REVIEW

On the development of optical peripheral nerve interfaces

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Funding: *This work was funded in part by the University of Colorado Medical Scientist Training Program and funds from the NIH SPARC initiative administered through the Office of the Director: 1OT2OD023852-01.*

Abstract

Limb loss and spinal cord injury are two debilitating conditions that continue to grow in prevalence. Prosthetic limbs and limb reanimation present two ways of providing affected individuals with means to interact in the world. These techniques are both dependent on a robust interface with the peripheral nerve. Current methods for interfacing with the peripheral nerve tend to suffer from low specificity, high latency and insufficient robustness for a chronic implant. An optical peripheral nerve interface may solve some of these problems by decreasing invasiveness and providing single axon specificity. In order to implement such an interface three elements are required: (1) a transducer capable of translating light into a neural stimulus or translating neural activity into changes in fluorescence, (2) a means for delivering said transducer and (3) a microscope for providing the stimulus light and detecting the fluorescence change. There are continued improvements in both genetically encoded calcium and voltage indicators as well as new optogenetic actuators for stimulation. Similarly, improvements in specificity of viral vectors continue to improve expression in the axons of the peripheral nerve. Our work has recently shown that it is possible to virally transduce axons of the peripheral nerve for recording from small fibers. The improvements of these components make an optical peripheral nerve interface a rapidly approaching alternative to current methods.

Key Words: *peripheral nerve interfaces; optogenetics; optical neural interface; optical peripheral nerve interface; GCaMP; ArcLight; adenoassociated viral vector; lentiviral vectors; viral vectors; implantable microscopy*

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doi: *10.4103/1673-5374.245461*

Received: *August 11, 2018*
Accepted: *September 19, 2018*

Introduction

Over 1.6 million Americans suffer from the loss of a limb (Ziegler-Graham et al., 2008), reducing their ability to function in society, and impacting their quality of life significantly. Approximately 130,000 new amputations occur each year (Dillingham et al., 2002), and by 2050, an estimated 3.6 million Americans will have undergone an amputation (Ziegler-Graham et al., 2008). While strides have been made in decreasing the numbers of amputations due to trauma and malignancy, little improvement has been made in decreasing the extent of congenital limb deficiency, and amputations due to vascular disease have increased as the population ages and the prevalence of diseases such as diabetes continues to rise (Dillingham et al., 2002; Ziegler-Graham et al., 2008). One study in France of both lower and upper limb amputees has shown a health related quality of life similar to that of people awaiting heart-lung transplants or with a diagnosis of a degenerative neurological condition, reporting significant physical disability and pain (Demet et al., 2003). Loss of a limb can impact an individuals ability to perform many basic activities of daily life such as driving, eating and tying a shoe, being able to cut food in meal preparation, or dry dishes. Additionally, one's ability to interact with technology such as computers, which may require significant amounts of typing, a task requiring dextrous manipulation, is impaired. While some of these tasks can be performed either with the other hand (in the case of unilateral upper limb amputees) or with the use of assistive technologies (such as automatic transmission vehicles or text to speech services) or by assistants, none of these solutions is a comprehensive solution for returning these individuals to their original abilities. Furthermore, some of these workarounds can have serious drawbacks, such as cumulative trauma disorder, also known as worn-limb syndrome, which is injury resulting from excessive use of a remaining limb (McFarland et al., 2010), which occurs more frequently in people who do not use a prosthesis. Other interventions may rely on resources that are not readily available or are too expensive. Prosthetic limbs have been produced with the goal of restoring function to these people and increasing their ability to interact in general ways with the ever changing human environment. While strides have been made in the development of prosthetic legs, enabling many people to return to walking, running and sports, due to the nature of the upper limb and the hand, success in developing multifunctional upper limb prostheses has been limited, leading to rejection and nonuse.

Additionally another 257,000–358,000 Americans have suffered a spinal cord injury (SCI) (National Spinal Cord Injury Statistical Center, 2018). Like persons suffering from limb loss, they also experience reduced life satisfaction (Fuhrer et al., 1992), have shoulder related overuse injuries that may impair their ability to perform activities of daily living and impact mobility (Ballinger et al., 2000), as well as increase their cardiovascular strain without increasing their level of physical fitness during such activities (Janssen et al., 1994). Furthermore, symptoms related to SCI also impair social integration (Jensen et al., 2007). Treatment of SCI is rarely curative for those with complete lesions, although

promising techniques for reestablishing function are currently being developed [for review see Ramer et al. (2014)], and the benefit of limb reanimation through surgical techniques has been established (Bernuz et al., 2011; Johanson, 2016). Limb reanimation can be expanded beyond surgical techniques to ones using a set of interfaces, one in the brain and another at the peripheral nerve, serving as a bypass around the damaged areas of the spinal cord. Such a bypass is made possible by the continued integrity of the downstream circuits – the lower motor neuron still innervates the muscle and can cause it to contract. These intact circuits make it possible to reanimate the limb through stimulation of the peripheral nerve, and similarly, through readout of sensory afferents; thus a closed loop system for neuroprosthetic limb reanimation can be developed.

