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REVIEW ARTICLE

In Vitro, *In Vivo* and *Ex Vivo* Models for Peripheral Nerve Injury and Regeneration

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Abstract: Peripheral Nerve Injuries (PNI) frequently occur secondary to traumatic injuries. Recovery from these injuries can be expectedly poor, especially in proximal injuries. In order to study and improve peripheral nerve regeneration, scientists rely on peripheral nerve models to identify and test therapeutic interventions. In this review, we discuss the best described and most commonly used peripheral nerve models that scientists have and continue to use to study peripheral nerve physiology and function.

Keywords: *Ex vivo* models, *in vitro* models, *in vivo* models, organotypic models, peripheral nerve, peripheral nerve injury, peripheral nerve regeneration.

1. INTRODUCTION

Peripheral Nerve Injuries (PNI) frequently occur secondary to traumatic injuries, making up approximately 2.8% of all traumatic injuries [1-3]. In developed countries, PNI affects between 13 and 23 people per 100,000 per year [4]. Annually 360,000 procedures are performed in the United States to treat PNI, with over $150 billion spent annually as a result [5]. Given the slow process of Wallerian degeneration followed by axonal regeneration at the rate of 1mm/day, higher level injuries take longer to recover than distal injuries. This makes peripheral nerve injuries costly both on an individual and societal level, resulting in $40,000-75,000 in lost earnings particularly if the patient is a primary earner of the family [5].

As such, many efforts have been made to investigate ways to optimize peripheral nerve regeneration. These have included advances in novel conduit materials and design, stem cells (derived from tissue sources or pluripotent stem cells), growth factors, cell fusion techniques, as well as the use of acellular therapies such as exosomes.

Despite these advances and the continued push for more innovative research, there remains another challenge in peripheral nerve research: the development of efficient, cost effective, reliable and valid peripheral nerve injury models. Current models that scientists use to study physiological processes can be arranged on a "physiological modeling ladder" as described by Geuna *et al.* [6]. On the lowest rung of this ladder are immortalized cell lines that are relatively low cost and help replace and obviate the need for primary cells, tissues and live animal subjects. However, given the neoplastic origin of many of these cell lines, they have a limited capacity to model the physiological processes being studied. On the next rung of the ladder are primary cell lines, which help reduce the number of animal subjects while providing primary cells that better model physiological conditions than their immortalized cell line counterparts. *Ex vivo* nerve models occupy the next highest rung and can emulate physiological processes and needs more closely with the addition of features that approximate the anatomic arrangement of primary cells. The production of these types of models involves higher levels of engineering and higher cost. At the highest level of this ladder are the *in vivo* models themselves, which more closely replicate the physiological processes being studied, second only to the human nerve. However, these models require a one-to-one ratio of animal subjects to interventions studied, making them costly and associated with higher ethical concerns.

The purpose of this review is to assemble the best described and most commonly used techniques to model peripheral nerve physiology and function.

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2. *IN VITRO* MODELS

In vitro studies are used to investigate cellular activities that are necessary for peripheral nerve regeneration. The main cell types include Schwann cells and neurons, which are the two critical cell types within peripheral nerve tissue responsible for the majority of nervous tissue function. As such, their functional responses to interventions can roughly reflect those of native peripheral nerve tissues. The origin of these two cell types can be either from an immortalized cell line or a primary cell line, each with its pros and cons.

2.1. Immortalized Cell Lines

Immortalized cell lines have a great ethical advantage over other models in that once the line is created, there is no further need for animal subjects. However, immortalized cell lines are often derived from neoplastic tissue, the properties of which are different from normal tissues and cells. This makes them less analogous to normal cells. Therefore, unless the scientific aim is to model neoplastic tissue within the peripheral nervous system, the science derived from immortalized cell lines may not accurately predict behaviors of native *in vivo* cells and tissues [6].

Immortalized Schwann cell lines that are commonly used to study peripheral nerve regeneration include RT4-D6P2T [7], JS-1 [8], RSC96 [9], R3 [10], and S16Y [11]. The RT4-D6P2T Schwann cell line, derived from rat schwannoma, has been shown to maintain the expression of key genes that characterize Schwann cells, making this cell line most similar to primary Schwann cells [12].

Because immortalized cell lines are not susceptible to cellular senescence, unlike primary Schwann cell cultures, proliferation assays performed on these cell lines have proven to be useful and reliable [6]. When studying nerve conduits and 3-dimensional constructs for peripheral nerve reconstruction, RT4-D6P2T Schwann cells have been used in assessing Schwann cell proliferation when seeded within scaffolds of various materials. These include conduits made from electrospun fibers [13] to peptide amphiphile nanofibers [14]. Furthermore, morphological alignment of RT4-D6P2T Schwann cells within 3-dimensional constructs has also been visualized [14], suggesting the utility of this cell line to study not only quantity (cellular proliferation) but also quality (cellular morphology) when placed in a 3-dimensional environment. One of the main drawbacks of the RT4-D6P2T Schwann cell line is the absence of the expression of the Neuregulin1/ErbB system seen in native Schwann cells [15]. This system has been shown to be key in both the growth of Schwann cell precursors and specific axonal-Schwann cell interactions. Given this, it should be noted that Schwann cells are not the only glial cell model that is being used to investigate peripheral nerve regeneration. In particular, the neonatal olfactory bulb ensheathing cell line, harvested from isolated rat neonatal olfactory bulbs with subsequent immortalization *via* retroviral transduction of the SV40 large T antigen, has been used in various experiments in both CNS and PNS regeneration [16]. In particular, this model does express the Neuregulin/ErbB system and serves as a meaningful adjunct to further understanding of peripheral nerve regeneration [17, 18].

In vitro studies involving neuronal cells are more tenuous than glial cell models, attributable to the need for an environment composed of a complex population of cells for primary neuron culture survival [6]. Given this, neuronal cell lines must be utilized to study environmental effects on neuronal behavior. One distinct advantage of immortalized neuronal lines is their longevity in culture and how much easier they are to work with than primary neuron cultures. At the same time, primary neurons and immortalized neurons can behave very differently. Therefore, immortalized neuronal lines have often only been used for screening purposes when studying interventions and their effects on neuronal behavior, and experiments performed on immortalized neuronal lines should be corroborated in primary neurons [6].

Immortalized neuronal cell lines that have been used include the PC12, 50B11 and NSC34 lines. The PC12 cell line is derived from pheochromocytoma from rat adrenal medulla. The main use of this line is to observe the pro-regenerative potential of varied substances in neurite outgrowth assays [19-21]. Even though the PC12 line has been used for these assays, these cells are not closely related to native PNS neurons; therefore, any data derived from these studies must be considered carefully. Both 50B11 and NSC34 lines are thought to be more closely related to native PNS neuronal cells [6].

The 50B11 cell line is derived from rat embryonic dorsal root ganglion (DRG) neurons immortalized by SV40-large T antigen transfection and serves to model nociceptive DRG neurons [6]. Largely undifferentiated in standard culture conditions, the 50B11 cell line displays multiple neuronal properties when subjected to a forskolin stimulus. These valuable properties include neurite extension, neuronal marker expression, action potential generation, and response to trophic factors and hormonal supplementation [22, 23]. These characteristics make the 50B11 cell lines similar to native DRG neurons. However, once stimulated and differentiated, the cells only survive for 72 additional hours, limiting their use to short term assays and precluding their utilization in the study of *in vitro* myelination, which typically necessitates cultures times of 10 or more days [21, 24]. Still, the 50B11 cell line does serve to alleviate ethical concerns when carrying out large-scale *in vitro* experiments by way of minimizing the need for animal subjects to obtain a reasonable substrate for neuronal assays.

