticipated. This was based on the work examining interscutularis muscle and nerve repair of Woollard and Lu (Fig. 3.1) [21], and deemed to be sufficient to capture an entire field capturing an entire a neurorrhaphy. The cross-sectional diameter of the facial nerve trunk from the sectioned samples measured between 0.9 – 1.1 mm (Fig. 3.4a) and methods were proposed: firstly, to image the data with the same, reliable and proven parameters as the CNS or secondly, adapt them to the PNS. Taking these factors into consideration, the volume calculations of the data were performed with the assumption that a nerve can be viewed a cylindrical object and thus $volume \ (V) = \pi r^2 h$ . The minimum volume of data acquisition would be 9.4 mm$^3$ exceeding the largest volume recorded of 0.13 mm$^3$. Two parameters were identified as key
Figure 3.4 Establishing the ideal imaging parameters.

A) Cross-section of the facial nerve trunk containing ~1200 axons.

B) 50nm thick sections C) 100nm thick sections and D) 150nm thick sections; sections thicker than 100nm had reduced resolution and diminished overall quality.

E) 50nm sized pixels F) 100nm sized pixels and G) 150nm sized pixels; the rapid drop off above 100nm pixels can clearly be seen.

H) 50ns dwell time (DT) I) 100ns DT and J) 200ns DT; no appreciable difference was observed between 100ns and 200ns.

The parameters chosen were 100nm thick sections, 100nm sized pixels and 100ns DT.
components in solving the volume problem: resolution size (pixels) and section thickness.

3.3.1 *Optimal thickness of epoxy-resin sections.*

As the previous experiments had demonstrated superior results with the softer epoxy-resin samples, these were chosen for subsequent studies. To examine the impact that changing the thickness of sections had on the quality of image acquired, all imaging parameters were kept constant as reported in Chapter 3.2.3. Twenty sequential sections were prepared at the following thicknesses: 50 nm, 70 nm, 90 nm, 100 nm, 150 nm and 200 nm. In electron microscopy, the best images are produced with the thinnest sections as these require the least stressful environment within the column for the electron beam to operate [22]. If thicker sections are used, greater beam power is required to produce a similar image at the cost of both resolution and potential damage to the material being imaged. To compensate for this, sections can be coated in thicker layers of carbon, but at the expense of a lower level of resolution. With this in mind, the aim was to establish how thick a section could be cut without sacrificing the information required to assess axonal regeneration.

Image analysis demonstrated that a 100 nm section produced results sufficient to yield adequate morphological data regarding nerve regeneration [23]. Larger thickness sections distorted myelin and made further segmental analysis more challenging whilst benefits of thinner sections were not applicable to this study as super-high resolution was unnecessary and would increase the datasets exponentially (Fig. 3.4 b-d).
3.3.2 Optimal pixel size

To acquire a cross sectional image two techniques can be used; to capture an image in one field-of-view or to take multiple smaller fields-of-view (FOV) and join these together as a montage. The benefits of being able to capture the specimen in a single FOV results in smaller data sets, however the disadvantage of increased processing time as stitching images to create a montage is time consuming and requires complex algorithms and computational power. Keeping all other imaging parameters constant (see Chapter 3.2.3) the pixel size was increased at staggered intervals from 50 nm to 200 nm to evaluate the ideal pixel size for data acquisition. The results demonstrated no appreciable differences when increasing the pixel size to 100 nm however, any increase greater than 100nm produced a drop in sharpness (Fig. 3.4e-g)). The maximum FOV of the Magellan SEM is 12k x 12k pixels and using a pixel size of 100 nm resulted in capturing a specimen measuring 1.2 mm in diameter in one image, exceeding the dimensions of the nerve specimens and negating the requirement on stitching images together to create a montage and therefore this was chosen as the preferred imaging modality.

3.3.3 Optimal dwell time

The final parameter that can be altered to change the speed of image acquisition is the dwell time (DT). This equated to the time the electron beam spends on each pixel. Similar to the above parameters, an increase in speed is at the expense of quality. For example, a 500 ns DT would take 72 seconds to image a 12k x 12k image, in comparison to 14 seconds if a 100 ns DT was used. For a single image the shorter DT may not be considered an appropriate trade-off for
the level of detail sacrificed, however, when imaging 150 sections on a single wafer, 500 ns DT would take 3 hours, in comparison to 36 minutes with 100 ns DT; this similarly becomes exponential when datasets are in the region of 10,000 sections.

Using 100 nm thick sections and a pixel size of 100 nm, the DT was decreased from 300 ns to 50 ns (Fig. 3.4h-j). Although the quality of the signal obtained with 50 ns DT in comparison to 200 ns was adequate for axonal assessment, the rate-limiting step was actually due to the auto-focus and auto-contrast inbuilt functionality of the SEM; this took approximately 25 seconds per section. The overall opinion was that the better image quality obtained at 100ns was the best compromise between speed and quality.

3.3.4 Pilot data capture of serial sections of facial nerve trunk

Having established the ideal parameters for sectioning and data acquisition, the next stage was to collect pilot data sets to train the computational segmentation algorithms. Five samples were embedded in the soft epoxy-resin and serially sectioned using the ATUM-tome system [17], collecting 300 sections per sample on Kapton® tape. In a similar fashion to the methods described in Chapter 3.2.2, the sections were mounted on silicon wafer, with each wafer holding 150 sections (Fig. 3.5). Using a modification of the technique described by Hayworth et al. [24], MATLAB coding was adjusted to accommodate the Magellan microscope. Firstly, the wafer required four copper reference finder TEM grids fiducials (style H6, Ted Pella, USA) of known precise dimensions to be added to the wafer to
Figure 3.5  Overview of the preparation of section for SEM imaging

A) The Automated Tape-collecting Ultra-Microtome (ATUM-tome)
B) Sections collected on Kapton tape and mounted on a 100mm silicon wafer with four fiducials to facilitate mapping
C) Thermo-evaporator (Edwards Auto 306)
D) Magellan 400L scanning electron microscope (FEI, USA)
spatially orientate the SEM and act as the points of a compass. The wafer was photographed against a background of known dimensions and orientation to ‘normalise’ the wafer in order to create an image that could be used by the SEM as a ‘Wafer Map’ (Fig. 3.6) [17]. The wafer could now be inserted into the chamber of the SEM and the system primed for data acquisition. The code written and modified in MATLAB communicated with the x,y stage axes of the microscope so that each section could be assigned co-ordinates so image acquisition of each section was then automated, allowing for the data capture of an entire wafer to run independently of an operator. The time taken for imaging each wafer (n=10, 150 sections per wafer) was approximately 160 minutes and broken down as follows:

- System loading and priming 10 minutes
- Wafer mapping 45 minutes
- Data acquisition 100 minutes
- System venting 5 minutes

The ATUM-tome cannot control the orientation of specimens as they are collected from the water reservoir onto the Kapton® tape and therefore the image stacks require alignment adjustment [25]. To achieve this, non-affine alignment was accomplished using the FijiBento alignment package allowing large data-sets to be aligned over a relatively short period ([https://github.com/Rhoana/FijiBento](https://github.com/Rhoana/FijiBento)) [18, 26]. Alignment was achieved using the Odyssey cluster supported by the Faculty of Arts and Science, Division of Science, Research Computing Group at Harvard University.
Figure 3.6  Wafer preparation and post-staining.

A) Normalised wafer image with approximately 150 sections used for wafer mapping.

B) Post-staining with lead citrate to improve the contrast during image acquisition
The aligned data sets were prepared for segmentation according to two methods; manual and automated. For manual segmentation, the images were loaded into an open-source program, VAST Lite (https://software.rc.fas.harvard.edu/lichtman/vast) created for volume annotation and segmentation. Ten sequential images from each nerve sample were selected and manually labelled to generate axonal counts. For the automated segmentation, the same methodology employed by Kaynig et al. [5] was used to focus on segmentation of cell membranes. Briefly, five images were chosen as a training set using the TrakEM2 ImageJ plugin (https://imagej.net/TrakEM2) with random forest classification being applied [27]. This process was required to account for the variability in the data, such as changes in image focus, contrast or image thickness, and allowed the user to manually correct the segmentation and the identification of axons. The interactively trained classifier was then applied to each data set for analysis (Fig. 3.7).

