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TITLE:

In vivo Measurement of Knee Extensor Muscle Function in Mice

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KEYWORDS:

knee extension, quadriceps, muscle strength, skeletal muscle, torque, non-invasive

SUMMARY:

Quantification of knee extensor maximal strength is imperative to understand functional adaptations to aging, disease, injury, and rehabilitation. We present a novel method to repeatedly measure in vivo knee extension isometric peak tetanic torque.

ABSTRACT:

Skeletal muscle plasticity in response to countless conditions and stimuli mediates concurrent functional adaptation, both negative and positive. In the clinic and the research laboratory, maximal muscular strength is widely measured longitudinally in humans, with knee extensor

musculature the most reported functional outcome. Pathology of the knee extensor muscle complex is well documented in aging, orthopedic injury, disease, and disuse; knee extensor strength is closely related to functional capacity and injury risk, underscoring the importance of reliable measurement of knee extensor strength. Repeatable, in vivo assessment of knee extensor strength in pre-clinical rodent studies offers valuable functional endpoints for studies exploring osteoarthritis or knee injury. We report an in vivo and non-invasive protocol to repeatedly measure isometric peak tetanic torque of the knee extensors in mice across time. We demonstrate consistency using this novel method to measure knee extensor strength with repeated assessment in multiple mice producing similar results.

INTRODUCTION:

Skeletal muscle is a highly adaptable tissue with compensatory alterations to mass and structure in response to a myriad of stimuli, such as exercise, nutrition, injury, disease, aging, and disuse. Many studies investigating skeletal muscle adaption in humans employ methods to measure both skeletal muscle size and impact on function, as gold-standard strength assessments are easily repeatable in human subjects.

Specifically, knee extensor and flexor strength, is most assessed in clinical research. Alterations to knee extensor strength have been widely reported in human studies of aging, exercise, orthopedic injury, knee osteoarthritis, chronic disease, and disuse[1-7]. However, methods to repeatedly and non-invasively analyze knee extensor muscle (quadriceps) strength in mechanistic rodent studies have been relatively limited. A method to determine in vivo quadriceps muscle contractility in rats was previously developed[8]; however, extensive construction of non-commercially available equipment is required. Given the breadth of rodent models developed to study musculoskeletal outcomes following knee injury/osteoarthritis[9-13] there exists a need for non-invasive assessment of quadriceps strength.

Furthermore, rodent studies investigating molecular mechanisms underpinning skeletal muscle adaptation often utilize mouse models due to the simplicity of genetic modification, as do many pharmacological intervention studies because of the decreased financial expense associated with lower weight-based dosing of a drug in mice compared to rats. We report a non-invasive method to repeatedly measure in vivo knee extensor function in the same mouse over time using commercially available equipment with minor modification, facilitating reproducibility among different laboratories, and providing more direct comparison to human strength outcomes.

PROTOCOL:

All experimental procedures were approved by University of Kentucky Institutional Animal Care and Use Committee.

1. Equipment setup

1.1. Confirm that machines are connected per manufacturer specifications.

1.2. If not already in place, attach the 300D-305C-FP motor with knee extension apparatus to the 809C animal platform.

1.3. Turn on the water pump to 37 ˚C to begin heating the platform.

1.4. If computer is not already on, turn the computer on, followed by the High-Power Bi-Phase Stimulator and 2 Channel Dual-Mode Lever System.

1.5. Pour isoflurane into vaporizer to maximum fill line.

2. Software setup

2.1. Open the software (details provided in **Table of Materials**).

2.2. To use the Instant Stimulation feature in conjunction with Live Data Monitor to optimize probe placement (step 4), select **Prepare Experiment** followed by **Configure Instant Stim** (**Figure 1**). Set the **Pulse Frequency** (Hz) as 125, **Pulse Width** (ms) as 0.2, **Number of Pulses** as 1, **Train Frequency** (Hz) as 0.5 and **Run Time** (s) as 120.

2.3. Select **File** and open **Live Data Monitor**.

2.4. To perform twitch (step 5) and torque-frequency (Step 6) experiments, select a previously programmed study that includes appropriate twitch and knee extension torque-frequency experiments (detailed below in step 5 and step 6).

2.4.1. Select the appropriate experimental mouse or **Add New Animal** and input corresponding mouse information to be stored with torque data.

2.4.2. Select **Next Experiment** or **Previous Experiment** to transition from twitch protocol to force-frequency sequence.

3. Mouse setup

3.1. Place individual mouse into the anesthetic chamber.

3.2. Release oxygen tank valve and set oxygen flow rate at 1 L/min with 2.5% isoflurane.

3.3. Ensure that mouse remains in the chamber with the lid securely closed until completely unconscious. Confirm complete loss of consciousness by absent foot reflex with toe pinch.

3.4. Place anesthetized mouse in a supine position with the head in the nosecone on the heated platform with oxygen flow rate at 1 L/min with 2.5% isoflurane.

3.5. Shave hair from the right hind limb using electric clippers. Clean removed hair away from the hind limb and the platform.

3.6. Securely clamp the upper hind limb, posterior to the knee (**Figure 2**).

NOTE: Ensure that knee range of motion is not impeded.

3.7. Place the lower hind limb into the knee extension apparatus with the anterior tibia lightly touching the adjustable plastic piece (The **Force In channel** reading should read between 0 and -1.0 mN*m). Depending on the size of the lower hind limb of the mouse, surgical tape may be wrapped around the bottom portion of the adjustable plastic piece to allow the leg to rest securely.

NOTE: Detailed images and dimensions of the custom-fabricated plastic piece are shown in **Supplementary Figure 1**.

3.8. Adjust knobs on platform to ensure the knee is bent at 60˚.

3.9. Lightly place a tape over the mouse torso onto the platform to prevent compensatory movement with maximal knee extension.

4. Electrode placement

4.1. Place electrodes subcutaneously 2-4 mm proximal to the knee directly above the quadriceps/knee extensor muscles (**Figure 2**). Electrodes should be approximately 1-2 mm apart.

4.2. To determine optimal placement of electrodes, utilize the **Instant Stimulation** function with **Live Data Monitor**. Set amperage/current at 50 mA for repeated twitches to confirm knee extension (the knee extensors will produce a negative twitch curve). Adjust the probes during **Instant Stimulation** to achieve maximal knee extension twitch torque as measured in the **Live Data Monitor** window.

NOTE: **Figure 3** shows a representative **Instant Stimulation** output, confirming knee extension. **Supplementary Video 1** and **Supplementary Video 2** show real-time and slow-motion knee extensor twitches without the motor arm in place, allowing for visual confirmation of knee extension.

4.3. During repeated twitches with **Instant Stimulation**, palpate knee flexor muscles with the index finger to confirm no activation of antagonist muscles. To maximally stimulate knee extensors, probe repositioning may be necessary depending on body composition of the mouse and slight anatomical differences in the exact location of the motor point of the femoral nerve and knee extensor muscles.

NOTE: A muscle motor point is the location where the motor branch of a nerve enters the muscle belly and is the point with the least resistance to electrical conductivity and subsequently the highest responsiveness to electrical stimulation[14,15]. In clinical applications using electrical stimulation, this point is identified by scanning with a pen electrode to find the location above the muscle at which a muscle twitch occurs with the lowest injected current[14,15]. Identification of the muscle motor point is essential to facilitate optimal neuromuscular electrical stimulation[15]. In human clinical trials, muscle motor points for quadriceps muscles have been identified in the distal half of the muscle[14]. To achieve optimal knee extensor stimulation in mice, this technique was recapitulated using electrode placement with **Instant Stimulation** to most closely approximate muscle motor point locations typically found in the distal half of knee extensors. There exists some variability in electrode placement (from relatively superficial to deep) that results in maximal torque, and the **Instant Stimulation** function facilitates optimal electrode placement.

5. Determination of optimal current

5.1. Once optimal probe placement is determined, perform a series of progressive twitches to determine optimal amperage/current to be used for the torque-frequency experiment, with the goal of determining the lowest current to achieve the maximal twitch torque output. Begin with current set at 50 mA and select **Run Experiment** to produce a single twitch. Select **Analyze Results** to display torque output. Record the twitch torque displayed under **Max Force** with baseline subtracted.

NOTE: Select the option to invert the **Force channel** to convert measurements from negative torque to positive.

5.2. Increase the current to 60-70 mA and repeat twitch experiment. Record the twitch torque displayed under **Max Force** with baseline subtracted.

5.3. Continue with a series of twitch experiments in this manner (increasing approximately 10-20 mA with each progression) until twitch torque no longer increases (either plateaus or begins to decrease). Example of twitch series is shown in **Table 1**.

5.4. Record the lowest current at which the highest twitch torque was achieved. This current will be used and remain constant during the upcoming force-frequency experiment. **Figure 4** shows a representative peak twitch.

6. Torque-frequency experiment to determine peak isometric tetanic torque

6.1. In the software (see **Table of Materials**), select the pre-programmed torque-frequency experiment for knee extension ensuring following setting. Stimulus duration: 0.35 s**,** Frequency sequence: 10 Hz, 40 Hz, 120 Hz, 150 Hz, 180 Hz, 200 Hz**,** Rest period between pulses/contractions: 120 s

NOTE: Sampling rate is 10,000 Hz (default setting).

