jove

Collection of Skeletal Muscle Biopsies from the Superior Compartment of Human Musculus Tibialis Anterior for Mechanical Evaluation

Anthony L. Hessel[1,4], **Daniel Hahn**[1,2], **Markus de Marées**[3]

[1] Faculty of Sport Science, Human Movement Science, Ruhr University Bochum [2] School of Human Movement and Nutrition Sciences, The University of Queensland [3] Faculty of Sport Science, Sports Medicine and Sports Nutrition, Ruhr University Bochum [4] Institute of Physiology II, University of Muenster

Corresponding Author

Anthony L. Hessel
anthony.hessel@uni-muenster.de

Citation

Hessel, A.L., Hahn, D., de Marées, M. Collection of Skeletal Muscle Biopsies from the Superior Compartment of Human Musculus Tibialis Anterior for Mechanical Evaluation. *J. Vis. Exp.* (), e61598, doi:10.3791/61598 (2020).

Date Published

September 1, 2020

DOI

10.3791/61598

URL

jove.com/t/61598

Abstract

The mechanical properties of contracting skeletal fibers are crucial indicators of overall muscle health, function, and performance. Human skeletal muscle biopsies are often collected for these endeavors. However, relatively few technical descriptions of biopsy procedures, outside of the commonly used musculus vastus lateralis, are available. Although the biopsy techniques are often adjusted to accommodate the characteristics of each muscle under study, few technical reports share these changes to the greater community. Thus, muscle tissue from human participants is often wasted as the operator reinvents the wheel. Expanding the available material on biopsies from a variety of muscles can reduce the incident of failed biopsies. This technical report describes a variation of the modified Bergström technique on the musculus tibialis anterior that limits fiber damage and provides fiber lengths adequate for mechanical evaluation. The surgery is an outpatient procedure that can be completed in an hour. The recovery period for this procedure is immediate for light activity (i.e., walking), up to three days for the resumption of normal physical activity, and about one week for wound care. The extracted tissue can be used for mechanical force experiments and here we present representative activation data. This protocol is appropriate for most collection purposes, potentially adaptable to other skeletal muscles, and may be improved by modifications to the collection needle.

Introduction

The study of human muscle physiology for clinical or research purposes often requires muscle biopsies. For example, a major challenge in human muscle physiology and biomechanics is to distinguish between and understand the various adaptations of muscle performance to exercise. Performance adaptations do not just include structural adaptations (e.g., changes in contractile proteins, muscle architecture) but also include neural adaptations[1], which

are very hard, if not impossible, to assess separately when testing intact in situ human muscles. Fiber-level experiments remove these higher-order components and allow for a more direct evaluation of muscle contraction and can be collected via biopsy techniques. Muscle biopsies have been collected since at least 1868[2]. Today, the predominant technique to collect muscle biopsies is the modified Bergström technique[3,4,5], although other techniques are available including the use of a Weil-Blakesley conchotome[6] or the so called fine-needle[7,8]. All these techniques use special needle-like instruments that are designed to pass into muscle and cut a piece of tissue. Specifically, the modified Bergström technique uses a large modified needle (5 mm needle size here; **Figure 1**) that has a window close to the needle tip and a smaller internal trocar that moves up and down the needle, cutting the muscle when passing over the needle window. Within this hallow trocar is a ramrod that moves up and down the shaft of the trocar and pushes the biopsy towards the needle window. To pull the muscle into the needle window, a suction hose is attached, which sucks air out of the needle and pulls the muscle into the needle window via negative pressure.