When considering motor neuron models, the NSC34 cell line is a hybrid line that is composed of a fusion of mouse neuroblastoma cells and motor neuron-enriched spinal cord cells [25]. While this cell line is typically used to study motor nerve degenerative diseases [26, 27], the maintenance of specific properties, including the generation of action potentials, expression of neurofilament triplet proteins, and acetylcholine synthesis, storage and release make this cell line good for the study of motor neuron regeneration following trauma and iatrogenic injuries. An example of this is a study on the effect extracellular vesicles from C2C12, a muscle cell line, have on the cell survival and neuronal outgrowth of the NSC34 cell line [28].

2.2. Primary Cell Lines

Slightly higher on the ladder of ethical costs and technical complexity is primary cell-based models. *In vitro* ex-

periments using primary cells more closely approximate conditions and outcomes *in vivo* than those that utilize immortalized cell lines. The main disadvantages of primary cell *in vitro* models are that the harvest of native cells inevitably causes neuronal and Schwann cell injury and results in the complete loss of the three-dimensional tissue framework [6]. These unavoidable consequences almost certainly induce significant alterations in cellular function and response and should be considered when interpreting any data obtained *via* these means. Of note, the conditions resulting from the harvest of primary cell cultures do appear to mimic the environment of Schwann cells and neurons post-trauma. Given this, primary cell-based models may serve to be uniquely qualified to model post-traumatic nerve regeneration [17].

Primary Schwann cell cultures have been harvested from mice, dogs and human nerves [29, 30]. While they possess similar qualities to native tissues, they have a limited lifespan in culture, and typically begin to show signs of senescence near passage 10. Although fibroblasts have been included in co cultures in *ex vivo* models as is discussed later, they are considered cellular contaminants in *in vitro* single cell type cultures. Techniques to remove contaminating fibroblasts begin by the immediate removal of the epineurium post-harvest, followed by one of several special culture conditions to further reduce fibroblast populations [6]. Strategies that have been used to this end include cytosine-B-arabinoside hydrochloride (an antimitotic agent) supplementation [31], antibody-mediated immunoselection [32, 33] and selective culture conditions utilizing the propensity of adult rat Schwann cells to metabolize D-valine to eliminate fibroblasts [34]. Adult human Schwann cells are a valuable resource that can be obtained either from cauda equina donors or from patients undergoing autologous nerve graft surgeries [35]. By utilizing techniques such as differential detachment in combination with selective culture conditions, purified adult human Schwann cells can be obtained [32, 35]. Another type of primary cell that is often used to model Schwann cells are olfactory ensheathing cells, which have several markers in common with native Schwann cells, including S-100 and low affinity nerve growth factor receptor p75 [15, 36, 37]. The ability of these cells to promote peripheral nerve remyelination in damaged axons [38] makes them of particular use in models that study peripheral nerve regeneration.

As previously discussed, primary neuronal cell cultures are difficult to obtain and purify and are limited in utility due to their short-term survival. Dissociated primary neurons of adult, neonatal or embryonic DRG or spinal cord origin can be co-cultured with Schwann-like cells, which serves to simulate the *in vivo* environment of a neuronal injury site [39]. On the other hand, the presence of Schwann cells in neuronal culture confound the picture when attempting to study specific factor modulation of neuronal behavior, making it impossible to distinguish whether various stimuli have a direct or indirect effect on neurons [6]. One example is the involvement of aquaporin-1 in axonal growth and regeneration [40]. An immunopanning-based method of rapid purification of DRG neurons *via* an antigen-antibody reaction was described by Zuchero *et al.* [41] and serves as one example of neuronal purification strategy. Another application of *in vitro* models of primary cell cultures is in the field of Chemotherapy Induced Peripheral Neuropathy (CIPN) research. Preparations of rat and mouse DRG sensory neurons are used to study CIPN. A recent study by Gornstein and Schwarz developed a DRG culture from mice that were used to study paclitaxel-induced neuropathy [42]. While the culture of embryonic spinal cord motor neurons has been successful [42], the culture of adult motor neurons remains elusive due to short-term survival and limited axonal outgrowth [6]. Research into this area is ongoing and there have been many suggested protocols to date with the goal of obtaining long-term cultures of healthy and functional adult motor neurons [43, 44].

2.3. Stem Cells

As a final note on *in vitro* models of peripheral nerve regeneration, the potential applications of stem cells in both conversions to neurons [45] or Schwann cells [46], as well as co-culture symbiosis is an exciting area of contemporary research [6]. Embryonic Stem Cells (ESCs) are pluripotent cells able to differentiate to form many types of cells in the body, except fetal cells [47]. Resulting differentiated Schwann cells express Schwann cell markers of glial fibrillary acidic protein, p75 and S100. In addition, these differentiated cells of embryonic origin are able to induce myelination when co-cultured with DRG neurons [48]. Neural crest cells derived from Pluripotent Stem Cells (PSCs), including ESCs as well as induced Pluripotent Stem Cells (iPSCs) also have been shown to stimulate neuronal outgrowth in co-culture [49-51]. PSCs can provide a practically unlimited source of cells for regenerative medicine and tissue engineering applications. ESCs are isolated from the inner cell mass of the blastocyst, have unlimited self-renewal capacity, and can differentiate into cell lineages derived from all three germ layers [52-54]. A recent development in PSCs eliminates the ethical concerns of the need for embryos entirely. Several groups have successfully reprogrammed adult cells (*e.g.*, fibroblasts) into iPSCs by the activation of four genes (transgenes), either Oct3/4, Sox2, c-Myc, and KLF4 or Oct3/4, Sox2, Nanog and Lin28 [55]. These iPSCs are similar to ESCs in their ability to maintain self-renewal and differentiate into cell lineages of all three germ layers [56], including differentiation into peripheral sensory neurons for modeling and studying CIPN [45, 57]. Disadvantages of use of pluripotent stem cells include potential tumorigenic properties [58, 59].

Adult stem cells may alleviate these concerns. Bone marrow mesenchymal stem/stromal cells have been shown to differentiate to Schwann-like cells and induce neuronal outgrowth in co-cultures [60]. Adipose stem cells can be induced to Schwann-like cells, which exhibit many of the same qualities, including secretion of neurotrophic factors that promote neuronal outgrowth and formation of myelin sheaths [61, 62]. Skin-derived precursor stem cells harvested from adult dermis tissue have also been shown to differentiate into Schwann-like cells that, when co-cultured with DRG neurons, induce myelination [63]. These properties, as well as the ease of retrieval paired with low ethical concern, make skin and adipose stem cells an ideal target for future research [47].

Common *in vitro* evaluations include assays for Schwann cell migration, myelination, secretion of neurotrophic factors, as well as neuronal axonal projection and proliferation.

While these cell attributes are necessary for peripheral nerve regeneration, they are not sufficient. Two-dimensional culture usually allows the investigator to observe the behavior of one cell type at a time, though studies have successfully demonstrated enhancement of neuronal viability and axonal growth when primary motor neurons and Schwann cells are grown in co-culture [64].

3. *IN VIVO* MODELS

In vivo models are invaluable in peripheral nerve research since they closely mimic the structural, cellular and extracellular chemical characteristics of human peripheral nerves and can be used to evaluate the return of function. However, each model has advantages and limitations, and none of them are suitable to study all aspects of PNI. The technical determinants of a model, species, strain, age and gender also affect peripheral nerve regeneration and need to be considered while choosing *in vivo* models. Finally, when selecting an *in vivo* peripheral nerve model, it is critical to consider the nerve type that is being studied. Therefore, in the following section, we present the various *in vivo* models widely used in peripheral nerve research as differentiated by animal type and nerve type, models of peripheral nerve injury creation, and finally techniques used to measure peripheral nerve recovery in animal models.