6.2. **Run Experiment**, **Analyze Results**, and manually record the torque displayed under Max Force with baseline subtracted (make sure the force channel is inverted, as knee extensor contraction will produce negative torque) at each frequency. Note the highest Max Force value as the peak isometric tetanic torque. Example of torque-frequency data is shown in **Table 2** and **Figure 5** shows a representative tetanus curve for the peak isometric tetanic torque output achieved at 120 Hz.

7. Termination of experiment

7.1. Upon completion of the torque-frequency experiment, perform a follow up twitch and compare with the initial peak twitch at the same current to assess damage/fatigue.

NOTE: In some models of injury and disease, increased fatigability of skeletal muscle is expected and does not constitute a problem with the experimental setup or the mouse.

7.2. When all torque measurements are completed, gently remove electrode probes, and unclamp the knee.

7.3. Turn off isoflurane and remove the mouse from the nose cone.

7.4. Place the mouse back into an appropriate cage placed on top of a warming pad. Monitor as the mouse recovers and regains consciousness.

NOTE: The mouse should be conscious and moving within 2-3 min.

8. Data analysis

8.1. Extract data after the experiment from analysis software (see **Table of Materials**).

8.1.1. Open analysis software.

8.1.2. Select **Get Data** from the software.

8.1.3. Select **Date** on which experiment was performed and appropriate mouse code.

8.1.4. Select the frequency of interest (all twitch experiments and each frequency of the torque-frequency experiment will be listed).

8.1.5. Select **Muscle Analysis**.

8.1.6. Confirm that **Use Baseline Correction** is checked.

NOTE: Baseline torque is calculated by the software as the average of the first 100 points sampled and subtracted from the absolute maximum torque value.

8.1.7. Record the torque value listed under **Maximum**.

NOTE: Data presented here are unfiltered; however, a filter may be selected in the software, if desired.

8.2. Alternatively, as described above in step 6.2, manually record the torque output displayed under **Max Force** in real time at each torque-frequency point/contraction through the **Analyze Results** window.

8.2.1. Confirm that the baseline is subtracted, and force channel is inverted.

8.2.2. Input data into a spreadsheet for body weight normalization calculations (torque/body weight in grams) and graphing and statistical analyses of interest. Statistical software was used for the purpose of graphing torque-frequency curves and calculating area under the curve.

NOTE: Torque data are measured in mN.m (milliNewton.meters).

8.3. To generate tetanus curves, export complete data from each frequency from the analysis software.

8.3.1. Repeat steps 8.1.1-8.1.4 above.

8.3.2. Select **Export Data**.

8.3.3. Select **Raw Filtered Data** and save in location of choice. MATLAB may be used to generate tetanus curves from the exported text file and/or for further analysis.

NOTE: MATLAB code to generate tetanus curve from text file is available upon request.

9. Dual-mode lever system calibration

9.1. Calibrate the system prior to the initial use to ensure accurate and reliable data, and repeat calibration periodically using the data collection software and known weights.

9.1.1. Open data collection software.

9.1.2. Click the **Setup tab** and select **Channel Setup**.

9.1.3. Select **305C-FP** listed under **My Instruments**.

9.1.4. Click **Calibrate Selected** to open the **Calibration Editor** window.

9.1.5. To calibrate length, input a series of test voltages including both negative and positive voltages (e.g., -3, -2, -1, 0, 1, 2, 3 V).

9.1.5.1. Click **Set** for the first line.

9.1.5.2. Click **Read**.

9.1.5.3. Measure the exact length of the lever arm in millimeters and input into the corresponding box.

9.1.5.4. Repeat for the next voltage.

9.1.5.5. Upon recording of all voltages, click **Calculate Cal Factors** (recorded in mm/volt).

9.1.6. To calibrate force in, utilize a set of known weights increasing in a linear progression.

9.1.6.1. Adjust the motor so that it is resting on the edge of the bench or table with the lever arm parallel to the benchtop and hanging over the edge to allow the weight to hang.

9.1.6.2. Hang the first weight from the lever arm using a rubber band. Under **Applied Force**, enter the known weight in grams accounting for the mass of the rubber band.

9.1.6.3. Select **Read**.

9.1.6.4. Repeat for at least 3 known weights.

9.1.6.5. Select **Calculate Cal Factor**.

9.1.6.6. To verify calculation, plot calibration data and curve fit by selecting **Plot Cal**.

9.1.7. To calibrate force out, enter calibration voltages (up to 10 volts)

9.1.7.1. Click Set directly next to the calibration voltage.

9.1.7.2. Repeat for each voltage line.

9.1.7.3. Gently apply pressure to the lever arm using a finger until the **Force Out** ceases to change and the motor arm begins to move.

9.1.7.4. Maintain this position. Select **Read**.

9.1.7.5. Repeat for each voltage line.

9.1.7.6. Select **Calculate Cal Factor**.

REPRESENTATIVE RESULTS

The torque-frequency curve utilizes lower frequencies to produce multiple isolated isometric twitches of relatively low torque and progresses through increasingly higher frequencies, resulting in fusion of twitches for an isometric tetanus contraction at which peak tetanic torque is obtained. The presented protocol for knee extension peak tetanic torque the force-frequency curve initiates at 10 Hz which elicits 3 isolated twitches. Partial fusion of twitches occurs at 40 Hz, and peak tetanic torque is reached between 120-180 Hz (**Figure 5**).

Figure 6 illustrates representative knee extension torque-frequency curves from female C57BL/6 mice. Three separate mice were tested at baseline, and the experiment was repeated in each mouse 2 weeks later for comparison to assess reproducibility. Torque-frequency curves are shown with raw torque values (**Figure 6A**), as well as raw torque values normalized to mouse body weight (**Figure 6B**). Repeated observations demonstrate comparable results in all 3 mice with a 2-week rest period between experiments. Body weight normalized torque data should be considered in addition to raw torque, as minor fluctuation in weight may impact functional output and is not considered with raw torque alone. Furthermore, body weight normalized torque data facilitates comparison of mice of varying sizes. Torque can also be normalized to muscle wet weight or myofiber cross sectional area, as we have previously shown[16].

Figure 7A shows the area under the curve using body weight normalized isometric torque data from complete torque-frequency experiments (10 Hz, 40 Hz, 120 Hz, 150 Hz, 180 Hz, 200 Hz) for 4 separate C57BL/6 mice, highlighting similar total torque output and coefficients of variation between 5.6% to 8.8% with repeated experiments within the same mice. Data are most simply reported as peak tetanic torque (**Figure 7B**) which is the maximal torque value from the repeated tetanus isometric contractions from 120-200 Hz. The peak tetanic torque output is comparable in 6-8-month-old female C57BL/6 mice (**Figure 7B**) with coefficients of variation between 4.8% and 8.7% with longitudinal assessment within the same mice. Peak tetanic torque is most comparable to the gold-standard strength assessment in human studies: maximum isometric toque.

Furthermore, the knee extensor peak tetanic torque protocol is a useful tool to detect strength differences in multiple mouse models. **Figure 8** demonstrates the stark contrast between knee extensor strength in a non-injured, healthy 6-month-old C57BL/6 female mouse (black line) and a transgenic mouse model of supraphysiological hypertrophy in which myostatin/GDF8 is knocked out (blue line). We also show a peak tetanus curve from a C57BL/6 mouse 7 days after surgical transection of the anterior cruciate ligament (ACL-T) (red line), demonstrating a nearly 50% decline in peak torque after injury which is well outside the coefficients of variation observed with repeat testing of uninjured mice. Concurrent with human data[17,18], strength is markedly diminished with ACL-T. All mice are female and of similar age (6-8 months).

FIGURE AND TABLE LEGENDS

Table 1: Example of twitch series. * denotes optimal amperage/current.

Table 2: Example of torque-frequency curve data. * denotes peak tetanic torque.

Figure 1: Data collection software setup. Illustration of setup for data collection software with Live Data Monitor.

Figure 2: Mouse setup and electrode placement. (**A-B**) Supine position of the mouse receiving anesthesia via a nose cone on the heated platform. Upper hind limb is securely clamped, posterior to the knee to allow for unrestricted movement at the knee joint. Motor arm is adjusted so that knee is bent at approximately 60˚. The femoral nerve motor point is stimulated by needle electrodes to activate contraction of knee extensors. Mouse setup is shown from a side view (**A**) and overhead view (**B**).

Figure 3: Determination of optimal electrode placement to achieve isometric knee extension. Representation of repeated negative twitches stimulated with 50 mA using the Instant Stimulation function and viewed in the Live Data Monitor. Red arrows indicate the first three knee extension twitches.

Figure 4: Representative twitch to determine optimal amperage. The lowest amperage to elicit the highest twitch isometric torque must be determined for the force-frequency experiment by repeated twitch experiments with progressively increased amperage.

Figure 5: Representative tetanic torque curves throughout a torque-frequency experiment for the same mouse. (**A**) Submaximal isometric tetanic torque produced at 10 Hz. (**B**) Submaximal isometric tetanic torque at 40 Hz. (**C**) Peak isometric tetanic torque output at 120 Hz. (**D**) Isometric tetanic torque at 150 Hz. (**E**) Isometric tetanic torque at 180 Hz. (**F**) Isometric tetanic torque at 200 Hz.

Figure 6: Representative torque-frequency curve data. (**A-B**). Torque-frequency curve at 2 different timepoints (week 1 and 3) in 3 separate mice, presented as raw peak torque (**A**) and raw peak torque normalized to body weight (**B**).

