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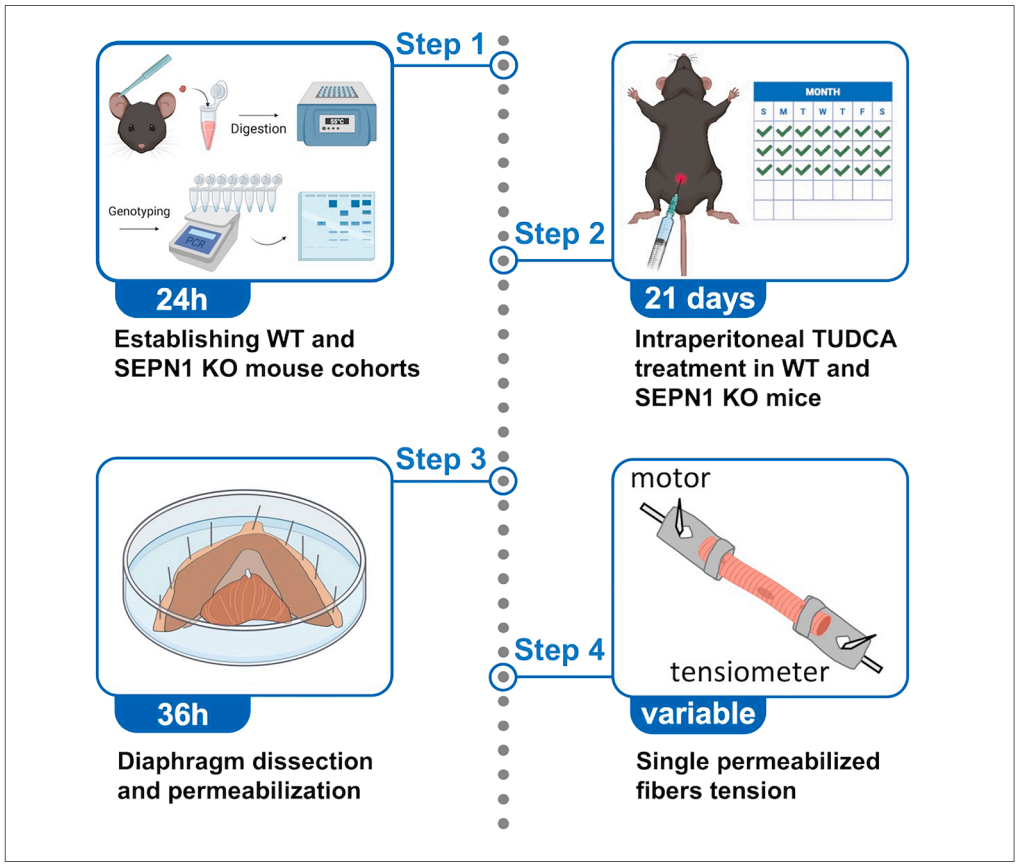
Protocol for measuring force in skinned diaphragm muscle fibers of myopathic SEPN1 knockout mice following chronic tauroursodeoxycholic acid treatment



Selenoprotein N1 (SEPN1) is a type II endoplasmic reticulum (ER) glycoprotein. Loss-of-function mutations in the gene encoding for SEPN1 give rise to myopathy. Here, we present a protocol for evaluating the contractility of diaphragmatic muscle fibers of SEPN1 knockout mice following chronic treatment with tauroursodeoxycholic acid (TUDCA). We describe steps for genotyping SEPN1 knockout mice, TUDCA *in vivo* treatment, diaphragm dissection, and chemical permeabilization. We then detail procedures for single muscle fiber isolation and tension measurement.

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Highlights

Instructions for genomic DNA extraction from mouse ears and genotyping

Protocol for chronic treatment with tauroursodeoxycholic acid in SEPN1 KO mice

Steps for chemical permeabilization and isolation of diaphragmatic muscle fibers

Guidance on measuring normalized tension force in diaphragmatic fibers

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Protocol for measuring force in skinned diaphragm muscle fibers of myopathic SEPN1 knockout mice following chronic tauroursodeoxycholic acid treatment

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SUMMARY

Selenoprotein N1 (SEPN1) is a type II endoplasmic reticulum (ER) glycoprotein. Loss-of-function mutations in the gene encoding for SEPN1 give rise to myopathy. Here, we present a protocol for evaluating the contractility of diaphragmatic muscle fibers of SEPN1 knockout mice following chronic treatment with tauroursodeoxycholic acid (TUDCA). We describe steps for genotyping SEPN1 knockout mice, TUDCA *in vivo* treatment, diaphragm dissection, and chemical permeabilization. We then detail procedures for single muscle fiber isolation and tension measurement.

For complete details on the use and execution of this protocol, please refer to Germani et al.[1]

BEFORE YOU BEGIN

Timing: 1 h preparing time, 24 h polymer settling time

Mechanical studies on permeabilized muscle fibers are historical techniques[2] currently employed for investigating muscle contractility and other fiber properties.[3] A standard experimental setup involves mounting a single fiber, typically with aluminum T-clips, onto a system equipped with a force transducer, a motor for precise length control, and a series of buffer-containing baths. Permeabilization facilitates buffer exchange within the fiber, enabling precise control over the calcium concentration surrounding the contractile machinery. By varying external calcium levels, it is possible to determine both calcium sensitivity (force developed at submaximal calcium) and maximal force production (achieved at saturating calcium). Force measurements are then normalized to the fiber cross-sectional area (CSA) to calculate the tension.

