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NON-INVASIVE ASSESSMENT OF DORSIFLEXOR MUSCLE FUNCTION IN MICE

--Manuscript Draft--

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Dr. Nandita Singh
Senior Science Editor
JoVE

Dear Nandita Singh,

Please find enclosed our manuscript entitled "***NON-INVASIVE ASSESSMENT OF DORSIFLEXION MUSCLE FUNCTION IN MICE***". In this procedure article, we show an *in vivo* mice muscle function assessment which is an efficient and helpful technic. We believe that this non-invasive assessment has advantages one to perform easily in a preserved physiological environment and allow following up measures in the same animal over different time points.

We also believe that this procedure article is appropriate for publication in Journal of Visualized Experiments (JoVE) because it clearly presents high-quality research findings that may be of interest to a diverse audience, including cell biologists and physiologists. Improving understanding in this field may aid in the management or strategy treatment of patients with muscle atrophy disease. This manuscript has not been published and is not under consideration for publication elsewhere.

We have no conflicts of interest to disclose. All authors have read and approved the final version of the manuscript. Thank you for your consideration, and we appreciate your further consideration of our manuscript and look forward to a decision to its publication in due course

Yours sincerely,

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- **X** Drafting of manuscript
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TITLE:

Non-Invasive Assessment of Dorsiflexor Muscle Function in Mice

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KEYWORDS:

Dorsiflexion, muscle function, *in vivo*, tibialis anterior, extensor digitorum longus, mice, non-invasive test

SUMMARY:

Measurement of rodent skeletal muscle contractile function is a useful tool that can be used to track disease progression as well as efficacy of therapeutic intervention. We describe here the non-invasive, *in vivo* assessment of the dorsiflexor muscles that can be repeated over time in the same mouse.

ABSTRACT:

Assessment of skeletal muscle contractile function is an important measurement for both clinical and research purposes. Numerous conditions can negatively affect skeletal muscle. This can result in a loss of muscle mass (atrophy) and/or loss of muscle quality (reduced force per unit of muscle mass), both of which are prevalent in chronic disease, muscle-specific disease, immobilization, and aging (sarcopenia). Skeletal muscle function in animals can be evaluated by

a range of different tests. All tests have limitations related to the physiological testing environment, and the selection of a specific test often depends on the nature of the experiments. Here, we describe an *in vivo,* non-invasive technique involving a helpful and easy assessment of force frequency-curve (FFC) in mice that can be performed on the same animal over time. This permits monitoring of disease progression and/or effectiveness of a potential therapeutic treatment.

INTRODUCTION:

Skeletal muscle is an important metabolic tissue that comprises approximately 40% of the total body weight. It plays a crucial role in the control of energy metabolism and homeostasis[1]. Skeletal muscle mass is maintained by a fine balance between the rates of protein synthesis and degradation[1]. Numerous disease conditions affect these processes in skeletal muscle, leading to a net loss in muscle mass (atrophy). These include, but are not limited to, cancer, AIDS, aging, fasting, and limb immobilization[2,3]. In the aging population, loss of strength is associated with a loss of muscle mass and is a predictor of all-case mortality[4]. In this context, assessment of muscle function provides an important measure when determining the effectiveness of therapeutic strategies to combat and/or prevent skeletal muscle wasting and loss of function.

Researchers have used many different approaches and animal models to understand the molecular pathways of muscle atrophy[5,6] and the implications of these mechanisms on muscle contractile function[2,3,7]. Therefore, correlating changes at the molecular level to differences in muscle function is imperative in understanding how molecular level changes can impact muscle functionality.

Skeletal muscle function, especially in small rodents, is typically performed using three well-described procedures[8,9] to detect impaired force production and/or monitor disease progression. (1) *Ex vivo*; where muscle is removed from the animal and incubated in a Ringer's bath solution to assess the muscle function using field stimulation[10]. (2) *In situ*; where the proximal attachment of the muscle remains in the animal and the distal tendon is connected to a force transducer, allowing muscle function to be performed by direct nerve stimulation[11]. (3) *In vivo*; where electrodes are placed subcutaneously to obtain nerve-evoked muscle force production[9,12]. While these three procedures are used for different purposes, they each possess advantages and disadvantages. Therefore, it is important to select an appropriate method based on the aim of the study. The main limitation with *ex vivo* experiments is the removal of muscle from its normal environment and the use of field stimulation. The *in situ* method maintains a normal blood supply and uses stimulation through the nerve, but normal anatomy is altered and the nature of the experiment is terminal; thus, this makes follow-up muscle function measurements impossible. The *in vivo* method described here most closely mimics normal physiology in that the anatomy is undisturbed, the neuromuscular bundle remains intact, and the experiment is not terminal, allowing follow-up measures within the same animal over time[8].

Here, we describe an *in vivo* procedure that allows multiple measurements of muscle function in the same animal over time. This procedure involves the assessment of muscles of the anterior crural compartment — including the tibialis anterior (TA), extensor digitorum longus (EDL), and

extensor hallicus longus (EHL) muscles, responsible for dorsiflexion — in a non-invasive procedure by fibular (also known as peroneal) nerve stimulation. The TA provides most of the force for ankle dorsiflexion[13], with only minimal contribution by the EDL and EHL that control movement of the toes. This non-terminal protocol ensures the preservation of nerve and blood supply. This allows for the investigation of disease evolution and treatment efficacy over time in the most physiological environment currently available in an animal model.

PROTOCOL:

All experimental procedures were approved by Deakin University Animal Ethics Committee (Ethical approval #G19/2014).

1. Equipment Setup

1.1. Ensure that all machines are properly connected.

1.2. Turn on the computer, the high-power bi-phase stimulator, and dual-mode lever system.

1.3. Set up the mouse knee clamp on the platform, as well as the mouse footplate on the transductor.

1.4. Turn on the heating platform to 37 ˚C.

1.5. Open the dynamic muscle control software on the desktop.

Note: This is the software needed to perform functional testing.

2. Software and Model Setup

2.1. Once the program is opened (**Figure 1**), calibrate the transducer and select **Setup | My Instruments | Calibrate**.

2.2. On the "Setup" button, select **InstantStim** and change the "Run Time" parameters to 120 s (**Figure 1A**).

Note: Optimal voltage can also be achieved by performing single twitches, manually setting up, or starting the InstantStim as many times as needed.

