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**Muscle function evaluation through isometric force measurement in mouse models of spinal muscular atrophy**

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Authors	Chien-Ping Ko Section of Neurobiology, Department of Biological Sciences, University of Southern California, California, USA Ming-Yi Lin Section of Neurobiology, Department of Biological Sciences, University of Southern California, California, USA
Working group members	John H. Caldwell (Cell and Developmental Biology/ Physiology and Biophysics, University of Colorado Denver, Colorado, USA9 Jean-Marc Raymackers (Universite Catholique de Louvain, Louvain, Belgium)
SOP responsible	Chien-Ping Ko
Official reviewer	Jean-Marc Raymackers



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## 1. OBJECTIVE

This document aims to provide instructions on how to measure isometric muscle tension elicited by nerve (indirect) and muscle (direct) stimulation from extensor digitorum longus (EDL) muscle in a mouse model (SMNΔ 7) of spinal muscular atrophy (SMA).

## 2. SCOPE AND APPLICABILITY

Muscle weakness is one of the major characteristics of SMA. It is thus critical to have a functional assay to evaluate the synaptic transmission at the neuromuscular junction (NMJ) and test muscle function. With the current protocol, one can assess whether there is synaptic transmission failure or if there are defects in muscle contractile properties in SMA mouse models. This can also provide a functional assay to validate the effectiveness of drug treatment.

The current protocol is focused on one hindlimb muscle, EDL, in SMNΔ 7 mice, one of the most widely used SMA mouse models. However, it should be applicable to other muscles and other mouse models of SMA. The *in vitro* preparation of EDL muscle with nerve intact allows the muscle to contract via indirect nerve or direct muscle stimulation. Comparison between the forces elicited by these two methods reveals whether synaptic transmission failures occur.

Related SOPs include: measuring isometric force of isolated mouse muscles *in vitro*. SOP ID DMD\_M.1.2.002; measuring isometric force of isolated mouse skeletal muscles *in situ*. SOP ID DMD\_M.2.2.005.

## 3. CAUTIONS

Dissection needs to be done carefully to ensure the integrity of the nerve-muscle connection. Avoid touching the muscle or nerve with the dissection tools and avoid pulling or stretching the nerve during the dissection. When connecting the muscle to the force transducer, avoid excessive pulling. The bath solution should be well oxygenated with 95%O<sub>2</sub>/ 5% CO<sub>2</sub> (for 3-5 min before use) and force transducer should be calibrated regularly according to the manufacturer's manual to ensure the accuracy of the measurement.

#### 4. MATERIALS

##### A. Force transducers

Typical force transducers include UC2, UC3, UTC3 (Gould and Statham) or FT03, FT10 (Grass). The resolution is usually 2-10mg and the maximum load is 20-60g for the force transducer.

##### B. Stimulator

The stimulators (S44, S88, Grass) provide adjustable voltage stimulations at variable frequencies. The duration of the square wave stimulation is usually between 0.1 and 0.5ms (0.1 ms is sufficient for stimulation of the nerve but not the muscle). If the force transducers are Gould UC series, one more stimulator will be needed to provide the DC power supply for the force transducer.

##### C. Electrode

###### a. Suction electrode for indirect nerve stimulation

Suction electrode is used to allow secure nerve-electrode contact. There are commercially available suction electrodes (A-M systems). You can also make your own suction electrode fairly easily (Johnson et al., 2007), and this is recommended since you should have a suction electrode that is tight fitting and thus is different in size for different ages of the mice.

