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TITLE:

Maximum Isometric Tetanic Force Measurement of the Tibialis Anterior Muscle in the Rat

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KEYWORDS:

Nerve injury, nerve regeneration, sciatic nerve, functional recovery, motor function, tetanic muscle force, rat model.

SUMMARY:

Evaluation of motor recovery remains the benchmark outcome measure in experimental peripheral nerve studies. The isometric tetanic force measurement of the tibialis anterior muscle in the rat is an invaluable tool to assess functional outcomes after reconstruction of sciatic nerve defects. The methods and nuances are detailed in this article.

ABSTRACT:

Traumatic nerve injuries result in substantial functional loss and segmental nerve defects often necessitate the use of autologous interposition nerve grafts. Due to their limited availability and associated donor side morbidity, many studies in the field of nerve regeneration focus on alternative techniques to bridge a segmental nerve gap. In order to investigate the outcomes of surgical or pharmacological experimental treatment options, the rat sciatic nerve model is often used as a bioassay. There are a variety of outcome measurements used in rat models to determine the extent of nerve regeneration. The maximum output force of the target muscle

remains the most relevant outcome for clinical translation of experimental therapies. Isometric force measurement of tetanic muscle contraction has previously been described as a reproducible and valid technique for evaluating motor recovery after nerve injury or repair in both rat and rabbit models. In this video, we will provide a step-by-step instruction of this invaluable procedure for assessment of functional recovery of the tibialis anterior muscle in a rat sciatic nerve defect model using optimized parameters. We will describe the necessary pre-surgical preparations in addition to the surgical approach and dissection of the common peroneal nerve and tibialis anterior muscle tendon. The isometric tetanic force measurement technique will be detailed. Determining the optimal muscle length and stimulus pulse frequency is explained and measuring the maximum tetanic muscle contraction is demonstrated.

INTRODUCTION:

Loss of motor function following traumatic peripheral nerve injury has a significant impact on the quality of life and socioeconomic status of patients[1-3]. The prognosis of this patient population remains poor due to minimal improvements in surgical techniques over the years[4]. Direct end-to-end tension-free epineural repair forms the gold standard surgical reconstruction. However, in cases with extended nerve gaps interposition of an autologous nerve graft has proven to be superior[5,6]. The associated donor site morbidity and limited availability of autologous nerve grafts have imposed the need for alternative techniques[7,8].

Experimental animal models have been used to elucidate the mechanism of peripheral nerve regeneration and to evaluate outcomes of a variety of reconstructive and pharmacological treatment options[8,9]. The rat sciatic nerve model is the most frequently used animal model[10]. Their small size makes them easy to handle and house. Due to their superlative neuroregenerative potential, the diminished time between intervention and evaluation of outcomes can result in relatively lower costs[11,12]. Other advantages of its use include morphological similarities to human nerve fibers and the high number of comparative/historic studies[13]. Although the latter should be approached cautiously, as a wide variety of different outcome measures between studies makes it difficult to compare results[14-18].

Outcome measures to assess nerve regeneration range from electrophysiology to histomorphometry, but these methods imply a correlation but do not necessarily directly measure the return of motor function[14,15]. Regenerating nerve fibers might not make appropriate connections which can cause an overestimation of the number of functional connections[14,15,19,20]. The best and clinically most relevant measurement to demonstrate correct reinnervation of end organs remains assessment of muscle function[21-23]. Creating motor function assessment tools for animal models is, however, challenging. Medinaceli et al. first described the walking track analysis, which has since been the most frequently used method to evaluate functional recovery in experimental peripheral nerve studies[21,24-28]. The walking track analysis quantifies the sciatic functional index (SFI) based on measurements of pawprints from walking rats[21,29]. Major limitations of the walking track analysis, such as toe contractures, automutilation, smearing of the print and poor correlation with other measures of reinnervation, have necessitated the use of other parameters for quantification of functional

recovery[30,31].

In previous studies in Lewis rats[32] and New Zealand rabbits[33], we validated the isometric tetanic force (ITF) measurement for the tibialis anterior (TA) muscle and demonstrated its effectiveness in the evaluation of muscle recovery after different types of nerve repair[34-39]. The TA muscle is well suited because of its relatively large size, innervation by the peroneal branch of the sciatic nerve and well elucidated biochemical properties[40-43]. When muscle length (preload force) and electrical parameters are optimized the ITF provides a side-to-side variability of 4.4% and 7.5% in rats[32] and rabbits[33], respectively.

This article provides a detailed protocol of the ITF measurement in the rat sciatic nerve model, including a thorough description of the necessary pre-surgical planning, surgical approach and dissection of the common peroneal nerve and the distal TA muscle tendon. Using predetermined values for the stimulus intensity and duration, the optimal muscle length and stimulus pulse frequency will be defined. With these four parameters, the ITF can subsequently be consistently and accurately measured.

PROTOCOL:

All animal procedures were performed with approval of the Institutional Animal Care and Use Committee (IACUC A334818).

1. Calibration of the force transducer

1.1. Ensure that the computer is properly connected to the USB-6009 multifunctional I/O data acquisition (DAQ) device, which in turn should be connected to the force transducer.

NOTE: Other rat strains and species may require a different load-cell force transducer as higher forces are to be expected [44].

1.2. Attach a custom clamp fashioned from a modified surgical hemostat to the force transducer that is mounted to a vacuum base adjustable lever arm.

NOTE: The custom-made clamp consists of a surgical hemostat modified with a tightening screw that allows for adjustment of the tension (**Figure 1**).

1.3. Position the custom-made acrylic glass testing platform, which contains two wooden blocks for fixation of the rat hind limb, on the table.

NOTE: Other materials such as urethane can also be used instead of wood as long as the K-wires are able to penetrate and fixate.

1.4. Attach the clamp, force transducer and adjustable lever arm combination vertically to the testing platform using its vacuum base.

1.5. Fasten a hook or loop to the clamp for the calibration weights.

1.6. Turn on the computer and open the software (e.g., Labview).

1.7. Once the software is opened, start the custom-made virtual instrument (VI) for ITF measurement (**Figure 2**).

NOTE: **Figure 2** contains the LabVIEW code in a VI snippet. This VI snippet can be dragged onto the block diagram in LabVIEW. It will automatically be transformed into a graphical code. For this experiment the sampling rate was set at 2000 Hz with 25 samples to read for each iteration.

1.8. Run the VI by pressing the white arrow in the left upper corner and select **New calibration**. A new window will open.

1.9. Start the calibration process with zero weight (only the clamp with an attached hook or loop) and press **OK**.

1.10. Consecutively, add 10, 20, 30 and 50 grams of weight and press **OK** in between each weight measurement.

1.11. Once all five measurements are collected, click on **Process**.

1.12. Only accept the values if the graph on the VI displays a positive linear curve (**Figure 3**).

1.13. Reposition the clamp, force transducer and adjustable lever arm combination horizontally on the testing platform. This will be the position used for measuring the ITF.

1.14. Click on **Zero** and the window will automatically close.

2. Animal subjects

2.1. Use male Lewis rats weighing between 300-500 g.

NOTE: For comparison of nerve regeneration, it is imperative to use the same rat strain in both the control and experimental groups, since weight and incidence of autotomy are strain dependent and can tremendously influence the results of the ITF[10,32,45-47].

3. Surgical preparation

3.1. Prepare all required surgical instruments prior to surgery (**Table of Materials**).

3.2. Weigh the animals to determine the required amount of anesthesia.

3.3. Induce anesthesia by placing the rat in a chamber gassed with 3% isoflurane in oxygen.

3.4. Deeply anesthetize the rat using a cocktail of ten-parts ketamine (100 mg/mL) and one-part xylazine (100 mg/mL) at a dosage of 1 mL/kg body weight via an intraperitoneal injection. Monitor the depth of anesthesia based on the response to a toe pinch and by observing the respiratory rate.

3.5. Approximately 30 minutes after the initial dosage of the ketamine/xylazine cocktail, administer a supplementary dose of 0.3-0.6 mL/kg body weight of only ketamine (100 mg/mL) intraperitoneally to maintain adequate anesthesia throughout the entire procedure, which is defined as a low respiratory rate and an absent response to a toe pinch.

CAUTION: It is important to meticulously administer the required anesthesia as an overdose cannot be counteracted.

3.6. Carefully shave the hind limbs of the rat using electric clippers.

3.7. Place the rat in prone position on a heating pad to maintain the body temperature at 37 °C. Optionally, the body temperature can be monitored using a rectal thermometer.

3.8. Inject 5 mL of 0.9% sodium chloride (NaCl) subcutaneously into the loose skin over the neck of the rat to preserve an adequate hydration status throughout the procedure.

3.9. Due to the non-survival nature of this procedure, the surgical field and instruments do not require to be sterile. The surgeon should use personal protective equipment (PPE) and surgical loupes are advised for proper visualization of the anatomical structures.

4. Surgical approach to the common peroneal nerve

4.1. Place the rat in either the right or left lateral recumbent position depending on which side will be measured first.

4.2. Create a 2-3 cm incision in the skin of the posterolateral thigh parallel to the femur starting at the greater trochanter using a surgical no. 15 blade.

4.3. Identify the plane between the biceps femoris muscle and the gluteus maximus and vastus lateralis muscles and perform a blunt dissection using tenotomy scissors to separate these muscles and expose the underlying sciatic nerve.