Figure 7: Representative area under the curve (AUC) and peak tetanic torque data. (**A**) AUC for 4 separate mice, presented as raw torque normalized to body weight. (**B**) Peak tetanic torque for the same 4 mice, presented as raw peak tetanic torque normalized to body weight.

Figure 8: Peak tetanic torque of knee extensors in multiple mouse models. Representative peak torque tetanus curves for an overt hypertrophy transgenic mouse model (GDF8 KO), an uninjured healthy C57BL/6 mouse (mouse 2), and a C57BL/6 mouse 7 days after anterior cruciate ligament transection (ACL-T).

Supplementary Figure 1: Dimensions of custom fabricated plastic. Inset in red shows dimension of depth.

Supplementary Video 1: Real-time knee extensor twitch without motor arm.

Supplementary Video 2: Slow-motion knee extensor twitch without motor arm.

DISCUSSION

Measurement and analysis of muscle function in rodent models is imperative to make translational and meaningful inferences regarding histological and molecular skeletal muscle adaptations observed with exercise, injury, disease, and therapeutic treatment. We demonstrate a method to assess knee extensor maximal strength reliably and repeatedly in mice using commercially available equipment, with the adjustable plastic piece to hold the lower hind limb at the anterior tibia being the only custom fabricated part that may be replicated.

Common functional assessment tools have been widely used to repeatedly evaluate physical performance within the same mouse, such as treadmill running to volitional fatigue, rotarod performance test, inverted cling test, and grip strength test. However, while informative, these assessments involve cardiopulmonary and behavioral component(s), which can obfuscate the interrogation of neuromuscular function associated with these physical performance measures. Furthermore, elements of endurance, coordination, and balance are present in many of these functional assessments to varying levels, limiting clear interpretation relative to muscle strength. The force producing ability of rodent muscle(s) can be measured in vitro, in situ, or in vivo. Each approach has relative advantages and limitations. Specifically, with in vitro assessment, the muscle is completely isolated and removed from the body of the animal so that there is no influence from perfusion or innervation[19]. This yields a well-controlled environment to ascertain contractile ability but limits the size of the muscle being studied through dependency on passive diffusion of oxygen and nutrients during testing. In situ testing maintains the innervation and blood supply of the muscle, but is limited to a singular terminal assessment, as with in vitro testing[20]. Finally, in vivo testing is the least invasive with the muscle remaining in its native environment with percutaneous electrodes inserted near the motor nerve to electrically stimulate the muscle. A strength of the in vivo approach is the potential for longitudinal testing across time[21-23].

In vivo evaluation of peak muscle contractility optimally measures maximal strength as the normal anatomy and physiology of the mouse remains intact and the method may be repeated on the same mouse before and after an intervention or throughout the lifespan. Specifically, in vivo measurement of knee extensor strength in mice is the murine strength assessment with the greatest translational relevance to human studies, as maximum knee extension torque is commonly measured and considered the gold-standard strength test in humans with correlation to various functional and health outcomes[24-27]. Moreover, knee extensor pathology is observed with aging as well as a myriad of injuries and diseases[1,2,4-6], but assessing the impact of these conditions on knee extensor strength longitudinally in mice has not been readily attainable.

Although this method offers utility to determine knee extensor peak torque in a longitudinal manner, certain limitations of the protocol should be considered. Lower frequencies between 40

Hz to 120 Hz were omitted from the torque-frequency protocol, which may limit the ability to detect left or rightward shifts in the torque-frequency curve with injury or disease. However, using this torque-frequency protocol, we have been able to detect alterations to peak tetanic torque in an ACL injury model and between C56BL/6 wild type mice and a transgenic mouse model of supraphysiological muscle mass (**Figure 8**). We note that it may be beneficial to secure the electrodes with helping hands or similar apparatus as muscle contractions may move electrodes slightly. We did not note any obvious displacement of electrodes with progressive contractions; however, the possibility of slight movement of the electrodes cannot be ruled out, which may impact muscle stimulation. Additionally, intramuscular electromyography (EMG) was not performed in conjunction with the stimulus protocol; however, inclusion of EMG measures may be feasible, if desired and appropriate for the experimental model of interest.

Assessment of knee extensor strength in murine models of orthopedic injury and disease will facilitate pre-clinical research with meaningful translational relevance to clinical strength measures. Our protocol enables precise and repeated assessment of maximal knee extensor strength in mice with commercially available equipment accessible to any laboratory.

DISCLOSURES

Matthew Borkowski is employed by Aurora Scientific Inc., a company that may potentially benefit from the research results and is also an executive of the company.

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Figure

Click here to access/download;Figure;62211_R1_Figure 1_1.27.21.pdf

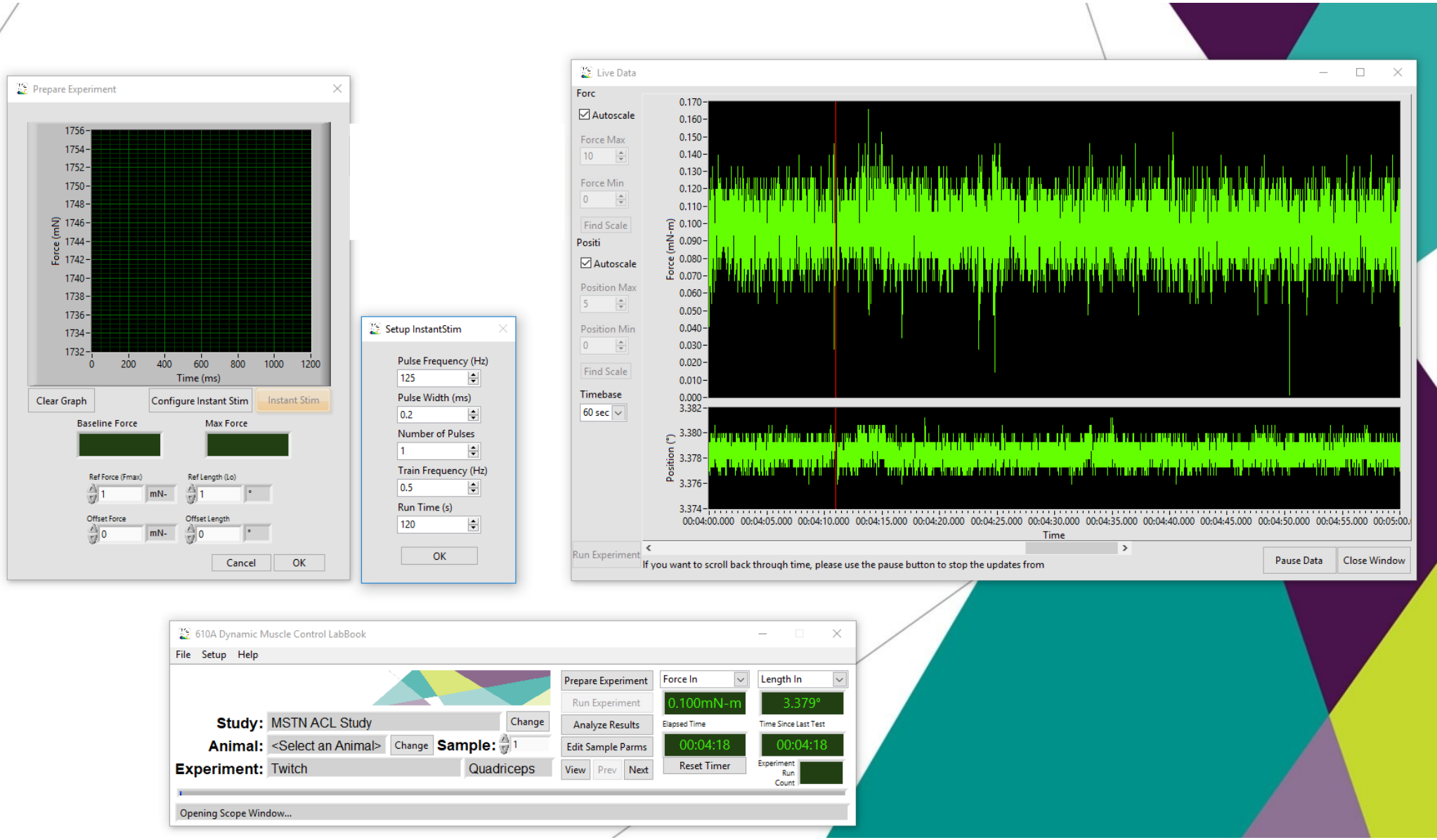
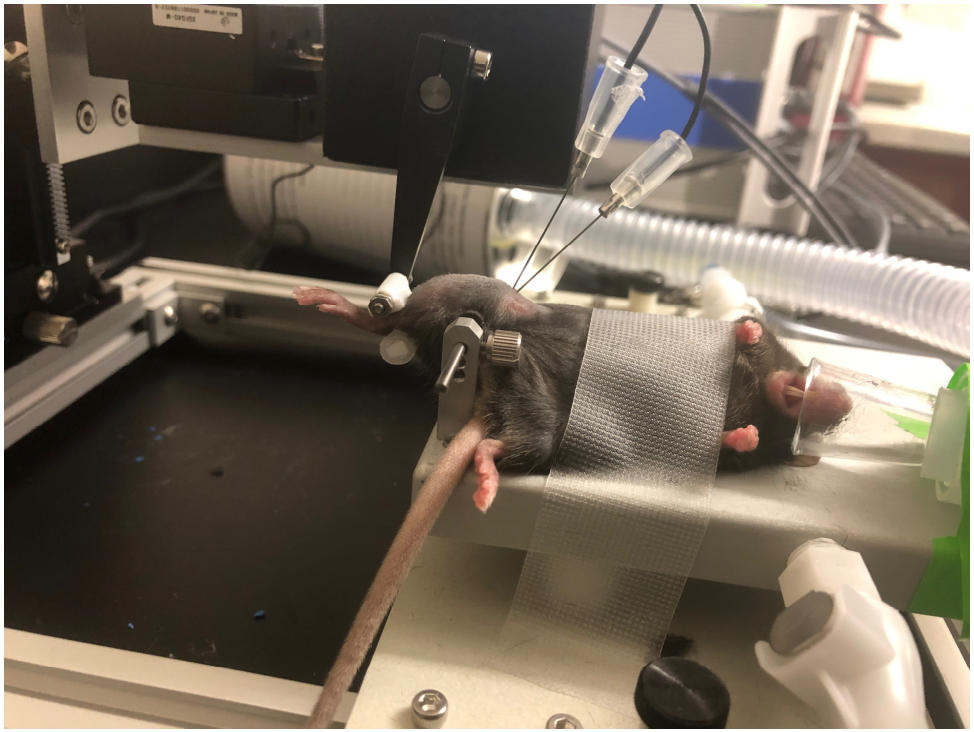


Figure 2

A.