###### b. Silver bipolar electrode for direct muscle stimulation

For smaller muscles during development, a silver bipolar electrode is sufficient to elicit contraction from all fibers. If adult muscle is used for the experiment, platinum plate field stimulation will be required (for more details, please refer to Measuring isometric force of isolated mouse muscles in vivo. SOP ID DMD\_M.1.2.002)

##### D. Ringer's solution bubbled with 95%O<sub>2</sub>/ 5% CO<sub>2</sub> before use.

Ringer's solution: 135 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 15 NaHCO<sub>3</sub>, 1 Na<sub>2</sub>HPO<sub>4</sub>, 11 D-glucose, 2.5 Calcium gluconate, in mM, pH 7.4. The CO<sub>2</sub> is essential and sets the pH with the bicarbonate. If the solution sits without bubbling, it will become basic.

E. Sylgard coated dish (Dow Corning: Sylgard).

F. Data acquisition system

It is preferred to have an analog-to-digital system to allow data to be recorded and analyzed in the computer. A typical system includes Digidata data acquisition system and Clampex software from Axon Instruments.

## 5. METHODS

A. Dissection

Mice are euthanized according to the animal protocol, usually by cervical dislocation or Nembutal injection (50 mg/kg bodyweight). Remove the skin, separate the hindlimb from the trunk by cutting between Pelvis and Femur, and place the hindlimb in the dish filled with oxygenated Ringer's solution. Pin the hindlimb to the dish with insect pins (Fine Science Tools, 26002-20). Remove other hindlimb muscles including gastrocnemius, soleus, and tibialis anterior. Carefully expose and isolate the sciatic nerve. The length of the nerve should be long enough (about 5mm) for the suction electrode. Care should be taken to keep the nerve-muscle connection intact without damage during dissection. The proximal tendon of EDL is kept attached to the bone. Tie two to three knots with 6-0 surgical silk at the distal tendons of EDL muscle. Make a loop with the other ends of the silk. Make sure the knots are tight. To confirm a successful dissection, stimulate the sciatic nerve with the suction electrode; the muscle should contract. In general, if the direct stimulation elicits a contraction, but the nerve stimulation is partially or not effective, the nerve may be damaged. Cut off proximal segments of the nerve, watching for muscle contraction when the nerve is cut with scissors. Keep testing the nerve as you cut closer to the muscle, until the nerve gives a full contraction.

*Force transducer*

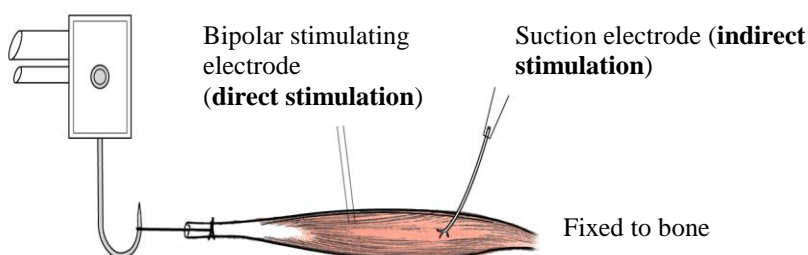


Fig. 1

B. Force measurement

- a. Pin the bone (proximal to EDL) firmly to the dish to prevent it from moving during tetanic contractions. EDL muscle is positioned horizontally and submerged in the Ringer's solution.
- b. Stimulate the nerve with the suction electrode and establish the threshold for eliciting muscle contraction (usually 0.2-0.5V, this depends on the tightness of fit of the suction electrode, stimulation duration 0.1-0.5ms, 0.5-1 Hz stimulation frequency). Suprathreshold stimulations should be used for following nerve stimulations. (Normally double the threshold voltage.)
- c. Connect the force transducer to the EDL via the loop at the distal tendons. See Fig. 1 for the schematic diagram of the setup.
- d. Stimulate the nerve with single twitches and adjust the muscle length by moving the transducer with a micromanipulator until the maximum tension is produced. This is typically about 20% greater than the slack length.
- e. Stimulate the nerve via suction electrode at various frequencies (up to 250 Hz for a period of 1-2 seconds). Rest the muscle for 30 seconds in between stimulations. Rest for two to three minutes before direct muscle stimulation. Note that the firing patterns of hindlimb muscles in awake, behaving rodents were described by Hennig and Lømo (1985).
- f. For direct muscle stimulation, the stimulation intensity may be higher than the nerve stimulation and the stimulus duration should be increased to about 1 ms. Adjust the stimulation intensity until the maximum tension can be elicited.
- g. Stimulate the muscle via direct stimulation electrodes at various frequencies. Rest the muscle for 30 seconds in between stimulations.
- h. Once the frequency-force relationship is established, the maximum tetanic force can be elicited at a given frequency (which elicits the plateau tension) via direct muscle stimulation for 2-3 times with 3-5 minutes rest between stimulations.
- i. Muscles can be fixed or frozen for following morphological or biochemical studies.

