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Fabrication of the Composite Regenerative Peripheral Nerve Interface (C-RPNI) in the Adult Rat

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Corresponding Author:	Stephen Kemp, Ph.D. The University of Michigan Ann Arbor, Michigan UNITED STATES
Corresponding Author's Institution:	The University of Michigan
Corresponding Author E-Mail:	swpkemp@med.umich.edu
Order of Authors:	Shelby R. Svientek
	Dan C. Ursu
	Paul S. Cederna
	Stephen W.P. Kemp, Ph.D.
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Cover Letter

PLASTIC SURGERY
UNIVERSITY OF MICHIGAN
HEALTH SYSTEM

Administrative Offices
2130 Taubman Health Center
1500 E. Medical Center Drive
Ann Arbor, MI 48109-5340
Phone (734) 936-5895
Fax (734) 763-5354

Chief
Paul S. Cederna, MD

Section Administrator
Nadine Lewis, MHSA

Adult Appointments:
Phone (734) 998-6022
Fax (734) 998-6696

Pediatric and Craniofacial Appointments:
Phone (734) 763-8063
Fax (734) 232-6973

Plastic Surgeons
David L. Brown, MD
Steven R. Buchman, MD
Paul S. Cederna, MD
Kevin C. Chung, MD, MS
Robert H. Gilman, MD, DMD
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M. Haskell Newman, MD
Riley S. Rees, MD
Melanie G. Urbanchek, MS, PhD

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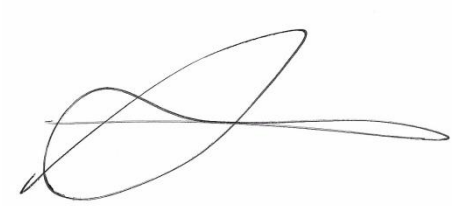
Kyle Jewhurst, PhD
Science Editor
JoVE

Dear Dr. Jewhurst,

Thank you very much for your letter regarding our submission to *JoVE* entitled "Fabrication of the Composite Regenerative Peripheral Nerve Interface (C-RPNI) in the Adult Rat". We are also very appreciative of the reviewer's very constructive and insightful comments regarding our manuscript, and we believe that these changes have strengthened our paper. We believe that we have addressed all of the reviewer's concerns, and we have subsequently incorporated these changes into a revised manuscript (changes are displayed in red and underlined in the revised manuscript). In our Response to Reviewers, we have set out a point-by-point list that highlights the changes that we have made with respect to each reviewer's comments. We look forward to the review of the revised manuscript, and if you have any further questions or require any additional information or clarification, please don't hesitate to contact me.

All authors of this paper have read and approved the revised version submitted. We look forward to hearing from you. Thank you very much for the opportunity to submit our revised manuscript for consideration for publication in JoVE.

Sincerely yours,



Stephen W.P. Kemp, Ph.D
Director, Neuromuscular Lab
Assistant Research Professor
University of Michigan, Section of Plastic Surgery
Department of Biomedical Engineering
1150 W Medical Center Drive
MSRB II, A570 Ann Arbor, MI, 48109-5456
P: (734) 615-2598
swpkemp@med.umich.edu

TITLE:

Fabrication of the Composite Regenerative Peripheral Nerve Interface (C-RPNI) in the Adult Rat

AUTHORS AND AFFILIATIONS:

Shelby R. Svientek[1] Dan C. Ursu[1], Paul S. Cederna[1,2], Stephen W.P. Kemp[1,2]

[1]Department of Surgery, Division of Plastic Surgery, University of Michigan, Ann Arbor, MI, USA
[2]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA

Corresponding Author:

Shelby Svientek (ssviente@med.umich.edu)

Email Addresses of Co-Authors:

Dan Ursu (danursu@umich.edu)
Paul Cederna (cederna@med.umich.edu)
Stephen Kemp (swpkemp@med.umich.edu)

KEYWORDS:

Peripheral nerve interface, prosthetic control, C-RPNI, neural feedback system

SUMMARY:

The following manuscript describes a novel method for developing a biologic, closed loop neural feedback system termed the composite regenerative peripheral nerve interface (C-RPNI). This construct has the ability to integrate with peripheral nerves to amplify efferent motor signals while simultaneously providing afferent sensory feedback.

ABSTRACT:

Recent advances in neuroprosthetics have enabled those living with extremity loss to reproduce many functions native to the absent extremity, and this is often accomplished through integration with the peripheral nervous system. Unfortunately, methods currently employed are often associated with significant tissue damage which prevents prolonged use. Additionally, these devices often lack any meaningful degree of sensory feedback as their complex construction dampens any vibrations or other sensations a user may have previously depended on when using more simple prosthetics. The composite regenerative peripheral nerve interface (C-RPNI) was developed as a stable, biologic construct with the ability to amplify efferent motor nerve signals while providing simultaneous afferent sensory feedback. The C-RPNI consists of a segment of free dermal and muscle graft secured around a target mixed sensorimotor nerve, with preferential motor nerve reinnervation of the muscle graft and sensory nerve reinnervation of the dermal graft. In rats, this construct has demonstrated the generation of compound muscle action potentials (CMAPs), amplifying the target nerve's signal from the micro- to milli-volt level, with signal to noise ratios averaging approximately 30–50. Stimulation of the dermal component of the construct generates compound sensory nerve action potentials (CSNAPs) at the proximal nerve. As such, this construct has promising future utility towards the realization of the ideal, intuitive prosthetic.

INTRODUCTION:

Extremity amputations affect nearly 1 in 190 Americans[1], and their prevalence is projected to increase from 1.6 million today to over 3.6 million by 2050[2]. Despite documented use for over a millennium, the ideal prosthetic has yet to be realized[3]. Currently, there exist complex prosthetics capable of multiple joint manipulations with the potential to reproduce many motor functions of the native extremity[4,5]. However, these devices are not considered intuitive as the desired prosthetic motion is typically functionally separate from the input control signal. Users typically consider these "advanced prosthetics" difficult to learn and therefore not suitable for everyday use[1,6]. Additionally, complex prosthetics currently on the market do not provide any appreciable degree of subtle sensory feedback for adequate control. The sense of touch and proprioception are vital to carrying out daily tasks, and without these, simple acts such as picking up a cup of coffee become burdensome as it relies entirely on visual cues[7-9]. For these reasons, advanced prostheses are associated with a significant degree of mental fatigue and are often described as burdensome and unsatisfactory[5,10-11]. To address this, some research laboratories have developed prosthetics capable of providing a limited degree of sensory feedback via direct neural interaction[12-15], but feedback is often limited to small, scattered areas on the hands and fingers[12-13], and sensations were noted to be painful and unnatural at times[15]. Many of these studies unfortunately lack any appreciable long-term follow-up and nerve histology to delineate local tissue effects, while noting interface failure on the scale of weeks to months[16].

For this population, the ideal prosthetic device would provide high fidelity motor control alongside meaningful somatosensory feedback from the individual's environment throughout their lifetime. Critical to the design of said ideal prosthetic is the development of a stable, reliable interface that would allow for simultaneous transmission of afferent somatosensory information with efferent motor signals. The most promising of current human-machine interfaces are those that interact with the peripheral nervous system directly, and recent developments in the field of neuro-integrated prosthetics have worked towards bridging the gap between bioelectric and mechanical signals[17]. Current interfaces utilized include: flexible nerve plates[14,15,18], extra-neural cuff electrodes[13,19-23], tissue penetrating electrodes[24-25,31-32], and intrafascicular electrodes[26-28]. However, each of these methods has demonstrated limitations with regards to nerve specificity, tissue injury, axonal degeneration, myelin depletion, and/or scar tissue formation associated with chronic indwelling foreign body response[16-19]. More recently, it has been postulated that a driver behind eventual implanted electrode failure is the significant difference in Young's moduli between electronic material and native neural tissue. Brain tissue is subject to significant micromotion on a daily basis, and it has been theorized that the shear stress induced by differences in Young's moduli causes inflammation and eventual permanent scarring[30-32]. This effect is often compounded in the extremities, where peripheral nerves are subject to both physiologic micromotion and intentional extremity macromotion. Due to this constant motion, it is reasonable to conclude that utilization of a completely abiotic peripheral nerve interface is not ideal, and an interface with a biologic component would be more suitable.