To assess the accuracy of the axonal counts generate by automated segmentation the results for each data set were compared to the manual counts. Three high-school summer students were trained using the VAST Lite software and asked to manually segment and produce axon counts for the five data sets; all students were blinded to the axonal counts produced by automated segmentation. There were no significant differences demonstrated between the manual counts and the automated counts, thus confirming that automated segmentation is capable of producing reliable axonal counts supporting other reported studies [5, 18, 19, 28-31].
Figure 3.7  The post-processing pipeline.

Once the images had been acquired and aligned, a small data set (10 section) was used to train random forest classifiers to segment using membrane classification. After four repeats of training, the algorithm could be applied successfully to the whole data volume to automatically segment the data and provide accurate axon counts.
Having established the accuracy of the segmentation algorithm it was applied to each data set for the facial nerve samples. The segmentation process assigns a unique colour to each axon to allow the pathway of each axon to be followed through the volume (Fig. 3.8, Video 3.2).
Figure 3.8  Fully automated segmentation of the facial nerve trunk.

The segmentation algorithm applies a unique colour code to each axon allowing each structure to be traced through the volume of tissue sectioned (see Video 3.2)
3.4 Discussion

To report on outcomes of nerve regeneration, an understanding of the changes taking place at an axonal level must be accounted for. With the advent of transgenic animals, observation of axonal regeneration has been revolutionised and the understanding of axonal behaviour during the regenerative processed has vastly increased. However, traditional histological techniques have not been entirely replaced as details seen at a cellular level are equally important.

Traditional cross-sectional analysis of the nervous system cannot evaluate changes occurring along the length of a regenerating nerve. In an attempt to bridge the gap between in vivo confocal fluorescence imaging of transgenic species and traditional cross-sectional analysis of the nervous system, the principals of serial section electron microscopy have been applied for the first time to the extra-cranial nervous system.

To be able to produce viable data sets, the methodology used to process cortical tissue was adapted to suit the extra-cranial nervous system. Although the level of cellular detail required is far inferior, the volume of tissue which requires processing is much greater, presenting a different size problem. To overcome this challenge, imaging parameters were adapted to process large tissue volumes by increasing pixel size and section thickness. Whilst imaging 100nm sized pixels is not using the SEM to the extent of its capability, the ability to automate the image capture of hundreds of sections mounted on wafers and capture the entire cross-section of a nerve efficiently and accurately streamlines this process.
Semi- and fully-automated nerve analysis is not a new concept [5, 28-31]. To be able to apply a fully automated segmentation tool to a nerve sample opens up the ability to track the regenerative process through a volume of tissue and produce three dimensional renderings of each axon within the volume [19]. This would allow for the analysis of associated behaviours of successfully regenerating axons in comparison with unsuccessful ones, which is currently not possible.
3.5 Conclusions from the pilot study

This pilot study has successfully demonstrated that SSEM can be applied to the extra-cranial nervous system. Fully automated segmentation algorithms can produce accurate axonal counts that can be used as an assessment of nerve regeneration through a large volume of tissue. By applying this technique to nerve regeneration the limitations of both confocal microscopy and traditional cross-sectional analysis can potentially be circumvented.
3.6 Bibliography.


Chapter 4

Evaluating the impact the axonal count of an autologous graft has on functional nerve regeneration

4.1 Introduction

The goal of facial reanimation surgery is to achieve a symmetrical spontaneous smile; the gold-standard technique used to achieve this is a two-stage procedure [1-3]. The first stage involves identifying a distal buccal branch of the functioning contra-lateral facial nerve and coapting this to an autologous cross-facial nerve graft (CFNG) that is tunnelled subcutaneously and banked in the pre-tragal region of the paralysed side. The second stage is performed after a minimum period of six months to permit adequate regeneration across the CFNG. A muscle is chosen to be transplanted into the paralysed side of the face and arterial and venous anastomoses are completed to restore perfusion of the transplanted muscle; next a neurorrhaphy is completed to connect the CFNG to the original nerve that supplied the muscle. Ideally after a period of 6 – 18 months, the transplanted muscle will become fully re-innervated by the contra-lateral buccal branch of the facial nerve via the CFNG and spontaneous symmetrical movement will be restored.

An excellent result is obtained in 60% of cases reported in the literature [2, 4-7]. Several studies have looked at the individual components that have the potential to have an impact on the outcome obtained by simulating the process in animal models. Particularly pertinent to the aims of this thesis are the results obtained
from using different nerves to act as donors for the transplanted muscle. Early work demonstrated that not every axon successfully regenerates across a neurorrhaphy [8-13], suggesting that a donor nerve with higher axonal counts would yield better results. It has been demonstrated in both animal and human studies that muscle contractility is increased when a donor nerve with a higher axonal count is chosen [14-17].

The purpose of this study was to investigate whether this principal can be applied to the CFNG (Specific Aim 3). To achieve this, the following hypothesis was assumed: autologous nerve grafts with higher axonal counts will yield a better outcome than grafts with lower counts. The rationale for this hypothesis is that an autologous graft with a higher native axonal count will offer a greater concentration of nerve growth factor (NGF), Schwann cells and neurotrophins (NT), as well as offer more structural channels to regenerate along.
4.2 Materials and Methods

4.2.1 Animal selection

As a continuation of the animal model developed in Chapter 2, six-week old YFP-16 female rats used for the experiments. The transgenic line was bred in-house and maintained according to local institutional guidelines. Experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) PHS Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act (7 U.S.C. et seq.), and an animal use protocol approved by the Standing Committee on the Use of Animals in Research and Training of Harvard University (Protocol 24-08). Genetically identical strains control the influence a genotype has on experimental outcomes and ensuring all animals were six-weeks old and female further controlled unwanted variables. YFP-16 transgenic mice were chosen for the axonal expression of YFP fluorescence that facilitated surgical dissection.

4.2.2 Selection of high myelinated axon count and low myelinated axon count autologous nerve grafts

To determine whether the axonal count of an autologous nerve graft affected nerve regeneration, it was important to select two nerves that reliably had a different axonal count. To accomplish this, ten female six-week old YFP-16 mice were selected for anatomical study. The animals were euthanized in a CO₂ chamber and death was confirmed via thoracotomies. The animals were perfused with 50 mls 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) via the left cardiac ventricle.
Following de-epithelialisation, a longitudinal incision was made over the posterolateral aspect of the proximal hind-limb. The subcutaneous tissue and biceps femoris muscle was split to expose the sciatic nerve trunk. The sciatic nerve was dissected out under the guidance of a fluorescent macro-zoom microscope (Axio Zoom, Zeiss) and using a Castroviejo surgical calipers (Fine Science Tools, USA), a 4 mm segment of the nerve was excised and placed in 4% PFA solution diluted in PBS. Using the same incision, the common peroneal nerve was isolated, skeletonised and a 4 mm segment of the nerve was excised and placed into 4% PFA solution. The same procedure was performed on the contra-lateral side and in total, 20 sciatic nerve (ScN) samples and 20 common peroneal nerve (CPN) samples were harvested. Using the same animals, bilateral posterior auricular incisions were made and dissections of the PAN nerve were completed (as described in Chapter 2.2). 4 mm sections of the PAN were harvested from each side and fixed in 4% PFA solution (n=20).

Following fixation, all samples were stained with 1% osmium tetroxide (OsO₄), serially dehydrated and embedded in epoxy-resin according to the protocol detailed in Chapter 3.2. Each block was prepared and 10 sections from each specimen were sectioned and prepared for imaging and analysis as described in Chapter 3.3. Automated axonal counts demonstrated that the PAN (n=20) had a mean axonal count of 947 axons, the ScN (n=20) had mean count of 2616 axons and the CPN (n=20) had a mean count of 1593 axons; these were significantly different to each group (Fig. 4.1).
Axon counts of the Posterior Auricular Nerve (PAN), Sciatic Nerve (ScN) and Common Peroneal Nerve (CPN)

Figure 4.1 Comparative axon counts of the PAN, ScN and CPN

The sciatic nerve (ScN) had a mean axon count of 2616 which was significantly higher than the mean for the common peroneal nerve (CPN) which was 1593. Both nerves had a significant higher axon count then the posterior auricular nerve that had a mean number of 947 axons.
The results of the automated axonal counts confirmed that there were significant differences between the ScN and CPN. Importantly, the axonal count for the PAN was lower than both of the other groups. The lower axonal count of the PAN avoids the possibility of bottleneck forming [18] where axons from the PAN would not have enough channels to regenerate through the graft.

