

**HOW TO MEASURE
ISOMETRIC FORCE OF
STRIATED MUSCLES IN
VITRO IN A 820MS OR
840MD MYOGRAPH**

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CHAPTER 1 - INTRODUCTION

This document is an example on how to perform isolated muscle function measurements using DMT Muscle Strip Myograph Systems; the 820MS Muscle Strip Myograph and 840MD MyoDYNAMICS Myograph system.

A description on the methodology performing function measurements for mouse extensor digitorum longus (EDL), soleus, and diaphragm is provided in this document as examples. Furthermore the document provides reference values for isometric contractile properties of muscles from mdx and C57 mice.

Many factors influence the accurate measurement of maximum muscle force-producing capacity (and power output) in vitro including surgical dissection, the use of accurate force recording equipment, and adequate muscle perfusion to prevent any part of the muscle becoming anoxic. Field stimulation with an electrode to contract muscles and muscle length can be maintained (isometric contractions). This 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 is an example on how to perform isolated muscle function and it 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.

NOTE: evaluation of muscle function in vitro requires careful surgical tendon-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.

CHAPTER 2 - MATERIALS

DMT SYSTEMS REQUIRED FOR FORCE MEASUREMENT IN STRIATED MUSCLES: MUSCLE STRIP MYOGRAPH SYSTEM - 820MS

The Muscle Strip Myograph System - 820MS represents a state-of-the-art 4-channel myograph system for muscle strips of up to 19 mm in length. The system was originally developed to give the skeletal muscle physiologist a highly sophisticated, easy-to-use, robust, high-throughput muscle myograph. The rectangular design of the chamber, however, gives this system the flexibility to mount.

In the 820MS chamber the muscles can be clamped directly to the mounting clips or by replacing the mounting clips with mounting hooks the muscle can be tied via suture to the immovable hook and the force transducer. Several types of mounting supports exist, depending on the user's preferences.

Customized mounting supports can be made upon request. In addition the myograph can also be used for the study of larger smooth muscle strips, because of the somewhat rectangular chamber.

MYODYNAMICS MUSCLE STRIP MYOGRAPH SYSTEM - 840MD

The MyoDynamics Muscle Strip Myograph System - 840MD represents a state-of-the-art 4-channel myograph system for muscle strips of up to 30 mm in length. The system was originally developed to give the skeletal muscle physiologist a precise, easy-to-use, high-throughput muscle myograph with the capacity to stretch and retract the muscle under a range of conditions including electric field stimulation. The 840MD system is optimal for fatigue, eccentric and concentric contraction studies using the build-in motors. The special design of the chamber, however, gives this system the flexibility to mount larger muscle strips of various organs and striated muscle up to 30mm.

In the 840MD chamber the muscles can be tied via suture to the immovable hook and the force transducer. Several types of mounting supports exist, depending on the user's preferences.

DMT STIMULATOR – CS4/ CS8

The CS4 and CS8 are 4-channel and 8-channel stimulators, respectively. The CS4 and CS8 stimulators combine a user-friendly interface with advanced electrical stimulation features required in electrophysiological experiments. Both stimulators are controlled by the MyoPULSE PC software where the stimulation protocol are made and transferred to the stimulators. The CS4 and CS8 have built-in trigger function making them able to trigger e.g. the motors on the 840MD system to start (Eccentric and concentric contractions) and they also initiate stimulation protocols be receiving external triggers e.g. from a data acquisition system as a Powerlab.

The CS4 and CS8 are modular, highly versatile voltage stimulators suitable for use with all DMT Myograph Systems.

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 (~0.8 cm) apart to ensure that the muscles are field stimulated and not by direct stimulation. Stimulation electrodes are available built into the chamber cover and can be used to activate the muscle via field stimulation with a stimulator.

DATA

DMT recommends the Powerlab/LabChart platform as the data acquisition system of choice for DMT Muscle Strip Myograph Systems.

BUFFER RECIPE

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.

Component	Consensus values (mM)
<i>Ions</i>	
NaCl	118-140
KCl	4.7-5.9
CaCl ₂	1.5 - 2.5
KH ₂ PO ₄	1 - 1.2
MgSO ₄	0.5 - 1.2
MgCl ₂	0 - 1.2
<i>Buffers</i>	
HEPES	0 - 25
NaHCO ₃	0 - 25
gas equilibration	0-5%CO ₂ ,95-100% O ₂
<i>Energy</i>	
Glucose	0 - 11
Pyruvate	0 - 1
<i>Inhibitors</i>	
D-Tubocurarine	0 - 0.3
<i>Solution Conditions</i>	
Temperature	20 - 30 °C
pH	7.3 - 7.6
Osmotic Strength	270 - 290 mOsm/L

Table 1. Buffer conditions for isolated muscle functional testing

First, some investigators add D-tubocurarine chloride to the buffer 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.