3.1. Models by Animal Types

Ensuring the safety and efficacy of newer treatment techniques is necessary before they can be used in clinical trials. Animals, especially smaller animals, offer the advantage of shorter length-dependent nerve regeneration and end-organ reinnervation, reducing study times. Animal models can be divided into rodents (rats, mice, guinea pigs, hamsters, *etc*.) and non-rodents (rabbit, sheep pig, monkey, horse, cat, dog, *etc*.). Large animal models (pig, sheep, dogs, monkey) are less commonly used in PNI research due to the cost of procurement and maintenance. Consequently, there is less data available on longer term large animal nerve regeneration [65, 66]. Furthermore, larger animal size leads to increased distances for reinnervation, which adds to study time, further increasing the cost of the project. At the same time, large animal models do have longer life spans and may offer a unique longitudinal view of peripheral nerve regeneration over a longer time span [65, 66].

3.1.1. Drosophila

Drosophila models of axon and dendritic regeneration have gained popularity in recent years. This model is useful for identifying critical neurobiological pathways facilitating the understanding of axonal regeneration that are also conserved in mammals [67]. One benefit of the *Drosophila* model is the lack of myelin and other known extrinsic inhibitors of regeneration thus facilitating regrowth studies of peripheral nerve [68]. Additionally, the thoroughly developed genetic lines and the availability of thousands of GAL4 lines allow for the intracellular mechanisms of PNI to be further explored. These components facilitate loss-of-function and gain-of-function as well spatial and temporal gene expression studies, respectively [67]. Both larva and adult *Drosophila* models have been used to develop a variety of injury models, including adult-head or larval Ventral Nerve Cord (VNC) injury, larval VNC or nerve crush, larval neuron laser axotomy, olfactory receptor neuron removal, brain explants injury, and peripheral nerve lesion by wing severance [69].

Embryonic and Larval *Drosophila* models are the preferred model for studying various aspects of neuronal development and function, including dendritic tiling, axon guidance, synapse formation, synaptic terminal growth, active zone development, mechanisms of neurotransmitter release, and axonal cell biology [67]. The transparency of the larval cuticle facilitates the imaging of neuronal axons and dendrites, allowing for the photo manipulations of neurons [70]. This model allows for the specific ablations of axons and dendrites with reduced collateral damage and preserved control neurites within the same neuron [70]. Because the larva's central nervous system is located ventrally, peripheral nerves are viable for very specific axonal or dendritic laser ablation [70]. Within the *Drosophil*a Ventral Nerve Cord (VNC), there are both motor and sensory neurons. The location of these neurons makes both cell types viable for larval laser axotomy model studies to investigate similarities and differences between these two neuron types. Additionally, sensory neurons located within the VNC are known as dendritic arborization (da) neurons, and by utilizing GAL4 lines stained with GFP, da neurons can be precisely visualized and ablated in laser axotomy studies [67]. The larval laser axotomy model has also been utilized to study an array of intracellular processes and their role in axonal and dendritic degeneration [71].

The larval nerve crush model is another model utilized to study PNIs and is remarkably similar to the sciatic nerve crush model commonly utilized in rodent studies. In the larval model, motor neurons and their individual axons can be visualized *via* the expression of GFP in available GAL4 genetic lines. This would allow for live imaging to be performed for visualizations of changes over time [68]. Xiong *et al.* established a larval nerve crush model, in which larval segmental motor nerves were visualized through the cuticle in third instar larvae under a standard dissection stereomicroscope [72]. Using a number 5 forceps, segmental nerves can be crushed leading to paralysis of the posterior segments [72]. The primary disadvantage associated with larval models is the intricacy associated with the injury preparation and the shortened study time duration. Since larvae pupate within 2-3 days, larval injury models can only be utilized for acute injury processes [70].

Drosophila larvae have a higher regenerative capacity compared to adults suggesting that molecular mechanisms controlling axon regeneration vary between larva and adult models [73]. Soares *et al.* developed an adult wing injury model that would allow for improved visualization of axon regeneration over the course of 14 days. Comparable to the larva model, in the adult model, GFP was used to visualize neurons and, with a pulse laser, specifically injure distinct L1 neuron cell bodies. Adult *Drosophila* varied in age from six hours to three days of age, and through GFP staining, the site of injury was monitored for axonal regeneration [73]. Purice *et al.* developed a peripheral injury model through sharp excision of the specimen's legs, wings and/or head and were able to identify a set of conserved genes that were acutely upregulated and downregulated in response to nerve injury

[74]. A notable benefit of the adult *Drosophila* wing injury model is the ease and speed at which nonlethal injuries can be created. Additionally, dissection is not required for visualization of the neural components of the wing. These factors allow for increased study size as many injuries can be created and processed in short periods of time [70]. Limitations of the adult drosophila model include the inability to perform antibody stains due to wing composition and the significant damage of adult cell bodies and dendrites during ablation.

3.1.2. Zebrafish

The zebrafish model has become a popular model organism in biomedical research due to multiple advantages, including their high physiological and genetic homology to mammals, external fertilization with high fertility rate and short gestation, rapid development, transparency of embryos and larvae, ease of genetic and other experimental manipulations, cost-and space-effectiveness, and remarkable capacity for nervous system regeneration [75, 76]. Additionally, the zebrafish genome has been found to be highly conserved in mammals, with a zebrafish ortholog identified for 70% of human genes and 82% of human disease-related genes, thus facilitating future clinical applications [75, 77]. Zebrafish are particularly useful in studying the intracellular processes that occur during peripheral nerve regeneration. Processes that have been studied include microtubule organization and axonal transport mechanisms, nerve immunology, regulator processes of axonal degeneration and regeneration, molecular motor proteins, synaptic receptors, and axon-Schwann cell interactions [78-82]. The posterior lateral line (pLL) of the zebrafish is a useful feature in the animal that can be used to evaluate the interactions between the peripheral nerve axons and the supporting cells during development and regeneration [78]. Because of the pLL's superficial and accessible location, all cell types in the system can be genetically, physically or chemically ablated, allowing for studying peripheral axonal structure and function *in vivo* [82]. Modes of injury delivery to the Zebrafish peripheral nervous system include electrically induced neurectomies [78] and laser ablation [83]. Zebrafish come in a wide variety of genetic lines and are useful for transgenic studies that use GFP, facilitating live imaging within embryonic and larval models. This allows for the mechanisms of axonal injury and regrowth to be viewed in real-time [81]. Distinct motor neurons can also be visualized in Zebrafish, allowing them to be methodically transected based on their location on larval hemisegments and distance from the spinal cord [79, 81]. However, as noted by Ceci *et al.*, the innervation process following pLL injury is a highly dynamic one and involves afferent neurons that have the ability to innervate multiple neuromasts simultaneously [78]. Therefore one disadvantage of this model is that nerve regeneration detected in Zebrafish following pLL injury may be secondary to promiscuous innervation, a phenomenon that is difficult to detect [78].

3.1.3. Rodents

The rat is the most common animal model used in peripheral nerve research, with the mouse being the second most popular model [84, 85]. This is secondary to their availability, lower care and maintenance costs, resistance to post-surgical infections, tolerance to captivity and societal acceptance for use in research. Furthermore, rodents are small, exhibit robust nerve regeneration, and come in many inbred strains that create more experimental options [86, 87]. They can be tested in large numbers allowing for sufficiently powered statistical analysis. However, despite the similarities between rodents and human peripheral neural anatomy, peripheral nerve regeneration is must faster than humans and longer nerve gaps must be produced to create a critical size defect.