4.2.3 Experimental design

Having established suitable nerves to use as autologous grafts animals were allocated into three groups requiring a total of 150 animals and 25 donor animals to provide the ScN (n=50) and CPN (n=50) nerve isografts:

1. Direct Nerve Repair (DNR, n=50)
2. Small Nerve Graft (SNG, n=50)
3. Large Nerve Graft (LNG, n=50)

Using the methodology described in Chapter 2, the restoration of ear movement would be used as an outcome measure of nerve regeneration. To compensate for the anticipated delay in clinical outcome, video recordings were collected over a 48-week period at the following post-operative week (POW) intervals:

1. Pre-intervention
2. POW01
3. POW03
4. POW06
5. POW12
6. POW24
7. POW48
Following surgical intervention, ten animals were allocated to a end-study time-point per intervention group. At each POW interval, each animal had their outcome assessment recorded. At the end of each animal’s study period, after completion of video recording, the animals were euthanized and the PAN graft was harvested and prepared for SEM. Therefore at each interval, the number of animals in each group decreased by ten. A summary for the organisation of the experimental groups can be seen in Figure 4.2.

4.2.4 Surgical technique

Before and after surgery, all animals were allowed access to food and water ad libitum. All animals were anaesthetized with an intra-peritoneal injection of ketamine and xylazine (87 mg/kg of ketamine and 13 mg/kg of xylazine, dose = 0.1ml/20g). Once adequate anaesthesia was confirmed the area surrounding the right auricle was prepared for surgery by removing the fur around the surgical field with application of Nair™ (Church & Dwight Co., USA) for 3 minutes. The surgical site was prepared using 70% ethanol solution. A 15 mm curvilinear incision was made extending from the posterior auricular region to the angle of the mandible. Surgical dissection proceeded as described in Chapter 2.2 using a fluorescent operating microscope to expose and define the PAN. In all groups the nerve was dissected freely and identification of the nerve was confirmed using a nerve stimulator (Vari-Stim® III, Medtronic, Xomed Inc.). The donor animals were euthanized using intra-peritoneal administration of sodium pentobarbital (432 mg/kg) and death confirmed by bilateral thoracotomies.

Direct Nerve Repair (DNR, n=50): After identification, the right PAN was sharply divided 3 mm from the origin of the facial nerve trunk using microsurgical
The study period ended at the time point when axon counts were made as the were transcardially perfused with 4% Paraformaldehyde solution. Each EMR recording was matched to individual animals.

**Figure 4.2**  The overview of the experimental groups and the intervention time points.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Description of Intervention</th>
<th>EMR¹</th>
<th>Axon Count²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isograft Donor (n=25)</td>
<td></td>
<td>Required for harvesting sciatic (ScN, n=50) and common peroneal (CPN, n=50) nerves for use in graft experimental groups</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ScN (n=50)</td>
<td></td>
<td></td>
<td>POW01,03</td>
<td>POW03</td>
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<tr>
<td>CPN (n=50)</td>
<td></td>
<td></td>
<td>POW01,06</td>
<td>POW06</td>
</tr>
<tr>
<td>Direct Nerve Repair (n=50)</td>
<td></td>
<td>Direct nerve repair following division of right PAN.</td>
<td>POW01,12</td>
<td>POW12</td>
</tr>
<tr>
<td>DNR03 (n=10)</td>
<td></td>
<td></td>
<td>POW01,24</td>
<td>POW24</td>
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<td>DNR06 (n=10)</td>
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<td>POW01,48</td>
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<td>DNR48 (n=10)</td>
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<tr>
<td>Small Nerve Graft (n=50)</td>
<td></td>
<td>Interposition of CPN graft into the divided right PAN</td>
<td>POW01,03</td>
<td>POW03</td>
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<tr>
<td>SNG03 (n=10)</td>
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<td>POW01,06</td>
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<tr>
<td>Large Nerve Graft (n=50)</td>
<td></td>
<td>Interposition of ScN graft into the divided right PAN</td>
<td>POW01,03</td>
<td>POW03</td>
</tr>
<tr>
<td>LNG03 (n=10)</td>
<td></td>
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¹ EMR - Ear movement Recovery video analysis
² Indicates end point of experimental group
dissecting scissors (Fig. 4.3c). The nerve was repaired using two epineural 11-0 Ethilon® sutures (Ethicon, USA) at the 12 and 6 o’clock positions (Fig. 4.3d). The surgical field was irrigated with 10mls of normal saline solution (0.9% NaCl).

**Small Nerve Graft (SNG, n=50):** The common peroneal nerve was prepared as described in Chapter 4.2.2 (Fig. 4.3a). Castroviejo surgical calipers (Fine Science Tools, USA) were used to excise a 4 mm segment of the nerve and one 11-0 Ethilon® suture (Ethicon, USA) was used to mark the proximal end of the graft and it was set aside in saline soaked gauze. The dissection proceeded to identify the right PAN of the experimental animal and it was transected 3 mm distal to the origin of the facial nerve trunk using microsurgical dissecting scissors, in the same fashion as described in previous groups. The graft was reversed to improve prevent regenerating axons from being misdirected down branches [19] and inset into the divided PAN using two epineural 11-0 Ethilon® sutures (Ethicon, USA) at the 12 and 6 o’clock positions at both ends of the graft. The surgical field was irrigated with 10mls of normal saline solution (0.9% NaCl) (Fig. 4.3e).

**Large Nerve Graft (LNG, n=50):** The sciatic nerve was prepared as described in Chapter 4.2.2 (Fig. 4.3b). Castroviejo surgical calipers (Fine Science Tools, USA) were used to excise a 4 mm segment of the nerve and one 11-0 Ethilon® suture (Ethicon, USA) was used to mark the proximal end of the graft and it was set aside in saline soaked gauze. The dissection proceeded to identify the right PAN of the experimental animal and it was transected 3 mm distal to the origin of the facial nerve trunk using microsurgical dissecting scissors, in the same fashion as described in previous groups. The graft was reversed and inset into the divided PAN using two epineural 11-0 Ethilon® sutures (Ethicon, USA).
at the 12 and 6 o’clock positions at both ends of the graft. The surgical field was irrigated with 10mls of normal saline solution (0.9% NaCl) (Fig. 4.3f).

In all animals, the surgical incision was closed using 5-0 Vicryl Rapide™ (Ethicon, USA) interrupted sutures. All animals received a subcutaneous injection of 0.5ml of warmed 0.9% NaCl and a subcutaneous injection of buprenorphine (0.05mg/kg) for post-operative pain control. Animals were recovered from anaesthesia in a heated chamber (32-36°C) with 95% oxygen and observed continuously until they become sternally recumbent, in control of their airways and could eat and drink on their own. Experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) PHS Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act (7 U.S.C. et seq.), and an animal use protocol approved by the Standing Committee on the Use of Animals in Research and Training of Harvard University (Protocol 24-08).

Following surgery, mice were allocated within their overall groups (DNR, SNG, LNG) to sub-groups to account for the POW they would be sacrificed to harvest the nerve biopsies. To achieve this, the mice were maintained in labelled cages (up to a maximum of four per cage) identifying the group they were part of, including at which POW euthanasia was planned along with the date of surgery. Toe clipping was used to identify individual animals in each subgroup.
Figure 4.3  The surgical technique for the interventional groups.

A) The exposure of the common peroneal nerve (CPN). The red lines indicate the 4mm segment used for the small nerve graft group (SNG)
B) The exposure of the sciatic nerve (ScN). The red lines indicate the 4mm segment used for the large nerve graft group (LNG)
C) The divided posterior auricular nerve (PAN)
D) Neurorrhaphy of the PAN in the direct nerve repair group (DNR)
E) The interposed CPN in the SNG group (between red arrow heads)
F) The interposed ScN in the LNG group (between red arrow heads)
4.2.4 Clinical outcome measure of nerve regeneration using the restoration of ear movement as an assessment tool.

Following the successful development of an outcome measure in Chapter 2, the same methodology was used with some minor alterations. For a comprehensive explanation of the methodology please refer to Chapter 2.4.3 as only a brief description is provided here. In summary, all animals in this experiment had video recordings taken pre-intervention. The study period was lengthened to take into consideration the anticipated increased time required for nerve regeneration; therefore, the sampling intervals were changed to POW 1, 3, 6, 12, 24, 48. During each session, each animal was recorded drinking from a water feeder on ten occasions per session; previous investigations had confirmed that the PAN was responsible for ear retraction in a measurable quantity. In the crush group, the recovery of ear movement was significant over the study period and the conclusion made from the pilot data was that this was a reliable assessment tool for measuring the regenerating nerve. All surgical manipulations were performed on the right ear, with the left ear serving as a control for normal ear movement.

