Please quote this SOP in your Methods.

Measuring isometric force of isolated mouse skeletal muscles *in situ*

SOP (ID) Number	DMD_M.2.2.005
Version	2
Issued	May 20th, 2009
Last reviewed	June 30th, 2016
Author	Gordon S. Lynch Basic and Clinical Myology Laboratory, Department of Physiology, the University of Melbourne, Victoria, Australia
Working group members	George Carlson (Department of Physiology, Midwestern University Glendale, AZ,USA) Elisabeth Barton (Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, USA) Dominic Wells (Department of cellular and molecular Neuroscience, Imperial College, London, UK)
SOP responsible	Gordon S. Lynch
Official reviewer	George Carlson

TABLE OF CONTENTS

1. OBJECTIVE

This document describes the methodology for performing measurements of muscle function on mouse tibialis anterior (TA) muscles, but the information is equally relevant for other hindlimb muscles, including the extensor digitorum longus (EDL), soleus or gastrocnemius muscles. Reference values for functional properties of TA muscles from C57BL/10 and *mdx* mice are provided. This document serves as a companion to the SOP entitled: "Measuring isometric force of isolated mouse muscles in vitro" (DMD_M1.2.002).

2. SCOPE AND APPLICABILITY

Critical to the evaluation of potential therapeutics for muscular dystrophy are accurate, sensitive and repeatable physiological assessments of muscle function in mouse models of dystrophy. The measurement of muscle contractile function is an important end-point for assessing the efficacy of potential treatments for muscular dystrophy, including: cell-, gene-, or pharmacologically based therapies.

The accurate evaluation of muscle contractile function *in situ* offers considerable advantages over *in vitro* assessments of isolated muscle preparations. With *in situ* measurements, the muscle being evaluated has its nerve and blood supply preserved meaning that recordings are physiologically relevant. This approach can be superior to recordings made *in vitro* which are performed at non-physiological temperatures (typically 25°C) and where inadequate muscle perfusion can quickly lead to the preparation becoming anoxic. An intact nerve and blood supply also provides a means for accurate assessment of muscle fatigue and neural stimulation of motor units. Problems or inaccuracies in the evaluation of muscle function *in vitro* can be identified easily with direct comparisons of the same functional measurements made *in situ*. Instead of stimulating isolated muscles *in vitro* using field stimulation between platinum plate electrodes, intact muscles can be stimulated to contract *in situ* by stimulating the muscle's nerve (using either surface electrodes or electrodes on an exposed nerve). As for muscle preparations examined *in vitro*, the length of muscles during measurements *in situ* can be either maintained (isometric contractions), shortened ("concentric" contractions), or lengthened (eccentric or lengthening contractions). The muscle can also be stimulated repeatedly (at different duty cycles) for assessment of fatigue.

3. CAUTIONS

Evaluation of muscle function *in situ* requires careful surgery on anesthetized animals. Typically these measurements are terminal experiments where the animals are killed after evaluation, however, this technique also allows for functional measurements to be performed on subsequent days (Brooks 1998). In these experiments, the surgical

intervention is kept to a minimum to allow for complete recovery between measurements. This document describes the procedures followed for terminal experiments (Consolino & Brooks 2004; DelloRusso et al. 2001; Schertzer et al. 2005; 2007; Schertzer & Lynch 2006).

As for all functional measurements on isolated skeletal muscles, the slightest damage to muscle fiber integrity during surgery can compromises the muscle's maximum force-producing capacity. It is suggested that those new to these techniques initially work with wild type (non-dystrophic, C57BL/10) mice until they can achieve values for maximum force that are comparable with published values before starting experiments with *mdx* mice.

4. MATERIALS

4.1 Function apparatus

Functional testing of mouse skeletal muscle *in situ* requires at least four components: a force transducer; a stimulator and electrodes to excite the nerve that controls the muscle being evaluated (using either surface electrodes or electrodes on an exposed nerve); a heated platform on which the anesthetized mouse can be secured and temperature maintained; and a data recording device. More sophisticated systems can be developed in-house provided there is resident workshop expertise. Alternatively, turnkey systems are now available commercially (e.g. Aurora Scientific, Aurora, ON) which provide standardized equipment and software that has been optimized for *in situ* evaluation of rat and mouse skeletal muscles. Several examples of commercially available components include:

4.2 Force-Position Transducers/Controllers

For isometric force only: The distal or proximal tendon of the muscle is tied directly between a fixed immovable hook and a force transducer (e.g. 1) Research Grade 60-2999, Harvard Instruments; 2) Grass FT03, Grass Instruments).

