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TITLE:

The Muscle Cuff Regenerative Peripheral Nerve Interface for the Amplification of Intact Peripheral Nerve Signals

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peripheral nerve, muscle cuff, exoskeleton, MC-RPNI, human-machine interface, neuromuscular interface

SUMMARY:

This manuscript provides an innovative method for developing a biologic peripheral nerve interface termed the Muscle Cuff Regenerative Peripheral Nerve Interface (MC-RPNI). This surgical construct can amplify its associated peripheral nerve's motor efferent signals to facilitate accurate detection of motor intent and the potential control of exoskeleton devices.

ABSTRACT:

Robotic exoskeletons have gained recent acclaim within the field of rehabilitative medicine as a promising modality for functional restoration for those individuals with extremity weakness. However, their use remains largely confined to research institutions, frequently operating as a means of static extremity support as motor detection methods remain unreliable. Peripheral nerve interfaces have arisen as a potential solution to this shortcoming; however, due to their inherently small amplitudes, these signals can be difficult to differentiate from background noise, lowering their overall motor detection accuracy. As current interfaces rely on abiotic materials, inherent material breakdown can occur alongside foreign body tissue reaction over time, further impacting their accuracy. The Muscle Cuff Regenerative Peripheral Nerve Interface (MC-RPNI) was designed to overcome these noted complications. Consisting of a segment of free muscle

graft secured circumferentially to an intact peripheral nerve, the construct regenerates and becomes reinnervated by the contained nerve over time. In rats, this construct has demonstrated the ability to amplify a peripheral nerve's motor efferent action potentials up to 100 times the normal value through the generation of compound muscle action potentials (CMAPs). This signal amplification facilitates high accuracy detection of motor intent, potentially enabling reliable utilization of exoskeleton devices.

INTRODUCTION:

In the United States alone, approximately 130 million people are affected by neuromuscular and musculoskeletal disorders, resulting in over $800 billion in annual economic impact[1,2]. This group of disorders is typically secondary to pathology within the nervous systems, at the neuromuscular junction, or within the muscle itself[3]. Despite the variety of pathologic origins, the majority share some degree of extremity weakness[1,3]. Unfortunately, this weakness is often permanent given the limitations in neural and muscle tissue regeneration, especially in the setting of severe trauma[4–6].

Extremity weakness treatment algorithms have classically focused on rehabilitative and supportive measures, often relying on harnessing the capabilities of the remaining intact limbs (canes, wheelchairs, etc.)[7]. This strategy falls short, however, for those whose weakness is not limited to a single extremity. With recent innovations in robotic technologies, advanced exoskeleton devices have been developed that restore extremity functionality to those living with extremity weakness[8–13]. These robotic exoskeletons are often powered, wearable devices that can assist with initiation and termination of movement or maintenance of limb position, providing a varying amount of force that can be individually tailored for the user[8–13]. These devices are classified as either passive or active depending on how they provide motor assistance to the user: active devices contain electrical actuators that augment power to the user, whereas passive devices store energy from the user's motions in order to release it back to the user when necessary[14]. As active devices have the ability to increase a user's power capabilities, these devices are utilized far more frequently in the setting of extremity weakness[[14]].

In order to determine motor intent in this population, modern exoskeletons commonly rely on pattern recognition algorithms generated from either electromyography (EMG) of distal limb muscles[8,15–17] or surface electroencephalography (sEEG) of the brain[18–20]. Despite the promise of these detection modalities, both options have significant limitations that preclude widespread utilization of these devices. As sEEG detects microvolt-level signals transcranially[18–20], criticisms frequently focus on the inability to differentiate these signals from background noise[21]. When background noise is similar to the desired recording signal, this produces low signal-to-noise ratios (SNRs), resulting in inaccurate motor detection and classification[22,23]. Accurate signal detection additionally relies on stable, low-impedance scalp contact[21], which can be significantly affected by the presence of coarse/thick hair, user activity, and even sweating[22,24]. In contrast, EMG signals are several magnitudes larger in amplitude, facilitating greater motor signal detection accuracy[15,18,25]. This comes at a cost, however, as nearby muscles can contaminate the signal, decreasing the degrees of freedom able to be controlled by the device[16,17,25] and an

inability to detect deep muscle motion[25–28]. Most importantly, EMG cannot be used as a control method when there is significant muscle compromise and complete absence of tissue[29].

In order to advance the development of robotic exoskeletons, consistent and accurate detection of motor intent of the intended user is required. Interfaces that utilize the peripheral nervous system have arisen as a promising interface technique, given their relatively simple access and functional selectivity. Current peripheral nerve interfacing methods can be invasive or non-invasive and typically fall within one of three categories: extraneural electrodes[30–33], intrafascicular electrodes[34–36] and penetrating electrodes[37–40]. As peripheral nerve signals are generally on the level of microvolts, it can be difficult to differentiate these signals from similar amplitude background noise[41,42], which reduces the overall motor detection accuracy capabilities of the interface. These low signal-to-noise (SNR) ratios often worsen over time secondary to worsening electrode impedance[43] produced from either degradation of the device[39,43], or local foreign body reaction producing scar tissue around the device and/or local axonal degeneration[37,44]. Although these shortcomings can generally be resolved with reoperation and implantation of a new peripheral nerve interface, this is not a viable long-term solution as foreign-body-associated reactions would continue to occur.

To avoid these local tissue reactions generated from peripheral nerves' interaction with abiotic interfaces, an interface incorporating a biologic component is necessary. To address this shortcoming, the Regenerative Peripheral Nerve Interface (RPNI) was developed to integrate transected peripheral nerves in the residual limbs of those with amputations with prosthetic devices[45–48]. Fabrication of the RPNI involves surgical implantation of a transected peripheral nerve into a segment of autologous free muscle graft, with revascularization, regeneration, and reinnervation occurring over time. Through the generation of milli-volt level compound muscle action potentials (CMAPs), the RPNI is able to amplify its contained nerve's micro-volt level signal by several magnitudes, facilitating accurate detection of motor intent[45,48,49]. There has been considerable development of the RPNI over the past decade, with notable success in amplifying and transmitting efferent motor nerve signals in both animal[50,51] and human[47] trials, facilitating high accuracy prosthetic device control with multiple degrees of freedom.

Individuals with extremity weakness but intact peripheral nerves would similarly benefit from high accuracy detection of motor intent through peripheral nerve interfaces in order to control exoskeleton devices. As the RPNI was developed for integration with transected peripheral nerves, such as in persons with amputations, surgical modifications were necessary. Building from experience with the RPNI, the Muscle Cuff Regenerative Peripheral Nerve Interface (MC-RPNI) was developed. Consisting of a similar segment of free muscle graft as in the RPNI, it is instead secured circumferentially to an intact peripheral nerve (**Figure 1**). Over time, it regenerates and becomes reinnervated through collateral axonal sprouting, amplifying and translating these efferent motor nerve signals to EMG signals that are several orders of magnitude larger[52]. As the MC-RPNI is biologic in origin, it avoids the inevitable foreign body reaction that occurs with peripheral nerve interfaces currently in use[52]. Furthermore, the MC-RPNI confers the ability to control multiple degrees of freedom simultaneously as they can be placed on distally dissected nerves to individual muscles without significant cross-talk, as has

been previously demonstrated in RPNIs[49]. Finally, the MC-RPNI can operate independent of distal muscle function as it is placed on the proximal nerve. Given its advantages over current peripheral nerve interfaces, the MC-RPNI holds substantial promise for providing a safe, accurate, and reliable method of exoskeleton control.

