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The Effect of Acetylcholine on the Reduction of the Required Voltage for Muscle
Contraction

by

Maral Gharib

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Joint Doctor of Philosophy

with the University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Dorian Liepmann, Chair

Professor Roger Cooke

Professor Liwei Lin

Spring 2012

Abstract

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Maral Gharib

Joint Doctor of Philosophy in Bioengineering

University of California, Berkeley

and

University of California, San Francisco

Professor Dorian Liepmann, Chair

Facial nerve dysfunction and the subsequent inability to blink is a common complication caused by numerous medical problems, such as stroke. Denervation and dysfunction of the orbicularis oculi, the muscle responsible for eye blinks can result in corneal abrasions and breakdown. Direct electrical stimulation of the muscle is not a viable solution, as the required electrical stimulus needed to induce a blink in denervated muscle is 3 mA while the human pain threshold is 1 mA. The insufficiency of electrical stimulation alone in triggering painless muscle contraction in denervated orbicularis oculi muscles indicates a need to investigate other methods of stimulation. The focus of this dissertation has been to investigate the response of skeletal muscle to combined electrochemical stimulation by initiating the intricate cascade that leads to muscle contraction with the application of acetylcholine (ACh) in order to reduce the required electrical stimulus. Initial *in vivo* testing in rabbits demonstrated that the required current to stimulate a muscle contraction could be reduced with the incorporation of ACh, supporting the feasibility of the concept of electrochemical stimulation. Focus was then shifted to *in vitro testing* using cultured C2C12 cells grown on microposts to determine the correlation between electrical stimulation to myotube excitability, indicated by the contraction force generated. Insufficient myotube growth and cell detachment upon electrical stimulation resulted in the decision to continue experiments using excised skeletal muscle from laboratory rats. Image analysis testing of excised biceps femoris demonstrated that the change in area of samples increased in response to electrochemical stimulation in comparison to electrical stimulation. Force analysis testing of excised extensor digitorum longus demonstrated that the application of ACh for electrochemical stimulation clearly enhanced the contraction force, thus allowing for a lower level of electrical stimulation. The influence of electrochemical testing in enhancing contraction forces in denervated muscle has been demonstrated. Thus potentially creating a new platform for the control of muscles that no longer have a functional neural control.

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Chapter 1: Introduction

Introduction

Facial nerve dysfunction is a physiological problem that affects both genders, all races, and all ages indiscriminately. It can be triggered by a variety of medical issues including stroke and seventh cranial nerve palsy. The seventh cranial nerve (CN-7) controls facial muscles and consists of around 10,000 fibers, 7,000 of which innervate the muscles of facial expression¹. Bell's Palsy, the most common form of facial paralysis, is characterized by facial drooping and affects approximately 25 in 100,000 people annually in the United States alone². Paralysis of the facial nerve does not impinge on the patient's vision but compromises control of the surrounding muscles needed to close the eyelid. Denervation and dysfunction of the muscles results in an inability to close the eyelid, loss of tone in the upper and lower eyelids, as well as drooping of the eyebrow. Impaired eyelid closure prevents movement of tears across the cornea by the normal lacrimal pumping action. The inability to blink consequently leads to problems such as disfigurement, inability to communicate emotions, excessive tearing, and, in severe cases, corneal breakdown³. In order to develop a treatment that restores function to a patient's eye, the basics of muscle function and the process of contraction must be understood.

Muscle

Muscle cells are responsible for the production of force and the generation of movement in the body. Three distinct types of muscle cells generate contractile force, but each varies in its function and structure. Cardiac muscle, found in the heart, is responsible for pumping blood and is controlled involuntarily. Smooth muscle is also considered to be involuntary, but is found in internal organs and is responsible for functions such as peristalsis. Skeletal muscle, on the other hand, allows for voluntary muscle control and is primarily responsible for the movement of bones. Together, these specialized muscle cells facilitate movement internally and externally. This dissertation focuses on the study of stimulating skeletal muscle; therefore within the introduction an overview of skeletal muscle structure and function is emphasized, along with an overview of muscle paralysis.

Skeletal muscle cells develop from precursor cells called myoblasts. As the myoblasts differentiate, they fuse together to form thin, elongated myofibrils. The differentiated cells are bundled in parallel with other myofibrils and fuse further to form a multi-nucleated muscle fiber with a common sarcolemma¹. Skeletal muscle fibers have a striated pattern that results from the sarcomere, the contractile unit of the myofibril. The sarcomere, an ordered arrangement of thick and thin filaments aligned in parallel, appears as a repeating pattern along the length of the myofibril⁴. Two Z lines define the boundaries of a sarcomere. Each thin filament is bound to a Z-line at one end, while the other end extends towards the center of the sarcomere and overlaps in parallel with thick filaments. Thin filaments are composed of actin filaments bound to nebulin, troponin, and tropomyosin. Nebulin is an actin binding protein that controls the length of the thin filament⁵. Tropomyosin, an actin binding protein, runs along the actin molecule and blocks the myosin binding site. Bound to both tropomyosin and actin is troponin, which is the calcium binding protein that regulates tropomyosin^{6,7}. Thick filaments are located in the center of the sarcomere and consist of intertwined myosin molecules, elongated contractile proteins

with globular heads that stick out in a staggered manner from the sides. Titin, a spring-like protein, extends from the Z lines and attaches to the ends of thick filaments to maintain thick filaments in the center of each sarcomere. Muscle contraction involves sarcomeric shortening that is generated by the thick and thin filaments sliding past each other, propelled by cross-bridge formation. The mechanisms behind muscle contraction will be further discussed later in the chapter.

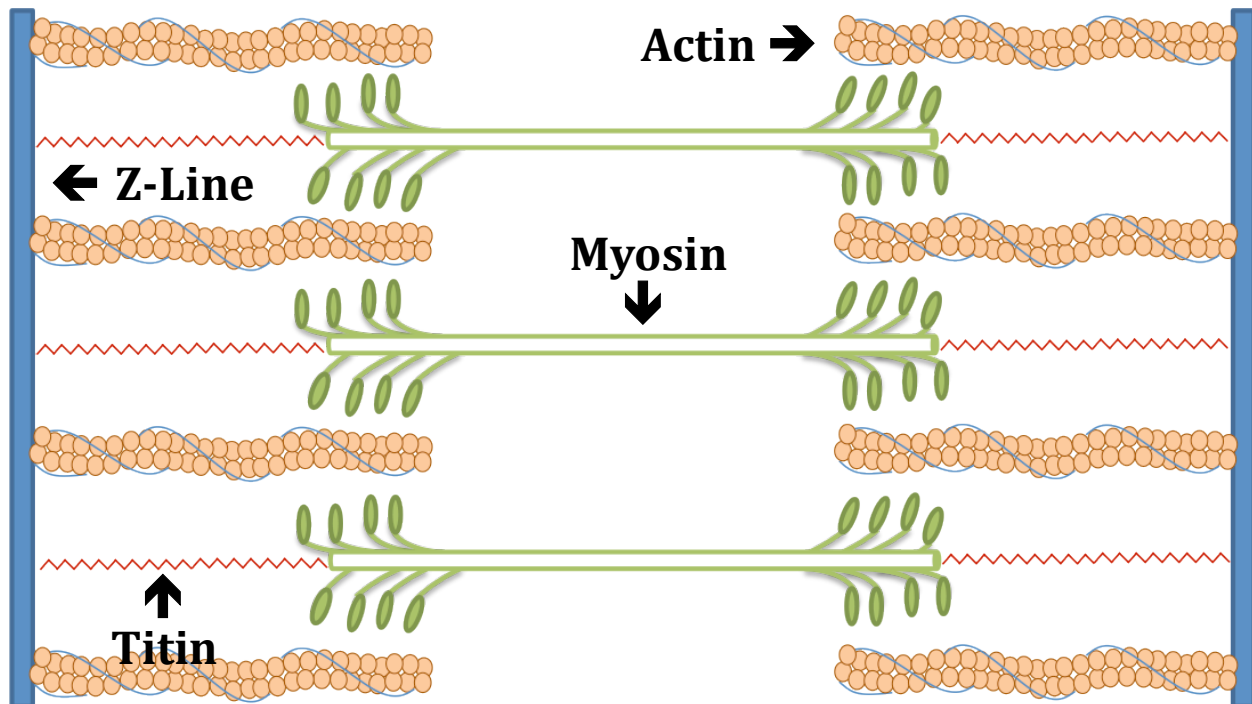


Figure 1. Sarcomere, diagram illustrating the ordered arrangement of actin and myosin filaments in parallel alignment.

Skeletal muscle consists of different types of fibers, which allow it to adapt to functional demands. The forms of skeletal muscle phenotypes are identified by their different protein isoforms, different ratios of sarcomeric proteins, and different metabolic rates⁴. Skeletal muscle is categorized as either type I or type II. Type I fibers are slow twitch fibers which produce small amounts of tension for a longer period of time. The fibers resist fatigue by maintaining an oxidative catabolism, which is assisted by the high density of capillaries surrounding the fibers. Type II fibers are fast twitch fibers and produce higher amounts of force for shorter periods of time. Type II fibers can be further classified into two subtypes, IIA and IIB. Type IIA fibers are fast oxidative fibers that maintain enough aerobic capacity to resist fatigue for several minutes. Type IIB fibers rely on anaerobic catabolism with a high glycolytic capacity for force output. Type IIB fibers maintain the highest rate of contraction which then causes them to fatigue more easily^{4,6,8,9}.

Nerve-Muscle Synapse

Motor neurons and muscle fibers meet at the neuromuscular junction (NMJ) and communicate through the neurotransmitter acetylcholine (ACh). The axon terminal of a motor neuron contains packed vesicles that contain ACh. The vesicles are clustered in dense patches at the active zone on the presynaptic membrane. The pre- and postsynaptic membranes are separated by a synaptic cleft that is approximately 50 nm wide^{10,11}. The synaptic cleft contains the basal lamina, an extracellular matrix mostly composed of collagenous glycoproteins, which contributes to the adhesion of pre- and post-synaptic components. The basal lamina most importantly contains high concentrations of acetylcholinesterase (AChE), the enzyme that hydrolyzes ACh¹². Neuromuscular connections typically occur near the center of the muscle fiber^{13,14}. The active zones of a motor neuron are positioned opposite an area of the muscle called the motor end plate. The motor end plate is the region of a muscle fiber that lies directly under the axon and contains junctional folds. Acetylcholine receptors (AChR) are located at the top of the folds while sodium channels are found in the depths of the folds^{1,15}.

Cholinergic sensitivity at the NMJ is associated with to the endplate region where AChRs are found^{13,16}. During myoblast fusion and differentiation, AChRs are distributed across the myotube. Agrin, a proteoglycan synthesized by the motor neuron is known to influence the clustering of AChRs. Upon innervation, agrin is released by the nerve causing the localization and activation of the MuSK receptor at the synaptic site. The binding of agrin to MuSK causes AChRs to cluster on the myotube surface^{1,17}. Nicotinic acetylcholine receptors (nAChR) are a specific type of AChR found at the NMJ. In the postsynaptic membrane of adult skeletal muscle, nAChRs can be found clustered at the crests of the junctional fold at a density of 10,000/ μm^2 ^{10,18}. nAChRs are transmitter ligand-gated ion channels composed of five transmembrane polypeptides arranged in a ring; two α , one β , one γ , one δ subunit. The two α subunits have ACh specific binding sites which regulate the opening of the channel. One activation site is located between the α and γ subunits and the other is between the α and δ subunits¹⁹. ACh attachment produces a conformational change in the ring that opens the channel to ion flow^{7,20,21}. When a channel closes, the ACh molecules dissociate and the channel will not open until two ACh molecules attach again.

As previously mentioned, acetylcholine (ACh) is a neurotransmitter synthesized and released by motor neurons at synaptic junctions. ACh binds to AChRs on skeletal muscle fibers to open the ligand-gated channel in order to initiate depolarization, which activates the muscle. ACh is synthesized by the acetylation of choline by acetyl coenzyme A (Acetyl-CoA). The enzyme choline acetyltransferase catalyzes the production of ACh. Following synthesis, ACh is packaged into vesicles where each vesicle stores one quanta of transmitter. The vesicles tend to cluster near the active zones of the presynaptic membrane²². Transmitter release is controlled by the depolarization of the presynaptic terminal. The presynaptic cell produces an action potential that opens voltage gated calcium channels at the active zone²³. An inward current of calcium causes the synaptic vesicles to fuse with the presynaptic membrane and to release the entire quanta via

exocytosis^{24,25}. Each quanta then produces a miniature end plate potential²⁶. The fused vesicle membrane is then taken into the cell via exocytosis and recycled for reuse²⁷. Upon its release, ACh diffuses across the synaptic cleft to the motor end plate region and binds to AChRs. Once ACh dissociates from the receptors, it is degraded by AChE.

Acetylcholinesterase (AChE) is an enzyme incorporated into the basal lamina of the NMJ and functions as a biomolecular off switch. The most common form of AChE is composed of three tetramers attached to a collagen stalk that is bound to the basal lamina at an approximate density of 1,500 per μm^2 ^{22,28}. The role of AChE is to terminate synaptic transmission by hydrolyzing ACh into choline and acetate once it has dissociated from the AChRs. AChE degrades ACh rapidly at an average rate of 3000 molecules per active site per second for efficient synaptic activity²⁸⁻³⁰. Once ACh has been hydrolyzed, the choline is then taken back into the axon terminal for reuse in the synthesis of ACh³⁰. If ACh molecules remain in the synaptic cleft upon their release, reattachment to the receptors would occur, thus causing the receptors to become desensitized eventually leading to the loss of new synaptic signals¹.

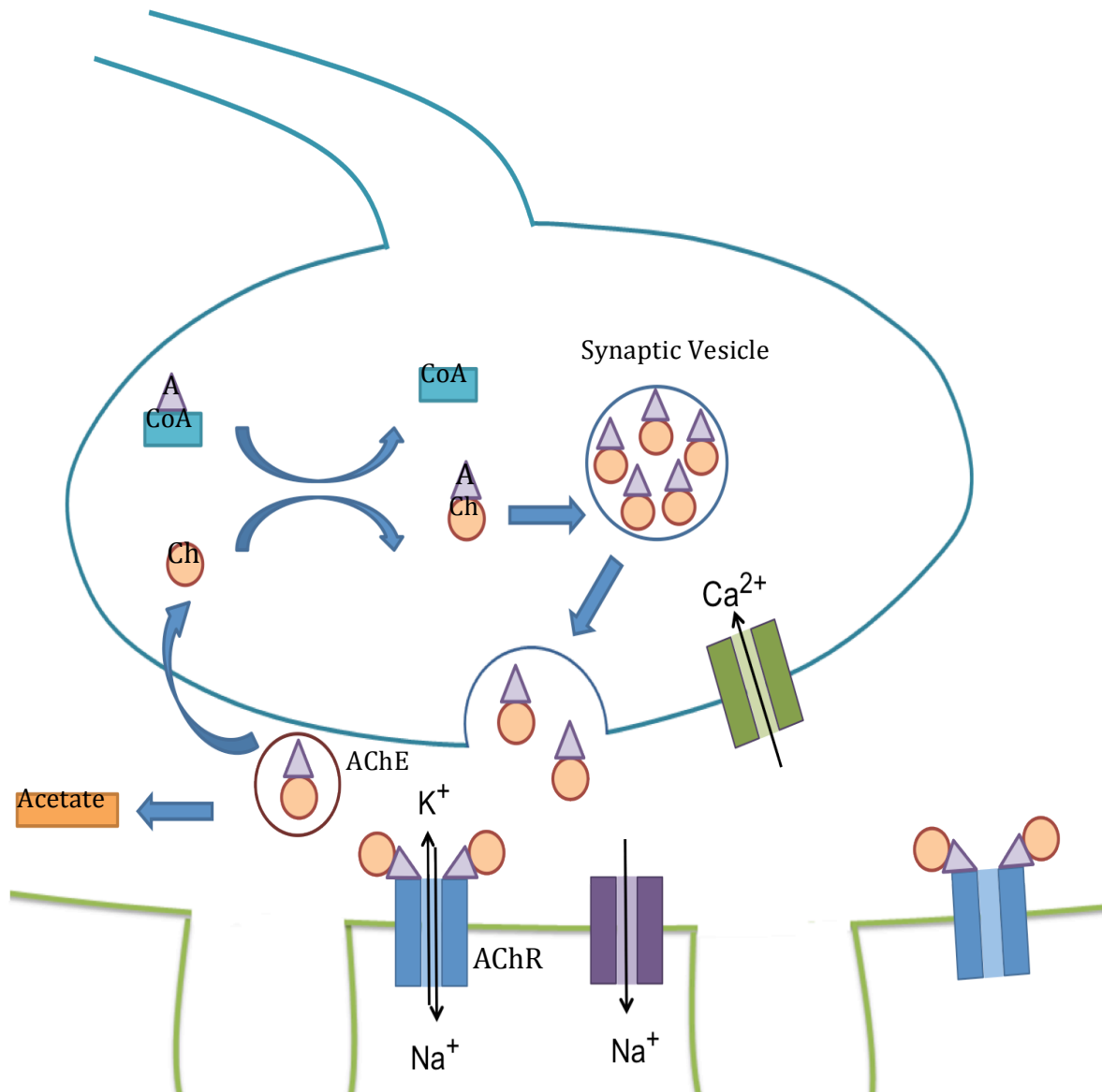


Figure 2. Synthesis and release of acetylcholine (ACh) at the neuromuscular junction. Depolarization of the presynaptic terminal leads to transmitter release. ACh then diffuses across the synaptic cleft to the motor endplate region. Attachment of ACh to acetylcholine receptors causes ACh gated ion channels to open, leading to an inward current of sodium ions and an outward current of potassium ions.

Excitation-Contraction Coupling

Excitation-contraction (EC) coupling is the process that links an action potential at the muscle surface to the mechanical response of contraction. A motor unit refers to a single motor neuron and the muscle fibers it innervates. The unit's contractile force depends on the muscle fiber type and the number of muscle fibers that are innervated. The formation of cross-bridges and their cyclic interaction generates force during muscle contraction^{31,32}. The amount of force a muscle can produce depends on its initial length, which determines

the amount of overlap between the myosin and actin heads³¹. The myosin heads function as a motor and slides the actin filament towards the center of the sarcomere³³.

At the NMJ, the nerve synapse secretes ACh which then binds to receptors on the post-synaptic membrane. The binding causes ACh gated ion channels to open, leading to an inward current of sodium ions and an outward current of potassium ions. The ion flow creates a local depolarization called an end plate potential (EEP), which activates neighboring voltage gated sodium channels to open, thus allowing more sodium in and further depolarizing the membrane. The increase in the inward sodium current converts the EEP into an action potential (AP). The AP is then propagated across the plasma membrane and further into the muscle via transverse tubules (T-tubules)^{34,35}.

In skeletal muscle, the depolarization of the T-tubules activates voltage sensitive calcium channels located on the membrane called dihydropyridine receptors (DHPRs)³⁶⁻³⁸. DHPRs control the activation of the sarcoplasmic calcium release channels known as ryanodine receptors (RyRs). RyRs and DHPRs interact at bridge junctions between the sarcoplasmic reticulum and T-tubules called junctional feet^{39,40}. When an AP reaches the DHPRs, there is a charge movement across the T-tubule membrane that houses the DHPRs. This charge movement then activates the RyRs via a direct molecular interaction by the junctional feet^{40,41}. Calcium is then released from the sarcoplasmic reticulum into the cytoplasm, where it proceeds to bind to troponin, located on the actin filaments. The binding of calcium to troponin causes tropomyosin to move, thus unblocking the myosin binding site on the actin filament and activating cross bridge cycling^{1,7}. Myosin heads are activated into their energized state by the attachment and hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and phosphate (P_i)⁴². With the entry of calcium into the cytoplasm, the energized myosin heads bind to the actin filament, creating cross-bridges. ADP and P_i are released from the myosin head upon cross-bridge formation, thus releasing energy and generating force via the production of a power-stroke³². At the end of the power-stroke, a new ATP attaches to the myosin head causing the actin and myosin to dissociate⁴². The new ATP is then split into ADP and P_i, thus reenergizing the myosin head. Cross-bridge cycling will continue for as long as calcium is bound to troponin. The removal of calcium from troponin allows tropomyosin to retain its blocking position⁴².

Orbicularis Oculi Muscle and Denervation

Blinking of the eyelid is controlled by the orbicularis oculi, a specialized skeletal muscle mostly composed of type II fast-twitch fibers²⁸. The signal to blink, or activation of the orbicularis, is initiated at the brainstem. Muscles that have lost their input from the nervous system due to nerve damage no longer receive ACh stimulus. Without the release of ACh from the axon terminal, there is no initiation of the intricate cascade that leads to muscle contraction, leaving the eyelid paralyzed and permanently open. Impaired eyelid closure consequently results in corneal damage.

Current management techniques focus on alleviating corneal dryness via viscous drops or ointments that can result in blurred vision and do not restore muscle tone and the ability to blink. Another approach to alleviating corneal dryness is to implant a one gram gold bar in the pretarsal region of the eyelid, allowing gravity to maintain closure in order to preserve

moisture^{43,44}. While the orbicularis oculi is innervated by CN-7, its antagonist, the levator palpebrae, is innervated by the third cranial nerve⁴⁵. Following a blink, the levator returns the eyelid to its open position. With the implantation of a gold weight, gravity pulls the eyelid closed when the patient is standing⁴³. Since the levator is still innervated and active, when the patient lies down, the eyelid is once again paralyzed open. Therefore, surgery results in a crude and static solution. Neither of these two techniques attempts to restore function to the patient's eye.

Electrical stimulation has been successfully utilized in existing medical devices to initiate muscle contraction. Electrical current of the proper parameters, applied directly to intact motor nerves generate muscle contractions. The stimulation depolarizes the muscle membrane and initiates the action potential that is normally generated by the secretion of ACh by the nerve at the NMJ. This approach, which is used in pace makers, uses the nerve to amplify the electrical stimulation. Denervated muscles do not have functional nerves attached to them and as a result the electrical stimulation must be applied directly to the muscle. As a result, the level and duration of stimulation required to create movement in a denervated muscle is at least ten-fold higher than that required for an innervated muscle^{46,47}. Attempts to use electricity to stimulate a blink in human patients with seventh nerve palsy have failed because the stimulation required for full eyelid closure induced significant pain. The human pain threshold is 1 mA, while studies indicated that at least 3 mA was needed to induce a blink^{47,48}. The insufficiency of electrical stimulation alone in triggering a painless muscle contraction in denervated orbicularis oculi muscles indicates a need for combining the electrical stimulation with neurotransmitter-based stimulation in order to bring the required current below the pain threshold. In addition, ACh should be incorporated not only to reduce the current, but also to maintain the integrity of the muscle and prevent atrophy⁴⁹⁻⁵¹.

Animal studies in rabbits have shown that electrical stimulation with implanted or percutaneous electrodes can successfully produce a functional blink in animals with surgically denervated orbicularis oculi. Rabbits are a good ocular model because of the resemblance of eyelid structure and function to that of humans. More specifically, the distribution of neuromuscular junctions and muscle fiber type of the orbicularis oculi in rabbits is remarkably similar when compared to humans. The required parameters for complete blink production in denervated rabbits is 3.7 – 6.5V with phase duration of 20-40ms, but signs of pain accompanied the level of electrical stimulation required to produce a full eyelid closure blink⁵². Others studies have shown that increasing the phase duration of the stimulus allows for a reduction in the amplitude of the stimulus⁴⁶.

Effects of Denervation

Following denervation, the NMJ's structure and function remain intact while the entire muscle becomes ACh sensitive⁵³. The heightened sensitivity to ACh is associated with a 5 to 50 fold increase in the number of AChRs⁵⁴. AChRs are found not only clustered at the NMJ but all over the muscle^{53,55}. It has been shown that electrical stimulation can decrease ACh sensitivity in denervated muscle by reducing the increased presence of AChRs, leading to the conclusion that muscle activity reduces AChR biosynthesis^{13,56-61}. Muscle inactivity, resulting from denervation, also causes muscle atrophy along with a decrease in AChE⁶².

The presence and activity of AChE decreases and eventually disappears from the muscle⁶³⁻⁶⁶. Interestingly, various species differ in their response to denervation. AChE activity in chicken and rabbit muscle increased after denervation, while rat muscle had a 70% reduction in AChE activity⁶⁶⁻⁶⁸. Electrical stimulation of denervated muscles has been found to maintain AChE levels. Stimulation initially induces AChE activity all over the muscle, but activity is then focused on the end-plate region⁶⁴. It can be concluded that muscle activity maintains the metabolic stability of the end-plate region, specifically the stability of AChRs and AChE⁶⁹.

C2C12 and Microposts

C2C12 cells, a mouse skeletal muscle cell line, have previously been used for in vitro studies as models for studying the molecular mechanisms involved with denervation⁵⁰. C2C12 cells develop organized myotubes upon differentiation and display measurable force upon stimulation⁷⁰. Electrical stimulation has been shown to induce contraction in the cells⁷¹. Differentiated C2C12 myoblasts contain membrane components and express membrane proteins that are brought about with proper development. Specifically AChRs, RyRs, AChE, and certain subunits of the voltage gated calcium channels^{21,72-74}.

Due to the fact that C2C12 generates measurable force upon stimulation and expresses AChR and AChE, the cell line was implemented in order to conduct electrical and electrochemical experiments. Following the methods demonstrated by Feinberg et al, diffusion and stimulation studies would be simplified⁷⁵. The authors integrated a monolayer of cardiac cells with a thin elastic film to create a muscular actuator. This technique created the possibility of growing myoblasts on a flexible substrate in order to obtain force measurements. By microcontact printing fibronectin lines, C2C12 cell surface adhesion properties would be enhanced and uniaxial cell alignment would be ensured. Cell alignment allows for internal organization, thus leading to an increase in force generated upon stimulation.

PDMS microposts have been used in previous cell studies to detect traction forces on substrates^{76,77}. The microposts provide a soft substrate that would allow for cell attachment, differentiation, and spatiotemporal deflection measurements. Microposts can be designed specifically to account for the properties of C2C12. McMahon et al. determined the maximum force developed by C2C12 to be 0.88 μN ⁷⁰. While Mathur et al. found the elastic modulus and spring constant of C2C12 to be 24 kPa and 0.0069 N/m, respectively⁷⁸. With assistance from the Lin lab, such properties were accounted for when designing the posts, thus creating an environment that supports cell growth along with the capability to conduct force measurements⁷⁹. Seeded cells attach and develop into myotubes in an aligned manner on top of the microposts. Alignment is controlled via micropost spacing and microcontact printing of micropost tops with fibronectin. Upon differentiation, cells would be stimulated which would result in muscle contraction. The contraction would cause the cell to pull on the top of the microposts, thus creating a displacement. The traction force exerted on the post is calculated via the deflection of the top of the post based on the properties of the post (Figure 3).

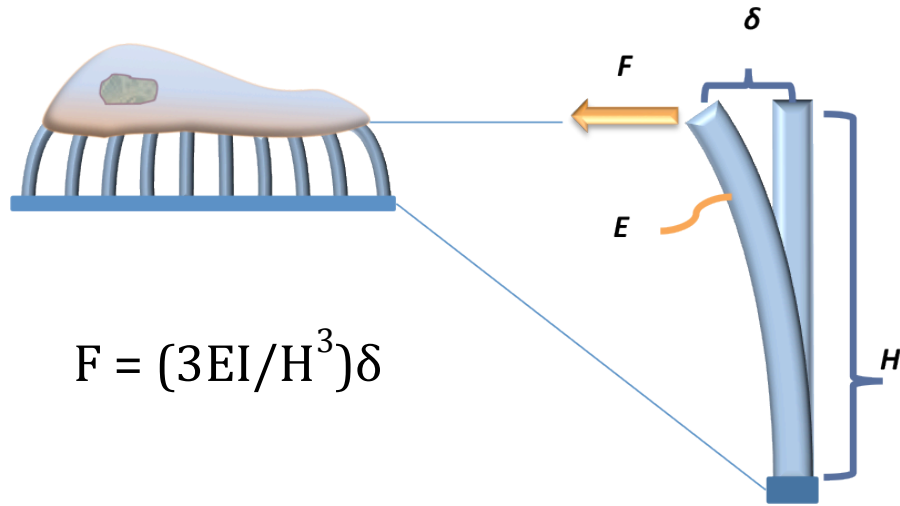


Figure 3. PDMS microposts for spatiotemporal deflection measurements. Cells attach and grow onto the tops of micropost arrays. Muscle contraction leads to micropost deflection. Force is calculated using the tip deflection (δ), Young's Modulus (E), the moment of inertia (I), and the height of the posts (H). Image adapted with permission from Sochol et al.⁸⁰

Muscle Stimulation

Excision, or primary harvesting, of skeletal muscle has been used in previous studies as a model for denervated muscle. The biceps femoris (BF) and the extensor digitorum longus (EDL), two hind leg muscles that are primarily type II skeletal muscle in rats, have been studied^{81,82}. Extensive studies on the EDL demonstrate the effects of electrical and electrochemical stimulation on denervated, or excised, samples. Gundersen et al. determined that atrophy due to 5 days denervation prior to excision resulted in an 18% reduction in muscle mass. A reduction in tetanic force was also observed due to atrophy⁸³. Dow et al. not only demonstrated that the loss of contractile activity due to denervation results in lower contraction forces when excised EDL was electrical stimulated, but also that the timing of the rest periods between stimulations can affect contractions⁸⁴. Another study by Dow et al. confirmed that consistent electrical stimulation of denervated muscle in a manner that is similar to normal muscle activity results in maintaining muscle mass and force⁸⁵⁻⁸⁸. Denervation of the EDL also results in the doubling of the length of time to reach the peak of contraction⁸³. This may be associated with Redfern et al.'s conclusion that denervation results in a decrease in the rate of rise of the action potential⁸⁹. Interestingly, fast twitch muscles are known to fatigue easily, however Gundersen et al. also determined that denervation improved the fatigue resistance of excised EDL^{83,87}.