In this review, peripheral nerve interfaces for the closed loop control of prosthetic limbs and reanimated natural limbs will be discussed. First, electrode based methods methods for interfacing with the peripheral nerve will be covered, from the surface electroneurogram to the cuff electrodes and intrafascicular electrodes. Second, we will show potential provided through an optogenetic peripheral nerve interface, and the components thereof. Third, we will discuss vectors for delivery of optogenetic proteins to the peripheral nerve in adult animals. Ultimately, we aim to show that an optogenetic peripheral nerve interface is a viable method for interrogation and stimulation of the peripheral nerve and could be used to provide a control interface for prosthetic limbs and for limb reanimation (**Figure 1**).

Interfaces with the Peripheral Nerve

The standard of care for prosthetic limb control is the surface electromyogram. In these systems, electrical activity produced during muscle activity is recorded and use to control the actions of a prosthetic limb. These systems often suffer from high latency, low specificity, and low numbers of control sites. Some of these problems have been addressed through a variety of pattern recognition algorithms, but they fail to provide sufficient control of a prosthesis to enable dextrous manipulation and other tasks that require simultaneous activation of many degrees of freedom (Jiang and Farina, 2014). When asked what changes prosthesis users would like to see, and may bring them back to prosthesis use, they have suggested things like improved dexterity, independent movement of fingers, sensory feedback, addition of fine motor skills, intuitive control, reliable precision, and additional degrees of freedom (Atkins et al., 1996; Kyberd et al., 1998, 2007; Biddiss and Chau, 2007; Biddiss et al., 2007). Many of these features will require an interface capable of both interrogation and stimulation, and with greater specificity than what is currently available with surface electromyogram.

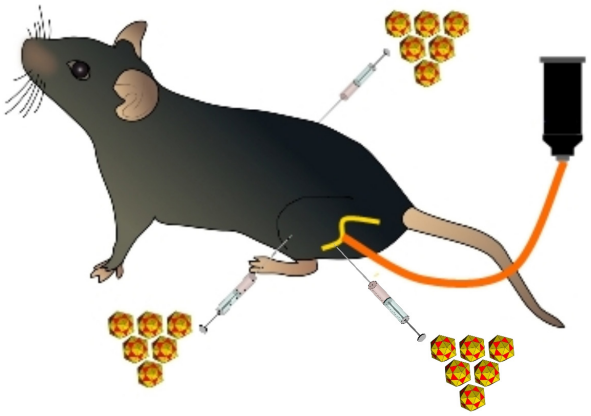

Figure 1 Elements of an optical peripheral nerve interface.
These elements are: (1) A vector, carrying an optogenetic transgene (2) injected into one of several sites, intramuscularly, intranerve, intrathecal and into the dorsal root ganglion being most common for targeted expression in the peripheral nerve. (3) A fiber optic or implanted microscope to interrogate and/or stimulate axons expressing optogenetic proteins.

The surface electroneurogram could provide an alternative means of interrogating motor intention, or sensory information, but it is only capable of interrogating the largest 20% of myelinated fibers, thereby ignoring information from smaller fibers such as nociceptors and thermoreceptors, and has only been deployed clinically as a part of the nerve conduction study wherein a large current is applied to generate a signal to read out (Mallik, 2005).

Interfacing directly with the peripheral nerve presents several advantages over both surface electromyogram and implantable EMG. First, because the interface is directly with the nerve, it is theoretically possible to shorten latency, no longer requiring the activation of a muscle before detecting a signal. Second, the potential for fatigue is reduced by no longer requiring the activation of muscles. Third, the interface itself can readily serve to read-in signals, providing sensory information from the prosthesis to the user. Fourth, because the interface is with nerve, complex surgeries such as those required for either targeted muscle reinnervation or regenerative peripheral nerve interfaces can be avoided. In general, the majority of these peripheral nerve interfaces fall into three categories (in increasing invasiveness): extraneural electrodes (best represented by cuff electrodes, but also including helical and button type), intraneural (the most widely used being intrafascicular) electrodes and sieve (or regenerative) electrodes [reviewed in Navarro et al. (2005), Grill et al. (2009), and Kung et al. (2013)].

Cuff electrodes consist of a cylindrical insulative sheath containing a set of contacts on the interior surface that fully encircles the epineurium (the exterior connective tissue of the nerve). They are often made of a flexible substrate such as silicone or polyimide. Stimulation can be directed to certain regions of the nerve using differing stimulus pulses and steering anodes (Navarro et al., 2001). Recording is often dominated by large fibers or those near the cuff. Snug fitting cuffs provide better stimulation characteristics and higher signal-to-noise ratio for recording, but lead to changes in nerve morphology and loss of larger nerve fibers. Despite these concerns, long term studies have been conducted in animals, and cuff electrodes have also been used to control foot drop in humans and to detect object slippage in interventions for persons with tetraplegia [reviewed in Navarro

et al. (2005) and Grill et al. (2009)]. In order to improve selectivity and better access deeper nerve fibers, the flat interface nerve electrode was developed (Tyler and Durand, 2002), which forces the nerve into a more elliptical shape, increasing surface area and the number of fibers accessible to the electrode. These electrodes can cause damage if high degrees of reshaping force are used, leading to scarring, axonal damage and demyelination (Leventhal et al., 2006).

