# In vivo Measurement of Knee Extensor Muscle Function in Mice

**Camille R. Brightwell**[1,2], **Ted G. Graber**[3], **Benjamin D. Brightwell**[4,5], **Matthew Borkowski**[6], **Brian Noehren**[5,7], **Christopher S. Fry**[1,2]

[1]Department of Athletic Training and Clinical Nutrition, University of Kentucky

[2]Center for Muscle Biology, University of Kentucky

[3]Department of Physical Therapy, East Carolina University

[4]Kinesiology and Health Promotion Graduate Program, University of Kentucky

[5]Biomotion Lab, College of Health Sciences, University of Kentucky

[6]Aurora Scientific

[7]Department of Physical Therapy, College of Health Sciences, University of Kentucky

## 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 are 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[1,2,3,4,5,6,7]. 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[9,10,11,12,13] 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.** Confirm that machines are connected per manufacturer specifications.

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

    **3.** Turn on the water pump to 37 °C to begin heating the platform.

    **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.

    **5.** Pour isoflurane into vaporizer to maximum fill line.

### 2. Software setup

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

    **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.

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

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).

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

    2. Select **Next Experiment** or **Previous Experiment** to transition from twitch protocol to torque-frequency sequence.

### 3. Mouse setup

1. Place individual mouse into the anesthetic chamber.

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

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.

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.

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

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

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

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.

8. Adjust knobs on platform to ensure the knee is bent at 60°.

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

### 4. Electrode placement

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.

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.

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**

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.

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

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.

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**

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).

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**

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.

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

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

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**

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

   1. Open analysis software.

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

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

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

5. Select **Muscle Analysis**.

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.

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.

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.

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

   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).

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

   1. Repeat steps 8.1.1–8.1.4 above.

   2. Select **Export Data**.

   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**

   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.

      1. Open data collection software.

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

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

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

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).

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

    2. Click **Read**.

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

    4. Repeat for the next voltage.

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

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

    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.

    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.

    3. Select **Read**.

    4. Repeat for at least 3 known weights.

    5. Select **Calculate Cal Factor**.

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

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

    1. Click Set directly next to the calibration voltage.

    2. Repeat for each voltage line.

    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.

    4. Maintain this position. Select **Read**.

    5. Repeat for each voltage line.

    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).