2.3. In the type-able window labeled "Autosave Base" to input the name of the auto save file location (*e.g.,* mouse1-date-timepoint1). Click the checkbox to the left of the "Autosave Base" window and change it to **Enable Autosave**.

2.4. At the top of the DMC control screen go to **Sequencer**, which will open a new pop up window. Select **Open Sequence** and select the premade protocol to be used (**Figure 1B**). Click **Load Sequence | Close Window**.

Note: This step is used to generate a force frequency-curve (FFC) test (1, 10, 20, 30, 40, 50, 60, 80, 100, 150, 200, 250 Hz).

2.5. Set the "RANGE" knob to 10 mA on the bi-phase stimulator.

Note: Ensure that the "ADJUST" knob (right next bottom) is on zero. This fine adjustment allows the setup of the electrodes.

3. Mice Setup

Note: All force measurements are performed on male wild-type mice (C57BI/6) at 12 weeks of age.

3.1. Place each mouse into the anesthesia chamber with an oxygen flow rate of 1 L/min with 5% isoflurane (*via* nosecone inhalation) until the mouse loses consciousness. Confirm adequate anesthesia *via* loss of the foot reflex.

3.2. Remove all hair on the right leg of the mouse by shaving with electric hair clippers.

3.3. Place the animal in a supine position on the heated platform and clean the right leg (either side can be used) with 70% alcohol and iodine. At this point, adjust the isoflurane to 2% (with oxygen flow at 1 L/min) and apply the conductive gel to the skin where electrodes will be placed.

Note: Use a rectal temperature probe to monitor the body temperature during the procedure and apply eye ointment to prevent any dryness and/or damage to the eye.

3.4. Place the foot onto the footplate and attach using medical tape. Clamp the knee to stabilize and immobilize the leg during the procedure.

Note: Some studies have described using a very thin pin inserted through the proximal tibia (posterior to the dorsiflexors muscles)[12] to provide stabilization. This protocol opts for a clamp, as this provides sufficient stabilization without unnecessary compression/damage to the knee. The clamp also avoids potential inflammation that a trans-osseous pin might create, while still allowing accurate assessment of muscle contractility. Furthermore, the mouse knee clamp has been successfully used[14].

3.5. At this point, use the knobs on the platform to position the mouse hindlimb so that there is a 90° angle at the ankle (**Figure 2**).

4. Optimization of the Electrodes Position

4.1. Once the mouse is placed on the platform, position the electrodes under the skin (subcutaneous) in the right leg.

Note: This is a crucial step, and some repositioning may be required to get the desired position during the setup in step 4.4.

4.2. Place the electrodes on the lateral side of the right leg; place one near the head of the fibula and the other electrode more distally on the lateral side of the leg (**Figure 2**).

Note: A custom-made electrode system is designed to optimize this step. However, this test can be performed with electrode needles provided by the manufacturer in this system.

4.3. Once these steps are achieved, on the high-power bi-phase stimulator adjust the knob labeled "ADJUST" as needed to obtain a stimulation of the peroneal nerve that results in maximum dorsiflexion torque.

Note: For adult wild-type mice, this range is less than 2 mA; however, this may be dependent on the size, age, and sex of the animal. The force production (peaks of curves) should be increased slowly until the maximum force is reached.

4.4. During stimulation, turn the transducer clockwise to yield negative values (**Figure 3**), which are important to ensure that the electrodes are stimulating only the dorsiflexor muscles by peroneal nerve. Once this step is achieved, stabilize the electrodes using a clamp, preventing any movement during the procedure.

Note: The peaks will slowly increase in magnitude, and the maximum amperage is determined as the level at which three or more consecutive stimulations result in identical contractility. Resist turning the amperage higher than necessary; the maximum amperage will stimulate the neighboring and potentially antagonist muscles to contract, causing co-contraction, which can generate peaks of positive values.

4.5. Stop the Instant Stim on the software.

4.6. On the main screen, turn on the button labeled "Start Sequence" to start the previous setup sequence (as described in step 2.4).

5. Ending the Procedure

5.1. Once the force measurements are finished, remove the electrodes, release the knee clamp, and remove the foot tape.

5.2. Turn off the isoflurane and maintain oxygen delivery for a few minutes aiding the animal recovery. Once the mouse starts moving and/or regains consciousness and can self-right, return the mouse to its cage.

Note: A nonsteroidal anti-inflammatory drug (NSAID) can be injected subcutaneously (1 mg/kg meloxicam) to prevent any discomfort and/or soreness after the procedure.

6. Data Analysis

6.1. Open the data analysis software.

6.2. Go to **High Throughput** (top-left on the screen). Select **Force Frequency** to analyze the above described setup sequence.

6.3. Select **Manual** and change the "End Cursor" value to 3. Also select **Remove Baseline**.

6.4. Click on **Pick Files** to access the previously performed procedure and then click **Analyze**. At this point the result can be accessed on the screen or exported to a spreadsheet for further analysis and/or calculations.

Note: The data were measured in mN; however, the torque can be calculated by multiplying the force value by the length of the lever arm (absolute force). If normalization is required (specific force), torque can be normalized to body weight, or terminal experiments can be performed to collect muscle mass of age-matched.

REPRESENTATIVE RESULTS:

The force-frequency curve is a useful test in which muscles can be stimulated by lower and higher frequencies to distinguish suboptimal and optimal force responses[15]. The force at lower frequencies can stimulate a single twitch, activating fewer and smaller motor units, and at higher frequencies a stable peak is reached, where isolated twitches fused (tetanus), reaching maximum force through activating all motor units[16]. In the test presented, the tetanic curve starts at ~60 Hz, where the potentiation can be visualized (**Figure 4A**) and the maximum force is determined at ~150 Hz (**Figure 4B**), when the plateau is reached with a completed fused curve[9,16].

Any variation of these results may indicate that the muscles are not being properly stimulated by the electrodes. Electrode placement is an important step in the preparation of this procedure, as the electrical stimulation must be correctly positioned to innervate the peroneal nerve and thus fully activate the muscles of dorsiflexion, which it supplies (TA, EDL, and EHL). Correct electrode positioning results in the generation of negative peaks (**Figure 3**) during this process, whereas misalignment of the electrodes or higher amperage can lead to the stimulation of surrounding muscles, causing co-contraction of the neighboring muscles and antagonist muscles, which in turn generates peaks of positive values.

