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Single muscle contractile measurements *in vivo* and *in situ*

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1. OBJECTIVE

The objective of this SOP is to provide a simple yet detailed method to test mouse skeletal muscle function *in situ* or *in vivo*. The procedures can be performed on the anterior crural muscles (ankle flexors or dorsiflexors), the posterior crural muscles (ankle extensors or plantarflexors) or the anterior thigh muscles (knee extensors or quadriceps femoris)[1-4]. For the sake of simplicity, we focus here only on the dorsiflexor muscles.

The *in situ* method provides contractile measurements of a single muscle (tibialis anterior) and is a terminal experiment. The *in vivo* method provides contractile measurements of a muscle group (dorsiflexors) and is not a terminal procedure, allowing measurements over time within one animal. The *in vivo* method also allows one to perform maximal lengthening (eccentric) contractions to assess susceptibility to injury.

2. SCOPE AND APPLICABILITY

Biological markers (e.g., serum creatine kinase levels) are typically elevated with muscle disease, but biological markers and histological findings do not always correlate with the loss of force production. Thus, the most comprehensive measure of the overall health of a muscle is likely contractile force. Here, we describe an *in vivo* animal model to measure torque and to produce a reliable muscle injury. With this approach, the extent of the torque loss immediately and the recovery of torque hours, days or weeks following the injury can be assessed. Note, torque is the application of a force at some distance from and perpendicular to an axis of rotation. We also describe our model for measurement of force from an isolated muscle *in situ*.

3. CAUTIONS

Some general cautions include:

- One should have a basic knowledge of rodent hindlimb anatomy and muscle physiology
- Excellent stabilization of the hindlimb, without impingement on the muscle being tested, is critical to accurate contractile measurements
- Animal positioning and electrode placement are just as important as settings used on the stimulator
- The body temperature of the animal should be maintained as temperature can affect muscle force output

4. MATERIALS

Some basic tools include:

- A method of anesthetizing animal (e.g., IP Injection or vaporized gas)
- A muscle stimulator and electrodes
- A load cell (see protocol)
- A method to stabilize the knee (e.g., a transosseous pin/27G needle and a tower to hold the pin/needle)
- Dissection tools

Specific commercial instruments are described below in the methods. A few of the specific tools we use are custom designed (e.g., footplate) and are also described, but can now be purchased from companies that sell equipment for muscle physiology.

5. METHODS

5.1 *In vivo* measurement of isometric torque

1. These procedures can be used for rats or mice. To begin, place the animal supine under inhalation anesthesia (~ 4-5% isoflurane for induction in an induction chamber, then ~ 2% isoflurane via a nosecone for maintenance, both with 1-1.5L/min 100% O_2) using a precision vaporizer (cat # 91103, Vet Equip, Inc., Pleasanton, CA). Be sure to keep the animal warm by use of a heat lamp or some other method (e.g.,a heated platform on which the animal rests (e.g., Model 809B, Aurora Scientific, Inc., Aurora, Ont. Canada).

2. Prep the skin by removing hair and by cleaning with alternating scrubs of betadine and 70% alcohol to prevent seeding skin bacteria into the soft tissue or bone. Confirm proper anesthesia by lack of a deep tendon reflex (no foot withdrawal in response to pinching the foot). A needle (27G) is manually placed through the proximal tibia to stabilize the limb on the rig. The needle should not enter the anterior compartment of the leg.

3. Lock the needle into a fixed position, such that the animal is supine and the toes are facing straight up. A custom-made device is used to secure the needle and thereby stabilize the leg. An alternative, if using a 1300A or 1305A system from Aurora Scientific, Inc. (see 4. below), is to secure the leg in a U shaped holder. A 25G needle is placed through one side of the holder so that ~1-2 mm is pushed through. The needle is secured with a set screw. The lateral tibia is pushed onto the needle, but not all the way through the tibia, and secured in place with a brace that comes from the other side of the U-shaped holder which is also held with a set screw.