To address this need for a biologic component, our laboratory developed a biotic nerve interface termed the Regenerative Peripheral Nerve Interface (RPNI) to integrate transected peripheral

nerves in a residual limb with a prosthetic device. RPNI fabrication involves surgically implanting a peripheral nerve into an autologous free muscle graft, which subsequently revascularizes and reinnervates. Our lab has developed this biologic nerve interface over the past decade, with success in amplifying and transmitting motor signals when combined with implanted electrodes in both animal and human trials, allowing for suitable prosthetic control with multiple degrees of freedom[2,34]. In addition, we have separately demonstrated sensory feedback through the use of peripheral nerves embedded in dermal grafts, termed the Dermal Sensory Interface (DSI)[3,35]. In more distal amputations, using these constructs simultaneously is feasible as motor and sensory fascicles within the target peripheral nerve can be surgically separated. However, for more proximal level amputations, this is not feasible due to intermingling of motor and sensory fibers. The Composite Regenerative Peripheral Nerve Interface (C-RPNI) was developed for more proximal amputations, and it involves implanting a mixed sensorimotor nerve into a construct consisting of free muscle graft secured to a segment of dermal graft (**Figure 1**). Peripheral nerves demonstrate preferential targeted reinnervation, thus sensory fibers will re-innervate the dermal graft and motor fibers, the muscle graft. This construct thus has the ability to simultaneously amplify motor signals while providing somatosensory feedback[36] (**Figure 2**), allowing for the realization of the ideal, intuitive, complex prosthetic.

PROTOCOL:

All animal experiments are performed under the approval of the University of Michigan's Committee on the Use and Care of Animals.

NOTE: Donor rats are allowed free access to food and water prior to skin and muscle donation procedures. Euthanasia is performed under deep anesthesia followed by intra-cardiac potassium chloride injection with a secondary method of bilateral pneumothorax. Any strain of rat can theoretically be utilized with this experiment; however, our laboratory has achieved consistent results in both male and female Fischer F344 rats (~200–250 g) at two to four months of age. Donor rats must be isogenic to the experimental rats.

1. Preparation of the dermal graft

1.1. Anesthetize donor rat in an induction chamber utilizing a solution of 5% isoflurane in oxygen at 0.8–1 L/min. Once the rat has been anesthetized, remove from induction chamber and place on a rebreathing nose cone, lowering the isoflurane to 2–2.5% for maintenance of anesthesia.

1.2. Administer a solution of 0.02–0.03 mL Carprofen (50 mg/mL) in 0.2 mL of sterile saline subcutaneously between the shoulder blades for analgesia.

1.3. Apply artificial tears ointment to both eyes to prevent corneal ulcers.

1.4. Using clippers, shave the entire lower hindlimb(s), ankle region, and sides of paw(s).

1.5. Cleanse chosen hindlimb and plantar surface of paw with alcohol, followed by iodopovidone solution, ending with a final cleanse with alcohol to remove residual iodopovidone.

1.6. Using a hand-held micro motor high speed drill with a removable round fine grit polishing stone (4000 rpm), burr the plantar surface of the paw to remove the epidermis. While burring, apply drips of saline as to not burn the skin. The underlying dermis will have a shiny appearance with pinpoint bleeding.

1.7. Apply a tourniquet to the lower extremity to slow blood flow.

1.8. Remove the plantar skin sharply with a #15 scalpel and place in saline-moistened gauze to prevent desiccation. Some tendinous and connective tissue will inherently be removed with the skin in this step and will be removed later.

1.9. Apply gauze wrap to the bleeding foot to slow hemorrhage. Repeat steps 1.5-1.9 if doing two constructs.

1.10. Under a microscope (20x magnification), remove the tendinous and connective tissue from the deep layer of the skin graft using micro-scissors. Take care not to make holes in the graft. The thinned dermal graft should be slightly opaque containing only dermis, measuring approximately 0.5 cm x 1.0cm in size.

1.11. Place in saline-moistened gauze until ready for C-RPNI construct fabrication. Grafts should be utilized within 2 h of harvest.

2. Preparation of the muscle graft

2.1. Make a longitudinal incision along the anterior aspect of the lower hindlimb from just above the ankle to just below the knee with a #15 scalpel. Dissect through subcutaneous tissue to expose the underlying musculature.

2.2. At the distal aspect of the incision, expose the tendinous insertions of the lower limb musculature. Tibialis anterior (TA) is typically the largest and most anterior of the muscles, and just underneath and posterior to this muscle lies the extensor digitorum longus (EDL). Isolate the distal EDL tendon from the other tendons in the area, taking care not to incise its insertion at this point.

2.3. Ensure isolation of the correct tendon by inserting both tines of a forceps underneath the tendon and exerting upward pressure by opening the forceps to cause tendon excursion. Manipulation of this tendon should cause all of the toes to extend simultaneously.

2.4. Perform a distal tenotomy with sharp iris scissors and separate the muscle from the surrounding tissues bluntly with tenotomies (or other blunt-tipped scissor) working proximally to find the tendinous origin.

2.5. Once the proximal tendon is visualized, again perform a tenotomy utilizing sharp iris scissors. Place the muscle graft in a saline-moistened gauze to prevent desiccation.

2.6. Once all desired grafts have been removed from a donor rat, euthanize primarily by intra-cardiac KCl injection (1–2 mEq K+/kg) followed by secondary euthanasia with bilateral puncture pneumothorax with a #15 blade.

3. Common peroneal nerve isolation and preparation

3.1. Anesthetize and provide analgesia to the experimental rat according to protocol outlined in steps 1.1–1.3.

3.2. Shave the desired thigh and cleanse with alcohol, betadine, ending with alcohol to remove traces of betadine.

3.3. Move animal from surgical prep table to surgical microscope table and place on heating pad with temperature probe for body temperature maintenance. Maintain isoflurane at 2–2.5% and oxygen at 0.8–1 L/min.

3.4. Mark the incision, extending from just distal to sciatic notch to the inferior portion of the knee. This marking should be inferior to, and angled away from, the femur. Make the incision with a #15 blade incising through the underlying biceps femoris fascia.

3.5. Carefully dissect through the biceps femoris muscle with either a hemostat or blunt-tipped micro-scissors to the space underlying biceps femoris.

NOTE: The sciatic nerve travels approximately in the same direction as the initial incision that was made. There are three branches, typically with sural nerve posterior and common peroneal and tibial nerve traveling superficial and deep to the knee, respectively.

3.6. Following identification of the common peroneal (CP) nerve, using a pair of micro-, fine-tipped forceps and micro-scissors, carefully isolate the CP nerve from the other sciatic branches and remove any lingering connective tissue distally.

3.7. At the point where the nerve crosses the surface of the knee, sharply transect the nerve with a pair of micro-scissors.

NOTE: Using sharp scissors is extremely important in this step as causing significant trauma to the nerve could increase the risk of neuroma formation.

3.8. Carefully free any remaining connective tissue from the CP nerve and work proximally to free the nerve to a length of approximately 2 cm.

4. C-RPNI construct fabrication

4.1. Remove the muscle graft from saline-moistened gauze and remove all central tendinous tissue as well as a small central segment of epimysium. Leave the tendinous ends intact.

4.2. Using an 8-0 nylon suture, secure the epineurium of the transected end of the CP nerve to the area of the muscle graft devoid of epimysium with two interrupted stitches on either side of the nerve.