The commonly used rat species are Wistar, Sprague-Dawley, and Lewis. The sciatic nerve is the most commonly used nerve for nerve injury experiments as it is the largest peripheral nerve in the animal, making experimental procedures easier to perform. It is also a mixed nerve, which allows for testing of both sensory and motor recovery. The forelimb nerves such as radial, ulnar and median nerves and hind limb nerves such as peroneal and tibial are smaller in size and are less commonly used [66]. Purely motor or sensory nerve injuries can be used if the experiment requires, however sensory nerves have a less efficient nerve regeneration compared to motor nerves and are more difficult to evaluate in rodents [88].

3.1.4. Sheep

Sheep models have been established as one of the most relevant animal models for experimental and pre-clinical human studies. Nerves in sheep and humans have similar size and regeneration behavior [89, 90]. Median nerve size is comparable to the ulnar and median nerves in humans, and the femoral nerve is the same length as the ulnar nerve in humans [91, 92]. Since age plays an important role in nerve regeneration, it is important to compare age groups between animals and humans. In sheep, one-week old lambs correspond well to young humans and one-year-old sheep are the closest equivalent to humans around 15-20 years of age [93]. Sheep tend to be the most appropriate large animal model compared to other large animals such as dogs and pigs because of availability, cost, simplicity of care and housing, and societal acceptance as a research animal.

The median nerve is the most studied nerve in sheep since they have the advantage of not affecting limb function, locomotion and weight-bearing. Furthermore, hoof desensitization, trauma and sepsis are uncommon problems [94, 95]. The median nerve originates from the eight cervical and first thoracic nerves. It trails along with the ulnar nerve within the same sheath until the middle of the forelimbs. The median nerve is approached *via* either a straight skin incision placed on the anteromedial aspect of the forelimb between the elbow and the wrist or a medial incision between the axilla and the elbow [96]. Care is taken while dissecting the nerve and the adjacent brachial artery since this will affect the outcome of nerve regeneration and function. After isolation of the median nerve, neurotmesis may be achieved by either an incision or removing a gap of the nerve. Nerve regeneration and outcomes can be studied for anywhere between 1-12 months.

For median nerve regeneration outcomes, nerve blood flow, electrophysiological studies, nerve morphometry and muscle weights have been used. Wet muscle mass of the flexor carpi radialis is specifically used since it is supplied solely by the median nerve [94, 97]. Compound nerve action

potential may be obtained with a stimulating electrode placed under the median nerve proximal to the site of injury, high in the axilla and a second bipolar recording electrode placed below the median nerve in the mid-forearm, distal to the site of injury [84, 98]. Latency, conduction velocity, amplitude and waveform are determined. Terminal branches of the median nerve are stimulated with two monopolar needle electrodes and latency, isometric twitch and tetanic tension of the flexor carpi radialis can be measured. A binary result can also be utilized with a stimulating current of 1-2 mA delivered to the median nerve proximal to the graft and observing the presence or absence of a hoof twitch after electrical stimulation.

In addition to the median nerve, the radial nerve in the forelimbs and the femoral, sciatic and tibial nerve in the hind limbs have been utilized. However, these interfere significantly with locomotion and weight-bearing and are therefore not commonly used [88]. Despite these advantages, the use of sheep to study PNI is relatively new and therefore not as validated as other animal models that have been used for PNI research, namely rodent and murine models [84].

3.1.5. Non-Human Primates

Despite their physiological similarities to humans, the Non-Human Primate (NHP) model is less likely to be utilized in peripheral nerve injury studies due to practical and ethical research limitations [65, 66]. Procurement, transportation, and maintenance expenses, shipping, equipment, animal maintenance during phases before and after the intervention, as well as the facilities required for surgery on these larger specimens, contribute to why NHP have been unpopular for use as *in vivo* models for PNI research [65]. Additionally, NHP show delayed nerve regeneration, lengthening the study period and increasing the cost, making them an inferior candidate for PNI research compared to smaller animal models [65].

NHP have been used to assess the functionality of intraspinal replantation of avulsed spinal nerve roots as a surgical treatment for motor deficits after severe brachial plexus injury. In a study by Carlstedt *et al.*, Cynomolgus monkeys underwent ventral root avulsion of C5, C6, and C7 with or without immediate implantation into the spinal cord. Behavioral and electrophysiological evaluations were conducted over one year. Immediately following surgery, there was flaccid paralysis in the shoulder and elbow with the apparent loss of hand function. Two to three months after surgery, electromyography (EMG) results suggested signs of reinnervation that coincided with evidence of clinical recovery and improved use of function assessed *via* eating habits. Over one year, animals whose EMG showed signs of reinnervation continued to display improved motor function, almost to the point of normalcy. Those with C5-T1 avulsed ventral roots also showed improved motor abilities but did not recover full hand function. Despite the notable reduced EMG activity, it was concluded that the ventral root implantation procedure should be considered as a potential therapeutic approach that can promote motor regeneration even after severe brachial plexus injuries [99].

Another promising NHP model is the rhesus monkey because of its morphological, physiological, and biochemical features resembling those found in humans. Despite these promising similarities, it is essential to note critical anatomical differences compared to humans. In a study of the cadavers of ten rhesus monkeys, it was discovered that there were notable variations of the nerve trunks in the upper brachial plexus of rhesus monkeys. Results of the study suggested that in future research projects, defects should be created at the C5 and C6 or C7 roots, due to inconsistency of distal cord formation. Through these results, future studies can better analyze the efficacy of different nerve grafts more applicable to human injuries [100].

4. MODELS BY NERVE TYPES

Selecting a peripheral nerve injury model requires the consideration of multiple variables, including both the diameter and length of the nerve of interest, the translational characteristics of the animal model to humans, and the possible behavioral sequelae of the animal in which the peripheral nerve injury is created and how that might affect post-injury and intervention testing of that animal. As described earlier, rat sciatic nerve is commonly used in PNI research given the many benefits of using rats combined with the large size of rat sciatic nerve, allowing microsurgical procedures to be performed with greater ease. However, with the sciatic nerve model, hindlimb paralysis is seen and can lead to self-injurious behavior (autophagy of an insensate limb), ulceration of an insensate limb, and joint contractures, all of which diminish the reliability of functional tests. Upper limb peripheral nerve injury models are of interest because of their relevance and potentially human clinical application. In this section, we discuss upper limb peripheral nerve models and the advantages or disadvantages of each.

4.1. Median Nerve

Median nerve models have been increasingly used in *in vivo* nerve regeneration studies because of numerous advantages such as the limited impairment of upper limb function and the remarkably similar anatomy and function between human and animal models, translating to increased clinical relevance [66, 101]. Limited impairment of the upper limb allowed for improved animal well-being and increased the reliability of various functional tests making the results obtained have heightened clinical translation.