4.4. Locate the trifurcation of the sciatic nerve and place a retractor to acquire better access. The three branches of the sciatic nerve include the common peroneal nerve, the tibialis nerve and the sural nerve.

4.5. Isolate the common peroneal nerve branch (usually the most ventral branch) of the sciatic nerve using a curved microsurgical forceps.

NOTE: In case of uncertainty, gently stimulate the isolated nerve with a surgical nerve stimulator and observe the motor response. Stimulation of the common peroneal nerve results in dorsiflexion of the paw.

5. Dissection of the distal tibialis anterior muscle tendon

5.1. In order to expose the TA muscle and its insertion, incise the skin at the anterolateral aspect of the lower leg, starting at the knee joint and descending to the mediodorsal side of the hind paw.

5.2. Dissect the distal TA muscle tendon from the surrounding tissue using a scalpel with a surgical blade no. 15.

5.3. Using a mosquito forceps, bluntly dissect the TA muscle tendon towards the insertion and cut the tendon as distal as possible. Leave the proximal TA muscle undisturbed, preserving the neurovascular pedicle.

NOTE: Regularly (approximately every 5 minutes), moist the TA muscle with heated 0.9% NaCl (37 °C) to prevent cooling and desiccation.

6. Isometric tetanic force measurement

6.1. Connect the bipolar electrode cables and the ground cable according to their color to a bipolar stimulator device.

6.2. Attach the other end of the bipolar electrode cables to a subminiature electrode.

NOTE: The reference electrode (red, anode) should be placed distal and the active electrode (black, cathode) proximal.

6.3. Transfer the animal together with the heating pad to the testing platform.

6.4. Fixate the hind limb of the rat to the wooden block using two 1 mm Kirschner wires through the ankle and the lateral condyle of the distal femur avoiding the posterior aspect of the knee.

CAUTION: Avoid vascular damage to the popliteal artery and vein which are located dorsally to the femur condyle.

6.5. Attach a holder with a custom clamp to the testing platform using its vacuum base.

6.6. Secure the distal TA muscle tendon to the clamp attached to the force transducer.

NOTE: The clamp and force transducer should be positioned parallel to the course of the TA muscle.

6.7. Place the retractor at the posterolateral thigh of the rat in order to access the common peroneal nerve.

NOTE: The sciatic nerve and its branches should be kept moist with heated 0.9% NaCl (37 °C) to prevent cooling and desiccation.

6.8. Insert the ground cable in the surrounding muscles (e.g., the vastus lateralis muscle).

NOTE: The Grass SD9 stimulator requires a ground cable to reduce electrical artifacts. Newer stimulators might not require an extra ground cable.

6.9. Hook the common peroneal nerve to the subminiature electrode and fix its position using the holder on the platform (**Figure 4**).

NOTE: Ensure that only the common peroneal nerve is hooked to the subminiature.

6.10. Optimization of the muscle length

6.10.1. Turn the bipolar stimulator device on and adjust the settings as follow: square monophasic pulse, delay 2 ms, stimulus pulse duration 0.4 ms, stimulus intensity 2 V.

NOTE: The delay determines the time between the sync out pulse and the delivery of the leading edge of the pulse.

6.10.2. Select **Parameter test** and turn on **Trigger collection** in the VI.

6.10.3. Increase the muscle length (preload) by adjusting the lever arm attached to the force transducer.

6.10.4. Start at 10 g of preload and use increments of 10 g until the maximum active muscle force is determined.

6.10.5. For each preload, apply two single twitches directly after each other using the button on the bipolar stimulator device. The output will be visible on the screen and the rat should show dorsiflexion of the paw.

NOTE: Before stimulating the nerve, always remove any excess 0.9% NaCl surrounding the nerve using cotton tipped applicators to ensure the signal is not conducted to the surrounding tissue.

6.10.6. To stop the measurement, hit **Trigger collection** again in the VI.

6.10.7. If the program automatically detects the two peak output forces click on **Accept**. In case the program does not automatically select these output forces, press **Decline** and select the peaks manually. The two peak output forces will be averaged to a mean peak output force (**Figure 5**).

6.10.8. Calculate the active muscle force by subtracting the preload from the mean peak output force.

6.10.9. Write down the active force for each preload to visualize the trend and recognize the maximum active force (**Figure 6**). A spreadsheet can also be used.

6.11. Measurement of isometric tetanic force

6.11.1. After determining the ideal muscle length, let the muscle rest at zero preload for 5 minutes prior to starting the tetanic muscle contractions.

6.11.2. Meanwhile, switch from **Parameter test** to **Frequency test** on the VI and adjust the stimulus intensity to 10 V on the bipolar stimulator device.

6.11.3. Keep the delay and stimulus pulse duration at 2 ms and 0.4 ms, respectively.

6.11.4. Measure the isometric tetanic muscle force using increasing stimulus frequencies starting at 30 Hz with increments of 30 Hz until the maximum force plateau is observed.

6.11.5. Click on **Trigger collection** and set to the predetermined optimal muscle length.

6.11.6. Press the **Repeat** button on the bipolar stimulator device to induce a tetanic stimulation for a maximum of 5 seconds or until a force peak is clearly observed.

NOTE: Before stimulating the nerve, always remove any excess 0.9% NaCl surrounding the nerve using cotton tipped applicators to ensure the signal is not conducted to the surrounding tissue.

6.11.7. To collect the data, press **Trigger collection** again and document the maximum output force. In case the program does not automatically detect the peak maximum output force, press **Decline** and select the peak manually.

6.11.8. Let the muscle rest again at zero preload for 5 minutes prior to starting the next tetanic muscle contractions.

NOTE: Regularly (approximately every 5 min), moist the TA muscle with heated 0.9% NaCl (37

°C) to prevent cooling and desiccation.

6.11.9. Continue increasing the stimulus frequency until the maximum force plateau is reached. The force plateau will be defined as the maximum isometric tetanic force.

NOTE: After this step, remove the K-wires, staple or suture the skin and repeat the entire procedure to the contralateral hind limb, starting at step 4.

REPRESENTATIVE RESULTS:

Five parameters are used to measure the ITF measurement. These include muscle tension (preload force), stimulus intensity (voltage), stimulus pulse frequency, stimulus duration of 0.4 ms and a delay of 2 ms. Prior to measuring the ITF, the optimal muscle tension has to be determined using two single twitch muscle contractions at an intensity of 2 V during the parameter test. These stimuli cause dorsiflexion of the paw and produce an output signal on the graph in the VI (**Figure 5**). These single twitch curves ideally have a rapid vertical upswing representing the contraction period directly followed by a slower vertical decrease period demonstrating the relaxation period. The program will average these two peak output forces, but the active force has to be manually calculated by subtracting the preload force from the mean output force. In the example in **Figure 5**, a preload of 10 g results in two peak output forces of 411.09 g (4.03 N) and 379.78 g (3.73 N), which is averaged to a mean peak output force of 395.43 g (3.88 N). When the active forces of each preload are plot in a graph, the maximum active force can be identified. These active forces usually produce a bell-shaped curve and the maximum active force for Lewis rats weighing 300-500 g should be around 30-40 g (0.29-0.39 N) (**Figure 6**).

For the tetanic stimulations during the frequency test, the stimulus intensity is increased to a supra-maximal voltage (10 V) to ensure maximal activation of all TA muscle motor units using increasing frequencies. The optimal tetanic curve increases and decreases sharply and has a slowly decreasing plateau phase with minimal oscillations. **Figure 7** depicts an example of a tetanic curve at a stimulus frequency of 30 Hz with an isometric tetanic force of 803.25 g (7.88 N). The highest force plateau is defined as the maximum ITF.

FIGURE LEGENDS:

Figure 1: Image of customized clamp fashioned from a surgical hemostat and modified with a tightening screw that allows for adjustment of the tension.

Figure 2: Graphical code for virtual instrument for isometric tetanic force measurement on LabVIEW.

Figure 3: Calibration of the force transducer. Successful calibration of the force transducer with five weights (0, 10, 20, 30 and 50 g) should result in a positive linear curve.

Figure 4: Schematic overview of experimental setup for isometric tetanic force measurement. (Copyrighted and used with permission of the Mayo Foundation for Medical Education and

Research; all rights reserved. Reprinted from: Shin, R. H. et al. Isometric tetanic force measurement method of the tibialis anterior in the rat. *Microsurgery.* 28 (6), 452-457 (2008)).

Figure 5: Representative single twitch curves for optimization of muscle length. For each preload measurement, two single twitches are applied. These single twitch curves have a rapid vertical upswing (contraction period) followed by a vertical decrease (relaxation period). The two peak output forces will be averaged to a mean peak output force. In this example with a Lewis rat, a preload of 10 g results in two peak output forces of 411.09 g (4.03 N) and 379.78 g (3.73 N), which is averaged to a mean peak output force of 395.43 g (3.88 N).

Figure 6: Optimal muscle length (preload). The active muscle force can be calculated by subtracting the preload from the mean peak output force. The active muscle force for each preload should be documented until a drop in active muscle force is visible. The preload yielding the highest active muscle force will be used to measure the isometric tetanic force. The optimal preload for Lewis rats weighing 300-500 g should be around 30-40 g (0.29-0.39 N) (N=10).