PROTOCOL:

All animal procedures and experiments were carried out with the approval of the University of Michigan's Institutional Care and Use of Animals Committee (IACUC). Male and female Fischer F344 and Lewis rats (~200–300 g) at 3–6 months of age are most frequently utilized in experiments, but any strain can theoretically be utilized. If utilizing donor rats instead of autologous muscle grafts, donor rats must be isogenic to the experimental strain. Rats are allowed free access to food and water both pre- and post-operatively. Following terminal endpoint evaluations, euthanasia is performed under deep anesthesia with intra-cardiac potassium chloride injection followed by a secondary method of bilateral pneumothorax.

1. Experimental preparation of the rat

1.1. Anesthetize the experimental rat utilizing a solution of 5% isoflurane in oxygen at 0.8–1.0 L/min in an induction chamber. Once adequate anesthesia is achieved and confirmed with the absence of corneal reflex, place the rat on a rebreather nose cone with isoflurane lowered to 1.75%–2.25% for maintenance of anesthesia.

1.2. Inject a solution of 0.02–0.03 mL Carprofen (50 mg/mL) in 0.2 mL of sterile saline with 27 G needle in the subcutaneous plane between the shoulder blades for peri- and post-operative analgesia.

1.3. Apply sterile eye ointment to both eyes to prevent corneal ulcers while anesthetized.

1.4. Using an electric razor, shave the lateral portion of the bilateral lower limbs, extending from the hip joint, over the thigh, and to the dorsal surface of the paw.

1.5. Sterilize the experimental area(s) by first wiping with alcohol prep pad, followed by povidone-iodine solution application, ending with a final cleanse with a new alcohol prep pad to remove the residual povidone-iodine solution.

NOTE: This can be a dermatological irritant; ensure the majority of the solution is removed.

2. Preparation of the muscle graft

2.1. Place the rat on a heating pad underneath a surgical microscope with an intraoral body temperature probe of choice for body temperature monitoring. Maintain isoflurane at 1.75%–2.25% and oxygen at 0.8–1.0 L/min.

2.2. Make a longitudinal incision along the anterior aspect of the desired donor hindlimb extending from just above the ankle to just below the knee with a #15 scalpel.

2.3. Dissect through the underlying subcutaneous tissue using sharp iris scissors to expose the underlying musculature and distal tendons just proximal to the ankle joint. Tibialis anterior (TA) is the largest and most anterior of the muscles; the extensor digitorum longus (EDL) muscle can be found just deep and posterior to this muscle. Isolate the EDL muscle and its distal tendon from the surrounding musculature.

2.4. Ensure isolation of the correct tendon by inserting both tines of a forceps or iris scissor underneath the distal tendon just proximal to the ankle joint. Exert upward pressure on the tendon by opening either the forceps or iris scissors. This motion should produce a simultaneous extension of all of the toes simultaneously. If isolated ankle dorsiflexion, ankle eversion, or single toe dorsiflexion occurs, the wrong tendon has been isolated.

2.5. Perform a distal tenotomy of the EDL muscle at the level of the ankle with sharp iris scissors and dissect the muscle free from surrounding tissues working proximally towards its tendinous origin.

2.6. Once the proximal tendon is visualized, perform a proximal tenotomy utilizing sharp iris scissors to free the graft.

2.7. Trim both tendinous ends of the muscle graft and cut to the desired length with sharp iris scissors.

NOTE: Grafts measuring 8–13 mm have been utilized with success; however, the most common length utilized is 10 mm.

2.8. On one side of the muscle graft, make a longitudinal incision along the entire trimmed length to facilitate placement of the nerve within the muscle graft and provide contact of the nerve with endomysium.

2.9. Place the prepared muscle graft in a saline-moistened gauze to prevent tissue desiccation.

3. Common peroneal nerve isolation and preparation

3.1. Mark the surgical incision, which will extend from a line ~5 mm from the sciatic notch, extending to just inferior to the knee joint. Ensure that this marking is inferior to, and angled away from, the femur that can be palpated below.

3.2. Incise through the skin and subcutaneous tissues along the marked incision line with a #15 blade. Carefully incise through the underlying biceps femoris fascia, taking care not to extend through the entire depth of the muscle as the sciatic nerve lies just below.

3.3. Utilizing blunt-tipped small scissors or a hemostat, dissect carefully through the biceps femoris muscle.

NOTE: The sciatic nerve travels in this space underlying the biceps, oriented in approximately the same direction as the incision marked on the skin. There are three notable sciatic nerve branches: sural (most posterior and smallest of the nerves), tibial (typically most anterior, but this nerve always dives deep to the knee joint), and common peroneal (typically located between tibial and sural, always travels above the knee joint).

3.4. Identify the common peroneal (CP) nerve and carefully isolate it from the surrounding nerves using a pair of micro-forceps and micro-scissors. Remove any surrounding connective tissue from the middle 2 cm of the nerve. Take care not to crush the CP nerve with forceps in this process, as crush injury can alter endpoint results.

3.5. Over the central-most portion of the freed CP nerve, perform an epineurial window by removing 25% of the epineurium along the length of the nerve that matches the desired length of the muscle graft.

3.5.1. To perform this hold the proximal epineurium with micro-forceps, cut into the epineurium immediately underlying with micro-dissection scissors, and remove ~25% of the epineurium traveling distally along the nerve. Take care to remove this segment in one piece, as multiple attempts can cause irregular epineurial removal, increasing the risk of nerve injury.

NOTE: The nerve tissue underlying the epineurium will have a goo-like texture; noting this quality of nerve ensures the correct tissue plane has been removed.

4. MC-RPNI construct fabrication

4.1. Remove the muscle graft from the saline-moistened gauze and place it under the central portion of the CP nerve where the epineurial window was created. Rotate the nerve 180° so the epineurial window section contacts intact muscle and does not underlie the eventual suture line.

4.2. Using an 8-0 nylon suture, suture the epineurium of the CP nerve both proximally and distally to the muscle graft within the groove created in step 2.8 using simple interrupted sutures to secure epineurium to endomysium.

NOTE: Place these stitches, ensuring the muscle is at normal resting length. Stretching or compressing the muscle too much can impact regeneration and signaling capabilities later on.

4.3. Circumferentially wrap the edges of the muscle graft surrounding the now-secured nerve and suture in place utilizing simple interrupted 8-0 nylon stitches (~4–6 depending on length).

4.4. Once hemostasis is achieved, close the biceps femoris fascia over the construct with 5-0 chromic suture in running fashion.

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

4.6. Cleanse the surgical area with an alcohol prep pad and apply antibiotic ointment.

4.7. Terminate inhalational anesthetic and place the rat in a clean cage isolated from cage mates and allow to recover with food and water *ad lib*.

4.8. Once the rat has suitably recovered, place it back with cage mates in a clean cage.

NOTE: These constructs require a maturation of three months at the minimum to produce adequate nerve signal amplification.

REPRESENTATIVE RESULTS:

MC-RPNI surgical fabrication is considered a peri-operative failure if rats do not survive emergence from surgical anesthesia or develop an infection within a week of the operation. Previous research has indicated a 3 month maturation period will result in reliable signal amplification from this constructs[42,45,48,49]. At that time or thereafter, surgical exposure of the constructs and evaluation can occur. If MC-RPNI fabrication was successful, revascularized muscle should be readily visible at the original MC-RPNI implantation site (**Figure 2B**). Successful MC-RPNIs will additionally contract following proximal nerve stimulation (**Video 1**). At times, significant scarring and atrophic muscle graft can be present (**Figure 2C**), indicating a failure of revascularization/regeneration typically secondary to too large of a graft, improper handling, or peri-operative tissue injury. These atrophic grafts commonly have some degree of contraction upon proximal nerve stimulation but produce lower signal amplification. Overall, it is considered a fabrication failure if, upon exposure, the MC-RPNI is found dislodged from the nerve or has no contraction upon proximal nerve stimulation.