The rat model is the preferred model and is the most common model used utilized in peripheral nerve research due to the larger dimensions of their nerves. The larger nerve size facilitates microsurgical procedures; however, there are fewer transgenic rat models available. In recent studies, the median nerve has been used in various studies to evaluate the effects of contralateral cervical 7 nerve transfer, methods of improved regeneration, use of *in vivo* growth factors, and regeneration and functional recovery of various nerve grafts [102-105]. As previously mentioned, the median nerve in animal models has similar anatomy and function as compared to humans. The median nerve in rats is known to exclusively innervate the finger flexors and therefore, muscle function recovery can be evaluated *via* feasible grip strength analyses [102]. Stößel *et al.* utilized various functional tests to evaluate the regeneration and recovery of the median nerve, including the grasping test, the staircase test, and non-invasive electrodiagnostic measurements assessing the recovery of fine and gross motor skills [105]. Yuan *et al.* uti-

lized the median nerve to evaluate the effect of nerve regeneration and motor function recovery after several types of nerve transposition for median nerve defects. Although the study found promising results in donor nerve translocation, it reported that despite utilizing functional wrist flexion tests, there were still shortcomings in the indicators and methods used to evaluate median nerve motor function, suggesting that various methods used to assess recovery have yet to be standardized [106]. As mentioned previously, unlike sciatic nerve models, median nerve models have fewer incidences of auto-injurious behavior or unwanted secondary changes in the musculoskeletal system [105]. The utilization of varied and refined functional tests and serial evaluation of thenar muscle reinnervation coupled with histological analysis allows for precise, improved easy-to-evaluate observation of the onset, progression, and completeness of functional recovery [105].

In the rhesus monkey model, the median nerve anatomy is very similar to human anatomy resulting in comparable sensory loss and loss of thumb opposition due to denervation of the thenar muscles and lumbrical muscles of fingers II and III [101]. Additionally, in comparison to the sciatic nerve model, there were significantly lower ulcerations and fewer joint contractions seen in the median nerve model [66]. The median nerve exclusively innervates the Abductor Brevis Pollicis (ABP) *via* the recurrent branch, which is an easy target muscle to evaluate *via* compound motor action potential electrophysical studies and muscle histology [107].

Behavioral assessment, including animal behaviors, such as finger agility, digital opposition of thumb to index fingers, fist clenching, and food-gripping can be evaluated along with electrophysiological assessments, fluorogold retrograde tracing tests, and histological and morphometric analyses were used to assess recovery nerve function. In addition to nerve conduction studies, muscle contraction force and wet weight of the APB muscle, apple pinch test (thumb abduction during grasping assess *via* photo analysis), and gross observation and histological observation of nerve regeneration can also be used for evaluation [108]. However, this model has associated difficulty in locomotive observation due to loss of adduction and flexion function of the metacarpophalangeal joints of the thumb [101, 109]. Additionally, the deteriorating function of the thumb, index and middle fingers innervated by the median nerve negatively affects the ability of the animal to self-aliment, leading to a poor functional recovery that is worsened and confounded by insufficient food intake [109].

4.2. Radial Nerve

Although not used frequently, the radial nerve has been used in PNI studies because of its innervation of the muscles that control elbow extensors. O'Daly *et al.* established a binary model in which both the radial nerve and musculocutaneous nerve are injured and evaluated by elbow extension and flexion function tests through the Montoya staircase test in the rat model [110]. Due to the location of these nerves, both could be surgically manipulated *via* various injury techniques. Radial nerve transection also denervates the wrist and digital extensors, however despite the injury, these actions were not needed for successful pellet retrieval in the staircase test which was used to assess functional recovery. This suggests that despite injury, eating and feeding habits were not grossly interrupting, leading to the improved well-being of the animal. This model allowed for functional assessment of the interaction of regeneration specificity and function across a single joint [110].

Wang *et al.* proposed the use of a radial nerve model injury in the rhesus monkey model due to its easily observable clinical behavioral test. The radial nerve was severed distally in the primates' forearms, and the designated nerve grafts were sutured to the cut end of the radial nerves. In addition to evaluating histomorphometry and electrophysiology, a wrist-extension test was developed as a behavioral method to evaluate neurological recovery [109]. It is essential to assess functional recovery in peripheral nerve regeneration studies as it is the most relevant outcome and does not necessarily coincide with histological and electrophysiological evidence [111]. The test entailed capturing photos of each animal extending its wrist from a fixed position to reach for food above its head. The photo processing technology was then utilized to evaluate the maximum wrist extension angle. Due to the feasibility of the test, this experiment was repeated ten times over the time span of 5 months. The results of this study demonstrated the functional recovery of the radial nerve defect was clearly shown by this wrist-extension test, which is very simple, non-invasive, and can be repeated as often as necessary in any research laboratory [109].

4.3. Ulnar Nerve

The ulnar nerve has also been utilized in rat peripheral nerve repair studies, although it is less popular. The ulnar nerve is selected because of its feasible functional assessment *via* finger flexion and grasping that can be evaluated through various behavior, functional and histological analyses [112].

Yu *et al.*, utilized the ulnar nerve to create a long segment defect which was then repaired by the transposition of the musculocutaneous, medial pectoral, muscular branches of the radial nerve or the anterior interosseous nerve in a rat model. Recovery was assessed *via* wrist flexion, electrophysiological testing, osmic acid staining, and hematoxylin-eosin staining of the flexor carpi ulnaris. This ulnar nerve injury modeled allowed for feasible electrophysiological testing of wrist flexion and different staining analyses. Despite the injury created all rats were able to self aliment [113]. The ulnar nerve has also been utilized to evaluate whether cutaneous sensory nerve regeneration induces motor nerves after end-to-side-neurorrhaphy. Although the study found that end-to-side nerve repair of a sensory gave rise to successful motor nerve reinnervation, the study was limited by the lack of testing of functional recovery of both motor and sensory fibers [114]. Additionally, the ulnar nerve and median nerves have been used to study different methods of functional recovery. Meyers *et al.* developed an automated behavioral assay that provides quantitative measurements of volitional forelimb strength in rats following median and ulnar nerve injuries. The study found that the isometric pull task is an effective method of evaluating forelimb function following PNI that could be utilized in future studies [115]. The ulnar nerve has also been used in nerve transfer models and was assessed *via* behavioral assessments *via* the Bertelli test (evaluating elbow flexion and abduction) and functional re-

covery [116]. It was selected in this study due to the anatomical similarity to humans, ventral accessibility, and sufficient length [116].

Ulnar nerve injury model has also been utilized in rhesus monkeys with challenges. Hu *et al.* excised 40 mm segments of the ulnar nerve located 10 mm distal to the medial epicondyle of the humerus and tracked nerve regeneration with electrophysiological assessment, neurofilament immunohistochemistry, and axon quantification [117]. The group also tracked neurofunctional recovery by observing the animals' recovery of flexion of the distal interphalangeal joints of the ring and small fingers [117]. Given that the animals were still able to flex the proximal and distal interphalangeal joints of the thumb, index, and middle fingers, as well as the proximal interphalangeal joint of the ring and little fingers (muscles innervated by the median nerve), the animals in this study were still able to grasp food freely. However, observation of functional recovery of flexion of only the distal interphalangeal joints of the ring and small fingers (muscles innervated by the ulnar nerve) was challenging, which presented one distinct disadvantage of this model [117].

4.4. Sciatic Nerve

The sciatic nerve is the most commonly used nerve in peripheral nerve injury studies rat, mice, rabbit, larger animal models and non-mammalian species as well due to its large size, mixed innervation, and easy surgical assessment [6, 66]. It has been used to evaluate different nerve injury models including compression lesions, transection lesions, chronic denervation, end-to-end neurorrhaphy, and nerve defect reconstruction with graft. Since the sciatic nerve is a mixed nerve, it can be evaluated for both motor and sensory functions. Some disadvantages of this model include the high anatomical variability of the sciatic nerve terminal branches as well as the significant limitation of movement following the injury that can lead to unwanted complications, notably serious self-injurious behavior [118].

