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Axon repair: surgical application at a subcellular scale

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Abstract

Injury to the nervous system is a common occurrence after trauma. Severe cases of injury exact a tremendous personal cost and place a significant healthcare burden on society. Unlike some tissues in the body that exhibit self healing, nerve cells that are injured, particularly those in the brain and spinal cord, are incapable of regenerating circuits by themselves to restore neurological function. In recent years, researchers have begun to explore whether micro/nanoscale tools and materials can be used to address this major challenge in neuromedicine. Efforts in this area have proceeded along two lines. One is the development of new nanoscale tissue scaffold materials to act as conduits and stimulate axon regeneration. The other is the use of novel cellular-scale surgical micro/nanodevices designed to perform surgical microsplicing and the functional repair of severed axons. We discuss results generated by these two approaches and hurdles confronting both strategies.

The utility of reconnecting nerves that have been severed was recognized in ancient Greek times by Paul of Aegina (625–690 AC) who first described the concept of nerve suture, with the first actual procedures performed in humans in 1864 by the French surgeons Laugier and Nelaton.¹ Since then, surgical technique and fundamental neuroscience have of course advanced greatly. However, the principle of reconnecting nerves to allow long-distance axonal regeneration across sites of injury still underlies modern nerve repair. To this day, repair in the peripheral nervous system (PNS) involving the major nerves that control limb muscles remains very difficult and often have suboptimal outcomes. Furthermore, damage to central nervous system (CNS) components such as the spinal cord, optic nerve, and brain results in permanent loss of neurological function as CNS axons have limited if any capacity for regrowth, and environmental factors further inhibit regenerative potential.

Currently, the major research paradigm for improving recovery from both PNS and CNS injuries is the promotion of axon regeneration. The goal of this regenerative strategy is to obtain the sufficient regrowth of injured axons from the damaged stumps back to their original targets and the reestablishment of appropriate synaptic contacts to regain neurological function. Axon regeneration is well suited as an application area for novel nanoscale materials and devices. New abilities to organize, display, or deliver biologically active compounds and biomaterials at small length scales can provide highly customized environments to regulate axon regeneration. Although the restoration of axonal connectivity is the key to functional recovery, not much attention has been paid to the alternative

possibility of direct 'surgical' repair of severed axons to achieve structural integrity after axon damage. The primary perceived obstacle to this approach is the technical inability to perform 'surgical' manipulations at the subcellular scale on axons. However, progress has been made recently in this area with the testing of technology principles and microdevices with nanoscale features to perform basic steps of axon repair. In this article, along with a brief discussion of nanomaterials used to foster axon regeneration, we will focus on the relatively new topic of axon microrepair and potential micro/nanosolutions to subcellular manipulations for the treatment of nervous system trauma.

The Challenge of CNS and PNS Injuries

A recent study by the Christopher and Dana Reeve Foundation provided a sobering statistic that in the United States, close to 1.3 million individuals suffer from some form of spinal cord injury and live with permanent neurological dysfunction (report available at www.christopherreeve.org). When the number of patients with impairment from PNS injuries is also added to this statistic, the true impact of axonal injury on our society can be appreciated. Severed axons in the adult CNS do not spontaneously regenerate from their axon stumps to reestablish appropriate neural circuits because of an inhospitable CNS tissue environment inhibiting axon regeneration.^{2,3} In addition, intrinsic properties of mature adult CNS neurons also do not favor axon regeneration as the axon growth potential present during nervous system development is dampened in the adult.^{4,5} Damaged PNS axons face a different set of difficulties. Unlike CNS axons, PNS axons can exhibit long-distance regrowth if they find their way into endoneurial sheaths that serve as microconduits extending all the way to the original targets. In current nerve reconstructive surgery, severed nerve stumps are aligned manually and precise axon alignment with endoneurial sheaths cannot always be achieved. As a result, the outcomes from major peripheral nerve injuries are often suboptimal.⁶