Figure 5A shows representative force frequency-curve data from a mouse across time, where the procedure was repeated once a week until 5 timepoints were completed. These observations have shown consistent force production values throughout the timepoints and/or observations measured. This procedure has also shown to be consistent between mice measurements, as **Figure 5B** shows the representative area under the curve of the FFC stimulated over 5 different observations in 6 mice tested once a week.

FIGURE AND TABLE LEGENDS:

Figure 1: Software system. A. Control software illustration of the steps for setting up the "Instant Stim" parameters. On the background photo, click **Setup | Instant Stim**. On the small popped up window (front photo), set up the parameters. **B.** Illustration of the "Sequencer" setup view.

Figure 2: Mouse setup. Overview of the position of the anesthetized animal. The right knee clamp is placed so that the knee is at 90° and so that the foot and the ankle are at 90° angles (dotted white line). Contraction of the dorsiflexors muscles is achieved by stimulation of the peroneal nerve, which is located just under (distal to) the head of the fibula. We use custom-designed electrodes (inset); however, needle electrodes that are provided with the unit, or purchased separately, are also sufficient.

Figure 3: Output from placement of the electrodes. Once the electrodes are positioned under the skin and the voltage is initiated, peaks with negative values are observed. At this point, reaching negative values (green lines) is a crucial step in making sure that the stimulation is achieved in the dorsiflexor muscles only (TA, EDL, and EHL). The real-time measurement is indicated between the two red lines.

Figure 4: Representative curves. **A.** Sample of the force curve at 60 Hz (mouse #06). **B.** Sample of the tetanic curve at 150 Hz (mouse #03).

Figure 5: Representative force frequency curve (FFC) and area under the curve data. A. FFC (x-axis) over 5 different timepoints (weeks 1, 2, 3, 4, and 5) in a sample mouse (#05). **B.** Area under the curve (AU, y-axis) of the FFC over 5 different timepoints (mouse #01, 02, 03, 04, 05, and 06, respectively; x-axis). Results are expressed as mean ± standard error of measurement (SEM) of five timepoints (tests) in 6 mice and were analyzed by one-way ANOVA test ($p < 0.05$).

DISCUSSION:

Measurement of maximal muscle contractile function in an accurate and repeatable manner is critical to the progressive assessment of genetic, metabolic, and muscle conditions[17]. Similarly, *in vivo* muscle contractile function allows for the assessment of novel treatments and therapeutics for debilitating muscle conditions. We demonstrate herein the measurement of force production of the dorsiflexor muscles of mouse lower hindlimb through an *in vivo* procedure.

Commercial apparatuses are efficient and helpful in performing this non-invasive procedure. This test provides important advantages related to the assessment of muscle contractile function

while preserving a native physiological environment, in which blood supply and innervation remain intact. On the other hand, its disadvantages are related to normalization of the force per unit of cross sectional area of muscle (specific force), which can only be ascertained in an isolated muscle that is harvested after experimentation. However, the non-invasive test allows multiple measurements of contractile function of the flexor muscles in the same animal over time, resulting in reduced numbers of experimental animals being required, especially if the goal is to assess relative changes (changes in absolute force over time).

There are important steps that must be considered during this procedure in order to achieve consistent data over the timepoints. First, one should attempt to standardize animal positioning whenever possible. Second, during the setting up it is important to be consistent with electrode positioning so that optimal stimulation can be reached *via* stimulation of the peroneal nerve. The location of the electrodes should be on the lateral side of the (in this case right) leg, near to the head of the fibula and other further down the lateral side of the leg (**Figure 2**). Based on this, the custom-made electrodes are designed as such that both can be placed at same position every time. However, sufficient stimulation can also be achieved using the electrode needles provided with the commercial apparatuses. Third, it is crucial to achieve negative peaks during the voltage setup by turning clockwise the transducer connected to the footplate. Correct positioning of the mouse leg electrodes with maximum voltage setup has shown to be a technique that can be performed on the same mouse over time.

The ability to assess and track muscle function at different timepoints on the same animal is an important assessment to characterize different muscle diseases as well as their progression. Furthermore, this measurement of muscle dorsiflexion in mice can be a tool to assess the efficacy of potential treatments in a native physiological environment, with minimum metabolic stress[12]. Thus, it provides a technique in assessing muscle disease, its progression and potential treatment.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