4.5. Tibial Nerve

Although less frequently used than the sciatic nerve injury model, the tibial nerve has been utilized in the mouse model to study the molecular mechanisms of motor axon excitability [119]. The rat tibial nerve model has been selected in an array of studies varying from assessing the repair capabilities of different engineered nerve conduits to exploring the molecular mechanisms of nerve damage. Tibial nerve injuries have been evaluated *via* the toe spreading reflex, walking track analysis, and force threshold analysis in rat studies [120] as well as functional, electrophysiological, and histological tests focusing on the gastrocnemius muscle as the target end-organ innervated by the tibial nerve [121]. While a motor deficit of the gastrocnemius muscle is a point of study following tibial nerve injury, the gait abnormalities following the injury can negatively affect the wellbeing of the animal model. Specifically, a flaccid hindlimb is more prone to ulceration, and the inability of the animal to walk to its food and water source can lead to failure to thrive for the animal and subsequent need for euthanization [122].

In the sheep model, the tibial nerve was selected because of easy accessibility by surgical intervention to evaluate spider silk constructs' enhancement of axonal regeneration and remyelination [90]. In this study, all animals were tested using the sensitivity of the hind limb. Following nerve injury, the fetlock was partially flexed and absent postural reactions and hypoalgesia of the limb distal to the stimulus were recognized. This notable functional impairment again raises ethical concerns similar to the sciatic nerve model.

4.6. Peroneal Nerve

The peroneal nerve injury model has been utilized in rats to further evaluate Wallerian degeneration and nerve regeneration after injury [123]. In a recent study, both tibial and common peroneal nerves were injured, and the difference in gene and protein expression were studied *via* RNA sequencing and proteomic techniques [122]. The study concluded that the biological process occurring after injury between the two nerves was distinct and these results should be considered in future studies. It is important to note that previous studies have found that the tibial nerve has superior recovery capacity compared to the common peroneal, which is likely why the tibial nerve is more commonly utilized in PNI studies [122]. Yan *et al.* used the peroneal nerve to investigate the impact of nerve repairs at different angles in the rat model assessed *via* muscle volume, tetanic force, the moist weight of extensor digitorum longus muscle, and histological analysis [124]. In addition to rats, the peroneal nerve has also been utilized in various rabbit studies to assess functional regeneration of the peroneal coaptation model and reverse end-to-side neurorrhaphy model [120, 125, 126]. In studies involving peroneal nerve injury, functional recovery was only able to be assessed *via* walking track analysis and toe spreading reflex [120]. This can be considered a disadvantage given the limited number of assessments that can be performed on a PNI model, compared to the sciatic or even tibial nerve injury model, which can be studied using more modes of testing [120].

5. MECHANISM OF INJURY

There are various types of mechanisms used to create injuries in PNI studies in animal models. Injury models should be selected based on the desired degree of severity as defined by the Sunderland classification system [127]. Grade I injuries correspond to neuropraxia and most often describe peripheral nerve compression caused by direct pressure on a nerve trunk or root compression. Grade II injuries entail the loss of axon continuity and demyelination, while Grade III injuries consist of damage to the endoneurium, and Grade IV injuries consist of damage to the perineurium [128]. Grade I injury reproduction in animal models have included compression-induced neuropathy model as well as chemical and cold injury models [127]. This form of injury can be utilized in studies looking to investigate the mechanisms of neuropathy without total loss of motor and sensory function [127]. One common form of injury is the compression lesion created either by ligation or crush methods; however, as will be later described, crush injury methods are mostly used to create more severe injuries.

Nerve crush injuries are caused by a sudden significant force applied to the nerve and are commonly used by researchers studying peripheral nerve regeneration to re-create Grade II-IV injuries [127]. Generally, experiments are performed on sciatic nerves of rodents using serrated or non-

serrated surgical forceps to inflict the injury [127]. However, the nerve crush model has also been adopted in other animal models as previously discussed. It is crucial to establish a uniform method of a crush injury to prevent variability in the duration of force application, the instrument used lesion size, magnitude, and reproducibility. A standardized clamp method that allows for standardized pressure and duration was described that creates a reproducible injury. There are notable advantages for animal well-being by using crush models, including improved nerve regeneration, limited functional impairment, and faster functional recovery [6, 129]. Additionally, self-injurious behavior, in studies specifically utilizing the sciatic nerve, was more frequently seen in protocols using ligation or transection than crush injury models [130]. Due to the mechanical creation of the injury, the crush injury is easier to create than lesions requiring microsurgery and requires less technical experience and oversight [128]. However, this can also lead to more variation between studies as different surgical instruments used in the studies are variable [127]. In crush models, there is an interruption of the continuity of axons leading to Wallerian degeneration [127]. This means that the crush model can also be utilized in studies further investigating the molecular mechanisms and biological processes of axon degeneration and even regeneration. Chemical models have also been constructed to create Grade II and Grade III nerve injuries. In addition, to crush models, excessive stretch injuries can also be used to recreate Grade II-IV injuries commonly seen in avulsion injuries of the brachial plexus and detachment of spinal cord rootlets [127, 131].

Lastly, the most severe nerve injury is Grade V defined by total loss of nerve trunk continuity. This grade of injury is most frequently seen in transection or laceration of peripheral nerves [127]. In this model, it has been found that neater the injury leads to improved recovery, suggesting that precise microsurgical methods should also be considered [127]. This type of injury can be used to investigate various autografts and implantation of tubular biological or synthetic reconstruction materials [127]. Additionally, transection models have also been used to evaluate axonal regeneration after complete nerve transection as well as denervation of distal nerve trunks and their associated consequences on skeletal muscle. Additionally, the transection model is relevant in investigating direct suture repair, tissue glues, and post-surgical scar formation [127]. Microsurgical end-to-end epineural suturing is the most common method of repair. In addition, repair with nerve conduits has been used, where nerve ends are placed near each other within conduits. Tissue glue can be used instead of epineural sutures to approximate the nerve ends. Nerve gaps of 3-5 cm have been studied with allograft or autograft nerve grafts [88].

6. OUTCOME ASSESSMENT

6.1. Functional Assessment

Motor functional assessment is one of the most common methods to evaluate outcome. Gait analysis includes walking track analysis, step length ratio, sciatic functional index, Sensory function assessment can be performed using thermal sensitivity testing and Von-Frey Test [132].

6.2. Electrophysiology

Compound nerve action potential may be obtained with a stimulating electrode placed under the median nerve proximal to the graft, high in the axilla and a second bipolar recording electrode placed below the median nerve in the mid forearm, distal to the graft [97]. Latency, conduction velocity, and amplitude can be determined.

6.3. Histology and Histomorphometry

Histomorphometric analyses such as total axon counts, myelinated axon counts, axon diameter, fiber diameter, myelin thickness, g-ratio (axon diameter/ fiber diameter), percentage of neural tissue (total nerve fiber area/ total area measured). The g-ratio is useful in measuring the maturity of reinnervated nerves. Additionally, histology and immunohistochemistry have been used to evaluate nerve regeneration. Wet muscle masses of muscles innervated solely by the injured nerve can be measured [98].

6.4. Imaging

Nerve blood flow of the injured nerve distal to the graft and that of the normal nerve proximal to the injury can be measured for comparison using Doppler ultrasound. *In vivo* imaging techniques such as MRI can be utilized to evaluate nerve regeneration. However, obtaining useful images with these tests are associated with technical difficulties and high cost [133].