SEPN1-RM loss-of-function mutations in the gene encoding SEPN1 give rise to an ultra-rare congenital myopathy SEPN1-RM, characterized by muscle weakness, fatigue and a life-threatening diaphragmatic weakness.[4,5] At the moment, two SEPN1 KO mouse models are available for the study

of this disease.[6,7] Both do not show an overt muscle phenotype. Still, we identified diaphragmatic muscle weakness in the ones characterized by Rederstorff et al., 2011.[6] Thus, we decided to further characterize the physio pathological basis of SEPN1 KO diaphragmatic weakness by implementing a protocol of fibers permeabilization.

The versatility of the protocol extends to several muscle tissues. While rabbit psoas muscle has historically served as a skeletal muscle model, murine hindlimb muscles have gained popularity due to the availability of numerous genetic models and the potential of fiber-type-specific investigations. Furthermore, this permeabilized fiber assay can be adapted to human-skinned muscle biopsies. The flexibility to analyze muscle fibers from different species, including humans, under consistent technical conditions, underscores the translational relevance of these analyses, rendering it a valuable tool for both fundamental and clinical research on muscle contractility.

Therefore, we applied this technique to examine the defect in the diaphragm function of the SEPN1 KO mouse and reveal the effect of drug treatment on the pathological phenotype. As ER stress was detected in the diaphragm of SEPN1 KO mouse models[7] and TUDCA, a pan ER stress inhibitor, was already safely employed as treatment in several neurological and non-neurological diseases,[8–10] we proceeded with a chronic TUDCA treatment for three weeks in SEPN1 KO mice.

The section below lists the Materials necessary to implement this protocol. The required equipment appears in the key resources table.

1. Prepare Sylgard bottom 60 mm petri dishes for diaphragm pinning and single fiber dissection.
   a. Supply materials: SYLGARD 184 Silicone Elastomer, 60 mm petri dishes (Sarstedt item 83.3901.500), 13 mm glass coverslip (Fisher Scientific 11588492),
   b. measure the two Sylgard components to obtain a volume of approximately 6 mL for each petri to be prepared,

   Note: The additional volume is required since the silicone elastomer is dense, and a certain amount of product will remain adherent to the mixing container walls.

   c. thoroughly mix the two components,
   d. pour about 6 mL of the combined mixture into the petri dishes, bubbles will come to the surface and disappear in about 10 min,
   e. when bubbles are gone, place a 13 mm round clean glass coverslip on a side over the polymerizing polymer,
   f. place the closed petri dishes to polymerize for 12 h at 37°C.

   Note: The inclusion of a glass coverslip provides a sturdy surface for securing aluminum T-clips in petri dishes utilized for single fiber preparation, while the soft polymer in the rest of the dish offers a soft material to pin diaphragm strips in place.

2. Prepare aluminum T-clips (Figure 1 and Methods video S1 T-clips making).
   a. Supply materials: prepare standard household foil aluminum foil sheet (thickness of about 0.016 mm), tweezers, stereo microscope with 8× magnification recommended, standard microscope glass slide.
   b. Wrap a small portion of the aluminum foil around the microscope glass slide,
   c. cut the squared centered hole by performing two small parallel cuts (Figure 1B) and two more perpendicular to those (Figure 1C),
   d. proceed with making two parallel cuts which will separate the T-clip body (Figure 1D),
   e. add two perpendicular cuts that will form the wings and the clip bottom (Figure 1E),

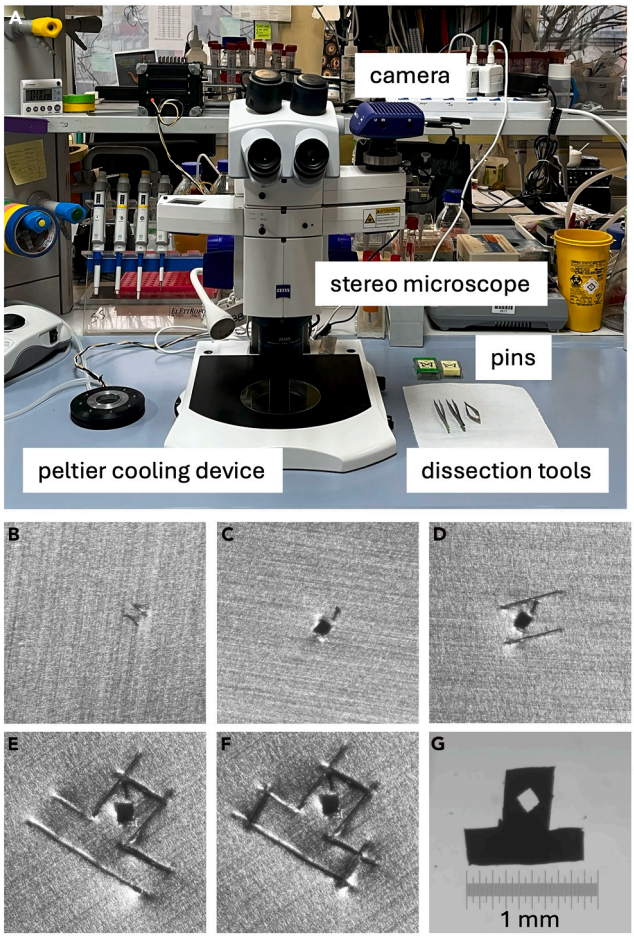

Figure 1. Stereo microscope setup and T-clip making process

(A) Stereo microscope ZEISS Discovery. V8 equipped with 1× lens (higher magnification) or 0.63× lens (larger field and larger working distance), camera Axiocam 208 color mounted on an Intermediate Photo Tube SteREO Discovery to equally split the view for teaching purposes, custom made Peltier cooling device, metal pins and dissection tools.