One minor modification to the reporting of the data was changed from using a ratio of recovery, to using percentage recovery. This in no way altered the measurements taken but was designed to only take into account the posterior movement of the ear a mouse exhibits whilst drinking from the water feeder (Fig. 4.4). In order to calculate this, the assumption was made that at the pre-intervention recording of ear retraction was the maximum, i.e. 100%, and that at POW01 the amount of ear retraction was the minimum, i.e. 0%. After this any movement could be displayed as a percentage of recovery and the following calculation was used:
Figure 4.4  Ear movement recovery index (EMRI) measurements.

A) Measurements from pre-operative recordings were used to measure the maximum expected ear movement ($x$).
B) Measurements were taken at POW01 to record the minimum movement ($y$), prior to appreciable nerve regeneration.
\[
Percentage\ Recovery = 100 \times \left[ \frac{POWx - POW01}{1 - POW01} \right]
\]

Where:

\(POWx\) is the post-operative week being analysed with the value being the amount of ear retraction, measured in pixels.

Although both methods of reporting the results are directly in proportion to each other, displaying the data graphically was improved. All other parameters of the data collection methodology used in the pilot study were kept the same. To increase the data capture process, an additional two recording suites were fabricated, as described in Chapter 2 so data could be collected from three mice synchronously.

4.2.5 Nerve biopsies and calculation of axonal counts

Having established that SSEM can be applied to the extra-cranial nervous system and provide fully automated axon counts, the same methods were applied for these experiments. With the exception of POW01, ten allocated mice were selected from each of the three groups (n=10 per group) following the video recordings of ear movement at each POW (3, 6, 12, 24 and 48). The selected mice were anaesthetised as per the methodology described in Chapter 2.4.2. Euthanasia was confirmed by performing bilateral thoracotomies and the animals were trans-cardially perfused with 50 mls of 4% PFA diluted in PBS. An incision using the previous scar was made to expose the previous surgical manipulation site of the PAN. An 8 mm section of the nerve was harvested ensuring 2 mm of proximal and distal PAN were included. The nerve specimens were placed in 1
ml vials containing 4% PFA diluted in PBS for twenty-four hours at 4°C and labelled as follows:

**Direct nerve repair group** - DNRxx/yy

**Small nerve graft group** - SNGxx/yy

**Large nerve graft group** - LNGxx/yy

Where ‘xx’ was the POW and ‘yy’ was the mouse number (1-10). This process was repeated at POW 3, 6, 12, 24 and 48 with ten animals being euthanised at each time point and nerve specimens were harvested, so that each animal’s ear movement recovery (EMR) was matched to a specimen.

The nerve specimens were processed for SEM as detailed in Chapter 3.2. To determine how axons regenerated through a nerve graft, analysis was planned at several points within the specimens. In the DNR group, 100 sections proximal and 100 section distal to the site of nerve repair were collected. For both graft groups, 100 sections proximal to the nerve graft, 100 sections within the nerve graft and 100 sections distal to the nerve graft were collected to assess how many axons successfully crossed a neurorrhaphy; a schematic can be seen representing this process in Figure 4.5. Each of the 100 section sets collected were mounted onto individual wafers and labelled as follows:

For the DNR group, two wafers were made for each time point and labelled as DNRxx/yy/p or DNRxx/yy/d where xx was the POW, yy was the animal identifier, p indicated sections proximal to the nerve repair, and d indicated they were distal to the nerve repair. For the LNG and SNG specimens, three wafers per specimen
Figure 4.5  Illustration demonstrating the regions sampled during sectioning (red vertical lines represent 100 sequential sections, blue lines represent location of neurorrhaphy).

A) DNR group. Sections were made proximal (PAC) and distal (DAC) to the neurorrhaphy
B) SNG group. Sections were made proximal to the graft (PAC), within the graft (GAC) and distal to the graft (DAC).
C) LNG group. Sections were made proximal to the graft (PAC), within the graft (GAC) and distal to the graft (DAC).
were created and labelled LNGxx/yy/p, LNGxx/yy/g and LNGxx/yy/d where xx is the POW, yy is the animal identifier, p indicated sections proximal to the nerve graft, g indicated sections from within the graft itself and d indicated they were distal to the graft. The same labelling was used for SNG specimens.

The wafers were post-stained and prepared for SSEM according to the methodology employed in Chapter 3.2. Similarly, the acquired data was aligned and auto-segmented to produce the axon count for each of the mini-stacks of sections.

Axon counts were made representative of where the sections were collected from within the biopsy specimen. In the DNR group, axon counts proximal to the neurorrhaphy were labelled ‘Proximal Axon Count (PAC)’ and distal axon counts were labelled (Distal Axon Counts (DAC)’. In the SNG and LNG groups, axon counts proximal to the graft were labelled ‘PAC’, axon counts from within the graft were labelled ‘Graft Axon Count (GAC)’ and axon counts distal to the nerve graft were labelled ‘DAC’ (Fig. 4.5).

Once axon counts had been obtained, the proportion of axons to successfully regenerate across a neurorrhaphy was calculated as a ‘Success Ratio (SR)’. The ‘Total Success Ratio (TSR)’ was the ratio of axons to regenerate across the whole specimen and calculated by, $TSR = \frac{DAC}{PAC}$
In the DNR group, in order to successfully regenerate, axons were only required to traverse one neurorrhaphy and therefore the TSR was the same as the SR. In the SNG and LNG groups, two neurorrhaphies were involved and therefore $SR_1$ was used for the ratio of axons successfully regenerating across the first neurorrhaphy into the graft and $SR_2$ was used for the ratio of axons successfully regenerating across the second neurorrhaphy from the graft and into the PAN,

$$SR_1 = \frac{GAC}{PAC} \text{ and } SR_2 = \frac{DAC}{GAC}$$
4.3 Results

All animals survived the initial surgery and no post-operative complications were encountered during the study period. Two animals were found dead in their cage during daily monitoring; one from the SNG group at POD75 (SNG12/03) and one from the LNG group at POD193 (LNG48/09), both appeared to result from complications of abdominal tumours.

4.3.1 Clinical outcome measure of ear movement recovery

The overall trend was that all groups demonstrated significant increase in ear movement recovery (EMR) during the study period (Appendix 1.1). All animals were successfully recorded drinking from the water feeder on ten occasions at each time interval, no malfunction of the recording suite was encountered. For all groups the greatest rate in EMR improvement was seen up to POW12, after which all groups failed to demonstrate any significant improvement. The best recovery at all POWs was seen in the DNR group, achieving 77% (SD13%) of baseline movement at POW24, followed by the LNG group, 49% (SD13%) at POW48 and the worst recovery was the SNG group, 30% (SD 13%) at POW12 (Fig. 4.6).

Trends within each group were analysed. Within the DNR group, paired t-tests were used to determine differences over the intervention periods during the study and confirmed significant improvement up until POW12 after which no significant change was demonstrated. Within the LNG group, there was a significant improvement up until POW12 and then recovery plateaued. Within SNG group, the only significant improvement was demonstrated between POW06 and
Recovery of ear movement following manipulation of the Posterior Auricular Nerve

**Figure 4.6** Comparison of ear movement recovery in all study groups

*Signifies a significant difference between groups (p<0.05)*
POW12. To determine whether time (measured in POW) and intervention group had a significant impact on recovery, two-way ANOVA testing was undertaken; both time and intervention group had a significant result. A summary of the results can be seen in (Figure 4.6).

The outcomes of EMR in the DNR group were significantly better at all time points than both the SNG and LNG groups. There were no significant differences between the SNG and LNG groups until POW12, after which the LNG group demonstrated a significantly better EMR than the SNG.

### 4.3.2 Axon counts

All groups demonstrated a significant increase in axon counts during the study period. The highest axonal counts were seen in the DNR group, followed by the LNG groups and the lowest in the SNG group (Appendix 1.2)

**Direct nerve repair:** The proximal axonal counts (PAC) were obtained from the sections proximal to the neurorrhaphy at all time points. There were no significant differences in the PAC axon counts at any time point and there were no significant differences between axonal counts of the PAC biopsies (1087, SD 211) in comparison to PAN biopsies (1039, SD 230) previously reported earlier in this chapter. Distal axon counts (DAC) were calculated from the sections distal to the neurorrhaphy. There was a significant increase between POW03 and POW12 and then no significant increases in axonal counts were demonstrated. At all recorded intervals, the DAC was significantly lower than the PAC.
The TSR was calculated for each time interval (Fig. 4.7). The highest TSR was recorded at POW12, indicating that 80% of axons had regenerated across the neurorrhaphy by this stage. Although the TSR was slightly lower for the remaining time interval, this was not statistically significant.