A

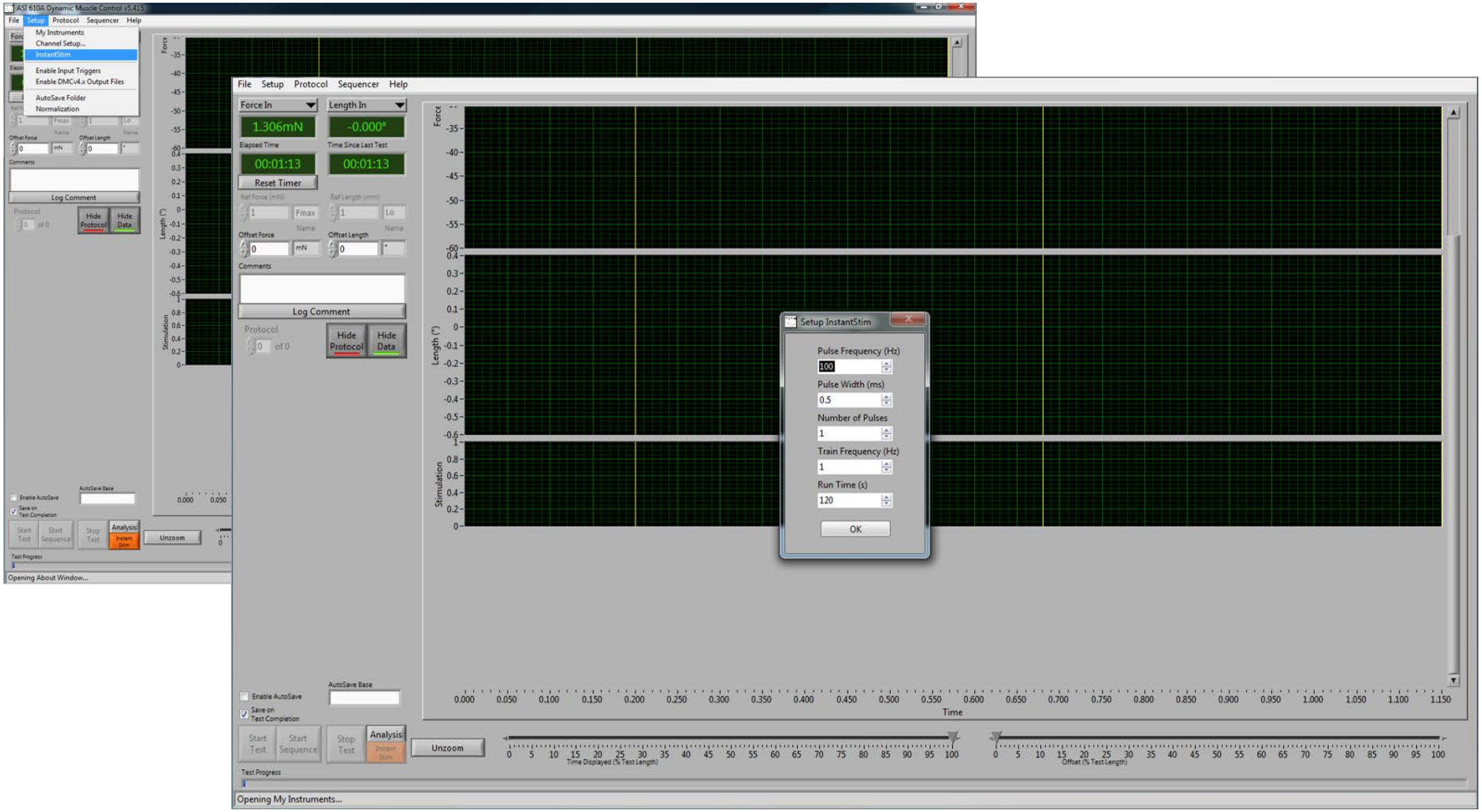
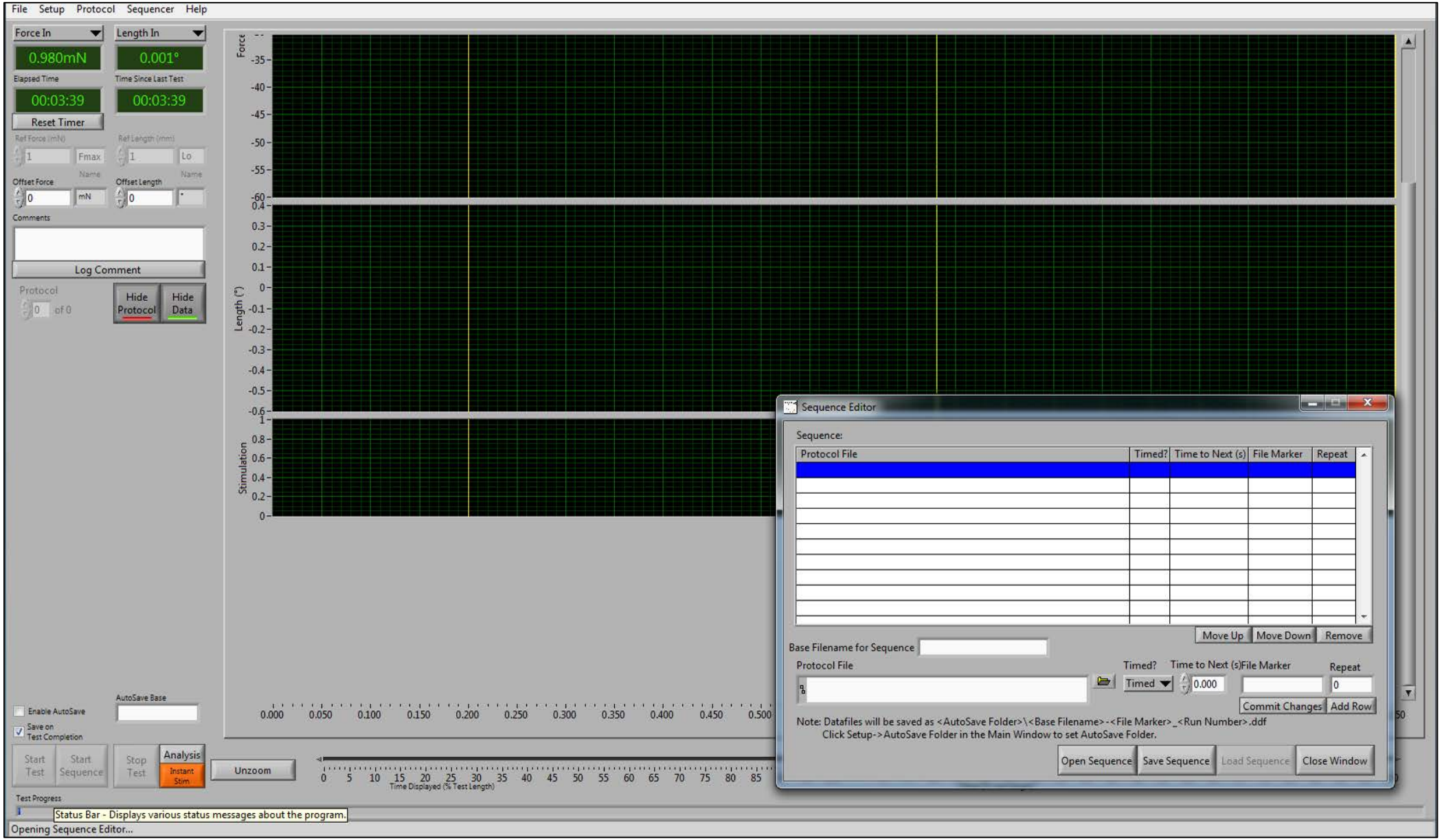


Figure 1

B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Move Down
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Commit Changes
Add Row
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Click Setup->AutoSave Folder in the Main Window to set AutoSave Folder.
Open Sequence
Save Sequence
Load Sequence
Close Window
AutoSave Base
Enable AutoSave
Save on Test Completion
Start Test
Start Sequence
Stop Test
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Instant Stim
Unzoom
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Test Progress
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Figure 2