Nielsen et al. demonstrated that muscle excitability is dependent not only on the membrane potential but also on the gradient for sodium and potassium. Specifically, electrical stimulation of excised EDL resulted in a decrease in intracellular sodium concentrations due to an increase in the sodium-potassium pump activity⁹⁰. Electrical stimulation has also shown to increase the influx and net accumulation of calcium.

Stimulation produced a 150% increase in calcium uptake in both isotonic and isometric contraction⁹¹.

The environment, or bath, in which excised EDL is tested has proven to be critical in contraction force studies. Ranatunga et al. found that a six to nine fold increase in shortening velocity, initiated by electrical stimulation, was correlated with an increase in bath temperature. Furthermore, the rate of tetanic tension also increased with temperature, while temperature sensitivity was found to be higher at lower temperatures⁹². Segal et al. determined that the time to peak tension decreased exponentially in correlation to an increase in temperature. Development of maximum tetanic tension required a higher electrical stimulation frequency with an increase in temperature⁹³. It can be concluded from these findings that muscle stability is dependent on incubation temperature.

Electrochemical stimulation studies on excised EDL have shown that muscle depolarization and contraction can be instigated via the application of ACh. Harborne et al. determined that contraction response increased with successive applications of ACh at 10 minute intervals⁹⁴. Further studies by Harborne et al. demonstrated that an increase in EDL contraction response could be elicited with an increase in incubation time in ACh. A 50 minute incubation with the application of 1 $\mu\text{g/mL}$ ACh every 10 minutes produced a peak response of an 8% increase from initial tetanic tension. It must be noted that samples were electrically stimulated between applications of ACh and that the response to electrical stimulation slowly decreased over time. Harborne et al. indicate that with electrochemical stimulation, the purpose of ACh is to potentiate the muscle's depolarization response. Specifically, the application of ACh seems to increase the flux of calcium in denervated muscles thus increasing the contraction response^{95,96}.

Scope of the Dissertation

The generation of an eye blink has never been demonstrated in humans with denervated orbicularis oculi using electrical, chemical, or electrochemical stimulation. The aim of this study has been to investigate the response of skeletal muscle to combined electro-chemical stimulation by initiating the intricate cascade that leads to muscle contraction with the application of acetylcholine in order to reduce the required electrical stimulus. This technique could provide a new approach, and demonstrates a potentially new platform, for the control of muscles that no longer have functional neural control and cannot use nerve stimulation to pace or control muscular behavior.

Chapter 2: Materials and Methods

A. *In Vivo* Pretarsal Orbicularis Testing

Electrical Stimulation in Human CN-7 Palsy Subjects

Five subjects with facial nerve palsy were recruited, three men and two women, ages ranging from 24 to 67. Testing was conducted by collaborators Cockerham et al. at UCSF⁴⁸. Research protocol approval was obtained from the institutional review board and informed consent was obtained from each participating subject. Topical proparacaine drops were placed in the palpebral fissure, and a scleral protector was placed. Topical lidocaine 5% was spread onto the skin overlying the orbicularis oculi on the affected side. Electrical stimulation of the orbicularis oculi was performed using Viking IV System disposable monopolar needle electrodes, 30-gauge diameter with 20 mm needle length (Viasys Healthcare, Yorba Linda, CA). Stimulation was accomplished using LabView software (National Instruments, Austin, TX) connected to a custom-made adapter in order to distribute electrical impulses to various sites simultaneously. Electrodes were then attached to the adapter and inserted directly into the muscle. Stimulation positions were designated at the upper lid 5 mm superior to the lacrimal puncta, upper lid 5 mm superior to the eyelid margin at mid pupil, upper lid 5 mm superior to the lateral margin, 5 mm directly lateral to the lateral margin, lower lid 5 mm inferior to the lateral margin, lower lid 5 mm inferior to the eyelid margin at mid pupil, and lower lid 5 mm inferior to the inferior lacrimal puncta (Figure 4). The level of electrical stimulation was varied while maintaining a pulse duration of 150 msec, in order to produce maximal movement of the eyelid. After each electrical impulse, patients were asked to rate the amount of pain caused by each electrical impulse using a scale from one to ten. A rating of one was defined as “no pain” and a rating of ten was defined as “the worst pain you can imagine”.

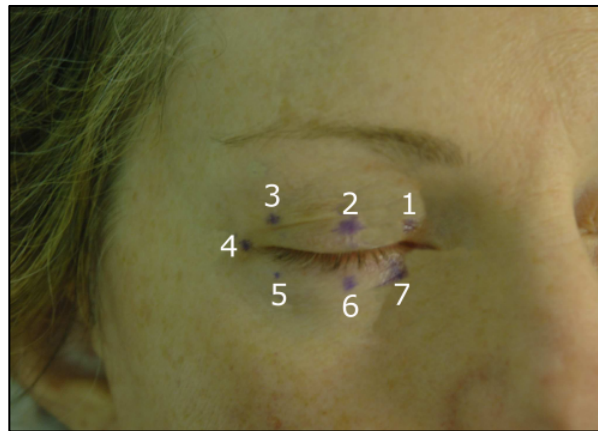


Figure 4. Stimulation points in human CN-7 palsy subjects. (1) Upper lid 5 mm superior to the lacrimal puncta (2) Upper lid 5 mm superior to the eyelid margin at mid pupil (3) Upper lid 5 mm superior to the lateral margin (4) 5 mm directly lateral to the lateral margin (5) Lower lid 5 mm inferior to the lateral margin (6) Lower lid 5 mm inferior to the eyelid margin at mid pupil (7) Lower lid 5 mm inferior to the inferior lacrimal puncta.

Microneedle Prototype

The tested device, a microelectromechanical systems (MEMS) syringe previously designed in the Liepmann Lab, consisted of arrays of 200 needles/cm² which delivered ACh uniformly across the pre-tarsal orbicularis in denervated New Zealand White Rabbits. Microneedles were 200 μ m long with a lumen diameter of 40 μ m (Figure 5A & B)⁹⁷. A reservoir backing for the microneedles was created by using a silicon mold to cast polydimethylsiloxane (PDMS) containers (Sylgard 185, Dow Corning, Midland, MI). PDMS backing was attached to the bed of microneedles via surface modification by oxygen plasma (50 Watts, 1 minute) (Figure 5C). Denervation of rabbits was performed by removing a 5 mm section of the CN-7. The device was then attached to a microsyringe and implanted on top of the pretarsal orbicularis (Figure 6A), and connected to a syringe pump for ACh delivery. Miochol®-E (Novartis, Basel, Switzerland), a parasympathomimetic preparation of ACh containing 10 mg/mL, was used in all of the following experiments. The efficacy of chemical stimulation alone was investigated by rapidly injecting different volumes (0.5 μ L, 1 μ L, 10 μ L, 25 μ L, 50 μ L, 100 μ L, and 200 μ L) of the ACh solution onto the pre-tarsal orbicularis using a standard medical syringe.

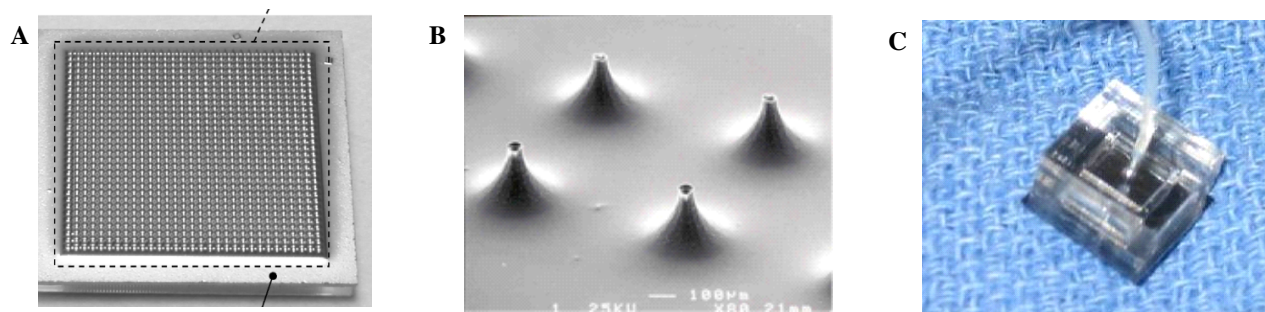


Figure 5. Microneedle Prototype (A) Array of microneedles 1 cm² (B) Close up of needles (C) PDMS backing attached to bed of microneedles.

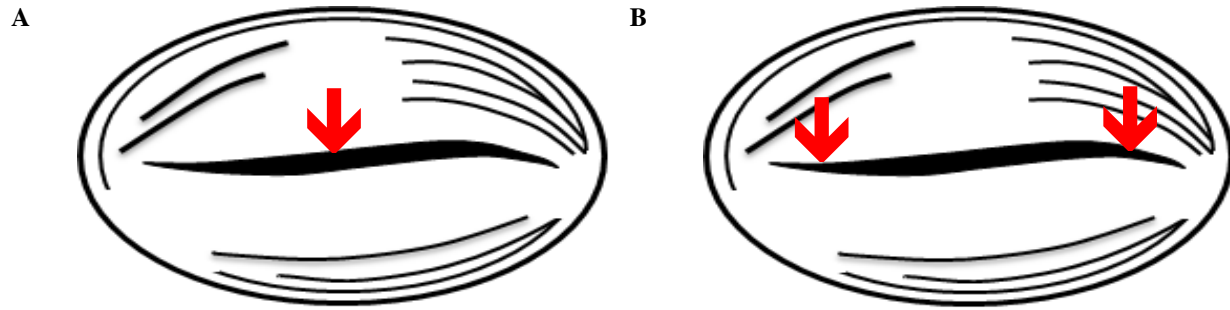


Figure 6. Diagram of the palpebral portion of the orbicularis oculi with stimulation delivery points. (A) Microneedle prototype and electrochemical prototype #1 delivered stimuli to the center portion of the pre-tarsal orbicularis (B) Electrochemical prototype #2 delivered stimuli to the pre-tarsal orbicularis approximately 7 mm and 20 mm from the inside corner of the eye.

Electrochemical Prototype #1

The first electrochemical prototype was created using SOMOS 9120, a liquid photopolymer, and titanium electrodes. Plastic parts were formed using a stereo lithography process. Upon construction, the prototype had 10 distributed orifices for ACh delivery and was 15 mm x 5 mm x 1.3 mm in size (Figure 7A & B). A cavity for ACh was located at the center of the device with tubing extending out for attachment to a microsyringe loaded onto a syringe pump. Two parallel titanium electrodes, 2 mm wide, were placed outside each row of orifices. The electrical portion of the device was controlled by lab view. The micro-syringe was manually operated for chemical delivery in order to create a spritz of ACh. The device was implanted on top of the pre-tarsal orbicularis (Figure 6A) and electrical testing was initially performed to obtain reference points. Testing consisted of implementing a square wave, 1V-10V, for 10 msec with a period of 1 second between starts. 2 μ L of saline were applied to the muscle between each stimulus application. Electrical testing was followed with electrochemical testing with the application of 6 μ L of ACh followed by a square wave pulse. Testing of electrochemical prototype #1 was performed twice in two different series on two different denervated White New Zealand Rabbits.

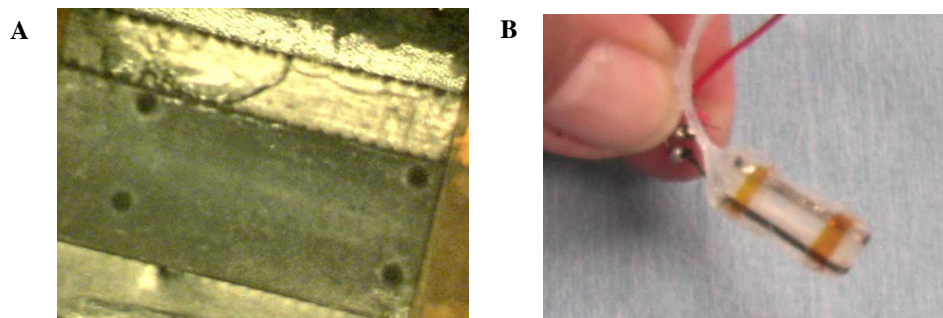


Figure 7. Electrochemical Prototype #1 (A) Close up of orifices and titanium electrodes (B) The final device.

Electrochemical Prototype #2

While the silicon-based micro-needle array provided good distribution of ACh across the muscle, the stiff planar structure may have affected the motion of the eyelid. For this reason, a second prototype (electrochemical prototype #2) was created from thin flexible tygon tubing (Cole Palmer, Vernon Hills, IL) with an inner diameter of 762 μm and an outer diameter of 2290 μm . Two platinum mesh electrodes (Sigma Aldrich, St.Louis, MO) were placed at the two ends of the device and were sized so that the electrodes would be placed approximately 7 mm and 20 mm from the inside corner of the eye (Figure 6B). Six orifices 350 μm in diameter were drilled into the tubing with 3 orifices distributed on both sides of the two electrodes (Figure 8). Instead of using a syringe-pump or manual control, a pressurized delivery system was designed to rapidly deliver precise volumes of ACh timed with the electrical stimulation (Figure 9). For the discussed experiments, the entire electrochemical stimulation occurred over a 200 msec period. The electrical stimulus was applied at 160 msec, after 80% of the ACh had been delivered (Figure 10). The current was applied in a series of eight biphasic pulses delivered over 40 msec. As with previous testing, the electrical stimulus was initially performed without ACh delivery to confirm a response from the rabbit.

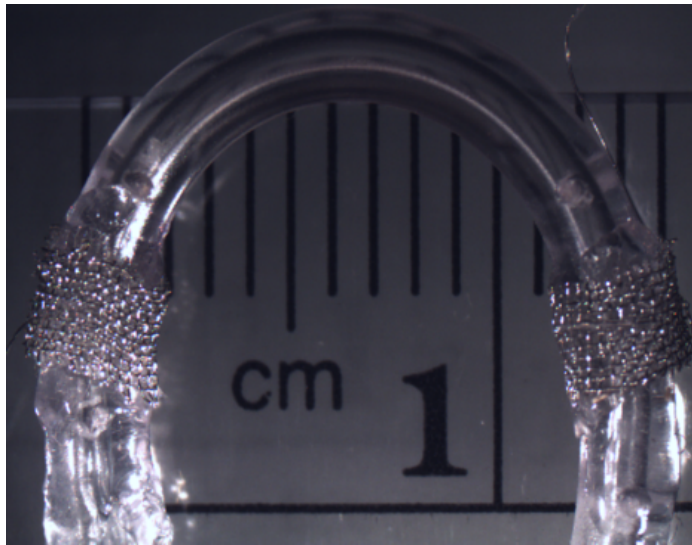


Figure 8. Electrochemical prototype #2, composed of tygon tubing and platinum mesh electrodes.

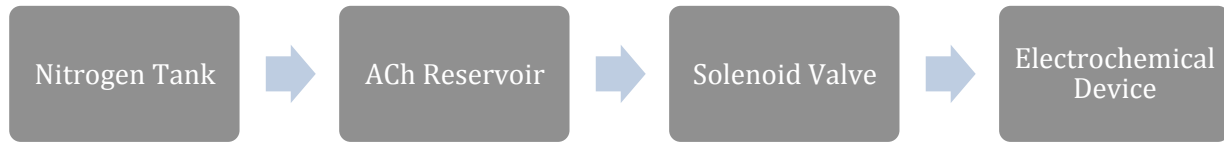


Figure 9. Pressurized delivery system. Set up consisted of a nitrogen tank with two regulators for controlled PSI. The nitrogen tank was attached to an ACh reservoir that led to a solenoid valve that controlled the rapid delivery of ACh via the electrochemical device.

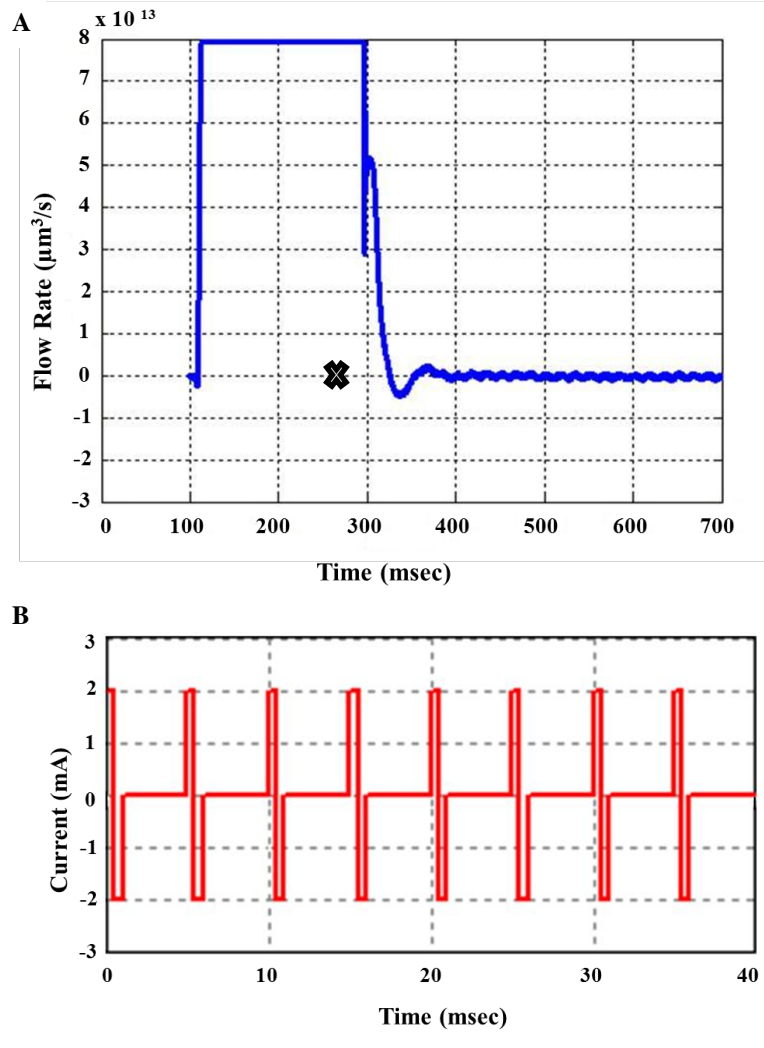


Figure 10. Application of stimuli by the pressurized delivery system. (A) Flow rate profile for the delivery of ACh with time point indicating application of electrical stimulation highlighted by X. Overshoot at 300 msec due to capacitance from the system. (B) Electrical stimulation consisted of 2 mA applied biphasically at 200 Hz with a pulse width of 0.5 msec

for 8 cycles of stimulation. Stimulation was applied when 80% of Ach had been delivered. A function generator, attached to a power supply, was set to trigger at the appropriate time point.

B. *In Vitro* Experiments on Myocytes

C2C12

C2C12 myoblasts were purchased from the American Tissue Type Culture Center (Manassas, VA). Undifferentiated myotubes were maintained in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 100 units/mL penicillin. Myotube differentiation was induced by replacing FBS with 2% equine serum (Hyclone). Cells were cultured in 75 cm² culture flasks in a humidified incubator (37°C, 5% CO₂) (Appendix 1). Prior to cell seeding, microposts were cleaned and sterilized. Posts were prepared for seeding with the application of 1 µg/mL fibronectin (Sigma Aldrich) along with 8 minutes of UV-Ozone. Cells were seeded onto the microposts at a density of 100 cells/ µL with the application of 10 µL to each pad. After an incubation period of 1-2 hours to allow for cell adhesions, microposts were mounted onto sterilized glass slides and placed in growth medium (Appendix 2). Differentiation was initiated approximately four days after seeding.

Electrical stimulation of C2C12 was conducted using two platinum mesh electrodes placed on either side of the micropost pad. Single pulse electrical field stimulation ranging from 4V-10V was generated with an isolated stimulator (Master-8, A.M.P.I., Jerusalem, Israel).

C. *In Vitro* Testing of Harvested Muscle

Image Analysis Testing

Biceps femoris (BF) were extracted from 6-7 week old female Sprague Dawley rats euthanized in a carbon dioxide chamber following guidelines and approval from the institutional review board. Upon removal, samples were placed in DMEM transport. Samples were cut to a standard size of 14 mm² using the minimal amount of cuts in order to fit into the experimental set up. A cassette pump (Manostat Carter Cassette Pump System, Thermo Scientific, Waltham, MA) was implemented during testing for fluid circulation set at a flow rate of 5 mL/min. DMEM was pre-incubated for baths and solutions and bath temperature was controlled at 37°C during testing. To prevent samples from moving out of the frame of view of the camera, a thin needle tip was inserted into the center of the sample, securing the sample to the testing platform.

Extensor digitorum longus (EDL) were extracted from 6-7 week old female Sprague Dawley rats euthanized in a carbon dioxide chamber following guidelines and approval from the institutional review board. EDL were excised without damaging the muscle via two small cuts at the tendons leaving the muscle intact. The standard incubation medium was Krebs-Ringer bicarbonate solution (K-R) containing 120.2 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 10 mM glucose. Upon excision, samples were placed in oxygenated K-R at room temperature for transport. A cassette pump was once again used during testing for fluid circulation set at a flow rate of 5 mL/min. K-R solutions were pre-incubated for baths and solutions. Incubation bath temperature during

testing were controlled at 30°C under continuous bubbling with a mixture of 95% O₂ and 5% CO₂. EDL samples were also secured in place to prevent movement out of the frame of the camera.

Deterioration of Excised Muscle

Thirty minute time-lapse studies of excised BF were conducted in both DMEM baths and 20 mg/mL ACh baths. EDL time-lapse studies were conducted in K-R baths and 0.01 µg/mL ACh baths. Samples were excised and prepared for testing per the above mentioned protocol and placed in their respective baths. Images were then acquired every minute for 30 minutes.

Electrical and Electrochemical Testing

Samples were excised and prepared for testing in their respective baths. Testing was conducted in DMEM, K-R, 0.01 µg/mL ACh, 1 µg/mL ACh, and 20 mg/mL ACh baths. Characterization of muscle response to electrical stimulation was conducted with the application of five cycles of 10 msec bipolar pulses applied as a train, or a 50 msec bipolar pulse. Muscles were stimulated via electrical field stimulation implementing bipolar platinum electrodes spaced 25 mm apart with the sample centered in the middle. Pulses were generated via an isolated stimulator (Master-8). Samples were stimulated each minute with the application of one train of stimulation followed by a second train eight seconds later, for a test period of 30 minutes. Testing was conducted using voltages ranging from 1-10V. Images were acquired at point of stimulation via a trigger from the stimulator set to activate the camera. Matlab was implemented for image processing to quantify the area change and contraction rates (Appendix 4).

Force Analysis Testing

EDL were excised from 6-7 week old female Sprague Dawley rats euthanized in a carbon dioxide chamber following guidelines and approval from the institutional review board. EDL were excised without damaging the muscle via two small cuts at the tendons leaving the muscle intact. The standard incubation medium was Krebs-Ringer bicarbonate solution (K-R) containing 120.2 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 10 mM glucose. Upon excision, samples were placed in either oxygenated K-R or ACh solution, at room temperature, for transport. For testing, suture string was tied onto the two ends of the EDL at a distance 10 mm to allow for mounting into the bath. One end of the muscle was attached to a force transducer and the other end to a stable mount (Figure 11). During testing the incubation bath temperature was controlled at 30°C under continuous bubbling with a mixture of 95% O₂ and 5% CO₂.

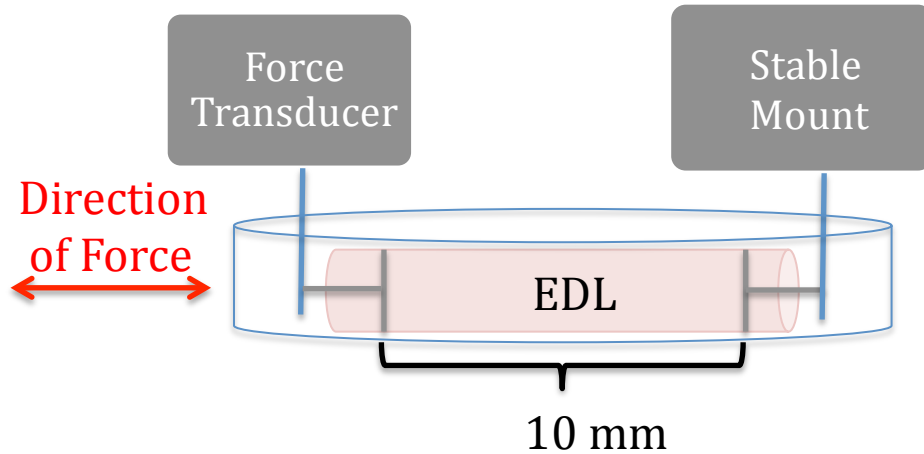


Figure 11. Depiction of EDL mounted onto the force transducer in a bath. Suture string was tied on at a 10 mm distance to allow for attachment to the force transducer and stable mount via hooks.

Electrical and Electrochemical Testing

Electrical field stimulation was carried out in order to characterize muscle response. Electrical field stimulation was again conducted with the application of 50 msec bipolar pulse trains applied to bipolar platinum electrodes spaced 25 mm apart with the sample centered in the middle. Pulses were generated via an isolated stimulator (Master-8, A.M.P.I., Jerusalem, Israel) for single voltage testing and short-term voltage sweeps. Long term voltage sweep testing was conducted using LabView software (National Instruments, Austin, TX) in conjunction with a DAQ card for recording. Long-term voltage sweeps were characterized as repeated extensions of the short -term sweeps. Samples were stimulated every 30 seconds with the application of one train of pulses. Testing was conducted with starting tensions of 0.2g, 0.5g, or 1g and stimulation voltages ranging from 0.5-5V. Testing was conducted in K-R, 1 $\mu\text{g/mL}$ ACh, 100 $\mu\text{g/mL}$ ACh, and 1 mg/mL ACh baths. Matlab was implemented for data processing and calculation of contraction force generated. (Appendix 5). For voltage sweep testing, four different stimulation patterns were tested (Table 1). Long-term voltage sweeps were repeated extensions of the short -term sweeps.

Time (min)	Pattern 1	Pattern 2	Pattern 3	Low Voltage Pattern
0.00	2V	4V	5V	0.5V
0.50	3V	2V	4V	1V
1.00	4V	5V	3V	1.5V
1.50	5V	3V	2V	2V
2.00	5V	3V	4V	0.5V
2.50	4V	5V	2V	1V
3.00	3V	2V	5V	1.5V
3.50	2V	4V	3V	2V
4.00	3V	5V	2V	0.5V
4.50	5V	4V	3V	1V
5.00	2V	3V	4V	1.5V
5.50	4V	2V	5V	2V
6.00	4V	2V	3V	0.5V
6.50	2V	3V	5V	1V
7.00	5V	4V	2V	1.5V
7.50	3V	5V	4V	2V

Table 1. Voltage sweep patterns for electrical and electrochemical testing of EDL.

Chapter 3: Results and Discussion

A. *In Vivo* Pre- Tarsal Orbicularis Testing

A series of *in vivo* experiments on electrical, chemical, and electrochemical stimulation of the pre-tarsal orbicularis muscle were conducted with the goal of developing a prosthetic system for generating eye blinks. The first set of experiments used only electrical stimulation and were on five human subjects suffering from CN-7 palsy [Cockerham, unpublished data]. The second sets of experiments were conducted to investigate the potential of using an electrochemical approach where both ACh and electrical stimulation are applied together.

For all of the experiments described below, movement responses of the eyelid to stimulation were graded using a standardized scoring system. Five categories were used to rate the orbicularis oculi movement:

- 1) No movement
- 2) Twitch: 1-2 mm of eyelid movement
- 3) Partial blink: movement greater than 1-2 mm but without complete eyelid closure
- 4) Complete closure: contraction inducing complete elimination of the palpebral fissure
- 5) A double pulsed blink: upper and lower eyelids touching twice in rapid successive movements

Electrical Stimulation in Human CN-7 Palsy Subjects

In order to determine the maximum current that is tolerable when applied transcutaneously to the upper orbicularis oculi, acutely and chronically denervated subjects were studied to determine if a functional blink could be created using electrical stimulation alone. Although a limited number of human subjects were examined, it is unlikely that tolerable amounts of electrical stimulation could produce complete blinks in patients.

Electrical stimulation in subjects #1, #2, #3, and #4 was unable to produce a blink, and resulted in either no movement or a muscle twitch. The levels of stimulation that caused a twitch in these four subjects approached the limits of patient tolerance. Complete functional blinks were not generated; rather a full body startle type movement was elicited at the upper limits of electrical intensity at all test positions. Subjects #1 and #2 exhibited identical twitch responses to maximum tolerable, yet uncomfortable, electrical current. The highest tolerated level ranged between 40-60 mA and caused an average pain response of 4.1 out of 10 (Figure 12). Subjects #3 and #4 also exhibited twitches at the maximum tolerated electrical potential (mV). Stimuli of 1.8V caused an average pain response of 5.7 out of 10 (Figure 13). Subject #5 exhibited a much higher tolerance level and a complete blink was created using 9V with 150 msec pulse duration (Figure 14).

