Assessing Mouse Muscle Function *In Vivo*, *In Situ*, and *In Vitro*

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The Author(s) of the SOP	Luke D. Flewwelling, Madison Garibotti, Dr. Arthur J. Cheng York University, Toronto, Ontario
Working Group Members	Jaryeon Lee
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1 Objective

The objective of this SOP is to demonstrate the ways to measure muscle function, force, and fatigue *in vivo*, *in situ*, and *in vitro*. More specifically, *in vivo* refers to non-invasive experiments that are performed on anesthetized living animals while all muscles are intact. *In situ* refers to invasive experiments that are performed on muscles that have been surgically isolated from the anesthetized animal (one tendon of the muscle is still attached to the animal, and the other side of the muscle is attached to a force transducer to measure force). *In vitro* refers to experiments done on muscles that have been completely removed from the animal. These techniques can allow for measures of muscle force, fatigue, strength, muscle damage, and other measures of muscle function across various muscles. Each of these three techniques allows for various levels of physiological relevance and isolation of specific muscles. Learning these techniques is greatly beneficial for individuals studying muscle function across various diseases, conditions, or other experimental models in animals. While these techniques are widely used in a variety of different muscles and animal models, how individuals perform these measurements can vary. This SOP provides standardized ways to measure basic muscle function assessments in mice. However, this can be expanded upon and specified depending on the needs of the experiments.

2 Applicability/Scope

In vivo muscle function can be measured whereby the animal is anesthetized and unconscious, their limb (typically the foot) placed in a footplate, and their muscles (typically the plantar flexors as they are easy to learn and applicable to disease and training studies) are electrically simulated at various frequencies and intensities. This can allow for measures of muscle force, fatigue, strength, muscle damage, and other measures of muscle function. *In vivo,* muscle function assessments are useful as the animal is still alive, and all muscles have an intact blood supply, allowing for physiologically relevant measures of muscle function. This technique can be used in chronic studies (e.g., training, disease progression, etc.) where muscle function can be tracked over a prolonged period due to its non-terminal nature. In addition, this method is very easy to learn and yields high applicability to research.

Studying muscle function *in situ* can be performed in various muscles, including, but not limited to, the soleus, tibialis anterior, and quadriceps muscles. The distal tendon of the muscle is isolated from the mouse and attached by a suture to a force transducer, while the proximal tendon is kept attached to its insertion point. Isolating the muscle of interest from the body while maintaining blood supply controls for possible confounding factors, such as the activity of different muscles or changes in limb angle, which may overshadow the role of the desired muscle. The target muscle is then electrically stimulated with needle electrodes at various frequencies and intensities, which allows for various muscle function assessments. Unlike the *in vivo* method in assessing muscular function, this procedure is terminal, limiting the assessment to one time-point

per animal. *In situ* muscle function assessments are beneficial for examining muscle weakness and fatigue at the endpoint of a disease.

Muscle function *in vitro* can be performed in many muscles, including, but not limited to, the extensor digitorum longus, soleus, and diaphragm. This measure requires the desired muscle to be dissected out of the animal after it has been euthanized. Once the muscles are removed from the animal, they are placed in an oxygenated buffer (Tyrode) to be kept alive and provide an environment that mimics the intracellular environment of the muscle cells. The tendons of the muscles are sutured into two loops, one is attached to a movement arm (attached to a force transducer) and the other to a stationary hook. The muscle is then electrically stimulated at various frequencies within the Tyrode bath by the two tungsten electrodes on each side of the muscle. Studying muscle activity in vitro is useful as this technique can be used to examine the effects of varying environmental influences, such as changing the temperature, O_2 content, or pH of the Tyrode bath, or adding a desired drug or supplement to the bath.