## 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[21,22,23].

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[24,25,26,27]. Moreover, knee extensor pathology is observed with aging as well as a myriad of injuries and diseases[1,2,4,5,6], 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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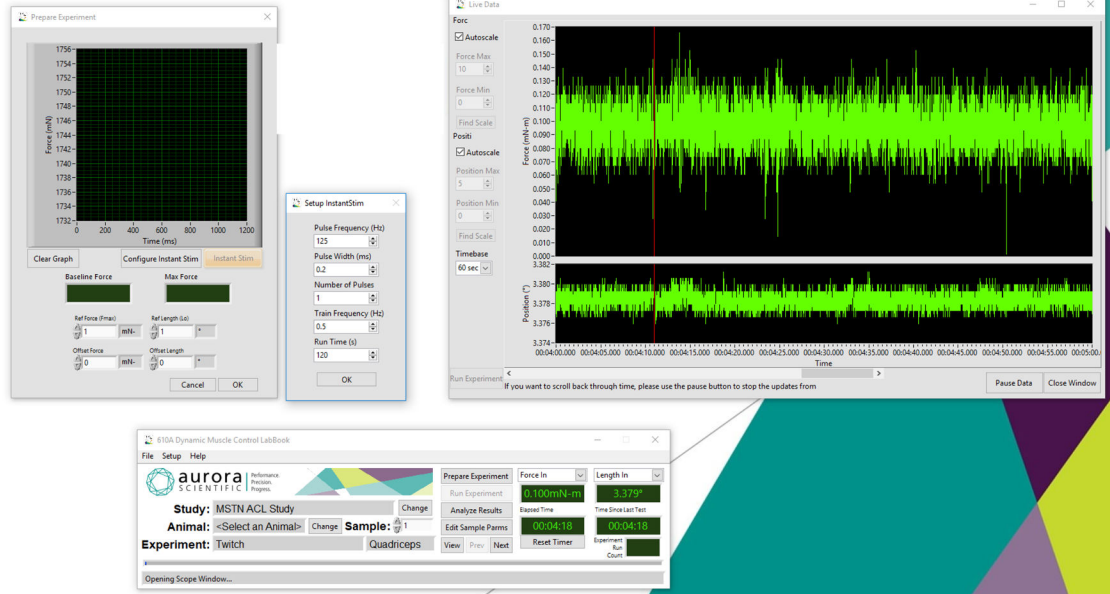
**Figure 1: Data collection software setup.**
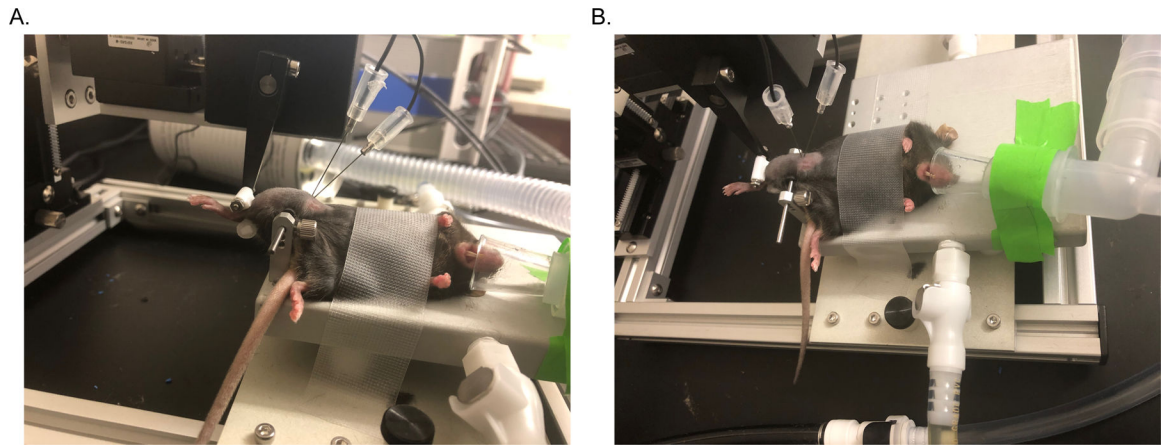Illustration of setup for data collection software with Live Data Monitor.