Figure 7: Representative isometric tetanic force curve. The optimal tetanic curve increases sharply, then has a slowly decreasing plateau phase followed by a sharp decrease. The highest force plateau is defined as the maximum ITF. This example depicts the tetanic curve at a stimulus frequency of 30 Hz with an isometric tetanic force of 803.25 g (7.88 N).

DISCUSSION:

This protocol describes a previously validated method for acquiring accurate maximum ITF measurements of the TA muscle in the rat model[32]. The recovery of maximum strength after experimental nerve reconstruction treatments is of primary interest in the clinical setting as it proves that the nerve not only regenerated, but also made working connections with the target muscle. The ITF can be used in a small nerve gap model, such as the rat sciatic nerve model[32], and with a few modifications to the protocol, it can also be used in a larger nerve gap rabbit model[33].

There are several critical steps that should be considered to ensure consistent and reliable maximum isometric muscle force measurements. The importance of carefully selecting the type of anesthesia to prevent skeletal muscle side effects has previously been described[32,33]. The use of isoflurane has demonstrated a time dependent decrease in muscle force, which can be explained by its ability to induce sarcoplasmic reticulum stimulated release of calcium[33,48]. The effect of ketamine/xylazine on the muscle force has proven to be minimal based on our experience and previous study[32]. Secure attachment of the distal TA muscle tendon to the force transducer is also of great importance for accurate measurements. Slippage or tearing of the tendon should be prevented or directly corrected. Therefore, a custom-made clamp was created from a surgical hemostat and modified with a tightening screw. Other research groups have described a technique of drying the tendon for about 30 minutes to mechanically strengthen the interface between the tendon and a clamp[49]. In order to maintain endurance of the muscle it is critical to avoid desiccation of the TA muscle and tendon with warm 0.9% NaCl

and implement a 5-minutes resting period between each tetanic stimulation. The resting period is based on the activity of the phosphagen system, also known as the immediate energy source, which is important for explosive muscle contractions. It consists of adenosine triphosphate (ATP) and creatine phosphate activity and provides energy for less than 10 seconds of maximal activity. It requires approximately 3-5 minutes to replenish 100% of the phosphagens[50].

We recognize the limitations of the method described in this video. The non-survival nature of the procedure does not allow for serial measurements over time. Additionally, it is a detailed and time-consuming testing protocol. During the 1 to 2 hour testing time, the nerve and muscle undergo a significant number of stimulations which may result in muscle fatigue with potential decrease in ITF. This has, however, proven to be less prominent in the rat model compared to the rabbit[33].

In conclusion, the ITF measurement described in this video is an invaluable tool in experimental peripheral nerve studies to quantify motor recovery. When presented with other outcome measures such as electrophysiology and histomorphometry, a global assessment of nerve function can be provided.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;JoVE61926 Figure 1 - Customized clamp.jpg

Figure 2

Click here to access/download;Figure;JoVE61926 Figure 2 .png

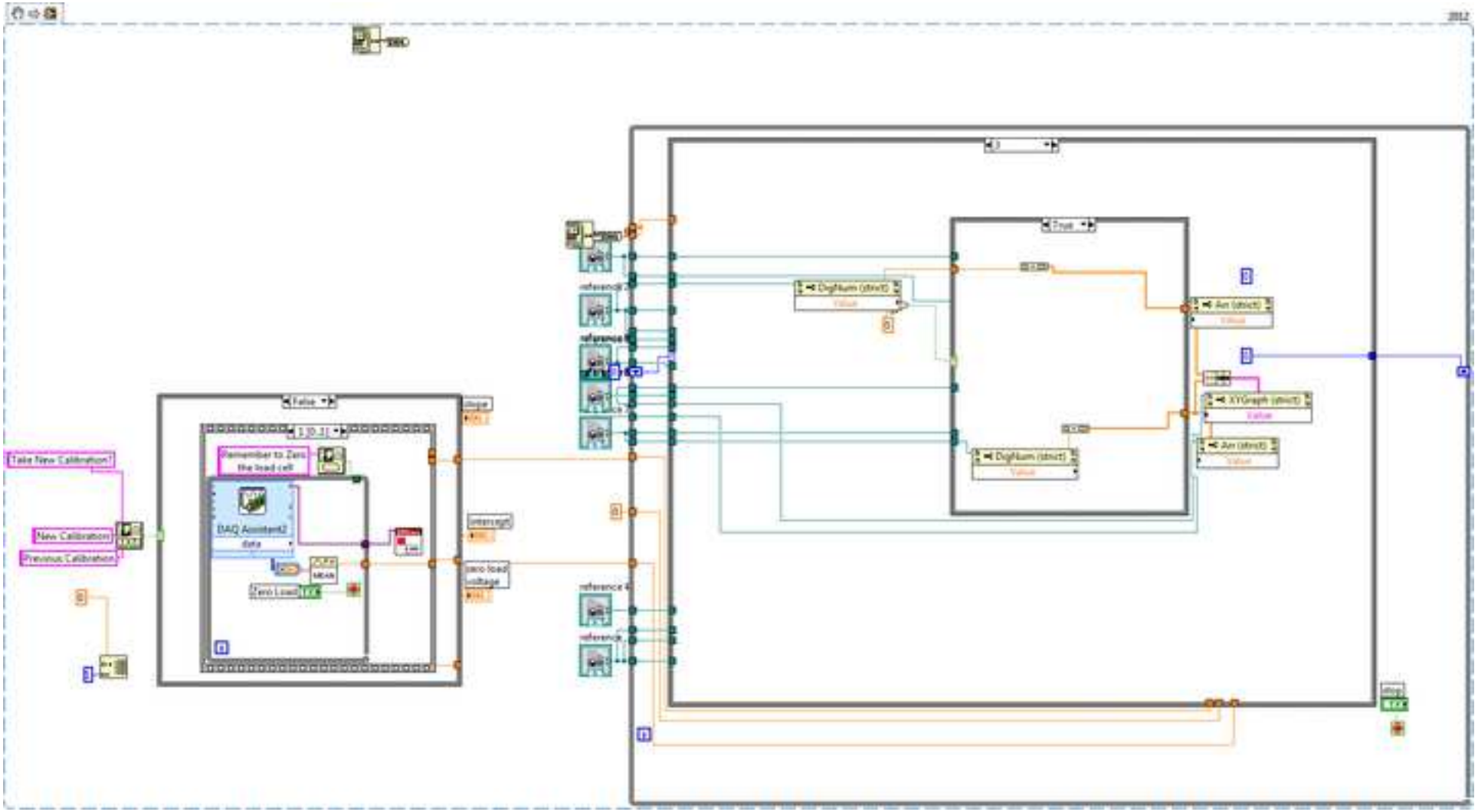


Figure 3

Click here to access/download;Figure;JoVE61926 Figure 3 - Calibration of the Force Transducer.png