3 Summary of the Procedure

Muscle Force Measurements

3.1 In-Vivo

In Vivo, force production is partially adapted from previous methods (Yamada et al., 2021). Mice are anesthetized using isoflurane inhalation, then moved to a nose cone and placed on a platform maintained at 37° C. The toe pinch reflex is performed to ensure the depth of the anesthetic. If the animal is in distress, the isoflurane flow/concentration is adjusted, and if required, the animal is returned to their cage to be monitored as the mouse recovers. Following hair removal from their legs (e.g., with Nair or shaving), one foot is secured to a footplate connected to a torque sensor (Aurora Scientific 3 in 1 system, model number: 15A-300LRFP, Ontario, Canada). Transcutaneous electrical stimulation via a simulator (Aurora Scientific Integrated Muscle Test Controller) can be employed by placing surface electrodes on the anterior and posterior side of one lower leg, or with bar electrodes on the bottom of the lower leg, or with needles placed into the muscle belly of the target muscle. Optimal length (L_o) is determined using single twitches (pulse width = 0.2ms) at varying muscle lengths. Once L_o is established, force as a function of stimulation frequency is measured during isometric contractions at varying stimulation frequencies. Thereafter, a force-velocity protocol can be performed, which involves stimulating the muscle to produce force during different velocities of contraction. Fatigue can be induced with electrical stimulation involving repeated maximal or submaximal contractions.

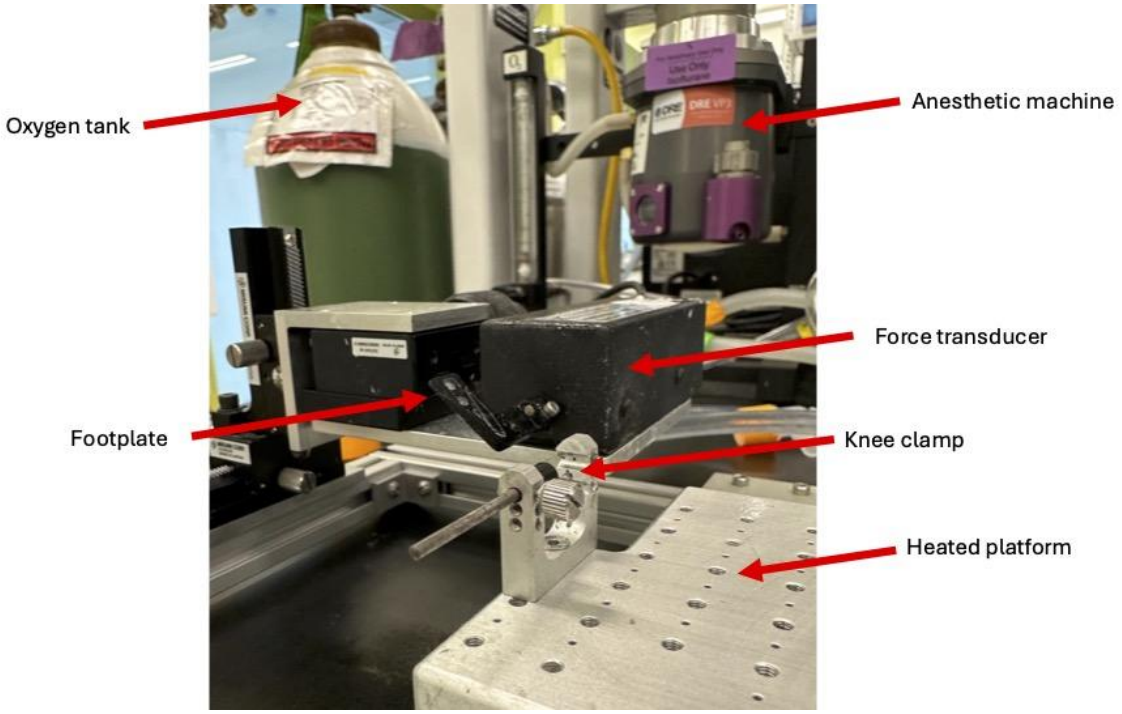

Figure 1. Example of *in vivo* system setup.

3.2 In-Situ

In situ, force production of the quadricep muscle is partially adapted from previous methods (Cohn and Campbell, 2000). Mice are anesthetized with isoflurane and placed on a platform at 37° C to maintain body core temperature. The toe pinch reflex is performed to ensure the depth of the anesthetic. There is also a need to continuously monitor the breathing frequency throughout the procedure. If the animal is under distress, the isoflurane flow/concentration is adjusted, and if required, the animal is euthanized if incisions have already been made or returned to their cage for monitoring if no incision has been made. After hair removal (ex., with Nair/shaving) from the area where the leg muscle of interest is located, an incision is made to expose the muscle. In the case of assessing the quadricep muscle, an incision is made above the patella to expose the femoral tendon. In the case of assessing the tibialis anterior, an incision is made, exposing the tendon of the tibialis anterior at the front of the leg just above the ankle. The tibialis anterior tendon is then secured with a suture and severed. In the case of assessing lower leg muscles, isolate the gastrocnemius and soleus by removing tissue just above the muscle. Once isolated, secure the gastrocnemius tendon with a suture just above the heel, sever the tendon by cutting through the bottom of the foot, leaving some bone attached. Once the knot is in place, the suture is attached to an Aurora Scientific 305C muscle lever arm with an S hook (Aurora