**Small nerve graft:** There were no significant differences in the PAC at any time interval within the SNG group or between the SNG and DNR groups. Between POW03 and POW12 there was a significant increase in the number of axons within the graft (GAC), however after this interval, no significant change was demonstrated. A similar pattern was observed in the DACs, where there was a significant increase up to POW12 and no significant change after this interval (Fig. 4.7).

A maximum of 64% of axons successfully regenerated across the proximal neurorrhaphy at POW12 (SR1 = 0.64, SD 0.07). This level was maintained throughout the remainder of the study period. Similarly at POW12, a maximum of 59% of axons successfully regenerated across the second neurorrhaphy (SR2 = 0.59, SD 0.01) with no significant change following this time interval. A maximum of 38% of axons regenerated across both neurorrhaphies, recorded at POW12 (TSR = 0.38, SD 0.04). Overall, in comparison to the DNR group, the TSR for the SNG group was significantly lower at all time intervals (Fig. 4.7).

**Large nerve graft:** There was no demonstratable difference in the PAC within the LNG group, or when compared to either the DNR or SNG groups. There was a significant increase in GAC between POW03 and POW06, thereafter
Figure 4.7  The comparison for the total success ratio for all groups. The TSR calculated the number of axons to successfully regenerate across the repair or graft.

* Signifies a significant difference between groups (p<0.05)
there was no change in the number of axons regenerating across the proximal neurorrhaphy into the graft. There was a significant increase in DAC between POW03 and POW12, thereafter no further change was demonstrated (Fig. 4.8).

A maximum of 78% of axons successfully regenerated across the proximal neurorrhaphy at POW06 (SR1 = 0.78, SD 0.03). This level was maintained throughout the remainder of the study period. A maximum of 73% of axons successfully regenerated across the second neurorrhaphy at POW24 (SR2 = 0.73, SD 0.04), however there was no significant change following between POW12 and POW48. A maximum of 56% of axons regenerated across both neurorrhaphies, recorded at POW24 (TSR = 0.56, SD 0.04). Overall, in comparison to the DNR group, the TSR for the LNG group was significantly lower at all time intervals.
Figure 4.8  The axon counts for each graft group during the study period.

A) – C) The counts for the SNG group over the 48-week study

D) – F) The counts for the LNG group over the 48-week study
Comparison of SNG and LNG groups: As stated, there was no significant difference between the PAC between the SNG and LNG groups. At all time intervals there was a significantly higher GAC in the LNG group, with the exception of POW12 (p=0.13). No significant difference was demonstrated in the DAC between the two groups until POW12, thereafter the LNG group had a significantly higher DAC (Fig. 4.9).

At all time intervals, the LNG group had a significantly better SR1. There was no significant difference in the SR2 until POW12 after which the LNG group had a significantly better SR2 then the SNG group for the remainder of the study period. A similar pattern was demonstrated for the TSR, as the LNG group had a significantly higher TSR than the SNG group.

4.3.3 Relationship between distal axon count and ear movement recovery

Having investigated the to outcome methods independently, the relationship between axon counts and EMR were investigated. The results were paired as for every EMR outcome, the axon counts were matched to individual animals. Both the PAC and GAC were deemed to be irrelevant to EMR outcomes as only the axons re-innervating the interscutularis muscle should affect movement of the muscle.
Figure 4.9  Comparison of axon counts in all the SNG and LNG graft groups.

* Signifies a significant difference between groups (p<0.05)
For each group, the EMR for each animal was paired with the DAC at each time interval. Within each group, the DAC was plotted on the y axis and EMR on the x axis and the Pearson's correlation coefficient was calculated. For the DNR group, $r = 0.63 \ (p<0.05)$, for the SNG group, $r = 0.58 \ (p<0.05)$ and for the LNG group, $r = 0.79 \ (p<0.05)$. After combining all the groups, $r = 0.80 \ (p<0.05)$ (Fig. 4.10), confirming that there was a significant direct relationship between DAC and EMR for this study.
Figure 4.10  A scatter diagram of the distal axon count plotted against ear movement recovery ratio. Pearson’s Correlation Coefficient demonstrated a significant linear relationship between the number of axons and the amount of EMR (p<0.05)
4.4 Discussion

Overall, the results from this study were successful and the methodology allowed for SSEM protocols to be adapted to calculate automated axonal counts. The results demonstrate detectable differences between the groups, and changes within each group. One untested parameter was the use of nerve isografts. The concept of using isografts is well established in the vascularised composite allotransplant literature. Isografts are frequently used from genetically identical animals as control groups to circumvent the requirement of immunosuppression. For these experiments, isografts were chosen as the impact on animal behaviour and stress caused by excising the sciatic nerve in comparison to the common peroneal nerve could be avoided and therefore controlled. There were no complications experienced from the surgical interventions or evidence of rejection of the isografts throughout the intervention period and the two fatalities were unrelated to the study.

The EMR outcome measure demonstrated that it was a reliable tool in measuring the amount of EMR in all study groups. In all groups the EMR plateaued prior to the end of the study period; for the DNR group at POW06 and for the SNG and LNG groups at POW12. This justifies that the study period was sufficient in length to capture the maximum EMR and indeed that future studies may warrant stopping at POW24.

The DNR group was included to provide information regarding axonal regeneration across a neurorrhaphy under ideal circumstances; the repair was immediate, no graft was required and the size match was identical. It is
unsurprising that the outcomes were best in this group, however EMR only reached a maximum of 75% during this study with no significant improvement over the last 36 weeks study. This suggests that no further improvement would be anticipated in a mouse model and that up to 25% loss of muscle power may be anticipated following direct nerve repairs.

Having established that a 25% loss of EMR could be anticipated following a DNR, it was expected that the same degree of loss could be anticipated for each neurorrhaphy in the graft groups. The results from this do not support this as there were significant differences demonstrated between the SNG and LNG groups after POW12. The LNG group demonstrated a maximum of 48% EMR in comparison to 30% EMR in the SNG group. Firstly, using the DNR data as an ideal environment, a 25% loss in EMR could be expected per neurorrhaphy. In the graft groups, 25% would be lost at each neurorrhaphy and therefore the ideal outcome would be 56% EMR \(100\times0.75^2\) however, both graft groups were lower than this implying that not only the use of a graft, but also the size of the graft influenced the outcome.

The axon count data had a similar result. The highest TSR for the DNR group was 80% implying that 20% of axons failed to regenerate across the neurorrhaphy and if this was extrapolated to two neurorrhapies the expected outcome would be a TSR of 64%, however the data did not support this. The maximum TSR for the SNG group was 32% and for the LNG group 54%. These results support the conclusions from the EMR data in that the use of a graft and the size of the graft influence the TSR.
Combining the two outcome measures demonstrated that there was a significant linear relationship between EMR and DAC. Although the concept that muscle function is directly related to the innervation of the muscle, such a significantly powerful study supports the use of the EMR outcome measure as a sensitive tool in monitoring successful nerve regeneration.
4.5 Conclusion

The EMR outcome measure is a useful tool in monitoring successful nerve regeneration. The results from the DNR repair group cannot be extrapolated to the nerve graft groups suggesting that the nerve graft itself has a negative impact on nerve regeneration. A nerve graft with a greater axon count (LNG) yielded superior nerve regenerative results, both clinically as a measure of EMR and at an axonal level with a higher DAC, than a graft with a lower axon count (SNG).
4.5 Bibliography


Chapter Five

Potential mechanisms explaining the findings of increased axonal regeneration in larger nerve grafts in comparison to smaller nerve grafts.

5.1 General overview

Nerve regeneration describes the process of either the repair or replacement of nerve cells that have been damaged by injury or disease. In the context of facial reanimation surgery, it describes the journey a distal buccal branch of the facial nerve takes as it regenerates through an autologous nerve graft, into a transplanted muscle in order to re-establish synapses at the neuromuscular level and thus innervate the muscle. The work presented in this thesis was stimulated by the desire to improve the clinical outcome for patients undergoing facial reanimation procedures. Excluding individual patients factors, there are three components that make up the gold-standard two-stage reanimation procedure:

1. The donor nerve
2. The autologous graft
3. The transplanted muscle

Of these three components, the impact of the donor nerve is the most extensively investigated [1-5]. Overall, it is agreed that the higher the number of motor axons within the donor nerve, the greater the force of contraction of the recipient muscle. In two-stage free functional muscle transfers (FFMT), the axonal count of the donor nerve is lower than the native axonal count of the FFMT [6]. The
'Bottleneck' theory suggests that there is an increase in axonal sprouting to re-establish synapses with motor units to compensate for this explaining the unpredictable outcome following FFMT. One way to address this issue is to only use a small segment of the overall muscle, reducing the number of motor units the donor nerve is required to innervate [7, 8]; clinically, this is not an exact science as muscular atrophy and irreversible end-plate degeneration must also be taken into consideration.