There are two major types of intrafascicular electrodes – longitudinally implanted intrafascicular electrodes and transversely implanted intrafascicular electrodes. Longitudinally implanted intrafascicular electrodes penetrate fascicles in the longitudinal direction, and their active sites lie parallel to the axons. They have been implanted for 6 months in animals, without damaging the nerve (Lawrence et al., 2002). Longitudinally implanted intrafascicular electrodes provide good selectivity for stimulation and recording from multiple fibers, but it is challenging to implant multiple electrodes, limiting their effectiveness in control of a prosthetic limb (Navarro et al., 2005). Longitudinally implanted intrafascicular electrodes have, nonetheless, been used to control prosthetic hands (Rossini et al., 2010). Transversely implanted intrafascicular electrodes are best known by the multielectrode arrays, which are arrays of needle electrodes that penetrate the nerve in a transverse direction. They are epitomized by the University of Michigan Array (Hoogerwerf and Wise, 1994) and the University of Utah Slant Array, which uses a grid of electrodes of varying length to achieve contact with multiple fascicles in the transverse direction of the nerve (Branner et al., 2004). However, in both animal studies and human studies, there are problems with long-term recording and stability of the interface (Branner and Normann, 2000). After 96 days in one study (Warwick et al., 2003), only three of an initial twenty functional electrodes were still functional. Such electrode arrays have had some success in recording from the dorsal root ganglion (Aoyagi et al., 2003; Weber et al., 2007).

Regenerative, or sieve electrodes attempt to interface with a large number of nerve fibers, by encouraging the regrowth of transected fibers through holes, each equipped to serve as an electrode. It is difficult to get the axons to grow through the holes, and once they do, they suffer from damaging compressive forces. Furthermore, axon numbers diminish over time due to trauma and scarring (Lago et al., 2005). Despite these drawbacks, stimulation and recording has been successfully implemented with them (Navarro et al., 1998).

The aforementioned methods also provide a means for stimulation for limb reanimation in the context of SCI. Functional electrical stimulation can activate distal muscles through the use of electrical stimulation either to muscles through surface electrodes (Rohm et al., 2013) or implanted electrodes (Kilgore et al., 2008) or through implanted nerve electrodes (Memberg et al., 2014) (for review see Loeb and Davoodi (2005)). These methods do not provide for sensory feedback, and depending upon the implementation used, vary in degrees of invasiveness (*e.g.* Rohm et al. (2013) have an implementation using electroencelogram and surface electrodes for stimulation, while other implementations utilize implanted electrodes, which could provide better selectivity and lower latency). While the end purpose is different (*i.e.*, stimulation instead of interrogation), the invasive peripheral nerve interfaces have many of the problems that affect recording electrodes, in addition to stimulation specific ones. For example, the recruitment of larger motor fibers before smaller ones increasing fatigue or special considerations that must be taken to prevent nerve damage from stimulating currents [for review see Navarro et al. (2005)].

Although there has been a proliferation of methods for interfacing with the peripheral nerve, there are many tradeoffs to be made between selectivity and damage or selectivity and robustness that make it difficult to justify the added risks with implanting a device to read out from the nerve directly. A less invasive method using rapidly developing optical technologies may provide a solution offering reduced invasiveness and damage alongside higher selectivity and reduced crosstalk.

Optical Neural Interfaces

Optogenetics and optical reporter proteins

The rapidly growing field of optically sensitive proteins provides a new means for interfacing with the nervous system. The discovery of optically sensitive ion channels, best exemplified by Channelrhodopsin-2 (ChR2) (Nagel et al., 2003), has opened new avenues for the stimulation of neural circuits including in peripheral nerves (Sharp and Fromherz, 2011; Towne et al., 2013) [for review see Montgomery et al. (2016)]. Some of these optogenetic proteins can be used for stimulation, and others, such as halorhodopsins (Gradinaru et al., 2008) and archaerhodopsins (El-Gaby et al., 2016) can be used for inhibition. ChR2 is a cation channel for sodium, calcium, potassium and hydrogen ions, activated by 460 nm wavelength light; Halorhodopsin is a chloride pump (Ordaz et al., 2017). New optogenetic proteins have been created with different excitation spectra from the blue-shifted ChR2, such as C1V1 (Prigge et al., 2012), a red-shifted variant, potentially allowing for coexpression with a spectrally non-overlapping reporter; other proteins, providing an extensive color palette for stimulation, continue to be developed – including Chrimson and Jaws [reviewed in Ordaz et al. (2017)]. These actuators have been deployed in the peripheral nerve, allowing for control of a rat hindlimb expressing ChR2 and stimulated using an implantable optical cuff, indicating its potential use in lieu of functional electrical stimulation for control of a natural limb (Towne et al., 2013). Optogenetic stimulation has also been used to restore diaphragm and bladder function in animal models of SCI (reviewed in Mallory et al. (2015)). Furthermore, optogenetic stimulation can be used to enhance motor skills (Gibson et al., 2014), and in the direct treatment of SCI, optogenetic stimulation has been shown to rescue diaphragmatic activity after C2 spinal cord hemisection (Alilain et al., 2008).