4. Place the foot of the limb onto a custom-machined footplate (see figure in appendix). The axis of the footplate is attached to a stepper motor (Model T8904, NMB Technologies, Chatsworth, CA) and a torque sensor (model QWFK-8M, Sensotec, Columbus, OH). The foot should initially be aligned so that it is orthogonal (at 90^{o}) to the tibia.

Alternatively, the Whole Muscle Test System, Model 1300A (mouse) or Model 1305A (rat), Aurora Scientific, Inc., Aurora, Ont. Canada can be used.

5. Use transcutaneous electrodes (723742, Harvard Apparatus, Cambridge, MA) or subcutaneous electrodes (J05 Needle Electrode Needles, 36BTP, Jari Electrode Supply, Gilroy, CA) to stimulate the fibular nerve near the neck of the fibula, where the nerve lies in a superficial position. Visually confirm isolated dorsiflexion by performing a series of twitches (0.1 ms rectangular unipolar pulse for the mouse and 1 ms rectangular unipolar pulse for the rat) before the foot is secured. Once the foot is secured to the footplate with adhesive tape, an increase in twitch amplitude in response to an increase in voltage confirms that opposing muscles (plantarflexors) are not being simultaneously stimulated.

6. The maximal force producing capacity of the dorsiflexors is recorded as the "maximal isometric torque". If lengthening contractions are used to induce injury, torque measurements are performed before and after injury on the same rig that is used to induce injury. Before recording maximal isometric torque, the pulse amplitude is adjusted to optimize twitch tension and the optimal position of the ankle is determined by giving twitches at different lengths of the dorsiflexors. After obtaining a torque-angle curve to determine the optimal length of the dorsiflexors (resting length, aka L_o, typically ~20^0 of plantarflexion from neutral), a torque frequency plot is obtained by progressively increasing the frequency of pulses during a 200 ms pulse train. A maximal fused tetanic contraction is obtained usually at 90-100 Hz. Three separate twitches and tetanic contractions are recorded and saved for further analysis.

7. When the experiment is completed, the stimulating electrodes are removed, the transosseous pin is removed and a small amount of betadine is applied to the exposed skin and the knee area of the tested leg. The animal should then placed in a warmed recovery chamber with 1L/min 100% O_2. When the animal can ambulate in the chamber normally, it can be returned to its cage.

5.2 *In situ* measurement of whole muscle tension

1. The animal is prepared and the tibia is stabilized as described above. All instrumentation is turned on at least 30 min prior to testing for proper calibration and to minimize thermal drift of the force transducer.

2. Incise the skin anterior to the ankle and sever the tendon of the tibialis anterior (TA) muscle. Carefully tie 4.0 Ethicon silk non-absorbable suture to the tendon and attach the suture to the load cell via the provided S-hook (weight = 0.1 g, model FT03, Grass Instruments, Warwick, RI). Alternatively, a custom clamp (weight = 0.5 g) can be used to attach the tendon to the suture.

3. The load cell is mounted to a micromanipulator (Kite Manipulator, World Precision Instruments Inc, Sarasota, FL) so that the TA can be adjusted to resting length and aligned properly (a straight line of pull between the origin and insertion). The TA is protected from cooling by a heat lamp and from dehydration by mineral oil. If the suture slips off because of the mineral oil, warmed PBS (phosphate buffered saline) can be used. Again, Models 1300 and 1305

4. Attach the TA to the load cell and apply single twitches (rectangular unipolar pulse of 1 ms) at different muscle lengths to determine L_o. Muscle resting length, measured using calipers, is defined as the distance between the tibial tuberosity and the myotendinous junction (can be adjusted by measuring length or setting passive tension ~ 10 g from TA). At this length, gradually increase the pulse amplitude and then the pulse frequency to establish a force-frequency relationship. A maximally fused tetanic contraction is obtained at approximately 90-100Hz (300 ms train duration comprised of 0.1 ms or 1 ms rectangular pulses). Use 150% of the maximum stimulation intensity to activate the TA to induce maximal contractile activation (P_o). Maximal tetanic contractions can be performed repeatedly and expressed as percentage of P_o, providing an index of fatigue at any desired point in time following the initial contraction.