(B–F) Steps for the making of an aluminum T-clip taken under the stereomicroscope.

(G) Final size of a T-clip in the range of 0.9 mm wide open.

f. connect the previous cuts to isolate the clip (Figure 1F),
g. carefully isolate the clip from the aluminum foil.

Note: To cut the aluminum foil a single arm of a tweezer is used. The tweezer is divided in half and the tip is sharpened to obtain a thin blade. The cutting edge will wear out with use and will need to be sharpened using a sharpening stone, such as the one provided by Fine Science Tools (item 29000-00). Reference of aluminum T-clip can be found in Goldman and Simmons.[11]

Institutional permissions

Procedures were conducted in conformity with ARRIVE 2.0 principles and the following laws; Italian Governing Law (D. lgs 26/2014, authorization number 19/2008-A issued 6 March 2008 by Ministry of Health; 485/2018, 774/2019, 764/2019-PR authorization to Ester Zito. All the experiments were performed on males. Animals were housed in IVC cages under standard laboratory conditions (22°C ± 2°C, 50% ± 10% humidity, 12 h light/dark cycle) and had ad libitum access to food and water.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Calcium chloride	Carlo Erba Reagents	328357
Calcium carbonate	Sigma-Aldrich	239216
Direct PCR (tail)	Viagen	102-T
Disodium adenosine triphosphate (ATP)	Sigma-Aldrich	A2383
Disodium creatine phosphate	Sigma-Aldrich	2380
Dithiothreitol (DTT)	Sigma-Aldrich	DTT-RO
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)	Sigma-Aldrich	03777
Glycerol	Sigma-Aldrich	G2025
Glutaraldehyde 25% aqueous solution	Electron Microscopy Sciences	16220
L-glutathione reduced (GSH)	Sigma-Aldrich	G6013
Hexamethylenediaminetetraacetic acid (HDTA)	TCI Chemicals	D2019
Imidazole	Sigma-Aldrich	I2399
Magnesium acetate	Sigma-Aldrich	M5661
Phosphatase inhibitor tablets	Sigma-Aldrich	PHOSS-RO
Potassium phosphate monobasic	Sigma-Aldrich	P0662
Potassium propionate	Sigma-Aldrich	CDS000543
Protease inhibitor tablets	Sigma-Aldrich	11836170001
Protein kinase K (PK)	Sigma-Aldrich	P8044
Quick-Load Taq 2× master mix	New England Biolabs	M0271L
Sodium azide	Sigma-Aldrich	S2002
Tauroursodeoxycholic acid, sodium salt	EMD Millipore	580549
Toluidine blue	Sigma-Aldrich	T3260-5G
Tris(Hydroxymethyl)aminomethane (Tris)	Carlo Erba Reagents	489983
100 bp DNA ladder	New England Biolabs	N3231S
Experimental models: Organisms/strains		
SEPN1 KO male mice 5-month-old	EMMA repository	(SELENON<tm1.2Mred>/Orl)
Software and algorithms		
Prism	GraphPad	version 10.0.2
Real-Time Muscle Data Acquisition and Analysis System	Aurora Scientific	600A
Other		
Aesculap rubber dam punch	B. Braun Aesculap	DF401R
Dumont #5 forceps	Fine Science Tools	11295-10
Electrophoresis package	Thistle Scientific Ltd.	MSMAXIDUO-PP300
Konus stereo microscope	Konus Italia Group S.p.a.	#5424
Mastercycler nexus	Eppendorf	EP6333000049
Metal pins 0.15 mm	Pin Service (Czech Republic)	01.03.01
Microcentrifuge tubes 1.5 mL	Eppendorf	HS4323
Permeabilized fiber system temperature jump	Aurora Scientific	1410A
STOCK Selenon$^{tm1.2Mred}$/Orl (mouse model)	EMMA repository	EM:02138
SYLGARD 184 silicone elastomer	Dow	
Thermomixer compact	Eppendorf	5350
Zeiss SteREO Discovery.V8	Carl Ziess Microscopy GmbH	
0.2 mL thin walled tubes with flat caps	Thermo Fisher Scientific	AB-0620
0.22 µm syringe filter	Starlab	E4780-1223
0.5 mL 30G syringe	Chemil	S005SSM30G
60 mm Petri dishes	Sarstedt	82.1194.500

MATERIALS AND EQUIPMENT

Proteinase K (PK) buffer for ear lysis

Reagent	Final concentration	Amount
Tris 1 M pH 8	50 mM	100 μL
$CaCl_2$ 1 M	3 mM	6 μL
ddH_2O	N/A	1894 μL
Proteinase K (PK)	0.2 μg/μL	20 mg
Total	N/A	2 mL

Divide in 200 μL aliquots and store at −20°C for up to 1 year.

Digestion buffer for ear lysis

Reagent	Amount per sample
Direct PCR	294 μL
Proteinase K (PK) buffer	6 μL

Reaction master mix for SEPN1 PCR genotyping

Reagent	Sequence	Amount
Quick Load Taq 2×	N/A	5 μL
Primer FW	TCCAATGACGTCAGGCTGTGACTTGC	0.35 μL
Primer RV	GGATCAGTAGAAAGTACC	0.35 μL
dd H_2O	N/A	3.3 μL
Total per sample	N/A	9 μL

TUDCA solution (0.05 mg/μL)

Reagent	Amount
Tauroursodeoxycholic acid powder (MW 521.7)	0.05 g
Water for injection	1 mL

Skinning buffer 1

Reagent	Final concentration
Potassium propionate	150 mM
K-EGTA	5 mM
Potassium phosphate	5 mM
Magnesium Acetate	5 mM
Sodium ATP	2.9 mM
DTT	2 mM
Sodium azide	0.25 mM
Glycerol	10%
protease inhibitor	as suggested by the producer
phosphatase inhibitor	as suggested by the producer

Adjust the volume in water, pH 7.1 at 10°C. Store at −20°C for up to 12 months.