Nanomaterials for Axon Regeneration

One strategy to support axon regeneration is the provision of artificial constructs to replace the endogenous inhibitory environment. This has been investigated both for CNS as well as PNS nerve regeneration. For PNS regeneration, an active area of investigation is nerve constructs that are inserted in between two PNS nerve stumps to replace a segment of damaged nerve.^{7,8} Axons do not need to be aligned specifically with endoneurial sheaths, and the desired molecular factors can be presented to stimulate axon growth. At the same time, axons within the artificial nerve can be shielded from inhibitory molecules. Earlier efforts in this area have mainly sought to incorporate biological molecules known to support axon growth. More recent work has utilized synthetic methods to produce an internal architecture within these constructs that mimics endogenous extracellular matrix nanotopography. An example is nerve constructs produced via electrospinning of biodegradable and bioactive polymers that can be chemically modified to display cell adhesion molecules in support of axon growth.^{9,10} Artificial nerve grafts can potentially bypass the current use of donor nerves from patients to bridge a gap between the viable proximal and distal stumps of a damaged nerve. This is especially true if artificial nerve constructs can be created to substantially speed up axon regeneration beyond the ~1 mm/day that is currently achieved under the most favorable PNS nerve reconstruction involving endoneurial repair.¹¹

Self-assembled peptides have been used as a nanoscale material to promote axon regeneration in the CNS.⁸ One approach known as nanoknitting utilizes 5 nm oligopeptides to form a complete environment through which CNS axons regenerate. As with the artificial nerve conduits for the PNS, nanoknitting is thought to provide a supportive environment

while eliminating any inhibitory factors normally present in the damaged CNS. Initial results from this nanoknitting strategy in a hamster model of CNS axon injury have been remarkable, as adult axons from the retina which do not spontaneously regenerate have been reported to grow past a lesion within the optic tract to reach their normal CNS target and form functional connections.¹² Nanoknitting has also been investigated for the treatment of spinal cord injury in rats.¹³

Subcellular Axon Repair as an Alternative to Regeneration

Microstructural reconnection of severed axons to reestablish axon continuity and functional neural circuits has been proposed as an alternative to regeneration. This approach considers nerve injury as a subcellular neurosurgical problem in which micro/nanodevices are used as instruments to accomplish the repair of individual axons. The central thesis is that the physical rejoining of two severed axon segments will restore axonal electrical conduction and cell biological mechanisms necessary for the long-term viability of neural circuitry.

Paradigm for Axon Repair

Although intervention at the level of individual axons is beyond existing surgical technology, it is nevertheless useful to consider axon repair as a procedure that is fundamentally surgical in nature. Surgical procedures may be broken down into the essential steps of cutting or removal of diseased tissue, the bringing together of healthy tissue, and the physical rejoining of healthy tissues to reconstitute normal function. The basic steps of axon repair can be similarly broken down into first, the removal of the injured ends of axons and the trimming back to healthy axon segments. Second, the axon segments must be physically brought together to achieve close membrane–membrane apposition. Third, the induction of biophysical fusion of the membranes of apposing axon segments to form a single structurally intact and functional axon. Injuries may also occur in which an entire axon segment is lost leaving a gap between the ends that must be rejoined. In this case, free segments of axons obtained from a donor nerve graft may be used to bridge the defect. Two sets of axon junctions will be involved, but the basic repair protocol can still apply.

Conventional surgery is typically performed using scalpels, forceps, and sutures or staples. In axon surgery, new methods and devices must be developed to accomplish the same steps taking into account the length scale and biological fragility of axons. Axon repair is also proposed to take place soon after injury to prevent the loss of synaptic circuitry. Within 1–2 days after injury, distal axon segments that are separated from the parent cell body undergo Wallerian degeneration characterized by the degeneration of the isolated axon together with its terminal arborization and synaptic connections. If axon repair can occur prior to Wallerian degeneration, the restoration of axonal continuity may take advantage of the fact that neuronal synaptic connectivity is preserved and function may be restored soon after surgical intervention. By comparison, conventional axonal regeneration occurs after Wallerian degeneration and requires axons to regrow substantial distances and completely reestablish appropriate synaptic connections with the correct partner cells. Below, we discuss the exploratory work that has been undertaken to investigate potential technologies and device foundations for axon repair. Alternative methods and principles will also be discussed as possible avenues for investigation.