5.3 Data collection

We use commercial software (Labview version 8.5, National Instruments, Austin, TX) to write a customized program used to initiate and record torque. This software can also be designed to synchronize contractile activation, onset of ankle rotation, and torque data collection during contraction-induced injury. Stimulation of the dorsiflexor muscles occurs while the computer-controlled motor simultaneously moves the footplate into plantar flexion, thus leading to a lengthening contraction (also called “eccentric” contraction, which causes injury of the muscle). The magnitude of injury can be regulated by manipulation of variables such as angular velocity, timing of muscle activation, %maximal activation, range of motion, and the number of lengthening contractions. The specific protocol depends on the magnitude of injury desired by the investigator.

We also use commercial software for the *in situ* measurements. The signals from the load cell (calibrated before each test) are fed via a DC amplifier (model P122, Grass Instruments, Warwick, RI) to an A/D board to be collected and stored by acquisition software (PolyVIEW version 2.1, Grass Instruments, Warwick, RI). Alternative software programs from Aurora Scientific, Inc., are Dynamic Muscle Control and Analysis Software Suite(Model 615A).

6. EVALUATION AND INTERPRETATION OF RESULTS

Data analysis. For either the *in vivo* or *in situ* experimental contractile data, typical variables determined by the automated software include: twitch peak torque, time to peak, half relaxation time; tetanic peak torque, the torque–time integral; percent of torque

loss/recovery following an eccentric injury protocol; percent torque loss during a fatigue protocol. Force is usually given in volts, but the manufacturer will calibrate the specific load cell so that volts can be converted into grams or newtons. Force clearly depends on proper methodology (limb stabilization, electrode placement, etc), but it is also subject to fiber type, the number of healthy fibers, and their arrangement within the muscle (muscle architecture). Clearly the absolute force will depend on the muscle(s) tested, as well as animal age, gender, and species/strain. The maximal force per unit of cross-sectional area ("specific force") is considered relatively constant in healthy skeletal muscle (~22.5 N/cm3). Thus, carefully harvesting muscles at the end of experiments allows the investigator to normalize the force to muscle weight, thereby allowing comparison of muscle contractility in different sized muscles.

Once these variables are determined they can be saved in Excel. There are numerous functions in Excel that provide for sorting, grouping, calculating additional variables, and plotting. These data can then be moved to a graphics program of the investigator's choice to produce additional high quality figures.

See figures below.

7. REFERENCES

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4. **Pratt SJ, Shah SB, Ward CW, Inacio MP, Stains JP and Lovering RM**. Effects of in vivo injury on the neuromuscular junction in healthy and dystrophic muscles. *J Physiol* 591: 559-570, 2013.

8. APPENDIX

TOP: *in vivo* apparatus.
Left: The tibia is stabilized and the foot attached to a motor-driven plate. The ankle dorsiflexors are stimulated via the fibular nerve. If injury is desired, lengthening contractions are produced when the dorsiflexors are stimulated while the footplate forces the foot into plantar flexion (dotted arrow).
Right: Representative trace recordings of torque from lengthening contractions. In this example, muscles are stimulated to induce a peak isometric contraction (filled arrow) before lengthening (open arrow) by the footplate through an arc of motion at an angular velocity of 900°/s.
BOTTOM: *In situ* apparatus
Left: The load cell is mounted to a micromanipulator so that the tibialis anterior muscle (TA) can be adjusted to resting length and aligned properly in the X, Y, and Z directions. The distal tendon of the TA is attached to the load cell and single twitches are induced at different muscle lengths in order to determine L_0. A maximal tetanic contraction is obtained to determine maximal contractile activation (P_0). Maximal tetanic tension can be performed repeatedly and expressed as percentage of P_0, providing an index of fatigue at a desired point in time.
Right: Representative data showing the decline in maximal isometric tetanic tension during repeated stimulation of the tibialis anterior muscle. In this example, the TA was isolated, adjusted to optimal length (L_0), and then stimulated with a 200 ms tetanic contraction once every second for 5 minutes.