Histological analysis of these constructs should demonstrate viable nerve and muscle tissue without any significant fibrosis or scarring (**Figure 3**). Immunohistochemistry can also be performed to confirm the presence of innervated neuromuscular junctions with neurofilament as a general nerve marker in combination with alpha-bungarotoxin as a marker for postsynaptic acetylcholine receptors (**Figure 4**). If the target implanted nerve fails to innervate the muscle component of the MC-RPNI, immunostaining would not demonstrate any collateral motor nerve sprouts traversing the construct, nor any innervated neuromuscular junctions.

Electrophysiologic testing can be performed on these constructs at any time following maturation, with results published demonstrating stable signals specifically in the MC-RPNI at 3 months[52] and up to 3 years in RPNIs in human subjects[47]. Electrophysiologic testing schematics can vary according to the area of interest and equipment available (**Figure 5**), but evaluations are most commonly performed with the provision of maximal stimulation to the proximal nerve with a hook electrode followed by a recording of compound muscle action potentials (CMAPs) generated at the MC-RPNI (**Table 1**). Recording electrodes can vary according to user preference, but epimysial patch/pad, epimysial bipolar probe, and penetrating bipolar electrodes have been

used experimentally with success. The average compound nerve amplitude (CNAP) recorded at the CP nerve following more proximal nerve stimulation was 119.47 μV ± 14.87 μV. Average CMAP amplitude recorded at the MC-RPNI following similar proximal CP nerve stimulation was 3.28 mV ± 0.49 mV, resulting in amplification of the nerve signal from 11–87x, with an overall average amplification factor of 31.8 ± 7.70. These generated CMAP waveforms are similar in appearance to native muscle, further supporting that they have become reinnervated by their contained nerve (**Figure 6B**).

To ensure MC-RPNI fabrication does not cause negative functional impact, electrophysiologic and muscle force testing can be performed on distally-innervated muscle. The majority of testing has been performed on the ipsilateral EDL muscle as it is easily accessible for testing and is innervated by the common peroneal nerve (the contralateral EDL is harvested for MC-RPNI fabrication and thus not assessed). CMAPs generated by physiologic EDL muscle following proximal CP nerve stimulation typically range from 20–30 mV[52]. When performing this test on rats with implanted MC-RPNIs, EDL CMAPs are not significantly different, averaging 24.27 mV ± 1.34 mV. Additionally, when comparing generated CMAP waveforms between these two groups, they are remarkably similar (**Figure 6C**). As an additional measure of distally-innervated muscle function, muscle force testing of the muscle of interest can be pursued (**Table 2**). Following proximal CP nerve stimulation, the average EDL maximal tetanic force generated in MC-RPNI subjects is 2451 mN ± 115 mN, similar to the average force of 2497 mN ± 122 mN obtained from EDL muscle in control subjects[52].

The overall purpose of the MC-RPNI is to amplify its contained nerve's microvolt-level signal by several magnitudes, increasing the SNR ratio and thus facilitating accurate detection of motor intent. This amplification has been demonstrated to occur in a reliable fashion in the range of 10–20 times[52], with more recent experiments achieving amplification factors of over 50 times; therefore, if a construct does not provide a similar level of amplification, it is considered sub-optimal. Sub-optimal results can typically be attributed to problems at the level of the muscle graft in the MC-RPNI, as incomplete regeneration and thus reinnervation can result in lower than standard CMAP, lowering the overall amplification abilities of the construct. The generated waveform is typically attenuated, with a noticeably abnormal appearance. If the muscle graft completely fails, the signal measured at the muscle component can either be non-existent (secondary to significant scar tissue) or mirror the CNAP generated at the upstream nerve.

FIGURE AND TABLE LEGEND:

Figure 1: Illustrative schematic of the MC-RPNI. The target peripheral nerve can be seen in yellow within the surrounding muscle graft. The MC-RPNI is able to amplify its contained nerve's motor efferent action potentials on the level of microvolts through the generation of compound muscle action potentials (CMAPs) several magnitudes larger. This facilitates the detection of motor intent that is easily differentiated from background noise.

Figure 2: MC-RPNI *in vivo.* The MC-RPNI is fabricated using an autologous extensor digitorum longus (EDL) muscle graft harvested from the contralateral limb. It is then circumferentially secured to the common peroneal nerve, with an example MC-RPNI outlined in white (**A**) at the

time of initial fabrication. This same MC-RPNI is again pictured in (**B**) at the time of endpoint evaluation 3 months following. The MC-RPNI has similar coloration as the surrounding muscle and has retained a good portion of the volume. An example of an atrophic muscle graft is shown in (**C**). The MC-RPNI has a similar appearance to surrounding scar and connective tissue and has lost considerable volume.

Figure 3: MC-RPNI histology. (A) H&E of a MC-RPNI cross-section with M indicating the muscle component, and N, the nerve. (**B**) Cross-section of the ipsilateral distally-innervated EDL muscle in a rat with a MC-RPNI. (**C**) Cross-section of EDL muscle in a control rat without a MC-RPNI.

Figure 4: Immunostaining of the MC-RPNI. The image at the top left shows a longitudinal section of a MC-RPNI specimen with nuclei noted in blue (DAPI) and nerve tissue in green (neurofilament). A close-up of another MC-RPNI is shown at the bottom right with multiple neuromuscular junctions present (alpha-bungarotoxin in red for acetylcholine receptors).

Figure 5: Endpoint electrophysiologic evaluation setup. Electrophysiologic testing requires at a minimum of three electrodes: (1) a grounding electrode – not pictured; (2) a nerve stimulating bipolar electrode; and (3) a bipolar recording electrode. In this setup, a bipolar stimulating hook electrode can be seen in white to the right of the image placed on the common peroneal nerve. The recording bipolar probe electrode is placed on the distal MC-RPNI. Signals are then recorded from the MC-RPNI following proximal nerve stimulation at the hook electrode until maximal CMAPs are achieved.

Figure 6: Standard electrophysiologic waveforms. These graphs depict typical waveforms captured during electrophysiologic analysis of a rat with an implanted MC-RPNI following proximal CP nerve stimulation. (**A**) In blue, a CNAP (*****) recorded from the CP nerve proximal to the MC-RPNI is pictured. The system artifact is indicated with a (******). (**B**) The representative CMAP recorded from the MC-RPNI following the generated CNAP in (**A**). (**C**) The resultant CMAP waveform recorded from the ipsilateral distally-innervated EDL muscle.

Table 1: Endpoint electrophysiologic analysis of MC-RPNIs. A selection of results obtained from rats undergoing endpoint analysis at 3 (Rats 1–9) and 6 (Rats 10–12) months post-fabrication. Following proximal common peroneal (CP) nerve stimulation, compound nerve action potentials (CNAPs) were recorded at the downstream CP nerve, and compound muscle action potentials (CMAPs) were recorded at the downstream MC-RPNI. The amplification factor for each test can be seen in the column at the right. Note: for Rats 10–12, the CNAP proximal to the MC-RPNI was unable to be measured given anatomical limitations that resulted from fabricating the MC-RPNI too close to the CP nerve's takeoff from the sciatic nerve. The average CNAP amplitude recorded was 119.47 µV ± 14.87 µV while the average CMAP amplitude was 3.28 mV ± 0.49 mV, producing an average amplification factor of 31.8 ± 7.70.