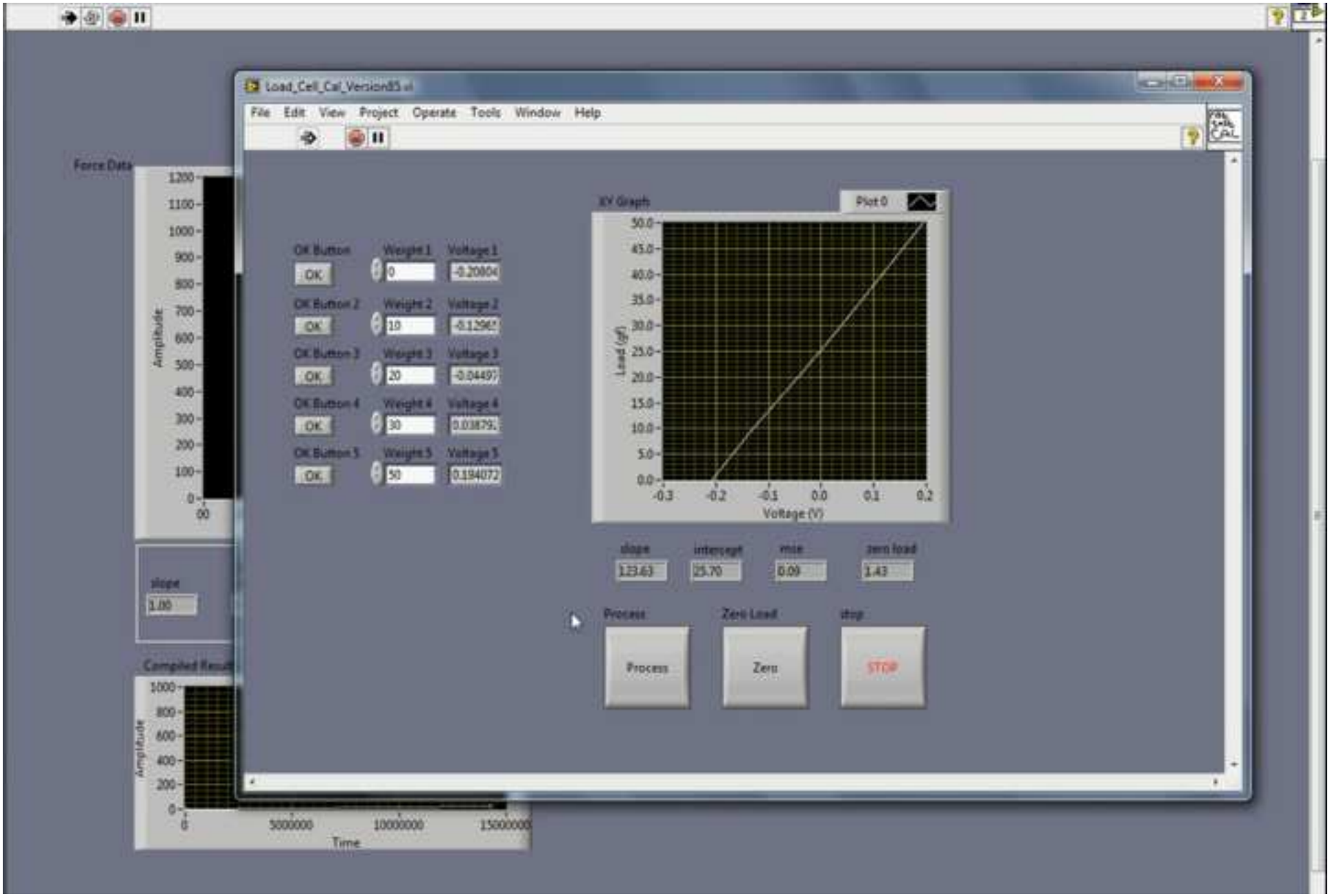


Figure 4

Click here to access/download;Figure;Figure 4 - Single twitch curves.png

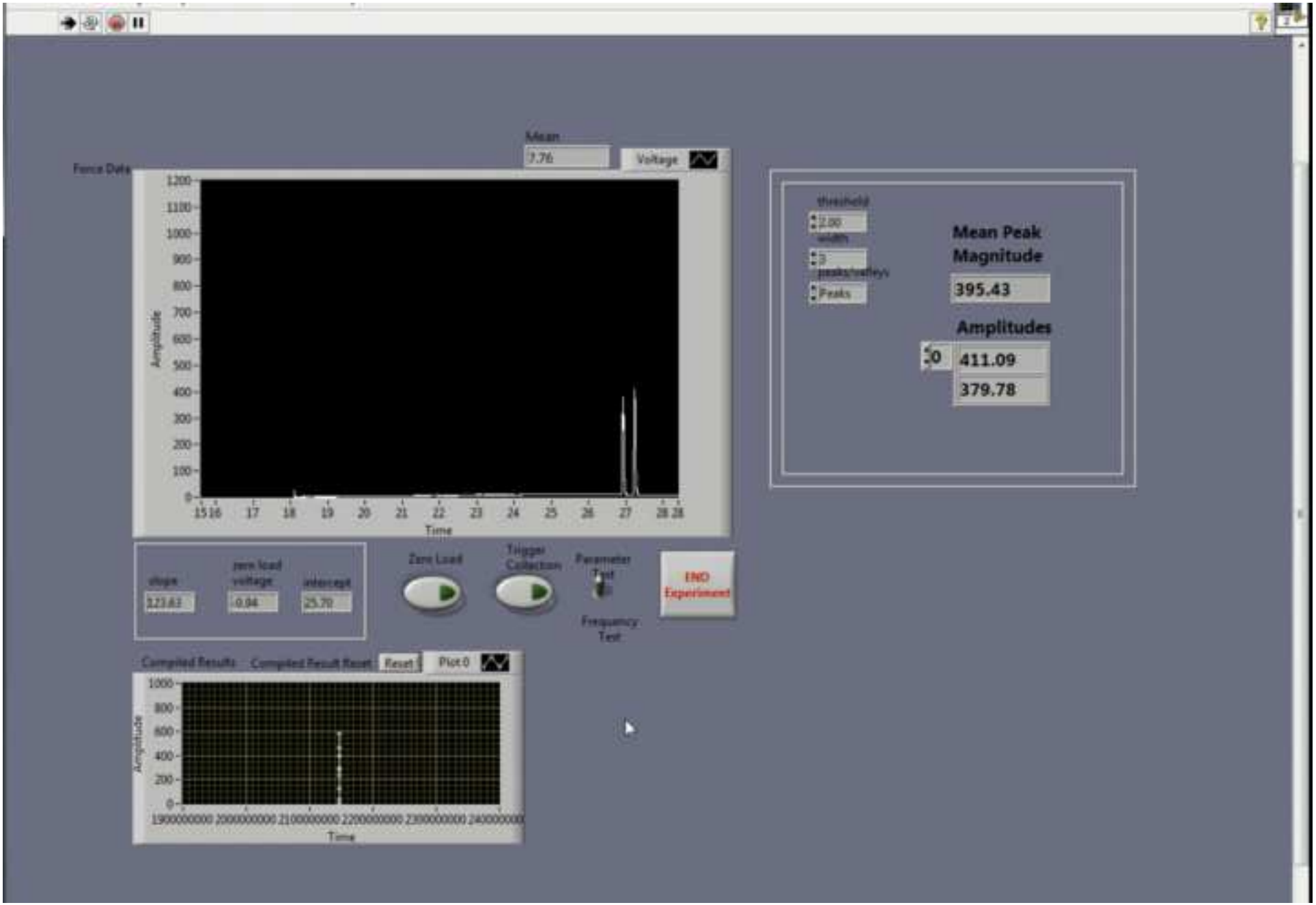


Figure 5

Click here to access/download;Figure;JoVE61926 Figure 5 - Single twitch curves.png

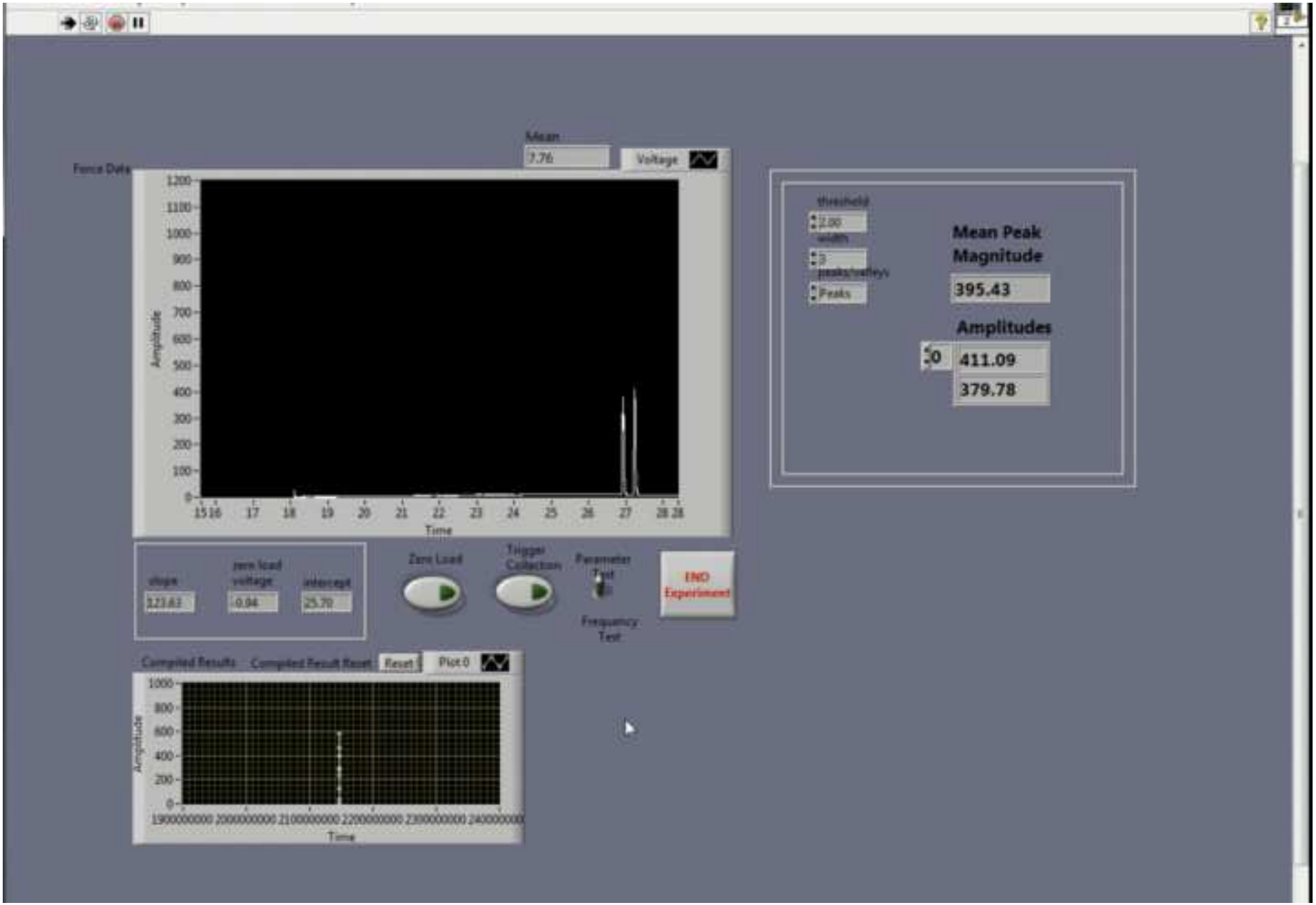


Figure 6

Click here to access/download;Figure;JoVE61926 Figure 6 - Optimal muscle length (preload).jpg