Subcellular-Scale Axon Cutting

Technologies for axon repair must be able to deliver precise cutting at specific locations along an axon without detrimental biological or structural effects on the remaining axon ends. A widely investigated method for biological cutting with micron- or submicron-scale precision is the laser microbeam or laser scissors.¹⁴ Lasers have been used to cut or ablate

whole cells and intracellular elements such as chromosomes, mitochondria, and microtubules. Laser cellular cutting typically involves short pulse times to minimize the heat-induced damage of adjacent structures whose biological function may be critical to retain¹⁵ and has been performed using nanosecond, picosecond, and femtosecond pulsed lasers. The resulting ablation zones from picosecond and femtosecond lasers are on the order of 300–500 nm in diameter.^{16,17} In the majority of instances, dye labeling was used to photosensitize the intended target prior to ablation. The microcutting of unstained chromosomes has been reported¹⁸ but required higher peak power than the cutting of dye-stained chromosomes. Femtosecond lasers have been used to sever single fluorescently labeled axons in nematodes.^{19,20}

An alternative that may be simpler, less costly to implement and does not require dye photosensitization, is miniaturized mechanical cutting that matches the axonal length scale. A nanoknife produced using silicon microfabrication and microassembly techniques has been demonstrated for the precise cutting of a variety of CNS and PNS axons both *in vitro* and, in anesthetized animals, *in vivo*.^{21,22} The initial version of the nanoknife was an assembly of two microfabricated silicon components consisting of a micromechanical suspension and a microscale cutting structure in the shape of an elongated pyramid formed from an optically clear shell of silicon nitride (Figure 1(a)). The mechanical suspension was a contiguous silicon planar structure consisting of a 1 mm² frame and a pair of serpentine flexures that permitted multiaxial motion for the centrally placed cutting shell and the right combination of compliance and stability to achieve subcellular-scale cutting. The apex of the elongated pyramid formed a nanoscale cutting edge with 20 nm radius of curvature (Figure 1(b) and (c)) and was oriented perpendicular to the plane of the suspension frame. The suspension allowed out-of-plane deflections in the nanoknife when it encountered any hard surfaces while completing its cutting stroke. The length of the cutting edge can be fabricated from one to several hundred microns depending on intended use (Figure 1(d)). In use, the assembled device was oriented with the suspension frame parallel to the underlying substrate and mounted onto a rod held by a micromanipulator to position the nanoknife and to deliver the cutting stroke (Figure 1(e)).

This nanoknife has been extensively tested and found to be highly effective in severing axons. In addition to axons grown in culture, it can also cut both myelinated and unmyelinated axons acutely harvested from adult animals (Figure 1(f)). The 20 nm radius of curvature at the cutting edge is about the diameter of a single microtubule or the width of a synaptic cleft^{23,24} and delivered precise cutting without distorting adjacent segments. With its small cutting footprint, the nanoknife can sever a single process within a complex field. Compared to glass micropipettes that have been used previously to sever axonal processes for basic biological investigations,^{25–27} the nanoknives avoid mechanical shearing that may lead to unintended cellular and molecular changes and produce more precise cuts at specific desired locations along axons.

In addition to *in vitro* demonstrations, axon nanoknives have also been used *in vivo* to address the question of whether miniaturized microfabricated cutting instruments can be effective under real surgical conditions. In a study involving sciatic nerve surgery in anesthetized mice, a single axon nanoknife, when used with due care, was found to be mechanically robust enough to make repeated cuts in a peripheral nerve.²² The progressive cutting of axons eventually completely severed all axons in a nerve as demonstrated by the elimination of the accompanying electromyographic signal recorded from the target muscle.²² The researchers also investigated microscale applications of the nanoknife and demonstrated the targeted excision of a short axonal segment from a living axon, mimicking one of the proposed steps of axon microrepair (Figure 2). It is of note that other than the

nanoknife, all other surgical components in this study consisted of off-the-shelf commercial components.