Skinning buffer 2

Reagent	Final concentration
Potassium propionate	150 mM
K-EGTA	5 mM
Potassium phosphate	5 mM
Magnesium Acetate	5 mM
Sodium ATP	2.9 mM
DTT	2 mM

(*Continued on next page*)

Continued	
Reagent	Final concentration
Sodium azide	0.25 mM
Glycerol	50%
protease inhibitor	as suggested by the producer
phosphatase inhibitor	as suggested by the producer
Adjust the volume in water, pH 7.1 at 10°C. Store at −20°C for up to 12 months.	

Rigor buffer for skinned fibers tension measurement	
Reagent	Final concentration
Imidazole	25 mM
Potassium Propionate	100.3 mM
K-EGTA	10 mM
GSH	10 mM
Magnesium Acetate	1.5 mM
Adjust the volume in water, pH 7.0 at 20°C. Store at −20°C for up to 6 months.	

Relax buffer for skinned fibers tension measurement	
Reagent	Final concentration
Imidazole	25 mM
Sodium creatine phosphate	20 mM
Potassium Propionate	11.8 mM
K-EGTA	10 mM
GSH	10 mM
Magnesium Acetate	6.89 mM
Disodium ATP	5.56 mM
Adjust the volume in water, pH 7.0 at 20°C. Store at −20°C for up to 6 months.	

Pre-Activating buffer for skinned fibers tension measurement	
Reagent	Final concentration
Imidazole	25 mM
Sodium creatine phosphate	20 mM
Potassium Propionate	11.7 mM
GSH	10 mM
HDTA	9.9 mM
Magnesium Acetate	6.48 mM
Disodium ATP	5.56 mM
K-EGTA	0.1 mM
Adjust the volume in water, pH 7.0 at 20°C. Store at −20°C for up to 6 months.	

Activating buffer for skinned fibers tension measurement	
Reagent	Final concentration
Imidazole	25 mM
Sodium creatine phosphate	20 mM
Potassium Propionate	11.7 mM
GSH	10 mM
Ca-EGTA	10 mM
Magnesium Acetate	6.39 mM
Disodium ATP	5.65 mM
Adjust the volume in water, pH 7.0 at 20°C. Store at −20°C for up to 6 months.	

Skinning buffers compositions were adopted from De Napoli et al.[12] Rigor, relax, pre-activating and activating buffers composition were adopted from Fusi et al.[13]

⚠ CRITICAL: Ca-EGTA solution should be prepared from a stock of Calcium carbonate solution to be added to an equimolar amount of EGTA powder. The solution needs to be heated (avoid boiling) to facilitate complete dissolvement of the chemicals, during the process a watch glass should be put on top of the beaker to avoid excessive evaporation and preserve volume.

Single fiber fixation solution	
Reagent	Final concentration
Glutaraldehyde	8% v/v
Toluidine Blue	5% v/v
Fixation solution is described in Hilber and Gallager.[14]	

⚠ CRITICAL: Some components can be harmful, toxic or irritant. It is recommended to inspect the related MSDS information and adopt the suggested handling modalities according to the specific substance considered.

STEP-BY-STEP METHOD DETAILS

SEPN1 KO mouse genotyping procedure

Timing: 20–24 h (from step 1 to step 11)

Timing: 16 h (for steps 2 and 3)

Timing: 45 min (for steps 4–6)

Timing: 4 h (from step 7 to step 11)

In this section we describe how to collect and extract genomic DNA from mice ears and how to genotype by PCR the SEPN1 KO mice using specific primers designed to target exon 3.[6]

1. Ears collection at 21 days of age,
   a. Immobilize each mouse following the correct handling.
   b. By using an ear punch for mice, remove a small piece of ear and put it in a 1.5 mL tube.

   Note: When removing the small piece of ear, pay attention to cutting in different positions of the ear, because ear holes are used to create an individual numbering system for a unique identifier of each animal.

2. The small pieces of ear are digested,
   a. Pre-heat the thermoblock at 55°C.
   b. Prepare the digestion buffer as indicated in the material section for the number of ears to digest and exceed the number by three.
   c. Gently mix the digestion buffer before using.
   d. Add 300 μL of digestion buffer in each 1.5 mL tube containing a piece of ear.
   e. Put the tubes in the thermoblock overnight at 55°C with shaking at 900 rpm.

   Note: The shaking is not fundamental, but it assures the digestion of the piece of ear. If a thermoblock with shaking function is not available, do make sure that the piece of ear is fully immersed in the digestion buffer before heating.