The purpose of the work contained in this thesis is to investigate the impact the final component has on nerve regeneration: the autologous nerve graft. This was not the first study to address nerve grafts and outcomes of nerve regeneration; extensive resources have been allocated to developing alternatives to autologous grafts either through bio-engineering or pharmacological manipulation of the graft environment [9-12]. There are established reports publishing the finding that longer grafts yield poorer outcomes; however, only one study has investigated the impact geometrical diameter of an autologous graft has on nerve regeneration [13, 14]. The results demonstrated that paradoxically, larger diameter grafts yielded poorer outcomes than smaller diameter nerve grafts, and based primarily on perfusion studies with no physical outcome measure over only forty days.

The hypothesis being tested in this study was that autologous grafts with a higher axon count will demonstrate better outcomes or nerve regeneration than autologous grafts with lower axon counts. Although this challenges previous studies [14], the primary focus is on the number of axons contained within the native nerve that is being used as an autologous graft, not purely a dimensional
study. The results from this thesis have successfully achieved the primary aim of this study by producing results to support the hypothesis. In order to achieve this, two secondary aims were accomplished: a new animal outcome measure for facial nerve surgery was successfully developed and the methodology from serial section electron microscopy was modified and adapted to analyse the nerve samples, producing automated axon counts.
5.2 Discussion of methodology

5.2.1 Justification of experimental design

Three experimental groups were included in the design of this study: Direct Nerve Repair (DNR), Small Nerve Graft (SNG) and Large Nerve Graft (LNG). Each experimental group used ten animals for each study interval. The group sizes allowed for adequate statistical analysis and for the possibility of loss of animal life. The DNR group was used to determine what the ideal recovery following a nerve repair was as there was no size discrepancy and no graft was used.

Two nerve graft groups were used which had significantly different axon counts. The common peroneal nerve (CPN, mean of 1593 axons) was used in the SNG group and the sciatic nerve (ScN, mean of 2616 axons) was used in the LNG group, both having a significantly larger axon count than the posterior auricular nerve (PAN, 947 axons); this was important to avoid a bottleneck effect at the neurorrhaphy between the PAN and graft.

Seven time-points were selected as study intervals. All animals were recorded pre-operatively and at post-operative week one (POW01) to capture the maximum and minimum ear movement. Subsequently, ten animals were pre-assigned per group to the following time intervals: POW 03, 06, 12, 24 and 48. Previously published studies report nerve regeneration across autologous grafts taking up to six months [15] therefore 48 weeks was chosen to accommodate
subtle differences between the two graft groups and ensure the maximal regeneration had been achieved.

5.2.2 Ear movement recovery index (EMRI)

The most important outcome of nerve regeneration is the restoration of function [16]. In clinical practice, the results of facial reanimation are ultimately judged on the function of the transplanted muscle to mimic the contra-lateral facial musculature in movement and symmetry. There are several animal models that address peripheral nerve regeneration however, far fewer specifically address facial nerve regeneration [17-21]. The studies that have tried to develop models for the facial nerve have largely focused on the zygomatic, buccal or marginal mandibular branches. The authors of these studies have subsequently published reports finding failures in their techniques either from alternative autonomic pathways, or through the cross-innervation patterns demonstrated within the facial nerve branches [22-24]. To circumvent these issues, proximal facial nerve lesions are required in combination with head-restraint systems that are not only expensive, but as the rat is smallest animal these can be used on, it excludes a large proportion of transgenic species.

The laboratory in which the initial methodology for this thesis was established had extensive experience in the study of transgenic mice [25-29]. For this reason, an outcome model that was applicable to smaller rodents was preferential so the models established in rats were excluded. A combination of two factors prompted further investigation into the function of the posterior auricular nerve. Firstly, the initial anatomical study of the facial nerve in YFP-16 mice demonstrated
extensive cross-innervation of the five major branches (Fig. 5.1). Whilst proximal facial nerve lesion remained a possibility, the axonal load of the proximal nerve is extremely high and finding two different nerve grafts that had both significantly different axon counts than the facial nerve would be exceedingly difficult. Secondly, a previous post-doctoral member of the laboratory had published the entire m. interscutularis connectome [30] (Fig. 5.2), providing clear evidence that the muscle was only innervated by the posterior auricular nerve, thus making it an ideal candidate for studying facial nerve surgery. Unfortunately, there were no reported descriptions of the actions of the m. interscutularis, therefore the first task was to establish the function of the muscle.

Experiments on anaesthetised mice confirmed that stimulation of the posterior auricular nerve caused the m. interscutularis to contract, retracting the ears posteriorly towards the midline (Fig. 5.3). Having confirmed that the nerve had an identifiable action, the challenge was how this could be measured. Initial experiments attempting to startle the animals failed, however it was coincidentally noticed that the animals would retract their ears whilst imbibing from the water feeder. The pilot studies demonstrated two key findings, firstly that a lesion of the posterior auricular nerve prevented the posterior retraction of the ear and secondly, crush injuries of the nerve had a measurable degree of recovery. The posterior auricular nerve was selected after not only taking these findings into consideration, but supported by animal behaviour studies reporting that ear posture is an expressive modality of emotion [31]; thus an ideal animal model for facial nerve surgery.
Figure 5.1  Anatomical dissection of the facial nerve of a mouse.

A) The superficial anatomy of the face of a YFP-16 mouse. The skin has been removed to expose the underlying facial nerve and musculature. The main branches of the facial nerve can be appreciated.

B) The same animal under fluorescent imaging. Under these conditions the cross-innervation patterns may be fully appreciated. Cross-innervation can be seen between the temporal and zygomatic branches, between the zygomatic and buccal branches and extensively between the buccal and mandibular branches; no demonstrable cross-innervation was seen with the posterior auricular nerve (PAN).

(Key: p – posterior auricular nerve, t – temporal branch, z – zygomatic branch, b – buccal branch, m – mandibular branch, dp – distal pes)
Figure 5.2  The *m. interscutularis* connectome.

The detailed report from Lu et al. demonstrated that the *m. interscutularis* has no innervation from any other nerve other than the posterior auricular branch of the facial nerve.

[Reproduced with permission from Lu et al.]

Figure 5.3  Function of the posterior auricular nerve in a mouse.

A) The anterior position of the right ear prior to stimulation of the posterior auricular nerve.

B) During stimulation of the posterior auricular nerve the ear can be seen to retract towards the mid-line.

C) The lateral position of the ear at rest.

D) During stimulation of the posterior auricular nerve, the ear is retracted posteriorly towards the midline.
The results from this thesis have established a new animal outcome measure for facial nerve surgery. The model has only been demonstrated to be effective in mice and further experiments must be undertaken to confirm reliability and reproducibility between investigators and different species; irrespectively, it is limited to animals with upwardly projecting ears so that detectable movement is possible. The model would benefit from modification to implement ear tracking, either with landmark tracking or tattooing, in order to improve sensitivity and reduce the number of repetitions required in the current design.

Overall, the ear movement recovery index (EMRI) is a reliable outcome measure for facial nerve surgery. The equipment required is inexpensive and the system is easy to implement making this a novel method for future investigations of facial nerve interventions.

### 5.2.3 Generating automated axon counts with serial section electron microscopy

Axon counts were used as the measure of successful cellular regeneration for this study. This is an accepted marker of nerve regeneration [13, 15, 32] and due to the high number of axons involved in the regenerative process, several techniques have been published to accomplish accurate counts using semi-automated or fully automated methods [13, 33, 34].

Electron microscopy was used as the tool for histological analysis of epoxy-resin embedded cross sections. Electron microscopy is widely used in the study of the peripheral nervous system as it is capable of imaging not only the large
myelinated axons but also the smaller unmyelinated fibres that are beyond the realms of traditional light microscopy. Further to this, the laboratory had excellent access to the use of scanning electron microscopes (SEMs) and experience with serial sectioning [35-38]. Many studies use nerve fibre counts to report outcomes following nerve regeneration and whilst this reduces the processing time as analysis of the whole cross-section is not required, it may be subject to sampling bias. The results from the experiments in this thesis demonstrated large areas of the cross section that were occupied with scar tissue and devoid of axons (Fig. 5.4); sampling this area would be unrepresentative of true nerve regeneration. The imaging parameters used for this thesis ensured that the whole cross-section was captured in one image circumventing this potential cause of inaccuracy.