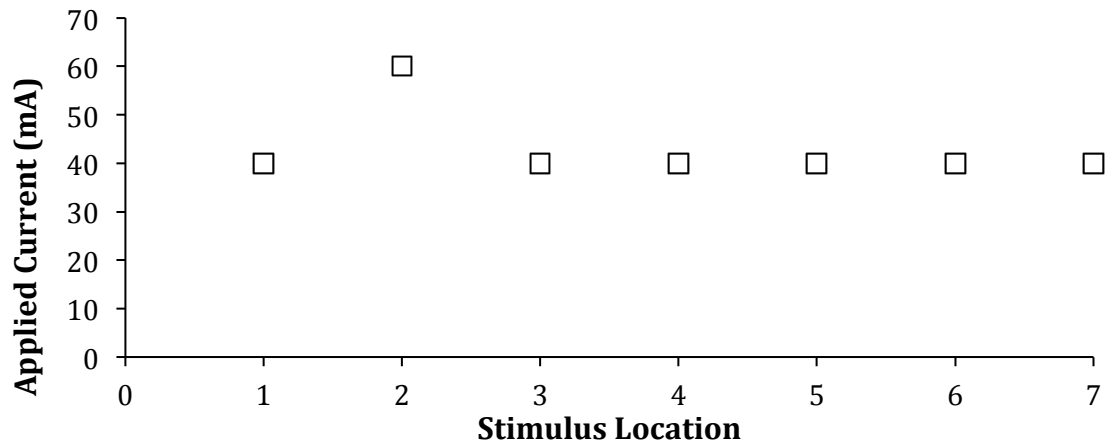


Figure 12. Maximum tolerable electrical current in human subjects #1 & #2

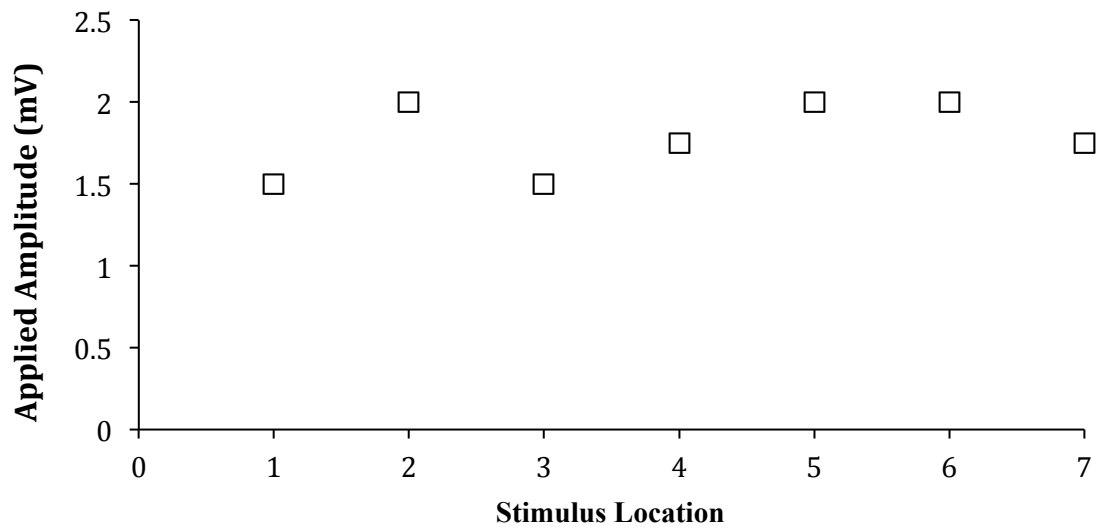


Figure 13. Maximum tolerable electrical potential in human subjects #3 & #4

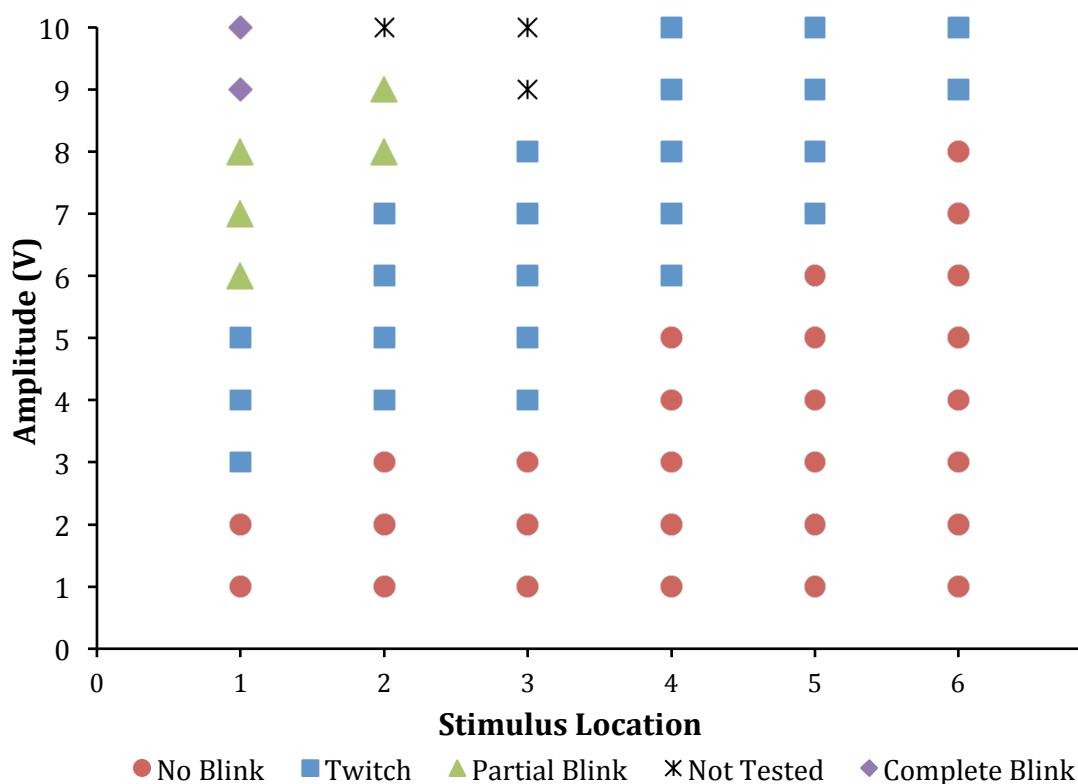


Figure 14. Electrical stimulation responses from human subject #5

Data from four out of five subjects demonstrated that electrical stimulation in any of the seven positions did not elicit a complete blink. It was only possible to generate complete blink in the last patient who demonstrated an abnormal level of pain tolerance at stimulation position #1. The insufficiency of electrical stimulation alone in triggering painless muscle contraction in denervated orbicularis oculi muscles limits the clinical applicability in humans. The pain induced by such stimulation indicated a need for using either chemical stimulation or combining electrical stimulation with neurotransmitter-based stimulation, in order to bring the required current below the pain threshold.

Chemical Stimulation in Rabbits using Microneedle Arrays

The next series of experiments were conducted to determine if chemical stimulation by a neurotransmitter could be used to create controlled eye blinks and avoid the pain caused by electrical stimulation. Acetyl Choline (ACh) was chosen for chemical stimulation because that is the neurotransmitter delivered by the neuron to signal muscle function. A MEMS syringe, previously developed in the Liepmann Lab, was used to deliver ACh to the pretarsal orbicularis muscle in denervated New Zealand White Rabbits (Figure 5). The microsyringe delivers a drug through an array of microneedles and was chosen because it could provide distributed dose of ACh across the surface of the pretarsal orbicularis muscle. The efficacy of chemical stimulation on muscle response was investigated by rapidly

injecting different amounts of ACh ranging from 0.5 μL to 20 μL onto the pre-tarsal orbicularis. Stimulation of the orbicularis with 85 μL of ACh produced a partial blink in the rabbit. The device hindered the production of a complete eyelid blink because the size and weight of the microneedle prototype not only weighed down the eyelid, but also prevented complete closure. The device was then removed and testing was conducted via topical chemical stimulation. Delivery of 200 μL of ACh resulted in tonic closure of the eyelid for one minute while all other volumes did not generate any response.

Electrochemical Testing Using Prototype #1

Because the chemical stimulation did not produce a blink but “all or none” results, it was decided to investigate if a hybrid prototype that provided both chemical and electrical stimulation to the pretarsal orbicularis could elicit a complete blink. The motivation for a combined stimulation was to reduce the electrical requirement by bathing the muscle with ACh. *In vivo* tests with various prototypes, which were continually refined, showed that the intricate cascade that leads to muscle contraction could be initiated at levels below pain thresholds by providing direct delivery of low electric stimulation coupled with the delivery of small volumes of ACh to denervated muscle.

Electrical testing was initially performed and a twitch was consistently obtained with 10-250 msec duration pulses of 5V (Figure 15). Synergistic testing was then performed with various levels of electrical stimulation being applied, between 1-4 V with an average pulse duration of 10 msec, simultaneously with 6 μL of ACh (Figure 16). The combined electrochemical stimulation was more effective and reduced the required electrical potential by half. As shown in the data below (Figures 15 and 16), the application of a 10 msec pulse at 7.5V was required to produce a twitch, while the same response could be generated electrochemically with half the potential (3.7V) when 6 μL of ACh was initially injected.

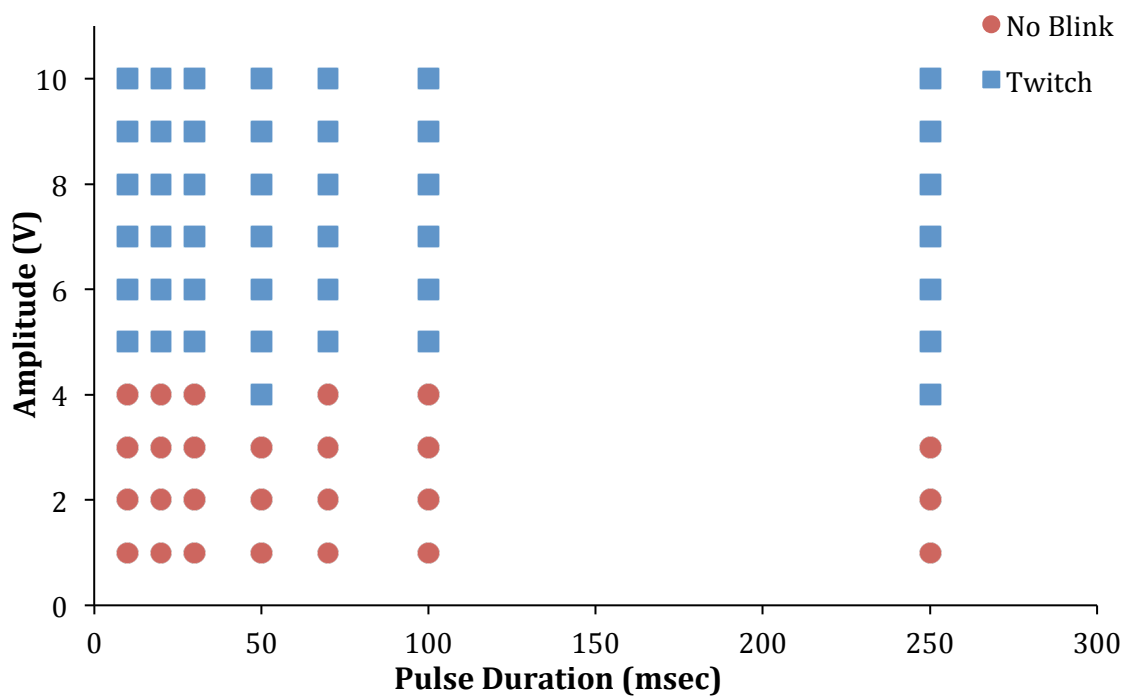


Figure 15. Electrical stimulation efficacy testing of prototype #1, experimental series 1. A twitch of the pretarsal orbicularis muscle was consistently obtained at 5V with pulse durations ranging from 10-250 msec.

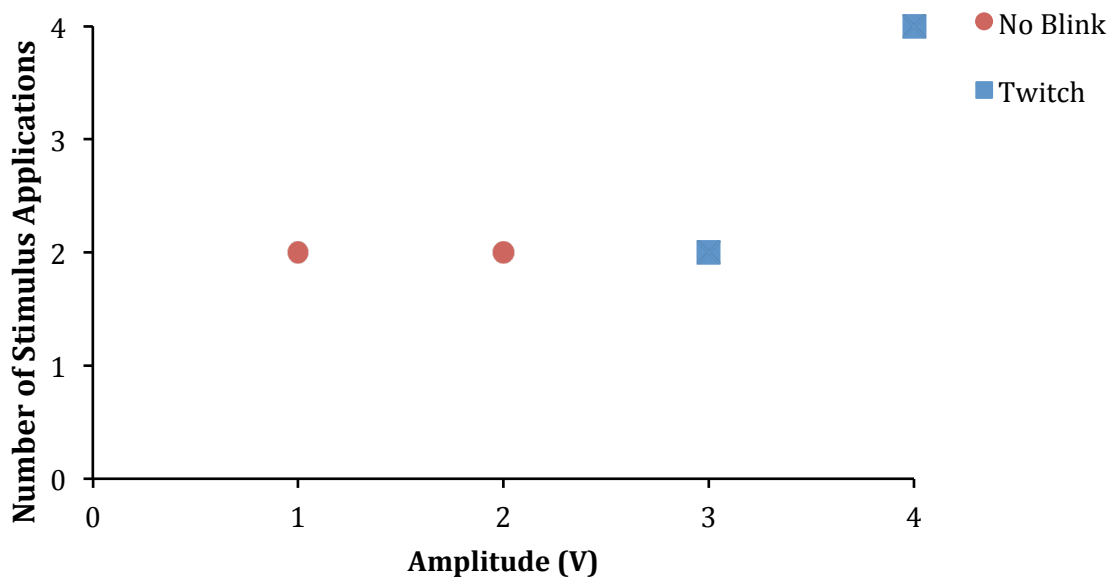


Figure 16. Electrochemical stimulation efficacy testing prototype #1, experimental series 1. A twitch was produced with the application of 6 μ L ACh prior to an average stimulation of 3.7V for 10 msec.

A second series of tests implementing the first electrochemical prototype began with initial testing with electrical stimulation to obtain reference points. Stimulation resulted in a twitch with an average application of 3V for 72 msec. A partial blink with only lower lid recruitment was produced with an average of 7.3V for 13 msec. A partial blink with lower and upper lid recruitment was produced with an average application of 6.8V for 78 msec. There were four occurrences of questionable full blinks where the lids touched but there were trains of twitches as opposed to singular twitches. These occurred with the average application of 9.5V for 50 msec. Complete and double pulsed blinks were produced with the average applications of 10V for 50 msec and 9.5V for 100 msec, respectively (Figure 17). The delivery of 2 μ L ACh coupled with electric stimulation elicited a twitch with the average application of 3.6V for 28 msec and a partial blink with the average application of 8.5V for 31 msec (Figure 18). It was decided to stop testing due to trains of twitches being produced as opposed to singular twitches.

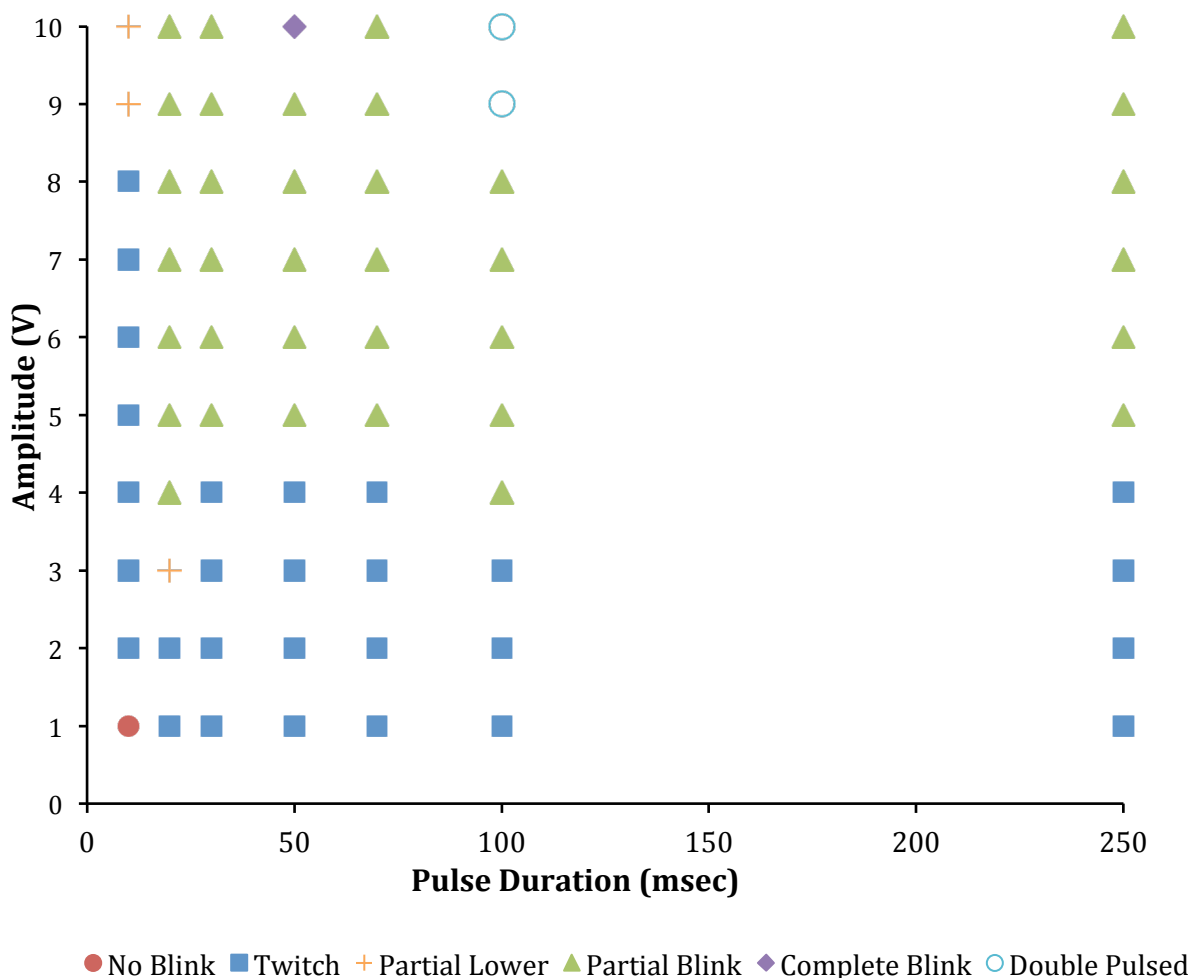


Figure 17. Electric stimulation efficacy testing prototype #1, experimental series 2. A twitch was consistently obtained at 3V with pulse durations ranging from 10-100 msec, while partial blinks were consistently achieved at 5-8V with pulse durations ranging from 20-250 msec.

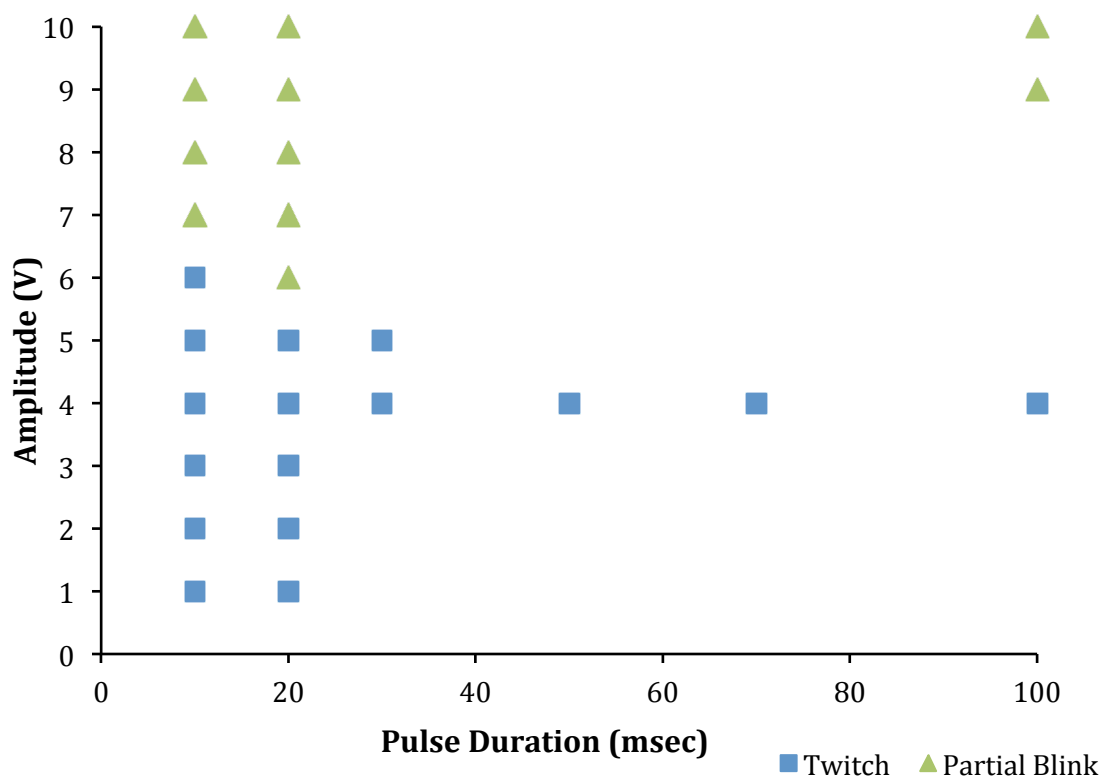


Figure 18. Electrochemical stimulation efficacy Testing prototype #1, experimental series 2. Muscle was bathed in 2 μ L of ACh prior to electrical stimulation. A partial blink was achieved with an average stimulation of 8.5V.

This first series of tests with prototype #1 proved that a blink response could be obtained from the pretarsal orbicularis oculi via a hybrid stimulus. It was demonstrated that the amplitude of the electrical stimulus needed to cause a muscular response could be reduced by incorporating ACh. The second series of experiments demonstrated that exposure of the muscle to ACh prior to electrical stimulation made it possible to use lower voltages with longer pulse durations.

Electrochemical Testing Using Prototype #2

The second prototype was designed in order to avoid activating the levator palpebrae with the delivery of a stimulus along the entire length of the pretarsal orbicularis (Figures 6 and 8). By delivering the stimuli to the inner and outer corners of the eyelid, a partial blink was obtained with electrochemical testing.

A full eyelid blink was obtained with 4.8 mA electrical stimulation alone. However, the application of 8 μ L of ACh and 2 mA at 200 Hz resulted in a synergistic response of a partial blink, or 20% eyelid closure (Figure 19 A-C). The application of 2 mA of electrical stimulus

after electrochemical testing resulted in a smaller twitch than was obtained with electrochemical testing. The inability to obtain a complete blink, even with electrical stimulus, was associated to issues with that specific rabbit. During previous testing, upon stimulation, the rabbit's nose twitched as opposed to its eyelid.

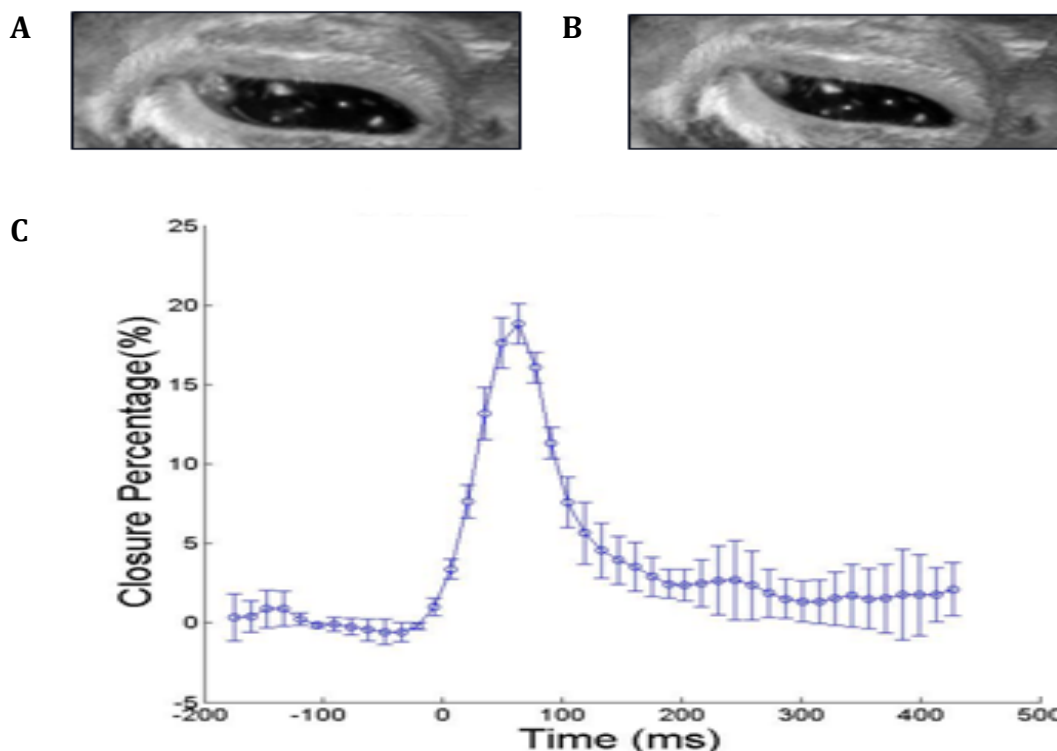


Figure 19. Electrochemical stimulation testing of prototype #2 (A) Eyelid open prior to stimulation at $t = 0$ ms (B) Twitch at $t = 42$ ms with the application of $8 \mu\text{L}$ of ACh and 2 mA stimulation (C) Average eyelid closure of 20%

Although electrochemical prototypes #1 and #2 were only tested a total of three times, it was clear that for all three rabbits the required current to stimulate a muscle contraction could be reduced with the incorporation of ACh, supporting the feasibility of the concept of electrochemical stimulation. However, *in vivo* testing with rabbits proved to be difficult and uncontrolled. Verification of complete CN-7 paralysis was not obtainable for multiple rabbits, which demonstrated partial reinnervation over time, thus making it hard to gauge the influence of the delivered stimuli. Furthermore, it was not possible to determine if acetylcholinesterase was fully responsible for hydrolyzing the ACh or if diffusion had allowed the ACh to run its course.

In addition, there was a time delay associated with the diffusion time of the ACh through the muscle sheath. The difference in response time between the electrical current and drug delivery makes it extremely hard to create a system that will provide the rapid response

needed for a physiologically functional eye blink. There remains the possibility that a prosthetic electrochemical system could be developed that keeps a constant ACh concentration in the muscle fibers. Electrical stimulation would then be used as the active control for eye blinks. The design of this device requires a better understanding of the interaction of chemical and electrical stimulation and, therefore, an experimental approach need to be developed that allowed for a more controlled environment.

B. *In Vitro* Experiments on Myocytes

The previous experiments showed that the characteristic time scales for the effects of electrical and chemical stimulation were significantly different. This results from the diffusion time of the ACh into the muscle. In order to understand the dynamics associated with the electrochemical stimulation, it was decided to investigate a simplified *in vitro* system using arrays of individual myotubes where the environment can be more easily controlled. The temporal response of myotubes to electrochemical stimulation was not affected by diffusion through tissue as in the *in vivo* experiments and the electrochemical environment could be more carefully controlled.

All factors considered, it was proposed to use cultured cells in order to conduct a similar set of electrical and electrochemical experiments. By growing myoblasts on a flexible substrate, force measurements could be obtained^{76,77}. The approach was to grow myoblasts on arrays of micro-posts made from PDMS with the goal of generating parallel muscle fibers supported by flexible posts. The arrays could be put into electrochemical baths with controlled ACh concentrations and electrical potentials. The application of either or both of the stimuli would cause the fibers to contract which could be optically observed and measured. Video images of contractions would be used to measure the speed and force of contractions. The displacements at the tops of the micro-posts could be directly related to the force generation. The main advantage of this experimental model is that the muscle fibers would not have a sheath or significant thickness so that the diffusion time for ACh would be minimized and the individual contributions of the electrical and chemical stimulation could be determined.

C2C12 Seeding and Attachment Density

Focus was initially placed on developing the model by determining the optimal environment for cell growth. Micropost arrays of various radii were seeded with C2C12 to determine the ideal radius and post spacing for cell growth and alignment. The alignment of cells in parallel with the array was critical so that the force measurements could be conducted. Cell alignment was highly favored with posts in array 18 which had a radius of 1.25 μm with a spacing of 3.125 μm between pairs of posts. Cell attachment and alignment was three times lower on posts in array 20 and 16, but higher than in all other arrays. The main characteristic differentiating posts in array 18 was the increased distance in spacing between sets of posts by removing a pair of posts (Figure 20). Proper cell attachment to post tops was further confirmed with SEM imaging of arrays 18 and 20.

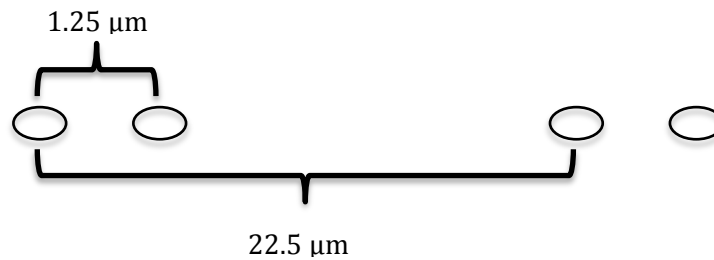


Figure 20. Micropost spacing and alignment for array 18. The distance between neighboring posts in the x-direction is $1.25\ \mu\text{m}$ with $22.5\ \mu\text{m}$ distance to the next pair of posts in the x-direction.