3. Inactivation of Proteinase K (PK),
   a. The following morning the pieces of ear will be fully digested. Before proceeding with the genotyping PCR, PK must be inactivated.
   b. Stop the thermoblock from shaking and set the temperature at 85°C.
   c. Leave samples at 85°C for 35 min.
   d. After inactivation, store samples at +4°C.
4. SEPN1 genotyping by PCR,
   a. Add 9 μL of PCR reaction master mix to each 200 μL PCR tube.
   b. Add 1 μL of the ear lysate to the 200 μL PCR tube.
   c. Close the tubes and spin them for 10 s.
   d. Load the samples into the PCR mastercycler and start the SEPN1 genotyping program.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	35
Annealing	56°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	10°C		

5. Prepare 1.5% agarose gel containing GelRed (1:30000).
6. Load 10 μL of each sample in the agarose gel wells and 5 μL of 100 bp DNA ladder.
7. Run the gel for 30 min at 150 V.
8. Detect the DNA bands using a UV trans-illuminator (Figure 2).

TUDCA *in vivo* treatment

Timing: 21 days

In Germani et al.[1] we highlighted the role of ER stress in the pathogenesis of SEPN1-RM. Thus, we decided to treat SEPN1 KO mice with the pan ER stress inhibitor TUDCA to see whether the reduction of ER stress might improve the muscle phenotype.

9. Mice randomization:
   a. Weight WT and SEPN1 KO mice at five months of age,
   b. Prepare the randomized sham and TUDCA-treated cohorts based on body weight.
10. Prepare the TUDCA solution.
   a. Dissolve TUDCA powder in sterile water.
   b. Filter TUDCA solution under a sterile hood using a 0.22 μm syringe filter.
   c. inject 0.5 mg of drug/g of mouse daily for three weeks.

Note: TUDCA resuspended in water for injection at a concentration of 0.05 mg/μL must be stored at +4°C (for a maximum of 48 h).

11. Intraperitoneal injection of water (placebo) and TUDCA treatment.
   a. Remove in advance the solution from the fridge to have it at room temperature by the time of the injection.
   b. Prepare the syringe with the amount of TUDCA solution based on mouse body weight (e.g. if the mouse's weight is 30 g, the syringe must be filled with 300 μL of solution).

⚠ CRITICAL: It is fundamental to remove all the bubbles that could have formed inside the syringe before proceeding with the intraperitoneal injection.

c. Handle each mouse following the correct handling procedures.
d. Proceed with the intraperitoneal injection preferring the lower right quadrant as the site of injection to avoid any possible damage to the abdominal organs.
e. After the injection, monitor the animal for a few minutes to make sure there are no complications.

Note: It is important to check on mice's health for the duration of the treatment. If wounds originate at the injection site, proceed to disinfect the wound and inject in the lower left quadrant. If mice undergo loss of weight due to the injection during the first week, be sure that the situation remains stable and that mice restart to gain weight during the other two weeks of treatment.

Diaphragm dissection and chemical permeabilization procedure

Timing: 24–36 h

12. Sacrifice the animal according to the ethics and regulations active in your Institution,
13. Carefully dissect the full diaphragm with the minimum amount of cage ribs needed to keep it flat,
    a. Rinse the diaphragm by gently submerging it a few times in a beaker containing cold Skinning buffer 1 to remove any blood remains,
    b. Secure the diaphragm on a SYLGARD coated 60 mm petri dish filled with fresh cold Skinning buffer 1 using 0.2 mm metal pins,
    c. Seal the petri dish with parafilm to avoid buffer leaks,

⚠ CRITICAL: Metal pins should be placed within the outer cage rib and not piercing the thin diaphragm fibers.

⚠ CRITICAL: All buffers need to be at 4°C to ensure that samples are not exposed to temperature shocks.

14. Place the petri dish at 4°C with gentle shaking for 24 h,
15. Skinning buffer 1 is removed and replaced with Skinning buffer 2,
16. Samples are gently shaken for 2 h at 4°C before moving them to −20°C.

⚠ CRITICAL: Samples in Skinning buffer 2 are viable for several weeks, but the evaporation of aqueous buffer at −20°C could cause osmotic stress. Check periodically the buffer level and replace it with fresh Skinning buffer 2 if evaporation occurs.

Note: The permeabilization process requires about 2 weeks in the storage buffer at −20°C to be complete. In case the samples need to be measured earlier, a wash in a cold relaxing solution containing 0.25% Triton X-100 for 5 min is recommended.

Single muscle fiber isolation and tension measurement

Timing: variable

Force measurement on single skinned skeletal muscle fibers of murine diaphragm. This procedure is performed using the Aurora Permeabilized Fibers setup 1410A. Clipped fibers are mounted to two

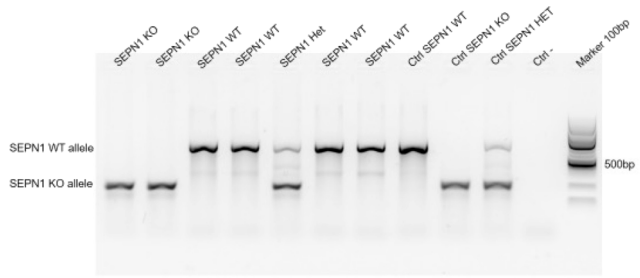

Figure 2. SEPN1 PCR genotyping representation

Representative results of a SEPN1 PCR genotyping samples of a cohort of seven weaned mice run on agarose gel (1.5%). SEPN1 WT allele refers to the WT 682 bp amplicon, SEPN1 KO allele refers to the KO 209 bp amplicon. Ctrl SEPN1 WT refers to a known SEPN1 WT sample, Ctrl SEPN1 KO refers to a known SEPN1 KO sample, Ctrl SEPN1 HET refers to a known SEPN1 HET sample, Ctrl- refers to a blank (a PCR sample without DNA) and Marker 100 bp is a DNA ladder.

hooks, one connected with the force transducer (Aurora 403C) and a motor (Aurora 315D). The fiber is still while a temperature controlled motorized bath system allows the change in buffer.