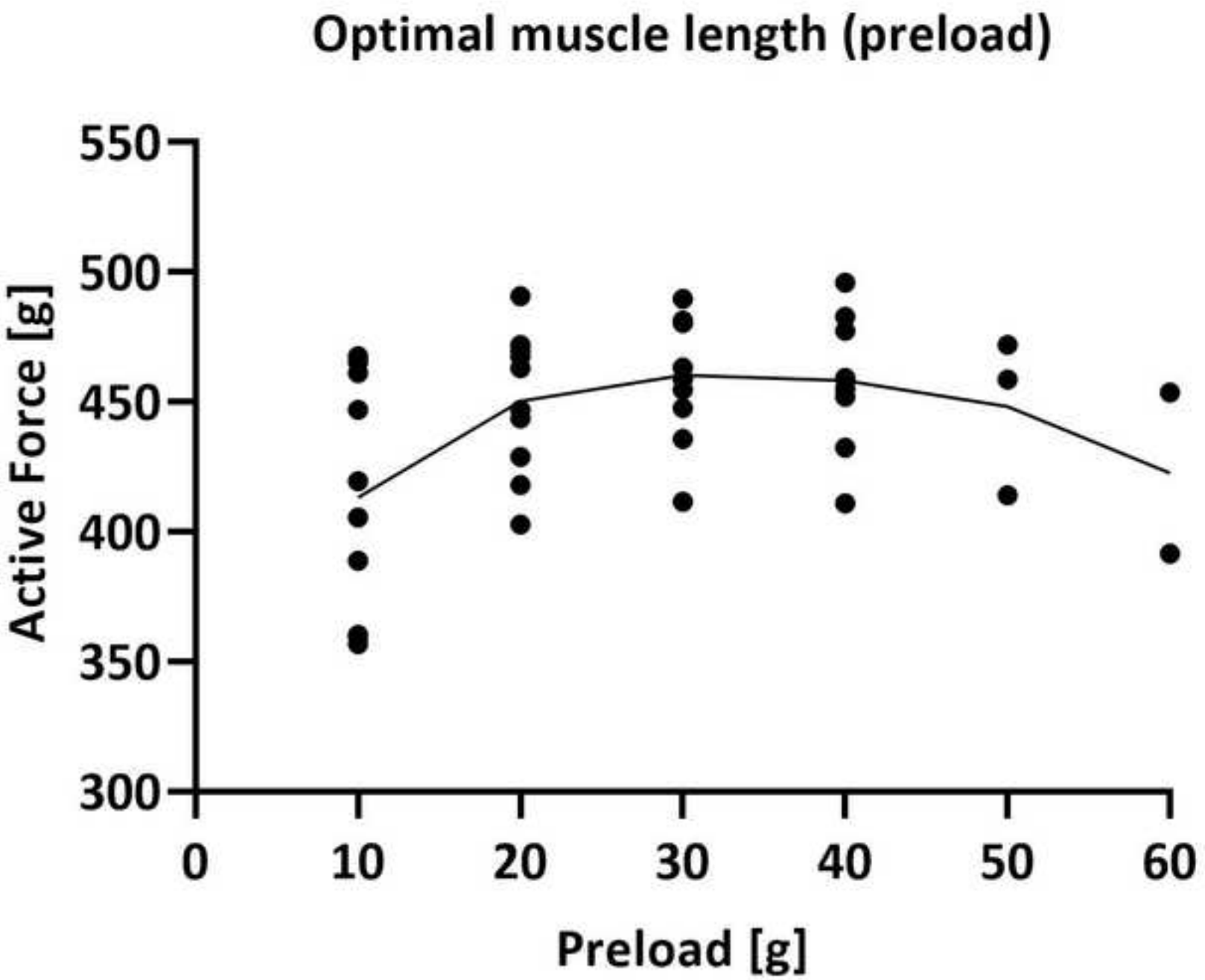
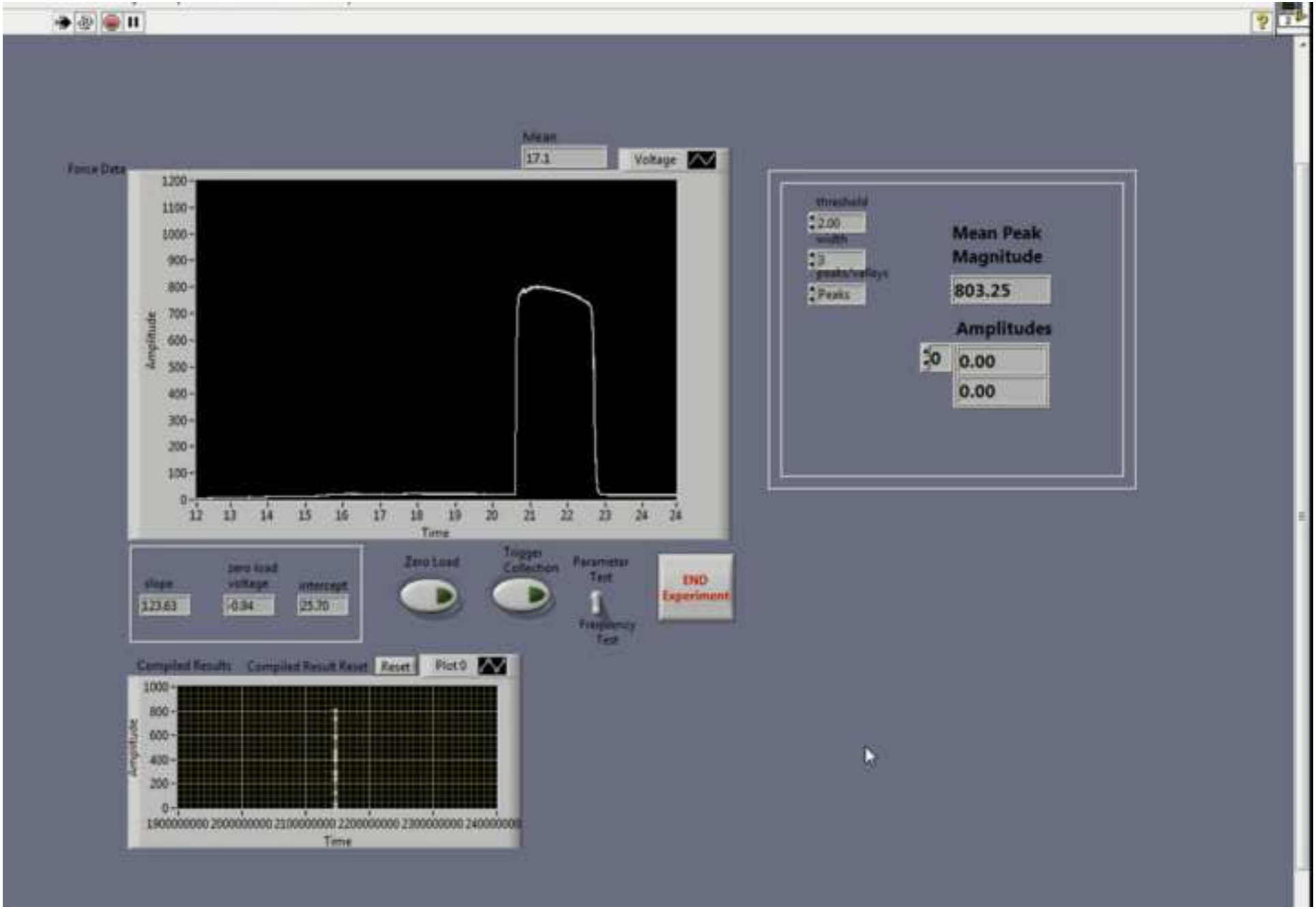


Figure 7

Click here to access/download;Figure;JoVE61926 Figure 7 - Isometric tetanic force curve.png



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.9% Sodium Chloride	Baxter Healthcare Corporation, Deerfield, IL, USA	G130203	
1 mm Kirshner wires	Pfizer Howmedica, Rutherford, NJ	N/A	
Adson Tissue Forceps	ASSI, Westbury, NY, USA	MTK-6801226	
Bipolar electrode cables	Grass Instrument, Quincy, MA	N/A	
Bipolar stimulator device	Grass SD9, Grass Instrument, Quincy, MA	N/A	
Cotton-tip Applicators	Cardinal Health, Waukegan, IL, USA	C15055-006	
Curved Mosquito forceps	ASSI, Westbury, NY, USA	MTK-1201112	
Force Transducer MDB-2.5	Transducer Techniques, Temecula, CA	N/A	
Gauze Sponges 4x4	Covidien, Mansfield, MA, USA	2733	
Ground cable	Grass Instrument, Quincy, MA	N/A	
Isoflurane chamber	N/A	N/A	Custom-made
Ketamine	Ketalar, Par Pharmaceutical, Chestnut, NJ	42023-115-10	
LabView Software	National Instruments, Austin, TX		
Loop	N/A	N/A	Custom-made
Microsurgical curved forceps	ASSI, Westbury, NY, USA	JFA-5B	
Microsurgical scissors	ASSI, Westbury, NY, USA	SAS-15R-8-18	
Microsurgical straight forceps	ASSI, Westbury, NY, USA	JF-3	
Retractor	ASSI, Westbury, NY, USA	AG-124426	
Scalpel Blade No. 15	Bard-Parker, Aspen Surgical, Caledonia, MI, USA	371115	
Slim Body Skin Stapler	Covidien, Mansfield, MA, USA	8886803512	
Subminiature electrode	Harvard Apparatus, Holliston, MA	N/A	
Surgical Nerve Stimulator	Checkpoint Surgical LCC, Cleveland, OH, USA	9094	
Terrell Isoflurane	Piramal Critical Care Inc., Bethlehem, PA, USA	H961J19A	
Testing platform	N/A	N/A	Custom-made
Tetontomy Scissors	ASSI, Westbury, NY, USA	ASIM-187	