C2C12 cells at a density of $200\ \text{cells}/\mu\text{L}$ were seeded on 15 separate sets of pads consisting of 24 post arrays of various radii. The results are shown in Figure 21 and resulted in the attachment and proper alignment of 45 myocytes to posts in array 18 which had a radius of $1.25\ \mu\text{m}$ with a spacing of $3.125\ \mu\text{m}$ between pairs of posts. Posts in array 20 and 16 resulted in the attachment and proper alignment of 15 and 14 cells respectively (Figure 21). Posts in array 20 had a radius of $1.5\ \mu\text{m}$ with a spacing of $3.75\ \mu\text{m}$ while posts in array 16 had a radius $1.25\ \mu\text{m}$ with a spacing of $3.125\ \mu\text{m}$. Increased spacing between posts, specifically arrays 18 and 23, resulted in the attachment and growth of cells at the base of the posts, or the floor of the pad (Figures 22-24). Myocyte attachment and alignment was further confirmed with SEM imaging (Figure 25.) Stiction between posts was noted upon SEM imaging. Stiction, along with micropost spacing and radius is crucial in creating the optimal environment for cell growth. Microposts spaced too close together will influence cells to grow across as opposed to in line with the arrays. Cell attachment to the micropost is also affected by the micropost diameter. If the radius of a micropost is too small, the cell will not have sufficient space for attachment. As a result, cell attachment to smaller microposts can only be accommodated if there is a closely positioned neighboring micropost. However, if microposts are not at their ideal spacing, stiction will result causing the microposts to attach to one another proving them useless in terms of applying them as cantilever beams for force measurements.

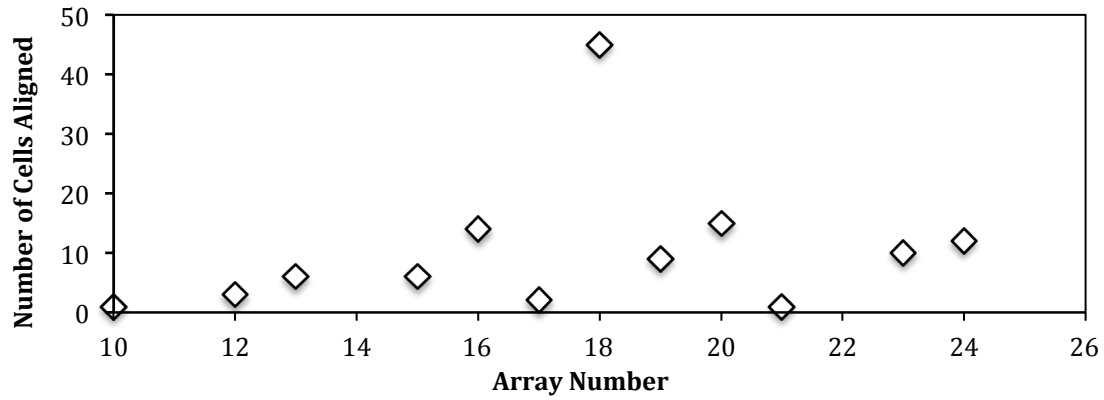


Figure 21. C2C12 cell alignment on micropost arrays with various radii and spacing.

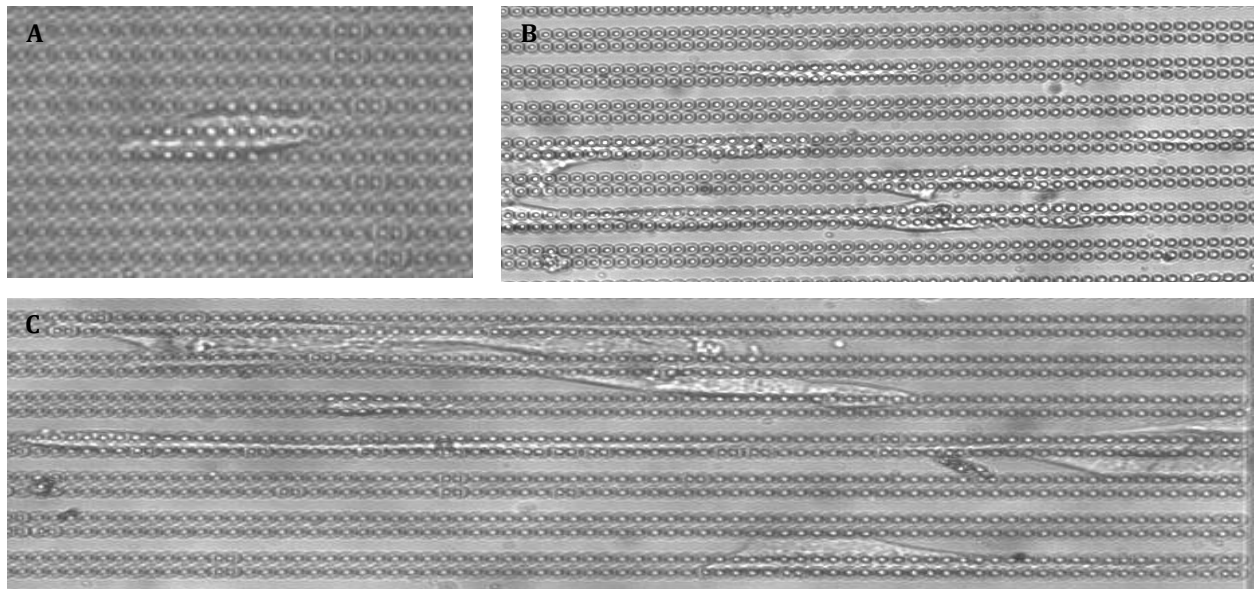


Figure 22. C2C12 attachment and alignment on microposts with varying radii and spacing. A) Myocyte alignment on top of posts in array 15, radius = $1.25\ \mu\text{m}$ and post spacing (center-to center in the x-direction) = $7.5\ \mu\text{m}$. B) Cell alignment on top of three different rows of posts in array 23, radius = $1.5\ \mu\text{m}$ and post spacing (center-to center in the x-direction) = $7.5\ \mu\text{m}$. C) Myocyte alignment and growth on top of posts in array 18, radius = $1.25\ \mu\text{m}$ and post spacing (center-to center in the x-direction) = $7.5\ \mu\text{m}$. Note cell attachment to base of pad in spacing between microposts.

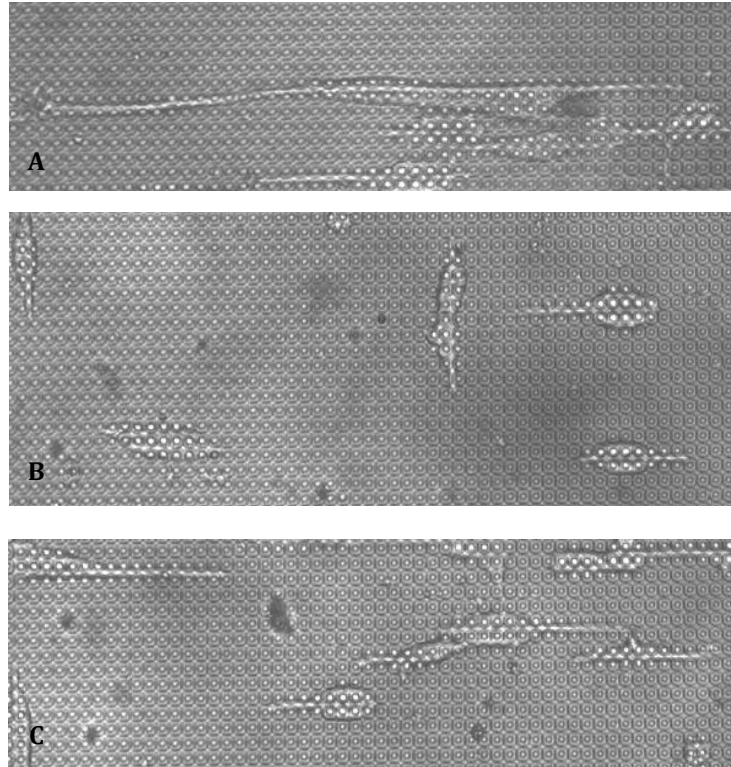


Figure 23 A-C. C2C12 attachment and alignment on microposts in array 20. Post were spaced $7.5\ \mu\text{m}$ (center-to center) with a radius of $1.5\ \mu\text{m}$.

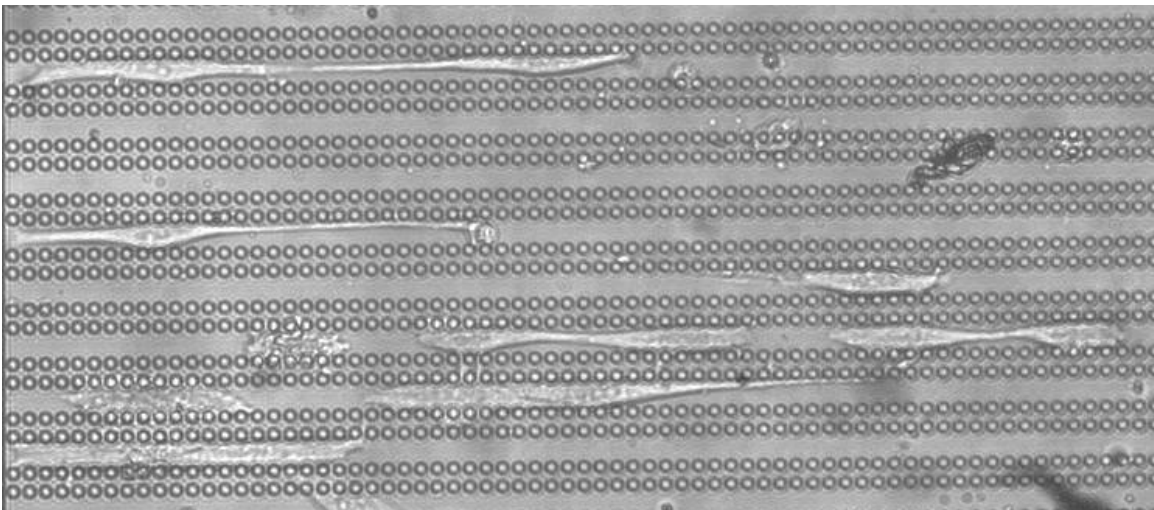


Figure 24. Myocyte attachment and growth was noted in arrays with increased spacing. Sets of posts were removed in array 23 to increase spacing between posts in order to assist in cell growth and alignment to the tops of the posts. However, cells had a tendency of attaching to the flooring of the pad.

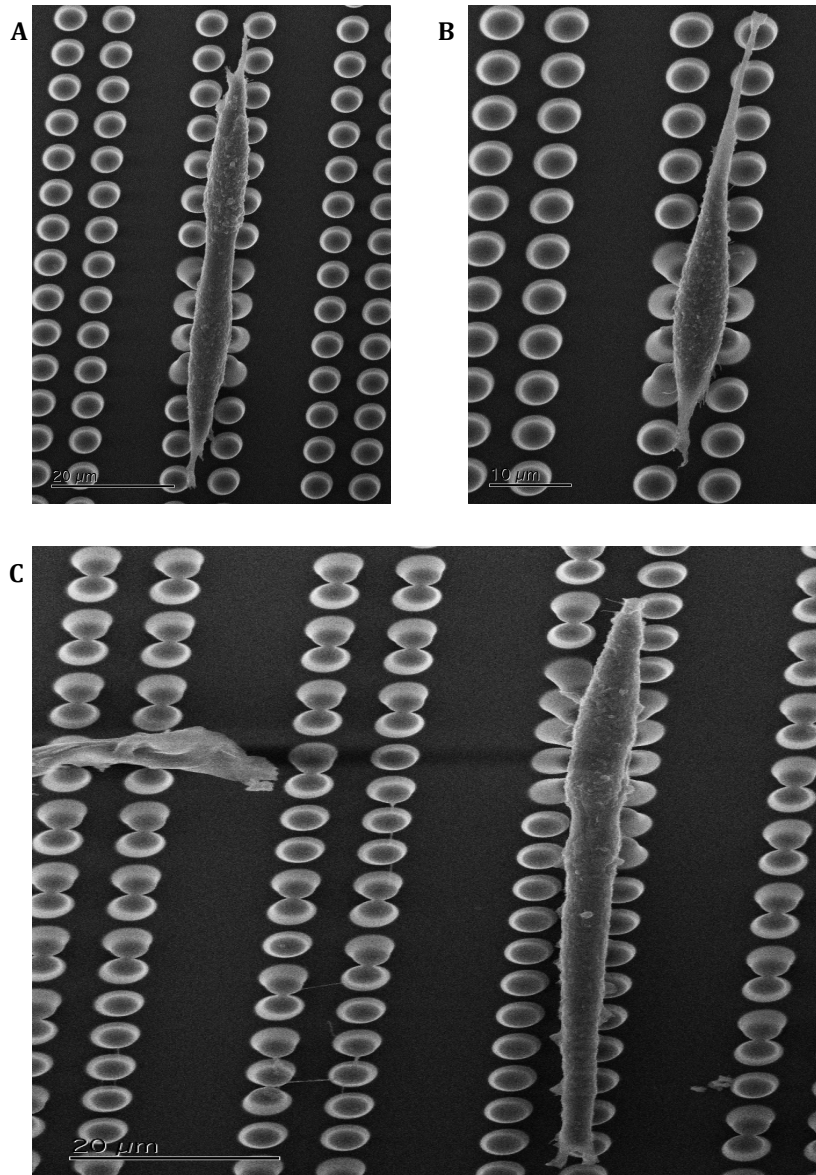


Figure 25. SEM images of differentiated C2C12 cells attached and aligned to microposts. A-B) Micropost from array 23, radius = $1.5\ \mu\text{m}$ and post spacing (center-to-center in the x-direction) = $7.5\ \mu\text{m}$ C) Microposts from array 18, radius = $1.25\ \mu\text{m}$ and post spacing (center-to-center in the x-direction) = $7.5\ \mu\text{m}$.

Testing focused on determining the correlation between electrical stimulation to myotube excitability indicated by the contraction force generated. Unfortunately, 4V-10V electrical field stimulation of C2C12 myocytes attached and aligned to the tops of the microposts generated sufficient contraction forces to detach cells from the posts. Further investigation determined that the focal adhesions to the microposts were not strong enough to stay attached and bend the microposts. Cell study experiments were then abandoned and focus was shifted to primary harvesting of skeletal muscle to investigate the response of skeletal muscle to combined electro-chemical stimulation.

C. *In Vitro* Experiments on Biceps Femoris

Because of the problems associated with growing sufficient myotubes and especially the insufficient adhesion to the microposts, the experiments were continued using excised muscle from laboratory rats. This system is not as ideal because the diffusion time of the ACh becomes more significant. In addition, there was variability between the muscle samples in terms of uniformity of muscle thickness and dimensions.

Deterioration of Excised Muscle

The biceps femoris, extracted from adult Sprague Dawley rats was used as a model for the orbicularis oculi. Instead of measuring post deflection resulting from linear contractions of single muscle cells, muscle area was analyzed to characterize the response of the muscles to electrochemical stimulation. The area of excised biceps femoris muscle changes even without electrical stimulation because the excised muscle deteriorates when it is removed from its natural environment (Figure 26). However, the consistency in the trend of area change of samples in DMEM indicates that the measurement technique is still valid for examining the viability of the tissue as a function of time. The area of samples in ACh change more rapidly as expected, since ACh induces muscle contraction (Figure 24). Samples tested in a DMEM bath without electrical stimulation resulted in an average change in sample area of 0.86% with a SEM of 0.002% (n=2). The average change in sample area in a 20 mg/mL ACh bath, without electrical stimulation, was 6.1% with a SEM of 0.015% (n=2). The normalized percent area change is calculated as the area of the sample at each time point divided by the sample area at $t = 0$.

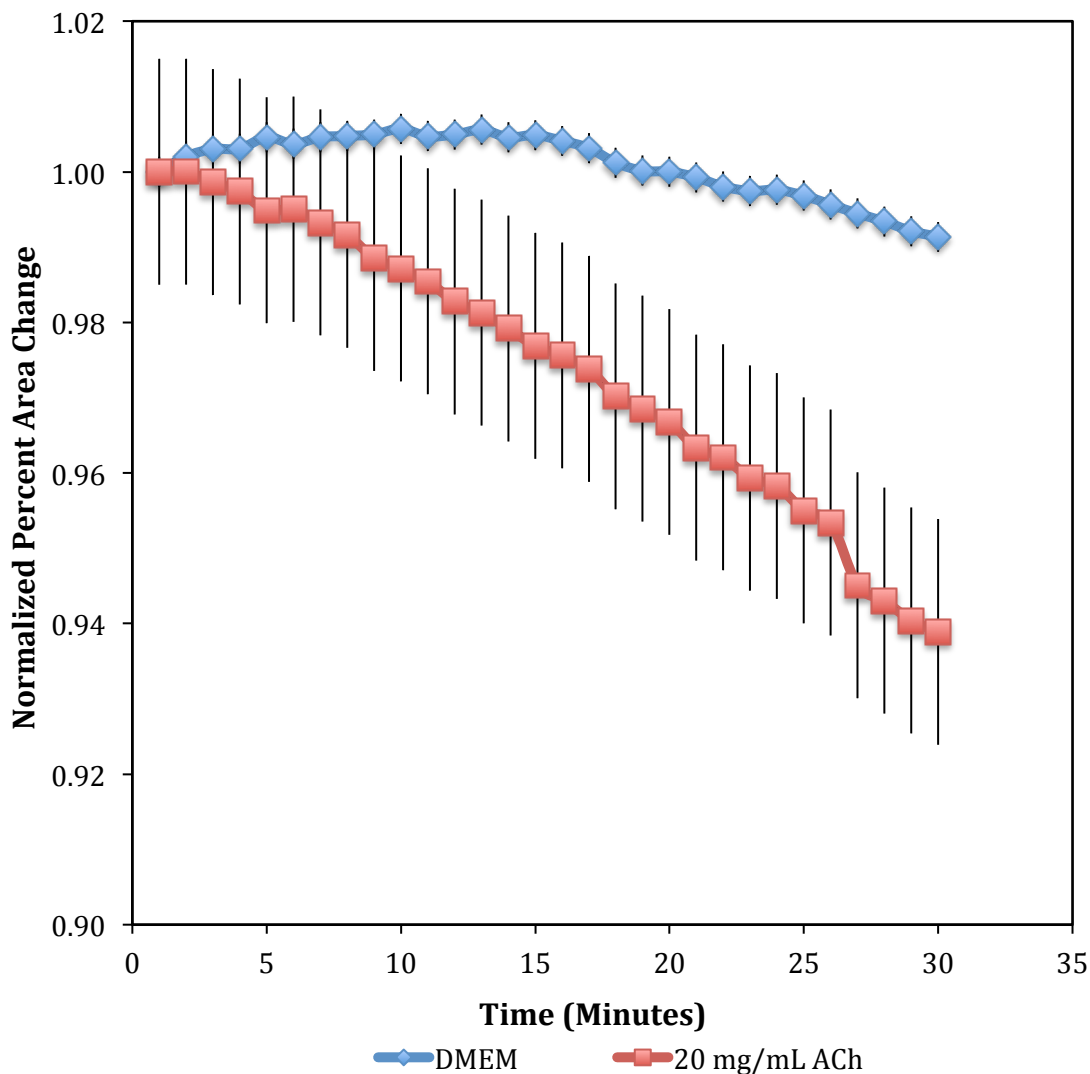


Figure 26. Area of excised biceps femoris muscle in various baths without electrical stimulation. Change in sample areas in DMEM (n=2) and 20 mg/mL ACh baths (n=2) over 30 minutes were 0.86% and 6.1% respectively.

After the muscle is excised, there is a time dependent decrease in area due to the onset of rigor mortis. The lack of ATP causes a build up of calcium, which binds with troponin creating cross-bridges between the actin and myosin. Due to death, the body is unable to complete the cross-bridge cycle that consists of unbinding the actin from the myosin, thus leaving the muscle in a constant state of contraction. This is demonstrated by the change in area over time in DMEM. The application of ACh increases the change in area by initiating contraction. Samples placed in the 20 mg/mL ACh bath had an area change of 6.1%, illustrating the combined effect of acetylcholine and the deterioration of the excised muscle.

Electrical Stimulation

The electrical response of the excised biceps femoris had to be characterized prior to electrochemical stimulation in order to establish a baseline. Various parameters such as the amount of area change and the rate of contraction at different levels of electrical stimulation are key parameters in understanding muscle dynamics. A range of excitation voltages were applied in order to identify the voltage which provided the most consistent response. Testing demonstrated that an increase in excitation voltage is correlated with an increase in the percent area change which makes sense because increased electrical stimulation increases the number of muscles cells activated, thus producing a higher contraction force (Figure 27). An excitation voltage of 5V produced the most repeatable response in terms of area change and the highest rate of contraction. The rate of change of area over time was found to consistently increase from 1V to 5V (Figure 28). However, unlike the trend in area change, the rate of change of area over time decreased at voltages higher than 5V. Indicating that 5V is the level of electrical stimulation that activates all of the muscle fibers in the bicep femoris, thus producing the maximum force output.

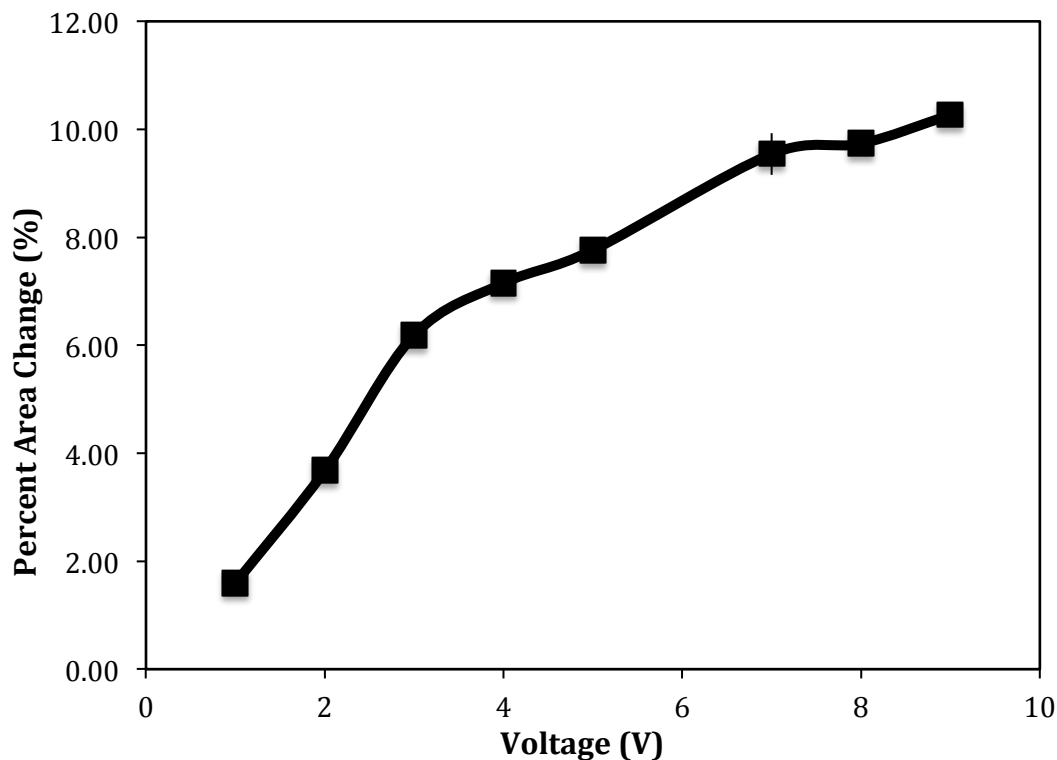


Figure 27. The percent area change in response to electrical field stimulation of the muscle, in DMEM, at various voltages displaying an average of four contractions with an eight second interval between pulses (n=4).

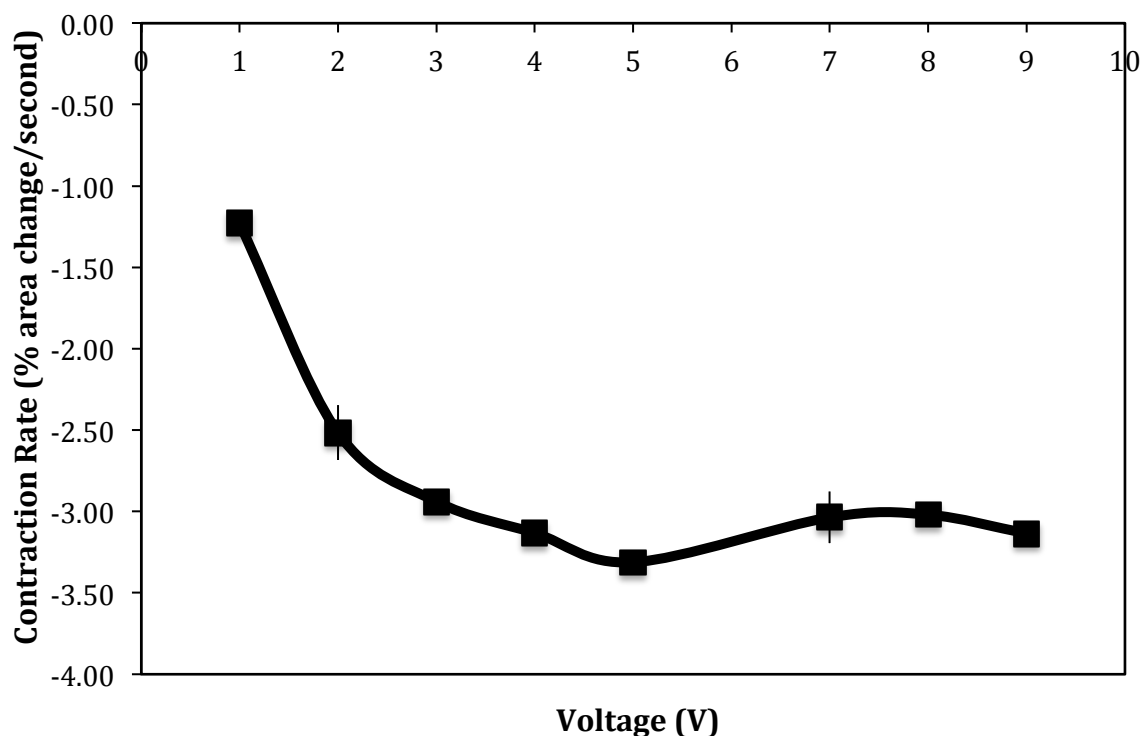


Figure 28. Rates of contraction with the application of electrical field stimulation. Maximum rate of contraction over time occurs with a 5V bipolar electrical field stimulation (n=4).

Electrochemical Stimulation

The application of ACh to the sample prior to electrical stimulation is expected to initiate the muscle contraction. Prior to reducing the amount of electrical stimulation required to complete the muscle contraction, it was critical to find a concentration for the ACh bath that allows for the initiation of the contraction without overloading the muscle into tetanic contraction.

Electrochemical testing of the excised biceps femoris in a DMEM bath with the application of 5V electrical field stimulation resulted in an average initial change in area of 10.74% with a SEM of 1.75% (n=2). The application of 5V electrical field stimulation in a 20 mg/mL ACh bath resulted in an average initial change in area of 9.39% with a SEM of 0.45% (n=2). A sample bathed in a 10 mg/mL ACh bath and stimulated with 5V electrical field stimulation had an initial change in area of 7.65% (n=1). The samples tested in DMEM demonstrate a faster rate of decline in comparison to samples in baths of various concentrations of ACh (Figure 29).

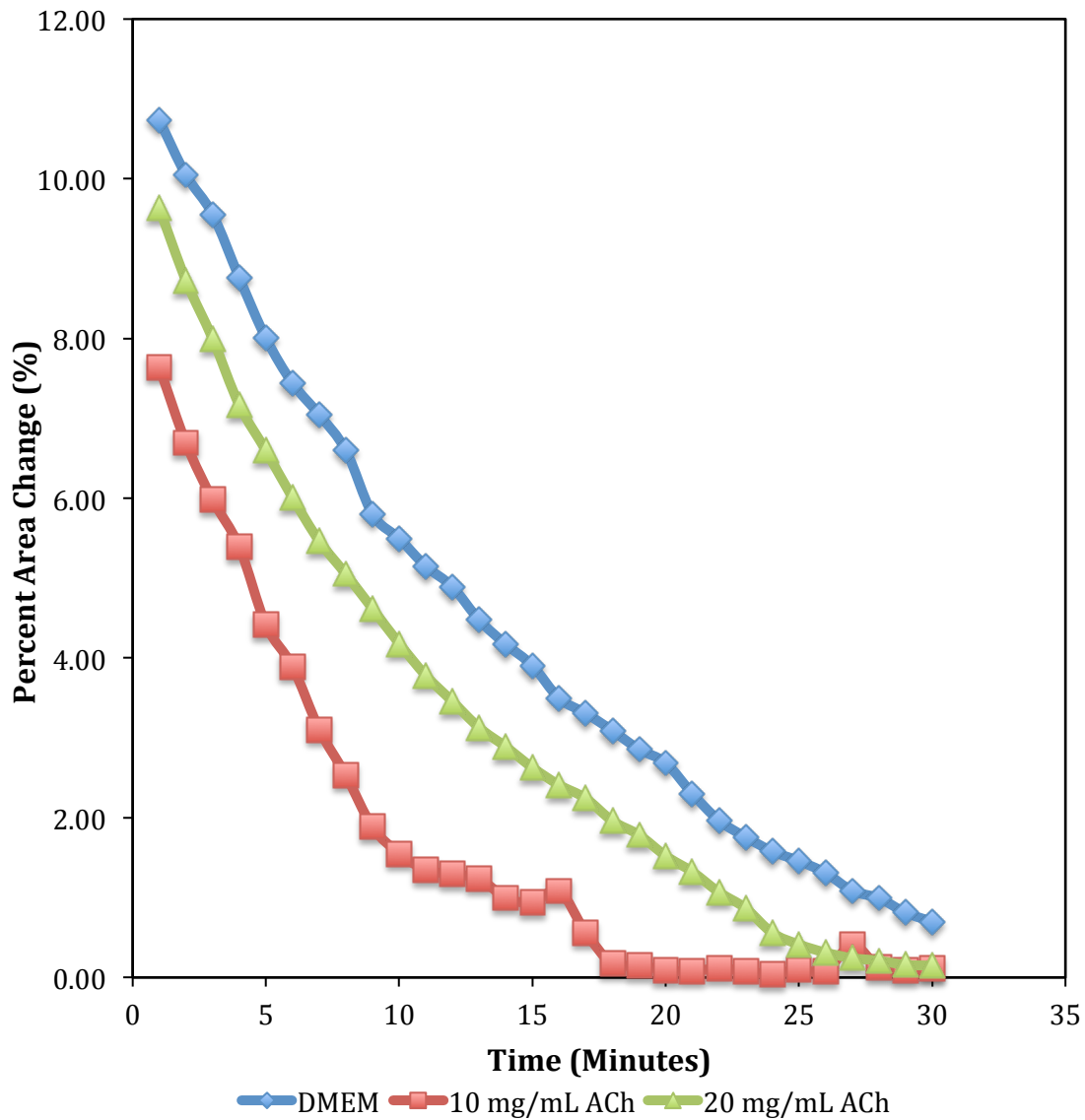


Figure 29. Electrical stimulation of the excised biceps femoris in various baths. 50 msec bipolar 5V electrical field stimulation was applied every minute for 30 minutes.