17. Mechanical isolation of single fibers,
    a. retrieve the skinned diaphragm preserved in glycerol storage skinning buffer at −20°C and let it warm to 4°C for 30 min,
    b. cut a strip along the fiber direction of about 2 mm in width in one of the two sides of the muscle,
    c. transfer the strip to a new Sylgard-bottom 60 mm petri dish,
    d. pin each end of the strip on Sylgard polymer covered bottom using the 0.15 mm metal pins to maintain it lightly stretched,
    e. mechanically dissect a single fiber using fine forceps by gripping a fiber end and gently pulling the fibers towards its length direction. The movement should alternate a slow pull and a gentle release until the fiber is released.

⚠ CRITICAL: Isolated fibers should only be moved in the petri by holding their extremities and with great care.

Note: Forceps might not be ideal for single fiber dissection as they are purchased, we encourage researchers to shape their forceps tips to their preference using a sharpening tool kit (e.g. Fine Science Tools item 29000-00, recommended forceps Fine Science Tools item 11254-20 or similar).

Note: The dissection/mounting process may require some time depending on the operator experience and skill, to avoid deterioration of the sample we recommend performing this step while maintaining the sample cool. This cooling can be achieved by placing the dish on an iced cooled aluminum surface or by using a cooling bath circulator. In our lab, we developed a cooling device that maintains the petri dish at low temperature using a Peltier module controlled by an Arduino (Figure 3). This system is built with circular elements that allows the background illumination of the stereo microscope base to reach the fibers.

18. Clipping a single fiber on aluminum T-clip,
    a. move the single fiber on top of the 13 mm glass coverslip that lies on the surface of SYLGARD bottom petri dish (see "Prepare aluminum T-clips" above),
    b. secure the fiber in the middle of the aluminum T-clip by gently closing the "wings" on both fiber ends (see Methods video S2 - fiber clipping procedure),

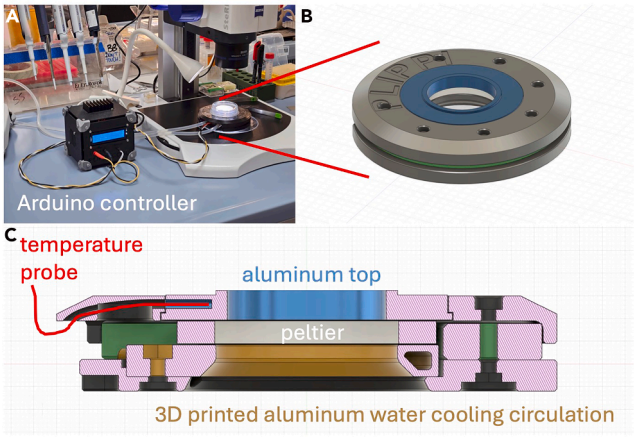

Figure 3. Peltier cooling device

Custom made cooling system designed to keep the 60 mm Petri dish at low temperature during single fiber dissection. The system is composed of a Peltier device, temperature sensors and an Arduino to be the PID controller (A). A 3D printed aluminum circular circuit (B and C) connected to a water chiller removes the excessive heat from the lower Peltier side. The system allows background illumination as both the Peltier and the cooling aluminum components are circular. Designed using Autodesk Fusion 360 licensed to LN.

Note: Ensure that the fiber is centered on the clip as shown in Figure 4, sharp aluminum edges may damage the fiber integrity and it could break during handling, mounting or activation.

19. Moving the fiber from the dissection stereo microscope to the Aurora setup,
    a. prepare the multi bath system of the Aurora setup by filling all baths with relax buffer except for the second one which will be filled with rigor buffer,
    b. set the baths temperature to 6°C,
    c. transfer the clipped fiber from the dissecting petri dish to the first bath of the Aurora setup using a thick aluminum foil bent as a small stretcher,
    d. using the forceps the T-clips are mounted on the transducer hook and on the motor hook (Figure 4).
    e. lightly stretch the fiber by moving the motor micrometer so that it is not slack (sarcomere length about 2.3 μm),
    f. set the bath controller to move in the second bath so the fiber is placed in rigor buffer,
    g. incubate the fiber in rigor buffer for at least 5 min, or until the developed tension stabilizes,

Note: Excessive tension increase can be avoided by gently shortening the fiber length.

20. Single fiber ends fixation,
    a. apply 0.6 μL of crosslinking buffer[14] (about 0.3 μL each side) to each fiber end, at the level of the aluminum T-clip,

Note: The crosslinking buffer is dense, and it will pour from the pipette tip into the solution as a continuous flow. Direct the descending flow on top of each T-clip while the excess will fall within the bath bottom.

    b. wait 5 to 10 s to allow the cross linking to stabilize the fiber ends,
    c. proceeds with moving the fiber in the subsequent baths to wash out the excessive crosslinking buffer, each wash is about 10 s while at each step, a down-up of the bath tray during this step improves washing efficiency,
    d. a blue shade should be visible at the fiber ends just after the procedure (Figure 5A).