For isometric and/or shortening/lengthening: Dual Mode (Force/Position) Servomotor (e.g. 300/305B-LR, Aurora Scientific); or force transducer (Grass) with separate length controller (Aurora). The servomotor controller allows for displacement of the level arm (to either shorten or lengthen the muscle or to keep it at a fixed length) and for the simultaneous measurement of force production.

The careful control of muscle length and simultaneous force recording requires appropriate computer applications such as LabView software (National Instruments, Austin,TX, USA) driving a personal computer with an onboard controller (i.e., PCI-MIO-16XE-10, National Instruments) interfaced with the transducer-servomotor control/feedback hardware. Suitable systems are commercially available (Aurora Scientific, Aurora, ON).

4.3 Stimulator

External stimulator (e.g. S44 or S88 stimulator, Grass Instruments) or Internal stimulator provided by Data Acquisition Controller hardware (e.g. PCI-MIO-16XE-10, National Instruments) to deliver square wave (0.2 ms) electrical pulses for direct stimulation of the

nerve. Since the muscle is being stimulated by its nerve, there is no need for power amplification of stimulator output (as for the stimulation of muscles *in vitro*). In fact, only relatively low voltages (5-15V) should be required for direct nerve stimulation that recruits all motor units. The requirement of higher voltages usually signifies that the preparation is damaged or that neurotransmission is compromised, as could be the case in very old animals.
Furthermore, it should be noted that under some circumstances, stimulating the muscle directly (using surface electrodes) will produce different responses than from stimulating the muscle via its exposed nerve. For example, a classical neurogenic disorder would be expected to produce a decrease to indirect muscle activation via nerve stimulation with normal levels of tension induced by direct muscle activation. In purely myopathic disorders such as dystrophy, one would expect that nerve and muscle stimulation would produce identical outcomes.

4.4 Platform

The anesthetized mouse lies on a platform made from Plexiglas or other material. The platform enables the mouse to be secured to prevent any movement during muscle stimulation. Such platforms are generally part of commercially available systems (e.g. Aurora Scientific, ON) or can be manufactured in-house, if workshop expertise is available. The platform often contains a recirculating water jacket that enables the platform surface to be kept warm throughout the experiments. The platform also allows for appropriate positioning of the servomotor. The mouse usually lies on the Plexiglas platform directly or in a sloping sunken trough built into platform. The trough can be filled with warmed mineral oil and maintained at ~35-36°C. The surgically exposed nerve can be kept moist by direct application of warmed saline or mineral oil. External heat sources can be used to maintain the animal's body temperature, but it should be noted that heat lamps, heating blankets and other devices must be used very carefully since anesthetized animal are prone to overheating and can die if their temperature is not monitored carefully.

4.5 Electrodes

A pair of wire electrodes is needed to stimulate the nerve directly.

5. METHODS

For all procedures, mice are anesthetized deeply such that there is no response to tactile stimulation. For *in situ* analysis (of an anaesthetized animal), usually the distal tendon of a hindlimb muscle such as the TA, EDL, soleus, or medial gastrocnemius muscle (of one leg) is isolated and the tendon for each muscle is either sutured directly to the lever arm of a force/position controller while still attached to the muscle or muscle group or the tendon is severed and then secured to the lever arm with suture. Alternatively, when using a position-only servomotor in conjunction with a separate force transducer, one end of the muscle is tied to the lever arm of the position feedback servomotor and the other end of the muscle

tied to a force transducer. Note that when the tendon is severed, repeated measurements are generally not possible and the experiments are terminal.

After the tendon is attached to the force-recording apparatus, the knee and body of the animal are secured, and the muscle in question stimulated by its nerve, e.g. the sciatic nerve. Accessory nerves to other muscles may also need to be severed to prevent co-contraction of those muscles surrounding the muscle being evaluated. This is especially relevant when assessing smaller muscles (such as the EDL or soleus muscle) in isolation. A major advantage of *in situ* evaluation of contractile function is that the muscle's nerve and blood supply remain intact and undamaged, allowing for functional evaluation of larger muscles (e.g. tibialis anterior, gastrocnemius) that cannot normally be evaluated *in vitro* because of the difficulties associated with maintaining adequate perfusion of such larger muscles. A potential disadvantage of the *in situ* approach is that since the muscle is stimulated via its nerve and so the accuracy of the functional recordings is dependent upon there being no interference with normal muscle innervation.

It is important that all aspects of muscle stimulation, servomotor control, and force assessments, are controlled by computer or automated appropriately. This ensures that all parameters can be reproduced and controlled accurately for all experiments. Furthermore, force transducers must be calibrated regularly and checked for accuracy.