4.3. Secure the muscle graft to the femur periosteum with a single 6-0 nylon interrupted stitch both proximally and distally with the nerve-muscle junction facing away from the femur.

NOTE: Secure the muscle so that it is at normal relaxed length. Try not to stretch the muscle significantly or leave too much laxity when securing.

4.4. Place an 8-0 nylon stitch at the inferior, central margin of the muscle graft epimysium, securing it to the CP nerve epineurium in a way as to create laxity in the nerve within the muscle graft and help to relieve any future tension it may be exposed to with later ambulation.

4.5. Remove the skin graft from the saline-moistened gauze and arrange it on the muscle graft in such a way to completely cover the nerve and the majority of the muscle. Ensure that the deep margin of the dermis is resting on the muscle. Trim any dermis that extends beyond the border of the muscle.

4.6. Secure the skin graft to the muscle graft circumferentially using 8-0 nylon interrupted sutures. Typically, 4–8 total sutures are used depending on the size of the construct.

4.7. Close the biceps femoris fascia over the construct in a running fashion with 5-0 chromic suture.

4.8. Close the overlying skin with 4-0 chromic suture in running fashion.

4.9. Swab the surgical area with an alcohol pad and apply antibiotic ointment.

4.10. Cease inhalational anesthetic and allow rat to recover with food and water sources separate from cage mates.

REPRESENTATIVE RESULTS:

Construct fabrication is considered unsuccessful if rats develop an infection or do not survive surgical anesthesia. Previous research has indicated these constructs require approximately three months to revascularize and reinnervate[2,3,17,36]. Following the three-month recovery period, construct testing can be pursued to examine viability. Surgical exposure of the constructs after three months will reveal revascularized muscle and skin if successful (**Figure 3**). At times, the free muscle and dermal grafts can consist solely of scar tissue, and/or the nerve will not be attached to the construct; these findings indicate an unsuccessful attempt. However, if

successful, gentle squeezing of the common peroneal nerve with forceps proximal to the construct will result in visible muscle contraction (**Video 1).** Histological analysis of constructs should demonstrate viable skin, nerve, and muscle (**Figure 4**). Immunostaining will also reveal motor and sensory nerve reinnervation to their neuromuscular junctions and sensory end organs, respectively (**Figure 5**). If the common peroneal nerve does not reinnervate those tissues, immunostaining will not demonstrate any individual nerve fibers within the construct with the exception of the implanted nerve itself.

Electrophysiologic testing can be performed on these constructs in vivo (**Figure 6**); previous research has been conducted at 3 and 9 months following C-RPNI fabrication[36] (**Table 1**). Following maximal stimulation with a hook electrode at the proximal common peroneal nerve just distal to its takeoff from the sciatic nerve, compound muscle action potentials (CMAPs) can be measured at the muscle component with visible muscle contraction. The type of electrode used at the muscle can vary according to preference, but epimysial patch, epimysial pad, and bipolar probe electrodes have been used successfully in this research. The average CMAP amplitude recorded at the muscle was 8.7 ± 1.6 mV at 3 months and 10.2 ± 2.1 mV at 9 months. The average conduction velocity was 10 ± 1.2 m/s at 3 months and 9.5 ± 0.6 m/s at 9 months. In comparison, CMAPs generated by physiologic EDL muscle typically range from 10–18 mV[37]. Following stimulation at the dermal component of the C-RPNI, compound sensory nerve action potentials (CSNAPs) were produced at the proximal common peroneal nerve, with average CSNAP amplitude measuring 113.7 ± 35.1 μV at 3 months and 142.9 ± 63.7 μV at 9 months. **Figure 7** illustrates single and summation CMAP and CSNAP signals obtained during electrophysiologic testing in a graphical format.

The C-RPNI serves to amplify a nerve's inherent microvolt signal, and previous research has demonstrated sufficient amplification from the microvolt to millivolt level[38]. Therefore, if a construct does not provide that level of amplification, it is not considered successful. If either the dermal, muscle, or both components of the C-RPNI fail, testing would result in recordings that mimic the stimulation signal utilized. For the muscle component specifically, a suboptimal result (but one that is still considered operational) would be one that has CMAP amplitude and conduction velocity in the range that falls between the signal stimulation value and that of physiologic EDL muscle. Additionally, these signals can become attenuated and lack the characteristic CMAP waveform (**Figure 8A**). Suboptimal results at the level of the dermal component can occur but are difficult to quantify given that rats cannot express the quality of sensation they experience. These suboptimal results usually involve dampening of the waveform with significant background noise (**Figure 8B**). However, if there is significant scarring or callusing of the skin graft, or minimal graft survived, no CSNAPs will be appreciated at the proximal common peroneal nerve regardless of stimulation value.

FIGURE AND TABLE LEGEND:

Figure 1: Illustrative schematic of the C-RPNI construct. The common peroneal nerve can be seen secured between the top dermal layer and bottom muscle layer. This construct is secured to the femur periosteum proximally and distally via EDL's tendinous junctions.

Figure 2: A pictorial representation of the C-RPNI in a patient with a trans-radial amputation. The user forms a desired motor intention at the cerebral level (e.g., pincer grasp), which is transmitted as an efferent motor signal to the C-RPNI via the implanted peripheral nerve. This signal generates a compound muscle action potential (CMAP) at the muscle component, which is recorded by implanted electrodes and recognized by the prosthetic device, generating the desired motion. Sensors on the device's fingertips recognize the amount of pressure generated, and relay that information to an electrode implanted in the dermal component of the C-RPNI. These signals activate the corresponding sensory end organs, generating an afferent compound sensory nerve action potential (CSNAP) transmitted through the peripheral nerve to the sensory cortex. An example signal generated at each component is pictured within the blue boxes pictured next to each component.

Figure 3: C-RPNI in vivo. (**A**) A C-RPNI immediately following fabrication and at (**B**) 3 months post-construction at time of electrophysiologic testing. The muscle component is the deep layer of the construct and the dermal, the superficial. Muscle tissue is marked by (M), dermis (D), and common peroneal nerve (N).

Figure 4: C-RPNI histology 6 months. C-RPNI H&E at 6 months in (**A**) cross-section and (**B**) longitudinal section. Muscle noted by (M), dermis (D), and nerve (N).

Figure 5: Immunostaining of the C-RPNI. (**A**) Representative example of a cross-section of muscle tissue, with red arrows identifying neuromuscular junctions. A higher magnification of the central neuro-muscular junction (NMJ) is pictured at the bottom-right. (**B**) Close-up of a neuromuscular junction noted in the sample. For (A) and (B), red staining (alpha-bungarotoxin) indicates presence of cholinergic receptors in muscle tissue; blue (neurofilament 200) specifies presence of neurofilaments within neuronal tissue; and green (choline acetyltransferase) notes specifically motor neuron presence. (**C**) Representative example of an iDISCO image focusing on the dermal junction, with red arrows marking sensory neurons (white) entering the dermis. (D) On-lay view of iDISCO demonstrating multiple sensory neurons (white, neurofilament 200)

Figure 6: Electrophysiologic testing schematic. The top image is an illustration of the standard electrode arrangement for testing the C-RPNI constructs. There is a patch and/or probe electrode placed on both the muscle and dermal components of the C-RPNI, with a double hook electrode placed at the common peroneal nerve proximally. The bottom image is an in vivo example of the testing arrangement on a rat subject.

Figure 7: Typical C-RPNI electrophysiologic signaling. (**A**) A single CMAP signal recorded at the muscle component following a 5.00 mA signal applied to the CP nerve. (**B**) 24 CMAPs generated by 5.00 mA stimulation at the nerve. (**C**) A single CSNAP signal recorded from the proximal CP nerve following dermal component stimulation at 900 µA. (**D**) A series of CSNAPs recorded from the proximal CP nerve following increasing stimulation at the dermal component from 500 µA to 1000 µA.