Traceable Big-Digit Timer/Stopwatch	Fisher Scientific, Waltham, MA, USA	S407992	
USB-6009 multifunctional I/O data acquisition (DAQ) device	National Instruments, Austin, TX	779026-01	
Vacuum Base Holder	Noga Engineering & Technology Ltd., Shlomi, Isreal	N/A	Attached clamp is custom-made
Weight (10 g)	Denver Instruments, Denver, CO, USA	820010.4	
Weight (20 g)	Denver Instruments, Denver, CO, USA	820020.4	
Weight (50 g)	Denver Instruments, Denver, CO, USA	820050.4	
Xylazine	Xylamed, Bimeda MTC Animal Health, Cambridge, Canada	1XYL002	

Manuscript JoVE61926 "Isometric Tetanic Force Measurement of the Tibialis Anterior Muscle in the Rat"

Response to Reviewers

Thank you for giving us the opportunity to submit a revision of the manuscript "Isometric Tetanic Force Measurement of the Tibialis Anterior Muscle in the Rat" for publication in the Journal of Visualized Experiments. We appreciate the time and effort that you and the reviewers dedicated to providing feedback on our manuscript.

Over the years, our laboratory has received many questions regarding the isometric tetanic force measurement. The purpose of this manuscript and video was to provide a detailed description of the method.

We have incorporated most of the suggestions made by the reviewers. Those changes are highlighted within the manuscript. Please see below, in red, for a point-by-point response to the reviewers' comments and concerns.

Sincerely,

On behalf of all authors,

Meiwand Bedar

Reviewers' Comments to Author:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
3. Please spell out journal titles in the references.

Thank you for your comments. These points are adjusted in the revised version of the manuscript. We have used the JoVE Endnote reference style. However, some journal titles are still abbreviated.

Changes to be made by the Author(s) regarding the video:
1. Please include a discrete representative results section after the protocol.

Thank you for your comment. Representative results are discussed within the protocol, since it is

important for understanding the protocol as well as the continuity. Therefore, discussing the results in a separate section would not be logical.

2. Please remove branding and watermarks.
• 0:25-0:50, 8:11-8:38 - "(C) Mayo Clinic" is visible in the bottom.
• 0:51-1:00 - The same "Mayo Clinic" tag is visible in the lower left, but the (C) and part of the M are cut off.
• 1:32 - The narration mentions "Lab View software".

Thank you for your comments. All branding has been removed from the video.

3. • 1:53-2:37 - The music does help this otherwise silent gap, but this is still a long time to go without narration. I recommend editing down the gaps between action by crossfading ahead in the shot. Another option may be to add a text list of the different weights instead of showing all of them.
4. • 1:53-2:37,5:54 - The music here comes in a bit loudly compared to the narration preceding it. I was also surprised by the music coming in two and a half minutes into the video, when we had not heard music until this point. I recommend adding music at the beginning to establish its presence.

Thank you for your comment. We have added the music in the introduction of the video and used the crossfade function combined with text to reduce the silent gap. We have also reduced the loudness of the music.

• 6:45-6:52 - I understand adding the camera audio here, but its presence makes the content (a rat's leg being drilled into) more unsettling than it needs to be. The audio should be removed.

Thank you for your comment. We have deleted this audio.

5. • 5:35, 9:56-10:02 - At these moments, there is additional compressed audio coming through the right audio channel. This needs to be corrected.

Thank you for your comment. We have recorded these parts again.

6. • 6:26-6:52 - Animal appears to be fully anesthetized, but its leg is being drilled into and there is a bit of blood underneath. It's a bit gruesome, considering the drill. Have a look and see what you think.

Thank you for your comment. Unfortunately, this part cannot be changed, since it is a crucial part of the protocol.

Reviewer #1:

Manuscript Summary:

The manuscript and related video demonstrate a possible measurement setup for measuring the tensile forces of the tibialis anterior muscle of male Lewis rats during isometric contractions at optimal muscle length. The authors intend with this contribution to introduce a "step-by-step" guide. The authors took great care in describing each step and also the video seems to offer good instructions for a general overview on how to perform this type of procedures. Further, it is worth mentioning that the team has many years of experience, which is reflected in several mentioned publications.

I think that the presented work fits well within the scope of JoVe and thus should be generally considered for publication as it could be useful for many researchers starting to measure isometric forces in rodents.

Nevertheless, the manuscript needs some major revisions in order to comply with scientific publication standards.

Major Concerns:

Novelty

Unfortunately the presented work does not contribute any novelty in terms of scientific results. All presented data is just exemplary and the method it self was already presented in (Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery. 2008;28:452-7). Although I think that the authors should present a complete set of measurement data for at least one animal, I acknowlegde that novelty is not a general aim of JoVe. Thus I think it also would be sufficient to publish a sound and detailed description of the method.

Thank you for your comment. We agree with the reviewer that the presented work does not contribute any novelty in terms of scientific results. However, due to the complexity of the technique and the questions we have received over the years, we deemed it necessary to provide a detailed description of the technique and visual content.

Calibration

The authors should perform calibration using weights for the entire measurement range. From a engineering point of view it is not sufficient to calibrate a load-cell for max 50g when measuring >800g.

Thank you for your comment. The load-cell force transducer is already calibrated by the company for 1134 g with an error of 0.567 g. We verify this calibration by measuring 0, 10, 20, 30 and 50 g. In case of a linear relationship, it is highly likely that measurements above the range of calibration are accurate.

Stimulator

The used stimulator is very old and should be replaced by a newer device. In particular the provided information regarding the used stimulation parameters is insufficient to repeat the measurements with another stimulator. For example - not knowing the used Grass stimulator - it is quite hard to understand why it requires an additional ground electrode when delivering bipolar (meaning two stimulating poles/electrodes) stimulation.

We appreciate the comment by the reviewer. Our device is functional and we do not have the funds for a new stimulator. In the revised manuscript, we have incorporated the additional information the reviewer suggested which will enable other researchers to repeat the measurements with a newer stimulator. We have also explained the reason for the need for an additional ground cable with our older stimulator.

Units
Forces are measured in Newton [N] not in gram [g].

Thank you for your comment. Our LabView VI is programmed to report the measurements in grams. The measurements can be converted to Newton. We have provided the converted measurements in Newton in the revised manuscript.

Slang terms
The 'playful' creator of their LabView VI uses rather unconventional names for his functions. While these terms are highly missleading in the manuscript they are simply
inappropriate for a scientific publication.

Thank you for your comment. We have adjusted this in the manuscript and video.

LabView implementation
The authors are using LabView for recording and visualisation of the force data. On the one hand they programmed certain routines (e.g. calibration) to automatize certain
processes, on the other hand most other steps involve additional manual interaction and manual noting. The authors should consider to automatize their procedures as good as possible in order to minimize human errors during the experiment. LabView is certainly a very powerful develop environment to achieve this.

Thank you for your comment. We will definitely consider further automating the process in the future.

Specific Comments:
L111: Please state the used settings somewhere. Which samplerate did you use for your recordings?

Thank you for your comment. We have added this to the revised manuscript. We use a sampling rate of 2000 Hz with 25 samples to read for each iteration. The maximum sampling rate of the DAQ device is 48 kilosamples per second.

L131: Calibration needs to be performed for the full measurement range. It is ok to start with 10g- but in this case also the weight of the attachment slings should be ackowledged.
The maximum calibration weight should be at least 1.134kg (2.5lb) to cover the entire measurement range of the load-cell. Further the authors should also mention that other strains of rats might require a different load-cell as higher forces are to be expected: (e.g. up to 1.69 kg (16.5 N) in adult male Wistar rats as reported in: Schmoll M, Unger E, Sutherland H, Haller M, Bijak M, Lanmüller H, et al. In-situ measurements of tensile forces in the tibialis anterior tendon of the rat in concentric, isometric, and resisted co-contractions. Physiol Rep. 2017;5)

We appreciate the comment by the reviewer. We would like to refer to our previous answer.
The weight of the attachment sling is acknowledged by the zero weight measurement. We have added the reviewer's suggestion to the revised manuscript.

L143: Other journals usually require a statement stating the compliance with the Animal Scientific Procedures Act of 1986 or the declaration of Helsinki. Nevertheless the number (or ID) for the ethical approval should be stated here.

Thank you for your comment. We have added the IACUC number.

L154: Please state isoflurane concentration and approximate duration for achieving induction. Also add isoflurance chamber and additionally required equipment to material list.

Thank you for your comment. We have added the required information to the manuscript: 3% isoflurane in oxygen.