Muscle biopsies are often acquired to study changes in protein content, gene expression, or morphology caused by disease or in a response to an exercise program[1,9,10,11]. Another critical use for muscle biopsies is mechanical experiments such as the measurement of fiber contractile force, muscle fiber stiffness, and history-dependent muscle properties[12,13,14,15,16]. Single fiber or fiber bundle mechanics are measured by attaching fibers between a length motor and force transducer on specialized rigs that control fiber length while simultaneously measuring force. By permeabilizing (e.g., skinning) fibers, the sarcolemma membrane becomes permeable to chemicals in the bath solution, allowing for activation control by varying calcium concentration. Furthermore, the effect of contractile properties on chemicals/pharmaceuticals/other proteins can easily be evaluated by adding the reagent in question to the bath solution. However, while this technique is highly used in other animal models, noticeably fewer studies conducted mechanical tests on skinned fibers from human muscle biopsies[17,18,19]. One reason is that the biopsy tools and protocols are designed to remove as much muscle tissue as possible with less regard for the level of structural damage sustained during tissue extraction. Indeed, a recent biopsy protocol suggests to drive the biopsy needle into the muscle and collect 2-4 chunks of muscle[3]. The process itself does little damage to the DNA or protein material, but often destroys fiber and sarcomeric structures in such a way that the activation of muscle fibers becomes unstable or impossible. Furthermore, the relative length of fibers within the biopsy are typically short (<2 mm) and not easily handled for mechanical testing. For mechanical testing, ideal fibers are long (3-5 mm) and not structurally damaged.

More advanced tissue extraction techniques can be used to limit fiber damage. For example, one group[20] took advantage of previously planned “open surgeries” of forearms (e.g., bone fracture repair), where the muscles were fully exposed and a surgeon was able to visualize the muscle structure and carefully dissect relatively large and structurally undamaged samples of muscle tissue (15 mm x 5mm x 5 mm). This “open biopsy” technique is favored when participants are undergoing a previously planned procedure, and so limits the pool of potential participants, especially for healthy adults, where no surgeries would otherwise take place. Thus, many biopsies conducted for research purposes are done as an outpatient procedure and the incision site is kept as small as possible to limit infection risk, scarring, and healing

time. Therefore, most biopsies are collected **blindly** (i.e., the operator is unable to see the collection needle as it passes through the fascia into the muscle). This implies that the quality of the biopsy is almost entirely based on the skill and experience of the operator. Every muscle has its own difficulties when collecting tissue, such as risks to violate nerves and blood vessels, selection of an ideal collection depth and location, and deciding on an appropriate body position to keep the muscle as slack as possible. Unfortunately, most of the muscle-specific skillsets are not written down and so each physician must "reinvent the wheel" when performing biopsies on muscles new to them. This lack of experience usually leads to several collections with low quality until the physician identifies the best practices for biopsies for that muscle. Novice physicians often learn the skill through conversations with their more experienced colleagues, but relatively few informative and peer-reviewed texts exist on the matter, especially for muscles that are not traditionally used for biopsy collection. If we consider the above information, along with the difficulty of recruiting human volunteers for biopsies, it is clear that more teaching information is needed that maximizes the chances of success for every participant.

Thus, the purpose of this paper was to present a muscle biopsy technique that provides protocols for the successful collection of muscle biopsies with long, undamaged fiber fragments for mechanical tests. Human muscle biopsies are usually carried out on, and the bulk of biopsy training material is on, the musculus vastus lateralis. Its relatively large muscle size and superficial location relative to the skin allows for the collection of adequate muscle tissue, while minimizing patient discomfort and physical trauma[1,21]. However, there are some limitations to using the vastus lateralis for longitudinal training studies. For example, during experimental protocols that include a training program, participants must refrain from additional training outside of the study for a period that often spans 2-6 months. For athletes, this is often not possible, as the vastus lateralis is usually trained during typical exercises (e.g., squats, jumps), or is generally used for the sport (e.g., running, cycling). These separate training experiences away from the study's aim can cause muscular adaptations that alter muscle mechanics, architecture, and physiology in such a way that it is difficult or impossible to know the true effect of the study's experimental protocol on muscle properties. For these types of studies, it would be ideal to select a target muscle that is often not the focus of training regiments. The musculus tibialis anterior (TA) is an ideal target muscle that satisfies the requirements above. In addition, training interventions can be targeted towards the TA using controllable approaches, such as with the use of a dynamometer. There is almost no training material pertaining to a TA muscle biopsy. Therefore, we developed a modified protocol to collect relatively undamaged muscle biopsies from the TA.

Protocol

NOTE: Below, we outline a protocol to harvest mechanically undamaged fibers from the TA of volunteers who were enrolled in a separate ongoing study. This protocol is similar to that described by Shanely et al.[3], who have described the modified Bergström technique in vastus lateralis. The information presented here has been refined by our research group but may not be ideal for all lab groups or organizational setups. We give only guidelines, and strongly suggests that laboratories new to biopsy collection consult experienced laboratory groups before attempting any human trials.