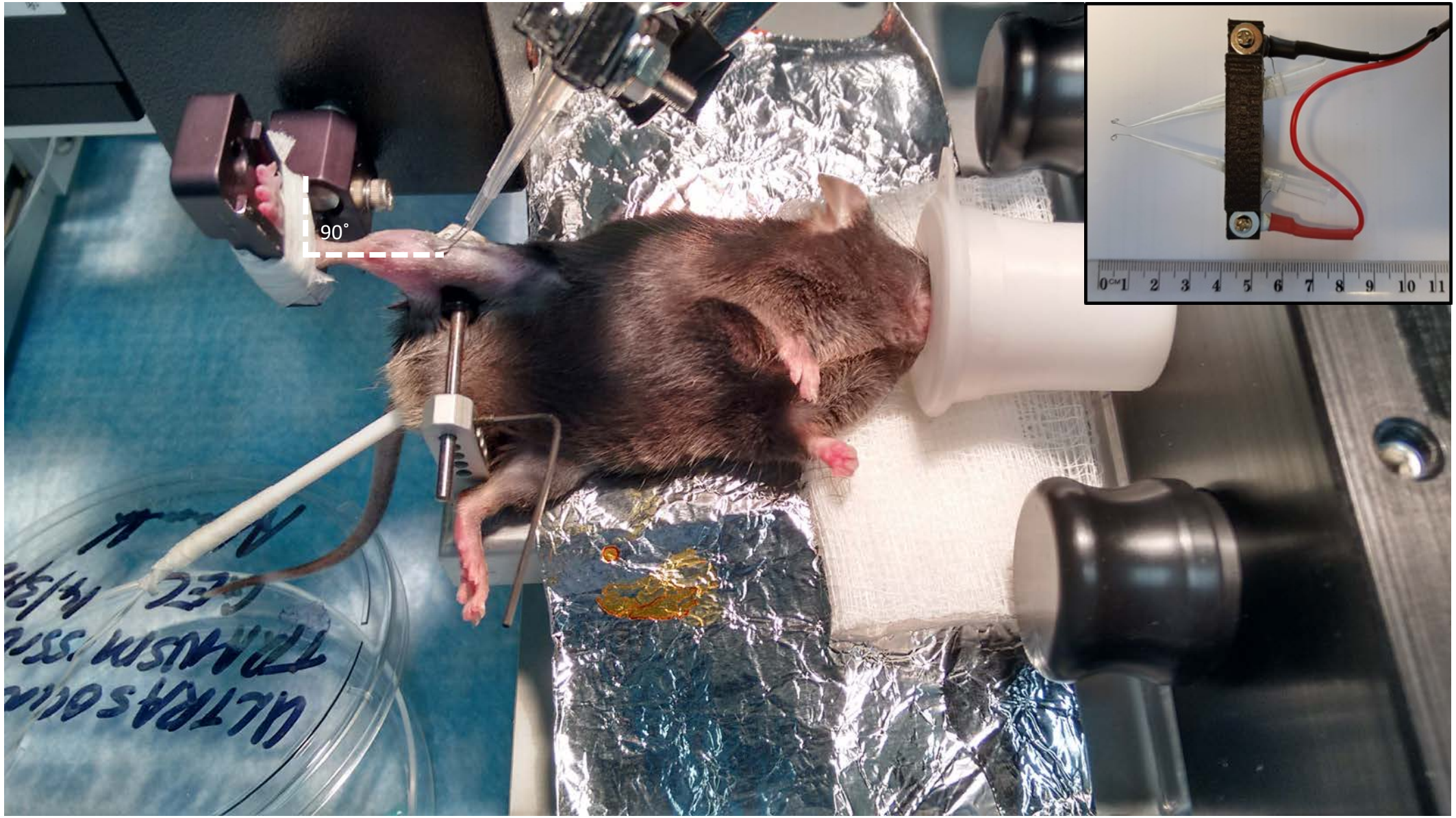


Figure 3

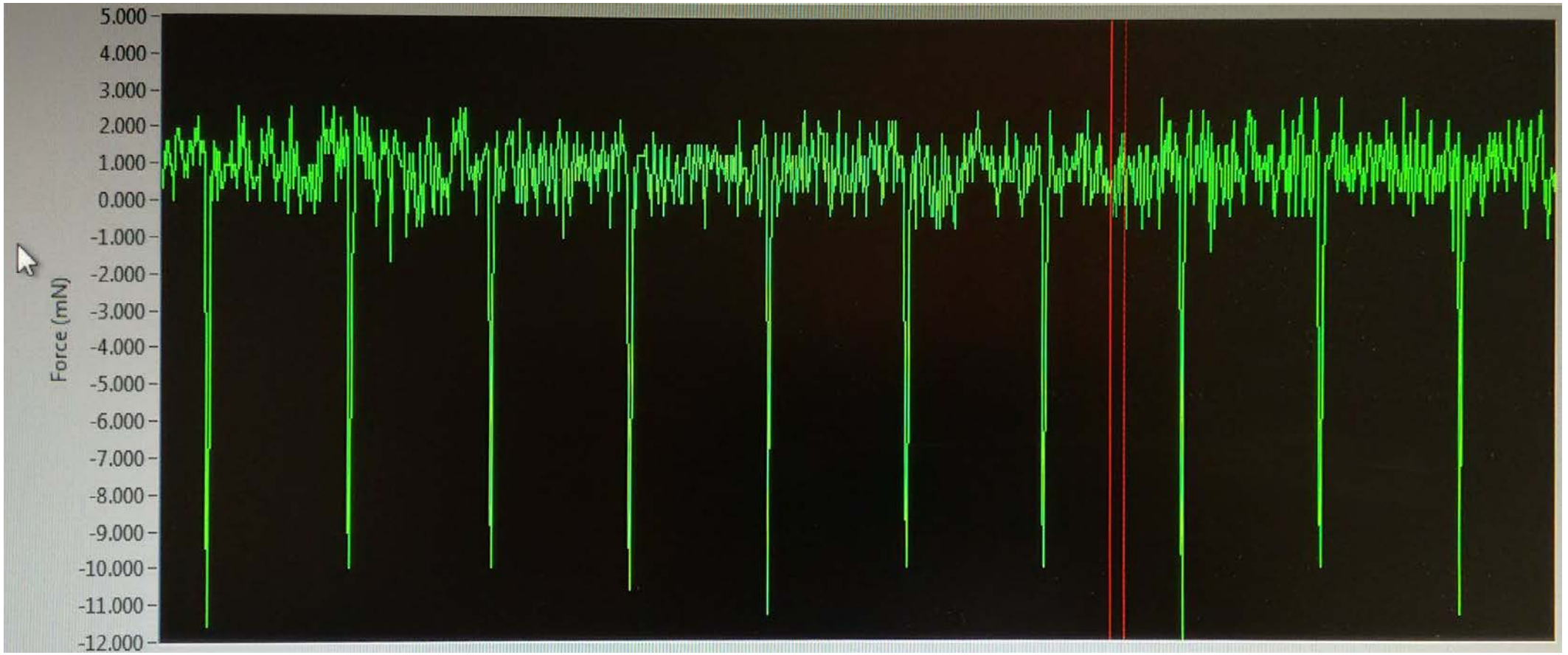
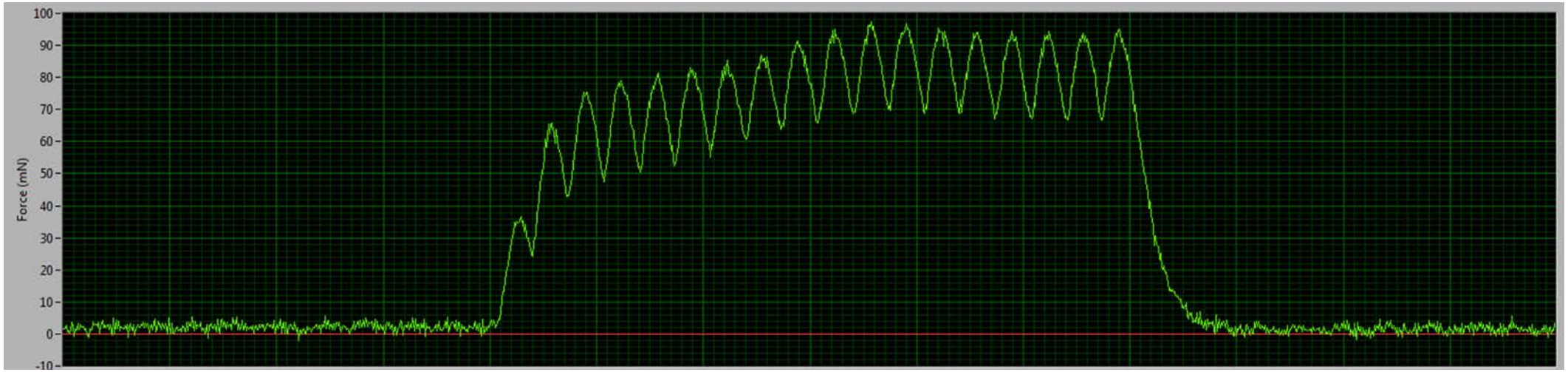


Figure 4

A