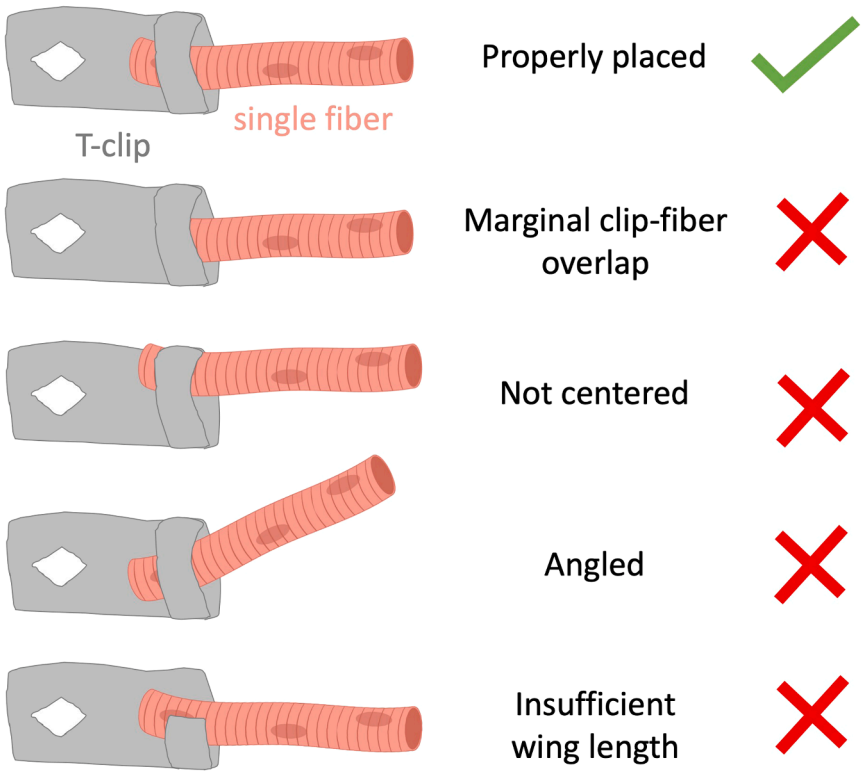

Figure 4. Single fiber T-clip placement scheme

The scheme is showing the proper fiber placement when clipping on aluminum T-clips. Fiber should be centered, straight and with an overlap of about the same width of the clip body. Designed using PowerPoint and BioRender licensed to LN (University of Padova).

Note: Fiber might be fixed also in regions that are not blue. Perform the fixation quickly avoids glutaraldehyde diffusion and nonspecific fiber cross linking. The blue color might only be visible right after the fixing procedure, while it will fade with washes and subsequent buffer exchanges.

21. Measurement of single fiber tension,
    a. move the bath system so that the fixed fiber is in a clean bath filled with relax buffer,
    b. clean and rinse with water all other baths while setting the low temperature to 6°C on baths 3 to 8, while the baths 1 and 2 are set to 21°C.
    c. evaluate sarcomere length (Figure 5B) using the Aurora camera system setting it to 2.6 μm adjusting the motor lever,
    d. measure fiber diameter for normalization purposes and fiber length,
    e. in the Aurora 600A calibration setting impose the current passive tension measured by the tensiometer to zero (Fin) and insert value for initial sample length (Lin),
    f. position the fiber in relax buffer in bath 6 to 8 while preparing subsequent baths with the following buffer order: relax (bath 1, 21°C), activating (bath 2, 21°C), activating (bath 3, 6°C), pre-activating (bath 4, 6°C), relax (bath 5, 6°C),
    g. incubate the fiber in pre-activating buffer for at least 1 min by moving to bathe 4,
    h. move the fiber in the low temperature activating buffer (bath 3) and observe the increase in tension,
    i. once the tension stabilizes to its maximal level, move the fiber to high temperature activation (bath 2),
    j. when the plateau is reached, impose a rapid shortening of 10% fiber length.

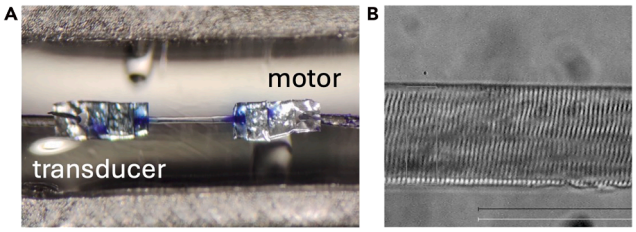

Figure 5. Single skinned fiber mounted on the Aurora 1410 permeabilized fiber setup

(A) A single fiber mounted on the aluminum T-clips and on the setup, on the left the force tensiometer hook while on the right is the motor hook. The blue color at each end is due to the crosslinking procedure described.

(B) Sarcomeres of a permeabilized fiber visualized on the microscope using the Aurora camera system.

k. move the fiber in a fresh relax buffer (bath 1).

Note: Fiber will develop maximal tension very quickly in high temperature activation buffer, set the transition timing and/or manual transition so that the time spent in high tension is reduced to the minimum once the plateau is reached to avoid sample rundown.

Note: Double check fiber sarcomere length after the development of maximal tension to ensure that it remained unchanged since the once set earlier. If this does not apply and the sarcomere length is significantly shorter, fiber might have slipped out of the clips. To solve that we suggest checking the troubleshooting section below.

⚠ CRITICAL: The rapid shortening of 10% fiber length should cause the tension to drop to nearly zero, if this does not verify check the troubleshooting section.