While optogenetic actuators show promise for stimulating peripheral nerves, and as such, returning information to the user about the state of a prosthesis, or to contract

muscles, another class of proteins are required for read-out of motor intent and sensory information from an intact limb. Bioluminescent proteins might be a method for read-out, but have yet to develop sufficient temporal and spatial resolution for this purpose (Lin et al., 2010). Thus, optical reporter proteins are likely the best means forward, at least in the present, for an optical neural interface. Such optical reporter proteins (sometimes referred to as optogenetic reporters) include reporters of voltage, pH (Miesenböck et al., 1998; Mahon, 2011), and calcium, which could all be used to varying effect to report neural activity [for review see Mutoh et al. (2011, 2012), Looger and Griesbeck (2012), Lin and Schnitzer (2016)].

Genetically encoded voltage indicators

Perhaps the most intuitive way of achieving read-out from an axon would be the use of a voltage sensitive protein that would change its fluorescence in response to voltage shifts, such as during an action potential [for review see Knöpfel et al. (2015)]. Numerous fluorescent proteins have been developed to respond to membrane voltage changes, including the (1) green sensors: ArcLight (Jin et al., 2012), ArcLightning (Treger et al., 2015), accelerated sensor of action potentials 1 (ASAP1) (St-Pierre et al., 2014), Bongwoori (Piao et al., 2015), (2) the red sensors fluorescent indicator for voltage imaging red (FlicR) (Abdelfattah et al., 2016), QuasAr (quality superior to Arch) (Hochbaum et al., 2014), and the VSD-FR (voltage sensitive domain-fusion red) series (Kost et al., 2017) and (4) the VSFP (voltage sensitive fluorescent protein) series (Perron, 2009) [for review see Miesenböck and Kevrekidis (2005), Mutoh et al. (2011)] which also offers a number of spectral variants (Perron et al., 2009). There also exist a series of Förester resonance energy transfer voltage indicators including VSFP-Butterfly (Mutoh et al., 2009) and the Mac voltage sensors (Gong et al., 2014). These sensors have been employed both *in vitro* and *in vivo* (Gong et al., 2014, 2015; Carandini et al., 2015). These sensors fall into two broad categories, those that are based on voltage sensing domains of ion channels (*e.g.*, ArcLight) and those that are based on archaerhodopsin (*e.g.*, QuasAr). The former generally decrease in fluorescence with electrical depolarization, often by quenching an attached fluorophore, while the latter generally increase in fluorescence following depolarization through the retinal molecule (Knöpfel et al., 2015). Archaerhodospin sensors, despite being faster, suffer from requiring higher intensity illumination than their fluorescent protein based counterparts (Hochbaum et al., 2014). Both of these types of proteins need to be membrane bound in order for successful operation, and they offer a wide variety of speed and sensitivity options.

Because of the low change in fluorescence upon depolarization, the limited area for expression of these proteins (*i.e.*, the membrane) and the kinetics of the action potential, their deployment *in vivo* has been limited [for a more detailed review of these problems see (Kulkarni and Miller, 2017)]. Ongoing efforts to improve these sensors have been undertaken, by either improving kinetics and brightness to better resolve action potentials (*e.g.*, with the Bongwoori variant Bongwoori-R3 (Lee et al., 2017b)), or to invert the negative fluorescence-voltage relationship (producing Marina from ArcLight (Platisa et al., 2017)). Inverting the fluorescence-voltage relationship is particularly important as it improves the signal-to-noise ratio of sensors as neurons are more often in a hyperporalized state than in a depolarized one (Platisa et al., 2017). Further developments have also been made with ASAP1, with ASAP-Y, increasing the magnitude of its $\Delta F/F_0$ (–9.49 ± 0.77% *vs.* –7.45 ± 0.80%) and improving its off-time kinetics (τoff 4.63 ± 0.44 ms *vs.* 6.52 ± 0.51 ms) through the elimination of an intermediate state (Lee and Bezanilla, 2017). New techniques, such as robotic screening, have been employed to screen new voltage indicators, yielding Archon1 and Archon2, red-shifted Arch-based sensors capable of $\Delta F/F_0$ of 81 ± 8% and 20 ± 2% in response to 100 mV depolarizations in human embryonic kidney cells, respectively (Piatkevich et al., 2018).

Recently, ArcLight has been used *in vivo* to monitor activity in the barrel cortex of mice; however, in that setting post-hoc subtraction of hemodynamic noise was required to recover the signal, a foreboding sign for deployment of ArcLight in the dynamic setting of a peripheral nerve interface (Borden et al., 2017).

While there have been significant recent developments in genetically encoded voltage indicators, the signal to noise ratio lags substantially behind that of the genetically encoded calcium indicators, and given recent developments in kinetics, calcium indicators remain the sensor of choice for reporting neural activity, especially in the *in vivo* setting.