A fully automated system of analysis of axon counts was developed to improve processing speeds and reduce the workload accrued with manual analysis. A total of 40,000 sections were collected for analysis as described in Chapter 4 therefore a fully-automated analysis system was imperative. The pilot study of the facial nerve samples confirmed that there was no difference between non-automated counts, generated by individual researchers, and those generated by the segmentation algorithm. Additionally, the segmentation algorithm averaged axon counts over a 100 section volume of tissue, equal to 10 µm, and avoided missing axons that may have not been counted if the cross-section was at the level of a Node of Ranvier.
Figure 5.4  **Scar formation within the nerve graft.**

Within this cross-section taken from the middle of a small nerve graft group at POW48. The red arrows indicate nerve fascicles, Blue arrow head indicates a arteriole within the graft and white arrow heads indicate areas of scarring and increased collagen fibril deposition.
5.3 Discussion of Results

The success ratio is an established simple method to evaluate the success of nerve regeneration [15, 32, 39, 40]. The number of myelinated axons at a distal point are expressed as a ratio of the number at a second point, usually measured proximal to the site of intervention. The main limitation of this technique is that it does not take into account the quality of the regenerated axons; unmyelinated axons are ignored and there is no differentiation between immaturely and maturely myelinated axons. However, the most important aspect of regeneration is to re-establish NMJs and re-innervate the target muscle; published reports evaluating the outcomes of alternatives to autologous grafts have demonstrated that this is an accurate measure of successful regeneration hence its selection as the cellular measure of successful regeneration [15, 39, 40].

5.3.1 The relationship between ear movement recovery index and axon count

Every animal in this study had the EMRI paired with the axon count at each time interval. This allowed for direct comparison of one outcome measured with the other. The purpose of having two modalities was important. The EMRI was a new concept that required validation so a second, established outcome measure was required to verify the reported outcomes. Using the Total Success Ratio (TSR) allowed each EMRI value to be compared to an accepted outcome measure of nerve regeneration. The overall results of this study demonstrated a statistically significant linear relationship between TSR and EMRI. At a cellular level this is not surprising as it would be reasonable to expect that a TSR would indicate more axons available to re-innervate the muscle, however, it was important to prove
this relationship statistically and validate the EMRI as an outcome measure so that the TSR in future studies may not be required, saving significant cost and time.

The additional benefit of measuring axon counts was to understand not only what the differences between then groups were, but where they were occurring within the process of axon regeneration. By sampling axon counts at two points in the direct nerve repair group (proximal and distal to the neurorrhaphy) and at three points in the graft groups (proximal, within and distal to the graft) the area where axons were being lost could be identified within the three groups.

The results in all three experimental groups demonstrated a slight delay between maximum distal axon count (DAC) and EMRI. Although the DAC confirmed the number of axons that successful crossed the neurorrhapy (DNR group) or the graft (SNG and LNG groups) it cannot provide information regarding re-innervation of the muscle. No significant difference was demonstrated in any of the groups after POW24 and therefore the explanation for this is the additional time required for the axons to complete regeneration and reach the NMJs, re-innervating the muscle.

This study has demonstrated a significant linear relationship between the DAC and EMRI. With this information it was possible to demonstrate the relationship between TSR and EMRI, as TSR is derived directly from the DAC. Making the assumption that no further re-innervation occurred after POW24, as there were no significant changes demonstrated in any group after this time point, the EMRI
for all groups was plotted against the TSR to calculate the linear relationship between the two variables and the following equation was derived:

\[ EMRI = 0.93(TSR) - 0.01 \]

Pearson’s correlation coefficient was 0.83 (p<0.05) confirming a similar relationship to EMSR and DAC (Fig. 5.5). The equation can be approximated to a 1:1 relationship between the TSR and EMRI, thus supporting the use of the EMRI as an independent measurement of axonal regeneration.

5.3.2 Interpretations of differences demonstrated between experimental groups.

The best results were demonstrated in the DNR group. As previously stated, the TSR was 80% after POW24 and was used as the benchmark for the best outcome. To test the null hypothesis that an autologous graft has no impact on axonal regeneration the assumption is made that the TSR for either nerve graft group could be calculated as the sum of two neurorrhaphies and the expected TSR = 0.8² = 64%. The TSR data was combined for both graft groups and after POW24 the mean was 37%, implying that the autologous graft has an independent negative effect on axonal regeneration and the null hypothesis is void.

Having established that an autologous graft has a negative outcome on axonal regeneration in comparison to a direct nerve repair, differences between the two graft groups were analysed. Overall, the LNG group yielded significantly better outcomes in both EMRI and TSR than the SNG group. In the graft groups the length of graft was 4mm, both groups contained a mixture of motor and sensory
Figure 5.5 The relationship between ear movement restoration index (EMRI) and the number of successfully regenerated axons.

A significant positive relationship was established between the number of regenerating axons and the degree of ear movement.
axons and all animals were the same age so that the only variable being altered was the axonal count of the graft.

Evaluating differences between the success ratio at the proximal graft neurorrhaphy (SR1) and the distal graft neurorrhaphy (SR2) demonstrated a significant difference between the two graft groups. There was no significant difference between LNG-SR1 (77%) and DNR-TSR (79%), however there was a significant difference between LNG-SR2 (72%) and DNR-TSR (79%). For the SNG group, SNG-SR1 (61%) was significantly worse than both LNG-SR1 and DNR-TSR and SNG-SR1 was significantly worse than SNG-SR2 (53%). These results suggest that the detrimental effect on regeneration occurred within the graft itself.

Two conclusions were made after analysing the results of the axon count data: firstly that the graft itself had a negative effect on nerve regeneration and secondly, a graft with a lower native axon count yielded poorer results. The sections used to calculate the axon counts were re-examined to observe morphological differences between the groups. In comparison to the DNR group, both graft groups had large areas without any axons present; the areas were filled with granulomatous and connective tissue (Fig. 5.6). This observation supports the study published by Best et al. [13], suggesting that a non-vascularized graft is subjected to ischaemia that results in central necrosis leading to scarring, and explains the difference demonstrated by the drop in SR1 and SR2 in both graft groups. However this concept does not explain why the results for SNG-SR2 were significantly worse than SNG-SR1, whereas there was no difference
Figure 5.6 Illustration to demonstrate the three areas sampled from within an SNG graft.

A) 100 sections were processed proximal to the nerve graft.

B) 100 sections sampled within the nerve graft show altered morphology and replacement of normal nerve tissue with connective tissue impeding axonal regeneration. [The red arrows indicate nerve fascicles, Blue arrow head indicates a arteriole within the graft and white arrow heads indicate areas of scarring].

C) Distal to the graft, a more organized appearance of the architectural structure of the nerve is restored.
between LNG-SR1 and LNG-SR2; the smaller graft would be expected to revascularise faster and the amount of scar formation to be less.

The difference between the SNG-SR1 and LNG-SR1 groups was the key to obtaining a better overall result. The mean native axon count for the CPN was 1679 and the ScN was 2612, approximately 1.6 times larger. If the results for POW24 and POW48 are used, the mean proximal axon count for the SNG group was 1005 and the LNG group 1044 implying that the SNG graft contained approximately 1.7x the number of axons than the PAN, and the LNG graft contained approximately 2.5x the number of axons. The better SR1 result seen in the LNG group is explained by the increased nerve growth factors and neurotrophins contained in, and released by the larger graft providing a better environment for the axons to regenerate across the neurorrhaphy. The mean graft axonal counts for the SNG group was 616 and the LNG group was 801, suggesting that 33% of native axons in the SNG group had been replaced in contrast to 30% in the LNG group. Even though the LNG graft had an axon count 1.6x greater than the SNG, it only contained 1.3x the number of axons within the graft supporting the notion that there is indeed a greater degree of ischaemia and scar formation within the graft (Fig. 5.7). The SR2 result was unexpected as the assumption was that there would be no difference between SNG-SR2 and LNG-SR2, given that the cellular environment at the time regeneration had reached the second neurorrhaphy would be similar to the DNR group, and therefore ~75% of axons should have been successful at crossing the neurorrhaphy.
Figure 5.7  Illustration of the three interventional groups. (proportional to axon count)