All studies conducted in this paper were approved by the Ethics Committee of the Faculty of Sport Science at Ruhr

University Bochum. Participants gave free written informed consent prior to participating in the study.

1. Experimental preparation

1. Assess exclusion criteria while taking the participant's detailed medical history during the participant consultation (see below).
    1. Exclude participants if they suffered an injury to the target muscle during the 6 weeks leading up to the biopsy. Ensure participants are generally healthy, aware of no muscle or coagulation disorders, and are not currently on medication that causes blood thinning (e.g., aspirin).
    NOTE: Here, we selected participants who were moderately active and instructed them to refrain from intensive or unaccustomed leg exercises at least 3 days before the biopsy. However, for other research questions, these criteria may change.
2. Adhere to sterilization and aseptic techniques, as regulated by German law and common practice and overseen by the team physician[22,23]. This procedure can often be conducted as a "bedside" procedure or in an outpatient surgical suite. Consult the local regulatory body for guidance.
3. Compose the biopsy team. We suggest that the biopsy team includes 4 people. A physician (or trained individual in biopsy collection), one medical assistant working with the physician, one assistant who monitors and interacts with the participant, and one assistant who handles the muscle biopsy immediately after extraction. With these numbers, quick patient care can be administered if a medical emergency occurs during the procedure. If comfortable with the procedure, then the team could be made of only two people: the physician and medical assistant, who would together take on patient care and tissue processing concurrently.
4. Have the participant meet with the project lead/physician to review, discuss, and sign the user consent form. Take a detailed medical history (allergies, injuries or surgeries to the lower limb and TA) and exclude the participant if they meet any of the exclusion criteria. Thoroughly discuss recovery and incision hygiene.
    1. Explain to the participant that they will be sore but able to walk around immediately after the procedure; walking down slopes or stairs is often uncomfortable for the first 48 hours, with full activity usually returning after 72 hours. Finally, explain that, to limit infection and mechanical abrasions, the incision site should remain bandaged for at least 1 week and kept clean.

2. Visualize the Anterior Tibialis with B-mode Ultrasound

1. Instruct the participant to lie down in a comfortable supine position and relax their leg muscles as much as possible. Use a custom-made device (see below) or have the assistant hold the ankle in a slightly dorsiflexed position to mimic that which will be done during the biopsy.
    NOTE: It is important that the participant has a relaxed TA so that it replicates the muscle characteristics during the procedure. During the exam, ask the participant to contract and relax the muscle so that the changes in muscle architecture can be noted.
2. Use an ultrasound probe to visualize the superficial and deep compartments of the TA, to survey the muscle architecture and decide on depth of insertion and needle angle of attack (**Figure 2A-B**). Indicate landmarks on the skin.

1. Give particular attention to the selection of a target area that avoids major veins, arteries, or nerves.
2. Assess the cross section of the muscle, with the goal of identifying the central aponeurosis within TA muscle belly (approximately 1/3 of the leg, distal to the knee, and 2 cm lateral of the tibial crest) (**Figure 2B**). Record the location and depth of the central aponeurosis (usually 1.5-3 cm) so that care can be taken to not drive the collection (Bergström) needle past this point.
3. Position the ultrasound probe in the proximal-distal orientation over the target location and visualize the fascicle pennation and muscle thickness (**Figure 2A**). Use this information to help successfully drive (blindly) the collection needle into the muscle belly. Save images of the target site in both planes for future reference during the surgical procedure.

3. With this information, create a plan for needle movement towards the target area.

B 1. Plan to make the incision 1-3 cm distal from the target biopsy area. After the needle is passed into the muscle, rotate the needle to a ~45% angle to the skin along the long axis of the limb, and then driven proximally towards the biopsy area. This strategy

C limits the changes chance of driving the needle into the central aponeurosis, if the needle is pushed too hard. Furthermore, the needle can be driven distally or

D proximally, depending on the handedness off the needle operator.

3. Biopsy procedure

1. Instruct the participant to lay supine on the operating table and relax their leg muscles. Make sure the participant's line of sight to the biopsy site is blocked by a curtain.
    1. Remove passive tension from the muscle belly by placing the participant's limb into a device that fixes the ankle into a slightly dorsiflexed position (0-5° from neutral; **Figure 3**). Ask the patient if they can still relax their muscle, as too much dorsiflexion can potentially make it difficult to relax.