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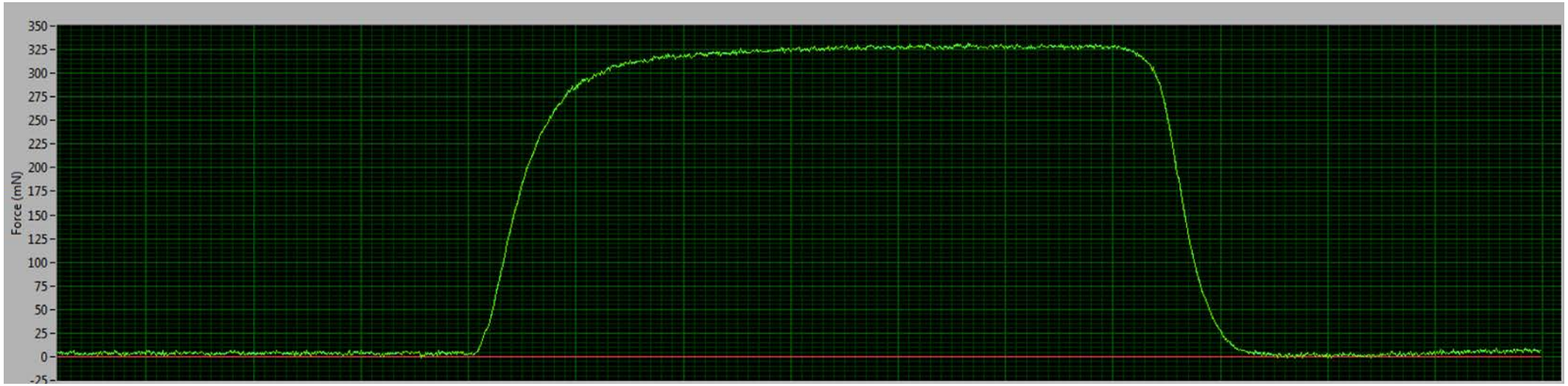
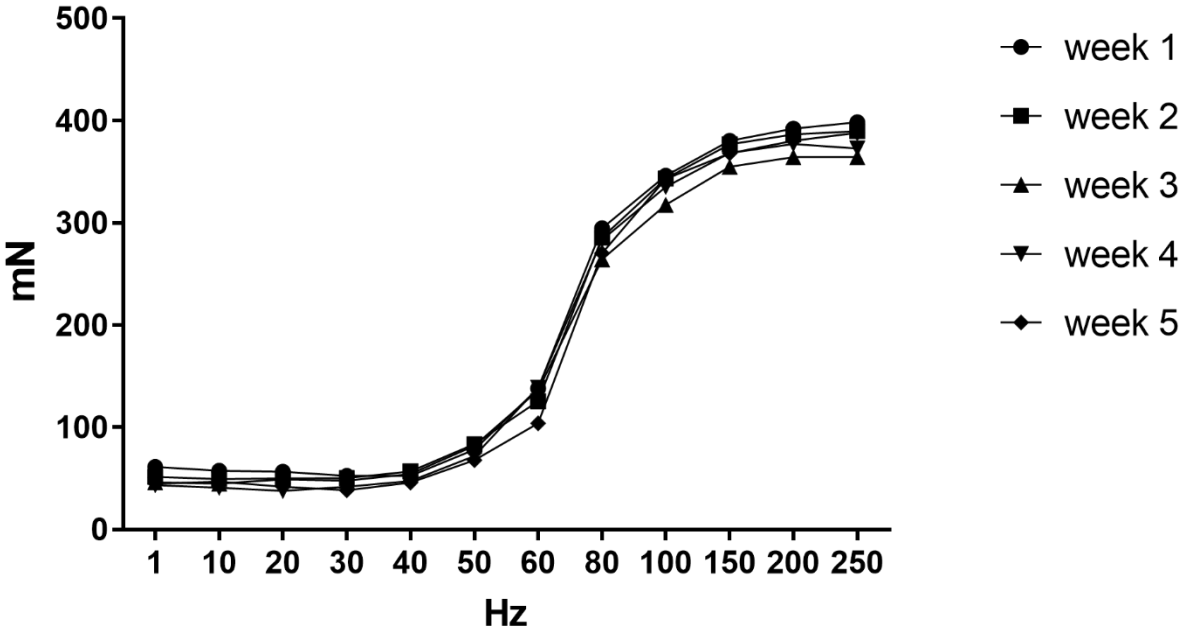
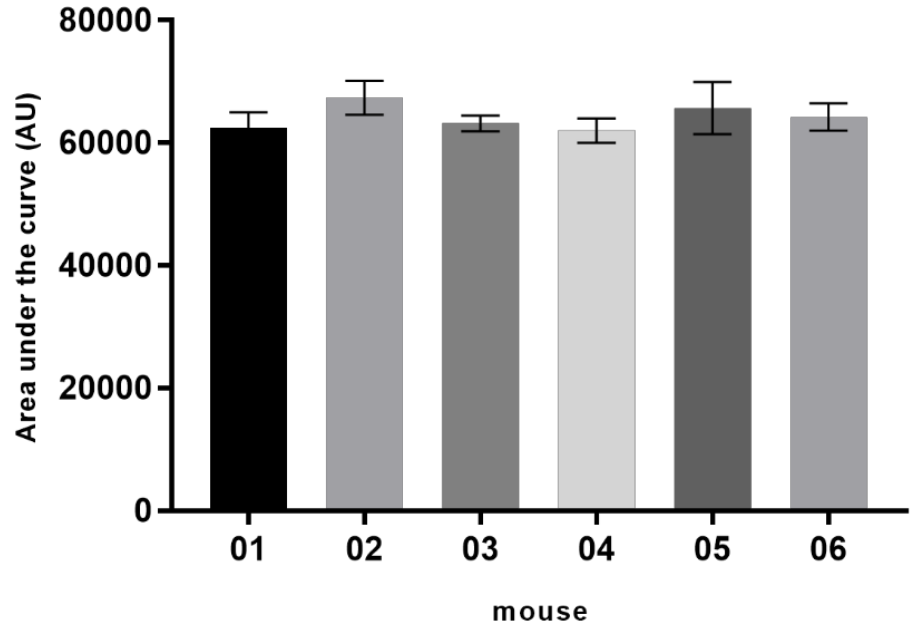


