

● PERSPECTIVE

Peripheral nerve regeneration monitoring using multilayer microchannel scaffolds

Over 200,000 Americans have peripheral nerve injuries annually that result in a loss of function and a compromised quality of life. Of these, a significant percent involves unsuccessful repair of peripheral nerve gaps that occur due to traumatic limb injury or collateral damage to peripheral nerves during tumor resection. The clinical gold standard to repair a nerve gap is to use sural nerve autografts. However, autografts are not ideal because of the need for secondary surgery to source the nerve, loss of function at the donor site, lack of source nerve in the event of diabetic neuropathies, neuroma formation, and the need for multiple grafts to bridge nerves. An alternative to autografting that has proved to have significantly less risks and sacrifices is a nerve conduit. While there are some nerve conduits approved for clinical applications (Pabari et al., 2010; Giusti et al., 2012), commercial nerve conduits for nerve repair are usually composed of type I collagen or biodegradable polymers, such that the conduit will degrade once the nerve has healed. Although possible complication from foreign materials is not negligible, nerve conduits have had success in bridging nerve gaps and restoring functionality to limbs. Unlike autografting, it does not require the sacrifice of the donor sural nerve.

In instances where nerve injury takes the form of long nerve gaps, the nerve regeneration, even with conduit supports, is insufficient to connect the proximal nerve ending to the distal stump. In order to reliably provide more physical support for cellular substrate formation, several types of microchannel scaffolds, occasionally combined with neurotrophic factors, have been developed to physically connect the proximal and distal nerve ends. Microchannel scaffolds have been developed to artificially provide the necessary physical support and direction to Schwann cell migration by further constricting the direction of outgrowth and increasing the surface area available for support. As opposed to nerve conduits that allow axon outgrowth within the guide itself to be relatively disorganized, microchannel scaffolds arrange axon outgrowth into a series of linear arrays, each one with the physical restraints necessary for reattachment to the distal nerve stump within the three dimensional *in vivo* environment. Although microchannel scaffolds have been used successfully in several nerve regeneration studies on rats and other small mammals (Lacour et al., 2009; Billiar et al., 2010), the details of the biological events during axonal regeneration have not been reported. In order to understand the process of peripheral nerve regeneration within microchannel scaffolds, longitudinal observation of axon outgrowth *via* the microchannel is crucial. Analyzing individual axonal growth patterns and tracing the gradual progress of nerve regeneration inside microchannel scaffolds may be required to further investigate and confirm microchannel functionality. For these functional and investigational purposes, multilayer microchannel scaffolds were developed to visualize and monitor the progress of axonal regeneration (Hossain et al., 2015; Kim et al., 2015). During the fabrication process, no special micromachining equipment was required and commercially available microwires were efficiently used to implement the microchannel structures. The multilayer PDMS microchannel scaffold consisting of individual layers of microchannels were manually stacked up to eight layers to form

the required implant size to match the approximate size of the rat sciatic nerve model (1.5 mm diameter). A schematic view of the multilayer microchannel scaffold is shown in **Figure 1A, B**. This approach allows a flexibility of sample size because the microchannels can be cut to any length from the initial length, which only depends upon the size of the molding structure and microwires. In this case, 100 mm long microchannel layers were developed and cut for the each designed 3 mm long microchannel scaffolds. These layers were not secured together using an adhesive, but were wrapped with a PDMS thin film which was anchored to itself with a small portion of PDMS as a glue, which made feasible to disassemble individual PDMS microchannel layers after explant from nerve tissue. The stacked microchannel layers could be extracted and separated after nerve regeneration without damaging the harvested regenerated nerve tissue as shown in **Figure 1B**.

A systematic study of the peripheral nerve regeneration through microchannel scaffolds has been performed using multilayer PDMS microchannel scaffolds in rat sciatic nerve model. One of the standard analysis techniques for nerve regeneration through an artificial conduit is immunohistochemistry using specific biomarkers, such as neurofilament (NF160, N5264, Sigma, St Louis, MO) (Gökbuget et al., 2015). NF160 is specific to the neurofilament which is a major component of the neuronal cytoskeletal structure and provide structural support for the axon and to regulate axon diameter. NF160 has been used as a major antibody to investigate nerve regeneration due to its strong specificity to the neurofilament. The red stained NF 160 lines in **Figure 1C, D** show neurofilament structures inside axons. The dash blue lines represent the PDMS microchannel walls and red colored regenerating axons are shown inside microchannels.

Surprising results were achieved after nerve regeneration using the multilayer PDMS microchannel scaffolds. We were able to trace growth cones from the regenerating nerves and observed axonal branching in the individual microchannels. Two major cellular responses within damaged nerves (transected in animal models) are growth cone migration and axonal branching. The former term refers to how a severed nerve navigates to the disconnected target muscles, and the latter term describes how an axon actively searches for local guidance cues. Due to high interest in regenerative medicine and neurodegenerative disease, studies of growth cone motility and axonal branching from the transected nerve have been recently emphasized.

