TREAT-NMD Activity A07: Accelerate preclinical phase of new therapeutic treatment developement

Work package 7.4: Develop standardised protocols and procedures for harmonising and accelerating pre-clinical studies (including standardised data analysis)

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1. OBJECTIVE

This document describes the methodology for performing isolated muscle function measurements for mouse extensor digitorum longus (EDL), soleus, and diaphragm, and provides reference values for isometric contractile properties of muscles from mdx and C57 mice.

2. SCOPE AND APPLICABILITY

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

The accurate measurement of maximum muscle force-producing capacity (and power output) *in vitro* is dependent upon many factors, including surgical dissection, the use of accurate force recording equipment, and adequate muscle perfusion to prevent any part of the muscle becoming anoxic. Muscles can be electrically stimulated to contract, and muscle length can be maintained (isometric contractions), shortened ("concentric" contractions), or lengthened (eccentric or lengthening contractions). Such *in vitro* analysis also facilitates the assessment of physiological parameters of the diaphragm muscle, the most severely affected muscle in the *mdx* mouse, as well as limb muscles (the EDL and soleus) which possess an ideal geometry for isolated muscle functional testing.

This document will provide a general description of dissection methods that have been utilized successfully in several labs for functional measurements, and a discussion of the potential pitfalls that can prevent optimal measurements.

These descriptions are limited to the evaluation of *isometric* forces of isolated mouse muscles *in vitro*. Protocols for other functional measures, including power of shortening and muscle susceptibility to contraction-mediated damage, are not described in detail. However, a general discussion of methods for evaluating susceptibility to contractile damage is provided in the appendix. Related SOPs describe the evaluation of muscle function capacity *in vivo* and *in situ.*

3. CAUTIONS

Evaluation of muscle function *in vitro* requires careful surgical tendon-to-tendon excision of muscles from anesthetized animals, and these techniques take time to perfect. The slightest damage to muscle fiber integrity during surgery compromises the muscle's force-producing capacity. This includes excessive pulling on the muscle, touching it directly, or

allowing it to dry out. It is suggested that those new to these techniques initially work with wild-type (non-dystrophic) muscles until they can achieve published force values for the muscles of interest. This will establish benchmark values for subsequent measurements in the mdx mouse

4. MATERIALS

Function apparatus

Functional testing of mouse skeletal muscles requires a minimum of four components: (1) a force transducer to monitor force production, (2) a stimulator and electrodes to excite the muscle, (3) a bath to superfuse the muscle with oxygenated Ringer's solution, and (4) a device to record force production. Systems can be developed in the lab, provided there is resident expertise. Alternatively, turnkey systems are available commercially (e.g., Aurora Scientific, Aurora, ON; Biopac Systems, Goleta, CA), which provide standardized equipment and software that has been optimized for monitoring function in small tissues. Several examples of commercially available components follow.

Force Transducers

For isometric force only: The muscles are tied directly or via small noncompliant rings between a fixed immovable hook and a force transducer (e.g., Research Grade 60-2999, Harvard Instruments; 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)

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.

Power Amplifier. Some stimulators require amplification of output by a heavy duty audio power amplifier (e.g., DC300A Laboratory power amplifier, Crown International, Elkhart, IN, USA; EP500B, Audio Assemblers, Victoria, Australia) in order to produce a sufficient field strength between the electrodes flanking the muscle preparation in order to recruit all motor units and produce a maximal isometric contraction.

Organ Bath

Typically, the organ baths are commercial varieties used primarily for pharmacological experiments that require a muscle be orientated vertically (e.g., Radnoti) Alternatively, custom-built Plexiglas chambers can be manufactured to allow for the isolated muscles to be orientated horizontally between the force recording equipment. Bath volumes range from 2-100 mL. It is important to have some method for controlling bath temperature. Commercial baths can have a water-jacketed chamber, which then requires a separate circulating temperature-controlled water bath for temperature maintenance. Alternatively, the

bathing solution can be pumped through a separate temperature controller and back to the organ bath.

Electrodes

A pair of platinum plate electrodes is needed to flank the isolated muscle on either side. The electrodes run the length of the preparation and are positioned a sufficient distance (~0.5 cm) apart to ensure that the muscles are field stimulated and not by direct stimulation (contact) of the muscle with an electrode.

Muscle Bathing Solution

Table 1 lists the components normally used to bathe isolated muscles for functional testing. The primary goal is to maintain physiological conditions to support muscle stability for the duration of the testing regimen, including ionic and osmotic strength, metabolites, pH, and there is variability in all of these conditions across a number of labs. Two differences deserve further explanation.