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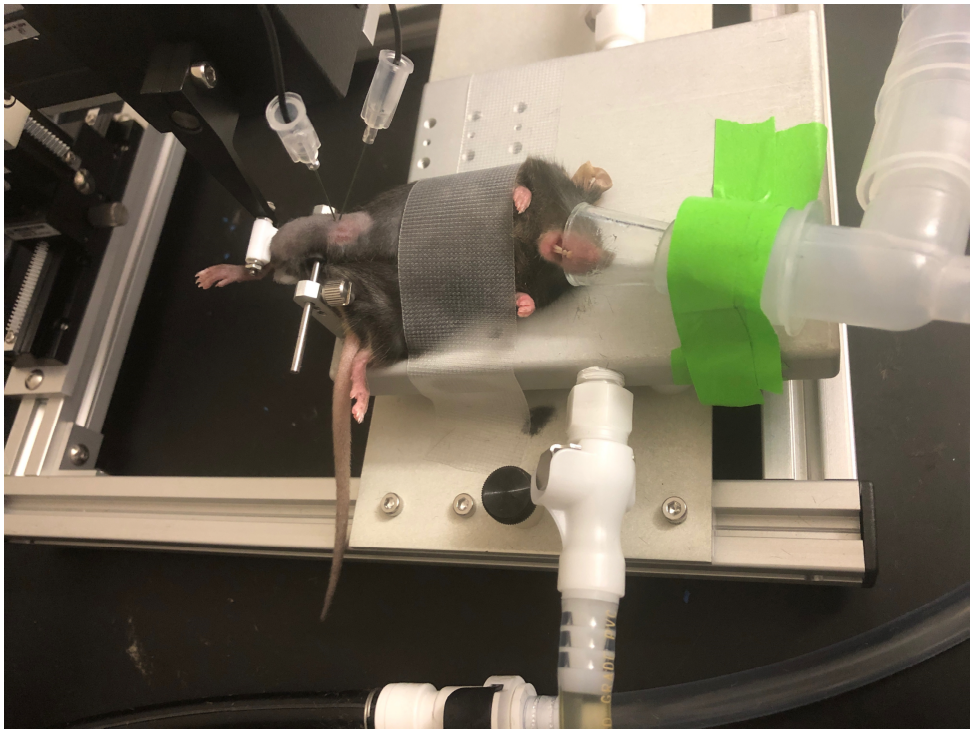

Figure 3

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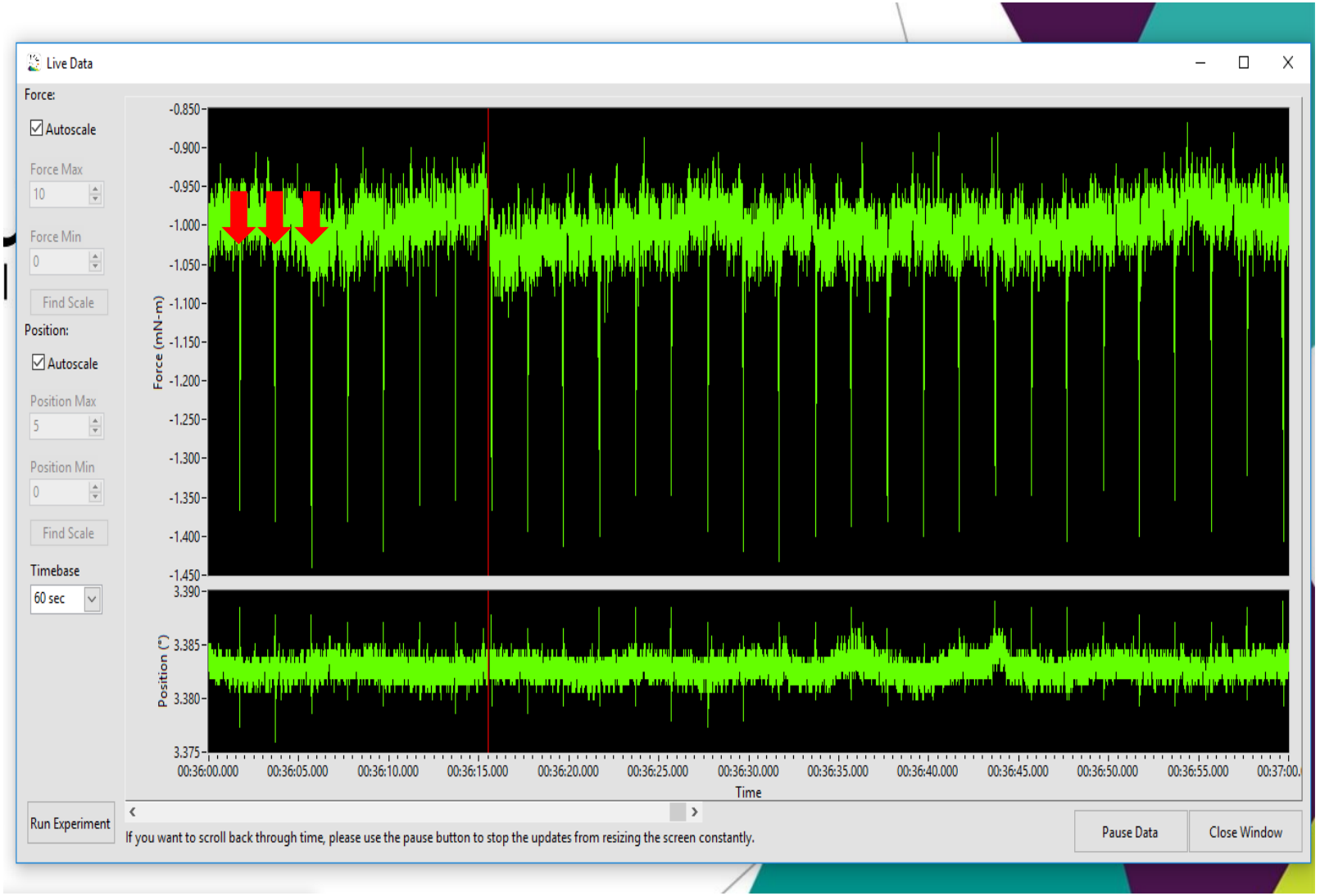

Figure 4

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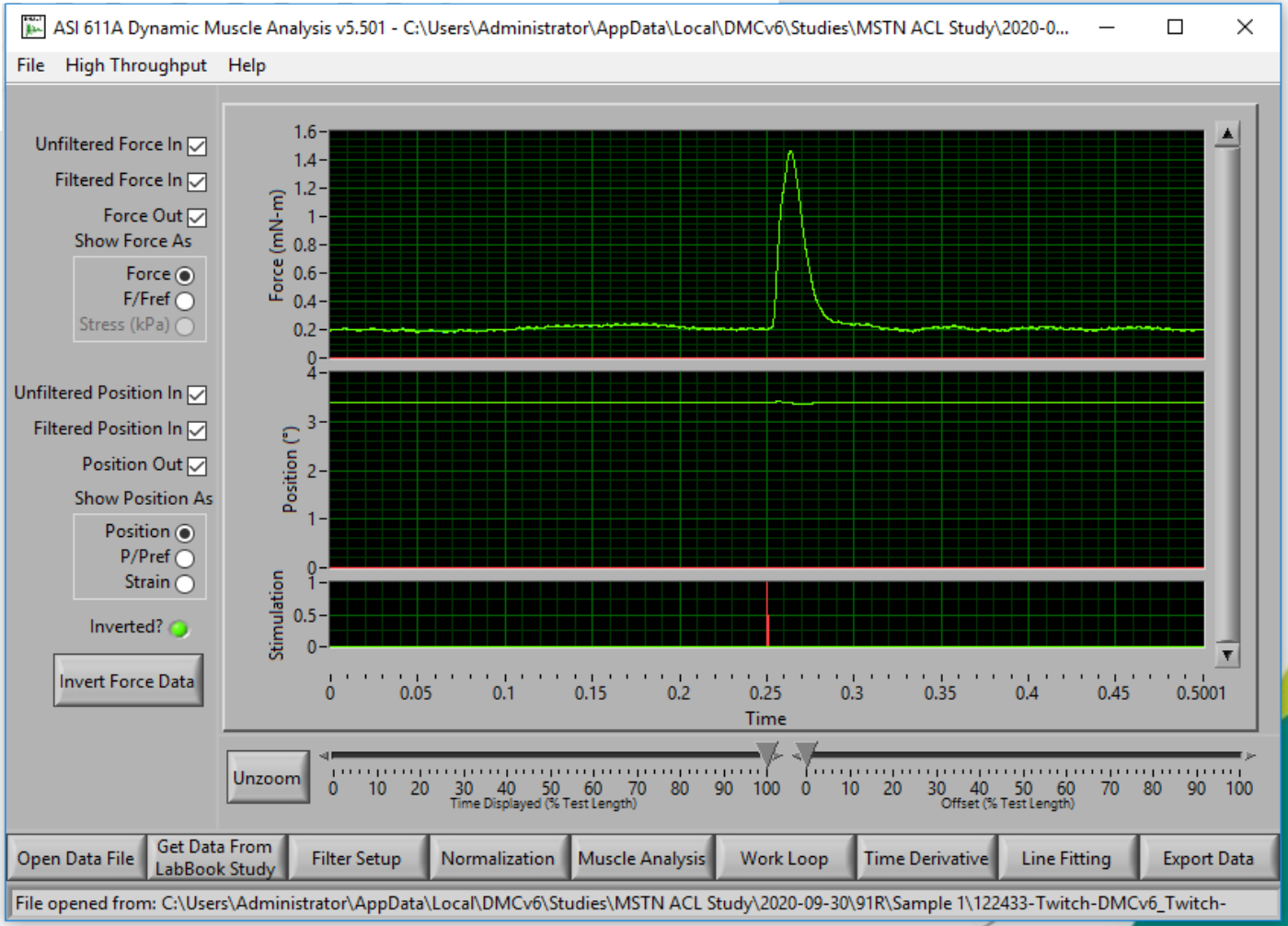