Scientific 3 in 1 system, model number: 15A-300LRFP). The knee is secured with a vertical knee clamp, immobilizing the knee joint with a 27 G needle. Contraction of the muscle is controlled with a stimulator (Aurora Scientific Integrated Muscle Test Controller) and through percutaneous stimulation of the nerve. Optimal resting length (L_o) can be determined using single twitches (pulse width = 0.2ms) at varying muscle lengths. Once L_o is established, force as a function of stimulation frequency is measured during isometric contractions at varying stimulation frequencies. Following the force-frequency protocol, a recovery period is provided, followed by a maximum torque test, which is performed at the frequency at which maximum force is achieved during the force-frequency curve. Thereafter, a force-velocity protocol can be performed, which involves stimulating the muscle to produce force during different velocities of contraction. Fatigue can be induced with electrical stimulation involving repeated maximal or submaximal contractions. Experiments involving the gastrocnemius or soleus muscle follow similar procedures to the above, except that the Achilles tendon is exposed, tightly secured with suture, severed and then attached to the muscle lever arm.

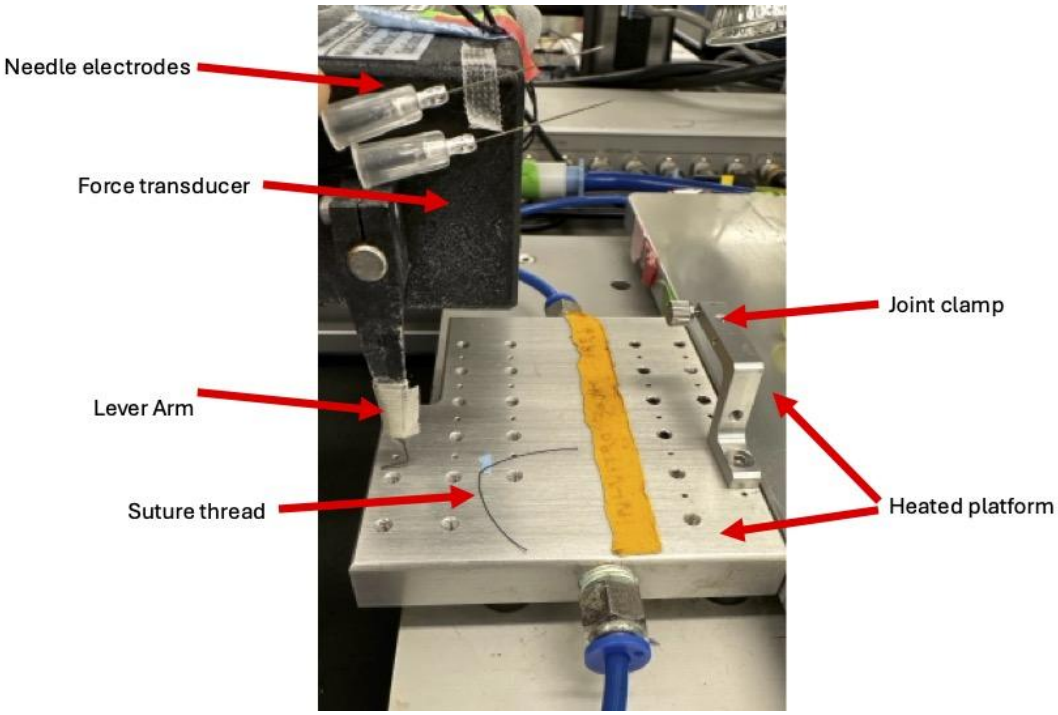

Figure 2. Example of *in situ* system setup.