Table 1. Solution conditions for isolated muscle functional testing

Component	Consensus values (mM)
Ions	
NaCl	118-140
KCl	4.7-5.9
$CaCl_2$	1.5 - 2.5
KH_2PO_4	1 - 1.2
$MgSO_4$	0.5 - 1.2
$MgCl_2$	0 - 1.2
Buffers	
HEPES	0 - 25
$NaHCO_3$	0 - 25
gas equilibration	0-5%CO2,95-100% O2
Energy	
Glucose	0 - 11
Pyruvate	0 - 1
Inhibitors	
D-Tubocurarine	0 - 0.3
Solution Conditions	
Temperature	20 - 30 ^{O}C
pH	7.3 - 7.6
Osmotic Strength	270 - 290 mOsm/L

First, some investigators add D-tubocurarine chloride to the bathing solution in order to prevent muscle stimulation via the neuromuscular junction. Its use is dependent upon the intent of the specific study. However, a recent publication demonstrated that D-tubocurarine chloride did not affect force output at high voltage stimulation (Cairns, et al,2007). Thus, for evaluation of maximal force production in mdx muscle, such blocking agents are not necessary.

Second, the temperature of the bathing solution can affect the stability of the muscle preparation, as well as a subset of the kinetic parameters associated with force production. Large muscles (~20mg), in particular, are susceptible to diminished force production and shortened stability when muscle superfusion is not longer sufficient to support increased

metabolic demand caused by high temperatures. Regardless of the temperature chosen, it is important for it to be maintained throughout the experiment.

5. METHODS

Muscle Dissection

For all procedures, mice are euthanized or anesthetized deeply such that there is no response to tactile stimulation. A commonly used anesthesia is a mixture of 10mg/mL ketamine + 1mg/mL xylazine, injected intraperitoneally at 10 mg/Kg body weight. In general, the muscle of choice (e.g. EDL, soleus, etc) is carefully dissected with tendons intact on both ends. The excised muscle is placed immediately into oxygenated Ringer solution (maintained at 24-25°C) and equilibrated for 5-10 min. Upon placing the muscle in a dish, it is gently stretched (not over stretched) with two pins (e.g., Fine Science Tools, cat #26002-20) on two tendons for further preparation. Alternatively, silk suture can be tied to the proximal and distal tendons during dissection and used as anchors to maintain resting length in the dish. Dishes can be made in the lab by adding a 5-10mm bed of silicone elastomer (e.g., Sylgard) to the bottom of a petri dish. Mice are euthanized according to the guidelines for the care and use of experimental animals in place at each institution.

EDL muscle. The EDL proximal tendon can be seen lateral to the knee under the distal end of the rectus femoris, and cut at this point to maximize the length of the tendon stump. The distal tendons are under the tibialis anterior (TA) tendon – ensure that all tendons leading to the toes are cut before removing the muscle.

Soleus muscle. The most straightforward dissection of the soleus requires cutting the Achilles tendon (which includes the distal soleus tendon) and carefully peeling all posterior muscles away from the rest of the limb. The proximal tendon is evident on the interior surface of the muscles close to the knee. Cut as close as possible to the back of the knee to maximimze the length of the tendon stump.

Diaphragm. For examination of the contractile function of the diaphragm, muscle strips composed of longitudinally arranged full length muscle fibers (2-4 mm wide) can be cut from the central region of the lateral costal hemidiaphragm and tied firmly with braided surgical silk at the central tendon at one end, and sutured through a portion of rib attached to the distal end of the strip at the other end (Figure 1A). Since damage always occurs on the fibers located at the sides

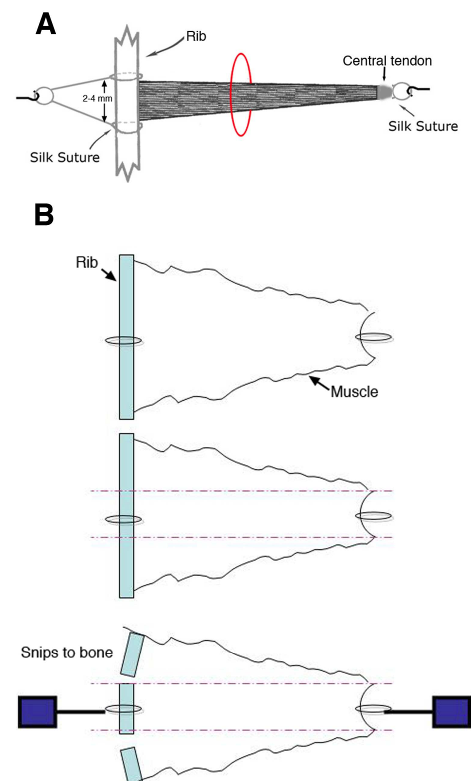

***Figure 1**. Diagram of diaphragm muscle dissection. A. Final muscle strip should be no more than 4 mm wide, and tied firmly at the central tendon and the rib. Red Circle indicates the region of muscle cross section taken for measuring damaged fibers in Figure 3. B. Alternate protocol for isolating functional strip of diaphragm without damaging edge of preparation*