It is important to keep the temperature of the bath solution constant throughout the experiment to avoid instability of the muscle preparation. Large muscles (~20mg), in particular, are susceptible to diminished force production and shortened stability when muscle superfusion is no longer sufficient to support increased metabolic demand caused by high temperatures.

CHAPTER 3 - 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 buffer (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) by tying a Vicryl suture (USP 6/0 FS-3 45 cm V387H, DMT product #100373) to the proximal and distal tendons during dissection and used as anchors to maintain resting length in the dish. (DMT Petri dish, Product No. 100103: 90 mm Glass Petri dish coated with 5 mm black Sylgard).

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 maximize 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).

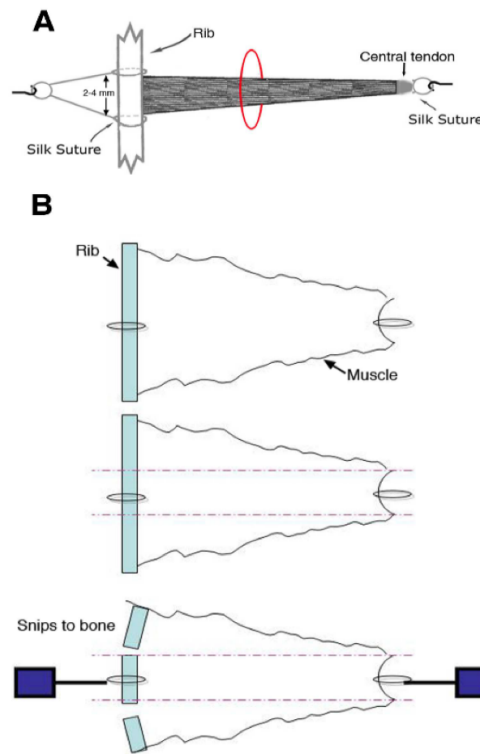


Figure 1. Diagram of Diaphragm muscle dis-section. A. Final muscle strip should be no more than 4 mm wide, and tied firmly at the central tendon and the rib. Red Circle indicates the region of muscle cross section taken for measuring damaged fibers in Fig 3. B. Alternate protocol for isolating functional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

Since damage always occurs on the fibers located at the sides 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.

CHAPTER 4 - FORCE MEASUREMENTS

ESTABLISH SUPRAMAXIMAL STIMULATION CONDITIONS

Measurements of maximum isometric tension require that all muscle fibers in a muscle are stimulated. 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_0) 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. A thumb of rule is applying 20volt/cm (measure the distance in cm between the two electrodes on the coverlid and multiply it with 20volts). An example if the distance between the two electrodes is 0.8cm the muscle should have 0.8×20 volts = 16 volts. Muscle length is adjusted very carefully and in small increments using the micrometer positioner on the 820MS or 840MD chamber (or decrements) to longer (or shorter) lengths. Rest the muscle for at least 30 s between twitch responses. Optimal muscle length (L_0) is achieved when twitch force is maximal. Record muscle length (the length between the myotendinous junctions) using Vernier calipers and monitor L_0 prior to and after the muscle is stimulated to ensure L_0 is maintained.

THE OPTIMAL VOLTAGE FOR MAXIMUM ISOMETRIC TENSION:

After the Optimal Length (L_0) have been determined as described in the above section the optimal voltage can be determined by doing twitch stimulation of the mounted muscle at the Optimal Length (L_0) for the given muscle. Start twitch stimulation on the mounted muscle at e.g. 10 volts and then increase the voltage by 1-3 volts per twitch and remember to have at least a 30 second pause between each twitch. The Optimal Voltage for maximum isometric tension is that which produce maximal twitch force.

NOTE: DO NOT INCREASE THE VOLTAGE AFTER REACHING A PLATEAU. TO HIGH VOLTAGE WILL DESTROY THE MUSCLE PERMANENTLY.