The deterioration of the excised bicep femoris upon the application of 5V electrical field stimulation occurs at a similar rate between samples in a DMEM bath or an ACh bath. Regardless of the presence of ACh, the similar rates of deterioration indicate a potential overload of electrical stimulation on the samples. A higher percentage of area change was expected for samples simulated in the ACh baths, however FIGURE X clearly illustrates a larger change in area when the samples were placed in a DMEM bath. This leads to the conclusion that the tested concentrations of ACh saturated the muscles. ACh samples also demonstrated a reduced ability to recover to their original areas, further suggesting that the tested concentrations of ACh overloaded the muscle samples into tetanic contraction.

Samples stimulated in a 20 mg/mL ACh bath demonstrated a similar trend as the 10 mg/mL ACh bath. However, the lower rate of decline of the sample in the 10 mg/mL ACh bath indicates that a lower concentration of ACh could potentially aid in delaying, or avoiding, tetanic contraction and assist in long term muscle response. It must be noted that the concentrations of ACh used for these series of tests were decided upon in order to mimic the concentration levels found in Miochol®-E.

Effect of Electrical Field Stimulation in Various Baths

The effect of electrical field stimulation in different baths was examined using a lower concentration of ACh in order to avoid tetanic contraction. It was once again observed that an increase in electrical field stimulation correlates with an increase in the percent area change for an excised biceps femoris. The percent area change of the initial contraction of a sample in DMEM stimulated with an electrical field stimulation at 3V was found to be 5.53% while a 5V stimulation resulted in 6.35% area change. The initial contraction of a sample stimulated at 3V in a 0.01 $\mu\text{g/mL}$ ACh bath resulted in a 7.71% change in area. A 5V stimulation of a sample in a 0.01 $\mu\text{g/mL}$ ACh bath produced a 11.42% change in area. 5V electrical field stimulation in both DMEM and ACh baths resulted in a higher rate of decline in comparison to 3V stimulation (Figure 30).

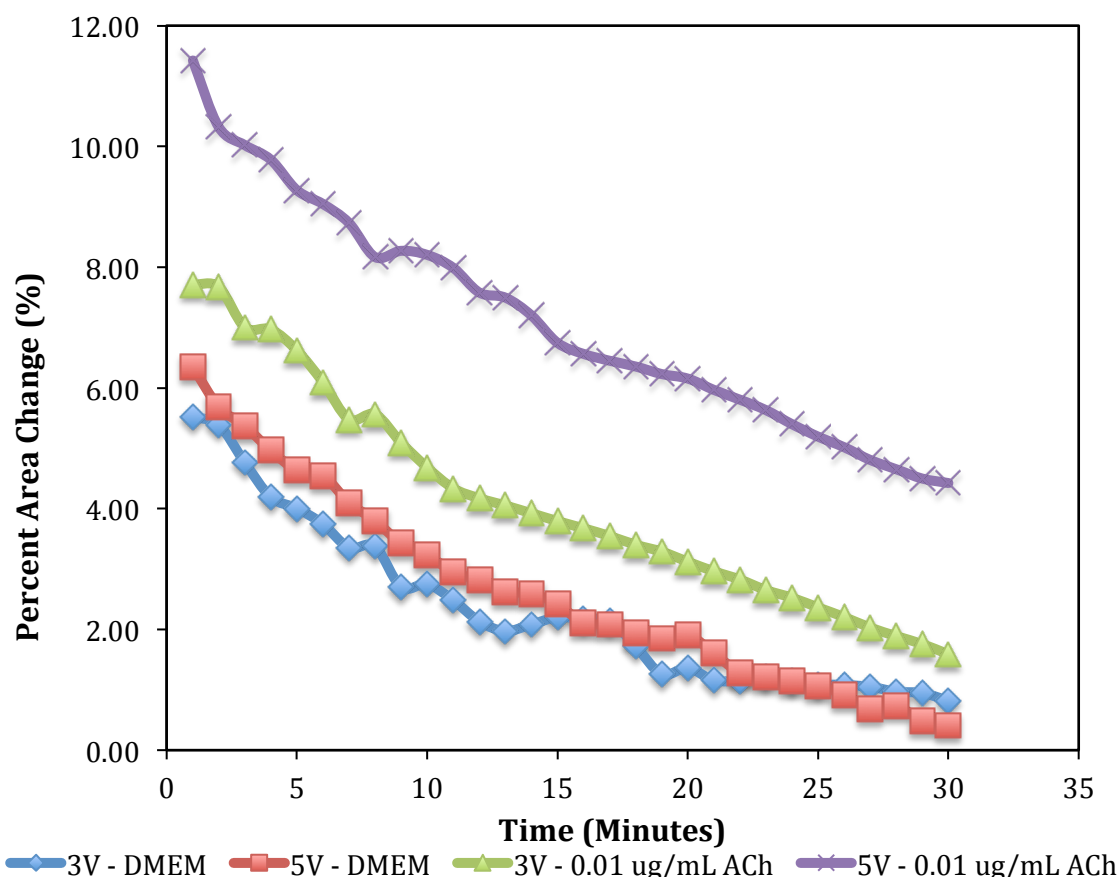


Figure 30. Electrochemical testing on excised biceps femoris implementing lower concentrations ACh baths (n=4).

Comparing 5V and 3V electrical stimulation in DMEM shows that an increase in excitation voltage is correlated with an increase in the percent area change. The increase in percent area change can be associated to an increased number of contractile units being activated. The percent area change of the initial contraction increased with both 3V and 5V stimulation when samples were placed in a 0.01 $\mu\text{g/mL}$ ACh bath. The percent area change of the initial contraction with 3V stimulation in the ACh bath was more than 1% higher than the percent area change of the initial contraction with 5V stimulation in DMEM. Thus illustrating how electrochemical stimulation, with the application of ACh, can be effective in not only initiating a muscle contraction but also increasing it as well while using a lower level of electrical stimulation. This is due to the fact that the application of ACh increases the number of cross-bridges recruited, thus compensating for the lower applied voltage.

When placed in a 0.01 $\mu\text{g/mL}$ ACh bath, the sample stimulated at 5V demonstrated a faster rate of fatigue. This may be associated with overloading the muscle both electrically and chemically. However, the sample stimulated at 3V demonstrates a less dramatic slope in the same concentration ACh bath, it can be concluded that the sample stimulated at 5V was being fully stimulated electrically indicating a need for testing at lower voltages.

D. *In Vitro* Experiments on Extensor Digitorum Longus - Image Analysis

Following the first set of image analysis experiments, it was realized that there are certain limitations with the bicep femoris in terms of uniformity of muscle thickness and the large size of the muscle. When testing with the bicep femoris muscle, samples must be cut down in order to fit into the experimental set up. Cutting the muscle may interfere with normal contractions and not reflect the true behavior of the muscle as a whole. Testing was then conducted using EDL samples that conferred several advantages. First, the EDL muscle is compact in size allowing for testing without cutting and damaging the muscle. Second, the EDL can be easily excised without damaging the muscle via two small cuts at the tendons leaving the muscle intact. Lastly, the EDL has a high degree of uniformity of thickness. With these advantages, testing was conducted using a lower stimulation voltage, to avoid electrical overloading of the muscle, and a lower concentration of ACh for the baths to avoid tetanic contraction.

Deterioration of Excised Muscle

Time-lapse studies of the EDL muscle in different baths without electrical stimulation provided a baseline of area change in response to the deterioration of the excised muscle. Samples placed in the 0.01 $\mu\text{g/mL}$ ACh baths displayed a smaller change in area over the 30 minute period than samples in K-R solution baths (Figure 31). Excised EDL muscle tested in a K-R bath without electrical stimulation resulted in an average change in sample area of 4.5% with a SEM of 0.004% ($n=4$). The average change in sample area in a 0.01 $\mu\text{g/mL}$ ACh bath, without electrical stimulation, was 1.7% with a SEM of 0.002% ($n=4$). The normalized percent area change is calculated as the area of the sample at each time point divided by the sample area at $t = 0$.

It was expected that samples in ACh would have a larger change in area due to the presence of ACh initiating muscle contraction. The lower change in area over time of samples bathed in 0.01 $\mu\text{g}/\text{mL}$ ACh illustrates that the concentration of ACh being applied did not limit the muscle's capability to contract and did not induce tetanic contraction. Thus confirming an effective concentration for testing.

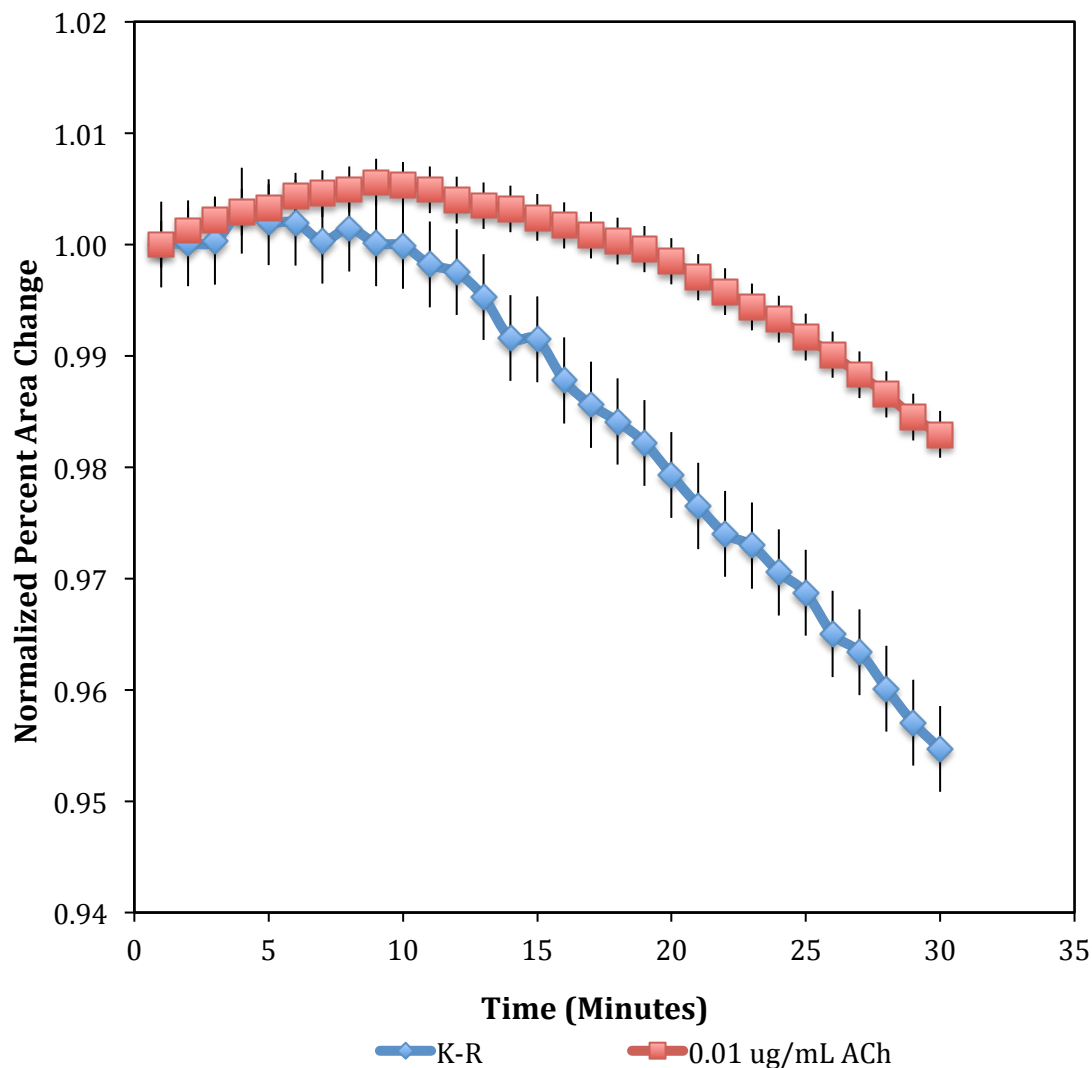


Figure 31. Area of excised EDL muscle in various baths without electrical stimulation. Change in sample areas in Krebs-Ringer bicarbonate solution (n=4) and 0.01 $\mu\text{g}/\text{mL}$ ACh baths (n=4) over 30 minutes were 4.5% and 1.7% respectively.

Electrochemical Stimulation

Electrochemical testing of the excised EDL in a K-R bath with the application of 3V electrical field stimulation resulted in an average initial change in area of 13.32% with a SEM of 2.20% (n=3). The application of 3V electrical field stimulation to samples in a 0.01 $\mu\text{g/mL}$ ACh bath resulted in an average initial change in area of 14.21% with a SEM of 0.75% (n=4). Samples bathed in a 0.01 $\mu\text{g/mL}$ ACh bath and stimulated with 3V electrical field stimulation demonstrated an average initial change in area of 13.43% with a SEM of 1.4% (n=2). The samples tested in K-R solution had a faster rate of decline in comparison to samples tested in baths of various concentrations of ACh.

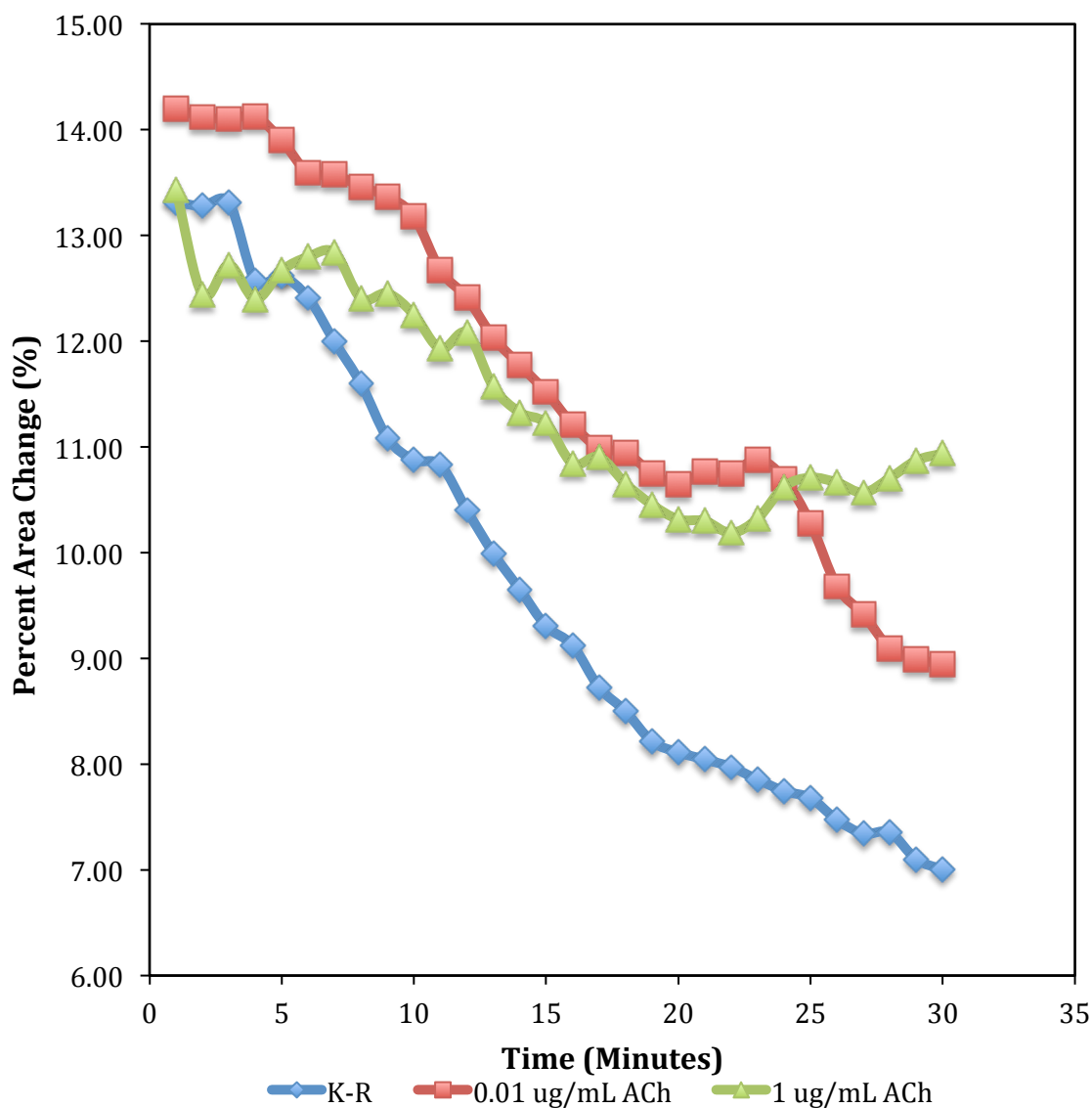


Figure 32. The effect of electrical field stimulation on excised EDL in various baths (n=9).

Electrochemical stimulation of samples with 3V electrical field stimulation in a 0.01 $\mu\text{g/mL}$ ACh bath produced a higher percentage of area change than samples stimulated at the same voltage level but in a K-R bath. The application of ACh increased the contraction area change, thus further confirming that the concentration of ACh is an important factor when conducting electrochemical testing. Unlike previous tests where the ACh saturated the muscle and hindered the muscle contraction, the ACh concentration of 0.01 $\mu\text{g/mL}$ enhanced the muscle contraction producing a larger change in area. The deterioration of the excised EDL samples with the application of 3V electrical field stimulation occurred at a faster rate in K-R baths in comparison to samples stimulated in an ACh baths. The lower rate of decline of samples in ACh solutions can be attributed to the efficacy of the ACh concentration initiating a muscle contraction without inducing tetanic contraction.

Image analysis EDL testing confirmed that the application of ACh initiates and enhances muscle contraction. However, limitations with the testing technique and the post-experimental processing highlighted the fact that they had to be revised. First, muscles must act against a force in order to generate work. The testing technique was modified so that the muscles were under tension and could thus act against a force. Second, fluctuations seen in the plots are most likely associated to the limitations of the image analysis algorithm. Unfortunately, the image analysis algorithm could not be further refined to reflect a more accurate calculation of the change in area.

E. *In Vitro* Experiments on Extensor Digitorum Longus– Force Analysis

Electrical Stimulation

Testing was changed from image analysis to force analysis, by implementing the use of a force transducer. This allowed for quantifiable force measurements along with the ability to place samples under tension. The electrical response of the excised EDL in K-R solution had to be characterized prior to electrochemical stimulation in order to establish a baseline. In response to electrical field stimulation in a K-R bath at a 0.2g starting tension, the excised EDL demonstrated a slight increase in average initial contraction when stimulated at 2V and 2.5V (Figure 33). Stimulation at 2V produced an average initial contraction force of 0.012N with a SEM of 0.001N ($n=3$) while stimulation of 2.5V produced an average initial contraction force of 0.014N with a SEM of 0.003N ($n=3$). The application of 3V electrical field stimulation resulted in an average initial contraction force of 0.012N with a SEM of 0.001N ($n=2$).

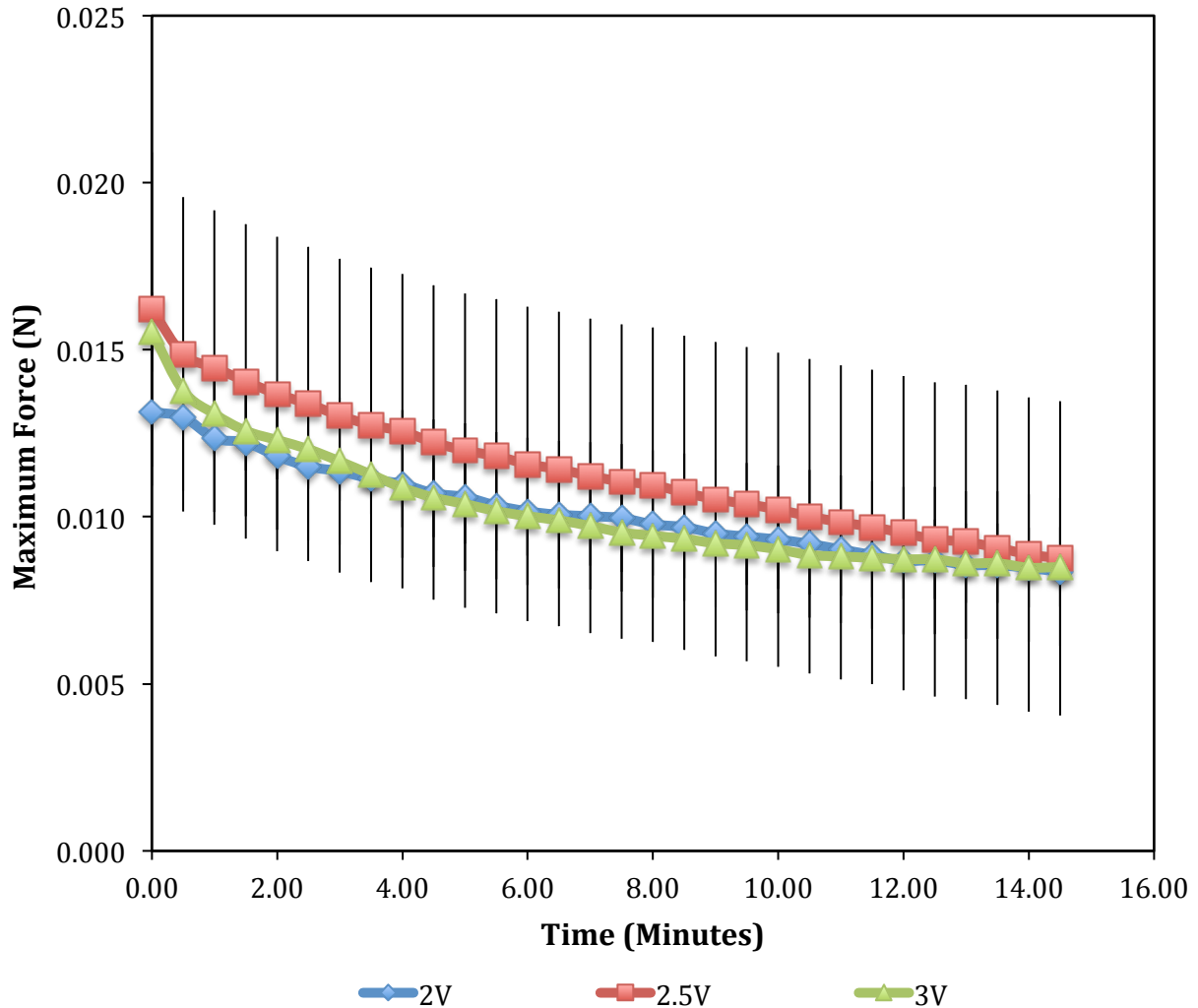


Figure 33. The effect of electrical field stimulation on excised EDL in K-R solution under 0.2g starting tension (n=8).

An increase in electrical stimulation from 2V to 2.5V resulted in a slight increase in contraction force. Since the electrical stimulation was only increased by 0.5V, only a small increase in force was expected. This was demonstrated by an increase of the average initial contraction force from 0.012N to 0.014N. The rate of decline of samples stimulated at 2.5V was slightly higher in comparison to samples stimulated at 2V. This is because higher forces were generated with the higher level of electrical stimulation, thus causing the muscle to fatigue at a faster rate. Surprisingly, samples stimulated with 3V electrical field stimulation responded in a similar manner as samples stimulated at 2V. A possible explanation for such behavior could be that the two samples tested at 3V produced contraction forces on the lower end of the range of potential contraction forces if more samples had been tested. Another possible explanation is that 3V electrical field stimulation was not activating an ideal number of contractile units due to the low starting tension.

Influence of ACh

Samples tested in ACh behaved the same as samples tested in K-R, when soaked in K-R upon excision (Figure 34). 3V stimulation of samples that had been soaked in K-R upon excision and tested in K-R resulted in an average initial contraction force that was equivalent to samples soaked K-R and tested in 1 $\mu\text{g}/\text{mL}$ ACh baths. Electrochemical testing of the excised EDL in a K-R bath with the application of 3V electrical field stimulation resulted in an average initial contraction force of 0.012N with a SEM of 0.001N ($n=2$). The application of 3V electrical field stimulation to samples soaked in K-R solution upon excision but tested in a 1 $\mu\text{g}/\text{mL}$ ACh bath resulted in an average initial contraction force of 0.011N with a SEM of 0.001N ($n=2$).

The similar response of the samples tested in ACh to those tested in K-R indicates that the ACh did not have a sufficient amount of time, the testing period, to diffuse properly into the muscle since there is no evidence of the ACh enhancing the contraction. This issue was not a problem in previous tests when a higher concentration of ACh was applied. The lower concentration solutions must be compensated with a longer soak period in order to allow the ACh enough time to diffuse into the muscle. The testing technique was revised so that samples that were to be tested in ACh would be soaked in ACh upon excision, providing enough time to allow the ACh to diffuse into the sample.

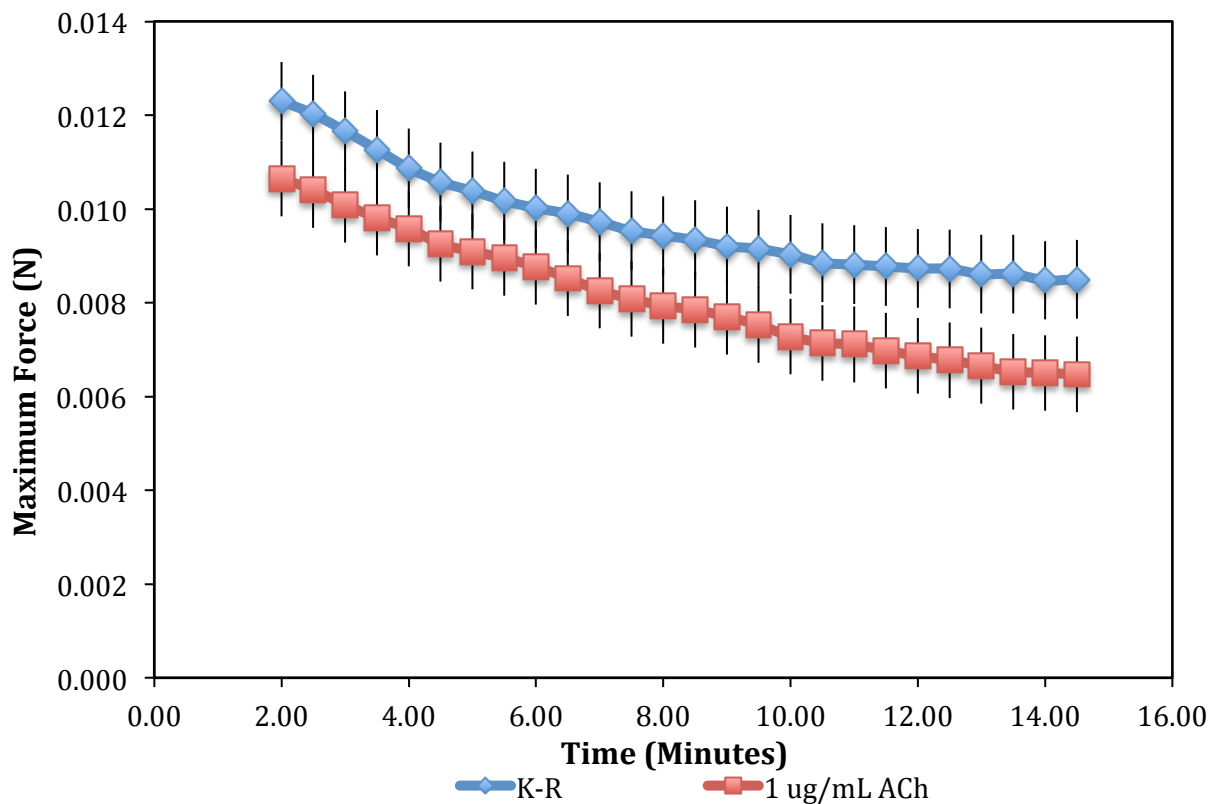


Figure 34. Electrical and electrochemical testing of excised EDL in various baths ($n=4$).

Effect of Starting Tension on Force Production

The amount of force a muscle can produce depends on its initial length. The length is correlated to the starting tension that is created by the muscle being stretched. An optimal length, or starting tension, must be determined for force production. If a muscle is too short or not under a sufficient amount of tension, the thick and thin filaments of the sarcomere interfere with one another and the muscle force generated will be low. Consequently, if a muscle is too long, or under too much tension, the thick and thin filaments will not be able to create as many cross-bridges therefore the force produced will be low once again.