Figure 8: Abnormal C-RPNI signaling. (**A**) A series of CMAPs obtained while ramping CP nerve stimulation from 0.2 to 4 mA. Waveforms peak at different points and fail to return to baseline, possibly indicating defective electrodes or inadequate overall construct function. (**B**) Summation of CSNAPs obtained while stimulating dermal component, ramping 0.1 to 5 mA. These findings can occur for a multitude of reasons, including malfunctioning electrode(s), dermal graft scarring, and/or nerve damage.

Table 1: Electrophysiologic testing of C-RPNIs at 3- and 9-months post-construction. To obtain CMAPs, a recording electrode was placed on the muscle with a stimulating electrode on the proximal common peroneal nerve. A series of stimulations increasing in amplitude was applied to the nerve until maximal CMAP values were obtained and results recorded. A similar methodology was applied to the dermal component but with the recording electrode placed on the nerve and stimulating electrode on the dermis. For the sensory evaluation of rat 4690 at 9 months, the dermal graft was found to be too scarred to allow for testing.

Video 1: Muscle contraction within a C-RPNI. A pair of forceps can be seen to the left of the video gently squeezing the proximal common peroneal nerve. This results in contraction of the muscle component of a 3-month-old C-RPNI that is visible to the viewer.

DISCUSSION:

The C-RPNI is a novel construct that provides simultaneous amplification of a target nerve's motor efferent signals with provision of afferent sensory feedback. In particular, the C-RPNI has unique utility for those living with proximal amputations as their motor and sensory fascicles cannot easily be mechanically separated during surgery. Instead, the C-RPNI utilizes the inherent preferential reinnervation properties of the nerve itself to encourage sensory fiber reinnervation to dermal sensory end organs and motor fibers to neuromuscular junctions.

As C-RPNI fabrication relies on the reinnervation abilities of the target nerve, careful handling of the nerve is paramount during the procedure. During dissection, avoid direct manipulation of, and trauma to, the target nerve. If the nerve must be handled, it is recommended to manipulate epineurium or surrounding connective tissue instead. Although our laboratory has not encountered neuroma formation within this construct, theoretically, significant nerve trauma could increase the risk. An additional key step in the process is the harvesting of the dermal grafts. All epidermal tissue must be removed from the hindpaw graft as retained epidermis can increase the risk of infection and inclusion cysts during the healing process. Furthermore, the dermal graft must be adequately thinned to promote imbibition and revascularization throughout the graft and avoid significant ischemia and necrosis.

Although the majority of studies conducted with the C-RPNI have been performed on the common peroneal nerve, any mixed sensorimotor nerve could be substituted. A pure motor or pure sensory nerve could be utilized, but the results are difficult to predict and would likely result in either largely muscle or dermal reinnervation, respectively. With regards to the muscle graft, as long as epimysium is removed from the portion contacting the nerve, any muscle graft similar in size could be utilized as long as it contained tendinous or fascial tissue at either end suitable

for anchoring to nearby periosteum. For the dermal graft, glabrous tissue is specifically used due to the potential for hair growth following grafting. Non-glabrous skin was previously attempted, but due to the difficulty of removing individual hair follicles, all resultant constructs had significant hair growth, inflammation, and scarring following the three-month maturation period. Additionally, other rat species can be employed, but Lewis and Fischer rats are recommended for this experiment as many other rat species will self-mutilate secondary to nerve transection[39-40].

Given the delay between procedure and results, it is difficult to know ahead of time if any alterations must be made to the method. Infection is a theoretical risk rarely encountered by our laboratory, but if infection occurs, it is typically responsive to antibiotics. Occasionally, rats chew on their incisions causing dehiscence, and this can be treated with washout, debridement, and re-closure. If, after three months at time of exposure, the construct is found to be non-functional and/or scarred, there are several potential causes. At times, if the nerve is not secured correctly to the construct with at least three sutures, the nerve can tear from the construct with ambulation. Additionally, the muscle and/or dermal grafts can necrose, causing failure. Typically, this is a result of either repeated infection, the dermal graft being too thick, or the muscle too damaged at harvest to recover properly. Additionally, if the muscle is not secured to periosteum at resting length, contraction can be impaired causing inadequate signals during testing. At times, the construct will appear viable but fail to produce adequate CMAPs/CSNAPs on testing (5–10% of constructs, on average). This could be secondary to failure in equipment, elevated electrode impedance, or significant skin callusing. Skin callusing can dampen and completely block signal transduction if the dermis is not thinned properly during fabrication. If any of the preceding described events are seen frequently during the testing process, one must return to the protocol and make appropriate alterations. In our laboratory's experience with over 90 successful C-RPNI constructs, our failure rate is <5% and typically attributed to surgical error during fabrication.

Methods commonly employed to amplify or record nerve signals include flexible nerve plates[18], extra-neural cuff electrodes[19-23], tissue penetrating electrodes[24-25,31-32], and intrafascicular electrodes[26-28], all of which have been associated with tissue injury, axonal degeneration, and/or scar tissue formation. This scarring is often attributed to chronic indwelling foreign body response[29] and shear stress induced by differences in Young's moduli[30]. The C-RPNI, however, is a biologic construct and thus does not induce foreign body response in neural tissue. Additionally, its mechanical properties are several factors closer to neural tissue than electrodes. Histologic analysis of these samples has not demonstrated any significant degree of scar tissue formation in the nerve with chronic use, thus allowing the C-RPNI to interface with the nerve for extended periods in comparison to the methods listed above. Although this method is highly effective at amplification of efferent motor signals, it is limited with regards to sensory afferent signal production. We have measured and characterized signal transduction produced with mechanical and electrical stimulation of the dermal component of the C-RPNI[36]; however, these rat subjects cannot qualify the type or degree of sensations elicited from stimulation of this construct. As such, at this time it is impossible to know what kind of effect the C-RPNI is producing with regards to sensation. Future directions for this construct will include characterization of signals produced in the proximal nerve following specific provided stimuli (e.g., heat, pain, pressure, etc.) as well as correlation with somatosensory evoked potentials generated in the sensory cortex of the

rodent brain. It is our laboratory's goal to establish a comprehensive foundation for C-RPNIs that will pave the way for clinical translation to human subjects.

The predecessor to the C-RPNI, the RPNI (regenerative peripheral nerve interface), consists of a free muscle graft attached to a transected nerve, with motor fibers reinnervating previously denervated neuromuscular junctions. The RPNI has demonstrated utility in human subjects, with several patients controlling advanced prosthetics from signals amplified by—and recorded from—these RPNIs[34]. Furthermore, these RPNIs have demonstrated beneficial treatment effects beyond prosthetic control, with several preliminary retrospective and prospective studies showing decreased neuroma formation, chronic pain, and phantom limb pain in those patients with extremity amputations. Despite these successes, a common complaint for those utilizing these advanced prosthetics, however, is the need to visualize the prosthetic during use as these prosthetics lack proprioception and provide minimal sensory feedback. The C-RPNI could be a solution to this common criticism by providing a way to deliver sensory feedback via the dermal component, leading to the realization of the much-desired, ideal prosthetic.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have no disclosures.