3.3 In-Vitro

In vitro force production is partially adapted from previous methods (Delfinis et al., 2022) and techniques used in our lab. Upon euthanization of the animal, the muscle of interest is isolated and attached to a muscle lever arm with an S hook (Aurora Scientific 3 in 1 system, model number: 15A-300LRFP). Contraction of the muscle is controlled with a stimulator (Aurora Scientific Integrated Muscle Test Controller). Optimal resting length (L_o) is determined using single twitches (pulse width = 0.2ms) at varying muscle lengths. Once L_o is established, force as a function of stimulation frequency is measured during isometric contractions at varying stimulation frequencies. Following the force-frequency protocol, a recovery period is provided, followed by a maximum torque test, which is performed at the frequency by which maximum force is achieved during the force-frequency curve. Thereafter, a force-velocity protocol can be performed, which involves stimulating the muscle to produce force during different velocities of contraction. Fatigue can be induced with electrical stimulation involving repeated maximal or submaximal contractions.

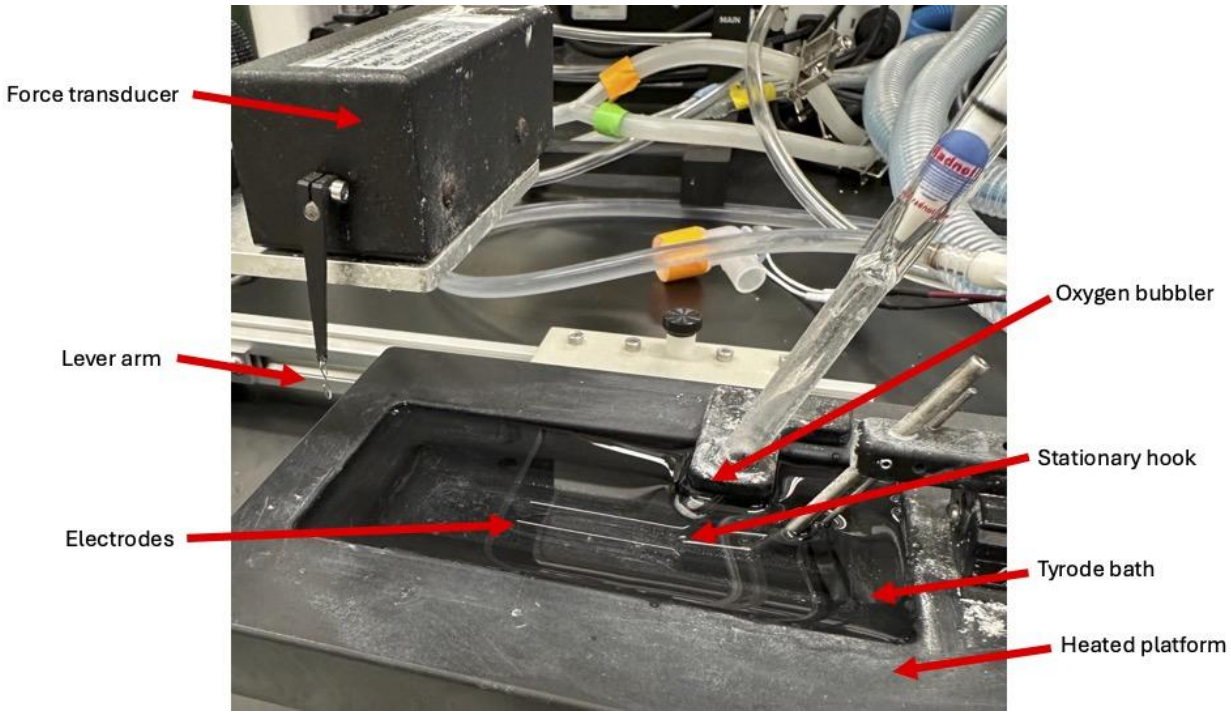

Figure 3. Example of *in vitro* system setup.