Table 2: Muscle force analysis of rats with implanted MC-RPNIs. Muscle force testing was conducted on the ipsilateral extensor digitorum longus (EDL) muscle to determine if the MC-RPNI had any impact on distally-innervated muscle function. Following proximal CP nerve stimulation,

force tracings were recorded and active force was calculated relevant to the test of interest. L_o was defined as the optimal muscle resting length that produced maximal force. Average maximal twitch force recorded from rats with implanted MC-RPNIs was 722.0 mN ± 32.11 mN and average maximal tetanic force recorded was 2451 mN ± 115 mN, similar to values obtained from control animals (maximal twitch: 822.2 mN ± 41.11 mN; maximal tetany: 2497 mN ± 122 mN).

Video 1: MC-RPNI contraction following proximal nerve electrical stimulation. Following proximal nerve electrical stimulation provided by the hook electrode at the right, visible muscle contraction of the MC-RPNI can be seen in the center.

DISCUSSION:

The MC-RPNI is a novel construct that allows for amplification of an intact, peripheral motor nerve's efferent action potentials in order to accurately control an exoskeleton device. Specifically, the MC-RPNI confers a particular benefit to those individuals with extremity weakness caused by significant muscle disease and/or absence of muscle where EMG signals cannot be recorded. Reducing already compromised muscle function would be devastating in this population; however, the MC-RPNI has the ability to provide this nerve signal amplification without detriment to distally-innervated muscle[52] (**Table 1**and **Table 2**). In those individuals with muscle-based or lower motor neuron disease, peripheral sensory nerves are typically unaffected by the disease process[53]. As sensation is preserved, it is imperative to keep the nerve in continuity and avoid injury, and the MC-RPNI appears to avoid any detriment to distally-innervated targets as a whole based on histology (**Figure 3**), immunohistochemistry (**Figure 4**), and evaluation of muscle function (**Table 2**).

The MC-RPNI relies on the concept of collateral axonal sprouting of the contained peripheral nerve, a concept readily demonstrated in both prior research[52] and also in the well-described surgical technique of end-to-side neurorrhaphy[54,55]. To ensure adequate reinnervation of the muscle graft during MC-RPNI fabrication and avoid negative impact to distally-innervated targets, meticulous handling of the nerve is imperative. During dissection of the nerve, trauma can be avoided through concise handling of either the epineurium or connective tissue only. However, the potential for nerve injury in MC-RPNI fabrication is the highest during the epineurial window step. To avoid sharp transection of nerve fibers, it is recommended to perform this step only under a high-power surgical microscope (at least 5x) after several opportunities for practice on non-experimental rats. This step can take several attempts to master, and it is not recommended to first perform this step on rats intended for experimental analysis. Theoretically, neuroma-in-continuity is a complication that could occur following MC-RPNI fabrication, especially in the presence of significant nerve trauma. However, this complication has not been encountered over the many years in development.

The majority of experiments performed with the MC-RPNI have been performed on the common peroneal nerve given its relative ease of access as well as evaluation of distally-innervated targets. Theoretically, any peripheral nerve with a motor component could be substituted. Pure sensory axons could be utilized as muscle tissue does have sensory components (spindle fibers, Golgi tendon organs, etc.), but these experiments have not been conducted thus far, and the

results are difficult to predict. For the muscle graft component of the MC-RPNI, grafts range from 20–150 mg depending on graft length and age of the rat, and any similar size muscle graft can be successfully utilized. Muscle graft regeneration relies in part on the ability to revascularize, and large/thick grafts are more likely to undergo necrosis and fibrosis, affecting overall signaling capability[56]. Research performed specifically on RPNIs has indicated successful muscle regeneration and maintenance of signal amplification in grafts up to 300 mg[56]. With regards to rat breed, Lewis and Fischer are recommended as the majority of other rats used for experimental purposes are known to self-mutilate secondary to nerve injury[57,58].

Overall, current experiences with MC-RPNI fabrication have produced a failure rate of <5%. The most common construct failures seen are typically attributed to the muscle graft segment, whereupon on the exposure they are noted to be either atrophic or dislodged from the nerve. Dislodged MC-RPNIs usually result from inadequate suturing at the time of fabrication, leading to "opening" of the circumferentially wrapped muscle graft and eventual partial extrusion of the contained nerve. However, these MC-RPNIs usually retain some degree (albeit reduced) of signal amplification capabilities as a portion of the graft still remains secured to the nerve. Atrophic MC-RPNIs are obvious on exposure as they lack the typical skeletal muscle appearance, often undistinguishable from scar tissue with light pink to grey/white coloration (**Figure 2C**). Atrophy of the muscle tissue can result from many factors, including infection, too large/thick of a muscle graft, acute blood loss anemia, muscle and/or nerve injury during fabrication, as well as the failure of the epineurial securing sutures causing pistoning of the graft on the nerve, reducing revascularization. On electrophysiologic testing, atrophic MC-RPNIs typically produce little to no signal amplification; if using high-sensitivity electrodes, recordings of the underlying nerve's CNAP can be recorded through the atrophic muscle. If significant atrophy is noted in multiple experimental subjects, one must return to the protocol and determine which steps require adjustment. Of course, if no signals are recorded when performing evaluations, it is important to troubleshoot and not assume the construct is a failure. Troubleshooting the device set up is paramount, as lack of signals can be secondary to damaged electrodes (recommend impedance <16 Ω), incorrect electrode configuration, or even inadequate proximal nerve stimulation (some nerves require 0.5–5 mA electrical stimulation to begin producing CMAPs at the downstream MC-RPNI).

Current methods of human-machine interfacing for exoskeleton use in those with extremity weakness typically rely on recordings obtained from either peripheral nerves or EMG from muscle tissue. As discussed previously, the MC-RPNI confers significant benefit with regards to exoskeleton control for those individuals with severely damaged or absent muscle tissue where EMG recordings are not possible[29]. The MC-RPNI also offers an advantage over current peripheral nerve interfacing options, including extraneural electrodes[30–33], intrafascicular electrodes[34–36] and penetrating electrodes[37–40]. As inherent nerve signals are commonly on the level of microvolts, the MC-RPNI has the ability to amplify these nerve signals over 30 times, facilitating accurate detection of motor intent from background noise and thus enabling reliable exoskeleton control. With chronic use, current electrode-based methods ultimately struggle to overcome complications inherent to material longevity *in vivo* and foreign body reaction, complications the MC-RPNI is able to avoid given its biologic origin. Over time, these foreign body reactions result

in tissue damage, scar tissue formation, and eventual axonal demyelination and degeneration. Experiments conducted up to six months have not revealed any evidence of neuronal injury, scar, or fibrosis/degeneration of distally-innervated muscle tissue (**Figure 3**), and in combination with RPNI stability noted in human subjects over a three year observation period[47], it is reasonable to conclude that MC-RPNIs could successfully interface with peripheral nerves on the scale of years to decades.

The MC-RPNI is intended to be utilized for exoskeleton control in a variety of pathologies, including those arising at the level of the nervous system as well as the muscle itself. For example, muscle-based pathologies can include conditions ranging from trauma, muscular dystrophy, inflammatory myopathies, and myasthenia gravis. Despite the profound muscular damage and weakness that can result in these conditions[1–3], the majority have functioning lower motor neurons that would facilitate MC-RPNI reinnervation and detection of motor intent. For those conditions that result in widespread muscle disease (muscular dystrophy, etc.), it is certainly possible that the free muscle graft component could be affected, thus limiting the amplification potential. However, given that detection of even a single motor unit (10–400 μV)[59] can provide amplification of peripheral nerve signals, it is reasonable to assume the MC-RPNI would contain enough motor units within its smaller, defined area to facilitate exoskeleton control in this population. A significant limitation of the construct, however, is in those pathologies that result in significantly reduced upper and/or lower motor neurons, such as in stroke, spinal cord injury, spinal muscle atrophy (SMA), and amyotrophic lateral sclerosis (ALS). Without a suitable peripheral nerve fiber population to reinnervate the MC-RPNI, it cannot regenerate and provide signal amplification, leading to construct failure. Experiments are being performed to determine the minimum population of functional peripheral nerve fibers required for adequate MC-RPNI function.