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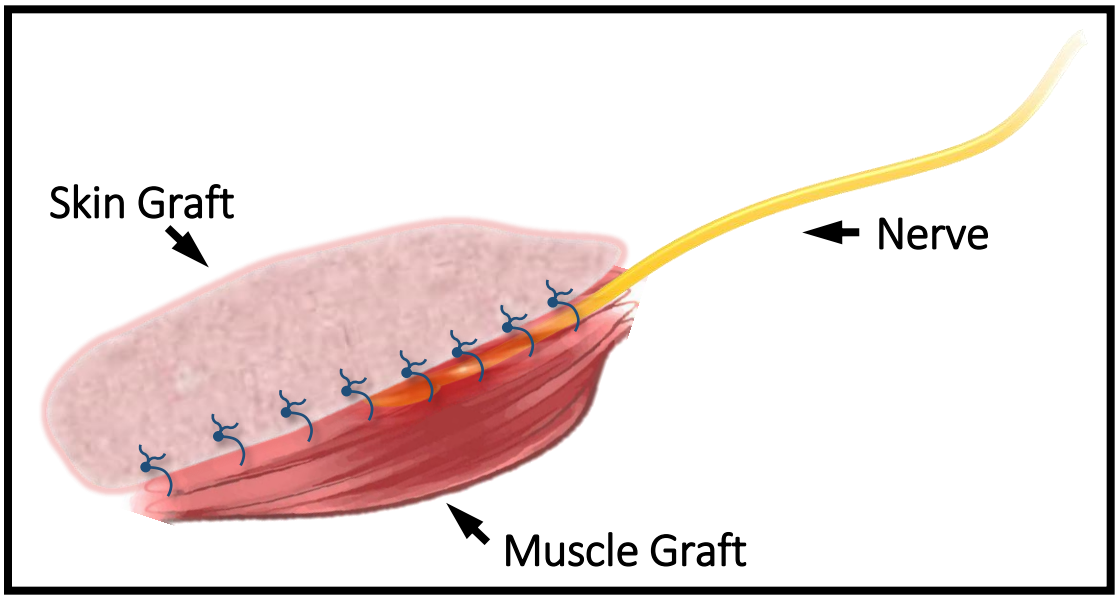

Figure 1: Illustrative schematic of the C-RPNI construct. The common peroneal nerve can be seen secured between the top dermal layer and bottom muscle layer. This construct is secured to the femur periosteum proximally and distally via EDL's tendinous junctions.

fig 1


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Video or Animated Figure
Figure 1 illustrative schematic.svg

fig 2

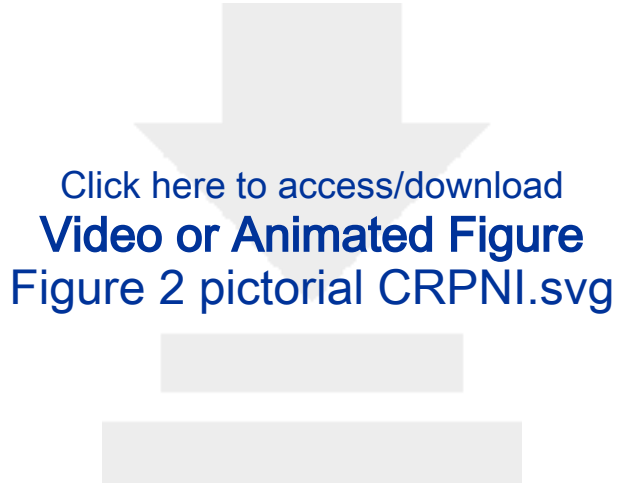
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Figure 2 pictorial CRPNI.svg

fig 3

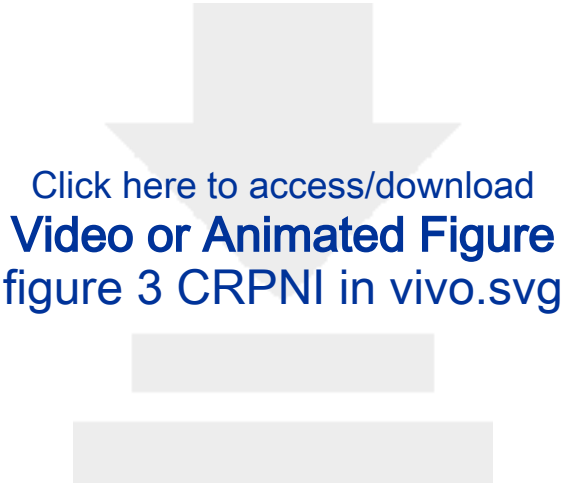

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figure 3 CRPNI in vivo.svg

fig 4

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Video or Animated Figure

figure 4 CRPNI histo.svg

fig 5

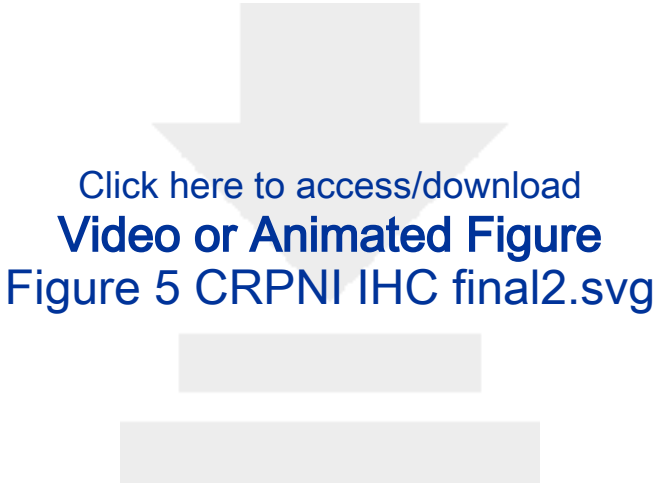

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Figure 5 CRPNI IHC final2.svg

fig 6

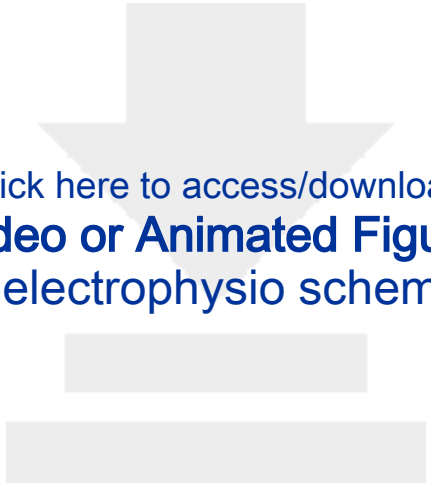

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figure 6 electrophysio schematic.svg

fig 7


Click here to access/download
Video or Animated Figure
figure 7 normal signaling.svg

fig 8


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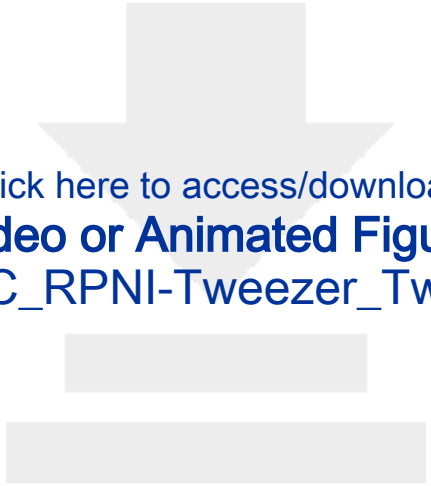
figure 8 abn signaling.svg

Video1



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Video or Animated Figure

Video 1 C_RPNI-Tweezer_Twitch.mov

Table 1

Click here to access/download;Table;Table 1 electrophys data at 3 and 9 months.xlsx

3 Month Data		CMAP Data (Stimulate CP nerve and record from muscle gra	
Rat ID Number	Construct Weight (g)	Stimulation Amplitude (mA)	Conduction Velocity (m/s)
4607	0.087	4.17	11.3
4608	0.15	1.65	11.1
4611	0.113	8.3	9.6
4613	0.116	3.18	10
4614	0.189	3	10.8
4616	0.122	5.2	9.4
4620	0.118	2.91	7.6