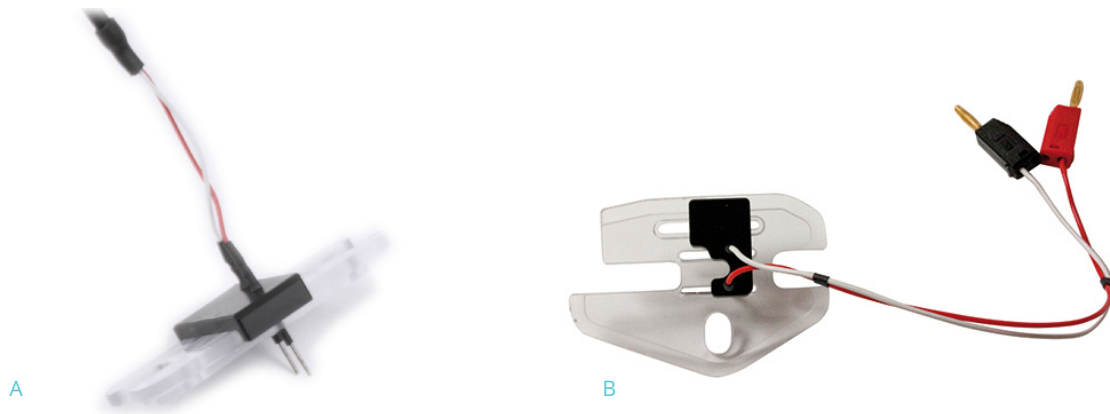


Figure 2. DMT Cover lids with or without electrodes. A) 820MS NFS Cover lid (DMT #100066) B) 840MD NFS Coverlid (DMT #100238)

The 820MS and 840MD NFS Cover lids have electrodes that can slide along the mounted muscle. Move the electrodes along the muscle and do a twitch stimulation at the different positions of the NFS electrodes to find the optimal position of the electrodes resulting the maximal force contraction.

ESTABLISH FREQUENCY-FORCE RELATIONSHIP

Once L_o (Optimal length) 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 L_0 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 mouse muscles are shown in the Table 2.

CHAPTER 5 - EVALUATION AND INTERPRETATION OF RESULTS

Typical values for maximum tetanic force for mouse 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.

	EDL		SOLEUS	
	Control (n = 6)	mdx (n = 7)	Control (n = 6)	mdx (n = 7)
Muscle mass (mg)	11.4 ± 0.3	18.0 ± 1.0a	7.9 ± 0.2	12.8 ± 0.5a
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 ± 15.9a
Specific force (kN/m ²)	243.4 ± 4.5	165.0 ± 7.7a	219.5 ± 9.6	180.1 ± 7.9a

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

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 $sP_o = P_o \times (\text{muscle mass}/L_f \times 1.06)$.

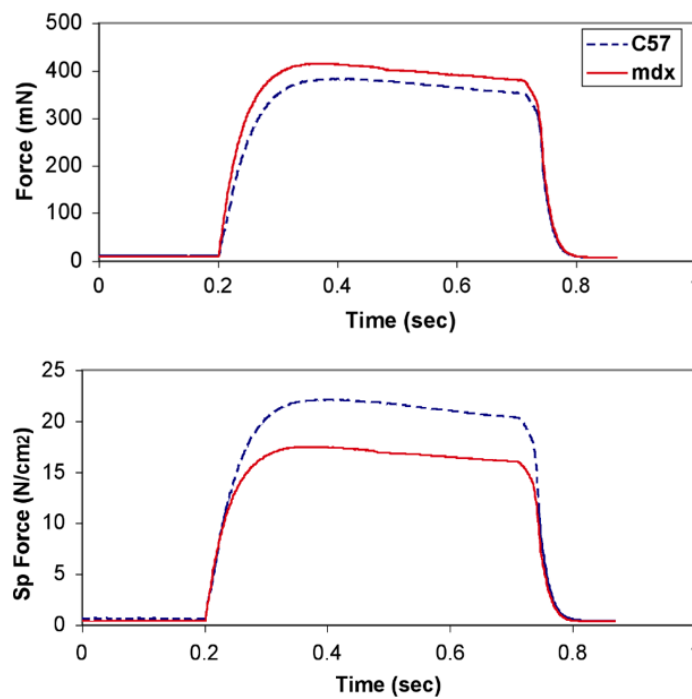


Figure 3. Comparison of mdx and C57 EDL muscles with absolute tetanic force (upper panel) and specific force (lower panel) demonstrates the need for normalizing force generating capacity by muscle cross-sectional area.

This can be very 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 3. 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 is 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/m²).

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.

ACKNOWLEDGEMENT

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