Figure 5

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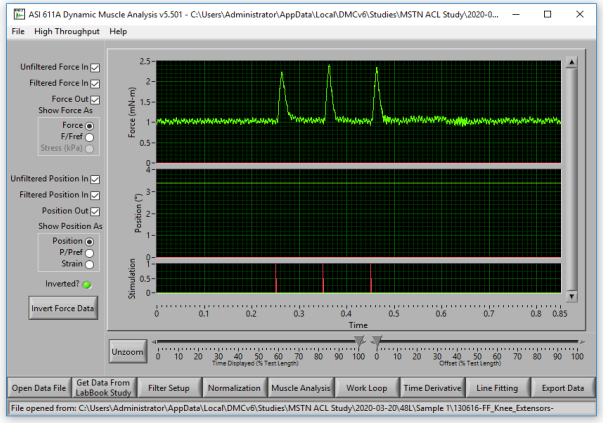
A.



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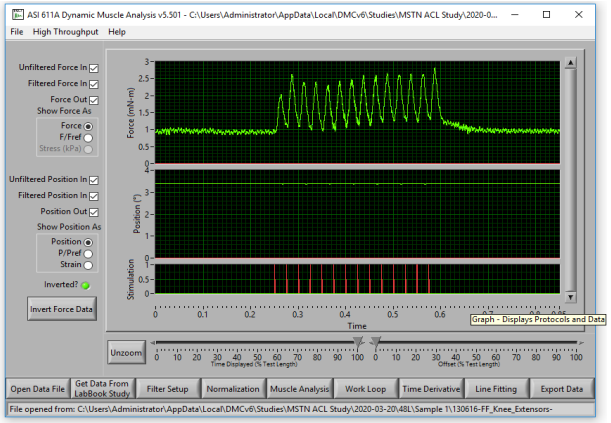

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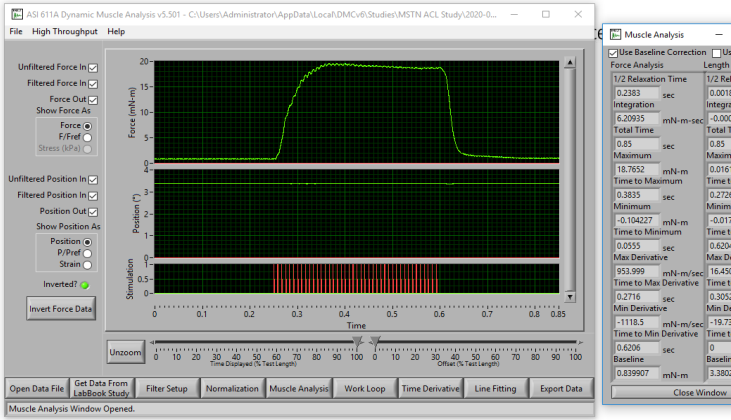
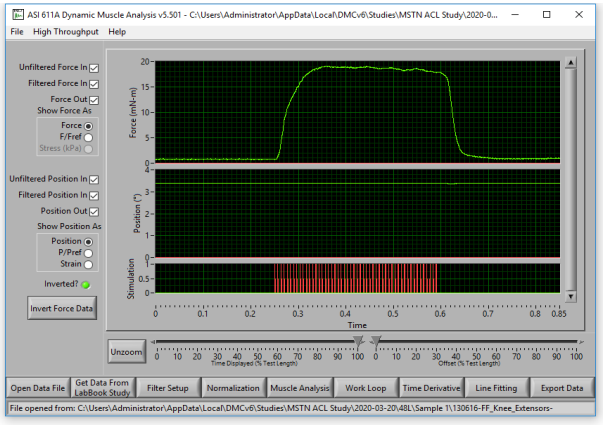
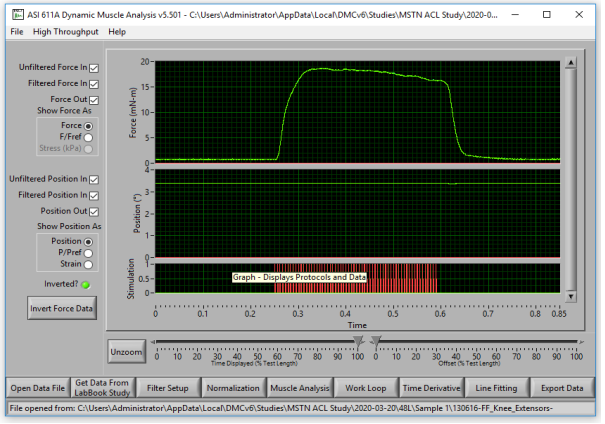


D.



E.



F.

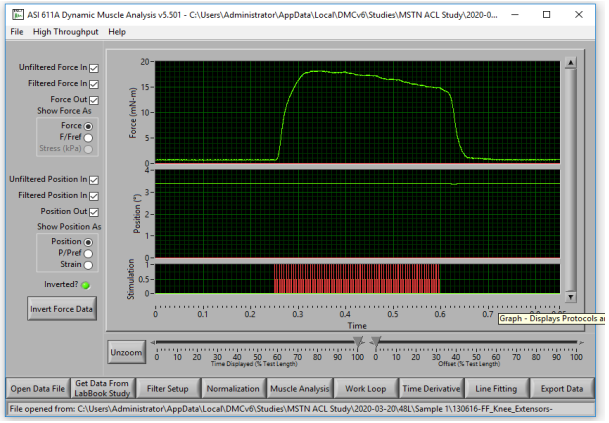


Figure 6

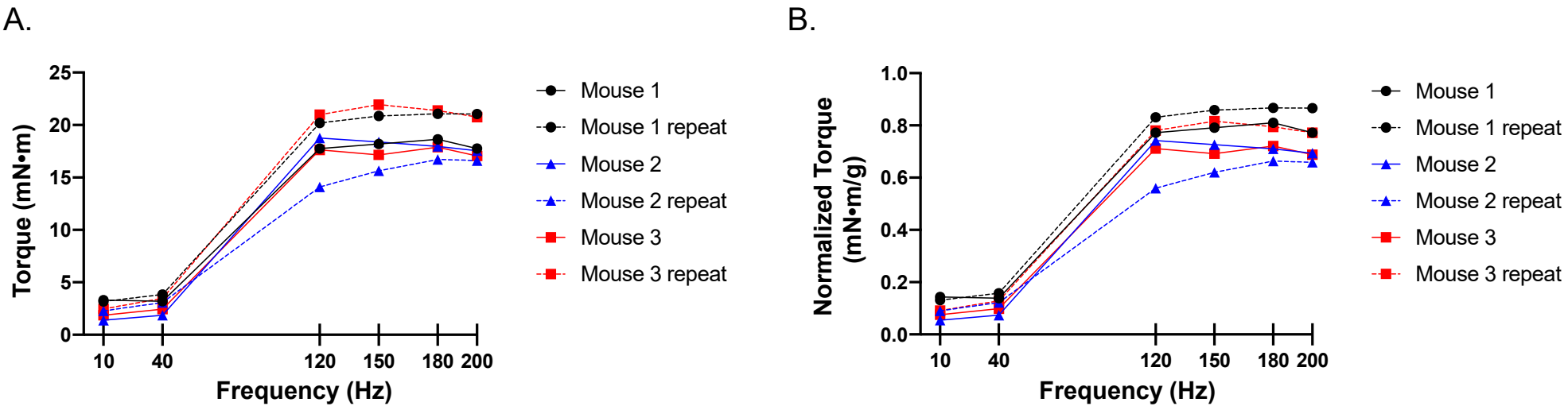
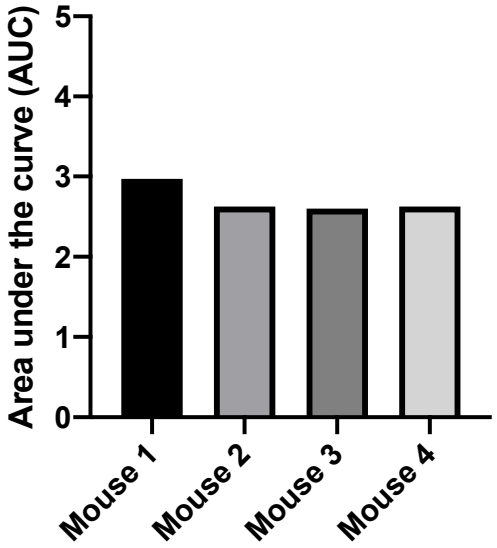


Figure 7

A.