9 Month Data		CMAP Data (Stimulate CP nerve and record from muscle gra	
Rat ID Number	Construct Weight (g)	Stimulation Amplitude (mA)	Conduction Velocity (m/s)
4687	0.238	1.35	9.6
4688	0.131	1.08	10
4689	0.26	1.26	9.6
4690	0.192	4.2	8.3
4691	0.213	1.38	10
4693	0.178	1.11	9.6

aft)	CSNAP Data (Stimulate skin graft and record from CP nerve	
V Peak-to-Peak (mV)	Stimulation Amplitude (mA)	Conduction Velocity (m/s)
10.3	18	11.1
17.1	7.7	6.5
11.2	10	10
9.6	1.44	8.3
9.6	7.39	9
14.9	1.8	9.1
7.4	8.7	10

aft)	CSNAP Data (Stimulate skin graft and record from CP nerve	
V Peak-to-Peak (mV)	Stimulation Amplitude (mA)	Conduction Velocity (m/s)
18.2	0.99	11
8.8	1.11	8
21.8	1.9	8.6
12.8	n/a	n/a
18.6	6.6	8
15.1	8.7	8.3

)

V Peak-to-Peak (µV)
121
136
121
134
151
100
219

)

V Peak-to-Peak (µV)
181
132
237
n/a
153
306

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
#15 Scalpel	Aspen Surgical, Inc	Ref 371115	Rib-Back Carbon Steel Surgical Blades (#15)
4-0 Chromic Suture	Ethicon	SKU# 1654G	P-3 Reverse Cutting Needle
5-0 Chromic Suture	Ethicon	SKU# 687G	P-3 Reverse Cutting Needle
6-0 Ethilon Suture	Ethicon	SKU# 697G	P-1 Reverse Cutting Needle (Nylon suture)
8-0 Monofilament Suture	AROSurgical	T06A08N14-13	Black polyamide monofilament suture on a threaded tape
Experimental Rats	Envigo	F344-NH-sd	Rats are Fischer F344 Strain
Fluriso (Isofluorane)	VetOne	13985-528-40	Inhalational Anesthetic
Micro Motor High Speed Drill with Stone	Master Mechanic	Model 151369	Handheld rotary tool; kit comes with multiple fine grit sto
Oxygen	Cryogenic Gases	UN1072	Standard medical grade oxygen canisters
Potassium Chloride	APP Pharmaceuticals	63323-965-20	Injectable form, 2 mEq/mL
Povidone Iodine USP	MediChoice	65517-0009-1	10% Topical Solution, can use one bottle for multiple surg
Puralube Vet Opthalmic Ointment	Dechra	17033-211-38	Corneal protective ointment for use during procedure
Rimadyl (Caprofen)	Zoetis, Inc.	NADA# 141-199	Injectable form, 50 mg/mL
Stereo Microscope	Leica	Model M60	User can adjust magnification to their preference
Surgical Instruments	Fine Science Tools	Various	User can choose instruments according to personal prefer
Triple Antibiotic Ointment	MediChoice	39892-0830-2	Ointment comes in sterile, disposable packets
VaporStick 3	Surgivet	V7015	Anesthesia tower with space for isofluorane and oxygen c
Webcol Alcohol Prep	Coviden	Ref 6818	Alcohol prep wipes; use a new wipe for each prep

red needle

nes

ical preps

·ence or from what is currently available in their lab

anister

Response to Reviewers

We would like to personally thank both the editors at JoVE and knowledgeable peer reviewers that took the time to thoughtfully review our manuscript titled “Fabrication of the Composite Regenerative Peripheral Nerve Interface (C-RPNI) in the Adult Rat.” We are very appreciative of the very constructive and insightful comments regarding our manuscript, and we believe that these changes have strengthened our paper. We believe that we have addressed all of the reviewer’s concerns, and we have subsequently incorporated these changes into a revised manuscript. Rebuttals are addressed point by point, are organized under reviewer number, and resultant changes to the manuscript are tracked in red and underlined.

Editor

We have made the following changes in response to the editorial comments:

1. Changes to protocol organization, missing information, and font are reflected in the draft
2. References were edited to follow protocol
3. Regarding references comment for superscripted citation number in paragraph two of the results, these were cited as such in the text because they were conference abstracts. Per the Instructions for Authors document received, “conference abstracts can be cited parenthetically in the text with the author’s last name, initials, and the year.”
4. Protocol step 1.2,3.3: Given the nature of the surgery, lack of foreign body material, and the species of animal, risk of infection is extremely low. Our laboratory practices aseptic technique, and we have not experienced a surgical site infection in these experimental animals to date. Aseptic technique during surgery is approved on our protocol overseen by the University of Michigan’s Committee on Use and Care of Animals.
5. From our perspective, the protocol is three pages long, not requiring highlighting.

Reviewer # 1

Major Concerns:

1. The last paragraph of the introduction could use a rewrite. It uses very 'branded' language and it's hard initially to grok what your 'interface' actually is. I think part of my problem is the flow from the previous paragraph made me expect the design was a full prosthesis instead of a grafted component system that can bolster the signal acquisition for various neuroelectric prostheses. It may also have to do with terming the system: 'interface', as 'interface' in this domain is typically used for a system that bridges the biological to the electronic, the latter of which this system does not explicitly include (I think of this system as more of a signal splitter/amplifier, though it does strictly 'interface' nerve to other tissues). I think all of the info is embedded in the paragraph, just a re-organization with explicit emphasis describing exactly what this system entails in plain language before going into detail or branding would help.

- We appreciate the comments from Reviewer 1. Several sections in the introduction have been rewritten to further clarify what the RPNI entails per the concern raised regarding what interface means in this context. We grok in fullness the point the reviewer was making with regards to using the term “interface” and agree that the term classically refers to a component that establishes the biotic-abiotic bridge. This bridge is oftentimes electrodes, but our use of the term interface refers to the ability of the RPNI/C-RPNI to directly interface with the target peripheral nerve, later bridging to an electrode. In the case of prosthetic device integration, our constructs are eventually implanted with electrodes which are able to record motor efferent nerve signals via compound muscle action potentials generated by the RPNI/C-RPNI. This manuscript was written strictly as a C-RPNI fabrication methods paper, and the electrode portion of the interface was not discussed as we considered it beyond the scope of this paper.

2. The claim "The C-RPNI, however, is a biologic construct and thus does not induce foreign body response" is not appropriate, as it still requires further interfacing to be useful. This claim implies that it solves the problem of foreign body response in PNS interface, though it merely passes the buck to the electrodes that must interface with the C-RPNI.

- The C-RPNI does not induce foreign body response in the nerve that it interfaces with, but the reviewer is correct in that the electrodes can cause varying degrees of scar tissue in the muscle/skin once they are incorporated with the C-RPNI. However, a subsidiary research component in our laboratory is currently investigating ultrasound technology to detect RPNI muscle component contraction to control prosthetic devices, thus negating the need for electrodes and the risk of foreign body response.

3. Further, the authors are using isogenic tissues so this is experimentally mitigated, but generally tissue grafts are not immune from foreign body response.

- For this protocol, isogenic tissues are utilized specifically to avoid the experimental effects that autografts can cause. Specifically, contralateral extensor digitorum longus (EDL) can be harvested in an experimental rat, but this affects many behavioral tests and gait analysis post-surgery. Unlike rats, humans can have a variety of muscle tissue harvested for autografts without any long-term functional effects. In humans, we typically utilize vastus lateralis due to its muscle bulk, ease of harvest, and minimal risk to the donor.

Minor Concerns:

1. The numbering in the protocols should go 1.9 -> 1.10 not 1.9 -> 2.0

- We appreciate the catch in improper numbering, changes were made in the manuscript to correct that.

2. For 1.11 & 2.6, how long are these constructs viable *ex vivo*?

- We have now added further clarification in the protocol regarding construct viability following harvest before implantation. Although we have not formally studied viability, we have successfully used these grafts up to the two hour time point following harvest.

3. For 3.8 is there any need to prevent elastic retraction of the transected nerve ends?

- There is no need to prevent elastic retraction of the transected nerve ends. The distal end is allowed to retract, and the proximal end is used to construct the C-RPNI under direct visualization.