6.5. Retrograde labeling

Retrograde labeling involved injection or application or placing the transected nerve in a conduit with a tracer such as fast blue, fluorogold or fluororuby. Stained axons are then counted, thus providing quantitative data of reinnervation across the injury site. It is also useful to determine misdirected regenerating axons, which will affect functional recovery [134].

7. *EX VIVO* MODELS

The line between *in vitro* and *ex vivo* cellular modeling is often hard to distinguish. Looking at the term "*ex vivo*," one can see the broad definitions that the term may entail. The word "ex" is taken from Latin, meaning 'out of', and "vivo" is also taken from Latin, meaning 'alive'. This suggests a definition of *ex vivo* as something taken out of something that is alive. An explanted tissue specimen, such as dorsal root ganglia, from a mouse that is then cultured and grows a population of primary sensory neurons and Schwann cells then would qualify as an *ex vivo* model: the specimen and cells it contains have been taken out of something alive.

However, as we have discussed above, primary cultures of cells taken from explanted tissues are considered in vitro if they do not exhibit any organizational characteristics that mimic critical *in vivo* conditions. The term "organotypic model" is perhaps more descriptive of such a model that allows cells to grow and function as if they were still growing and functioning within the organ from which they were explanted. Geuna *et al.* have defined the *ex vivo*/organotypic model as in vitro culturing of neurons with three-dimensional organization without completely removing the anatomic and molecular environmental stimuli that are critical for cellular function [6].

We refine this definition by describing an *ex vivo*/organotypic peripheral nerve model as an in vitro culturing of peripheral neurons in a system that recreates the anatomic, chemical, and cellular interactions of *in vivo* nerve, while allowing for real-time assessments of the nerve model without adversely affecting the growth processes that the cellular components undergo while within the *ex vivo* system. They can be either 2 dimensional or 3 dimensional. *Ex vivo* peripheral nerve models should significantly reduce the number of animal subjects needed in a research study, and to this end, they are ideally high throughput, allowing for numerous simultaneous and independent assessments. Finally, *ex vivo* peripheral nerve models should allow for study of both sensory and motor neurons.

There is no consistent methodology of categorizing the various types of *ex vivo* peripheral nerve models that exist, likely due to the great variety of approaches to produce these models. However, we can differentiate *ex vivo* models by characterizing them based on 1. the type of substrate upon which they are based, 2. allowed dimensions of growth (2 dimensional versus 3 dimensional), 3. neuron type (motor, sensory, mixed), and 4. co cultures (neurons, Schwann cells, fibroblasts) and growth factors involved.

7.1. Substrate Types

There have been many substrates used in the fabrication of *ex vivo* peripheral nerve models. These have included organic hydrogels such as collagen, microfluidic devices based on polydimethylsiloxane (PDMS), and other inert polymer matrices.

Collagen is a common substrate choice for *ex vivo* peripheral nerve models. The principles that drive design in peripheral nerve guides and constructs for peripheral nerve regeneration are effectively the same as those of *ex vivo* nerve models: both seek to promote and direct axonal growth. Collagen is a critical component of the extracellular matrix found in peripheral nerve, and its inclusion in these models help recreate the extracellular milieu for neurons and other cellular components in the model. Design complexity varies when using these biomaterials, ranging from longitudinally aligned, highly oriented porcine collagen scaffolds [135] to 3-layer substrates consisting of ventral nerve root graft, fibrin hydrogel, and longitudinal micro-structured collagen scaffolds [136].

The use of PDMS is also popular with *ex vivo* peripheral nerve modeling, as these devices can be etched with microchannels that can behave like aligned fascicles to direct axonal growth. Not only can PDMS microchannel widths vary (as narrow as 20μ [137]), but the viscoelastic properties of the PDMS can be modified as well. Cellular elements in peripheral nerves are surrounded by different tissues varying from adipose tissue, muscle, dermal elements, and cartilage, each with a different viscoelastic property. Matching the viscoelastic properties of the PDMS gel to the origin tissue type helps optimize the growth environment of the neural cells native to that tissue [138].

Blasiak *et al.* developed a 3D-printed surface to model peripheral nerve growth using Veroclear, a material similar to acrylic, and designed channels into the surface ranging from 2500 μm to 500 μm wide, and 500 μm deep [139]. Batth *et al.* have described a 3-dimensional substrate constructed from nylon mesh to coculture a neuronal cell line and primary rat sciatic nerve Schwann cells and investigate their cellular interactions including recording action potentials [140].

There have also been models that have used a combination of the aforementioned materials, such as a PDMS based platform and collagen membrane overlay connected to a peripheral nerve graft, described by Siddique *et al.* [141]. Their design allowed for access to perform microsurgery and image spinal cord sections as they grew ventral root axons on the collagen surface [140]. The growth of axons from the collagen membrane into the peripheral nerve graft simulated a repair of a motor nerve using a peripheral nerve graft [142].

Finally, incorporating other critical Extracellular Matrix (ECM) components onto the substrate itself such as laminin and fibronectin allows neuronal adhesion and neurite outgrowth [143, 144]. Chen *et al.* found greater arborization of neurons when growing on PDMS substrate coated with laminin [138] and Sundararaghavan *et al.* designed into their *ex vivo* model a gradient of bioactive oligopeptide sequences from laminin, YIGSR, or IKVAV [145].

7.2. Dimensional Quality

When discussing 2D versus 3D *ex vivo* models, it is important to understand that the reference of growth pertains to cross sectional axonal organization. While a 2D *ex vivo* model involves directed axonal growth along a biocompatible surface such as a microchannel made from PDMS, a 3D *ex vivo* model involves directed axonal growth usually within a biocompatible gel, such as collagen. The 3-dimensional orientation of extracellular matrix components and architectural orientation of peripheral nerve is very difficult to model *in vitro* [140]. Three-dimensional organization is important because release of cellular growth factors and appropriate interaction of these factors with neighboring cellular elements following nerve injury occurs within a three-dimensional structure *in vivo*. Of the *ex vivo* models we encountered, Siddique *et al.* [141], Allodi *et al.* [146], Gerardo-Nava *et al.* [136] and Gingras *et al.* [147] all describe 3D modeling, and all have relied on a collagen-based substrate to create this 3D growth feature.

7.3. Neuron Types

From a clinical standpoint, motor recovery takes precedence over sensory recovery, and as such, modeling motor axon growth and behavior should prioritize that of sensory neurons. However, the *ex vivo* models that exist use a spectrum of neuron types, including models of purely sensory, purely motor, or mixed motor-sensory neurons.

DRG sensory neurons are easy to dissect, and those sensory neurons can be cultured for extended periods of time [148, 149]. This is contrast with primary motor neuron cultures obtained from embryonic sources, which are much more difficult to culture and maintain. However, sensory neurons are distinct from motor neurons and their behavior cannot be extrapolated to model motor neuron activity.

Motor neurons can be obtained from spinal cord slices taken from 7-day old rat pups. From these spinal cord slices,

the ventral root motor neurons can undergo neurite outgrowth [150] which can be maintained for as long as 3 months [151]. Gerardo-Nava *et al.* designed a 3D model of motor neuron growth based on rat pup spinal cord slices and tested motor axonal growth from the ventral portion of the spinal cord slices into 3 scaffold materials as mentioned above: ventral nerve root graft, fibrin hydrogel, and longitudinal micro-structured collagen scaffolds [136]. They found that spinal cord slices can reproduce the growth of motor axons along each of these biomaterials. As mentioned above Vyas *et al.* developed an *ex vivo* model of peripheral motor nerve repair also using spinal cord sections from mice [142].