Measurements of maximum force require all fibers within a muscle to be recruited – i.e. all motor units are stimulated. Muscles are adjusted to the optimum length (L_o) for the development of isometric twitch force. The muscle is stimulated with a single electrical pulse to produce a twitch response. Stimulation voltage is adjusted to produce a maximal twitch response. Muscle length is adjusted very carefully in small increments (or decrements) to longer (or shorter) lengths. Muscles should be rested for at least 30 s between twitch responses. Optimal muscle length (L_o) is achieved when twitch force is maximal. Record muscle length using Vernier calipers and monitor L_o prior to and after the muscle is stimulated to ensure that L_o is maintained throughout the recording. It should be noted that it is sometimes difficult to measure L_o using calipers while the muscle is attached to the transducer. It is also possible to measure L_o using a straight length of suture placed alongside the muscle and then cut to represent the length between its attachment and the tendon that is secured to the transducer.

After determination of L_o, the frequency-force relationship should be established. The muscle is stimulated at increasing frequencies, typically 10, 20, 30, 40, 50, 75, 100, 120, 150, 200, 250, 300 and 350 Hz. It should be noted that for evaluations performed at physiological temperatures, fusion occurs at higher frequencies compared with evaluations performed in vitro at bath temperatures of 20-30°C. Stimulation is delivered for a period of 300-1200 ms depending on the muscle being evaluated. The muscle is rested for 2 min between successive stimuli. Maximum absolute isometric tetanic force (P_o) is determined from the plateau of the frequency-force relationship. Alterations in the frequency-force relationship can provide important insight into changes in motor unit recruitment and other parameters and it is recommended that a frequency-force relationship should be established for all muscles under investigation.

After determination of maximum force, the muscle can then be subjected to a variety of different protocols, to either evaluate muscle power, muscle fatigue, or perhaps the muscle's susceptibility to contraction-mediated damage. These protocols will vary between laboratories and are not described here. After determination of the muscle's contractile properties, the muscle is excised, blotted on filter paper (Whatman No. 1) and then weighed on an analytical balance. Muscles can be covered in OCT (Tissue Tek), snap frozen in thawing isopentane (cooled in liquid nitrogen) and then stored at -80°C for later biochemical or histological experiments. Muscle mass is required for determination of the muscle's cross-sectional area and for the calculation of specific (normalized) force – i.e. force per cross-sectional area.

Total muscle cross-sectional area (CSA) is calculated based on either morphometric measurements made after the experiments or approximated mathematically by dividing the muscle mass by the product of optimum fiber length (L_f) and 1.06 mg/mm^3, the density of mammalian muscle. L_f is determined by multiplying L_o by previously determined muscle length to fiber length ratios; e.g. 0.44 for the EDL and 0.71 for the soleus muscle (Brooks & Faulkner 1988), and 0.6 for the TA muscle (Burkholder et al. 1994). It should be noted that measurements of muscle length to fiber length will vary depending on whether external muscle length is measured from the point of attachment to the transducer or measured directly at the myotendinous junction. If morphometric measurements are used to measure CSA, it is useful to ensure that sections are cut perpendicular to the long axis of the muscle and that sections are cut at the mid-belly region. If mathematical approximations are used to calculate CSA, it is useful to ensure that neither the muscle density or pennation angle have been altered as a consequence of the therapeutic intervention, since the calculations rely on these factors being constant. Since absolute P_o is dependent upon muscle size, P_o values are normalized for muscle CSA (P_o is divided by the calculated total muscle CSA) and expressed as specific force (sP_o; kN•m^2), where $sP_o = P_o \times$ (muscle mass/$L_f \times 1.06$).

Assessment of Contractile Function of Mouse Tibialis Anterior Muscles in situ

1. After the mice have been anesthetized adequately, the right (or left) hindlimb should be shaved and minor surgery performed to expose the full length of the TA muscle.
2. The exposed tendon, muscle and nerve should be kept moist by application of warmed isotonic saline.
3. Cut the exposed tendon of the muscle several mm distal to the myotendinous junction and tie secure knots on the tendon using 5-0 surgical suture.
4. Immobilize the knee by passing a stainless steel pin or syringe needle behind the patellar tendon without damaging the surrounding tissue. The pin should affix to the base of the platform. The anesthetized animal must be secured firmly to prevent any movement during contraction.
5. Using the suture from step 3, tie the tendon of the muscle to the lever arm of the dual-mode servomotor.
6. Immerse the lower portion of the animal's body in warmed mineral or paraffin oil. The exposed TA muscle should be covered in mineral oil. The oil is used to

maintain the muscle preparation at the desired temperature for experimentation (i.e. 36-37^{o}C).