A) Direct nerve repair (DNR), the results demonstrated a 79% total movement restoration.
B) Small nerve graft - Significant differences were noted between the two graft groups.
C) Large nerve graft – The larger yielded the best results from the experiments confirming that autologous grafts with higher axon counts should be preferentially chosen over nerve grafts with a lower axon count.
This assumption held true for the LNG group, however the SNG group had a SR2 of 52% for which no clear physical cause could be determined; there are no reports cited in the literature with similar results for comparison. It would seem that at the time of initial surgery, when the graft is interposed into the nerve gap, a preferable interface must be established between the larger nerve graft and the PAN in comparison to the smaller graft group. The results from this study cannot prove this as the neurorrhaphy was not examined directly and unfortunately the tissue containing the information was discarded during the sectioning process. Future work using vascularized nerve grafts may be a prudent direction to pursue, however the reports in the literature are inconclusive and a well-designed study is indicated [41].
5.4 Conclusion

The hypothesis tested in this study was that autologous nerve grafts with higher native axon counts would yield better regenerative outcomes than autologous nerve grafts with lower axon counts. The results support the hypothesis, as better outcomes were demonstrated in the large nerve graft group in comparison to the small nerve graft group, both by a clinical outcome measure and at a cellular level. Although the assumption is that the larger graft group had a higher level of ischaemia resulting from the increased time taken for revascularisation, the benefits of higher levels of nerve growth factors and neurotrophins outweigh this by providing a superior environment to support axonal regeneration in the short term. In the longer term, the results obtained could not provide a clear cause for the greater axonal regeneration at the distal neurorrhaphy demonstrated by the LNG group in comparison to the SNG group; this may result from the interaction between the graft and the nerve at the initial time of surgery that could not be determined in this experimental design.
5.5 Bibliography


Chapter 6

Final conclusions and future research directions

6.1 Future directions of research

Peripheral nerve regeneration is an exciting area of current research ranging from stem cell technology to bioengineering and even robotics; one sub-category can easily provide enough work for a lifetime’s worth of research. Having embarked on the exploring the topic of nerve regeneration through autologous grafts, it would be pertinent to consider how to take the results of this thesis and develop them to investigate new areas of scientific interest. The two most significant findings were:

1) There were changes within the autologous grafts over the study period that lead to significantly poorer outcomes compared to the direct nerve repair group.

2) That an autologous graft with a higher native myelinated axon count supported better axonal regeneration than an autologous graft with a lower native myelinated axon count.

To address these points, two pathways of investigation are proposed.

6.1.1 Prevention of scar formation within the peripheral nervous system

The explanation used to support the findings that the graft groups had poorer outcomes was based on scarring and granuloma formation within the grafts. In
the central nervous system, glial scarring is an active area of stroke research. Following an ischaemic brain injury, reports have demonstrated a disproportionate scarring response and have suggested that the glial cells switch from a myelin producing state to a scar forming state; fibroblasts play a key role in promoting scar formation and may also warrant attention. This same theory has been applied to the peripheral nervous system and in particular, spinal cord injury. Incidentally, when human autologous nerve grafts are imaged by transmission electron microscopy, the appearance is strikingly different to rodent tissue. The appearance of increased amounts of collagen fibres is most apparent (Fig. 6.1) and there is no clear understanding as to the mechanism of this process. One suggestion is that the collagen fibrils are likely from higher levels of chondroitin sulphate proteoglycans (CSPGs) that in turn, prohibit axonal regeneration through a mechanism akin to scar formation [1-3]. Studies of the central nervous system demonstrate nerve regeneration is improved with treatments that target scar formation by either reducing collagen formation (chondroitinase) or preventing Schwann cells from forming myelin (Fingolimod) however, little data exists for the peripheral nervous system [4-6]. The enzyme ‘chondroitinase’ is not native to humans but animal studies have demonstrated improved outcomes in spinal cord regeneration, supporting this mechanism of action [7-9].

Future experiments are required to investigate the mechanism of action of these drugs and develop a local release system, such as hydrogel delivery, so that potentially every nerve repair or graft could be locally treated at the time of surgery, avoiding the potentially toxic side effects of systemic administration. If scar formation can be reduced, better outcomes would be expected.
Figure 6.1  Morphological differences between human and mouse nerve graft specimens

Human nerve specimen taken from a cross facial nerve graft (A). Formation of collagen fibrils is clearly evident in contrast the mouse autologous sample (B). In both samples areas sparsely populated by axons represent scar formation that may be the target for future investigation.

[Key: red arrow head – Collagen fibril deposition/scar formation, green – Schwann cells, Blue – Myelinated axons]
6.1.2 Detailed assessment of axonal behaviour during regeneration

To analyse axonal behaviour, individual axons need to be viewed over larger volumes of tissue. Ideally every axon in a neurorrhaphy would be identified and the journey it takes as it regenerates would be traced through the volume of tissue. The goal would be to determine any associations between ‘successful’ axons in comparison to ‘unsuccessful’ axons.

In order to achieve this, the methodology from this thesis was used to determine the feasibility when applied to a direct nerve repair. A pilot study used serial section electron microscopy to image 900 sections, sampled from within a neurorrhaphy. The sections were imaged, the stack was aligned and auto-segmented. The data was then rendered to produce a three-dimensional reconstruction of every axon (n=748 axons) within the volume (Fig. 6.2). Using 3ds Max® (Autodesk, USA) a viewer can now take an immersive journey through the axons, providing a new way of examining the regeneration process (Video 6.1). Individual axons can be rendered in high levels of detail to demonstrate the relationship between the axon, myelin, Schwann cell and Nodes of Ranvier (Fig. 6.3, Video 6.2).

The next stage is to serially section the whole of a nerve repair. It is anticipated that approximately 40,000 sections will be required to capture the whole of a neurorrhaphy (~4 mm at 100nm thick sections); this is an unprecedented size of data. The time taken to image the sections will take in the region of 6 weeks of continuous data capture followed by the additional time taken for alignment and
Figure 6.2  Axonal regeneration across a neurorrhaphy

A 3-D reconstruction of 748 axons regenerating across a neurorrhaphy. 900 serial sections were aligned and auto-segmented to demonstrate an overview of axonal behaviour. Larger sparse areas within the tissue volume can be seen surrounding the nylon suture material used for the neurorrhaphy. Some axons can be seen to be spiralling around whereas others have a more direct pathway.
segmentation however, once the dataset is complete full analysis of axonal regeneration will be possible. For example, it will be possible to select only successful axons and analyse behavioural patterns and associations with the surrounding environment to help predict which axons will be successful. This level of detail will further our understanding axonal behaviour during regeneration and help determine individual factors that currently impair this process.
Figure 6.3  3-Dimensional reconstruction of a single axon

900 sections were accurately segmented to produce a rendered representation of an axon (pink) surrounded by its myelin sheath (purple) and the associated Schwann cells (green and yellow)
6.2 Conclusion

The work presented in this thesis has sought to test the hypothesis that autologous grafts with higher native myelinated axon counts would yield better nerve regeneration outcomes than autologous grafts with lower native myelinated axon counts. To prove this, a novel animal model was successfully developed assessing the Ear Movement Recovery Index (EMRI). Myelinated axon counts were used as the cellular outcome measure being presented as the ‘Success Ratio’ of axons that successfully crossed the site of intervention. The methodology used for serial section electron microscopy was modified to automate this process by implementing segmentation protocols. Overall the study supports the hypothesis and the results demonstrated a significant improvement in nerve regeneration in the larger graft group than the smaller graft group. Further research is required to determine the reason why autologous grafts have a poorer associated outcome than simple direct nerve repairs. The rationale suggested by this thesis is the formation of scar tissue within the autologous graft and that future work investigating how to manipulate this process may improve regenerative outcomes.
6.3 Bibliography


Appendix 1.1: Raw data for Ear Movement Recovery (EMR) measurements. Measurements are reported as ratios of increasing ear movement towards the maximum.

[POW – Post-operative week, DNR – Direct nerve repair group, SNG – Small nerve graft group, LNG – Large nerve graft group]
<table>
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<th>Group</th>
<th>n</th>
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<th>POW06 Proximal Graft Distal</th>
<th>POW12 Proximal Graft Distal</th>
<th>POW24 Proximal Graft Distal</th>
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Appendix 1.2 Axon count results for all specimens sectioned in this study.

[POW – Post-operative week, DNR – Direct nerve repair, SNG – Small nerve graft, LNG – Large nerve graft]