Although miniaturized mechanical cutting of axons is possible and can be used in real operative environments, there is substantial room for improvement. In particular, microsuspensions with additional points of fixturing will provide additional stability to the microdevice in use. Another major advance in usability and precision can be achieved by the incorporation of on-board knife actuation to deliver the cutting stroke. Rather than depending on an external micromanipulator for the downward cutting stroke with its attendant problem of mechanical vibration, a self-actuated nanoknife will allow the system to maximally benefit from the precision inherent in microdevices.

Controlling Axon Movement

In conventional macroscale surgery, tissue manipulation usually relies on physical gripping and the application of mechanical forces. The specific surgical tool used for gripping and the amount of force applied depends on the size and fragility of the tissue to be manipulated. At present, we do not have instrumentation that can direct the movement of subcellular processes such as axons using microscale gripping and mechanical movement. However, mechanical translocation of cells in fluidic environments has been demonstrated in which MEMS polymer grippers have been used to maneuver single cells or cell clusters.^{28,29} It is unclear whether this principle can be scaled down to effectively manipulate axons. Furthermore, although the axonal cytoskeleton composed of structural protein complexes such as neurofilaments and cytoskeleton should provide some mechanical stiffness and support, axon manipulation via direct physical contact will require complex control of grasping forces that are low enough to avoid potential axon damage, but strong enough to maneuver axons.

Manipulation by Optical Trapping

A robust method of microscale biological manipulation that has wide application in biological research is laser-mediated optical trapping.^{30,31} Based on the concept of momentum exchange between photons and physical objects, the forces generated are typically very small in the piconewton range, but corresponds exactly to the forces useful for some types of cellular manipulation and biological studies.^{32,33} Optical trapping is easiest for objects with high refractive indices as the change in the direction of light through the object is balanced by momentum imparted onto the object. Low refractive index objects such as mammalian cells are challenging but have been demonstrated.^{34–36} The issues related to the use of laser trapping for axon manipulation and repair include selection of a suitable wavelength to minimize biological damage, the application of minimal power to minimize temperature elevations while ensuring sufficient laser power to generate a trap that can move the target axon at a reasonable velocity (e.g., 5–10 $\mu\text{m/s}$). Some of these issues can be mitigated by the use of ‘handles’ of suitably sized optically refractive beads that are tethered to the axonal membrane. Laser trapping of the beads may then also move the axon to which the beads are tethered. Axon movement via optical trapping has not yet been demonstrated and methods that require the tethering of particles on axons are less desirable from a therapeutic point of view. Laser-based manipulation systems for axon repair will also require multiple beams that are steered independently. Such systems have been described using acousto-optic deflectors to time-share the light beam and generate multiple trap locations,³⁷ and by using spatial filtering phase contrast approaches.³⁸

Dielectrophoretic Manipulation of Axons

Dielectrophoresis (DEP) has been shown to be a highly effective means of noncontact biological manipulation that generates sufficient force for whole cell translocation in fluidic environments.^{39–41} DEP is the movement of polarizable objects in a nonhomogeneous electrical field. In an electrical field, objects including cells experience dielectric polarizations resulting in the opposing poles to be pulled in opposite directions. In a homogeneous electrical field, the opposing forces will exactly cancel each other, and no net movement of the object will occur. However, in nonhomogeneous electrical fields with gradients of field strengths, the object will invariably experience a net force in one direction, causing it to move toward one side of the electrical field.³⁹ DEP force is directly related to the magnitude of the applied electrical field and the AC frequency. The size and shape of the object and the dielectric properties (permittivity and conductivity) of the object and of its surrounding medium also greatly influence the DEP force. In practice, the magnitude of the voltages that can be applied to objects in a fluid environment is limited as high voltages damage cell membranes and can lead to electrolysis of water when the electric field frequency is low. As a result, DEP forces are most useful for manipulation of small objects such as bacteria (0.5–2 μm) and cells (5–30 μm) at frequencies above at least 5 kHz. An important advantage of DEP is that cells move within DEP fields based on their intrinsic properties without the need for some form of extrinsic labeling.