4 Definitions

- *in vivo* (whole animal)

- *in situ* (isolated muscle still attached to an animal),
- *in vitro* (isolated muscle)
- L_o (Optimal resting length)

5 Materials and Equipment Required

5.1 Equipment

- Aurora Scientific 3 in 1 system
- Aurora Scientific Integrated muscle test controller
- Computer
- Anesthetic Machine
    - Associated tubing/nose cone
    - Anesthesia induction box
- Microscope (if needed for in vitro dissection)
- Dissection tools
    - Small spring scissors
    - Forceps

5.2 Consumables

- Oxygen cylinder (95-99.5% O^2)
- Nair
- Ultrasound gel
- Veterinary eye drops
- Electrodes
    - Pads or needle
- 4.0 Suture thread
- Isoflurane
- Tyrode solution:
    - 121mM NaCl, 5.0mM KCl, 1.8mM $CaCl_2$, 0.5mM $MgCl_2$, 0.4mM NaH_2PO_4, 24mM $NaHCO_3$, 0.1mM EDTA, and 5.5mM glucose

6 Step-by-Step Procedure

6.1 Hair removal (If needed) - (Day(s) Before the Experiments)

1. Take a mouse from the cage and place it in the anesthesia box under ~2% isoflurane

2. Once the mouse is anesthetized, transfer the mice (prone) to a nose cone on a heated pad/plate with a paper towel over the top (easier to clean)
3. Add veterinary eye drops to eyes and ensure breathing is stable
4. Rotate the mouse to supine while keeping the nose in the nose cone to maintain the level of anesthesia
5. Shave the leg, or generously apply 'Nair' or other hair removal product to the lower leg(s) and ensure it penetrates deep enough by using a Q-Tip or gloved finger against the direction of the hair
6. After ~7 min (or what is recommended on the hair removal product), wipe product and hair off the leg with a paper towel
    a. This can be done while the mouse is still under anesthetic or can be done quickly while holding the mouse (this will take some practice)
7. If the hair does not come off completely or is patchy, leave the product on for a bit longer
    a. Ensure you remove as much 'Nair' as possible from the leg(s), as it can cause skin irritation and or damage if left on too long
8. Return the mouse back to its cage, and if required, re-Nair the following day
    a. If this is a continuous protocol, you will need to re-Nair the leg(s) every week or two to ensure the hair does not grow back

6.2 *In-Vivo* Experimental Setup (Planter Flexors):

1. Take a mouse from the cage and place it in the anesthesia box under ~2% isoflurane
2. Once the mouse is anesthetized, transfer the mice (prone) to a nose cone on a heated pad/plate
3. Add veterinary eye drops to eyes and ensure breathing is stable
4. Rotate the mouse to supine while keeping the nose in the nose cone to maintain a level of anesthetize
5. Clamp the knee at ~90° and place the foot onto the footplate and tape the foot for security.
    a. The easiest way to do this is to first clamp the knee with the footplate out of the way and then move the footplate closer to the foot while GENTLY holding/pulling the foot slightly back with forceps until the heel of the mouse is touching the footplate
    b. The foot should rest at the bottom of the footplate
6. Add tape over the body where it is securing the animal, but not to the point that it is limiting breathing
7. If using surface electrodes, set the voltage at ~45V to get supramaximal levels of contraction
8. Placing the electrodes:

a. Liberally add ultrasound gel to the desired area of the skin and electrodes. Ensure surface electrodes are cut to the desired size
    i. A sufficient amount of gel is easily visible and will disperse once electrodes are placed and pushed to the limb. The more gel, the better.
b. When placing the electrodes on the surface of the skin, it is best to have the two electrodes taped together on top with a gap between them so they can sit on either side of the muscle
c. Once the electrodes are sitting on the desired location of the muscle, use a long, thin piece of tape going around the electrodes and the leg so the electrodes remain in place.

Figure 4. Image of *in vivo* muscle function measurement on the planter flexors with surface electrodes.

9. Optimize Resting Length
    a. To set optimal length, use twitches (pulse width = 0.2 ms) at varying muscle lengths with at least 10 seconds of rest between twitches
        i. This could also include adjusting the joint angle (vertical and horizontal). However, ~90° for both is generally close to the optimal joint angle
    b. Increase/decrease length until maximal twitch force is achieved and wait about 30 seconds between twitches to avoid twitch potentiation.
    c. On average, ~10-week-old female mice have ~5-20 mN baseline tension for reference. This can change with age, sex, breed, disease, etc..