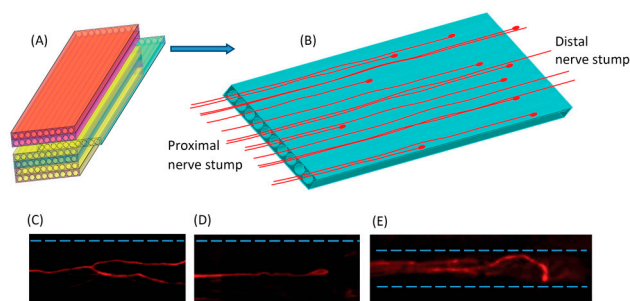


Figure 1 Schematic view of a multilayer microchannel scaffold and neurofilament histology profile two weeks after implantation.

(A) Multiple growth factors can be tested concurrently. Each layer has a different biochemical coating and represented by different colors. (B) Separated one layer out of the multilayer scaffold. A cyan color coated layer will have its own unique nerve regeneration profile. (C) Axonal branching. Dashed blue lines are the side walls of the 120 μ m diameter microchannel. (D) A regenerating growth cone. (E) A unique structural profile of a growth cone when it hit a side wall while regenerating through the microchannel.



Growth cone pathfinding can be attracted or repulsed by chemotropic cues, adhesive/anti-adhesive surface molecules, morphogens, and growth factors. Most of the previous studies have used *in vitro* cell culture systems, such as dissociated sensory neurons from dorsal root ganglia or *in vivo* brain models (Cebrián et al., 2005; Schmidt and Rathjen, 2010). Although cultured neuronal networks have shown a variety of mechanisms of neuronal functionality, individual axonal regenerations has not yet been introduced during *in vivo* animal studies. Recently, some *in vivo* peripheral nerve regeneration models have shown axonal branching aspects. However, the topographical views of peripheral nerve branching are mainly available in zebra fish models due to the optical transparency of zebrafish embryos (Bouquet et al., 2004; Schmidt and Rathjen, 2010). It is still scarcely reported in rodent models because of the requirement for advanced biotechnology to image the growth cones and axonal branching in the peripheral nervous system. Now we have the capability to demonstrate the guided growth cone motility and axonal branching in the *in vivo* rodent peripheral nerve model. The multilayer microchannel scaffolds have effectively addressed these fundamental neuroscience questions and could handle a variety of experimental approaches including use of chemotropic cues.

Initial studies in other literatures described the temporal status of regenerating axons using the proof of cross-sectional histology pictures. Using the multilayer microchannel scaffolds, the pictures of longitudinal sections of the regenerating axons were captured at different temporal points to see the progress of the axonal growing. The longitudinal nerve regeneration patterns are crucial to understand the temporal and structural responses of the regenerating axons. The multilayer microchannel scaffold gives an unprecedented method to understand the characteristic of regenerating peripheral nerves. The fundamental usage of the multilayer microchannel scaffold will be monitoring the growth cone motility profiles while severed axons start to grow from the proximal nerve stump and go through microchannels and reach the other end of the distal nerve stump. Each temporal study frame can be designed to evaluate the growth cone motility and axonal growth. **Figure 1E** shows a traveling route of a single axon growth two weeks after the implantation surgery. The diameter of the microchannel was 120 μ m and the length of the regenerating axon from the proximal end of the microchannel was 3 mm. Histological analysis can show the initial growth cone trajectory in the microchannel. We observed random direction of the growth cone traveling pathway. When it touched the wall of the microchannel it bounced back with almost same angle. When the regenerated nerve had formed in the microchannel for a longer time period such as six weeks, the angle of regenerating axons touching the wall were smoother and aligned to the wall. The initial wide angle of the axonal growth trajectory was barely observable six weeks after the implantation surgery. This characteristic presents a case we can continue to pursue with the regenerating axon in microchannel scaffolds that could be crucial in addressing a variety of biological questions of the peripheral nerve regeneration. The structural and temporal variances taking place in the scaffolds make the final shape and arrangement of the nerve regeneration for a more secure and firm connection between proximal and distal ends.

After a systemic study with a wide range of the temporal and structural variation, promising clinical applications can be pursued using this temporal structural nerve regeneration; for

instance, a guided nerve regeneration from the proximal nerve to the severed target distal nerve. This is dependent on a proper nerve regeneration where enough number of axonal growth should be initiated and guided to the target nerve stump using scaffolding materials and finally reinnervate to the target muscles. For final clinical uses, the scaffolding material will be switched with biodegradable materials using the same fabrication technique. While the well guided regenerated nerves regain the functional control on the target muscles, the biodegradable scaffolds will also disappear. The other benefit of the temporal and structural guidance of the growth cone and axonal branching is the selective nerve regeneration to the specific nerves and proper sensory and motor axonal connection to improve misdirection of the regenerated nerves. This could be achieved with an additional capability of the multilayer microchannel scaffolds with biochemical guidance.

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