Controlled axon movement in DEP fields has been demonstrated *in vitro*. Using microfabricated electrode pairs of various configurations designed to create inhomogeneous electrical fields, isolated sciatic nerve axons have been observed to move in response to the presence of DEP fields.⁴² Of note, observations from these studies indicate that DEP fields influence axons within a range of about 30–40 μm . Axons beyond this distance are not affected. This localized effect is a potential advantage for directly controlling the movement of specific axons without influencing others nearby. Axon movement in DEP fields occurs at a velocity of about 5 $\mu\text{m/s}$ (Figure 3). The speed with which axons move in response to a particular method of translocation is an important parameter as repair may involve a large number of axons, and induced axon movements that are too slow may prolong procedural time.

The demonstration of axon DEP raises two important questions. First, will DEP be sufficient to generate the close axon membrane apposition between axon pairs necessary for biological fusion? Second, is DEP compatible with axonal viability and if so, what is the parameter space for safe axonal DEP? Studies of whole cells in DEP fields have shown that cells, when polarized by an external electric field, can behave like tiny electrodes. As two cells approach one another, they couple to the electrical field and exhibit mutually attractive end-forces to ‘pearl-chain’ together end-on-end along the electrical field lines as the plus side of one cell attracts the minus side of another.^{39,43–45} Neighboring cells within a pearl-chain thus have regions of close membrane apposition with one another. Given that axons are simply extensions from the cell body with similar membrane biophysical properties, axons should likewise exhibit a ‘pearl-chaining’ phenomenon in DEP fields. However, as they differ in their highly elongated geometry and much smaller dimensions, the type of pearl-chaining that can result and the specific field configurations to best induce such effects remain to be determined.

DEP does not appear to have gross deleterious effects on cells. DEP parameters typically used for cell manipulation (10 kHz to 1–3 MHz, in suspending media with conductivities of 50 mS/m or less) have been shown not to affect cell viability and membrane integrity.^{46,47} Cortical neurons that have been subjected to DEP have been reported to continue axon outgrowth.^{48,49} Hawkes and colleagues have recently examined whether DEP fields influenced subtle aspects of axonal biology such as mitochondrial trafficking. Mitochondria

serve as the source of cellular energy, mediate calcium homeostasis, and mitochondrial dysfunction underlies neurodegenerative diseases.^{50,51} The results showed that axons can be safely exposed in a low conductivity medium (100 mS/m) to DEP fields of V/m at 100 kHz for at least 60 s, without any detectable alteration in mitochondrial dynamics such as average speed, the presence of anterograde and retrograde transport (Hawkes et al., in preparation).

Light Activated DEP

As DEP is an electrokinetic phenomenon, DEP cellular manipulation is typically performed using fixed microfabricated electrodes. As a result, DEP might be effective if axons only need to be moved short distances for membrane apposition, but long-distance translocation over hundreds of microns or more may not be easily achieved even using DEP electrode arrays. By comparison, optical trapping is independent of fixed positions and may be better suited for this purpose. An interesting combination of optical and DEP methods of manipulation has been demonstrated.⁵² Rather than fabricated electrodes that are fixed in position on a surface, optical DEP utilizes a photoconductive surface and generates DEP at sites corresponding to an impinging light pattern, which produces a transient local 'virtual' electrode on the photoconductive surface. Movement of light along this surface is then tracked by movement of the DEP cell trap(s). As LED light sources can be used, much less optical intensity is required compared to laser-based manipulation. At the same time, optical DEP also provides for much greater flexibility in maneuvering cells compared to conventional electrokinetic methods.