**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**).
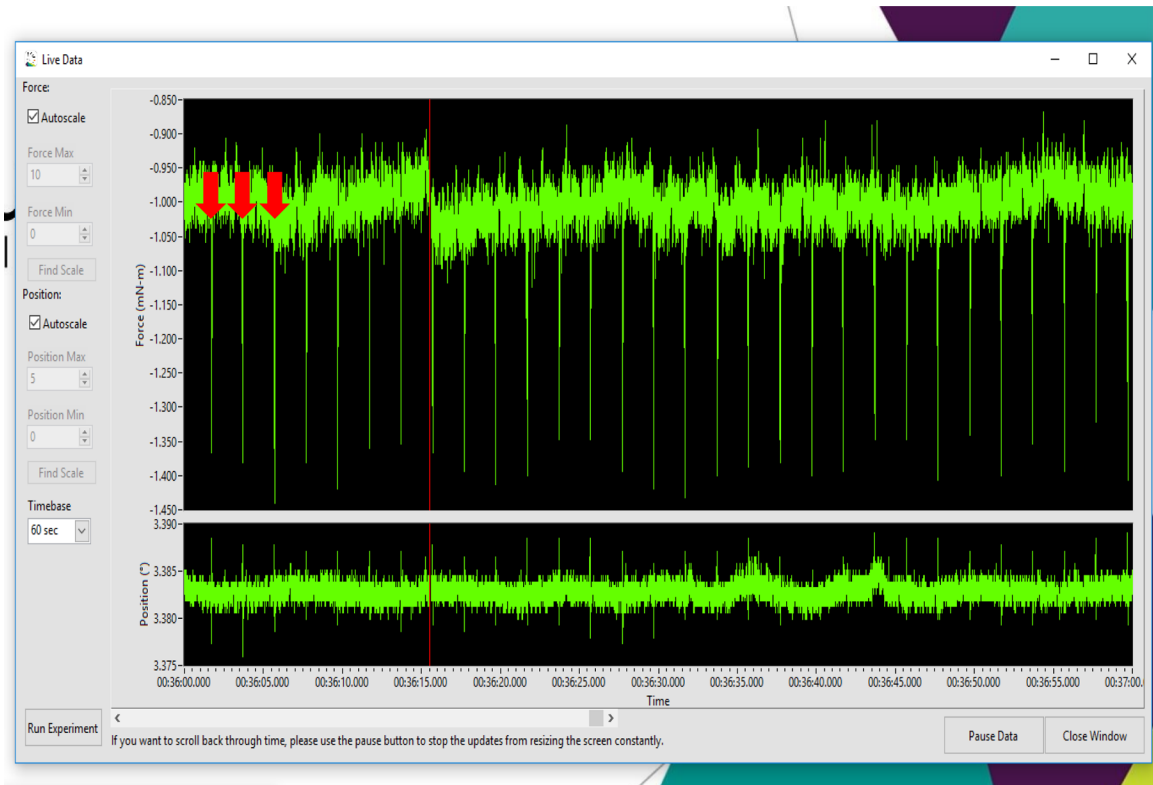
**Figure 3: Determination of optimal electrode placement to achieve isometric knee extension.** Representation of repeated negative twitches stimulated with 50 mA using the Instant Stimulation function and viewed in the Live Data Monitor. Red arrows indicate the first three knee extension twitches.
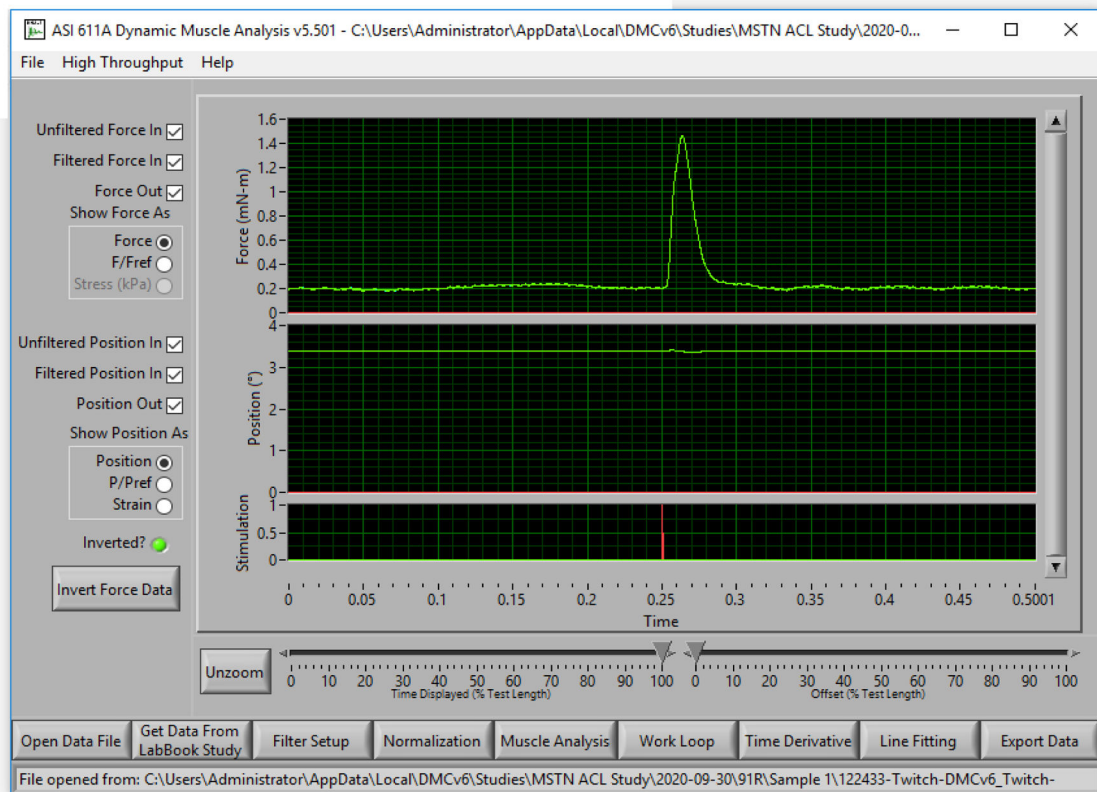
**Figure 4: Representative twitch to determine optimal amperage.**
The lowest amperage to elicit the highest twitch isometric torque must be determined for the
force-frequency experiment by repeated twitch experiments with progressively increased
amperage.
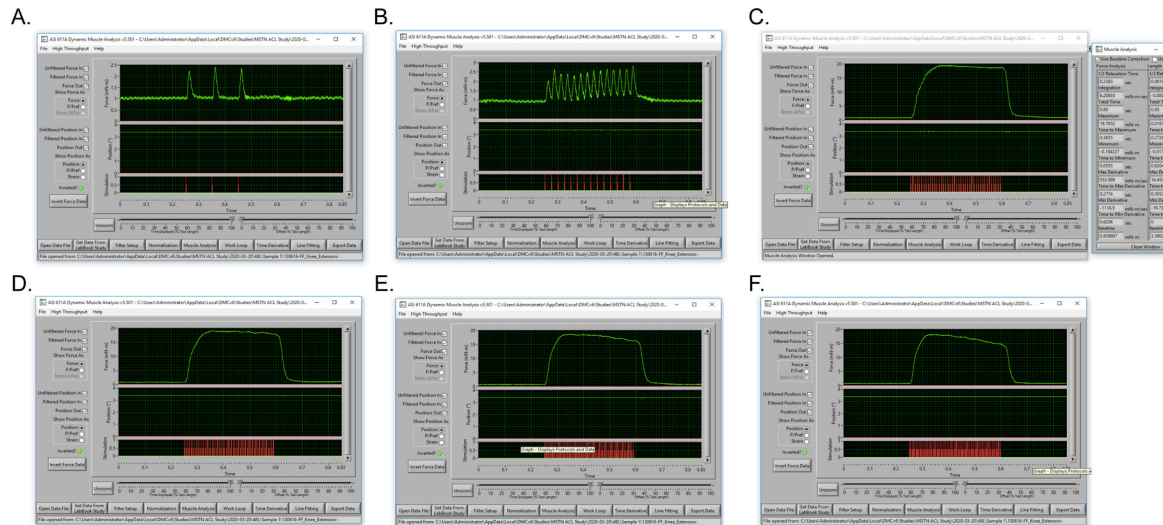
**Figure 5: Representative tetanic torque curves throughout a torque-frequency experiment for the same mouse.**