Note: Activating fibers in low temperature allows calcium to flow in while limiting shear damage due to the progressive contraction. Then, transferring the activated fiber to high temperature allows the measurement of physiological tensions. The technique is referred to as "temperature-jump" and it has been classically introduced by Ranatunga.[15]

EXPECTED OUTCOMES

The outcome of this analysis is the measurement of normalized tension produced by a skinned fiber under saturating calcium concentration. In addition to this, fiber diameter and cross-sectional area (CSA) can be measured. These values can be compared with cryosections obtained from analogous samples to ensure that the subset of measured fibers is representative of the tissue. It must be noted that skinned fibers are susceptible to swelling,[16,17] so appropriate corrections need to be considered. In this regard, an interesting comparison between skinned and intact muscle preparations has been reviewed by Lewalle and coworkers.[18] Typical diameter values for mouse diaphragm fibers are around 30 μm leading to a cross-sectional area in the range of 730 μm^2, while fibers from tibialis and gastrocnemius are larger at around 50 μm, with a CSA around 1960 μm^2. Absolute force produced by skinned diaphragm fibers is in the range of 0.08 mN. Normalized force of permeabilized diaphragm fibers should be expected to be around 100 mN/mm^2, while tibialis fibers are usually stronger at around 120–150 mN/mm^2. Permeabilized fiber sarcomere length is usually at 2.1 μm while slack, and it should be stretched to 2.5–2.6 μm before tension measurement as indicated previously. In our analysis, tension is estimated by dividing the force produced by the fiber (mN) for its cross-sectional area (mm^2). GraphPad Prism (version 10.0.2 for Mac, GraphPad Software, Boston, Massachusetts USA) was used to perform the statistical analysis and graph representation.

LIMITATIONS

A limitation of the current approach is that skinned fibers might be selected during the mechanical isolation based on their structural integrity. Consequently, damaged fibers are less likely to be

extracted from the muscle and more susceptible to damage during handling and measurement. Therefore, the quality of the skinned sample and the operator experience are crucial for obtaining reliable results. This limitation is particularly relevant in heterogeneous samples, such as those with dystrophy, where the presence of both healthy and deteriorated fibers may necessitate several measurements from multiple animals to ensure robust findings (see troubleshooting problem 5).

TROUBLESHOOTING

Problem 1

Amplicon bands on the agarose gel are barely visible, or absent.

Potential solution

Barely visible bands can be due to the low purity of the sample. Ensure to inactivate PK by keeping the samples at 85°C for 35 min and start counting them when the thermomixer temperature reaches 85°C, not before. After digestion, keep the samples at +4°C for optimal storage. Another cause of low purity can be insufficient Mg^{2+}, the Taq polymerase indicated in the key resources table is supplied with additional $MgCl_2$ if required.

Problem 2

Mice lose weight during the first week of treatment.

Potential solution

Mice may lose weight for two main reasons: stress due to human handling and puncture, or the effects on body weight and lipid metabolism of TUDCA administration.[10,11] If weight loss is related to any of these reasons, it should be minimal and recovered in the next two weeks of treatment.

Problem 3

Skinned fibers slip out of the aluminum T-clips during full activation.

Potential solution

Fibers must be clipped using the right amount of pressure to secure them, but without smashing the fiber underneath by excessive force. Damaged/crushed fibers are not as stable as safely secured ones, even if the crosslinking procedure is properly performed. The process of dissecting and mounting single fibers is quite delicate and it requires the acquisition of a set of skills that might need a substantial amount of time and practice to consolidate. In addition to this, the problem might be caused by the fixing solution. Old glutaraldehyde might not readily fix the fibers end, causing instability and fiber detachment from the clip. To avoid this, aliquots the solution to be kept at −20°C while only thawing the amount needed in a measurement session.

Problem 4

Tension does not reach zero after the 10% fiber length rapid shortening.

Potential solution

The issue could be related to an error in estimating fiber length which causes a too small shortening. If the tension is evaluated using the Aurora 600A software Scope, keep in mind that function only records live data at 100 Hz, and the shortening event might be faster than that. To assess this, check on the raw data (usually sampled at 10 or 20 kHz) if the tension is still higher than zero. Also, extra compliance might be introduced by an inadequate fiber end cross linking or large aluminum T-clips, causing the tension to not properly drop. We suggest either repeat the cross-linking step and/or replace the crosslinking buffer[15] with a fresh one.

Problem 5

Fiber selection is biased due to the mechanical isolation procedure.

Potential solution

The process of single fiber isolation exerts a bit of mechanical force to extract the fibers from the small muscle bundle. This leads to the possibility that weak and damaged fibers are harmed by the user during the pulling releasing process, this issue could become more relevant in muscles of myopathic models. A proper skinning buffer and procedure would help in loosening the extracellular matrix to facilitate fiber extraction. A gentle pulling and releasing movement will provide unharmed fibers for analysis. In Germani et al.,[1] we successfully measured very small fibers with a diameter of about 30 μm, proving that the procedure is feasible. To obtain a proper representation of muscle contractility we suggest: i) a randomized fiber selection sampling several locations within the diaphragm, ii) analyze diaphragms of different animals iii) consider a blinded methodological approach.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Ester Zito (ester.zito@marionegri.it).

Technical contact

Questions about the technical specifics of the protocol should be directed to and will be fulfilled by the technical contacts: Serena Germani (serena.germani@marionegri.it) for animal genotyping and *in vivo* TUDCA treatment and Leonardo Nogara (leonardo.nogara@unipd.it) for the muscle permeabilization procedure and single fibers tension analysis.

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data generated by this study are available within this work and the related publication.[1] Thumbnails for the graphical abstract and figures for this study were prepared using BioRender.com, Microsoft PowerPoint, and Autodesk Fusion 360, licensed to L.N. (University of Padova).

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AUTHOR CONTRIBUTIONS

Conceptualization, L.N. and S.G.; writing – original draft, L.N., S.G., and C.D.N.; writing – reviewing and editing, L.N., S. G., C.D.N., B.B., and E.Z.; funding acquisition, B.B. and E.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103918.

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