4. Is there any indication why particular grafts fail or have attenuated responses prior to your electrophysiology? I.e., the authors mention scarring/callusing/survival of the graft-Is there any particular reason why these occur in some animals and not others? Or are there histological measures that indicate fewer innervations regardless of scar status?

- In our experience, the majority of severe scarring is secondary to improper surgical technique, and callusing is often due to inadequate burring down of existing calluses during skin graft harvest. Unfortunately, prior to electrophysiology, we have no way to identify which animals have failing grafts. For those experimental animals with electrodes implanted at time of construct fabrication, electrophysiologic testing during gait studies could give an early indication of a failed construct if attenuated signals are obtained, but these situations are rare, making it difficult to formally draw conclusions. As far as determining degree of innervations, this is typically accomplished through multiple evaluations requiring sacrifice of the animal including immunohistochemistry, histomorphometry, CMAP/CSNAP amplitude +/- peak-to-peak values.

5. Since you presumably have a mapping of many nerves to many nerve endings, it may be interesting (but not necessary for this publication) to show a format of ephys that would exhibit

whether (a) you can stimulate different regions on the dermal interface to show effectiveness as a spatial sensor; and (b) sensitivity/stimulus ramps to show the dynamic range of stimulation/sensation on the graft constructs.

- We greatly appreciate the reviewer recognizing this potential for the grafts to operate as a spatial sensor as this is an area of great interest in our lab. We have been investigating several different electrode concepts (including carbon fiber and silicon arrays) in order to target specific sensory end organs, muscle spindles, etc, in discrete dermatomal arrangements, and we are very excited to pursue this in the future.

6. It may be helpful to provide in the author's Fig 1 schematic how this would interface beyond just grafting, or more pictorial representation of how this is used. It would be helpful if Figure 7 also included a visual schematic for the author's ephys strategy.

- To further clarify how the C-RPNI can be used in real-life applications, an additional Figure 2 was created. An additional figure was added (Figure 7) to demonstrate our electrophysiologic testing set up.

Reviewer #2

1. Please discuss the number of rats this has been performed on? What is the inter-animal variability and success?

- We appreciate the reviewer's interest and compliments for our construct as well as its success in an animal model. We added further detail to paragraph four in the Discussion section regarding our laboratory's experience and success with the construct. Overall, we have created over 90 of these constructs in rats, with a <5% failure rate (with the unspoken added benefit that the majority of these constructs have been fabricated by senior surgical residents). Regarding inter-animal variability, please refer to Figure 6 which details typical electrophysiologic values obtained from our most recent unpublished study utilizing C-RPNIs.

2. Were the proximal sensory signals from the C-RPNI compared to a group which just contained the motor component? I am wondering how essential the skin component is - or if stimulation of the muscle alone would activate sensory fibers that would be conducted proximally. One thought would be to prove the technology utilizing thin-film electrodes on the brain, placed over the sensory cortex and the collection of SSEPs. I understand that this is out of the scope of the current report - and not necessary for publication - but it is worth discussing as a potential future direction.

- For the data reported in this manuscript, proximal sensory signals (CSNAPs) generated in the proximal nerve following dermal stimulation of the C-RPNI were not compared to a muscle-only RPNI during the course of this study. However, we have previously stimulated muscle-only RPNIs in years prior, with CSNAPs generated at the more proximal nerve. When comparing CSNAP generation patterns following elicited stimulation in rat models, there is a noticeable difference in peak-to-peak values, allowing us to differentiate between whether the signal is of dermal or muscle origin. We have theorized that the muscle component CSNAP generation is secondary to activation of muscle spindles, Golgi tendon organs, Golgi-Mazzoni corpuscles, etc; however, when RPNIs have been stimulated in human subjects, they have reported reproducible sensory effects at specific areas in their hands and wrists. It is possible that the muscle-only RPNIs can transmit some sensory information, but we believe that information is incomplete and would benefit from the addition of the dermal component of the C-RPNI. We are additionally grateful for the idea for future study by correlating C-RPNI stimulation/signaling with collection of SSEPs from intra-neural electrodes in the sensory cortex and look forward to incorporating this into our next project.

Reviewer #3

1. What could improve this paper, would be some additional research in state of the art of neural electrodes, since many developments have been made in the recent years. I would further be very careful to state that the here presented method provides high selectivity. This is a very sensitive issue, and many researchers have been working on improving this issue by using intraneural and multi-cuff electrodes. The same accounts for the restoration of sensory feedback in amputees! The procedure itself is described very well. Also the histological results are well performed.

- We agree with your assertion that the field of neural electrodes has seen great progress, especially with regards to selectivity. However, the innovation with the C-RPNI is not the electrode component itself, but the ability to amplify a signal, requiring less capability from the electrode of choice. We do not explicitly state that the C-RPNI provides higher selectivity than advanced neural electrodes at its current state, but we do believe it has the capability when combined with highly selective electrodes. Due to funding limitations and that advanced electrode research is not one of the core focus areas of our program, our laboratory only uses 1-2 channel electrodes that are fabricated in-house, including epimysial patches, pads, and penetrating barbs. We feel that the C-RPNI has a potential for high selectivity via targeting individual sensory end organs at the level of the skin (not afferent axons), and we are currently working with another lab at the University of Michigan to utilize carbon fiber micro-arrays to target individual sensory end organs and motor units. There has been recent research on the topic of specific pulse trains to activate specific sensory end organs, and we plan to utilize this to bypass the need for neural interfacing, directly interfacing with the skin and muscle components of the C-RPNI, instead.

2. Personally I would wonder if this procedure is more/ or as effective as the ones presented by Dr. Kuiken from the rehabilitation institute Cgo. Did the authors study this to a sufficient extent?

- Dr. Kuiken's group has conducted excellent, clinically proven research with TMR, and we consider it a different strategy to achieve similar aims in a specific amputation population. Unlike TMR, we do not have to re-calibrate skin sensory and muscle control regions with each use which is a noted limitation. We have not looked into comparing the effectiveness of these two techniques, and it is an interesting target for future study.

3. Regarding the state of the art section, the authors included relevant publications in this field. However there are several statements which could be questionable. The authors state that "complex prosthesis do not provide … subtle sensory feedback for adequate control". Recently many papers have been published tackling this problem (e.g. Zollo, Sci-Rep. 2019: Restoring tactile sensations via neural interfaces for real-time force-and-slippage closed-loop control of bionic hands).

As the author states it is very true that an optimal prosthesis & interface would integrate sensory feedback, as well as prosthesis control using efferent motor signals (closed- loop neuroprostheses). The authors state that extra-neural cuffs, tissue penetrating electrodes or intrafascicular electrodes are, until today, not specific enough. Considering the dates of the citations I would suggest to do some further research regarding these points. It is of great importance to understand the problems, before providing a solution to them.

- We appreciate the updated resources the reviewer has provided regarding closed-loop prosthetics and advanced neural electrodes, and we have added these to the manuscript. However, many of these studies are lacking with regards to reproducing a natural sensory dermatome with natural sensations. Additionally, these studies are conducted on the time scale of weeks-to-months, and they lack long-term follow-up. As is mentioned in Jung et. al, for the limited samples that perform histology, many groups utilizing intra-fascicular electrodes note reduced axonal diameter and myelin, scar tissue build up, and traumatic

fibrosis. These groups often wrongly conclude that because the electrodes functioned for the indicated time-scale despite these “minor” histologic findings, their local tissue effect is negligible. This is incorrect as it is well-known that many compressive neuropathies and peripheral nerve disorders in humans begin with demyelination, producing a cascade of inflammatory and ischemic effects if demyelination continues over time, with signaling functionality being one of the last characteristics to be affected. In our laboratory, we have demonstrated RPNI functionality on the time-scale of years with continued function and lack of local tissue effect.