6.3 *In-Situ* Experimental Setup:

1. Take a mouse from the cage and place it in the anesthesia box under ~2% isoflurane
2. Once the mouse is anesthetized, transfer the mice (prone) to a nose cone on a heated pad/plate
3. Rotate the mouse to supine (if needed to access the muscle) while keeping the nose in the nose cone to maintain the level of anesthetise

4. Once the muscle (e.g., TA) has been sutured (4.0 surgical silk) at the distal tendon and tied to the force transducer, insert needle electrodes in the fascia underneath the muscle (see photos below)
    a. Do not insert needles directly into the muscle belly.

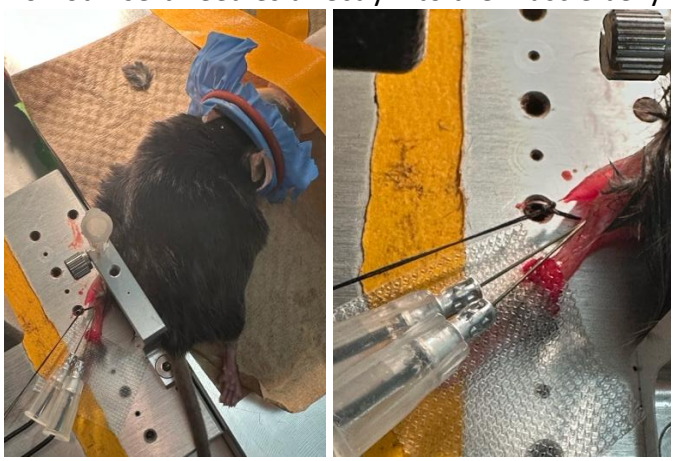

Figure 5. Image of *in situ* muscle function measurement on the tibialis anterior with needle electrodes.

5. Optimize Current
    a. Range:10-100 mA (if you can get the right placement, this can go to 10 mA, but if you struggle, it can increase to 100mA). If you place the electrodes well, you can stimulate the sciatic nerve with very little current (20% of 10mA).
    b. Run instant stim protocol for single twitch
    c. Adjust the stimulation intensity to ensure all muscle fibres are being recruited – obtain three twitches in a row where force does not increase (run instant stim and increase current between twitches (wait about 30 seconds in between twitches)
    d. Set supramaximal current by increasing current by 15%
6. Optimize Resting Length
    a. Increase/decrease length until maximal twitch force is achieved and wait about 30 seconds between twitches to avoid twitch potentiation.
    b. On average, ~10-week-old female mice have ~10-50 mN baseline tension for reference. This can change with age, sex, breed, and disease.

6.4 *In-Vitro* Experimental Setup:

1. Ensure your bath is filled with Tyrode solution and oxygenated for at least 30 minutes with an attached 95% O_2/ 5% CO_2 tank, with the water circulator turned on and maintained at 25°C before the start of surgery.

   a. This ensures the bath and muscle have enough oxygen since we rely on oxygen diffusion into the muscle and not perfusion through the blood supply. 5% CO_2 provides the necessary buffering of pH in the Tyrode solution.
2. Dissect out desired muscle with tendon lengths as long as possible and tie suture thread (4.0 surgical silk) loops onto each end of the tendon
   a. Specific for diaphragm
      i. Limit muscle (diaphragm) strip width to 2mm (maximum 4mm) as wider strips have difficulties with diffusion limitations of O_2.
      ii. 25°C is used over 37°C as O_2 has poor solubility at higher temperatures.
3. Attach both ends of the suture thread loops onto the force transducer and the stationary hook as below:

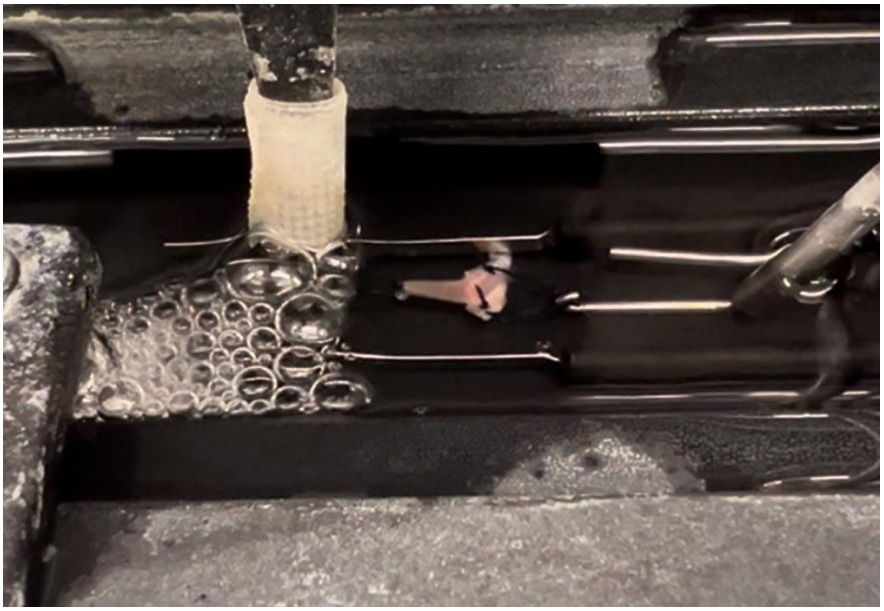

Figure 6. Image of *in vitro* muscle function measurement on an isolated strip of the diaphragm.

4. Let muscle acclimatize for 20-30 min with some tension applied (resting tension will decrease during this acclimation)
   a. This will allow resting tension in the muscle to relax and will reduce background tension; give it a tetanus stimulus to fully relax the fibres before optimizing length for full data collection.
5. Optimize the current
   a. Range: 1A (50-100%) (in vitro force stimulation requires a much higher amperage or voltage than in-situ or in-vivo force stimulation since this is a field stimulation)
   b. Run Instant Stim and check force development

c. Increase stimulation intensity to ensure all fibres are recruited- if you see an increase in force development, then more fibres have been recruited, and you are not at a supramaximal current. Use three twitches in a row with increasing current (30s rest between) where force does not increase.
d. Set supramaximal current by increasing current by 15% (this will ensure you have all fibres being activated in the muscle strip).

6. Optimize the length (necessary to make sure the maximal amount of cross-bridges are forming)
    a. Gradually increase the length of the muscle and apply instant stim until you obtain maximum force. Provide 30s of rest to avoid fatigue.
        i. Be careful of drastic changes in length this will increase basal tension of the muscle. Use ¼ turns of the fine adjustment knob and use gross adjustment knob only when necessary.
    b. Allow some time (approx. 5min) for the muscle to rest before starting the force-frequency curve

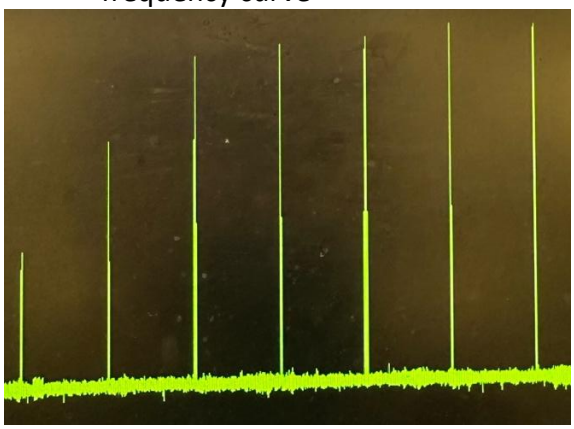

Figure 7. Example of what optimizing current/length looks like.

7. Measure the muscle length before and after the stimulations, as you will need that to normalize the data

6.5 Force-Frequency:

1. After determining optimal muscle length, it is typical to measure the Force-Frequency relationship.
    a. Typically, this looks like 10 isometric contractions at varying stimulation frequencies (10, 20, 30, 40, 50, 60, 80, 100, 150, and 200 Hz) with 1-2 minutes rest in between each contraction