of the strip, an alternative approach is to prepare a slightly triangular strip, and then giving two tiny incisions perpendicular to the rib (avoiding the muscle), in order to be sure that the force is only generated from sane muscle fibers restricted in the so determined quadrilateral portion (Figure 1B).

The nerve and blood supply to each muscle are severed just prior to excision to ensure optimum condition of muscles. Tendon sutures should be as close to the myotendinous junction as possible, but not in contact with the muscle fibers. Optimum placement of the sutures ensures less contribution of the tendon to compliance, and minimizes failure of this attachment during stimulation. It is essential that all attachments are secured very tightly since the muscles produce considerable forces. If the attachments to the muscle or to the equipment are not secured, the contracting muscle can tear away from these points of weakness.

Force Measurements

Muscle Attachment to Apparatus. When using a dual mode (force-position) servomotor, one end of the muscle (usually the distal tendon) is tied to an immovable pin and the other end of the muscle (usually the proximal tendon) is attached to the lever arm of a position feedback servomotor. 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.
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 in all subsequent experiments. Force transducers should be calibrated regularly to ensure accuracy.

Establish Supramaximal Stimulation Conditions. Measurements of maximum isometric tension require than all muscle fibers in a muscle are stimulated. Because there is tremendous variability in the components of a function apparatus, this must be determined for each individual set up. For example, bath size or the type of stimulator can affect the intensity of stimulation. Twitch stimulation is a reasonable way to determine supramaximal stimulation conditions.

Establish Optimum Length. Muscles are adjusted to the optimum length (L_o) for the development of isometric twitch force. To this end, the muscle is stimulated with a single electrical pulse to produce a twitch response. Stimulation voltage is that which produces a maximal twitch response. Muscle length is adjusted very carefully and in small increments (or decrements) to longer (or shorter) lengths. Rest the muscle for at least 30 s between twitch responses. Optimal muscle length (L_o) is achieved when twitch force is maximal. Record muscle length (the length between the myotendinous junctions) using Vernier calipers and monitor L_o prior to and after the muscle is stimulated to ensure Lo is maintained.
An alternative method is to establish Lo using tetanic stimulation (se below). If this approach is used, then one must allow the muscle to rest 3-5 minutes between bouts of stimulation to minimize the contribution of fatigue to the measurements

Establish frequency-force relationship. Once L_o has been achieved, the frequency-force relationship can be established. The muscle is stimulated at increasing frequencies, typically 10, 30, 50, 80, 100, 120, 150, 180, 200, and 250 Hz. Stimulation is delivered for a period of 500-900 msec. The muscle is rested for 3-5 min between successive stimuli. Maximum absolute isometric tetanic force (P_o) is determined from the plateau of the frequency-force relationship. The plateau for EDL muscles is typically achieved with 150 Hz, and for soleus muscles with 100 Hz. This forms the basis for determining maximum isometric force. For any given study, once the frequency-force relationship is established, it is not required to perform the range of frequencies on all muscles. However, this relationship can be extremely useful to evaluating therapies that alter muscle fiber properties or calcium handling (refs here), which are reflected in shifts in the frequency-force relationship.

Maximum Isometric Tetanic Force. One is now ready to determine optimum force generation of a given muscle. Muscles should be stimulated at supramaximal voltage at Lo at a plateau stimulation frequency. Muscles are typically stimulated 2-3 times with rest periods of 3-5 minutes between stimulation bouts. Muscles can then be removed from the apparatus for further processing. Muscle mass is needed to determine the cross-sectional area and specific force calculations.

Absolute maximum force of isolated mouse muscles in vitro will vary depending on which muscle is being investigated and on the size of the muscle. Typical values for normal and dystrophic muscles are shown in the Table 2 below.

6. EVALUATION AND INTERPRETATION OF RESULTS

Typical values for maximum tetanic force are included in Table 2. Values are for 10-12 week old C57BL/10 and *mdx* mice (see Gregorevic et al. *Muscle Nerve* 30: 295–304, 2004). Experiments performed in vitro at 25°C. N.B. There will be variation in the size/mass of muscles depending on the age of the mice and so absolute forces will vary accordingly. Differences in forces are expected depending in the equipment and protocols employed but it is important that experiments on muscles from control and mdx mice are done together to ensure accurate comparisons.