       NOTE: We have found that collecting biopsies from a dorsiflexed foot, no more than 5° of neutral (i.e., the sole of the foot perpendicular to the shank) produces more consistent and larger biopsies than more plantar flexed ankle angles. The device that keeps the ankle

E dorsiflexed is a custom-made device. However, any number of (cheap) devices can be fabricated that still produce the desired result.

2. Shave, clean, and disinfect the selected incision area, as per standard practices[24].

   NOTE: The participant's "clean" area is about 20 cm proximal-distal and 10 cm medial-lateral of the proposed incision site. However, always consult the institution's and/or national regulations (if any) on this topic. The disinfection protocol includes scrubbing the skin clean and then disinfecting four times with liberal use of medical-grade disinfection spray. If the participant leaves the table for any reason, the disinfection protocol must be restarted.

3. Administer a suprafascial injection of 1.5 cc of 2% Xylocitin with Epinephrine at the biopsy site, which

functions as a local anesthetic and vasoconstrictor. Wait for the allotted affect time of ~20-30 min.

NOTE: These drugs are myotoxic and thus must never be injected into the muscle, only the subcutaneous tissue. As a reaction to the vasoconstriction, the area of the injection site may turn white (on lighter skin tones) or gray (darker skin tones).

4. Confirm drug effect with skin pitches and gentle pokes with a sterile scalpel.

5. At the previously marked biopsy site, make a 1 cm proximal-distal incision with a sterile scalpel that cuts through the skin and fascia, exposing the muscle belly. Take care to cut the fascia fully because the needle is blunt and will not pass through the fascia.

6. Push the biopsy needle 0.5-1.0 cm into the muscle with an orientation perpendicular to the skin (**Figure 2C, 2E**).

NOTE: The operator will feel a change in the tension needed to drive the needle through the different tissue types. The fatty tissue is easy, the fascia is the toughest, and the muscle is in between (but can be variable, based on the participant).

7. Orient the needle to a position of ~45° angle to the skin, along the long axis of the leg (**Figure 2D, 2F**). Push the needle another 1-2 cm into the muscle until the needle tip is at the target location within the muscle.

NOTE: The physician should utilize the saved ultrasound images to account for individual variation of muscle dimensions. Because the incision is only large enough to insert the needle, the physician drives the needle blindly through the skin. There is a “feel” that the biopsy operator gains with experience. A novice must learn the skill from a trained biopsy operator (more on this in the discussion).

8. Attach the 100 mL syringe and hose to the biopsy needle (**Figure 1G**). Apply suction to the Bergström needle by pulling the plunger of the syringe by about 15-20 mL to produce a negative pressure in the needle and sucking the muscle tissue into the needle window. Then, excise the muscle by a quick push(es) of the trocar over the needle window.

NOTE: Before and during suction, it is sometimes helpful to place light pressure on the skin immediately above the needle window to help push the muscle into the needle.

9. Gently remove the needle from the leg, rotating slowly. There should only be light resistance while extracting the needle. If there is more resistance, this may indicate a partial biopsy cut. It this occurs, return the need to the target location, and reattempt tissue collection.

10. Push the excised tissue towards the needle window using the internal ramrod.

11. Carefully remove the sample from the needle.

NOTE: Submerging the needle into collection solution (see fiber preparation section) often dislodges the biopsy from the needle. Additionally, the syringe can be used to drive air through the needle and push out the sample. These techniques remove the need to physically touch the biopsy with tweezers and reduces the possibility of damage. If tools, hands (gloved or not) or non-sterile solutions come in contact with the needle, the needle cannot be used furthering during the procedure. Thus, if a second immediate biopsy is needed, then a new sterile needle must be used. This often occurs, so it is a best practice to maintain several sterile needles in reserve.

12. Identify the tissue as muscle and not adipose or connective tissue. Muscle tissue is easily identified from other tissue because of its deep red color (**Figure 4A**).

Sometimes, the collected tissue is not muscle, but fat or connective tissue.