Induction of Axon Fusion

At its fundamental level, axon repair is based on the concept of biological fusion. Fusion of two separate cells to create one functional entity is not a new concept in biology and in fact occurs *in vivo* as myoblasts fuse to form differentiated muscle cells and has been noted for the fusion of bone marrow cells with adult cerebellar neurons to form stable heterokaryons.^{53–55} Fusion also occurs at subcellular levels such as synaptic vesicle fusion during neurotransmission⁵⁶ and fusion of enveloped viruses to gain entry into cells.^{57–59} In addition to naturally occurring cellular fusion, induced-cell fusion is also an established methodology in biotechnology and has most commonly been used in the creation of hybridoma cells for monoclonal antibody production.⁶⁰ It is of interest to note that axons in invertebrates that have been severed have been reported occasionally to undergo spontaneous fusion with their separated distal segments. This phenomenon has been reported for axons from the California sea slug *Aplysia californica* cultured *in vitro*,⁶¹ and *in vivo* in axons from the leech *Hirudo medicinalis*⁶² and the nematode *Caenorhabditis elegans* following femtosecond laser ablation of axons.^{63,64} Fusion events occurred in only 1–10% of time and the molecular mechanisms at work are not currently understood.

Axon Microelectrofusion

One method for inducing fusion in mammalian axons is microelectrofusion. Electrofusion involves the use of electrical pulses to create the transient breakdown of the cell membrane and the formation of pores that are unstable and eventually reseal.⁴⁵ If the pores are formed in the region of contact between neighboring cells, fusion between the two cells will occur. The threshold voltage across a membrane required for this electrical breakdown is ~1 V. In cell culture, the typical field strengths required to generate 1 V across membranes are in the range of 2–8 kV/cm,^{44,45} usually delivered using 2–4 rectangular pulses of 10–100 μ s each. Electrofusion has been used extensively to create mammalian cell hybrids^{65,66} and has also been used to fuse embryonic or fetal somatic cells with enucleated oocytes to successfully clone both sheep and mice.^{67,68} The ability of fused embryo cells to develop to term and subsequently to grow into adult animals, argue for the safety and efficacy of electrofusion.

Early studies of axon microelectrofusion in culture have provided examples of mammalian axon fusion. In initial studies on mouse axons, relatively crude probe-like microelectrodes 12.5 μm in diameter were positioned within 50–100 μm of axons using a micromanipulator, to induce electrofusion between axons that contained soluble cytoplasmic green fluorescent protein (GFP) with axons that did not. Successful axon fusion was observed as GFP spread from one fusion partner into another (Figure 4).⁴² Although useful as an initial proof of principle, the time-consuming positioning of microelectrodes near target axon pairs is not precise, leading to poor control of fusion parameters and is thus impractical. One of the challenges in using electrofusion for axon reconstruction is the need for close membrane apposition between the axon fusion partners. In cell fusion, this can be achieved via a membrane apposition step using DEP to first bring two cells into contact or pearl-chained with each other. Similarly, efficient axon fusion may require a seamless progression from axon DEP to appose two axons, followed by axon fusion. In addition, novel ways of bridging two axons might provide the conditions necessary for high fusion efficiency.

Other Methods for Fusion

Cellular membrane fusion requires some means of inducing a breakdown in the bilayer structure. In addition to the use of electrical fields for this purpose, chemicals that disrupt lipid bilayers have also been used. As a result of its low cost and ease of use, polyethylene glycol (PEG) is a popular fusogen for hybridoma cell production in which immortal myeloma cells are fused with antibody producing B cells to produce immortal antibody producing hybridoma cells. Fusion of brain tumor cells with neurons to create neuronal cell lines has also been reported using PEG.⁶⁹ From its earliest days as a fusogen, however, PEG is known to have toxic effects on cells (e.g., Ref 70). Since PEG is typically used in instances in which millions of cells are available for fusion, substantial cell loss and low viability after fusion are acceptable as long as a few immortal cells remain.