Figure 5

A



B



Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
1300A: 3-in-1 Whole Animal System – Mouse	Aurora Scientific Inc.	305C-LR: Dual-Mode Footplate; 605A: Dynamic Muscle Data Acquisition And Analysis System; 701C: Electrical Stimulator and 809C: in-situ Mouse Apparatus	Complete muscle function system
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Eye ointment	Alcon	Poly Visc	pharmaceutic product (ophthalmic use)
nonsteroidal anti-inflammatory drug (NSAID)	Ilium	Metacam	veterinary medicine (injectable 5mg/ml)
Isoflurane	Zoetis	Isoflo	veterinary inhalation Anaesthetic

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Title of Article:	NON-INVASIVE ASSESSMENT OF DORSIFLEXOR MUSCLE FUNCTION IN MICE
Author(s):	Frederico Gerlinger-Romero, Alex Addinsall, Richard M.Lovering, Victoria C. Foletta, Chris van der Poel, Paul A. Della Gatta, Aaron P. Russell

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Title:	Associate Research Fellow
Signature:	
Date:	10/08/2018

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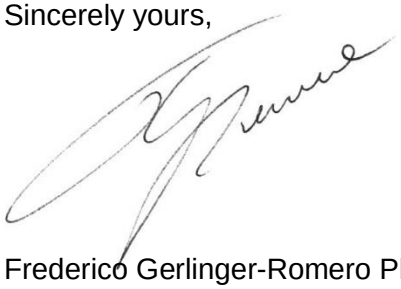
August 6th, 2018

Dear
Editor-in-Chief
Journal of Visualized Experiments (JoVE)

Re: Manuscript No. JoVE58696

I would like to submit the revised version of the manuscript entitled “NON-INVASIVE ASSESSMENT OF DORSIFLEXOR MUSCLE FUNCTION IN MICE” by Frederico Gerlinger Romero et al, to be re-evaluated for publication in Journal of Visualized Experiments (JoVE). We have considered the reviewers comments and recommendations, as well as performed several modifications to improve the manuscript. The modifications in the text are included in the revised version of the manuscript highlighted in red font. We are also submitting the letter of responses to the reviewers, addressing all the comments point-by-point and indicating where in the text the modifications were made. We do appreciate the valuable suggestions that were made and hope to have adequately addressed all the raised points. Once again, we thank the opportunity to have our manuscript evaluated by Journal of Visualized Experiments (JoVE).

Sincerely yours,



Frederico Gerlinger-Romero PhD
Associate Research Fellow
School of Exercise and Nutrition Sciences, Faculty of Health

Answers to Reviewers' comments

We are very grateful for the opportunity to have our manuscript evaluated by Journal of Visualized Experiments (JoVE) referees. We thank the reviewers for careful and critical discussion of our manuscript and helpful comments. We have made all the requested changes. We have addressed each criticism below and have made corresponding changes in the attached manuscript. A 'redlined version' of the revised manuscript has also been uploaded for convenience. We believe that the protocol reads more clearly due to the suggested revisions.

EDITORIAL COMMENTS:

Changes to be made by the Author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

A: We have revised the text accordingly.

2. Please note that both Standard Access and Open Access are checked in the uploaded ALA. Please select one type only. Note that in the Questionnaire Responses Open Access is selected. Please be consistent.

A: The correction was made in this document.

3. Figure 1: Please increase the resolution of this figure. Currently the text is difficult to read. Probably expand panels A and B and arrange them vertically.

A: We have revised the figure.