Table 2. Typical values for isometric contractile properties of EDL and soleus muscles from wild-type and *mdx* mice

	EDL		Soleus	
	Control (n = 6)	***mdx*** (n = 7)	**Control** (n = 6)	***mdx*** (n = 7)
Muscle mass (mg)	11.4 ± 0.3	18.0 ± 1.0[a]	7.9 ± 0.2	12.8 ± 0.5[a]
Optimum muscle length (mm)	11.7 ± 0.3	12.3 ± 0.3	10.9 ± 0.2	11.6 ± 0.3
Time to peak twitch (ms)	16.0 ± 0.3	15.9 ± 0.3	32.0 ± 1.5	34.1 ± 0.9
Half relaxation time (ms)	18.3 ± 0.4	18.3 ± 0.6	41.0 ± 3.0	44.0 ± 1.0
Max. isometric force (mN)	507.1 ± 14.0	520.9 ± 38.8	211.1 ± 6.4	264.9 ± 15.9[a]
Specific force (kN/m^2)	243.4 ± 4.5	165.0 ± 7.7[a]	219.5 ± 9.6	180.1 ± 7.9[a]

It is very important to check the accuracy and validity of values for absolute and specific force since this impacts the evaluation of the efficacy of any treatment for ameliorating functional deficits in muscular dystrophy. If the accuracy of functional data is questionable, so too is the validity of the evaluation of compound efficacy.

Specific Force Calculations

Total muscle cross-sectional area is either calculated based on morphometric measurements made after the experiments or mathematically approximated 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, 0.44 for the EDL and 0.71 for the soleus (Brooks & Faulkner Contractile properties of skeletal muscles from young, adult and aged mice. *J. Physiol.* 404:71-82, 1988). If morphometric measurements are used to measure CSA, it is useful to ensure that sections are cut perpendicular to the long axis and that sections are made at the mid-belly of the muscle. If mathematical approximations are used to measure CSA, it is useful to ensure, that neither the density or pennation angle changed due to the therapeutic intervention, as the method relies on these factors being constant.

Since absolute P_o is dependent upon muscle size, P_o values are normalized for cross-sectional area (P_o is divided by the calculated total muscle cross-sectional area) and expressed as specific force (sP_o; kN/m^2), where $sP_o = P_o \times$ (muscle mass/$L_f \times 1.06$). This can be very

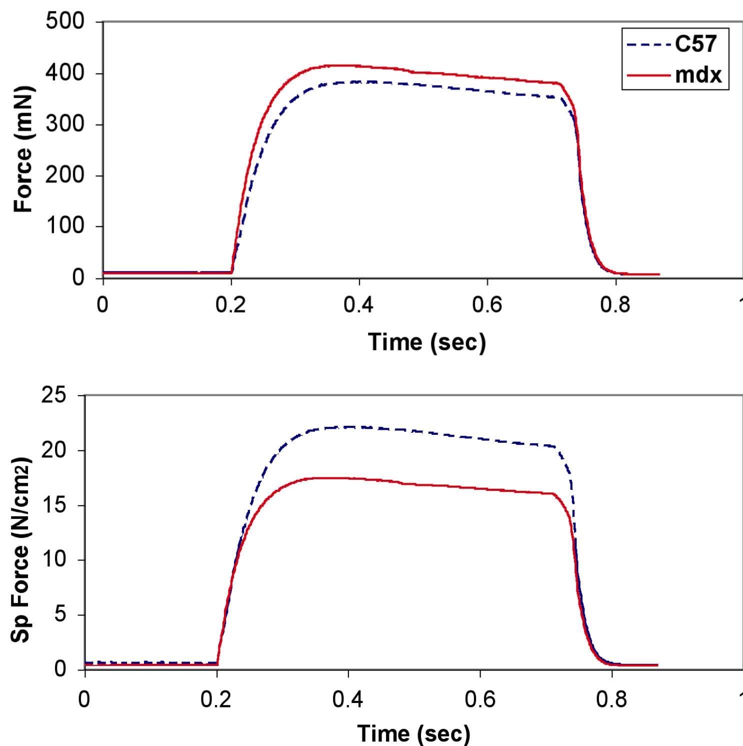

Figure 2. *Comparison of mdx and C57 EDL muscles with absolute tetanic force (upper panel) and specific force (lower panel) demonstrates the need for normalizing force generating capacity by muscle cross-sectional area.*

important in the assessment of mdx muscle function, particularly because mdx muscles tend to be larger than those in age-matched control mice (Table 2). An example of this is shown in Figure 2. In the top panel, absolute muscle force is shown for a representative EDL muscles from an mdx and C57 mouse. Note that tetanic force production in the mdx EDL is higher than that in the C57 muscle. However, when absolute force is normalized for muscle cross-sectional area, specific force ins lower in mdx than C57 EDL muscle (lower panel), highlighting the intrinsic weakness of dystrophic muscle.