L159: change to: "toe pinch and by observing respiratory rate."

L155: The description of the cocktail is slighly missleading. I assume the authors mean: 80mg/ml and 10mg/ml instead of "mg/kg".
L160: Please be more accurate here. Of course the correct doses vary from rat to rat (sometimes even from day to day). I´m aware that the appropriate level of aneasthesia is usually maintained by "feeling", but as you are going to present step-by-step instructions it would help a lot of new investigators to have clear guidelines on that. Please try to answer and incorporate the following questions according to your experience (or in reference to literature):
In which time interval should supplementarty doses be administered?
What is an appropriate dose (another 80 mg/kg)?
How do you define "adequate" aneasthesia?
What do you do in case you adminstered the supplementary dose too early (i.e. how to counteract an overdose)?

Thank you for your comment. We have adjusted the manuscript accordingly (L159-171).

L163: Do you measure the body temperature? Or how do you verify that the bodytemperature remains at 37°C? Please elaborate.

Thank you for your suggestion. We do not measure the body temperature. We have added the option of measuring the body temperature with a rectal thermometer to the revised manuscript.

L168: "personal protective equipment" -> please revise

L185: Although the nerve reflects a rather stable structure I would highly recommend to use a electrical stimulator instead of "gently" pinching the nerve with a foreps. Even if the propability is rather low, it´s not good practise to risk the chance of a new nerve damage when willig to access the ITF of a muscle.

Thank you for your suggestions. We have revised the manuscript accordingly.

L198: In our experiments on the EDL (extensor digitorum longus) muscle we used a drip of liquid parafin at 30°C to keep the muscle moist. Higher temperatures reduces the force response significantly. This is why a constant drip of 37°C seems a bit high to me. Nevertheless, the required equipment to provide a constant heated dripping of 0.9% sodium is not shown in the video nor in the material list. Please revise.

Thank you for your comment. We do not use a constant drip, but regularly (approximately every 5 minutes) moist the muscle with heated 0.9% NaCl. We have adjusted this in the manuscript.

L210: Which type of wood do you recommend for best fixation? Also - please elaborate a bit further on the specific dangers when drilling through the bone for fixation. Out of experience I know that there are big blood vessels very close to the location where to fixate the knee joint. A lot of practise is required to perform a safe fixation with K-wires. Please give the reader some guidance on which anatomical structures to orientate in order to avoid vascular damage.

Thank you for your comment. Out of experience, we know that the specifics of the material are not important as long as the K-wires are able to penetrate and fix. Other materials such as urethane can also be used.
We have added extra instructions on how to safely fixate the K-wires.

L215: Please elaborate a bit how you secured the Tendon. In our experiments we often had the problem that the tendon slipped under higher loads. By just additionally tightening our clamp we sometimes had the problem that the tendon was damaged (due to the clamping) and broke. We therefore introduced an additional step of drying the tendon until it became stiff and transparent. This was at the very last part of the tendon in order to avoid drying of the muscle itself. Might be worth to mention in case other researchers find similar problems. (REF: Schmoll M, Unger E, Bijak M, Stoiber M, Lanmüller H, Jarvis JC. A novel miniature in-line load-cell to measure in-situ tensile forces in the tibialis anterior tendon of rats. PLOS ONE. 2017;12:e0185209)

Thank you for your comment. We have added this suggestion to the discussion section of the revised manuscript. We have not experienced any issues with our custom-made clamp, since the tightening screw allows for adjustment of the tension.

L222: I´m not very familiar with the Grass SD9, but why does it need a ground electrode during bipolar stimulation? Unfortunatelly I didn´t find a manual of the SD9 online due to its age. But generally the definition of bipolar stimulation is to have 2 poles (electrodes) so I´m a bit confused on why the stimulator needs a ground.

Thank you for your comment. The Grass SD9 stimulator requires a ground cable to reduce electrical artifacts. Newer stimulators might not require an extra ground cable. We have clarified this in the revised manuscript.

L224: Figure 3 was used previously in your previous work: (Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery. 2008;28:452-7). Please state the reuse. Further it is not very clear that the subminiature electrode actually consists of the 2 bipolar stimulating electrodes (red and black). In the figure it looks

more like one bit metal hook which grabs the peroneal nerve and thus needs the GND electrode to function. Please clarify the figure - eventually with a separate drawing of the "subminiature electrode" tip.

Thank you for your comment. We have made adjustments accordingly. The subminiature electrode consists of 2 electrodes, but this is not clearly visible since the electrodes are covered by one plastic hook. We have adjusted the image to make the 2 electrodes more visible.

L229: Sorry for being really picky here but in order to reproduce experiments with another stimulator it is important to describe stimulation parameter carefully. I would kindly invite the authors to integrate the answers for the following questions:
Which pulseform? -> rectangular or other?
Which pulse type? -> monophasic or biphasic?
Assuming biphasic pulses, are the 0.4ms per phase (0.4ms negative phase + 0.8 ms positive phase) or for the entire pulse (0.2ms negative phase + 0.2ms positive phase)?
What do you mean by "delay 2 ms"? The inter-phase-gap between negative and positive phase of the stimulus? If so -> 2ms would be quite a lot.

We appreciate the feedback. We have added this information in the revised manuscript. We generated a square monophasic pulse with 0.4 ms duration. The delay is the time between the sync out pulse and the delivery of the leading edge of the pulse.

L232: On your LabView Software? Please specify.

L235: Although [g] is clinically used more often I highly recommend the use of [N] for any scientific purposes. You can perform calibration using 10g, 100g, 500g and 1000g weights but the readout should be in Newton as you are aiming to measure force. Please also give a clear recommendation on the increments to use.

Thank you for your suggestion. Our LabView VI is programmed to report the measurements in grams. The measurements can be converted to Newton. We have provided the converted measurements in Newton in the revised manuscript.

L237: Please give specific recommendations on how to determine the correct "preload increments" or remove this note.

Thank you for your comment. We realize that this note is confusing; therefore we have removed the note.

L239: This is why I was asking to be more precise in my comment for line 229. Before you set the stimulation frequency to 10Hz (10 pulses per second) and now you apply single pulses. Please revise the settings section in L229 to fit the delivery of single pulses. Also, please state how much time there was between the two single pulses.

Thank you for the comment. We have adjusted the described settings in the manuscript. The 2 single twitches are delivered directly after each other.

L242: To moisten the nerve liquid parafin might have certain advantages, as it avoids an electrical shortcut of the stimulation.

Thank you for the suggestion, we will definitely consider this technique for our future experiments.

L246-250: Although I´m personally a big fan of a "playful" development process - I would highly recommend to stay away from terms like "You betcha" and "Not so much". The manuscript is supposed to be presented to a wider scientific community. Please pay your respect and revise your naming in official documents like this. Nevertheless, I would encourage the authors to keep their curiosity and playfulness! Just not in a paper - it makes your work look less professional than it is.

We have adjusted this in both the manuscript and video

L253: LabView is a powerful programming environment. I´m sure the playful VI creator is able to calculate the active muscle force via an additional button.
L255: I would suggest to implement this as a feature in order to support the user. With LabView you have all the required tools for logging and visualisation available.

Thank you for your comment. We will definitely consider further automating the process in the future.

L261: The priorly determined preload is reflecting the muscluar load required to put the muscle at its optimal length (-> muscle length with the highest force output). If the muscle is at its optimal length, I would assume that it is sensible to maintain this length (i.e. preload) also during rest. Why are you suggesting to let the muscle rest at zero preload?

Thank you for your comment. We let the muscle rest at zero load, because the tension to the muscle could potentially cause muscle fatigue, even though it might be negligible. Moreover, after inducing a stimulus the muscle length can be slightly altered.

L262: I know that the 5min are common practise in your lab. Can you provide any justification for this number? In our experiments we also achieved highly reproducible results when waiting 30s to 1min. Especially after single twitches the muscle will not be fatigued. I´m not claiming that a shorter duration is better - I´m just curious on how you determined your resting duration. Could you comment on that?

Thank you for your comment. We have provided an explanation in the revised manuscript: The resting period is based on the activity of the phosphagen system, also known as the immediate energy source, which is important for explosive muscle contractions. It consists of adenosine triphosphate (ATP) and creatine phosphate activity and provides energy for less than 10 seconds of maximal activity. It requires approximately 3-5 minutes to replenish 100% of the phosphagens.

L265: Please specify "delay"

L267: Please be clearer on the used frequencies. Please just directly state the used frequencies. For tetanic force measurements I would suggest to use a fixed stimulation frequency of 100Hz (or higher if

prefered). The force-frequency relationship is very similar between different animals of the same strain so and the platau will be found soon after or arround 100Hz.
Otherwise please clarify:
"frequency starting at 10 or 30 Hz" -> Which one did you use?
What do u mean by "a resolution of 10 Hz"? Like a setting of 30Hz will result in a frequency between 20 and 40Hz? If so -> please buy a newer stimulator

We appreciate the reviewer's comments and we have adjusted the manuscript.

L269: Please give specific recommendations on "how to select the right starting frequency" or remove this note.

Thank you for your comment. We realize that this note is confusing; therefore we have removed the note.

L273: It seems that the 5s are "timed" by hand. Why didn´t the authors control the timing with the LabView software (i.e. one digital output of the USB-6009 connected to the trigger input of the Grass stimulator)?