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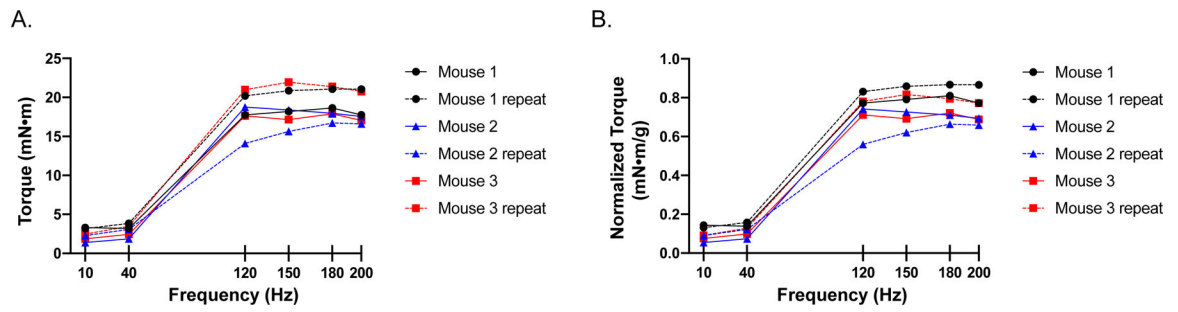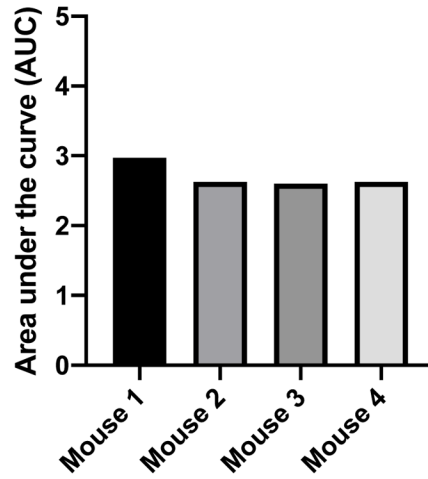
**Figure 6: Representative torque-frequency curve data.**
(**A-B**). Torque-frequency curve at 2 different timepoints (week 1 and 3) in 3 separate mice, presented as raw peak torque (**A**) and raw peak torque normalized to body weight (**B**).
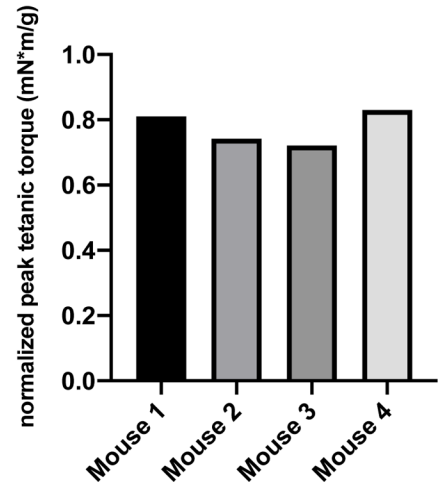
A.