For diaphragm muscle strips, the width and thickness and consequently mass, vary unpredictably among animals at the discretion of the individual excising them. Therefore, absolute forces developed by diaphragm muscle strips have no physiological meaning, and comparisons can only be made after the forces have been normalized for total fiber cross-sectional area (kN/m^2). In *mdx* mice, a considerable portion of the cross-sectional of the diaphragm muscles can be composed of necrotic and connective tissue and so the area of viable muscle should also be taken into consideration for the normalization procedures. Further, preparation of the diaphragm strips inevitably results in damage of fibers along the plane of dissection from the central tendon to the rib insertion. Immersion of muscle strips in oxygenated Ringers with procion orange (0.2-1.0%, Sigma, R-8254) helps to identify damaged fibers, and the dye is membrane impermeant and can only enter fibers through sarcolemmal tears (Petrof BJ, Shrager JB, Stedman HH, Kelly AM, Sweeney HL. Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc Natl Acad Sci USA* 90:3710–3714, 1993). An example of procion orange uptake is shown in Figure 3.

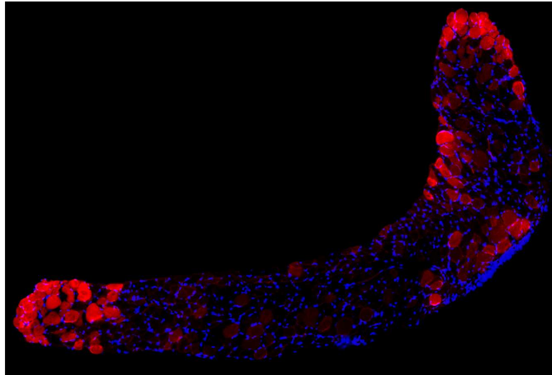

Figure 3. *Cross section of a dystrophic diaphragm that had been incubated in 0.2% procion orange during functional measurements. Red fibers are indicative of muscle damage, which is evident along the borders of the muscle strip, and within the muscle cross section. Muscle section was counterstained with DAPI to identify nuclei.*

Advantages:

The assessment of isolated muscle function in vitro offers the advantage of investigating contractile properties of dystrophic and normal muscles in the absence of any influences from the nerve or blood supply.

Disadvantages:

The size of the muscles to be analyzed by in vitro methods is limited. Muscles larger than 25 mg cannot be adequately supported by superfusion for long periods of time, and tend to develop an anoxic core diminishing functional output. In these cases, alternate methods of functional assessment should be utilized.

The absence of the nerve and blood supply, despite being advantageous for some investigations, also raises questions as to whether functional deficits could be attributed to compromised innervation, failures at the neuromuscular junction, or issues related to altered metabolism or circulation. This is especially relevant when interpreting the effects of muscle fatigue.

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8. APPENDIX

Evaluation of Susceptibility to Contractile Damage.

For the muscular dystrophies, the ultimate cure would return dystrophin function to the muscle. Thus, an important extension of evaluating isolated muscle function includes examining changes in the contractile fragility of the muscle. There is tremendous variability in the methods to measure susceptibility to contractile injury, and still more variability in the interpretation of these results. However, there are a few key points that need to be considered before adopting one of the existing methods or developing new protocols.

First, the protocol must invoke a significant and reproducible loss in force in the mdx muscle of study.

Second, the same protocol must also invoke measurable loss in force in age-matched wild-type muscle. This establishes a dynamic range between wild-type and mdx muscles, and enables evaluation of the efficiency of any therapy to protect against contractile damage. If there is no loss in force in wild-type muscles, then the protocol is not severe enough to truly evaluate differences.

Third, the same protocol may not be relevant for the different muscles. Because the EDL, soleus and diaphragm do not share identical muscle geometry, one must tune the damage protocol for each muscle group. This can include changing the rate or extent of lengthening during stimulation, the frequency of stimulation during the protocol, or the number of stimulation bouts imposed on the muscle.

Fourth, not all therapies will change susceptibility to contractile damage. Therefore, prior to investing in the needed equipment, such as a dual mode servo motor, consider the goal of the therapy of interest.

In situ muscle function

The development of a second SOP for in situ function is underway.