B.

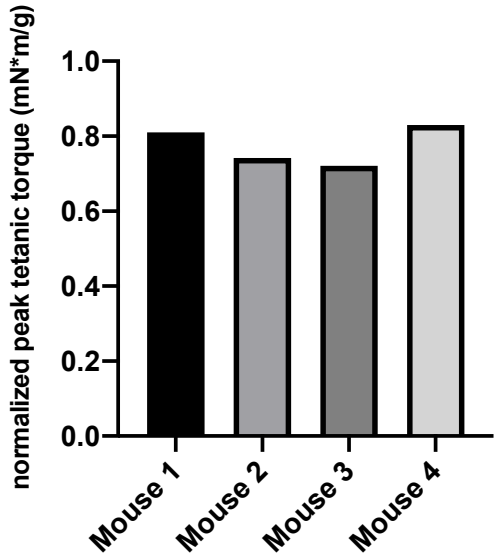


Figure 8

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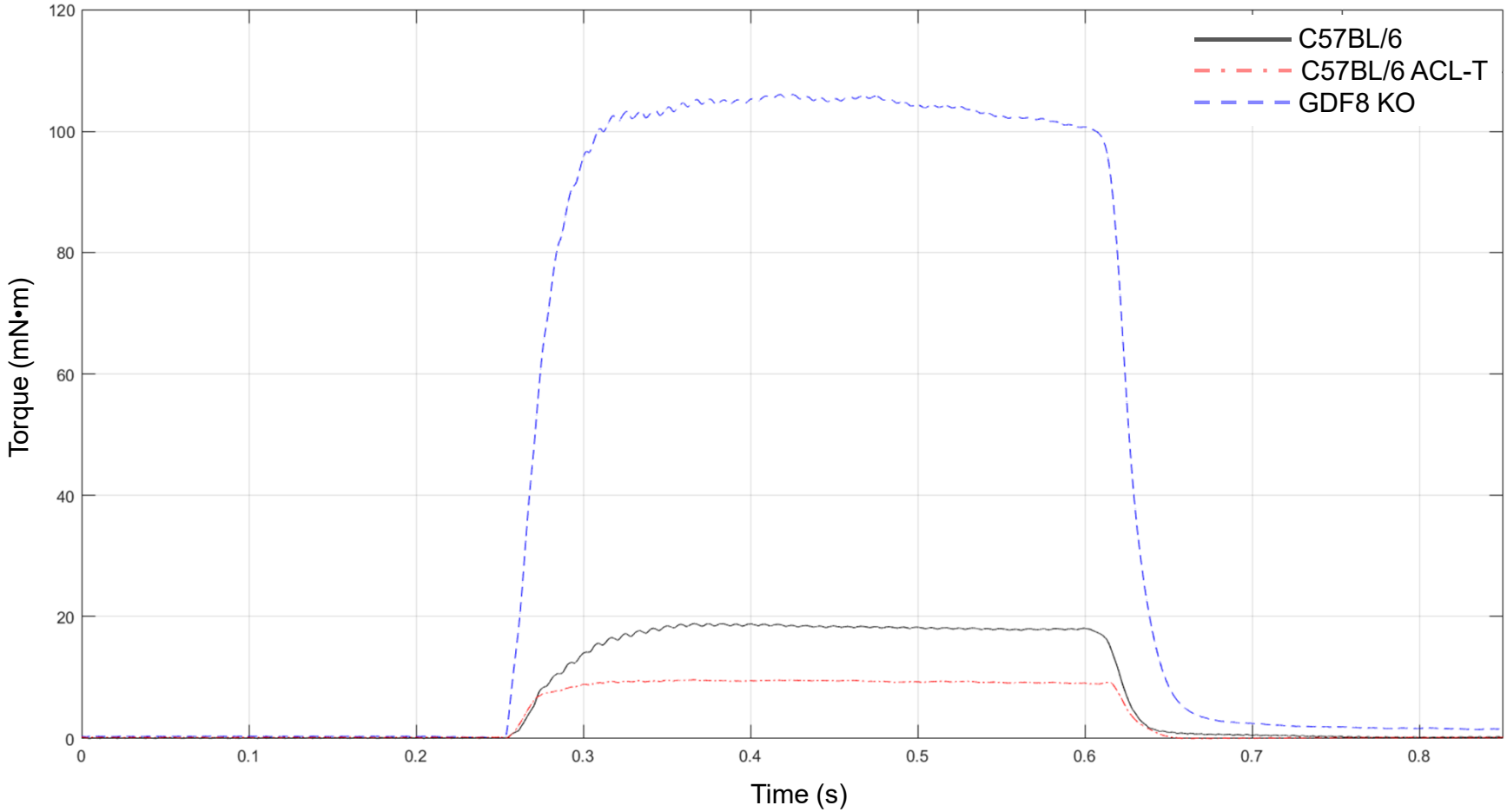

Supplemental Video 1

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Video or Animated Figure

Supplementary video 1.mov

Supplementary Video 2

Table 1

Twitch experiment	Amperage/Current (mA)	Torque (mN•m)
1	50	1.279
2	70	1.341
3	90	1.36
4	110	1.362
5	***130**	**1.449**
6	150	1.436
7	140	1.333

Table 2

Frequency (Hz)	Torque(mN•m)
10	1.385
40	1.869
120	***18.765**
150	18.375
180	17.97
200	17.548

Name of Material/Equipment	Company
1300A: 3-in-1 Whole Animal System- Mouse	Aurora Scientific Incorporated
6100 Dynamic Muscle Control LabBook software	Aurora Scientific Incorporated
611A Dynamic Muscle Analysis	Aurora Scientific Incorporated
BravMini hair clippers	Wahl Clipper Corporation
Eye Lube	Optixcare
Isoflurane	Covetrus
V-1 Tabletop Laboratory Animal Anesthesia System	VetEquip Inhalation Anesthesia Systems
Prism 8	GraphPad Software, LLC

Catalog Number

300D-305C-FP: dual-mode motor with custom knee extension apparatus, 605A: Dynamic Muscle Data Acquisition and Analysis System, 701C: Electrical Stimulator, 809C: *in-situ* Mouse Apparatus

DMC v6.000

DMA v5.501

ASIN: B00IN24ILE
Item Number: 142422
NDC: 11695-6777-2

Item Number: 901806

Version 8.3.0 (328)

We thank the reviewers and editor for their constructive critique of our manuscript. We have addressed individual comments below in red. Briefly, we have amended the manuscript per editorial comments, and we have added a limitations section to the discussion, improved the premise of the study in the introduction, added new representative images/data to our manuscript and provided additional clarity to several needed methodological considerations. We feel the additions and edits to the manuscript strengthen it and increase the impact of the work. We hope the reviewers and editors find our revised manuscript suitable for publication.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We confirm that we have thoroughly proofread the manuscript again to address any spelling or grammar issues.

2. Please provide the institutional mailing address of all the authors.
The institutional mailing addresses have been added to all author affiliations.

3. JoVE cannot publish manuscripts containing commercial language. 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: e.g., Eppendorf, Accellerase, Thermo, Dynamic Muscle Control LabBook software, etc.
Commercial language has been removed as appropriate. This protocol is specifically designed for use with the Aurora physiology system and Dynamic Muscle Control LabBook software (DMC v6.000) as click-by-click instructions are provided that are unique to this software, so only necessary references to specific commercial software to ensure clarity regarding how to use the apparatus and corresponding software remain. We have removed “Dynamic Muscle Control LabBook” and “Dynamic Muscle Analysis/DMA”from the text and simply reference the Table of Materials for details regarding the software mentioned in the step-by-step text.

4. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please do not use " Dynamic Muscle Control LabBook " within your text and use generic language instead. Please also remain neutral in tone throughout (reduce the usage of words like novel, fast, simple, etc.)
We have removed mention of specific software in the text and instead refer to the Table of Materials for details regarding appropriate software. We have also toned down language to reflect a more neutral tone throughout the Introduction and Discussion.

5. Please include any strain, age, or sex-specific bias if any.
Strain and sex of mice were previously included, and age has now been added to the manuscript in Representative Results.

6. 6.2: Please include more details, how the results are analyzed?

"Analyze Results" refers to the clickable button in Dynamic Muscle LabBook. When opened, this window displays torque output under "Max Force" which should then be manually recorded, as described in 6.2. The word "manually" has been added in the text preceding "record" to clarify this point.