Despite its potential toxicity, chemical fusion of nerves using PEG has been reported. Up to 50% PEG was applied to the ends of whole nerves in an attempt to reconnect axons to obtain functional recovery.^{71–74} Electrical conduction was reported across the junction suggesting that some axons were indeed reconnected, presumably by fusion. Given the application of a nonspecific fusogen such as PEG to whole nerves, the numbers of axons fused were noted to be small and fusion was noted to be fragile; an observation that may be due in part to membrane toxicity caused by PEG. PEG-mediated repair of whole nerves awaits confirmation by additional research. Furthermore, repair strategies that are applied manually to whole nerves are likely to suffer from poor control compared to methods that specifically target the individual axons that act as the basic units of neuronal function.

The application of laser cell trapping combined with laser induced membrane fusion^{75,76} has been demonstrated. In this approach, the two cells to be fused are trapped using lasers and brought into contact with one another. Once in position, a different laser is used to induce breakdown of the cell–cell membrane contact region. A fusion rate of 38% was reported and hybridoma cells producing monoclonal antibody were generated using this technique. This laser fusion technique, however, still required the addition of 5% PEG, as the fusion rate without PEG was 0%. The use of laser trapping for cell fusion requires a great deal of operator skill and has not been attempted for axons.

Lastly, as membrane fusion is an integral part of biology, highly regulated and successful molecular mechanisms have evolved to mediate this process. It is possible that aspects of biological fusion can be harnessed to work in concert with micro/nanodevices in axon repair. Two types of biological fusion have been extensively studied. The first is the fusion between intracellular synaptic vesicles with the presynaptic axon plasma membrane occurring during neurotransmission.⁵⁶ As a result of this fusion, neurotransmitter molecules

inside vesicles are released extracellularly into the synaptic cleft to activate a postsynaptic neuron. Synaptic vesicle fusion requires SNARE (Soluble NSF Attachment Protein Receptor) and SM (Sec 1/Munc 18-like) proteins with SNARE proteins on apposing membranes interacting in alpha-helical bundles to pull together membranes for fusion. SM proteins bind to SNARE complexes to support the fusogenic events. Another well-studied type of biological fusion is the one used by enveloped viruses to gain entry into mammalian cells. Although viral fusion proteins can be grouped into three distinct classes based on protein structure, they all use a strategy of conformational change to bring together lipid membranes for fusion.^{57–59} Both vesicular fusion and viral fusion mechanisms should be explored for potential utility in axon fusion. For example, it may be possible to transfer the biological activities or the potential for conformational change inherent in individual proteins into the new cellular context of axons to support a novel form of biological fusion. Two challenges facing such an approach include the integration of fusion proteins into axon repair without the use of genetic manipulation, and the sufficient control of fusion to ensure an orderly repair process.

Management of the Subcellular-Scale Operative Environment

Cellular-scale surgical intervention will require new methods and instruments to manage the operative environment. Currently, conventional neurosurgery is performed within an exposed surgical field. Repair of single cells and axonal processes requires a more stringent intraoperative environment in order to maintain cell survival, operation of micro/nanosurgical devices, cell monitoring, and mechanical stability. This may require miniaturized neurosurgical platforms placed within the larger surgical field, to isolate the environment within which cellular-scale manipulations can take place. As cells require a fluidic environment, such surgical platforms require fluidic control, while retaining maximal accessibility for cellular intervention and imaging. The design of such mini-surgical platforms may vary considerably to suit the anatomical constraints of the specific neurosurgical task at hand.