Genetically encoded calcium indicators

Calcium influx in response to action potentials is well established in neurons of the central nervous system (Lev-Ram and Grinvald, 1987; Schiller et al., 1995; Helmchen et al., 1996; Zhang, 2006) in both somas and axons of motor and sensory neurons innervating the periphery (Barrett and Barret, 1976; Wächtler et al., 1998; Mayer et al., 1999; Jackson et al., 2001; Zhang et al., 2010; Zhang and David, 2016; Fontaine et al., 2017) As such, it may be possible to image calcium transients as a reporter of neural activity. In order to accomplish this goal, a group of engineered calcium responsive fluorescent proteins has been developed. Perhaps the best known of these is the GCaMP family (Nakai et al., 2001; Muto et al., 2011; Akerboom et al., 2012; Ohkura et al., 2012; Chen et al., 2013; Sun et al., 2013; Badura et al., 2014; Helassa et al., 2015, 2016) of sensor. These sensors utilize the binding of calcium ions to calmodulin (CaM) resulting in an interaction with a target peptide (*e.g.*, M13), which subsequently causes a conformational change in a circularly permutated green fluorescent protein, restoring its fluorescent state. Other variants, such as GCaMP6s (GCaMP6-slow), are capable of ~5-fold fluorescence changes in response to 10 action potentials (Chen et al., 2013). There also exist red-shifted calcium sensors, such as RCaMP (Akerboom et al., 2013) and RGECO (Zhao et al., 2011), but they have yet to accomplish the same kinetics and signal strength of the

latest GCaMP sensors (Dana et al., 2016). Genetically encoded calcium indicators have been deployed *in vitro* and *in vivo* (Muto et al., 2011; Chen et al., 2012; Emery et al., 2016; Wang et al., 2016) and alongside optogenetic actuators (Guo et al., 2009; Tian et al., 2009).

Recent developments to these sensors include GCaMP6f$_u$ (GCaMP6f-ultrafast), which boasts a $t_{1/2, on}$ 9 times faster (1.3 ± 0.02 ms) than GCaMP6f and a $t_{1/2, off}$ 22 times faster (2.8 ± 0.1 ms), making it competitive in speed with the faster genetically encoded voltage indicators, while retaining a 4.1-fold fluorescence change between the calcium bound and unbound states (Helassa et al., 2016)). GCaMP3$_{fast}$ is another high-speed calcium sensor, with an 8.8-fold fluorescence change between bound and unbound states and a $t_{1/2, on}$ of 2.2 ms and a $t_{1/2, off}$ of 0.9 ms (Helassa et al., 2015). These sensors may be able to outstrip the speed of the underlying calcium dynamic (Helmchen et al., 1996), which may become the limiting factor for resolution of neural activity with calcium sensors.

None of these sensors or optical proteins would be of much use in an optical neural interface without a means of generating long-term expression of them in peripheral nerves. While transgenic animals can be created and can stably express proteins such as GCaMP and ChR2, this would not be a solution for peripheral nerve interface. Viral and nonviral vectors provide a means of genetic delivery to even mature, nondividing cells such as a neurons.

Vectors

Transgenes, such as those expressing optogenetic proteins, require a means of delivery to cells and cell regions of interest – in this case, the axons of sensory and motor neurons of the peripheral nerve. A variety of non-viral methods have been employed for transgene expression *in vivo* [for review see (Montgomery et al., 1994; Kantor et al., 2014)]. Naked plasmid DNA injections have been successfully used for retrograde labeling of motor neurons in mice following intramuscular (IM) injection, albeit at a modest level (Morris et al., 2004). Immunogene nanoparticles (PEGylated polyethlenimine DNA carriers conjugated to antibodies against receptors expressed on cells of interest) have also been used to label motor neurons following intraperitoneal injections in neonatal mice (Rogers et al., 2014). While these methods may have reduced immunogenicity and reduced production costs compared to viral vectors, it is unclear if these methods would provide stable long term expression at high enough levels for use in a peripheral nerve interface. Because of these constraints, viral vectors remain the best method of ensuring sufficient, long lasting expression of transgenes for a peripheral nerve interface. Several different viruses have been employed to transfer genetic material to cells *in vivo*, including: adenovirus, lentivirus, herpes simplex, rabies, and adenoassociated virus [AAV; for review see Lentz et al. (2012) and Kantor et al. (2014)]. Adenoviral vectors have a large carrying capacity of ~30 kb [for a detailed review see Lee et al. (2017a)], but tend to cause substantial immune responses (Thaci et al., 2011), and require additional handling requirements (*i.e.*, Biosafety Level 2) (U.S. Department of Health and Human Services, National Institutes of Health (NIH), 2016). These drawbacks hurt their utility in the context of transgene delivery for an optical peripheral nerve interface. Rabiesvirus vectors, while capable of transsynaptic transport, tend to reduce cell viability, and are often only used for short term studies (Wickersham et al., 2007; Osakada et al., 2011). AAVs, lentivirus and herpes simplex virus have all been used to induce expression in peripheral nerves and will be discussed below.