7. 8: How do you determine the baseline torque, maximal torque etc.? How do you filter raw torque data? What kind of statistics is used here?
Baseline torque and maximal torque are determined automatically in the Analyze Results window within Dynamic Muscle LabBook. There is no need for manual calculation as the software automatically calculates/generates the data. We have added a clarification that normalized data are calculated in a separate spreadsheet as torque/body weight in grams. These data are unfiltered. A NOTE line has been added to more clearly reflect this fact. We did not run statistical analyses on these data, but instead report representative results generated using this protocol. We have added a sentence in 8.2.2 to indicate statistical software was used to graph torque-frequency curves and calculate area under the curve, as shown in Representative Results figures. We have added GraphPad Prism to the Table of Materials.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We have included very specific details throughout all steps, specifically regarding button clicks in the data collection and analysis software. We have included specific numerical parameters for all components of experimental design, including pulse frequency, pulse width, number of pulses, train frequency, and run time for Instant Stimulation along with stimulus duration, frequency sequence, and rest period between pulses for the torque-frequency experiment. We believe the detailed steps provided are sufficient to clearly replicate the experiment in a step-by-step manner.

9. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
A one line space has been included between each protocol step (steps 1-8), but not between sub-steps (ex. between 1.1 and 1.2). Please advise if a one line space is needed between sub-steps as well. Steps 1-7 (approximately 2.5 pages) have been highlighted for inclusion in the protocol section of the video.

10. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
All figure and table legends have been moved to directly after the Representative Results, preceding the Discussion.

11. In the discussion section, please include some limitations of the protocol as well.
We have added a limitations/considerations paragraph to the Discussion section.

12. In the reference section, please use the following format for the authors: last name, first and middle initials (if available). For six or more authors, show only the first author's name followed by et al. Do not use a comma before et al. Example: Kioh, L. G. et al. Physical Treatment in Psychiatry. Blackwell Scientific Pubs. Boston, MA (1988).
We have amended the reference style noted.

13. Please do not use any abbreviations for journal titles and book titles. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
We have amended the bibliography to reflect the citation style specified.

14. Table 1 and 2: Use the multiplication dot for compound units. Example mN·m,
Torque units have been changed to mN·m, using the multiplication dot, in both Table 1 and Table 2.

15. Figure 1, Figure 4, Figure 5: Please remove the commercial branding: aurora scientific.
The Aurora Scientific desktop background in the screenshot images demonstrating use of data collection and data analysis software have been removed in Figures 1, 4, and 5.

16. Figure 6, Figure 7: Please define the X and Y-axis and mention the units in brackets.
The X and Y-axes are defined in Figures 6 and 7 with corresponding units denoted in parentheses.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Brightwell and colleagues detail a non-invasive technique to assess knee extension torque in anesthetized mice. This non-invasive technique allows for longitudinal testing in the same animal during disease progression, recovery from injury, and/or during treatment. Muscle torque (strength) in general is the primary function of skeletal muscle and is the most clinically relevant outcome measurement. While knee extension torque methodology has been described in rats, methods in mice are lacking.

Major Concerns:
Rationale: The current rationale for this technique is weak in two areas. First, in vivo approaches have been available to assess muscle strength non-invasively since 1985 with McCully and Faulkner, so while commercialization of the equipment may have been limited, the methods to do so were not. Second, the plantarflexors of the mouse hindlimb collectively represent ~200mg (gastrocnemius, soleus, plantaris muscles), and therefore current mouse in vivo techniques are not limited to relatively small and/or specialized musculatures. The rationale for this study relating to a reliance in human research on knee extensors is strong. I would also recommend the authors lean into their own research on ACLs and highlight the knee injuries and osteoarthritis

mouse models cannot be appropriately assessed without a knee extension technique, thus this technique is expanding access to the muscle-bone-cartilage-tendon field.

We agree with the reviewer that our rationale should better reflect lacking knee extensor measures in mice given translational relevance to ACL injury, OA, etc. We also agree that methods have existed for repeated non-invasive assessment of muscle strength for some time, and we have removed comments to that effect that the reviewer correctly notes. We have significantly reformatted our introduction to emphasize translational relevance to ACL injury, OA, etc. as the premise to the current methodological study.

Cellular and technical discussion: There is some confusing language regarding how this technique works throughout the manuscript. First, lines 66-69 mention neural control then say muscle architecture and size largely determine strength. Motor unit recruitment and the frequency of action potentials to those motor units is the primary determinant of strength, not taking into account length-tension and/or force-velocity circumstances. This is evident in individuals with central and/or peripheral nerve damage that have muscle mass but cannot generate muscle torques due to impaired recruitment. Second, lines 238-240 suggest the torque-frequency data changes due to motor unit recruitment. This electrophysiology approach stimulates the voltage-gated sodium channels along the axon and therefore all motor units are recruited. There is no motor unit recruitment with in vivo electrophysiology. What determines torques changes is that the frequency of action potentials is being modulated by your protocols (frequency) and more frequency leads to greater intracellular calcium concentration, greater cross-bridges, and more force production by the muscle. I think the manuscript could be improved with more discussion on these topics with the in vivo approach and adding details to what Frequency, stimulus duration, pulse durations are exactly in the stimulation protocol. Aurora should be able to provide you with an image of their square-wave pulse, and that would be helpful to include as well.

We agree with the reviewer that there was a lack of clarity in the introduction and discussion. We have largely rewritten the Introduction to address comment #1 from Reviewer 1, and subsequently the confusing language in the lines regarding neural control have been removed. Additionally, we have added a brief discussion of various methods (in vitro, in situ, in vivo) to assess neuromuscular function in rodents. We have also added specific details per your comment in our protocol:

- pulse width (0.2 ms)
- pulse frequency (125 Hz)
- torque-frequency protocol sequence (10 Hz, 40 Hz, 120 Hz, 150 Hz, 180 Hz, 200 Hz)
- stimulus duration (0.35 s)
- rest period between pulses (120s)

We have added representative images on the square wave pulse in our new Figure 5 that also shows representative tentani as well. The square pulse wave is seen in red in the representative images in Figure 5.

Data accuracy: The data provided is a little concerning. Figure 5 and 8 are not representing fully fused tetany as there is clearly still relaxation events occurring, i.e., the plateau portion of the

curve is not flat. In Figure 6 it is not clear why frequencies between 40 and 120 Hz would not be included considering these frequencies are the most common ones for detecting left/right shifts with disease/injury. Also, the torques generated are quite low. 20mNm and ~0.7 mNm/g BM are what the plantarflexors muscles generate in C57Bl6 mice. Perhaps the quadriceps in mice are the same strength as the plantarflexors. This is OK but again requires you to revisit the rationale above. However, it is also possible that your force transducer is not capturing all the torque generated. In Figure 2, it appears that some of the torque is going into the lever arm. The lever arm is sensitive to rotational torque not compressive torque. The more appropriate configuration of this would be with the lever arm parallel with the limb such that knee extension is applied at a mostly perpendicular/rotational manner. It also might be beneficial to secure the electrodes with helping hands as muscle contractions may move them slightly and also limit full tetanic contractions.

We wish to clarify that these data are not filtered, but instead are raw torque data. The data collection and analysis software allow for application of a filter, if desired; however, we selected to not apply a filter to the data. We have amended the manuscript to more clearly reflect this point (see NOTE under Step 8. Data Analysis). Our frequency sequence for knee extension is as follows: 10 Hz, 40 Hz, 120 Hz, 150 Hz, 180 Hz, 200 Hz. We chose this sequence as we consistently observe peak tetanic torque occurring between 120-180 Hz for knee extension. We acknowledge the potential limitation to detect left/right shifts in the force-frequency curve by omitting frequencies between 40-120Hz. We have added a limitations section to the Discussion where we acknowledge this point. We have added graphical representative data (displayed in the analysis software) from each of the frequencies to demonstrate the torque-frequency pattern more clearly. This addition to Figure 5 displays torque from each frequency from a torque-frequency experiment in the same mouse (mouse 2). We also note the reviewer comment regarding fully fused tetanus. The representative images selected in Figures 5 and 8 occur at a frequency of 120Hz, where very subtle relaxation is visually evident. We have added representative images at 150Hz that show complete tetanus and updated Figure 5 accordingly.

We acknowledge the reviewer's comparison of quadriceps vs plantarflexor torque. However, we note that the peak tetanic knee extensor torque that we currently report in young healthy mice (≈20 mN•m) is approximately double that which our lab[1] and two independent labs[2,3] (≈10 mN•m) have reported for in vivo peak tetanic plantar flexion using a similar protocol. A study by Pratt and Lovering reported maximal isometric quadriceps torque in C57BL/6 mice of 11±1 N•mm[4] (2-3 month old animals). While these reported values are lower than our reported values, our mice are several months older (6-8 month old). Body weight and sex for the mice in that study were not reported[4] to directly compare BW-normalized quadriceps torque to data we report, but given the difference in age (and likely body weight/muscle mass), our values are likely comparable to those published by Pratt and Lovering[4].

Regarding the issue of rotational versus compressive torque, the motor arm is designed to measure torque (rotational force). The material of the plastic piece in which the tibia rests and directly presses against with isometric contraction is not compliant, so there is no loss of torque from the tibia pressing into this piece. To ensure consistent and appropriate placement, we observe the "Force In" measurement displayed in the data collection software and adjust the placement of the motor arm so that the "Force In" is approximately -1.0 mN•m or less—

indicating that the tibia is lightly resting against/touching the plastic piece (i.e. not hovering in 'dead space' which would require a concentric contraction preceding the isometric contraction) but not being driven into the plastic piece with high force prior to the isometric contraction. We have amended Step 3 of the manuscript to clarify this point. We acknowledge that we could perform a full range of motion analysis at different knee angles to identify the angle at which peak torque occurs for the mouse, as others have done, but we chose to utilize the consistent placement displayed in Figure 2 and detailed in Step 3. We have included this discussion in our limitations and consideration section of the discussion.