The MC-RPNIs predecessor, the RPNI, has shown immeasurable success with accurate control of powered prosthetics in human subjects through the amplification and recording of signals generated from transected peripheral nerves. Most notably, it is able to do so on the scale of months to years without reoperation or recalibration of the prosthetic device. Common complaints with current methods of human-machine interfacing for exoskeleton control center on signal contamination from cross-talk and the need for frequent recalibration in EMG-reliant methods[26–28], and peripheral nerve interface instability over time necessitating secondary surgeries[37,39,44]. The MC-RPNI, however, is able to avoid these complications given its biologic makeup as well as strategic placement capabilities. It is imperative to establish a thorough understanding of this construct in order to pave the way for use in human subjects and the eventual widespread utilization of accurate, reliable exoskeleton devices in those living with extremity weakness.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no disclosures.

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Figure 1

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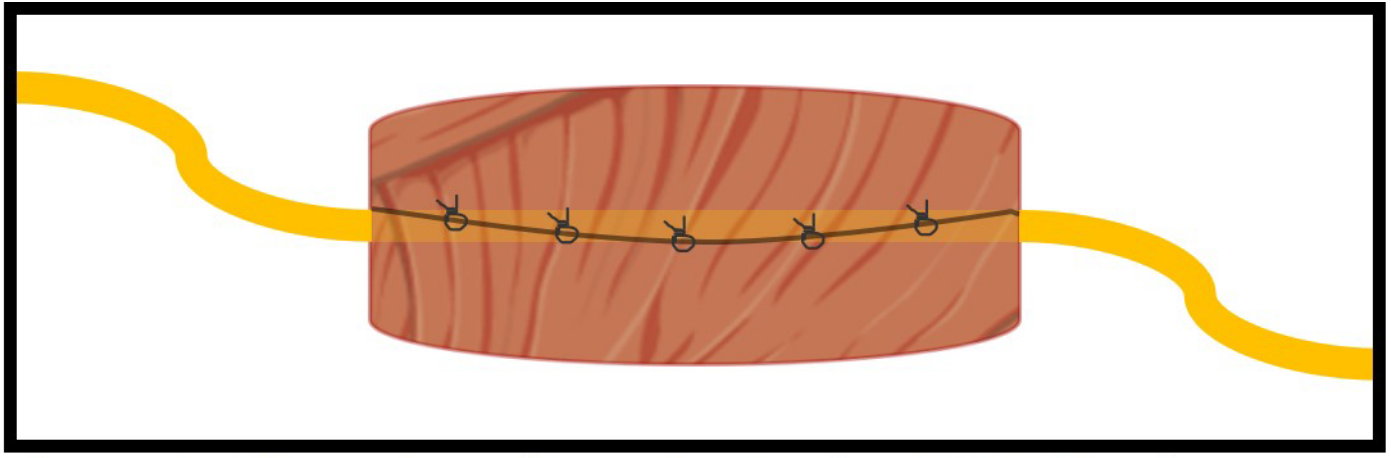

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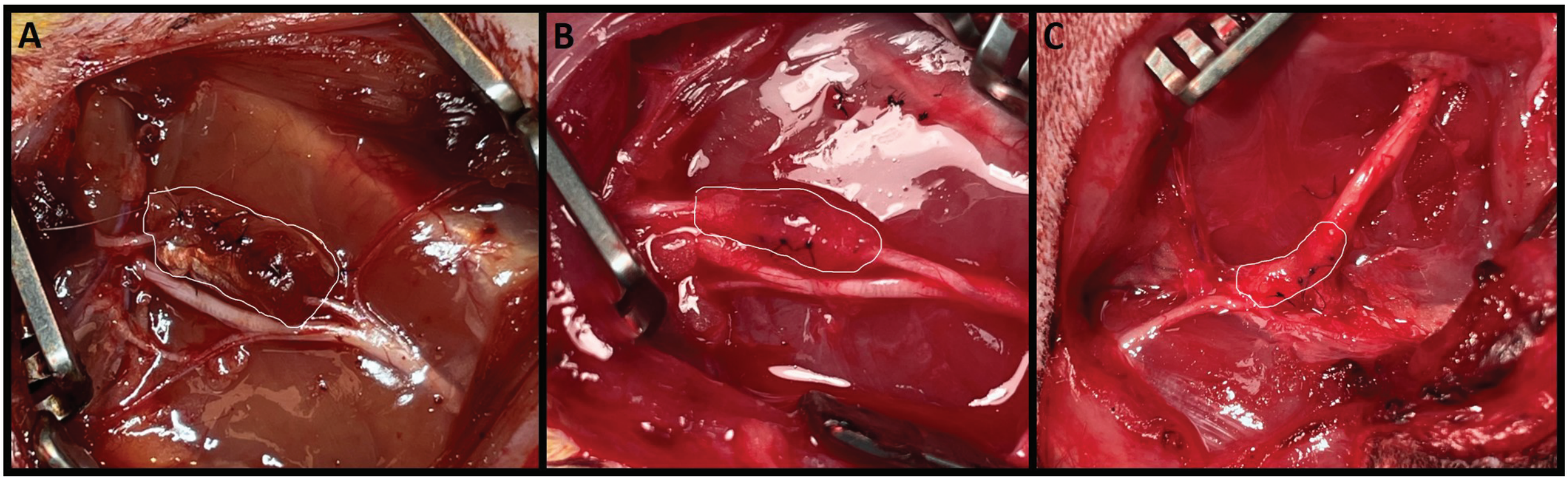