Thank you for your comment. We appreciate the feedback, but this is unfortunately not possible since the Grass S9 stimulator is not connected to the DAQ device (USB-6009). We will definitely consider further automating the process in the future.

L278: Please be more clear here on the possibility to save data.
L286: Please define your criteria for accepting the force to be at its plateau.
L316: Calibration needs to be done over the entire measurement range.

Thank you for your comments. We removed the option for saving the data, since we document the results in an excel file. By increasing the frequency, the highest maximal isometric tetanic force will be detected (the force plateau). We would like to further refer to our previous answers regarding the calibration process.

L296: Assuming that the dorsiflexion of the paw is produced by the EDL muscle, can the authors exclude the possibility that this movement influenced the measurements?

Thank you for your comment. The tibialis anterior muscle tendon is detached from the paw and attached to the customized clamp. Any ankle motion during the test does not interfere with the results.

L303: Please use Newton when refering to forces - not [g]. Also: Figure 5 is just a black box. At this point it is unclear how many rats have been used, how old and how heavy the rats have been. Without knowing the content of figure 5 it is also not possible to evaluate the quality of the data. Please revise.

Thank you for your comment. This figure will be uploaded properly with the revision.

L306: Assuming an impedance less than 1000Ohm with the 2V we can assume a current of ~2mA which for direct nerve stimulation can be assumed to be supra-maximal already. Why did you use different intensities for your twitch and tetanic experiments? In both cases it should be your aim to stimulate supra-maximally.

We appreciate the reviewer's comments. However, we do not understand the question. During the optimization of muscle length, we provide a single twitch at 2 V until the optimal muscle length is obtained. During the isometric tetanic force measurement, we provide tetanic stimuli with 10 V to ensure maximal activation of all TA motor units.

L308: Please provide more data on your actual measurements. Only like this it is possible to verify wheter your setup is producing correct data. Also, the statement "plateau phase with minimal oscillations" is wrong, as you are also testing at lower frequencies (starting 10 Hz) you will severe oscillations as the TA muscle is a very fast one. The oscilations get less from 30Hz upwards -> see some force traces here: (Schmoll M, Unger E, Sutherland H, Haller M, Bijak M, Lanmüller H, et al. In-situ measurements of tensile forces in the tibialis anterior tendon of the rat in concentric, isometric, and resisted co-contractions. Physiol Rep. 2017;5)
L323: Please integrate these numbers in the results section and use [N] as unit of force.
L337: Please integrate these number in the results section and use [N] as unit of force.

L341: The examplary data shown does not validate the presented method. You can argue that the method has been validated in your previous paper (Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery. 2008;28:452-7), but then please refer to it.

Thank you for your comment. We have adjusted the manuscript and referenced the mentioned paper.

L347: Preload is to be determined individually for each animal anyway - so how is this influencing your procedure? For a rabbit you certainly need a different sensor because 1.34kg (2.5lb) will not be enough to measure ITF in rabbits. Also please elaborate why you think that stimulation frequency is effecting the maximal ITF in different animals. If you select a fixed very high frequency (e.g. 100Hz or above) you should be always at plateau level regardless of the strain or species - assuming the choosen stimulation frequency is sufficiently high.

Thank you for your comment. We realize that this note is confusing; therefore we have removed the note.

L356: The authors state that based on their previous experience and the previous study the combination ketamine/xylazine is only having a minimal impact on the functioning of the skeletal muscle. If you could make a comment in the discussion of the maximal experimental duration possible with this anaesthesia in comparision to isoflurane, this would be certainly interesting to other readers.

Thank you for your comment. We have only used isoflurane in experiments with rabbits and it had a significant influence on the muscle contraction. We recommend not to use isoflurane as stated in the manuscript.

L357: Regarding "slippage" might consider comment L215

L358: Replace "endurance" with "force production capability". Endurance of the muscle itself will not be the problem. You are rather refering to the general viability of the muscle. Personally I think it would be appropriate to discuss the resting period of 5min in contrast to other resting values used in literature.

Thank you for your comment. We have provided an explanation in the revised manuscript.

L362-370: Please remove entire paragraph. Considering the aim of your work - gait analysis is not compareable with the measurement of ITF. To mention it as a potential method for nerve recovery is very welcome in the introduction. But in the discussion you should focus on interpreting your results and comparing your work with literature.

Thank you for your suggestion. We have adjusted the manuscript.

Reviewer #2:
Manuscript Summary:
This manuscript provides a step-by-step instruction of isometric tetanic force measurement of the tibialis anterior muscle in the rat for evaluating motor recovery after nerve injury or repair. Overall, the paper is well-written, but minor revisions are still needed.

Major Concerns:
In this protocol, the distal tibial anterior muscle is secured to "a custom clamp fashioned from a modified surgical hemostat" attached to the force transducer. The custom clamp is an extremely critical part, however, the detailed procedure of fashioning a modified surgical hemostat to a custom clamp is missing. Therefore, it is imperative to provide this detailed procedure.

Thank you for your comment. We have added an image of the customized clamp to the revised manuscript to provide some assistance in manufacturing comparable clamps.

Minor Concerns:
①The title of this manuscript should be changed to "The maximum isometric tetanic force measurement of the tibialis anterior muscle in the rat". Because the maximum isometric tetanic force authentically reflects motor recovery after nerve injury or repair, not the isometric tetanic force. Moreover, the ultimate goal of the adjustment of various parameters in this manuscript is to measure the maximum isometric tetanic force. Therefore, "the maximum isometric tetanic force" is more precise and more suitable.
② In the abstract, On the 50th line, the expression "repair or injury" should be changed to "injury or repair". Because repair happens after injury.
③In the figure 1 legend, the full name of ITF, isometric tetanic force, should be added.
④ In the figure 2 legend, 0 should be added before the 10 in brackets. Because there are five weights totally.
⑤ In the figure 5 legend, On the 329th line, the "maximum active muscle force" should be changed to

"optimal preload".

Thank you for the useful feedback. We have adjusted all 5 points in the revised manuscript.

Reviewer #3:
Manuscript Summary:
As someone who has performed numerous muscle force measurements in rats following nerve injury this protocol provides a very nice overview of the methodology employed in these types of measurements. The illustrations are excellent and provide a good overview of the orientation of the components involved. One of the most important things to consider is that the hardware/software setup amongst researchers is not ubiquitous resulting in a myriad of custom solutions for measuring muscle force. This makes articulating the various parameters used in this protocol very important. That being said, understanding the choice of stimulation parameters is important and not explained in the protocol. For instance, not everyone has a grass SD9 stimulator especially considering the company making it no longer exists and the unit is no longer manufactured. Parameters such as delay is not explained and may not be present in other isolated stimulators. Characteristics that warrant more comment are the pulse width (why 400us), frequency (why 10Hz to start and how high do the frequencies go during tetanic measurements (i.e. what is max frequency?)). Also the use of a the Grass SD9 means it is a constant voltage stimulator, what are the pros/cons of using this type of device vs a constant current stimulator? Perhaps an explanation on the relative settings needs to achieve contraction (i.e. increase amplitude until a contraction is noticed and for final measurements increase further 3-5x threshold amplitude). Furthermore, the connection of the stimulation contacts is also important. There needs to be an explanation on if the black contact is the cathode or anode. Typically, for force measurements, the cathode should be placed distal so as to not block any propagating signals towards the muscle (anodal block). Additionally, what is the function of the ground electrode? Just because the Grass SD9 has a ground output connection does not mean that the ground connection is needed. In a bipolar stimulation setup only two contacts are needed (anode+cathode). Please explain the use of this contact. Lastly, it is critically important to explain why the rest period is necessary. I see it was written in the manuscript but the video omitted this. Overall, an excellent protocol that hopefully will benefit other researchers in this field.

Thank you for your comments. We have adjusted the manuscript accordingly and provided more information about the exact settings that we have used. The parameters used in this manuscript have previously been validated (Shin et al. 2008).
The red electrode is anode and the black electrode the cathode. The Grass SD9 stimulator requires a ground cable to reduce electrical artifacts. Newer stimulators might not require an extra ground cable. We have clarified this in the revised manuscript.
We have also provided an explanation for the resting period: it is based on the activity of the phosphagen system, also known as the immediate energy source, which is important for explosive muscle contractions. It consists of adenosine triphosphate (ATP) and creatine phosphate activity and provides energy for less than 10 seconds of maximal activity. It requires approximately **3-5 minutes** to replenish 100% of the phosphagens.

Shin, R. H. et al. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery. 28 (6), 452-457, doi:10.1002/micr.20520, (2008).

Major Concerns:
none

Minor Concerns:
Some glitches in the video while I was watching where audio was not present: Timestamp 1min 15sec and 10 min 5 sec. Perhaps providing an executable version of the LV program may be of benefit as potential users may not own the LV software platform (it's pricey).

Thank you for the feedback. We have adjusted the video.

Reviewer #4:
This manuscript merits publication in for the following reasons:
- The fit of this research with current knowledge and its practical application or its role in filling current knowledge gaps are clearly stated.
- The methods used are adequately described.
- Results reported are in agreement with methods.
- Discussion is strong enough and flow of the discussion is clearly integrated with those of concepts of other published works.

Thank you for your positive feedback.