Lentiviral vectors

Lentiviral vectors [see Parr-Brownlie et al. (2015) for review] are retrovirus based vectors capable of integrating into the host chromosome, enabling stable long term expression (Kantor et al., 2014). They are capable of transducing both dividing and non-dividing cells (*e.g.*, neurons). Integration can occur at random locations in the genome, including in coding regions of non-dividing cells (Bartholomae et al., 2011), which may present a problem if concern of insertional mutagenesis is warranted. Integration deficient lentiviral vectors which do not integrate into the host genome can provide long lasting expression of at least three months in post-mitotic cells (Apolonia et al., 2007), and have similar transduction efficiencies to integration competent vectors in motor neurons, neurons of the dorsal root ganglia (DRG) and interneurons (Peluffo et al., 2013). Lentiviral vectors are often pseudotyped using the vesicular stomatis virus glycoprotein (VSVg), which allows for wide tropism, including in glia and neurons (Jakobsson et al., 2003). Pseudotyping with rabies glycoprotein or a chimera of rabies and VSVg can allow for retrograde transport of viral particles. Lentiviral vectors boast a packaging size of 8-10kb and are approximately 100 nm in diameter, which limits their spread through extracellular space, either limiting unwanted expression or reducing strategies available for enhancing spread (*e.g.*, convection and mannitol for AAVs) (Parr-Brownlie et al., 2015). Lentiviral vectors, however, unfortunately require higher biosafety requirements (Biosafety Level 2) reducing their ease of use and safety profile (Pauwels et al., 2009).

In the context of spinal cord injury, lentiviral vectors have been used to express chondroitinase ABC, which degrades scar tissue that may form, blocking axonal regrowth (Jin et al., 2011; Wang et al., 2017). They have also been successfully used to target neurons of the DRG (Ogawa et al., 2014), including both large myelinated fibers and smaller unmyelinated fibers, such as nociceptors, following direct DRG injection (Yu et al., 2011). Retrograde transport to the DRG was also seen following sciatic nerve injection (Yang, 2012), a less invasive procedure than DRG injections, and to the trigeminal ganglion following injection into the temporomandibular joint (Kyrkanides et al., 2004). These studies demonstrate the ability of the lentiviral vector to transduce neurons of interest of the peripheral nerve. Thus, lentiviral vectors could be used to express optogenetic proteins in these sensory fibers, for read-in or read-out as part of a

bi-directional interface for control of a prosthetic limb or in the context of limb reanimation.

Herpes simplex viral vectors

Herpes simplex virus is an enveloped dsDNA virus, capable of carrying a payload up to 16.5 kb (Miyagawa et al., 2015), and has been used for viral delivery to DRG neurons following peripheral innoculation for short term expression (2–4 weeks) in sensory fibers (Goss et al., 2001; Chattopadhyay et al., 2003). Reinnoculation has been shown to reestablish transgene expression (Liu et al., 2004). Motor neurons may also be transduced through footpad or direct nerve injection (Palmer et al., 2000). Long-term expression of at least 6 months may be possible with the JΔNI6 vector, which also boasts reduced cytoxocity (Verlengia et al., 2017), a substantial problem that can result in significant neuron loss (O'Shea et al., 2018). While herpes simplex virus may prove a viable vector for transduction of peripheral nerve axons, it has not yet reached maturity as a means for stable, safe, long term transgene expression for this cell population.

AAVs

Of viral vectors, adenoassociated viral vectors may be the most promising, offering a combination of low biosafety requirements (Biosafety Level 1) (U.S. Department of Health and Human Services, National Institutes of Health (NIH), 2016), a diverse, selectable cell tropism, the ability to transduce nondividing cells, low immunogenicity, low tendency to integrate into the host genome, and long-term expression [for review see Burger et al. (2005)]. Because of the advantages of AAVs, their use in clinical trials (Ojala et al., 2015; Saraiva et al., 2016), and their ability to transduce a variety of tissues including neurons of the periphery, they will be explored below.

Wild-type AAV is a small (20 nm) parvovirus, enveloping a 4.7 kb single stranded DNA genome, encoding four replication proteins (*rep* genes), three capsid proteins (*cap*), and the assembly activating protein [reviewed in Kantor et al. (2014)]. It requires co-infection with a helper virus (such as adenovirus (Atchison et al., 1965) or herpes simplex (Buller et al., 1981)) in order to replicate; otherwise it can integrate into the host cell genome or can be retained in an episomal form (Schnepp et al., 2005). Long-term expression is possible post-AAV injection: eight years in dogs (Niemeyer et al., 2009), over six years in non-human primates (Rivera, 2005) and 1.5 years in mice (Xiao et al., 1996) (for review see Lentz et al. (2012)). Recombinant AAVs (rAAVs), such as those currently used for clinical gene transfer, have the entire AAV genome replaced except for the inverted terminal repeats, and are packaged using helper virus free systems (Xiao et al., 1998; Collaco et al., 1999). Pseudotyping, swapping the capsid protein from one serotype with another or engineering the capsid protein, allows for the tailoring of expression to specific organs and cell types. AAV2 (Xu et al., 2003), AAV5, AAV6, AAV8 and AAV9 (Yu et al., 2016) have all shown tropism for sensory neurons in the periphery. For motor neurons, AAV1 (Hollis II et al., 2008; Homs et al., 2014), AAV6 (Towne et al., 2013), AAV7, AAV9, AAVrh8, AAVrh10, AAVrh39, and AAVrh43 show promise (Yang et al., 2014). Engineered capsids such as AAVPHP.B (Deverman et al., 2016) and AAV-B1 (Choudhury et al., 2016 p.1) have been specifically designed for neuronal tropism and show improvements over serotypes such as AAV9 for that purpose. Recent improvements to engineered capsids include AAV-PHP.S which has enhanced expression in DRG neurons (Chan et al., 2017). Route of administration also can allow for additional tailoring of expression sites, with localization to the peripheral nerve possible following intranerve (Boulis et al., 2003; Xu et al., 2003; Pleticha et al., 2014), intramuscular (Martinov et al., 2002; Hollis II et al., 2008; Towne et al., 2010; Zheng et al., 2010; Towne et al., 2013), direct DRG injection (Yu et al., 2013), intravascular (Duque et al., 2009; Schuster et al., 2014), and intrathecal injections (Xu et al., 2012; Homs et al., 2014). Lastly, choice of promoter can also restrict cell expression with the ubiquitous cytomegalovirus promoter showing strong expression in motor neurons, the chicken beta actin promoter capable of enhanced expression in DRG neurons at the expense of motorneurons (Gray et al., 2011), and the synapsin promoter restricting expression to neurons (Holehonnur et al., 2014; Dashkoff et al., 2016). These features of capsid, promoter and route administration allow for targeting to specific types of neurons in the peripheral nerve. Given the relatively small size of fluorescent reporter proteins, AAVs provide a feasible means of long-term expression in the axons of the periphery.