4. Again many papers have been published on this topic. I would suggest to cite also more recent works from several authors. One of the authors published a very nice paper (citation regarding a PEDOT coated stainless steel electrode. In how far is this electrode more selective than currently available intraneural or cuff electrodes? Maybe this point could be considered in the text, or the claim that "nerve specificity" is higher using RPNI could be removed.

- We agree with your point that some publications were out of date. We have now included in the revised manuscript additional sources that have publication dates in the 2016-2019 range, including your recommended sources (one of which, Frost et.al. is from our laboratory). Regarding the PEDOT electrode, it is not more specific than currently available intraneural or cuff electrodes. It is beneficial in that it has a higher conductance and signal secondary to low impedance which works well with our constructs.

5. Maybe this point could be considered in the text, or the claim that "nerve specificity" is higher using RPNI could be removed.

- We do not feel that the C-RPNI has higher specificity than currently available methods at present, but we hope to further develop this characteristic and fully investigate how it compares to existing neural electrodes in the future.

6. The amount "multiple degrees of freedom" should be mentioned for scientific reasons. In citation 26, 4 is mentioned to be the number of available DOFs.

- Degrees of freedom are not explicitly stated in this manuscript because published data is not reflective of capability. For the study referenced in the comments (source 26, Kubiak et. al), the protocol was developed to specifically test only four degrees of freedom secondary to time constraints and testing demand on participants. The DEKA Luke prosthetic arm utilized in our research is constrained to six degrees of freedom at the wrist and hand, as the middle through small fingers move as a unit. However, the RPNIs when used in conjunction with a virtual hand have far more wrist/finger movements available, with human trials demonstrating the ability to move a thumb to any desired target in a 3D space.

7. The surgical procedure seems to have the potential to promote proliferation into the dermal graft and motor fibers, innervating them eventually. Can this considered to be an interface?

- This paper details the fabrication of a biologic nerve interface. It is the intention that there is an abiotic component (e.g. electrode) to this interface, but that was considered to be beyond the scope of this paper detailing C-RPNI fabrication.

8. The electrophysiological testing does not clearly state where exactly, and especially with which electrode (needle, wire, hook, etc.) the proximal common peroneal nerve is stimulated. In how far is this procedure consistent? Is the location always the same?

- Detailed electrophysiological testing protocol was considered to be beyond the scope of this paper, but further detail was added to representative results in addition to Figure 7 to demonstrate our set-up. We typically test with a paired hook wire electrode (Nicolet-

VIASYS) just distal to the common peroneal nerve's takeoff from the sciatic nerve, which is proximal to the C-RPNI. This location is relatively consistent, as the anatomy is rarely discrepant in rats.

9. Finally, if someone would like to reproduce this study it could be very interesting to know which EMG electrodes you were using in this case. Did you use epimysial electrodes as suggested in the long-term studies?

- Further detail regarding successful EMG electrodes utilized was added to the representative results section. We have used indwelling epimysial patch and pad electrodes (Grass Technologies) for chronic studies, and typically utilize penetrating needle electrodes or bipolar probe for studies utilizing an open surgical approach to evaluate electrophysiological outcomes.

Reviewer #4

1. The Introduction states that RPNIs allow revascularization and reinnervation of previously denervated neuromuscular junctions (NMJs). Is there evidence that the NMJs remain and then are reinnervated? Is it possible that new NMJs are formed? If evidence with either conclusion is known, please cite it.

- We appreciate the reviewer addressing this concern regarding RPNI reinnervation and neuromuscular junction formation, and you have an excellent point. At this time, we have not been able to develop a study that has the capability to assess to what degree old neuromuscular junctions are re-innervated or new NMJs are formed. We have previously found that these RPNIs are capable of poly-innervation, indicating that new NMJ formation is possible in select circumstances. However, we are unable to quantify this, and instead, we assume that there is likely a combination of the two phenomenon occurring with graft incorporation. The statement regarding "reinnervation of previously denervated NMJs" was misleading and removed from the manuscript.

2. Under the Protocol for c-RPNI fabrication, is there a difference between steps 4.2 and 4.4? They seem identical?

- Steps 4.2 and 4.4 are similar, in that they involve suturing of the epineurium to the muscle component of the C-RPNI. However, step 4.2 entails the initial securing of the nerve to the central portion of the muscle, and step 4.4 is an additional securing stitch at the outside margin of the muscle that allows for a segment of nerve within the construct to serve as a tension relief (e.g. during ambulation, any tension on the nerve will be directed at first to this inferior margin "stress relief" stitch instead of the central securing stitches).

3. In the Representative Results, use present verb tense rather than future tense.

- We thank the reviewer for this comment. The tense has been changed in the revised manuscript to the present tense in the representative results per suggestion.

4. Figure 5. Could you provide a higher magnification image of panel A? Do you have better images of alpha-bungarotoxin staining? It doesn't look characteristic, but is in the correct location, in the provided image.

- Figure 5 has been changed to include a higher magnification image of an NMJ in panel A. We agree with the reviewer that the junction displayed in this image is not totally characteristic, and we believe that is secondary to the cutting and preservation process we employ.

5. In Table 2, are the upper and lower halves of the table different timepoints? In the PDF, they have the same headings.

- The table was uploaded as an excel file per JoVE's request, and we believe the format was changed with the automatic conversion to PDF format. The table is detailing two different time points, 3 months at the top and 9 months at the bottom. The table is pasted plain text below

3 Month Data		CMAP Data (Stimulate CP nerve and record from muscle graft)			CSNAP Data (Stimulate skin graft and record from CP nerve)		
Rat ID Number	**Construct Weight (g)**	**Stimulation Amplitude (mA)**	**Conduction Velocity (m/sec)**	**V Peak-to-Peak (mV)**	**Stimulation Amplitude (mA)**	**Conduction Velocity (m/sec)**	**V Peak-to-Peak (µV)**
4607	0.087	4.17	11.3	10.3	18	11.1	121
4608	0.15	1.65	11.1	17.1	7.7	6.5	136
4611	0.113	8.3	9.6	11.2	10	10	121
4613	0.116	3.18	10	9.6	1.44	8.3	134
4614	0.189	3	10.8	9.6	7.39	9	151
4616	0.122	5.2	9.4	14.9	1.8	9.1	100
4620	0.118	2.91	7.6	7.4	8.7	10	219

9 Month Data		*CMAP Data (Stimulate CP nerve and record from muscle graft)*			*CSNAP Data (Stimulate skin graft and record from CP nerve)*		
Rat ID Number	**Construct Weight (g)**	**Stimulation Amplitude (mA)**	**Conduction Velocity (m/sec)**	**V Peak-to-Peak (mV)**	**Stimulation Amplitude (mA)**	**Conduction Velocity (m/sec)**	**V Peak-to-Peak (µV)**
4687	0.238	1.35	9.6	18.2	0.99	11	181
4688	0.131	1.08	10	8.8	1.11	8	132
4689	0.26	1.26	9.6	21.8	1.9	8.6	237
4690	0.192	4.2	8.3	12.8	n/a	n/a	n/a
4691	0.213	1.38	10	18.6	6.6	8	153
4693	0.178	1.11	9.6	15.1	8.7	8.3	306

6. Inclusion of more clinical points, such as additional indications/applications of RPNI and a discussion of what patients are candidates for the procedure, would be nice.

- We appreciate the reviewers request for further RPNI indications in this manuscript, and further detail has been added to the discussion. We have several promising ongoing studies regarding RPNI and dermal sensory interface (DSI) use in the treatment of chronic pain, phantom limb pain, and neuroma formation. These interfaces can be used in any population of patients that has neuropathic-based pain and/or neuromas in the setting of previous nerve transection/injury. We anticipate the C-RPNI will be particularly beneficial for those with more proximal (e.g. transhumeral) amputations, but we have not yet reached the human trials stage at present.