Allodi *et al.* have used 3-D culturing of spinal cord slices (primary motor neurons) and DRG explants (primary sensory neurons) embedded in 60 uL aliquots of rat-tail collagen gel (made at a little over 3 mg/mL) [146]. Their goal was to determine the effects that specific neurotrophic growth factors have on sensory versus motor axonal regeneration and arborization. Their results suggested a difference in arborization response between sensory versus motor neurons when exposed to different neurotrophic factors. For example, sensory axons arborized significantly more than motor axons when exposed to GDNF, whereas the reverse was true when both were exposed to BDNF. Their model demonstrated the ability to study the effects of diffusible molecules on growth patterns of motor versus sensory axons.

An interesting approach to modeling a mixed motor-sensory nerve in vitro was demonstrated by Brushart *et al.* [152]. They used spinal cord slices taken from mice expressing Yellow Fluorescent Protein (YFP) to create an in vitro peripheral nerve regeneration model with motor axons that were able to be visualized clearly as they grew from a the ventral root [140]. The group has expanded this model by coculturing these YFP expressing motor neurons with Red Fluorescent Protein (RFP) expressing sensory neurons taken from mice DRG [151]. The group used a reversed femoral nerve graft with a Y-branch to allow motor neurons to grow down one branch, and the sensory axons to grow down the other branch; the subsequent mixing of the two axon types then offered a color-coded mixed motor-sensory nerve. Interestingly, they found an inhibitory effect on motor axon growth when motor and sensory axons were grown together. By contrast, motor axons demonstrated normal and much higher growth rate when grown alone.

Sharma *et al.* have recently demonstrated simultaneous growth of human motor and sensory neuron cell lines within fabricated spheroids that were then cultured within a hydrogel micro well system in an aligned fashion [153]. Through this system they were able to perform electrophysiological studies as well as confocal microscopy, immunohistochemistry imaging, and transmission electron microscope imaging, demonstrating aligned and myelinated peripheral nerve growth.

7.4. Co-cultures and Growth Factors

Many cellular elements are necessary for *in vivo* axonal function. These include, but are not limited to, Schwann cells, immune cells, fibroblasts, and the most distal end organ, at the neuromuscular junction.

Conveniently, primary Schwann cells are readily cultured along-side neurons and axons they myelinate within the same primary tissue specimen, such as with dorsal root ganglia. Bozkurt *et al.* used hemisected DRG to grow axons and Schwann cells (after 21 days) into their *ex vivo* models [135]. The growth patterns were noted to resemble the "Bands of Büngner" structures that have been implicated in the guidance of regenerating axons.

As mentioned above, Blasiak *et al.* used a 3D printed substrate to direct axonal growth when embryonic mouse DRGs were plated on mouse NIH3T3 fibroblasts precultured for 3 days [139]. They discuss that fibroblastic secretion of matrix proteins created the molecular surface needed to help with axonal growth attachment and guidance along the channel.

Gingras *et al.* have produced a 3D model based on scaffolds made from collagen-chitosan cultured for 21 days with both fibroblasts and Schwann cells [147]. Following this, primary motor neurons were seeded onto this construct and cultured for an additional 14 days. During this period, the growth medium was supplemented with a neurotrophic factor cocktail containing human NT-3, BDNF, GDNF, CNTF. They found that the addition of fibroblasts as well as the neurotrophic growth factor cocktail were the most effective elements in the model that promoted neuronal survival and growth. Without fibroblasts, the motor neurons were not able to survive more than 14 days when seeded alone on the scaffolds. The group describes that fibroblasts were critical in the survival of the neurons, likely secondary to their production of ECM elements as well as secretion of growth factors. The addition of neurotrophic growth factors underneath the scaffold allowed for deep axonal growth along a gradient towards the area of the highest factor concentration. Finally, the group was able to detect Myelin Basic Protein (MBP), a marker for myelination, after 14 days of culture of the neurons along with Schwann cells and fibroblasts.

There have been efforts to develop models to study the end-organ of the peripheral motor nerve: the neuromuscular junction (NMJ) [154]. The study of the NMJ over the past few decades was meant to understand synaptogenesis. However, with the progressive awareness of peripheral neuromuscular degenerative diseases and their prevalence, such as ALS and myasthenia gravis, comes the need also to produce models that study the NMJ site where denervation eventually leads to NMJ fragmentation and eventually the irrevocable loss of motor innervation. Models of the NMJ have ranged from 2D models to more complex 3D microfluidic systems. Examples include 2D nerve-muscle cocultures and the subsequent formation of NMJs when mice embryonic stem cells differentiated into motor neurons were cocultured with myotubes [155]. More complex systems have included motor neuron and myocyte aligned compartmentalization within an ECM hydrogel-filled microfluidic device with a gradient of bioactive oligopeptide sequences, as mentioned previously [143].

8. FUTURE DIRECTIONS FOR *EX VIVO* MODELS

Microchannel arrays have already been used for the purposes of peripheral nerve interfacing in the advancement of bioprosthetics control [137]. Along those same lines, Froeter *et al.* have been advancing neural-electric interfaces by making the systems smaller and more controllable. They have

investigated single neuron axonal guidance and growth on biocompatible semiconductor surfaces, the guidance provided by external cues in the form of microtubes (2.7 - 4um in diameter) fabricated from self-rolled silicone nitride film. They found that mouse-derived cortical neurons exhibited 20 times the rate of axonal growth compared to growth outside of the nanotubes on the same silicone oxide (glass) surface coated with poly D-lysine [156]. The application of their work in peripheral neurons and axonal growth is currently being considered.

A fundamental characteristic of the *ex vivo* nerve model is to allow for efficient screening for effective treatments in peripheral nerve injuries before committing to time and resource-intensive *in vivo* models, surgeries and post-operative evaluations that follow intervention. As such, an *ex vivo* model should be high throughput, capable of simultaneously assessing hundreds or even thousands of interventions. There has yet to be a high throughput *ex vivo* peripheral nerve model described that can allow for this number of simultaneous assessments.

An *ex vivo* nerve model should also allow for real-time assessments of axonal growth without affecting the growth process itself, such as with use of live cell imaging techniques and ion-sensitive dyes to study action potential generation. Alternatively, whole-cell patch clamping has been used previously by Lin *et al.* in their study of action potentials in mouse dorsal root ganglia sensory neurons on elastic PDMS gel following mechanical stimuli with glass pipettes [157]. Although a terminal testing procedure for the neurons, whole-cell patch clamping is still a viable technique to measure action potentials, given that the *ex vivo* model is high throughput.

Other limitations with current *ex vivo* nerve models are the lack of incorporating vasculature, immune cells, and directed and organized end-organ targeting, namely the neuromuscular junction [135].

CONCLUSION

Peripheral nerve injuries represent challenging clinical problems, the sequelae of which can be devastating. Therefore, the study of peripheral nerves is of great importance, including the use of *in vitro*, *in vivo*, and *ex vivo* models. *In vitro* models employ cell culture and are the most basic form of peripheral nerve modeling. These types of models usually are inexpensive and have lowest ethical burden. *In vivo* models represent the gold standard in peripheral nerve modeling and have the highest ethical burden and cost, which includes the animals themselves along with the associated surgeries/ interventions and testing. Furthermore, *in vivo* modeling is limited that a single animal can usually only provide a pair of peripheral nerves that can be studied, one of which is often used as an internal negative control. *Ex vivo* models attempt to bring together the strengths of *in vitro* and *in vivo* models by recreating the anatomic, chemical, and cellular interactions of *in vivo* nerves within a device. A great variety of *ex vivo* models have been created and studied, each having strengths and weaknesses.

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CONFLICT OF INTEREST

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