An Optical Peripheral Nerve Interface

An optical neural interface ultimately relies on a means of transforming the electrical activity of an axon into an optical change. While it is possible to use intrinsic optical signals to report action potentials and neural activity in isolated optic nerves (Macvicar et al., 2002) and spinal cord (Sasaki et al., 2002) preparations in cultured cells (Stepnoski et al., 1991), and to stimulate using near infrared (Wells et al., 2005; Wininger et al., 2009), these methods are sensitive to motion and would not be feasible in the context of the highly mobile environment of a prosthetic or reanimated limb. Furthermore, it may be difficult to localize the neural activity. Thus, some sort of optical transducer is required, most readily filled by the long-term expression of an optical reporter protein.

Fiber Optic and Miniaturized Microscopy

Once an AAV has successfully transduced a motor axon with an optical reporter protein, a means of reading out the fluorescence change in response to neural activity is needed. This problem has been explored with fiber optic cables (Gmitro and Aziz, 1993; Flusberg et al., 2005; Piyawattanametha et al., 2007; Engelbrecht et al., 2008; Shin et al., 2010; Ozbay et al., 2015) [for review see Mehta et al. (2004) and Goetz et al. (2007)] and fully implantable devices (Ng et al., 2008; Ohta et al., 2008; Tamura et al., 2008; Murari et al., 2009, 2010; Park et al., 2011) particularly in the brain. Skull windowing techniques have been shown to permit imaging

from a Förester resonance energy transfer-based voltage sensors (VSFP-Butterfly) for 13 months in transgenic mice (Song et al., 2018), an encouraging development in long-term stable optogenetic reporter imaging. Two-photon imaging of AAV2/1 delivered calcium reporters GCaMP6s and RGECO through a skull window have also been demonstrated (Kwon et al., 2018). [For a detailed review of calcium imaging techniques in the murine brain, including the use of gradient-refractive index lenses, head mounted microscopes, fiber photometry and calcium signal analysis techniques see Girven and Sparta (2017)].

In the periphery, size constraints make microscopes of the head-mounted size unviable, and musculoskeletal anatomy makes transcutaneous imaging of nerves challenging. Furthermore, the scattering properties of peripheral nerve myelin severely limits optical imaging depth, resulting in a 20-fold greater attenuation (Fontaine et al., 2018), and significant wavefront distortion (Futia et al., 2018) compared to cortex, even with two photon imaging. Thus it may require the employment of either nerve shaping techniques (*e.g.*, cuffs), dissection of complete nerves into fascicles and subsequent fascicular imaging, or more advanced imaging solutions, such as three photon microscopy (Horton et al., 2013), before the entire depth of the peripheral nerve can be imaged. Nonetheless, imaging technological window into the peripheral nerve that can be exploited in a peripheral nerve interface, and research continues to make inroads in improving optical imaging depth.

For read-in, optical stimulation cuffs have also been demonstrated (Liske et al., 2013; Towne et al., 2013) and it seems readily feasible to extend such technologies to the stimulation of sensory afferents or motor neurons. Combining an implantable or fiber optic microscope with an optical stimulation cuff would allow for a complete optical neural interface.