C. Specific force calculation

To calculate the specific force, after the force measurement, muscles can be weighed or sectioned to allow the maximum tetanic force to be normalized to the muscle weight or the cross-section area. Histological examination is recommended for muscle sections. In some diseases, muscle fibers atrophy and muscle is occupied by fat and connective tissue. Although this has not been reported in current SMA mouse models, it is important to examine the muscle morphology to avoid error in estimating muscle size. If necessary, count

the total number of muscle fibers and measure the size of individual muscle fiber to allow a better estimation of muscle size.

## 6. EVALUATION AND INTERPRETATION OF RESULTS

In healthy muscles, synaptic transmission will be faithfully converted to muscle contraction; thus, muscle tension elicited by indirect nerve or direct muscle stimulation should in theory be equal. In practice, the ratio of indirect / direct stimulation evoked muscle force will be close to one. However, for diseases that exhibit loss of NMJs or severe defects in NMJ synaptic transmission, failures in nerve-elicited muscle contraction may occur, and the indirect / direct stimulation evoked muscle force will be less than one.

This method is useful for assessing overall NMJ transmission in a given muscle in SMA mouse model. Intracellular recording often reveals a reduction in synaptic efficacy in SMN $\Delta$ 7 mice (Kong et al., 2009, Ruiz et al., 2010; Ling et al., 2010). Since NMJs have a high safety factor, meaning they normally release 2-10 times more neurotransmitter than required to depolarize the muscle to threshold for a muscle action potential (Wood and Slater, 2001), the effect of the reduced synaptic efficacy needs to be evaluated with this protocol. For example, in EDL muscle of SMN $\Delta$  7 mouse model, the quantal release is reduced by 25%, but it doesn't result in transmission failure since the ratio of indirect / direct stimulation evoked muscle force is close to one (Ling et al., 2010). This method also provides a functional readout for denervated muscles and could serve as a quick functional assay for evaluating the effectiveness of drug treatment.

SMA muscles are usually much smaller and produce less force compared to WT mice. Maximum tetanic force is around 1.5 - 3g for non-SMA and 0.5 -1.5g for SMA mice (P12-P14, SMN $\Delta$  7 mouse model). The force variations arise from variations in muscle size and animal disease progression. To calculate the specific force, we normalize the maximum tetanic force to the muscle cross-section area (muscles are usually too small to obtain accurate weight). The EDL cross-section area is typically around 0.5 mm<sup>2</sup> for non-SMA and 0.2 mm<sup>2</sup> for SMA mice. By calculating the specific force, one can assess whether the reduced muscle force is attributed to difference in muscle size or to defects in muscle contractile property. The specific force of EDL is around 4 g/mm<sup>2</sup> (or 40 kN/m<sup>2</sup>) for both non-SMA and SMA mice. The typical value of specific force in adult mice is around 400 kN/m<sup>2</sup> (Gonzalez et al., 2000). The smaller specific force in P12-14 mice reflects weaker force production from the immature muscle fibers (Dangain et al., 1992; Dangain et al., 1993). Thus, in EDL muscle of SMN $\Delta$  7 mouse model, the decrease in muscle force by direct muscle stimulation is not due to defects in intrinsic muscle contractile capability, but due to the smaller size.

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