7. Expose a small portion of the sciatic/femoral nerve by making an incision in the thigh muscles.
8. Place two wire (simulating) electrodes on (or hooked under) the nerve and stimulate the muscle to contract using a supramaximal voltage (i.e. 10-14 V) of 0.2 ms square wave pulses of 350 ms train duration.
9. All stimulation parameters and contractile responses are controlled and measured using appropriate computer software.
10. It is common to determine optimal muscle length by progressively increasing the length in small increments until maximum twitch force is obtained.
11. After determination of L_o, stimulate the muscle at increasing frequencies to construct a full frequency-force relationship. The muscle should be rested for 2 min between success contractions. Maximum force is determined from the plateau of the frequency-force relationship.
12. Once maximum force has been determined, the muscle can be subjected to different protocols to determine power of shortening, muscle fatigue, or susceptibility to contraction-induced damage.

After determination of contractile parameters, the muscle should be excised, blotted on filter paper and weighed on an analytical balance. The muscle can then be frozen for later biochemical or histological analyses.

6. EVALUATION AND INTERPRETATION OF RESULTS

Typical values for *in situ* measurements of an undamaged TA muscle in a 12-16 week old C57BL/6 or C57BL/10 mouse are:

- Maximum twitch force can vary and is usually in the range ~350-500 mN.
- Maximum (tetanic) force occurs at a stimulation frequency of 200-300 Hz and is usually in the range ~1500-1700 mN.
- Maximum tetanic force corrected for the cross sectional area of the muscle (i.e. specific force) is usually in the range ~260-300 kN•m^{-2} A lower value for specific force of muscles from control mice generally indicates some form of damage has occurred to the muscle.

Ballpark values of isometric contractile properties of TA muscles from 8 week old C57BL/10 and *mdx* mice are presented in Table 1.

Table 1. Morphological and isometric contractile properties of tibialis anterior (TA) muscles in 8 week old C57BL/10 (n = 6) and *mdx* (n = 6) mice.

Parameter	**control**	***mdx***
mass (mg)	37 ± 1	64 ± 4
P_t (mN)	389 ± 19	599 ± 45
dP^{twitch}/dt (mN/ms)	63 ± 3	88 ± 6
TPT (ms)	16 ± 1	14 ± 1
½ RT (ms)	12 ± 1	14 ± 1
P_o (mN)	1539 ± 31	1652 ± 86
sP_o (kN•m^2)	320 ± 7	212 ± 5

P_t, isometric twitch force; dP^{twitch}/dt, rate of twitch force development; TPT, time to peak twitch; ½ RT, half relaxation time; P_o, maximum isometric force; sP_o, specific or normalized maximum isometric force (force per muscle cross-sectional area).

7. REFERENCES

Brooks, S.V. & Faulkner, J.A. (1988). Contractile properties of skeletal muscles from young, adult and aged mice. *J Physiol*. 404: 71-82.

Brooks, S.V. (1998) Rapid recovery following contraction-induced injury to *in situ* skeletal muscles in *mdx* mice. *J Muscle Res Cell Motil*. 19: 179-187.

Burkholder, T.J., Fingado, B., Baron, S. & Lieber, R.L. (1994) Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. *J. Morphol*. 221: 177-190.

Consolino, C.M. & Brooks, S.V. (2004) Susceptibility to sarcomere injury induced by single stretches of maximally activated muscles of *mdx* mice. *J Appl Physiol*. 96: 633-638.
Dellorusso, C., Crawford, R.W., Chamberlain, J.S. & Brooks, S.V. (2001) Tibialis anterior muscles in *mdx* mice are highly susceptible to contraction-induced injury. *J Muscle Res Cell Motil*. 22: 467-475.

Schertzer, J.D., Plant, D.R. & Lynch, G.S. (2006) Optimizing plasmid-based gene transfer for investigating skeletal muscle structure and function. *Mol Ther*. 13: 795-803.

Schertzer JD, Lynch GS. (2006) Comparative evaluation of IGF-I gene transfer and IGF-I protein administration for enhancing skeletal muscle regeneration after injury. *Gene Ther*. 13: 1657-1664.

Schertzer, J.D., Gehrig, S.M., Ryall, J.G. & Lynch, G.S. (2007) Modulation of insulin-like growth factor (IGF)-I and IGF-binding protein interactions enhances skeletal muscle regeneration and ameliorates the dystrophic pathology in *mdx* mice. *Am J Pathol*. 171: 1180-1188.