4. Figure 3: Please explain the red line and green line in the figure legend.

A: We have revised the figure legend.

5. Figure 5: Please line up the panels better and ensure that each panel has the same dimension if possible.

A: We have revised the panels in this figure legend.

6. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc.

A: The corrections were made.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

A: We have revised the text

8. Please include single-line spaces between all paragraphs, headings, steps, etc.

A: The corrections were made.

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

A: we have highlighted the essential steps on the manuscript (in yellow).

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

A: The highlight steps were made on the manuscript.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

A: The highlight steps were made on the manuscript.

12. References: Please do not abbreviate journal titles.

A: We have revised the references.

13. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

A: The correction was made in the Table of Equipment and Materials.

REVIEWERS' COMMENTS:

Reviewer#1:

1. The authors stabilized the leg of the mice by clamping the knee. While, a needle was reported to be used to stabilize the limb of the animal (Lovering et al., 2011). It would benefit from providing a more detailed discussion of the differences and/or advantages between these two procedures.

A: Lovering et al has shown a similar procedure using a custom-made apparatus that required to use a thin pin through the proximal tibia to achieve optimal stabilization. However, we have used a different step-up without any intervention on the hindlimb. Besides, we feel the clamp is able to avoid any unwanted inflammation that a trans-osseous pin might create, while still allowing accurate assessment of muscle contractility. This is now addressed in the revised text (lines 170-175).

2. Line 176, As mentioned by the authors that the optimized amperage for stimulation may be different among mice, so is there a process to determine the specific amperage for each assessed animal before stop the Instant Stim? If so, it should be described in the Protocol Section.

A: Thank you. This is now discussed in the revised text as recommended (lines 139-142).

3. A close-up figure or diagram of the custom-made electrodes and the position where the electrodes put on the mice leg should be provided to further aid other investigates to better perform this method. I think this figure or diagram is necessary since the video that will describe this step may be not clear enough.

A: Based on the reviewer's suggestion we have included a close up of the electrodes (inset in Fig 2)

4. The authors provided various figures to describe the normal contractility of the wildtype mice. As discussed, a normalization step is required for accurate evaluation of muscle contractility. In practice, researchers often compare muscle contractility between non-treated and treated muscle, or between wild-type and genetic modified mice, of which, the muscle masses or fiber areas are usually not comparable. A detailed discussion of 'Normalization' should be provided. As for measurement of force from different time points using the same animal, how to normalize without sacrificing the mice? In addition, dorsiflexion involves contraction of three types of muscle, of which the cross sectional area should be used to generate the 'specific force'?

A: The reviewer raises an interesting question. We have mentioned that is important to select

the appropriate method based on the aim of the study because all different procedures have some advantages and disadvantages. This procedure can be used to track and monitoring the hindlimb [absolute] force production over different time points. Using the first measurement as a reference would allow monitoring relative muscle function over disease progression or with treatment. The notion of measuring the "percent loss" after injury and the "percent recovery" over time is not new (e.g., PMCIDs 28056487; 25920768; 24947322). If the normalization is required torque can be normalized to body weight, muscle mass of age-matched mice sacrificed at the same time points, or terminal experiments can be performed. We and others have shown the majority of torque produced by the dorsiflexors is from the TA (PMID: 14672973), and we have shown previously that this model results in injury to this muscle (PMID: 24066899; 16205165; 14522817; 17466731). This has been clarified in the revised text (lines 229-231; 273-274).

Reviewer#2:

Major Concerns:

1. The Key point of the protocol is to correctly place the electrode. I'd strongly recommend adding a carton diagram in either Figure 2 or 3 to help readers better understand the placement of the electrode.

A: We thank the reviewer for this suggestion and we have made some modifications on this figure. The description of electrode placement has been expanded in the manuscript (item 4) and additional details will also will be on the video.

2. Section 2.2, the authors set the "Run time" to 2 min. This needs justification. Have they tested different run time?

A: *We have described this time based on the previous experiments where 2 minutes was long enough to achieve the optimal voltage. However, if this time is not enough to achieve the optimal voltage it can be either changed to a longer time or the instant slim can be started again as many time as needed. This has been clarified in the revised text (lines 139-142).*

3. Section 2.5, the authors suggest "RANGE" knob to 10 mA. However, later on, the author indicated that for WT mice, the range should be less than 2mA. This needs clarification.

A: We have described this procedure using a commercial apparatus and it can be used for a range of different experiments. The range on the high-power bi-phase stimulator can be used in 10 mA, 100 mA, 1 A, 20 V and 80 V. The RANGE (left bottom) knob has to be on 10 mA and during the optimizing of the electrodes the fine adjustment should be achieved using the

right next bottom labeled ADJUST which means by using this bottom the voltage can be adjusted between 0 and the maximum 10 mA. We have added the corrected description on the article (line 152-153; 191).

4. The authors mentioned about two other commonly used mouse muscle function assays, ex vivo and in situ. These protocols have been described in several JoVE papers. I suggest to cite these papers (see below).
Hakim CH, Wasala NB, Duan D (2013) Evaluation of muscle function of the extensor digitorum longus muscle ex vivo and tibialis anterior muscle in situ in mice. J Vis Exp: e50183
Moorwood C, Liu M, Tian Z, Barton ER (2013) Isometric and eccentric force generation assessment of skeletal muscles isolated from murine models of muscular dystrophies. J Vis Exp: e50036
A: We thank the reviewer for these suggestions. We have added these two references.

Minor Concerns:
1. The authors stated that their protocol can reduce the number of mice. This may not necessarily true for some applications. For example, the total absolute force of mdx mice are not altered. Only specific force can be used to distinguish the difference between normal and affected.
A: We have clarified this in the text. We know that absolute force is not reduced in mdx and the difference can only be detected if force is normalized to muscle mass. However, if one is studying muscle injury and recovery, this can be done in the absence of killing animals at each time point to obtain muscle mass. The "percent loss" in the force production (torque) we are interested in, not normalized force. As mentioned above, the notion of measuring the "percent loss" after injury and the "percent recovery" over time is not new (e.g., PMCIDs 28056487; 25920768; 24947322); (lines 273 and 274).

2. The authors provided an example using the right leg. It should be mentioned that this approach can be used for both limbs.
A: We thank the reviewer for bringing this to our attention and we have also included this information (lines 162 and 163).