In Pursuit of an Optical Peripheral Nerve Interface

Our lab and our collaborators have actively pursued the combination of these three elements – a fluorescent reporter of neural activity, a vector for delivery and the development of an implantable optical device for the stimulation and recording of neurons in the peripheral nerve. Toward that end, we injected mice intramuscularly with AAV1 expressing GCaMP6f under the control of the CAG promoter (Anderson et al., 2018) or ArcLight (unpublished results) under the control of the Synapsin promoter (**Figures 2** and **3**). GCaMP6f expression was widely seen in small fibers, with 48% of detected fibers being below 1 μm in size, indicating likely transduction of nociceptors, metaboreceptors and thermoreceptors, all key fibers for sensory reporting from a reanimated limb. These fibers demonstrated calcium influx along their entire length unlike in motor neurons, where it was confined to the node (Fontaine et al., 2017). They responded linearly with changes in fluorescence at low numbers of stimuli before plateauing with increasing numbers of stimuli. Given that these were small fibers that convey information that does not necessarily require a high frequency response, this type of read-out may be acceptable for use in a peripheral nerve interface. In future work, we plan to express optogenetic actuators and reporters in both sensory

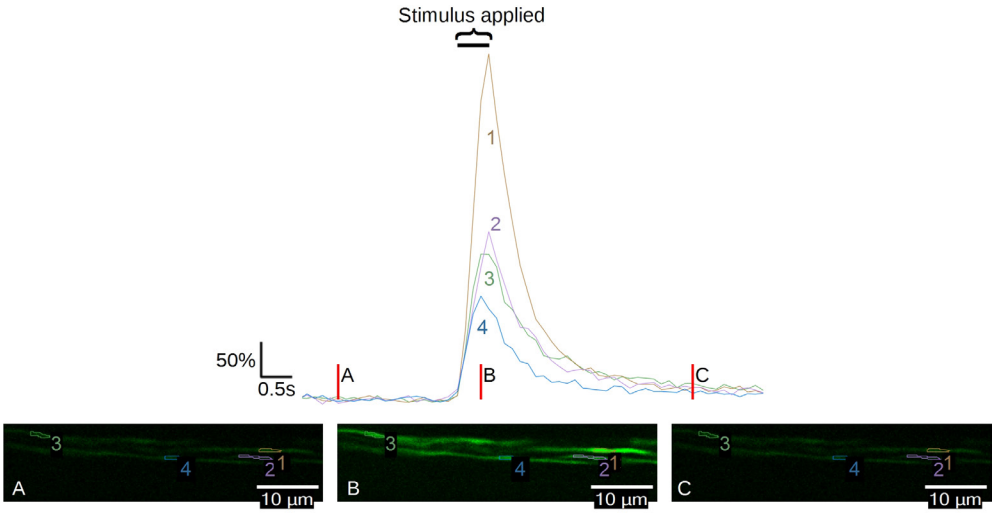

Figure 2 Intramuscularly injected AAV1-CAG-GCaMP6f causes expression of GCaMP6f in small fibers of the peripheral nerve.
When excised nerves are stimulated electrically *ex vivo* (here 50 Hz for 500 ms), GCaMP6f reports the activity. (A–C) Correspond to time points indicated in traces. Adapted from Anderson et al. (2018). AAV: Adenoassociated virus; GCaMP6f: GCaMP6-fast.

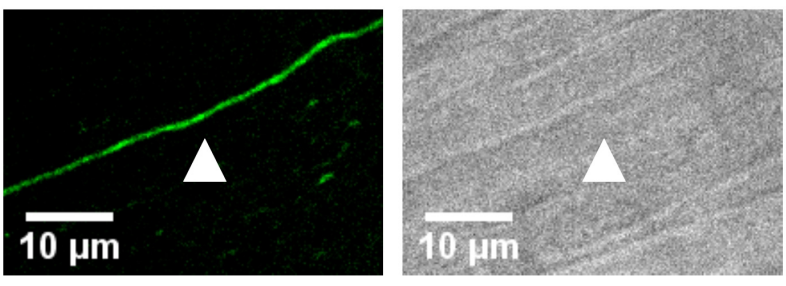

Figure 3 Injections into the anterior tibialis muscle of AAV1-Syn-ArcLight cause expression of ArcLight in the common peroneal nerve of mice.
488 nm excitation on left panel, brightfield image on right panel. Arrowheads indicate ArcLight labeled common peroneal nerve axon. AAV: Adenoassociated virus.

and motor axons in the hope that we can demonstrate the viability of such an approach as a means of selectively interrogating and stimulating the peripheral nerve as would be necessary for limb reanimation or prosthesis control.

As developments in vectors, optogenetic reporters and actuators, and implantable microscopes continue, many of the challenges faced by an optical peripheral nerve interface recede. New vectors reduce immune responses, enhance selectivity and provide for long-lasting expression in cells of interest. New optogenetic proteins diversity the color palette, decreasing spectral overlap, and improve kinetics and signal-to-noise ratio of reporters. New microscopes increase flexibility, stability, and imaging depth. While much work remains, an optical peripheral nerve interface is on the horizon.

Acknowledgments: *HEA would like to thank John Caldwell (University of Colorado, Anschutz Medical Campus, USA) for his assistance in revising the manuscript.*
Author contributions: *Manuscript writing and experiment performment: HEA. Direction of research: RFW.*
Conflicts of interest: *The authors report no conflicts of interest.*
Financial support: *This work was funded in part by the University of Colorado Medical Scientist Training Program and funds from the NIH SPARC initiative administered through the Office of the Director:1OT2OD023852-01.*

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C-Editors: Zhao M, Yu J; T-Editor: Liu XL