A miniaturized surgical platform with some of these features has been tested for sciatic nerve surgery on anesthetized mice.²² The goal of this study was to test the performance of a surgical microdevice (axon nanoknife) in a live animal setting. The platform consisted of a glass substrate onto which a branch of the sciatic nerve was draped over and mechanically isolated from movements induced by the animal's breathing and heart pulsations. The platform was also divided into two fluidically isolated compartments and a stimulating electrode was built into one end. A recording electrode was placed into a calf muscle to record the electromyogram resulting from electrical stimulation of the nerve. With this basic setup, the authors were able to demonstrate the use of an axon nanoknife to systematically cut all of the axons within the nerve branch (~500 axons). Other than the axon nanoknife, all of the surgical, electronic, microscopy components used in the study were assembled using off-the-shelf components, demonstrating that existing technologies can be harnessed to support the *in vivo* use of a microdevice specifically designed for axon repair.

CONCLUSION

Although the concept of direct axon surgical repair is a departure from the more conventional approach of axon regeneration, it theoretically offers a number of advantages including rapid functional recovery and the maintenance of existing patterns of neural and synaptic circuitry. The ability to manipulate and physically reconstruct single cells has long been in the imagination of scientists seeking to pursue new biological insights and therapeutic avenues. While examples of cellular 'surgical' manipulations have been demonstrated including the ablation of organelles, collagen fibrils, and even the physical

stretching of axons, a cell-scale paradigm that systematically takes apart and puts together subcellular elements has not been pursued. Technological advances in micro- and nanoscience are beginning to provide the tools necessary for such purposeful microscale intervention. As we are at the earliest stages of technology and biological proof of principle for axon repair, much needs to be learned. Important areas of research include identification of the best principles for each stage of axon repair, the development of efficient cellular surgical microsystems, assessment of the biological consequences of axon repair, and eventually the integration of subcellular-scale surgery into clinical practice.

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FIGURE 1.

Nanoknife for microscale axon cutting. (a) Schematic planar view of microsuspension and cutting shell microassembled into a nanoknife. Black arrow, serpentine flexures acting as compliant microsuspension. White arrow, 1- μm thick silicon nitride cutting shell. Footprint of entire structure is 1 mm^2 . (b) Scanning electron microscope (SEM) view of pyramidal shaped cutting shell with apex serving as cutting edge (scale = 20 μm). (c) SEM showing cutting edge with an ~ 20 nm radius of curvature (scale = 100 nm). (d) Image of assembled nanoknife (scale = 200 μm). (e) Nanoknife mounted at an angle to a rod and held by a micromanipulator (not in view). For axon cutting *in vitro*, the nanoknife is positioned and angled, so that its planar footprint is parallel to the cell culture dish. The cutting stroke is executed as a downward movement delivered via the micromanipulator (scale = 500 μm). (f) Examples of cuts made by a nanoknife in an unmyelinated axon (arrow) and a myelinated axon (arrowhead) (scale = 25 μm).

FIGURE 2.

Single axon surgery in an anesthetized mouse. (a–f) Images showing the isolation of a short segment from a single axon using a nanoknife (black profile in ‘b’ and ‘c’). The arrows in ‘d’ and ‘e’ show the location of the first cut. The arrow in ‘f’ points to the isolated axon segment (scale a–f = 200 μm).

FIGURE 3.

Axon movement induced by dielectrophoresis (DEP). (a–d) A myelinated axon from the sciatic nerve of an adult mouse was positioned in between microfabricated electrodes (white arrows). Upon application of DEP, the axon was bent toward the electrode on the right, moving at a velocity of $\sim 5 \mu\text{m/s}$.

FIGURE 4.

Axon microelectrofusion demonstrated by the passage of soluble cytoplasmic green fluorescent protein (GFP) from one axon fusion partner into another. (a) Bright field image showing hippocampal axons in culture. Single arrow points to location where microelectrofusion was induced. (b) Prefusion image of the same field as in 'a' showing axons containing soluble cytoplasmic fluorescent GFP. (c) After microelectrofusion, GFP passed from the axon at the bottom left into an axon fusion partner that did not originally have GFP, indicating successful fusion of the two axonal compartments.