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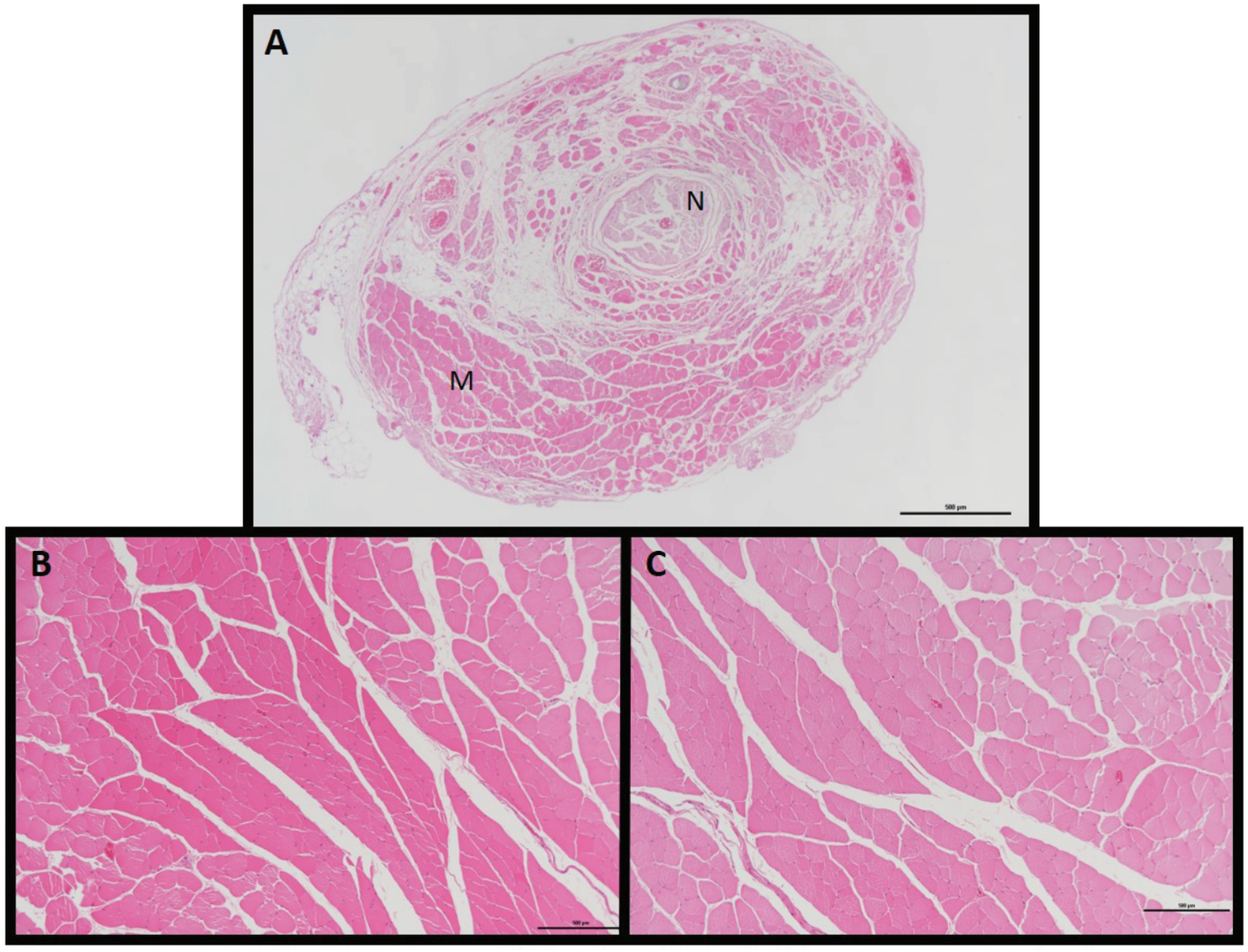

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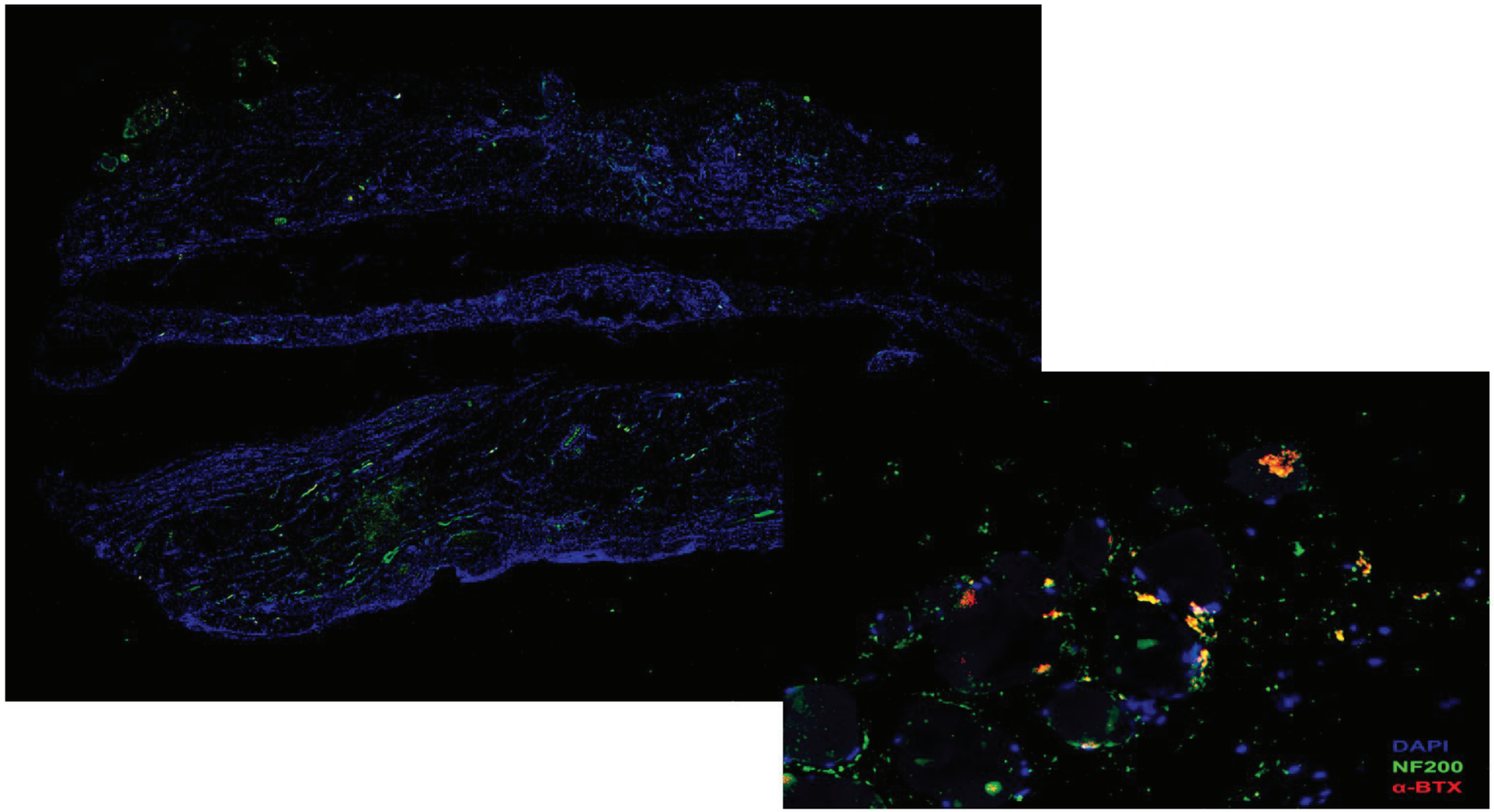