The application of 3V electrical field stimulation to samples in K-R solution under 0.2g starting tension produced an average initial contraction force of 0.012 N with a SEM of 0.001N (n=2). A sample tested in K-R under 0.5g starting tension had an initial contraction force of 0.023N (n=1). Samples soaked in K-R solution upon excision and tested in 1 $\mu\text{g/mL}$ ACh bath under 0.2g starting tension had an average initial contraction force of 0.011N with a SEM of 0.001N (n=2). A sample soaked in K-R solution upon excision and tested in 1 $\mu\text{g/mL}$ ACh bath under 0.5g starting tension had an initial contraction force of 0.030N (n=1).

The response of excised EDL to 3V electrical field stimulation at different starting tensions was examined in both K-R solution and ACh (Figure 33). Testing demonstrated that an increase in starting tension is correlated to an increase in maximum contraction force. With a 0.2g starting tension, samples tested in 1 $\mu\text{g/mL}$ ACh baths did not have an increase in response as expected. However, a sample with a 0.5g starting tension tested in a 1 $\mu\text{g/mL}$ ACh bath showed a jump in contraction force when compared to testing in K-R. The increase in contraction force with ACh is correlated to the presence of ACh in combination with the sample being at its optimum length with the 0.5g starting tension. Samples demonstrated a similar rate of decline to their counterparts under the same amount of tension, indicating the presence, or absence, of ACh does not influence the rate of fatigue. However, the presence of ACh did result in a higher final contraction for the sample tested in ACh under a 0.5g starting tension.

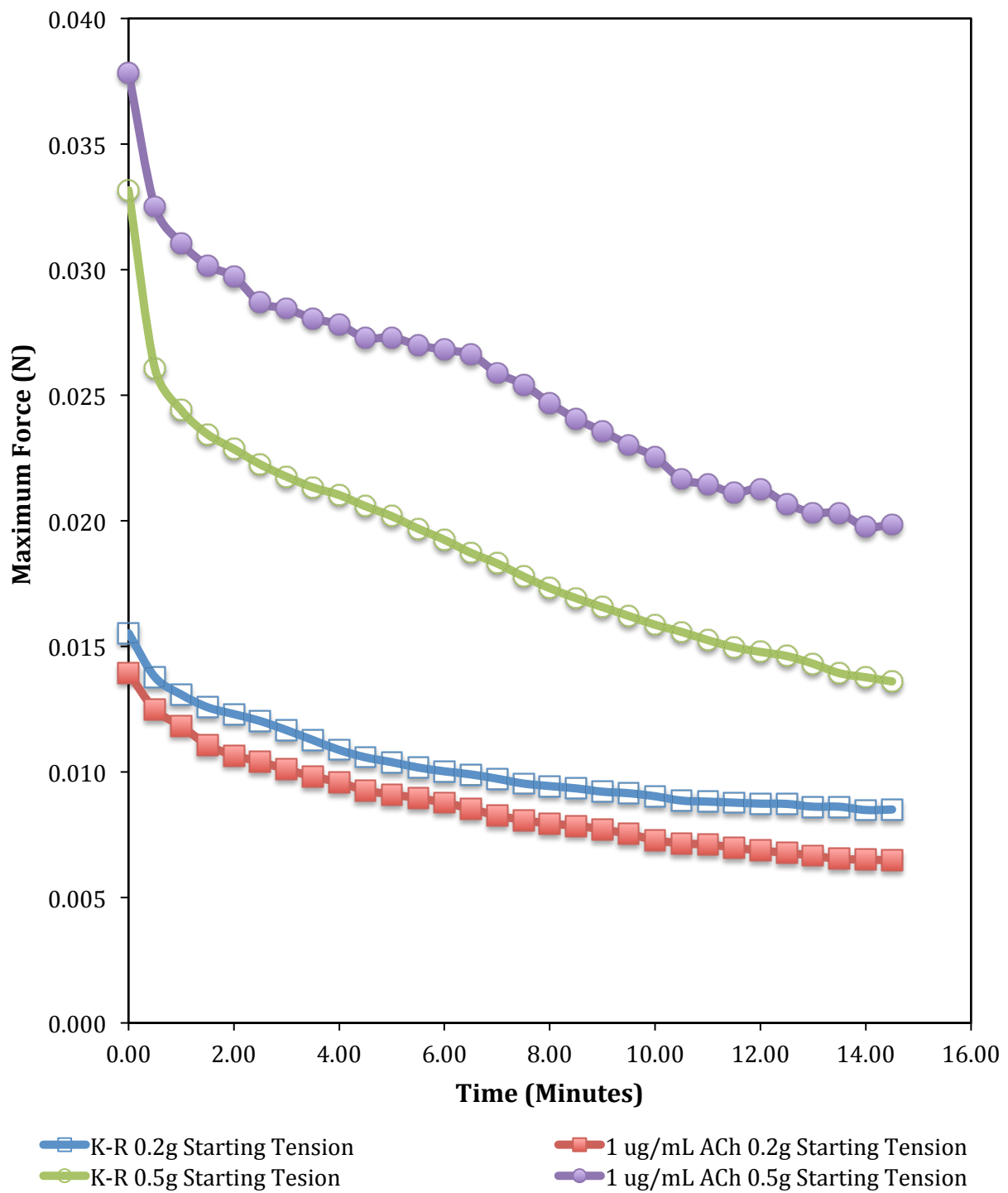


Figure 35. Contraction force produced by excised EDL in various baths under different starting tensions.

Electrochemical Stimulation and the Effect of Soaking

The ACh in the solutions needs a sufficient amount of time to diffuse into the sample so that it can initiate a muscle contraction. To study the effect of diffusion, samples were placed either in K-R or ACh solutions upon excision prior to testing. Testing was then conducted on all samples with the application of 3V electrical field stimulation. EDL samples soaked in K-R upon excision but tested in 1 $\mu\text{g}/\text{mL}$ ACh baths resulted in an average initial contraction force of 0.011N with a SEM of 0.001N ($n=2$). Samples soaked in 1 $\mu\text{g}/\text{mL}$ ACh upon excision and tested in baths of the same concentration produced an average initial contraction force 0.011N with a SEM of 0.001N ($n=3$). The average final contraction forces for samples soaked in K-R and samples soaked in ACh were 0.006N and 0.009N, respectively (Figure 36). Soaking samples in ACh demonstrated significantly less fatigue over time in comparison to samples soaked in K-R. Both conditions produced a similar initial contraction force. However samples soaked in ACh responded more consistently over time while samples soaked in K-R had a drop in response. This leads to the conclusion that ACh has a positive effect in terms of overall muscle response over time.

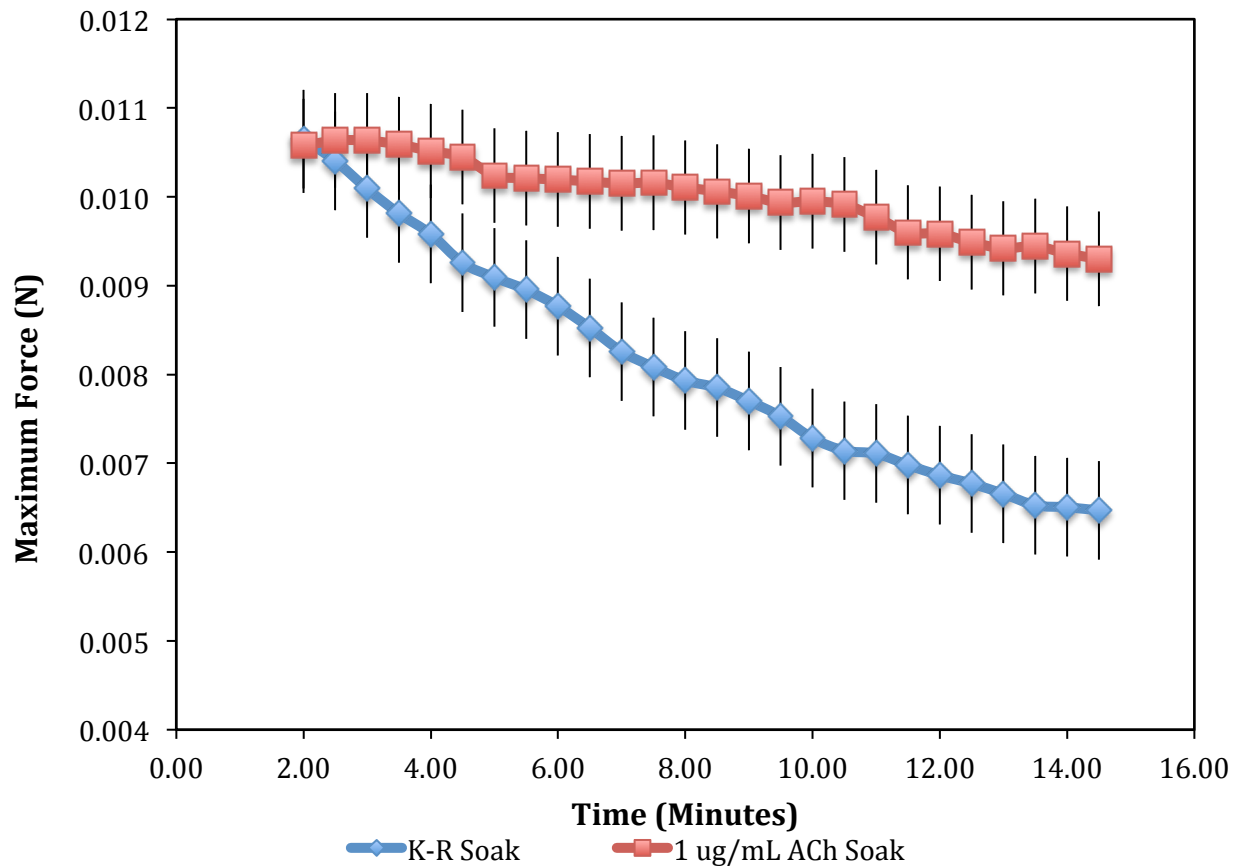


Figure 36. The effect of soaking samples in different baths prior to testing in ACh. Contraction force produced by 3V electrical field stimulation of excised EDL soaked in a K-R bath or an ACh bath upon excision under 0.2g starting tension. All tests were conducted in 1 $\mu\text{g}/\text{mL}$ ACh baths.

The effect of ACh was examined with the application of different levels of electrical stimulation on the excised EDL. Samples were soaked in 1 $\mu\text{g}/\text{mL}$ ACh upon excision and then tested in an ACh bath of the same concentration. Testing of EDL samples with the application of 2V electrical field stimulation resulted in an average initial contraction force of 0.012N with a SEM of 0.001N ($n=5$). The application of 2.5V electrical field stimulation produced an initial contraction force of 0.014N with a SEM of 0.001N ($n=3$). 3V electrical field stimulation resulted in an average initial contraction force of 0.011N with a SEM of 0.001N ($n=3$) (Figure 37).

The application of ACh did not result in an increase in contraction force even though samples were soaked in ACh upon excision to provide sufficient time for the ACh to diffuse into the muscle. The initial contraction force and the rate of decline for all samples at various voltages were found to be the same as their respective counterparts tested in K-R under the same starting tension. Once again, samples tested at 3V behaved in a similar manner as samples stimulated at 2V. Due to the muscle response being inconsistent, various factors had to be considered, specifically the starting tension and the concentration of ACh in the solutions.

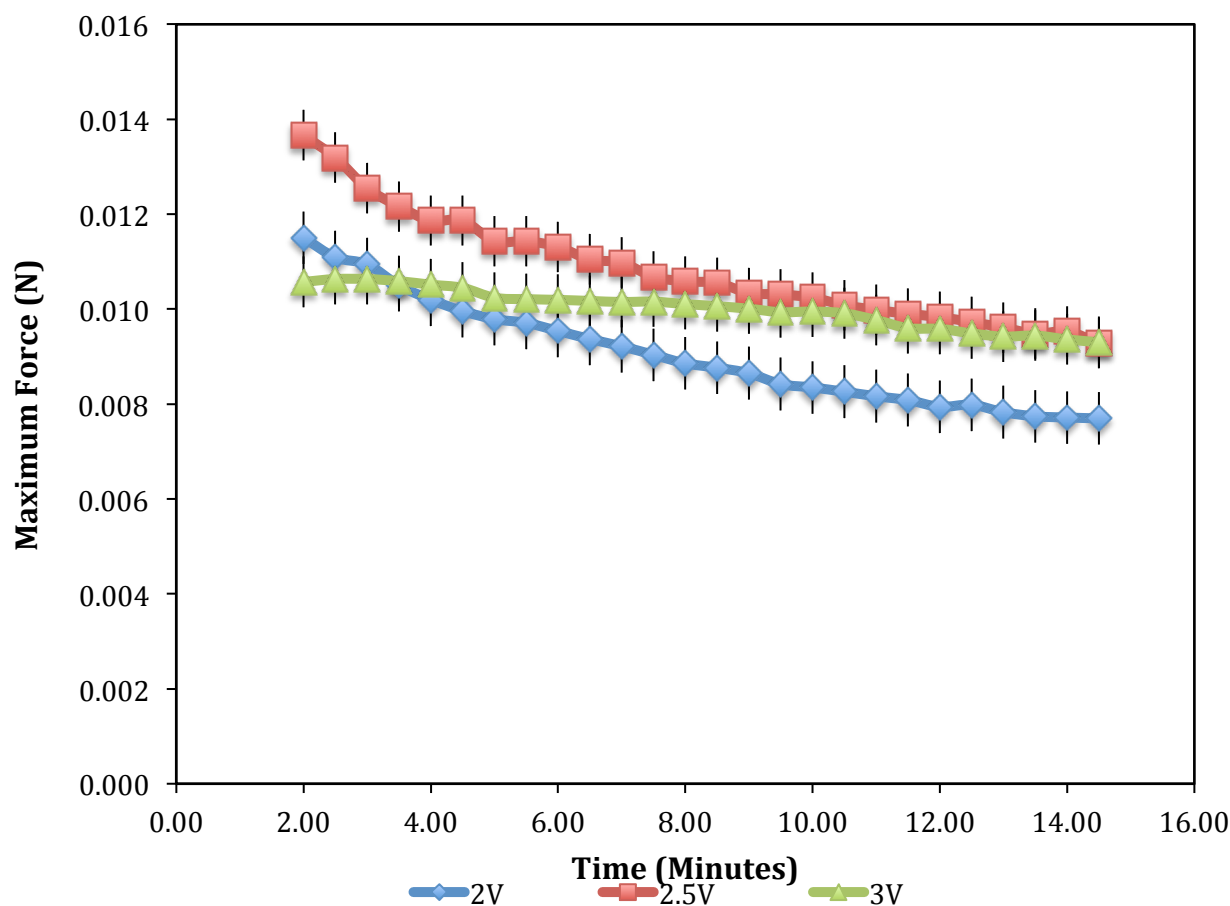


Figure 37. Contraction forces generated during electrochemical testing. Electrical stimulation of EDL soaked and tested in 1 $\mu\text{g}/\text{mL}$ ACh baths with 0.2g starting tension ($n=11$).

Effect of ACh Concentration

The effect of various ACh concentrations combined with soaking samples in ACh upon excision was studied to determine the ideal ACh concentration for electrochemical testing (Figure 38). The application of 3V electrical field stimulation to samples soaked and tested in 1 $\mu\text{g/mL}$ ACh baths, with 0.2g starting tension, resulted in an average initial contraction force of 0.011N with a SEM of 0.001N ($n=3$). Testing at the same level of electrical stimulation on samples soaked and tested in 100 $\mu\text{g/mL}$ ACh baths produced an average initial contraction force of 0.009N with a SEM of 0.004N ($n=3$). Samples soaked and tested in 1 mg/mL ACh baths and stimulated at 3V had an average initial contraction force of 0.011N with a SEM of 0.007 ($n=2$).

Testing determined that an increase in ACh concentration is not correlated with an increase in contraction force. Initial contraction force was found to be equivalent at various concentrations of ACh with the application of 3V electrical field stimulation. The rate of decline of sample response over time was found to be the least with a concentration of 1 $\mu\text{g/mL}$ ACh. Furthermore, a concentration of 1 $\mu\text{g/mL}$ ACh demonstrated a slight increase in contraction force at lower stimulation voltages. Thus further confirming the efficacy of the concentration used.

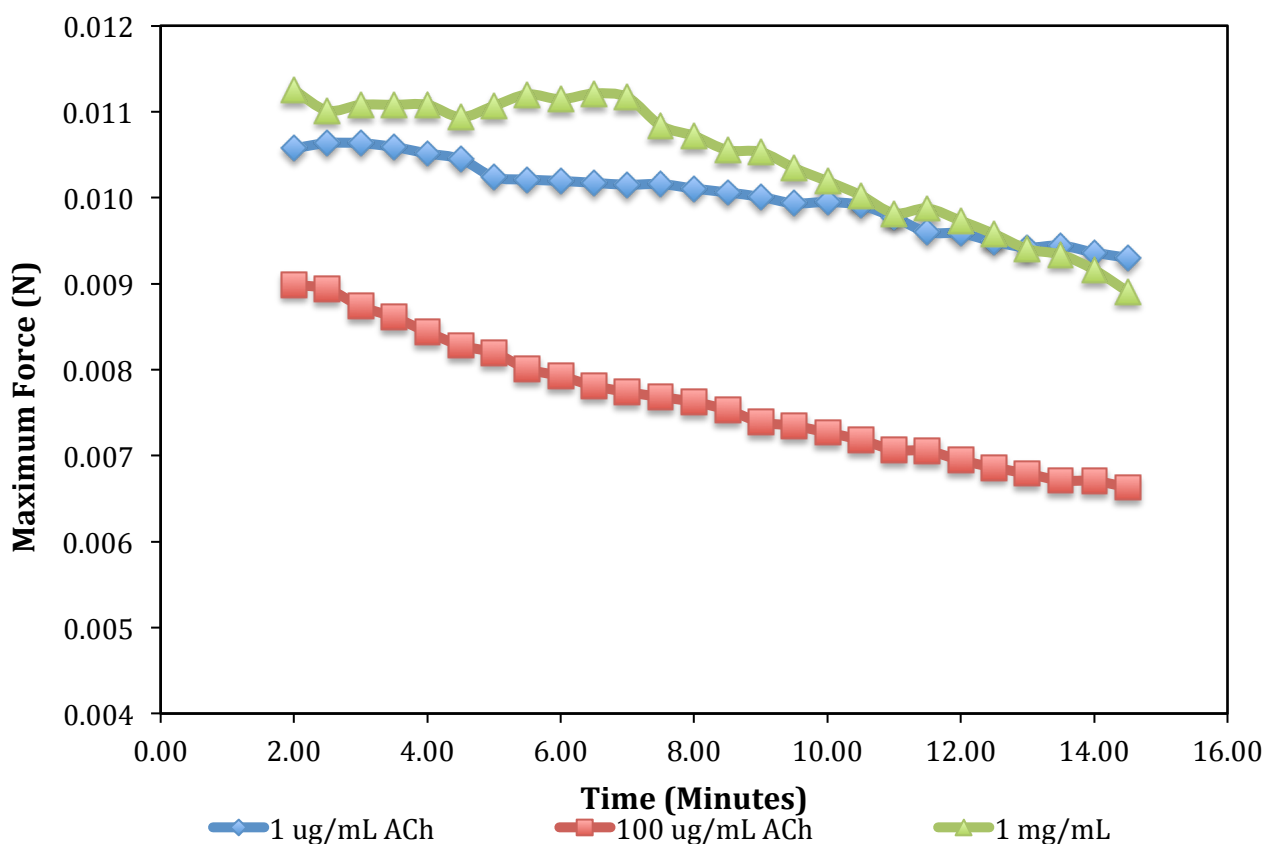


Figure 38. Contraction force resulting from 3V electrical field stimulation on samples soaked and tested in baths of various concentrations of ACh.

Electrochemical Testing, Higher Starting Tension

Earlier testing led to the conclusion that samples needed to be tested under a higher starting tension with an ACh soak if they were to be tested in ACh. With that in mind, the effect of electrical stimulation under a higher starting tension was examined using 5V and 10V electrical field stimulation. It was observed that an increase in electrical field stimulation is correlated with an increase in contraction force for EDL samples tested in K-R baths with 1 g starting tension (Figure 39). The initial contraction force with an electrical field stimulation of 5V was found to be 0.057N with a SEM of 0.005N (n=4). The initial contraction force of samples stimulated at 10V was 0.065N with a SEM of 0.003 (n=3). As expected, samples stimulated at 10V produced a higher average initial contraction in comparison to samples stimulated at 5V due to an increased number of muscle cells being recruited by the higher electrical stimulation. Samples stimulated at 10V demonstrated a higher rate of fatigue and a lower final contraction due to the faster rate of decline.

The same trend was not observed with samples stimulated at lower electrical levels with 1g starting tension (Figure 40). The initial contraction force of samples tested in K-R with 4V electrical field stimulation was found to be 0.130N with a SEM of 0.011N (n=5) while a 5V stimulation resulted in an initial contraction force of 0.057N with a SEM of 0.005N (n=4). The initial contraction of samples stimulated at 4V soaked and tested in a 100 μ g/mL ACh bath was found to be 0.062N with a SEM of 0.005 (n=6). 5V stimulation of samples soaked and tested in a 100 μ g/mL ACh bath was found to be 0.081N with a SEM of 0.024 (n=6).

Samples stimulated at 4V under 1g starting tension in K-R produced an initial average contraction that was more than double the force produced by samples stimulated at 5V. Thus indicating that 4V may be the optimal electrical stimulation level for excised EDL. When soaked and tested in 100 μ g/mL ACh baths, samples stimulated at 4V produced an equivalent force as samples stimulated at 5V in K-R. It must be noted that 5V samples soaked in ACh demonstrated higher contraction forces than samples stimulated at 5V in K-R. Even though 5V samples tested in ACh had a faster rate of decline than samples tested in K-R, they continued to produce higher contraction forces over time. Samples stimulated at 4V in ACh produced lower contraction forces than samples tested at 4V in K-R. A possible explanation of such behavior can be that with the application of ACh, motor unit recruitment is initiated. Without ACh, the 4V electrical field stimulation produces the maximum force by activating all of the muscle fibers in the muscle. However, it is possible that if the ACh is already recruiting the units, the 4V electrical stimulation is limited in terms of the number of units it is able to recruit. Therefore resulting in lower contraction forces.

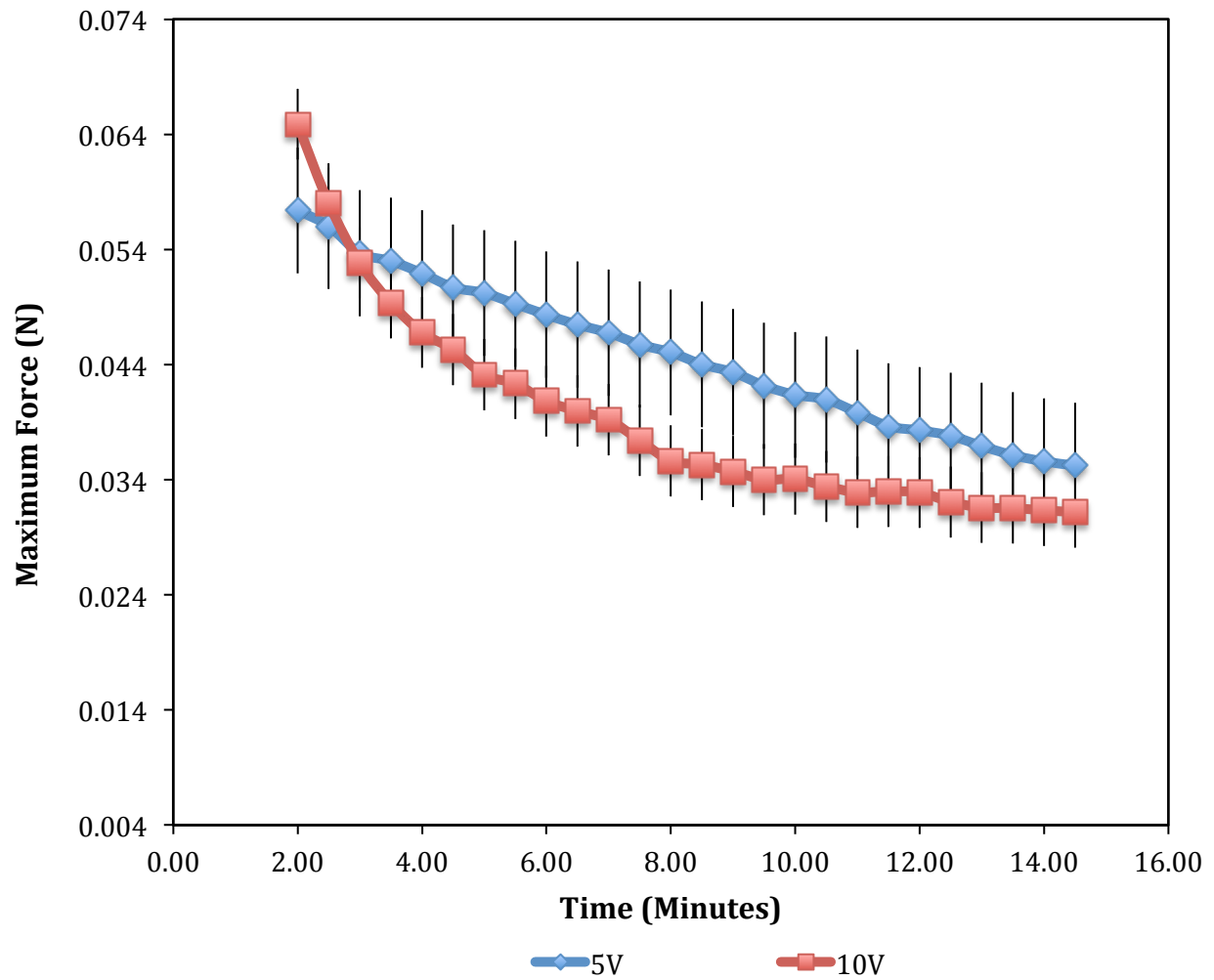


Figure 39. Electrical field stimulation of EDL samples with 1g starting tension. Testing in K-R solution demonstrated that an increase in electrical field stimulation is correlated with an increase in contraction force.

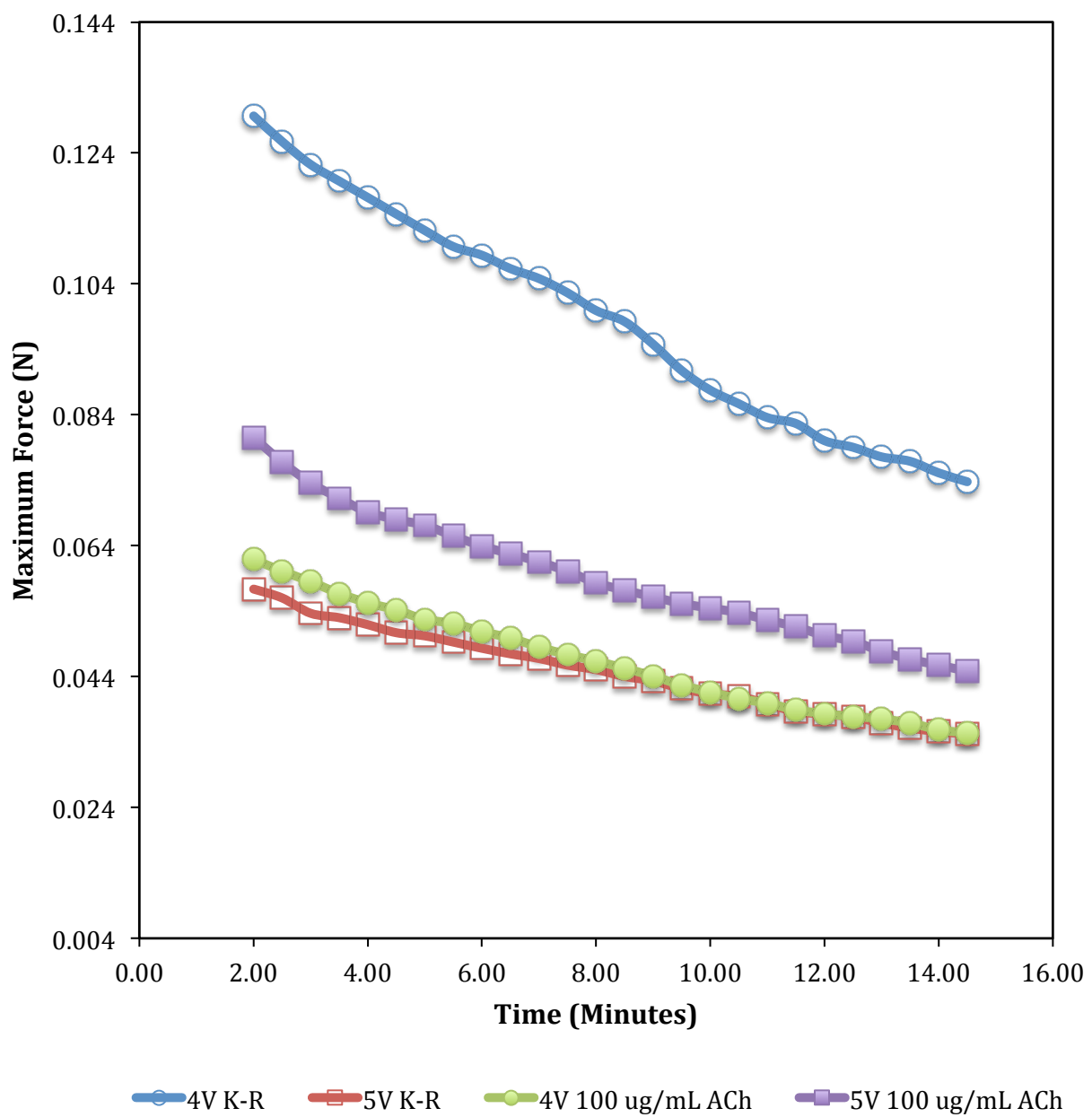


Figure 40. Electrochemical stimulation of EDL samples in various baths.