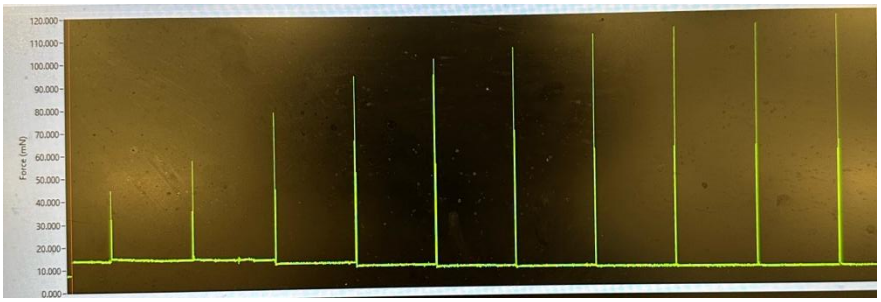

Figure 8. Example of force-frequency graph

6.6 Fatigue Test:

1. Let the mouse/muscle rest for 5 minutes after the Force-Frequency measures, it is typical to do a measurement of fatigue
    a. Fatigue is typically tested with 100-360 repeated contractions every second (70Hz, 0.2 ms pulse width, 0.3 ms duration).

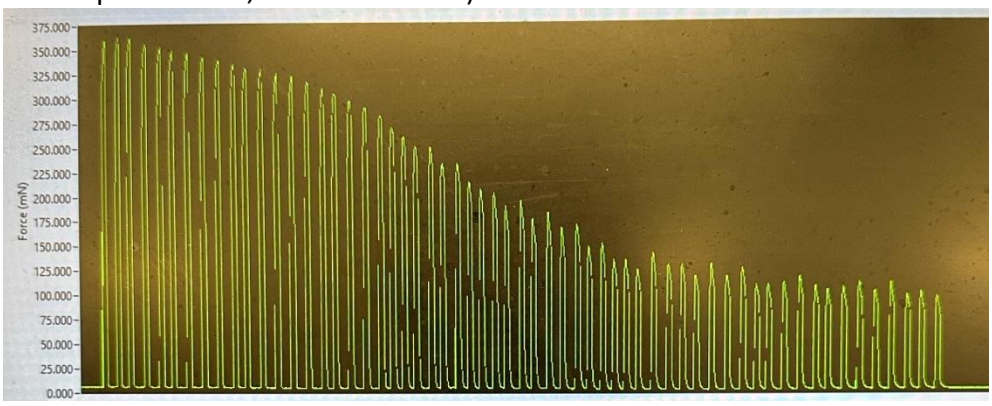

Figure 9. Example of fatigue graph.

6.7 Force Normalization:

1. Fatigue can be normalized as the % difference from the first contraction
    > In-Vivo
        Force production can be normalized to the body weight of the animal
    > In-Vitro/In-Situ
        Force production can be normalized ("specific force") to the calculated CSA of the muscle strip (m/l×d, where m is the muscle mass, l is the length, and d is mammalian skeletal muscle density: 1.06 mg/mm^3).

7 Quality Assurance and Quality Control

These procedures take time to learn and optimize, and it is recommended that 6+ animals be included per group/condition. We also recommend using both legs/muscles when learning to ensure similar force production between both legs/muscles to ensure proper optimization occurs (there should be similar force production between each side). During experiments, you can use the animal's non-stimulated leg as a control, or, depending on the experiments (disease, genetically modified, drug group, saline group, etc.), you can also use a different group of mice as a control group.

8 Safety Precautions

- All personnel performing activities with this SOP must have basic training in laboratory safety procedures and wear appropriate PPE.
- All personnel performing activities with this SOP must have basic animal handling training and be comfortable euthanizing and dissecting tissues.
- An active or passive scavenging system should be connected to the anesthetic system to minimize exposure to isoflurane.

9 References

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10 Examples & Tips

- For *in vivo* force production, if the baseline value is negative, it may be due to where the foot is taped. If the tape is too low on the foot, it can result in the toes being lifted off the footplate and, therefore, increasing the force on the heel of the foot. It is recommended to tape from

the bottom to the top of the foot while still leaving the nails exposed to ensure visual confirmation that the toes are contracting when stimulating the plantar flexors muscles.

- Be sure to double knot any suture ties as they can slip during contractions.
- Some muscles are very sensitive to oxygen (eg: soleus) and should be dissected out as quickly as possible and then rested in oxygenated Tyrode for longer than most muscles (~1hr).
    - Some muscles (eg: soleus) may also have better force production when the animal is euthanized without isoflurane and therefore, other methods of euthanizing may be better for some muscles.
- Blinding the scientist performing force measurements on mice treatments/models is good practice, but this may not always be feasible given the clear physical phenotypes in some experimental design.