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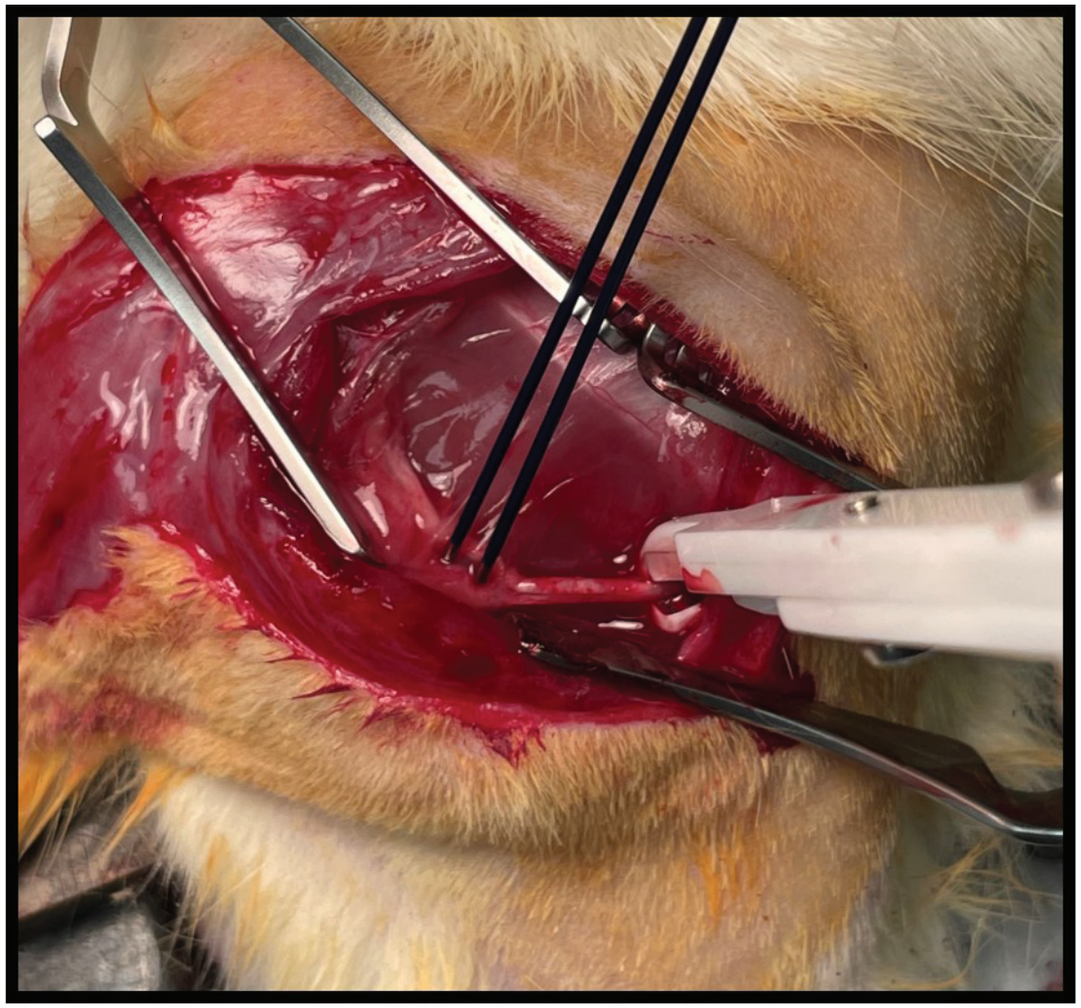

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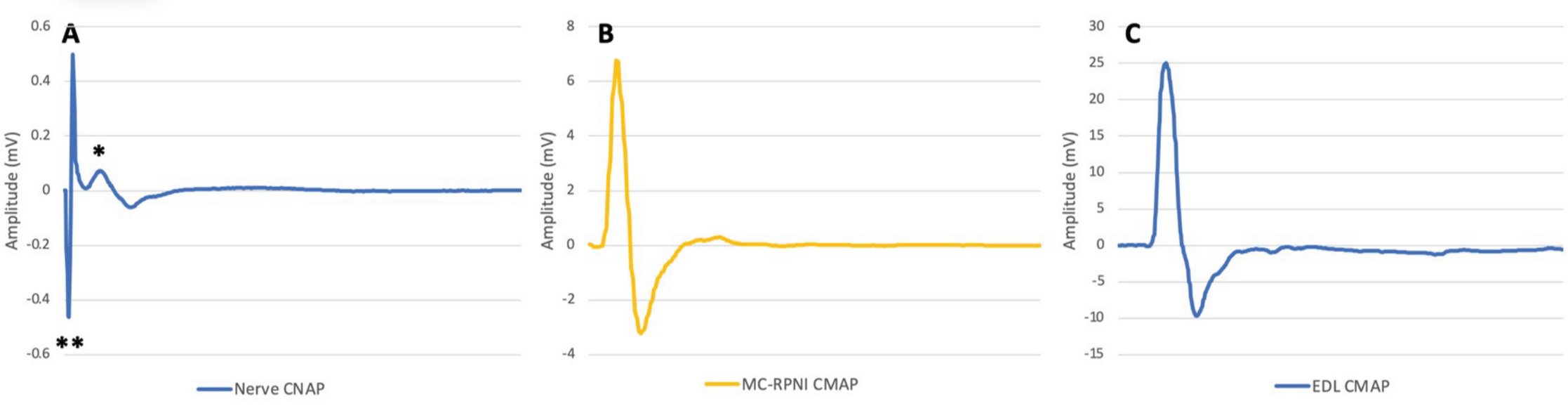

Video 1

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Video or Animated Figure

Video1.mov

Table 1

Rat ID	Rat Weight (g)	Nerve CNAP Amplitude (µV)	MC-RPNI CMAP Amplitude (mv)	Nerve Signal Amplification factor
1	421	123.3	1.4	11.35
2	368	65.6	1.6	24.39
3	390	110.7	4.5	40.65
4	482	217.2	3.61	16.62
5	417	144.6	1.39	9.61
6	417	156.1	3.4	21.78
7	381	82	7.2	87.8
8	393	87.9	2.3	26.17
9	378	87.8	4.2	47.84
10	459	n/a	5.3	n/a
11	380	n/a	2.1	n/a
12	415	n/a	2.4	n/a

Latency (ms)
0.8
1.05
1.45
0.95
0.9
0.95
0.9
1.15
1
1.55
0.75
1

Table 2

Rat ID	Maximal Twitch (mN)	V	Maximal Tetany (mN)	V
1	927.13	3	2668.29	3
2	768.22	3.5	2677.85	3.5
3	646.99	3	2164.84	3
4	863.62	3.5	3109.67	3.5
5	774.48	1.5	2723.24	2
6	558.19	4	1930.22	4
7	753.97	1	2605.64	1
8	768.38	2	2897.08	2
9	559.9	1.5	1984.17	1.5
10	600.6	5.5	2416.09	5.5
11	770.27	5.5	2496.89	5.5
12	672.22	2.5	1740.04	2.5

Hz	Lo (mm)
80	30.64
80	31.15
80	28.36
150	31.07
80	28.83
120	29.46
100	31.13
100	31.86
100	31.11
80	32.51
80	31.89
50	31.34

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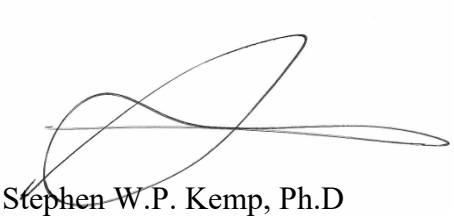
November 12, 2021

Vidhya Iyer, PhD
Science Editor
JoVE

Dear Dr. Iyer

Thank you very much for your letter regarding the review of our submission to *JoVE* entitled, "**The Muscle Cuff Regenerative Peripheral Nerve Interface (MC-RPNI): A Surgical Method to Facilitate Amplification of Intact Peripheral Nerve Signals**" (Manuscript #: JoVE63222). We are also 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 track changes). We include not only our response to each comment, but the specific portion of the manuscript at which any changes were made. We have set out a point-by-point list that highlights the changes that we have made with respect to each reviewer's comments in our Response to Reviewers. We look forward to the review of the revised manuscript, and if you have any further questions or require any additional clarification, please don't hesitate to contact me.

Sincerely yours,



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Response to Reviewers

Editorial comments:

Changes to be made by the Author(s):

1. 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

2. Please avoid abbreviations in the title and shorten the title to something along the lines of "The Muscle Cuff Regenerative Peripheral Nerve Interface for the amplification of intact peripheral nerve signals."

-We have removed the abbreviation and have changed the title to the one suggested by the Editor.

3. Please provide an email address for every author.

4. Please keep the summary within 50 words.

-Summary was shortened to 48 words

5. Please revise the following lines to avoid previously published work: lines 150-153, 197-199, 200-201, 202-204, 254-255, 295-297.