Voltage Sweep Force Analysis

In order to account for differences in muscle response when testing was conducted on one sample at a time using a single voltage, testing was modified so that a range of voltages was applied to the same sample. Three different patterns, using stimulations ranging from 2V to 5V, were applied to investigate if the pattern of stimulation had an effect on muscle response. Short term testing, implementing the Master-8 for stimulation, demonstrated an increase in contraction force with increased electrical field stimulation for samples soaked and tested in K-R for all three stimulation patterns with 1 g starting tension.

Samples soaked and tested in K-R showed that an increase in excitation voltage is correlated with an increase in contraction force for the three different patterns. The average rate of decline in force over time was more prominent at higher voltages in all three patterns. Testing in 100 $\mu\text{g/mL}$ ACh baths, after soaking in K-R, resulted in the generation of higher contraction forces in comparison to testing in K-R. Tables 2-4 display the resulting contraction forces from electrical and electrochemical stimulation implementing three different stimulation patterns. The effect of ACh was found to be more prominent with 4V and 5V electrical field stimulation. The rate of decline over time was higher with ACh testing than in K-R testing due to the generation of higher contraction forces that caused the muscle to fatigue faster (Figure 41). Samples soaked and tested in ACh produced much higher contraction forces in comparison to the two previously mentioned tests. Once again, the generation of larger forces led to higher rates of decline in contraction force over time. The addition of ACh at minute 4 to samples soaked and initially tested in K-R produced a general increase in contraction force for all three stimulation patterns. For stimulation pattern one, contraction forces were comparable if not higher to the contraction forces of the sample soaked and tested in ACh. The addition of ACh demonstrated an increase in contraction force with 5V stimulation for stimulation pattern two. Stimulation pattern three generated an increase in contraction force with the addition of ACh at 3V and 4V.

Time (min)	Stimulation Voltage	Krebs Soak/ Krebs Bath	Krebs Soak/Ach Bath
2.00	5V	0.068 N	0.092 N
2.50	4V	0.055 N	0.084 N
3.00	3V	0.033 N	0.047 N
3.50	2V	0.011 N	0.007 N
4.00	3V	0.031 N	0.046 N
4.50	5V	0.057 N	0.084 N
5.00	2V	0.009 N	0.009 N
5.50	4V	0.047 N	0.078 N
6.00	4V	0.046 N	0.079 N
6.50	2V	0.008 N	0.009 N
7.00	5V	0.050 N	0.084 N
7.50	3V	0.028 N	0.079 N

Time (min)	Stimulation Voltage	ACh Soak/ ACh Bath	Krebs Soak/ Krebs-ACh Bath
2.00	5V	0.123 N	0.120 N
2.50	4V	0.107 N	0.108 N
3.00	3V	0.061 N	0.076 N
3.50	2V	0.017 N	0.028 N
4.00 *	3V	0.056 N	0.071 N
4.50	5V	0.096 N	0.109 N
5.00	2V	0.017 N	0.020 N
5.50	4V	0.088 N	0.098 N
6.00	4V	0.083 N	0.093 N
6.50	2V	0.015 N	0.018 N
7.00	5V	0.086 N	0.089 N
7.50	3V	0.047 N	0.060 N

Table 2. Resulting contraction forces generated using voltage sweep stimulation pattern 1 testing in various baths (n=4). Asterisk (*) denotes the time point ACh was added into the bath for the Krebs-ACh bath testing.

Time (min)	Stimulation Voltage	Krebs Soak/ Krebs Bath	Krebs Soak/Ach Bath
2.00	3V	0.020 N	0.070 N
2.50	5V	0.025 N	0.124 N
3.00	2V	0.004 N	0.007 N
3.50	4V	0.023 N	0.109 N
4.00	5V	0.023 N	0.111 N
4.50	4V	0.022 N	0.101 N
5.00	3V	0.017 N	0.054 N
5.50	2V	0.003 N	0.003 N
6.00	2V	0.003 N	0.003 N
6.50	3V	0.017 N	0.046 N
7.00	4V	0.022 N	0.085 N
7.50	5V	0.023 N	0.088 N

Time (min)	Stimulation Voltage	ACh Soak/ ACh Bath	Krebs Soak/ Krebs-ACh Bath
2.00	3V	0.041 N	0.056 N
2.50	5V	0.052 N	0.078 N
3.00	2V	0.009 N	0.018 N
3.50	4V	0.047 N	0.070 N
4.00 *	5V	0.046 N	0.011 N
4.50	4V	0.043 N	0.074 N
5.00	3V	0.032 N	0.055 N
5.50	2V	0.007 N	0.006 N
6.00	2V	0.007 N	0.004 N
6.50	3V	0.029 N	0.050 N
7.00	4V	0.039 N	0.064 N
7.50	5V	0.040 N	0.064 N

Table 3. Resulting contraction forces generated using voltage sweep stimulation pattern 2 testing in various baths (n=4). Asterisk (*) denotes the time point ACh was added into the bath for the Krebs-ACh bath testing.

Time (min)	Stimulation Voltage	Krebs Soak/ Krebs Bath	Krebs Soak/Ach Bath
2.00	4V	0.045 N	0.062 N
2.50	2V	0.016 N	0.003 N
3.00	5V	0.054 N	0.065 N
3.50	3V	0.025 N	0.034 N
4.00	2V	0.014 N	0.002 N
4.50	3V	0.023 N	0.033 N
5.00	4V	0.040 N	0.058 N
5.50	5V	0.045 N	0.062 N
6.00	3V	0.021 N	0.031 N
6.50	5V	0.041 N	0.060 N
7.00	2V	0.009 N	0.002 N
7.50	4V	0.034 N	0.051 N

Time (min)	Stimulation Voltage	ACh Soak/ ACh Bath	Krebs Soak/ Krebs-ACh Bath
2.00	4V	0.073 N	0.073 N
2.50	2V	0.009 N	0.009 N
3.00	5V	0.077 N	0.077 N
3.50	3V	0.037 N	0.035 N
4.00 *	2V	0.008 N	0.005 N
4.50	3V	0.034 N	0.013 N
5.00	4V	0.057 N	0.023 N
5.50	5V	0.060 N	0.026 N
6.00	3V	0.031 N	0.016 N
6.50	5V	0.056 N	0.025 N
7.00	2V	0.006 N	0.000 N
7.50	4V	0.049 N	0.024 N

Table 4. Resulting contraction forces from voltage sweep stimulation pattern 3 testing in various baths (n=4). Asterisk (*) denotes the time point ACh was added into the bath for the Krebs-ACh bath testing.

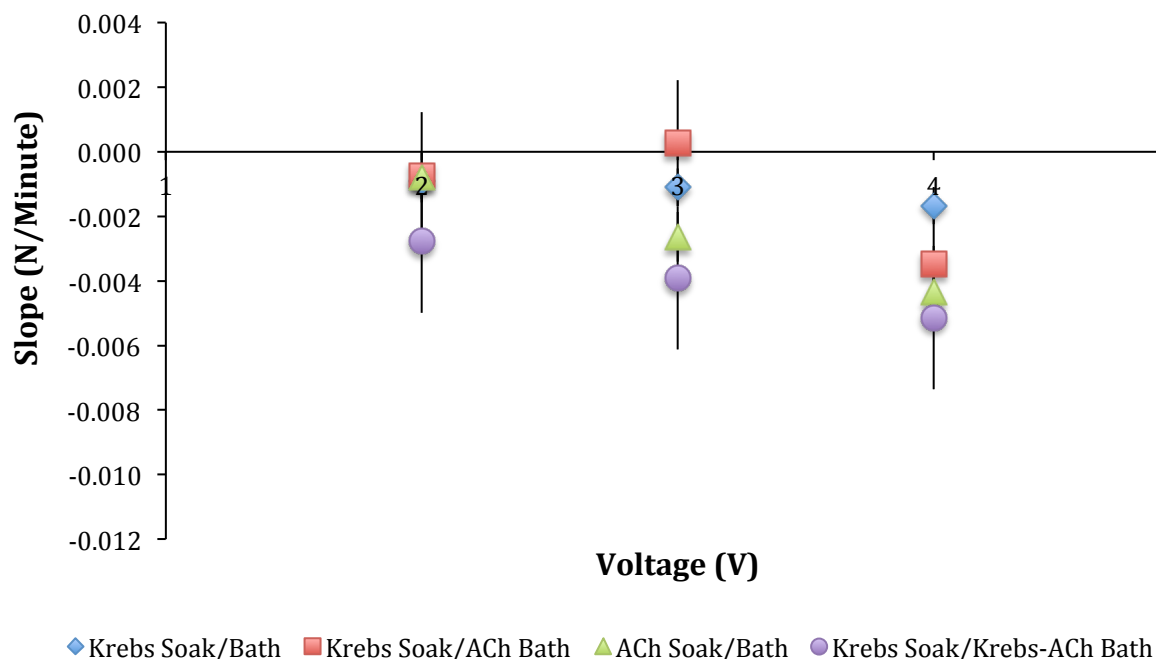


Figure 41. Average contraction force rate using three different stimulation patterns in various baths (n=12). An increase in the rate of decline is evident for testing conducted with ACh.

In order to perform long term voltage sweep testing, a new stimulation system had to be implemented using Labview and a DAQ card. The same three stimulation patterns were applied, but for 32 minutes as opposed to 8 minutes to study the effect of stimulation in various baths over a longer period of time. Testing was conducted with samples soaked and tested in K-R and with samples soaked in K-R, but tested in 100 $\mu\text{g/mL}$ ACh baths. Testing of samples produced similar results for all three stimulation patterns. Stimulation at 3V, 4V, and 5V did not produce distinct contraction forces with the increase of stimulation voltage for tests conducted with samples soaked and tested in K-R and for samples soaked and initially tested in a K-R bath and then in a 100 $\mu\text{g/mL}$ ACh bath starting at minute 4 (Figures 42-44). Testing with all three patterns revealed a general trend of an increase in contraction force with the addition of ACh at minute 4. Reviewing the results, it was noted that the muscle response was not varied at different voltages in both sets of experiments. Investigation into the new stimulation set up determined that the system was limited by the conductivity of the bath, which was limiting the drive needed to increase the voltage in a short period of time from 0V to the desired voltage. Further investigation determined that the voltage would begin to ramp starting at 2.4V (Figure 45), therefore testing was revised and the range of electrical stimulation was change to 0.5V to 2V.

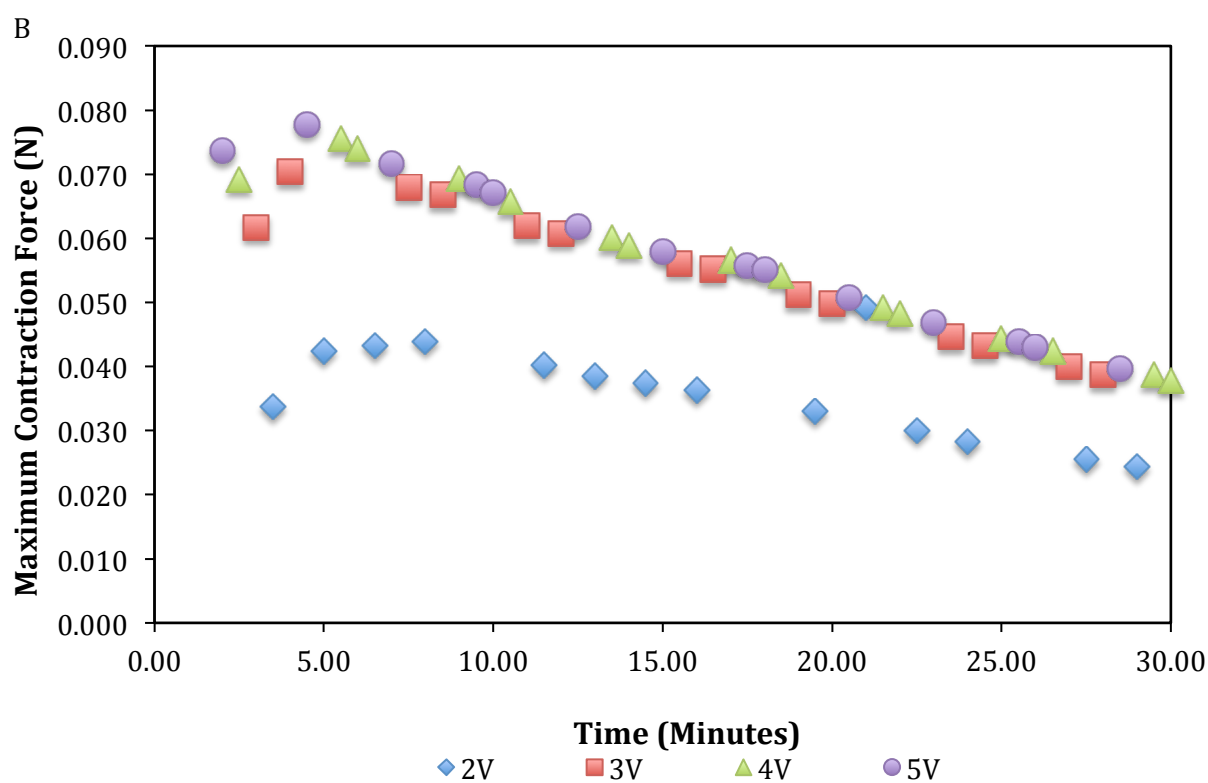
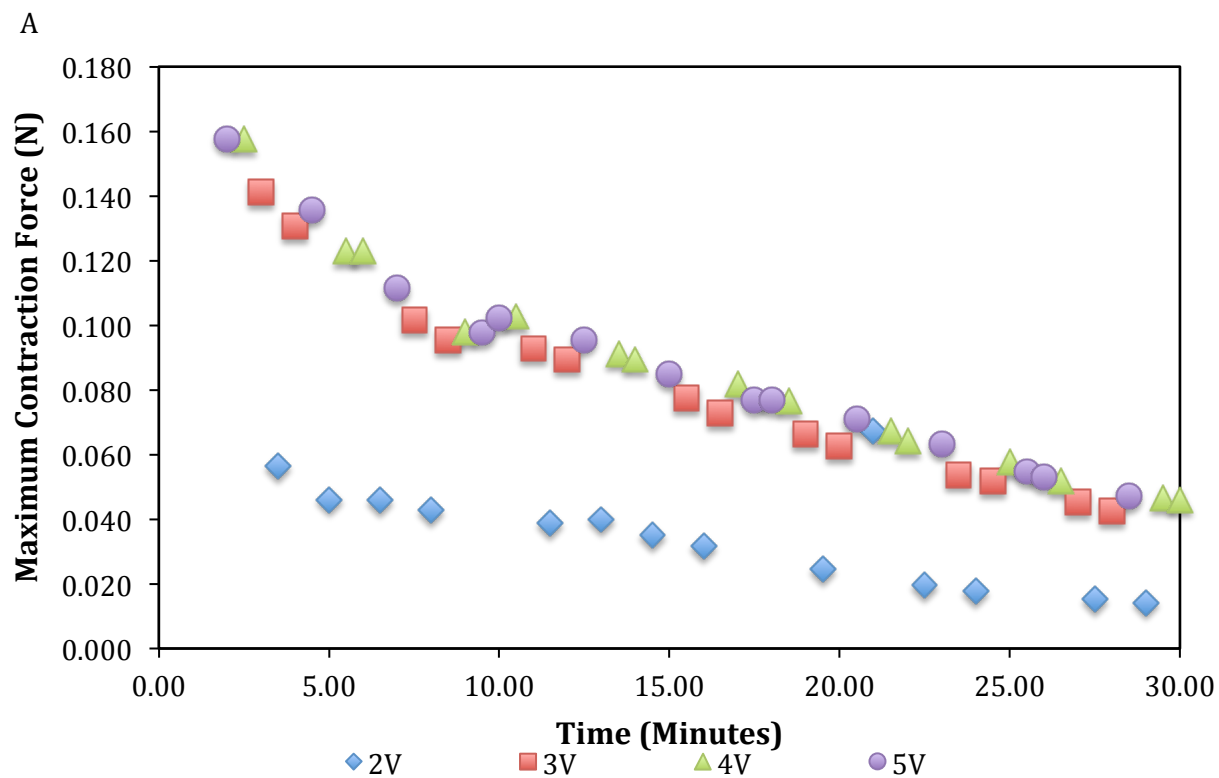


Figure 42. Stimulation pattern 1 (A) Electrical stimulation in K-R solution (n=1) (B) Electrochemical stimulation with the addition of 100 µg/mL ACh at minute 4 (n=1)

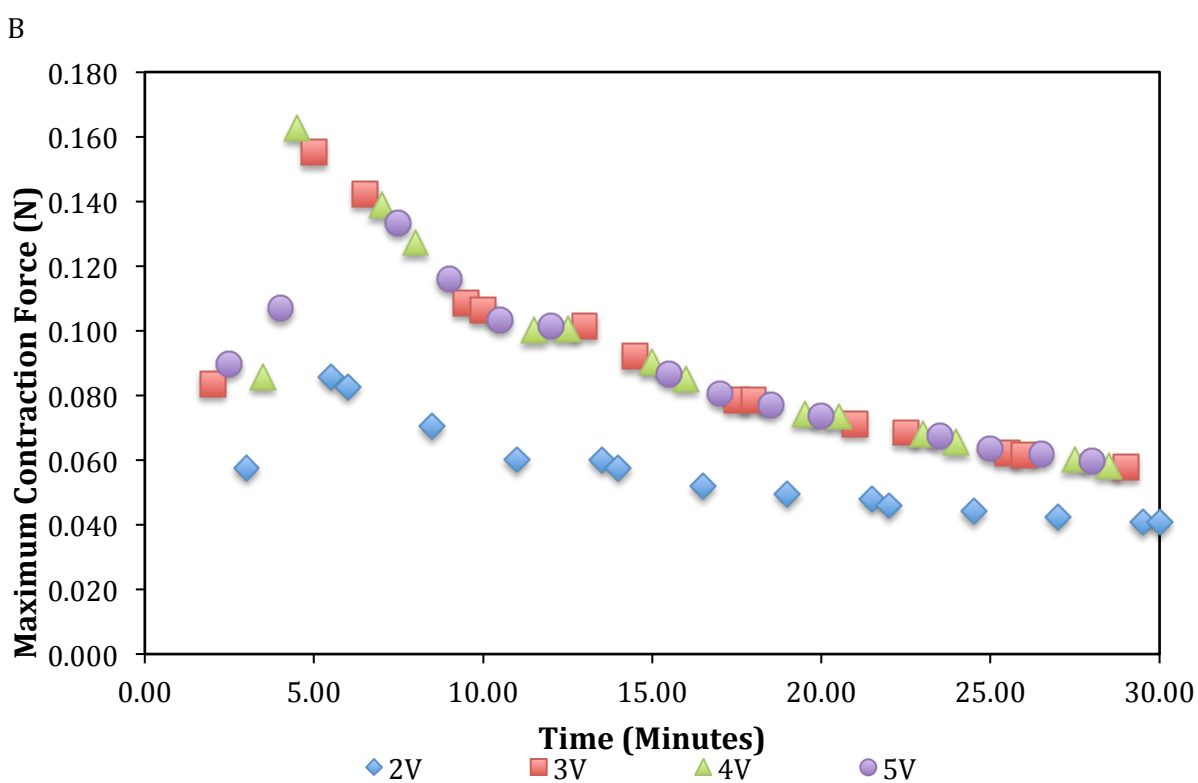
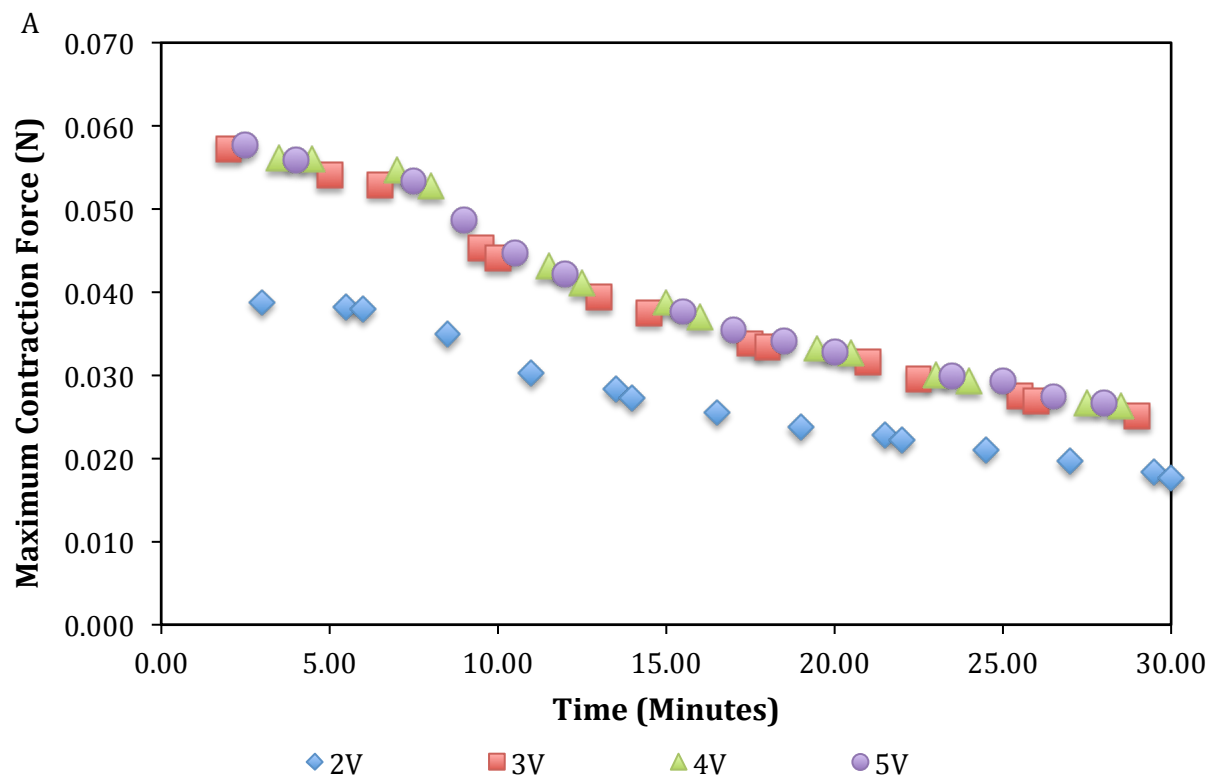


Figure 43. Stimulation pattern 2 (A) Electrical stimulation in K-R solution (n=1) (B) Electrochemical stimulation with the addition of 100 $\mu\text{g/mL}$ ACh at minute 4 (n=1)

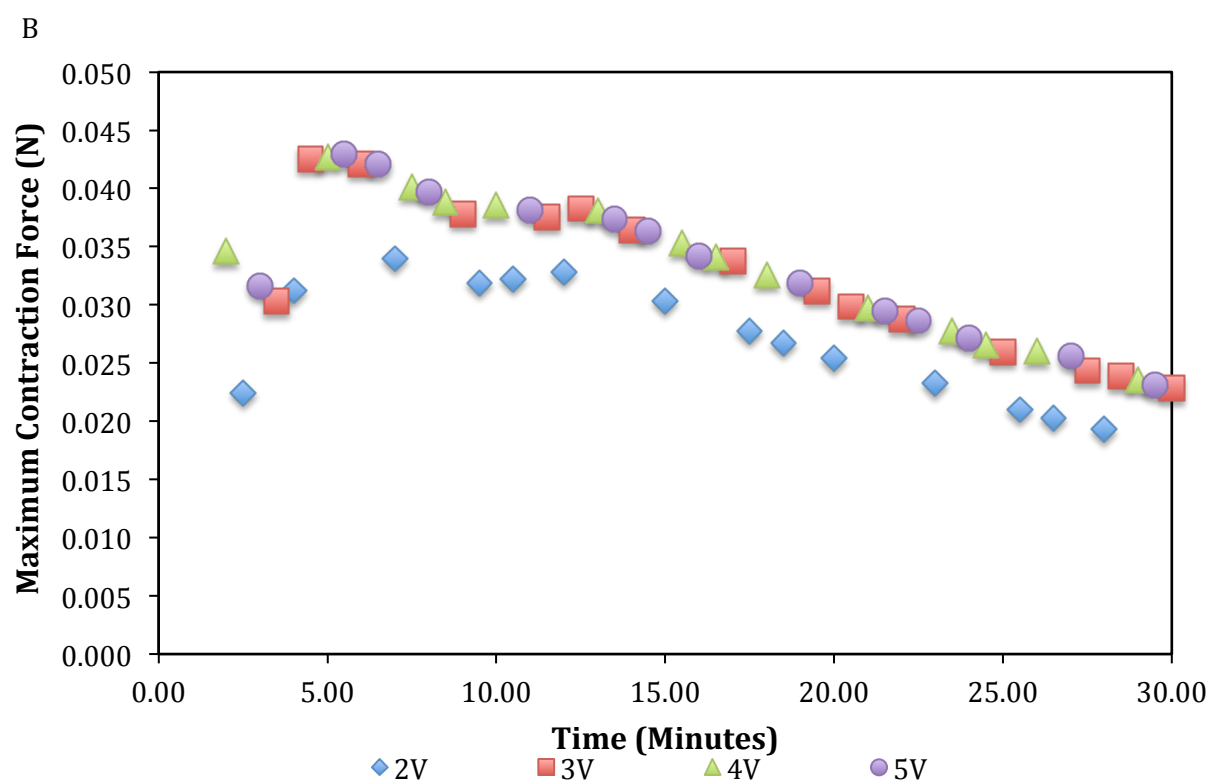
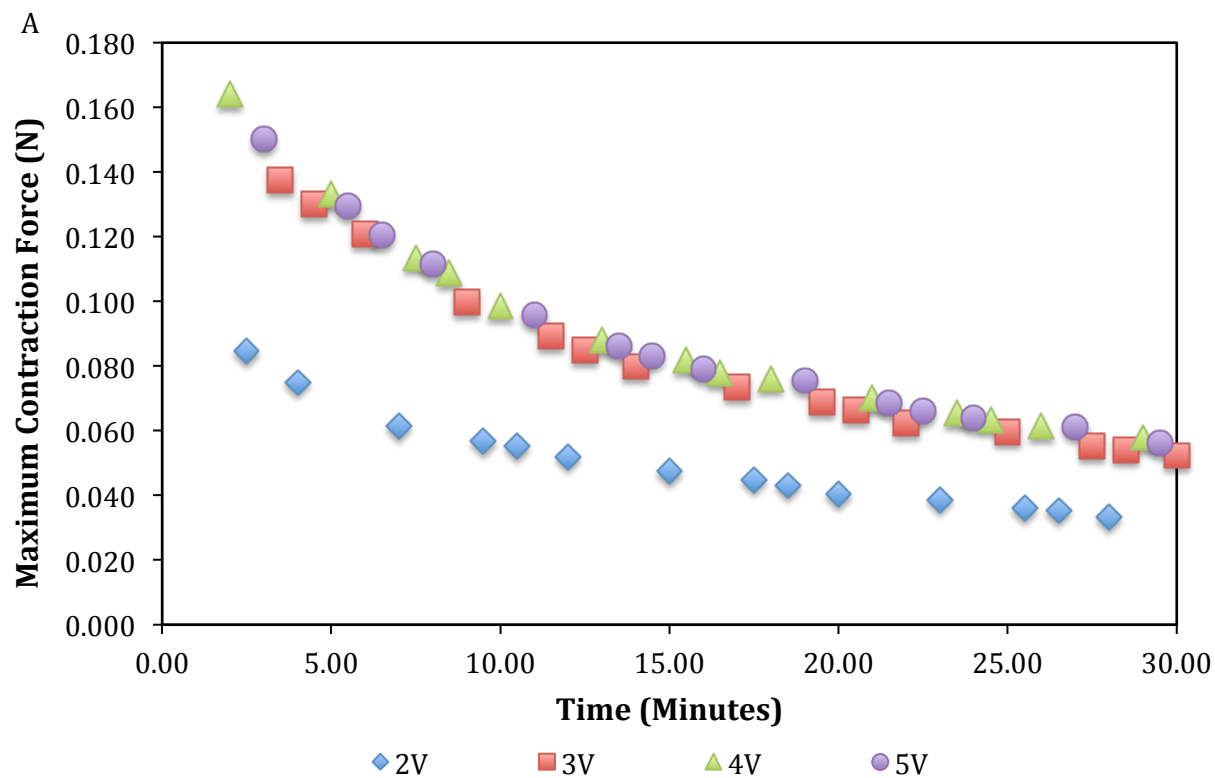


Figure 44. Stimulation pattern 3 (A) Electrical stimulation in K-R solution (n=1) (B) Electrochemical stimulation with the addition of 100 µg/mL ACh at minute 4 (n=1)

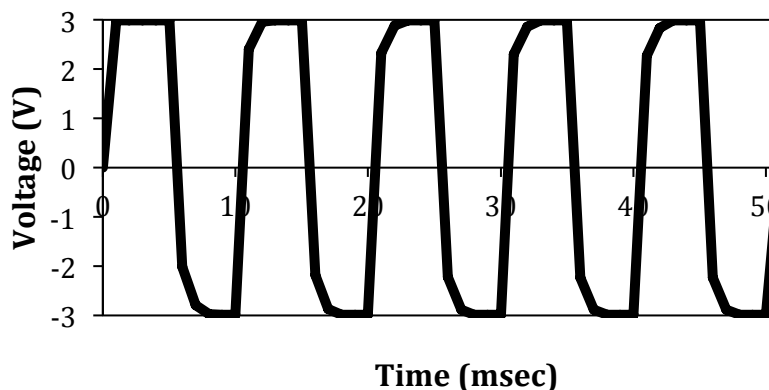


Figure 45. 50 msec bipolar electrical field stimulation generated implementing Labview/DAQ card set up. Testing revealed that the stimulation begins to ramp starting at 2.4V.