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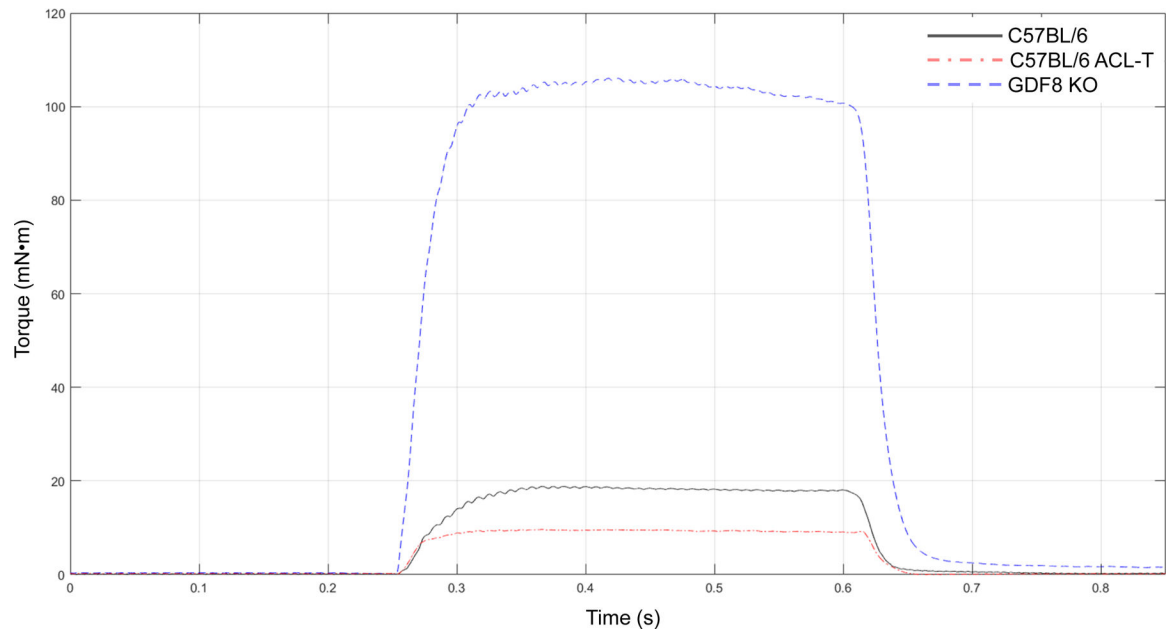
**Figure 8: Peak tetanic torque of knee extensors in multiple mouse models.**
Representative peak torque tetanus curves for an overt hypertrophy transgenic mouse model (GDF8 KO), an uninjured healthy C57BL/6 mouse (mouse 2), and a C57BL/6 mouse 7 days after anterior cruciate ligament transection (ACL-T).

**Table 1:**

Example of twitch series.

| 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 |

*
denotes optimal amperage/current.

**Table 2:**

Example of torque-frequency curve data.

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

*denotes peak tetanic torque.

Table of Material

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 1300A: 3-in-1 Whole Animal System-Mouse | Aurora Scientific Incorporated | 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 |
| 6100 Dynamic Muscle Control LabBook software | Aurora Scientific Incorporated | DMC v6.000 |
| 611A Dynamic Muscle Analysis | Aurora Scientific Incorporated | DMA v5.501 |
| BravMini hair clippers | Wahl Clipper Corporation | ASIN: B00IN24ILE |
| Eye Lube | Optixcare | Item Number: 142422 |
| Isoflurane | Covetrus | NDC: 11695-6777-2 |
| V-1 Tabletop Laboratory Animal Anesthesia System | VetEquip Inhalation Anesthesia Systems | Item Number: 901806 |
| Prism 8 | GraphPad Software, LLC | Version 8.3.0 (328) |