-Lines were all changed to avoid previously published work; however, lines 197-204 all describe how we follow institutional protocol for euthanasia, rat strain selection, etc and as these steps are identical to previous work, it cannot be altered without causing misrepresentation of the experiment.

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

7. JoVE policy states that the video narrative is objective. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item or laboratory. To this end, we ask that you please reduce the number of instances of " Our laboratory has developed/we developed the RPNI " within your text.

-All mention of our laboratory developing these constructs was removed

8. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

-All instances were removed

9. Please ensure that all text in the protocol section is written in the imperative tense; any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

-Text was altered as needed

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. The video must present the main message of your paper clearly, as indicated by your title.

11. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions,

numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
i) Line 208: please specify how to determine that anesthetization is adequate.
ii) Line 210: please specify the injection-needle gauge
iii) Line 221: Please specify the type of temperature probe.
iv) Lines 316, 334, 340, 345: "[**MCRPNI CITE HERE]" - please cite the appropriate references.

-Requested items were specified/changed.

12. Please submit individual figures (PDF, TIF, or JPEG) and tables (.xls) without associated legends in your editorial manager account.
13. Figure 3: Please increase the font size of scale for legibility.

-Increased font size was added

14. Figure 4: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure or its legend.

-Scale bar added

15. Please define the abbreviation Lo in the last column in Table 2.

-Defined in legend

16. As we are a methods journal, please ensure the Discussion explicitly covers any limitations of the technique and indicates when this technique would be useful (current and future applications).

-Have made some elaborations when addressing other reviewers comments in the Discussion

17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
18. Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

-Added additional materials including some requested by Reviewer #2

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
this work challenges to the problem of having a clear signal of motor intent for communication between neuromuscular system of the patient and an exoskeleton. This approach works through a muscle graft that envelops the nerve and its activation amplifies the nerve signal.

Major Concerns:
This work tackles a really important problem of peripheral nerve interfaces. Therefore I consider it very interesting and useful.

Despite this a have some concerns:

1) I would like to better understand which are for the Authors the clinical settings in which an exoskeleton with this technology could be used. As it needs the graft muscle activation, I imagine it cannot be used in neuromuscular disorders in which the muscles cannot contract. Therefore, I would like the Author to state for which clinical problems it should be used (spinal cord injuries? Strokes? Traumas? Muscular dystrophies? Neuropathies?...).
-An additional paragraph was added towards the end of the discussion to provide additional clinical context for the function of this construct. A significant limitation of the MC-RPNI is when there is complete absence of lower motor neurons, as in severe stroke and complete spinal cord injury. A complete lack of functional lower motor neurons would prevent MC-RPNI reinnervation and thus signal amplification. In the majority of other neuronal diseases, some degree of lower motor neurons are present which would enable MC-RPNI function, albeit at lower degrees of signal amplification (studies are currently underway to better define the required minimum). Similarly in muscular dystrophy, motor units are functional although reduced in number and weakened/damaged, similarly producing lower signal amplification. As for neuropathies, although we have not explicitly studied the MC-RPNI in that scenario, it is assumed that given more proximal placement on the nerve and reliance on motor neuron reinnervation, neuropathies would be less likely to affect MC-RPNI function except in the most extreme of cases.

2) In which way the Authors imagines the recording of the signal in an in-vivo setting and the following communication with the exoskeleton (Wireless I imagine; in this case maybe wirelessly rechargeable sensors should be used)?
-At present in rats we utilize bipolar electrodes fabricated in our laboratory that are hooked up to either an implanted headcap or routed and buried at the level of the scapula. The wires that are buried are left in until time of eval when they are surgically explanted and secured externally to the rat. In human studies with the MC-RPNIs predecessor, the RPNI, we use implanted bipolar electrodes routed through the skin. We have recently received approval for implanted wireless electrodes that will be utilized in future human experiments. Specifically for the MC-RPNI, we anticipate utilizing wireless electrode systems in eventual human experiments. At present in rats, the wireless devices available are too expensive and not small enough in scale, unfortunately.

3) Do the Authors think that this technology could be used even for inside body artificial muscles in the future or just for exoskeletons? I imagine this technology useful for detecting the nerve activation and then communicating with a sensor implanted in the natural muscle for triggering activation or entirely communicating with an artificial actuator.
-This is an incredibly interesting point, and we don't see why this couldn't be an eventual application given that the neuromuscular interaction is the same in both cases. We have utilized the RPNI with muscle grown in vitro with success, and it is reasonable to assume this word work with the MC-RPNI as well. We have also discussed a variety of other applications, including its potential use for chronic pain, CRPS, and SCI-related muscle spasm. It will be interesting to see how its use will continue to develop over time.

Reviewer #2:
The article describes a protocol to create a muscle cuff regenerative peripheral nerve interface. The methods are clearly described, and a good fit for a JoVE article. I have only minor suggestions for improvements.

_In terms of flow, I would have liked to see a description of standard methods to validate the interface included in the protocol, rather than scattered in the representative results section. Likewise, the length of time required for the construct to be mature and functional should be in the protocol rather than the results.
-The length of time for maturation was added to the protocol. The primary focus of this manuscript is to detail the fabrication of the construct with data from our prior experiments to support its viability. Detailing the validation of this construct is an additional protocol that lies outside of the scope of this manuscript, but it is an excellent point about more detail being needed. We would be happy to write an additional paper detailing all of our validation methods in an additional JoVE manuscript.
_In the Table of Materials, little detail is provided about the surgical instruments. The protocol involves removing a window of epineurium, which sounds like a very delicate procedure. Readers may appreciate more details about the appropriate tools for this task.
-Details regarding the tools utilized to handle the nerve and perform the window were provided in the table of materials. The remainder of tools utilized in this protocol are general surgical tools that don't require special consideration/purchasing.
_In figure 6, multiplying the amplitude of nerve recording by 10 is potentially confusing. It would be preferable to have separate axes if the range of amplitudes is too large to be visualized on a single axis.
-We have altered this figure to hopefully lessen confusion

_Some discussion might be helpful of whether the technique would apply in any cases where there is some impairment of the lower motor neuron. The authors could be a little more specific about what would and would not be appropriate indications for this approach.
-The particular injury/ratios of affected lower motor neurons that are required for MC-RPNI function is currently being investigated. It is our assumption that if some functional lower motor neurons are present, they will to some degree reinnervate the construct and thus provide amplification. This would be beneficial over utilization of electrodes in distal muscle tissue and the functioning/reinnervated motor units would have a higher density within the smaller surface area of the MC-RPNI. We have added an additional paragraph towards the end of the discussion to provide some clinical scenarios for its use as well as its limitations.

Minor:
_There are some missing references through the text ("[**MCRPNI CITE HERE]").
-Apologize, the incorrect final version of the document was submitted that omitted the finalized citation (we had been waiting on the final publication date for that particular citation). This is corrected in the current version.
_l. 331: I suggest clarifying here that the EDL being harvested is contralateral, otherwise it is confusing since the ipsilateral EDL is the muscle being used for testing.
-Added the above clarification
_Table 2: undefined abbreviation Lo.
-Lo is a standardized term utilized in muscle physiology assessments, its definition was added to the table legend to provide necessary definition/clarification.
_The authors may wish to note that the video provided includes audio of someone saying "we'll trim this for the final video"…

-Apologize, the powerpoint version of the figures that was sent with peer review did not correctly strip the audio. The final .mov for the figures has the audio correctly stripped

_In the abstract, the term "notoriety" does not seem to match the intended meaning, given its negative connotation.

-Appreciate this mention as it does cause ambiguity/confusion, changed wording to better fit intended meaning.

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