Voltage sweep testing with three different stimulation patterns determined that the pattern does not influence muscle response. Testing was modified and conducted with a single pattern with voltages ranging from 0.5V to 2V. Testing of the excised EDL in K-R using lower stimulation voltages once again demonstrated an increase in contraction force with an increase in stimulation voltage (Figure 46). Samples soaked and tested in K-R, with a 1g starting tension, demonstrated an increase in contraction force as the stimulation voltage was increased. 0.5V stimulation generated an average initial contraction force of 0.004N with a SEM of 0.002N. Stimulation at 1V, 1.5V, and 2V generated average initial average contraction forces of 0.013N with a SEM of 0.004N, 0.037N with a SEM of 0.007N, and 0.062N with a SEM of 0.009N (n=3). Samples soaked in K-R, but tested in 100 $\mu\text{g/mL}$ ACh baths did not produce higher contraction forces in comparison to samples soaked and tested in K-R (Figure 47). However, samples tested in ACh demonstrated a lower rate of decline in contraction force over time due to the presence of ACh, which causes an increase in the number contractile units, engaged. Testing of samples with 0.5V and 1V in 100 $\mu\text{g/mL}$ ACh baths after they were soaked in K-R produced initial average contraction forces of 0.002N with a SEM of 0.001N and 0.006N with a SEM of 0.004N, respectively. The application of 1.5V and 2V produced initial average contraction forces of 0.023N with a SEM of 0.009N and 0.047N with a SEM of 0.013N (n=6). Testing conducted on samples soaked in K-R and initially tested in K-R demonstrated the influence of ACh in enhancing the contraction force. Testing was initially conducted in K-R for 16 minutes, or 32 contractions, at which point the bath was changed to a 100 $\mu\text{g/mL}$ ACh bath. Testing demonstrated a general decline in force over time. An increase in contraction force was demonstrated with 2V electrical field stimulation (Figure 48). At minute 15, while still in K-R a 2V stimulation produced an average contraction force of 0.031N with a SEM of 0.010N. Once the bath was changed to an ACh bath, a contraction force of 0.045N with a SEM of 0.016N was generated at minute 17.5 (n=6). A similar effect was seen with 1.5V stimulation. An average contraction force of 0.012N with a SEM of 0.006N was produced in K-R, while a force 0.015N with a SEM of 0.006N was produced in ACh (Figure 46).

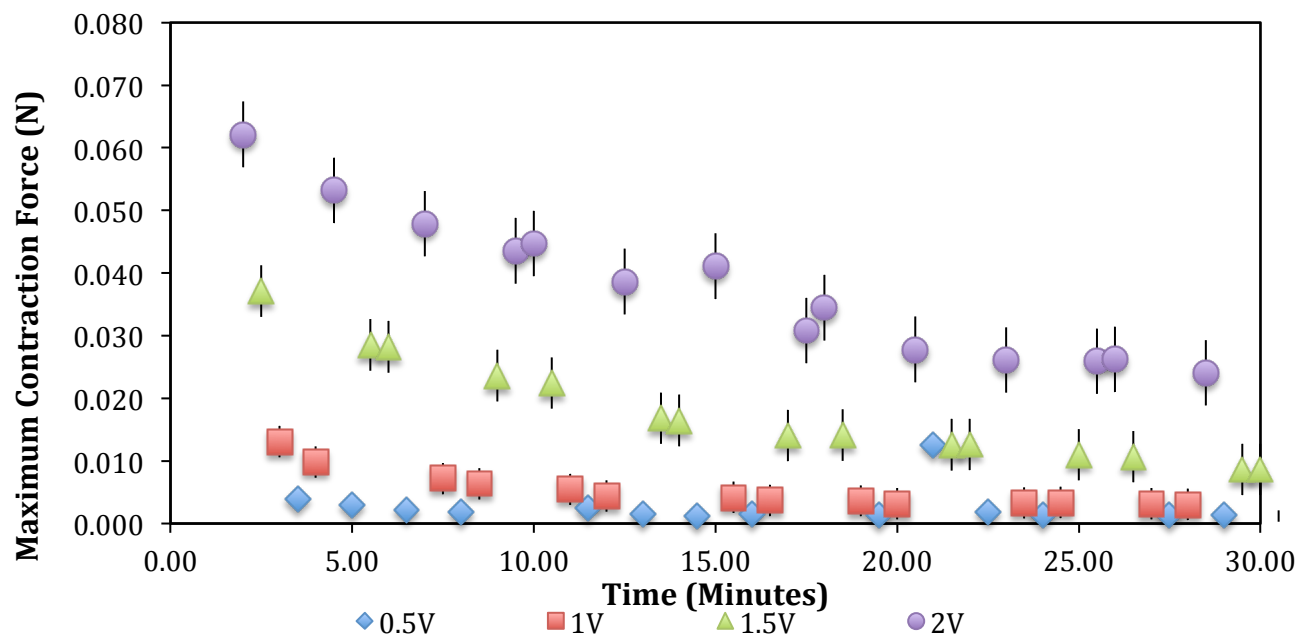


Figure 46. Voltage sweep testing of samples soaked and tested in K-R with stimulation voltages ranging from 0.5V to 2V (n=3).

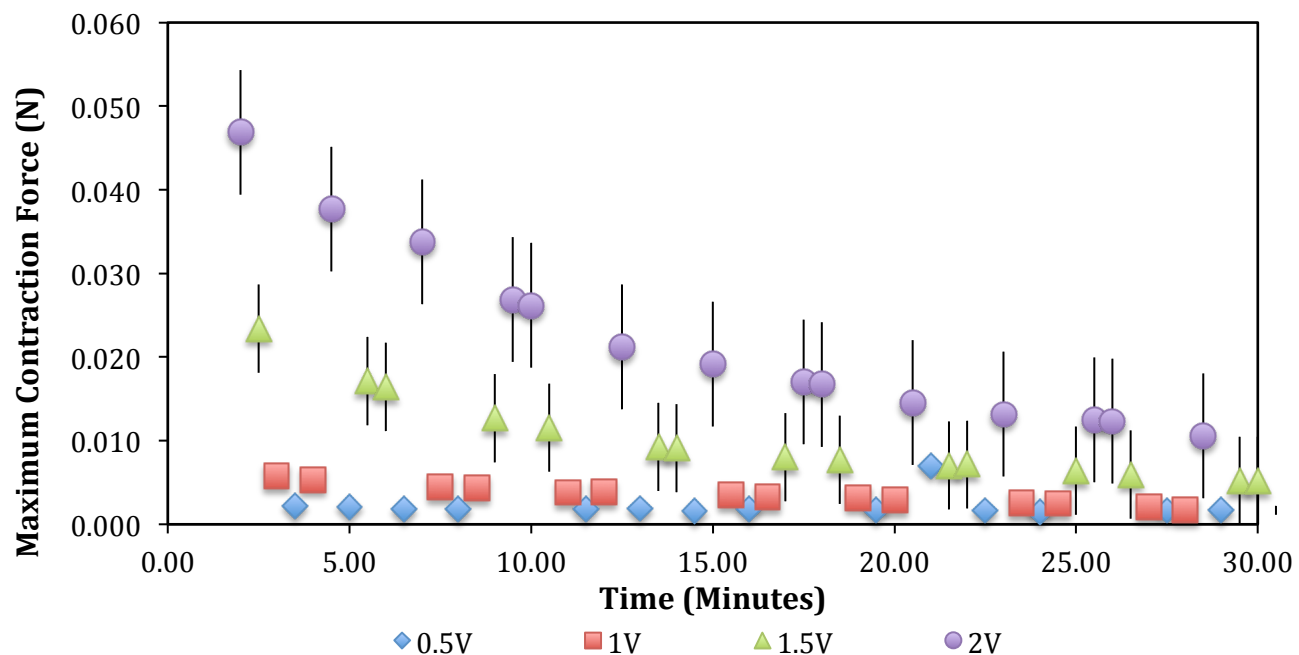


Figure 47. Voltage sweep testing of samples soaked in K-R and tested in a 100 µg/mL ACh bath with stimulation voltages ranging from 0.5V to 2V (n=6).

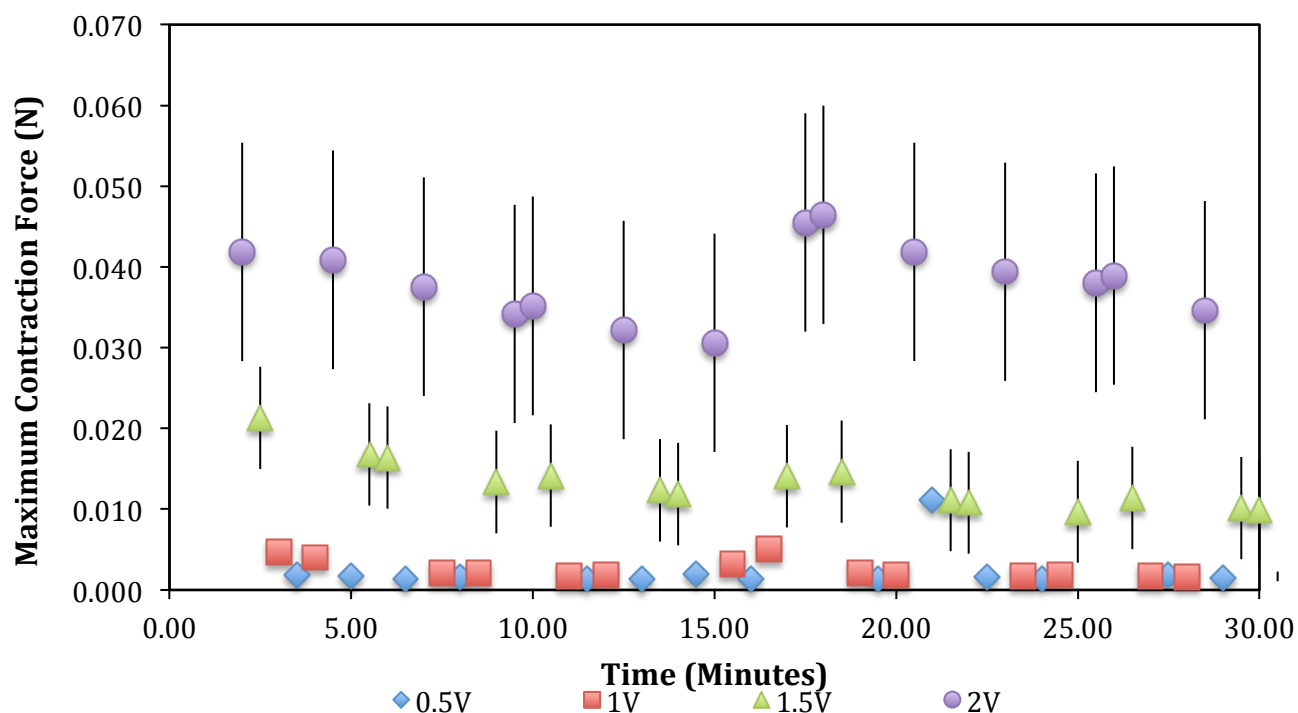


Figure 48. Electrochemical voltage sweep testing. Voltage sweep testing of samples soaked in K-R and initially tested in K-R for 32 contractions. At minute 15.5, testing was then conducted in a 100 $\mu\text{g/mL}$ ACh bath. Stimulation voltages ranged from 0.5V to 2V (n=6).

The influence of ACh was clearly demonstrated with a 0.014N jump in contraction force with the application of 2V. A similar effect was observed with a 1.5V stimulation, which produced a smaller jump in response to ACh. As seen in previous tests, the contraction response of the muscle to electrical stimulation decreases over time. However, with the application of ACh, the contraction force can be restimulated to produce a stronger response. Thus demonstrating the influence of ACh in not only initiating a muscle contraction but also enhancing the contraction force while using a lower level of electrical stimulation with electrochemical testing.

Chapter 4: Conclusions and Future Work

Conclusions

The insufficiency of electrical stimulation alone in triggering painless muscle contraction in denervated orbicularis oculi muscles indicates a need to investigate other methods of stimulation. Current management techniques focus on minimizing corneal damage and are not effective in restoring the ability to blink or the resting muscle tone. These techniques have minimal benefits and do not restore function to the patient's eye.

The focus of this dissertation has been to investigate the response of skeletal muscle to combined electrochemical stimulation by initiating the intricate cascade that leads to muscle contraction with the application of acetylcholine (ACh) in order to reduce the required electrical stimulus. *In vivo* testing in rabbits demonstrated that the required current to stimulate a muscle contraction could be reduced with the incorporation of ACh, supporting the feasibility of the concept of electrochemical stimulation. *In vitro* testing of excised biceps femoris, implementing image analysis, demonstrated that the change in area of samples increased in response to electrochemical stimulation in comparison to electrical stimulation. Force analysis testing of excised extensor digitorum longus demonstrated that the application of ACh for electrochemical stimulation clearly enhanced the contraction force. Overall, testing has demonstrated the influence of electrochemical stimulus in enhancing contraction forces in denervated muscle, thus allowing for a lower required electrical stimulation.

Future Work

A new platform for the control of muscles that no longer have functional neural control has been demonstrated. This new platform implements a novel technique in comparison to current muscle stimulation methods. Electrical stimulation has been used widely in order to restore muscle control. However, no existing technology has focused on stimulating a muscle. Rather, the current focus has been on generating a muscle response through neural stimulus. One example of this current focus is the artificial cardiac pace maker which paces the heart by stimulating the sinoatrial node⁹⁸. Another example is cochlear implants that provide electrical stimulation to the auditory nerves in order to improve hearing⁹⁹.

The generation of an eye blink has never been demonstrated in humans with denervated orbicularis oculi using electrical, chemical, or electrochemical stimulation. In order to reanimate denervated orbicularis oculi in a painless manner, this study has shown that the usefulness of ACh application. The efficacy of ACh in electrochemical stimulation is due in large part to the fact that denervated muscles have an increased number of acetylcholine receptors, thus heightening sensitivity to ACh^{53,100}. The long-range objective of this research is to develop an artificial synapse chip system that can be used in a novel therapeutic strategy for denervated muscles. Development of a microstimulator with the ability to mimic the natural stimulation that occurs at the neuromuscular junctions would create a platform technology that can be applied to control other muscles that no longer have functional neural control.

The key components of this long-range project are to use microelectromechanical systems (MEMS) technology to create an implantable microstimulator device that can deliver a

combined electrical and chemical stimulation, by providing direct delivery of low electrical stimulus coupled with the delivery of ACh, when placed on a denervated muscle. The device would need to be small enough so that it is not only implantable but also cosmetically acceptable⁹⁹. The application of MEMS technology would aide in providing controlled delivery of ACh to desired locations¹⁰¹. This work will also require the design and fabrication of a sensing chip to be placed on a nearby functional muscle to signal the artificial synapse chip to induce a synchronous response in the affected muscle. A synchronous reponse is critical so that the paralyzed eyelid is restimulated in conjunction with the fully functioning eyelid, thus creating a simultaneous blink. The microstimulator would have to deliver painless stimulus while at the same time being biocompatible and implantable. The final goal is an integrated wireless subdermal system implenting integrated circuits and MEMS technology, consisting of an electrode array, microfluidics and electronics for control. The device would function at the level of the neuromuscular junction by initiating muscle contraction and thus potentially becoming a valuable platform for muscle reanimation.

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Appendices

Appendix 1: C2C12 Cell Culture Protocol

A. Thawing Frozen Cells

1. Warm media to 37°C by incubating in water bath
2. Quickly thaw the cryo-preserved cells (agitate in the 37°C bath)
3. Sterilize the external surface of the tube with 95% ETOH
4. Transfer to a new sterile tube using a P1000 pipet with filtered tips
5. Pellet the cells gently using a balanced centrifuge, not exceeding 2000 rpm for 30-60 seconds at room temp (21°C)
6. Sterilize the tube with EtOH again
7. Remove the medium on top
8. Re-suspend in 1mL of 37°C medium by gently pipetting up and down
9. Transfer to a 25 cm² flask
10. Add 14 mL of warm medium to suspension
11. Close the lid
12. Place in 37°C incubator with 5% CO₂

B. Standard Passage of C2C12 Cells

1. Warm C2C12 cell growth media & trypsin in 37°C water bath for 30 minutes
2. Aspirate old media from flask
3. Pipette 15 mL of PBS
4. Place flask down and swish gently back and forth.
5. Aspirate PBS holding flask sideways and tipped towards you
6. Add 1 mL of Trypsin
7. Cap off flask and gently rock back and forth so that the trypsin is evenly distributed
8. Allow to sit for *3-5 minutes*
9. Gently tap both sides of the flask to loosen cells
10. Add 14 mL of media
11. Gently pipette cell suspension up and down to mix properly
12. Try to avoid creating bubbles
13. Split cell suspension into new T-75 flask
 - a. For *1:2 splitting*: Add 10 mL of media and 5mL of cell suspension
 - b. For *1:6 splitting*: Add 12.5 mL of media and 2.5 mL of cell suspension
14. Aspirate any remaining cell suspension left in the old flask

C. Differentiating C2C12 Cells

1. Warm C2C12 differentiation media in 37°C water bath for 30 minutes
2. Obtain desired flask from incubator
3. Aspirate cell growth media from flask
4. Pipette 15 mL of PBS into flask to wash cells.
 - a. Place flask down and swish gently back and forth.
5. Aspirate PBS
6. Add 15 mL of differentiation media
7. Transfer flasks back to incubator

Appendix 2: Microcontact Printing Microposts

A. Preparing Microposts for Seeding

1. Cut micropost to appropriate size & soak in 100% EtOH by sonicating for 10 minutes
2. Cut out PDMS stamps of appropriate size
3. Soak stamps with 100 μ L of 1 μ g/mL fibronectin for 1 hour
4. Sterilize 3 standard glass slides
 - a. Soak in 70% EtOH and leave in hood & under UV for 15 minutes
 - b. Flip slides and soak for another 15 minutes
5. Sterilize double stick tape
 - a. Place tape in petri dish as shown
 - b. Soak in 70% EtOH in hood & under UV for at least 15 minutes
6. Obtain microposts from sonicator
 - a. Dry microposts by aspirating them using an aspirator a Pasteur pipette
1. UV-Ozone microposts for 8 minutes
2. After 1 hour, aspirate fibronectin from PDMS stamps
3. Place stamps, fibronectin side down, on top of microposts
 - a. Leave on for 15 minutes
4. Aspirate EtOH from the petri dish with tape
5. Rinse the tape with 30 mL of DI water
 - a. Fill petri dish up to rim to ensure rinse
 - b. Swish around petri dish to dilute remaining EtOH
6. Aspirate DI water & dry the tape as much as possible
 - a. Allow tape to dry overnight in hood & under UV
7. Aspirate EtOH from the petri dish with slides
8. Rinse slides with DI water
9. Remove slides from water & aspirate meticulously
10. Lay out 5 large petri dishes in hood & label accordingly
 - a. 70% EtOH
 - b. DI H₂O #1
 - c. DI H₂O #2
 - d. 0.2% Pluronic
 - e. PBS
11. Fill appropriate petri dishes accordingly
 - a. 30 mL 70% EtOH
 - b. 30 mL DI H₂O
 - c. Combine 24.5 mL DI H₂O with 0.5 mL Pluronic
 - d. 30 mL PBS
12. Lay out one small petri dish for every micropost pad and fill with PBS
 - a. Fill with enough PBS so that the microposts are submerged
13. Remove PDMS stamps from microposts
14. Place microposts, face up, in the large petri dish with EtOH
 - a. Soak for 1 minute ensuring posts are submerged
15. Remove microposts from EtOH & soak micropost face down in DI H₂O #1

- a. Soak for 1 minute
- 16. Remove microposts from DI H₂O #1 & soak in DI H₂O #2 for 1 minute
 - a. Micropost face down
- 17. Transfer microposts to pluronic solution
 - a. Micropost face up
 - b. Soak for 30 minutes
- 18. Transfer microposts to the large petri dish filled with PBS
 - a. Soak for 1 minute
 - b. Micropost face down
- 19. Place each micropost pad, face down, in its own individual small petri dish filled with PBS.
- 20. Place petri dishes in incubator
- 21.

B. Seeding C2C12 Cells on Microposts

- 1. Pass cells as usual
 - a. Trypsinize and add media
- 2. Transfer cell suspension to 15 mL centrifuge tube
- 3. Centrifuge at 1000 RPM for 4 minutes
 - a. Removes trypsin completely
- 4. Aspirate media
- 5. Add 13 mL of fresh cell growth media
 - a. Break up the pellet by pipetting up and down
- 6. Determine base concentration
 - a. Count cells using hemacytometer
 - b. Want ~ 200 cell/ μ L
- 7. Dilute cell suspension appropriately
- 8. Obtain microposts from incubator
 - a. Remember, they were left soaking micropost face down
 - b. Flip microposts up and aspirate PBS using a Pasteur pipette making sure to dry the microposts
- 9. Add 10 μ L of diluted cell suspension to each pad
 - a. 1 pad = 16 arrays
- 10. Add 1 mL of media to the dish
 - a. Add the media on the side and not on the pads so as to not disrupt the cell solutions on the pads. This will prevent the cell solution from drying out
- 11. Incubate at least 1-2 hours

C. Micropost Mounting

- 1. Wet a Kimwipe with EtOH and lay in back of hood
- 2. Score sterilized microscope slide at midpoint
- 3. Fold wet Kimwipe over slide and snap it in half
- 4. Rinse split slides in DI water
- 5. Dry slides thoroughly
- 6. Cut 2 small pieces of sterilized tape & place on bottom of large petri dish

7. Place split glass slides on tape and position onto bottom of petri dish
8. Remove microposts from incubator
9. Aspirate media from petri dish
10. Aspirate cell suspension from microposts
11. Aspirate the backside of the microposts thoroughly
12. Place seeded PDMS micropost pad on a new sterilized glass slide
 - a. Make sure the microposts face up
 - b. If there are air bubbles under the PDMS, create shear by shifting the PDMS with tweezers
13. Add 10-13 mL of media to the large petri dish with the split slides
14. Place the slide with the microposts face down between the split slides
15. Add a little more media to ensure that the microposts are soaking in it

Appendix 3: Area Analysis Matlab Code

```
clear all
clear memory
close all

FPS = 500;           %frame rate of the image acquisition
numimgs = 400;       %number of images in the final directory
scale = 56.036;      %conversion factor in px/mm

%free variables
Area = [];
Max = [];
Max_mean = [];
Max_std = [];
Min = [];
Min_mean = [];
Min_std = [];

location = '/Users/maralgharib/Desktop/Rat Muscle Stimulation Data/Data
2~7~11/Raw Data/';
prefix = '2_7_11_S1_';

%Filters for processing
h = fspecial('average', 3);           %Smoothing
[b,a]=butter(2, 125/FPS, 'low');      %Filter 1

datavoltagevec = [2];

for i =1:length(datavoltagevec)

dirname = sprintf(['S1_' num2str(datavoltagevec(i)) 'V/']);
imgdir = strcat(location, dirname);

    for j = 1:numimgs
        fname = sprintf('ImgA%06d.tif', j-1);
        file = strcat(imgdir, fname);

        im=imread(file);
        ims = imfilter(im,h, 'replicate'); %Smoothing filter
        level = graythresh(ims);
        levelbin(j) = level;
        BW = im2bw(ims, 38/255); %Pixel threshold parameter
        BW2 = bwareaopen(BW, round(100*scale^2)); %Removes areas smaller
than 100 mm^2*scale [px^2]
        whitepx = find(BW2>0); %White pixel count based on 1 square px
being min sensitivity
        numpix = length(whitepx);
        Area(j,i) = numpix/(scale^2);
    end
end
```

```

Cycle_1 = Area(1:100,i);
Cycle_2 = Area(101:200,i);
Cycle_3 = Area(201:300,i);
Cycle_4 = Area(301:400,i);

Cycle_1filt = filtfilt(b,a,Cycle_1); %FILTER #1 butter
Cycle_2filt = filtfilt(b,a,Cycle_2);
Cycle_3filt = filtfilt(b,a,Cycle_3);
Cycle_4filt = filtfilt(b,a,Cycle_4);

Max_1 = max(Cycle_1filt);
Max_2 = max(Cycle_2filt);
Max_3 = max(Cycle_3filt);
Max_4 = max(Cycle_4filt);

Max = [Max; Max_1, Max_2, Max_3, Max_4];

Min_1 = min(Cycle_1filt);
Min_2 = min(Cycle_2filt);
Min_3 = min(Cycle_3filt);
Min_4 = min(Cycle_4filt);

Min = [Min; Min_1, Min_2, Min_3, Min_4];

time_1 = linspace(0, length(Cycle_1filt)/FPS, length(Cycle_1filt));
time_2 = linspace(0, length(Cycle_2filt)/FPS, length(Cycle_2filt));
time_3 = linspace(0, length(Cycle_3filt)/FPS, length(Cycle_3filt));
time_4 = linspace(0, length(Cycle_4filt)/FPS, length(Cycle_4filt));

figure(i);

plot(time_1, Cycle_1filt/Cycle_1filt(1,1), 'ro-', 'LineWidth',
2,'MarkerSize',4)
hold;
plot(time_2, Cycle_2filt/Cycle_2filt(1,1), 'bx-', 'LineWidth',
2,'MarkerSize',4)
plot(time_3, Cycle_3filt/Cycle_3filt(1,1), 'kd-', 'LineWidth',
2,'MarkerSize',4)
plot(time_4, Cycle_4filt/Cycle_4filt(1,1), 'm*-', 'LineWidth',
2,'MarkerSize',4)

xlabel('Time (sec)')
ylabel('Area')
title(['Total Area - ' num2str(datavoltagevec(i)) 'V Stimulation'])
legend('C1','C2','C3', 'C4','Location', 'SouthEast');

pname = sprintf([prefix num2str(datavoltagevec(i)) 'V.tif']);
fileloc = strcat('/Users/maralgharib/Desktop/MatlabPlots/',pname);
print('-dtiffnocompression','-r150', pname);

end

indices = 1:numimgs;

```

```
Areawithindices = [indices' Area];
Max_mean = mean(Max,2);
Max_std = std(Max,0,2);
Min_mean = mean(Min,2);
Min_std = std(Min,0,2);

rname = sprintf([prefix 'results.mat']);
rloc = strcat('/Users/maralgharib/Desktop/', rname);

save(rloc);
```

Appendix 4: Force Analysis Matlab Code

```
close all
clear all
clc

f = 1000;          %Labview sampling frequency
spt = 200;         %Sample per trigger
numtrig = 64;      %Number of triggers
m = 0.4124;        %Slope of calibration line
b = 0.004;         %y intercept of calibration line

load('/Users/maralgharib/Desktop/Rat Muscle Stimulation Data/Data
12~16~11/S3_KrebsAChBATH_16min_P1_1.txt')
vdata = S3_KrebsAChBATH_16min_P1_1;      %data from labview (voltages)
prefix = ' S3_KrebsAChBATH_16min_P1_1_';

ii=1;

colors = ['b*-'; 'mo-'; 'rd-'; 'kx-'; 'g*-'];
ref = ['C01'; 'C02'; 'C03'; 'C04'; 'C05'; 'C06'; 'C07'; 'C08'; 'C09'; 'C10'; ...
      'C11'; 'C12'; 'C13'; 'C14'; 'C15'; 'C16'; 'C17'; 'C18'; 'C19'; 'C20'; ...
      'C21'; 'C22'; 'C23'; 'C24'; 'C25'; 'C26'; 'C27'; 'C28'; 'C29'; 'C30'; ...
      'C31'; 'C32'; 'C33'; 'C34'; 'C35'; 'C36'; 'C37'; 'C38'; 'C39'; 'C40'; ...
      'C41'; 'C42'; 'C43'; 'C44'; 'C45'; 'C46'; 'C47'; 'C48'; 'C49'; 'C50'; ...
      'C51'; 'C52'; 'C53'; 'C54'; 'C55'; 'C56'; 'C57'; 'C58'; 'C59'; 'C60'; ...
      'C61'; 'C62'; 'C63'; 'C64'];
time = linspace(0, spt/f, spt);

gconversion = (vdata(:, :)-b)./m;          %Volts converted to grams
fconversion = (gconversion(:, :)/1000)*9.81; %converts data into force
burstforce = transpose(fconversion);

burstmax = reshape(max(burstforce), numtrig, 1);
burstmin = reshape(min(burstforce), numtrig, 1);

last_p = 0;

for p=1:size(burstforce,2)
    jj = mod(p,5);
    if jj==1
        figure(ii)
        hold on
        ii = ii+1;
        clear legend_text;
        last_p = p-1;
    end

    disp(p)

    plot(time, burstforce(:,p), colors((mod(p,5)+1),:), 'LineWidth',2,
'MarkerSize',4)
    xlabel('Time (sec)')
    ylabel('Force (N)')
```

```

        title(['Contraction Force - EDL in  Krebs Bath & 100 ug/mL ACh
Solution Bath with 0.5V - 2V  Stimulation - C' num2str(5*(ii-2)+1) '-C'
num2str(5*(ii-1))])

        pname = [prefix, ['C' num2str(5*(ii-2)+1) '-C' num2str(5*(ii-1))],
'.tif'];
        print('-dtiffnocompression','-r150', pname);

end

save  S3_KrebsAChBATH_16min_P1_1_results.mat;

```