We acknowledge the benefit of helping hands to secure the electrodes during muscle contractions and we have added this to limitations/considerations in the Discussion as well.

Reviewer #2:

Manuscript Summary:

The manuscript submitted by Brightwell and colleagues presents a method to measure in vivo knee extensor muscle function in mice. In general, the manuscript is well written and the methods are nicely detailed. The development of an ergometer to quantify twitch and tetanic torque in mice in vivo is a useful tool for the field. However, for the field at-large to adopt this approach, more detail is needed on the calibration of the strain gauge and signal processing. I have also provided some minor comments to help improve the clarity of the manuscript.

We thank the reviewer for their feedback and agree that methods required greater development for adoption in the field. We have addressed each point and feel that the inclusion of greater detail strengthens our methods manuscript.

Major Concerns:

1. The authors should include data and instructions for calibrating the strain gauge.

We agree that these data are needed for proper method adoption. A detailed blog regarding calibration written by one of this paper's co-authors is available on the Aurora website at https://aurorascientific.com/how-to-calibrate-your-dual-mode-lever-system-using-dmc/. Step-by-step instructions for calibration have been added to the manuscript, Protocol section #9. From our recent calibration preceding the data collection reported in the manuscript:

- Length in scale factor: 1.3959 mm/V
- Length out scale factor: 1.3959 mm/V
- Force in scale factor: 500 mN/V
- Force out scale factor: 500 mN/V

2. Please include the sampling frequency for the torque and position channels in the methods.

The sampling rate was 10,000 Hz. This has been added as a NOTE under Step 6.1.

3. More information is needed to understand how the raw torque and position data are filtered. Specifically, please provide details on the type of filter used and what frequency the data were filtered at.

The data presented (Fig 5, Fig 6, Fig 7, Table 1, Table 2) are not filtered but instead represent raw data. The software provides the option to set a filter (by toggling on the Filter option box in the analysis software); however, we did not select this option, as it was not necessary. We have amended the manuscript to more clearly communicate the fact that the data are unfiltered (see NOTE under Step 8. Data Analysis).
The data exported as a text file for Matlab processing (simply for the purpose of visually overlaying 3 different tetanus graphs from different mice in Figure 8) are, by default, filtered using a low pass filter (set to filter only high frequency noise). The peak torque with baseline subtracted from these filtered data is equal to 18.7650 mNm, compared to 18.7652 that we report using the raw unfiltered data taken from the analysis software—a difference of 0.001066%.

4. Methods section 8. Please provide more detail on how the maximal torque is determined. For example, is the absolute peak from the filtered data used or does this represent the average over a given period of time? Additionally, is the baseline torque factored into the calculation of the maximal torque? Including the equations for these calculations would be useful.
The peak torque data that we report is unfiltered, which we have now clarified in the text. The peak torque then represents the absolute maximum point (not an average of the plateau). Baseline torque is subtracted (see Step 8.1.6). A NOTE has been added under Step 8.1.6 to clarify calculation of peak torque with baseline subtracted.

Minor Concerns:
1. Strength is an ambiguous term. Please consider using torque or power where appropriate throughout the manuscript.
Thank you for this suggestion. We have used the term torque when referring to recorded data and now only use strength when referring to translational relevance to physical function.

2. The authors use force and torque interchangeably throughout the manuscript. Please revise to torque as you are reporting the force produced about the knee.
We thank the reviewer for this note as this has improved clarity of the manuscript as a whole. We have amended the manuscript throughout to use the term torque (specifically “torque-frequency curve”) and only use force when referring to “Max Force” which is the section within the software in which peak torque is displayed (i.e. Step 5.2. “Record the twitch torque displayed under Max Force with baseline subtracted.”).

3. Please revise 'amperage' to current throughout the manuscript.
We have added the word current (i.e. “amperage/current”) when the term is initially introduced in text (Step 4 and 5) and in Table 1 and replaced the term “amperage” with “current” in the remaining text.

4. Methods section 2.2. Please revise to state "enter desired parameters for each of the following variables in the dialogue box" or similar.
We thank the reviewer for this suggestion. We have included the specific pulse frequency, pulse width, number of pulses, train frequency, and run time input into the software for this experimental protocol to align with editorial comments regarding clarity to facilitate step-by-step reproducibility.

5. Methods section 3.6. Please refer to Figure 2 here to point readers to the correct position for the upper hind limb.
We thank the reviewer for this excellent suggestion to highlight correct hind limb position. We have added reference to Figure 2 on Step 3.6.

6. Line 176: Please revise 'max force' to 'maximal torque'. This appears elsewhere throughout the manuscript but torque is measured, not force.
We thank the reviewer for pointing this out, as stated in the previous response to comment 2. We have replaced the term “force” with “torque” throughout the manuscript, except when referring directly to the “Max Force” display box within the software (but we have clarified that torque is displayed under “Max Force” in this software).

7. Line 186: Please remove "be used for and".
We have amended the language used to improve clarity and readability.

8. Methods section 6. Please revise 'force-frequency' to 'torque-frequency' because force is not measured with this apparatus.
We have replaced the term “force-frequency” with “torque-frequency” throughout the manuscript and figures.

9. Methods section 6.2. The reference to mouse 2 is not necessary. Please remove.
For clarification, “mouse 2” was included to denote that this is the same mouse shown in Figures 6 and 7; however, we have removed reference to mouse 2 in Step 6.2.

10. Line 241 does not read well. Please revise for clarity.
Thank you for this suggestion. We have revised the Discussion to improve clarity.

11. Lines 254-259: The authors refer to body-weight normalized torque data in Figure 7A, but these data are in Figure 7B, and the non-normalized data are in Figure 7A. Please revise these Figure numbers to be consistent with the way the data are reported.
Figure 7A shows the area under the curve (AUC) using body weight normalized isometric torque data for each mouse, to consider the impact of differential size on peak torque output. This represents the normalized torque output across the entire torque-frequency experiment (10 Hz, 40 Hz, 120 Hz, 150 Hz, 180 Hz, 200 Hz), instead of the peak torque alone (from a single frequency between 120-180 Hz). We have added text to this portion of the Discussion in which Figure 7 is referenced to clarify this distinction. Figure 7B shows the single peak tetanic torque (occurring between 120-180Hz) normalized to body weight for each mouse.

12. Lines 273-274: A detailed image with the dimensions of the custom-fabricated plastic part would be useful for other research groups.
We agree with the reviewer that this is important information to facilitate replication of the experiment and have added a new Supplementary Figure 1.
Under step 3.7, we have added a new note which states:
NOTE: Detailed images and dimensions of the custom-fabricated plastic piece are shown in Supplementary Figure 1.

13. Reporting the coefficient of variation for the torque-frequency curve would be advantageous to statistically demonstrate the robust repeatability of these data.
We thank the reviewer for this insightful suggestion. For area under the curve (AUC) of the torque-frequency experiment using body weight normalized torque data (Fig 7A), below are the coefficients of variation for the 3 mice on which repeat testing was performed:
Mouse 1: 5.6%
Mouse 2: 8.1%
Mouse 3: 8.8%
For body weight adjusted peak torque, below are the coefficients of variation for the same 3 mice on which repeat testing was performed:
Mouse 1: 4.8%
Mouse 2: 7.8%
Mouse 3: 8.7%
These data have been added to the Representative Results section of the manuscript.

14. Please indicate somewhere in the discussion section whether it would be feasible to include intramuscular EMG measures to obtain measures of CMAP with stimulation as done in human studies.
Intramuscular EMG has been performed in rodent models by many groups, particularly in models of neuromuscular disease, and is sometimes used as a clinical diagnostic tool of neuromuscular disease in humans. We have not previously performed EMG studies in rodent models; however, this seems to be a feasible option for other groups to add to this protocol for assessment of CMAP, if desired. We have added a sentence to the limitations/considerations section of the Discussion to acknowledge this.

15. Please mention in the whether the plastic piece is adjustable for different shank lengths.
The custom-fabricated plastic piece is not adjustable for tibial length, although the motor arm can be adjusted/positioned as desired in relation to the platform on which the mouse rests in the supine position. We did not design the plastic piece to be adjustable in this regard as we don't expect and have not observed significant variability in the tibial length between mature mice in our lab.

16. Figures 1, 3, 4, and 5: Please remove the background image (Aurora Scientific desktop image) for greater clarity.
The Aurora Scientific desktop image has been removed from all screenshot Figures.

17. Figure 3. Please include an arrow to show where knee extension occurred in the torque trace.
Red arrows have been included in Figure 3 to denote the first three knee extension twitches, and the Figure 3 legend has been updated to reflect this addition.

References cited

1 Englund, D. A. *et al.* Satellite cell depletion disrupts transcriptional coordination and muscle adaptation to exercise. *Function.* doi:10.1093/function/zqaa033, (2020).

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4 Pratt, S. J. P. & Lovering, R. M. A stepwise procedure to test contractility and susceptibility to injury for the rodent quadriceps muscle. *J Biol Methods.* **1** (2), doi:10.14440/jbm.2014.34, (2014).

Supplementary Figure 1

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