1. If an adequate amount of muscle tissue is collected, continue the protocol. If there is not enough muscle, attempt the biopsy again.
2. If a second biopsy is needed, carefully monitor the participant, as a second needle push occasionally makes the participant more uncomfortable than the first.

13. Wash muscle samples immediately in a collection solution and prepare for single fiber experiments (see muscle biopsy handling and storage).
    1. Have an experienced assistant check the sample quality (see below) and assess the need to perform a second biopsy. A separate assistant takes the biopsy for processing, while the rest of the team continues with the participant.
14. Close the incision site.
    1. Close the incision wound with sterile Leukostrip tape. Use one or more pieces to join the edges of the incision site by laying them perpendicular to the long axis of the incision, and then lay further strips in a starshaped pattern to protect against multi-directional loading.

       NOTE: Proper handling of this step will reduce scarring. Suturing the wound can be done but is not necessary. Other options include wound glue.
    2. Place sterile wound dressing (e.g., Leucomed T plus) over the incision site to protect against infection.
    3. Wrap the leg with cohesive elastic bandages (e.g., Unihaft) to limit initial bleeding and protect against external mechanical impact.
    4. Wrap the leg with acrylastic compression bandages to prevent bleeding and protect the deeper bandages from becoming loose or destroyed.

4. Post-biopsy care

1. Ask the participant to walk around immediately after the procedure. There will be localized soreness. Instruct the participant to walk as normally as possible.
2. Instruct the participant to not remove the bandages or let water soak the bandages. They must be kept on for at least: one days for the acrylastic bandage, three days for the cohesive elastic bandage, and seven days for the wound dressing. Inform the participant that they can be rebandaged if needed.
    1. Tailor the post-biopsy care of a participant to the needs of the individual. Have a trained assistant or physician assess the participant and make an appropriate post-biopsy care plan. For this procedure, we suggest that any further in vivo neuromuscular testing of the TA is separated by at least a week from the biopsy.

5. Muscle biopsy handling and storage

1. After tissue extraction, immediately place the tissue into a 5 mL vial containing rigor collection solution (in mM: Tris (50), KCl (2), NaCl (100), $MgCl_2$ (2), EGTA (1), protease inhibitor tablet (1), pH 7.0) and lightly shake for 4-6 min to wash out blood.
2. Exchange the Rigor solution for fresh rigor, lightly shake for 4-6 min, and then store at 4 °C for 4-6 h to allow the exchange of protease-inhibitor storage solution and blood.

3. Exchange Rigor solution for overnight rigor (in mM: Tris (50), KCl (2), NaCl (100), $MgCl_2$ (2), EGTA (1), protease inhibitor tablet (1), 50:50 glycerol, pH 7.0), and store at 4 °C for 12-18 h.
4. Exchange overnight rigor for 50:50 collection rigor:glycerol and stored at -20 °C for up to 3 months, or one year in a -80 °C freezer.

   NOTE: This process permeabilizes the fiber membrane which allows for manual addition of calcium into and out of the cell. This process takes time and could be different between different muscles and species.

Representative Results

The entire time commitment for a participant was about one hour (10 min consultation, 10 min ultrasound, 20 min surgery preparation and anesthetic administration, 10 min surgery, and 10 min recovery). Often, participants unconsciously activated their TA and needed consistent reminders to keep the muscle as relaxed as possible. When the biopsy needle was inside the muscle, participants usually reported a unique “pressure” sensation in the area around the biopsy needle, with occasional periods of moderate to intense discomfort. Once, a participant’s toes slightly cramped during the procedure, but immediately stopped after the needle was removed. Biopsy sizes were usually ~50-100 mg (wet mass). The participants’ reactions to the procedure was often unpredictable. Sometimes, the participant expected to be unaffected during the procedure but then showed signs of fainting, while others were nervous but completely unfazed during the procedure. Thus, we found it to be good practice to keep the participant busy with a conversation or let them use their mobile phone, so that their full attention was not focused on the ongoing procedure. The assistant who talked with the participant also monitored them for signs of distress, pain, or fainting. Sometimes, a biopsy contained only adipose or connective tissue (identified by a pale-white color of the tissue, **Figure 4A**). In these cases, a second biopsy was immediately taken (after approval was given by the participant). Usually, a successful biopsy will yield >80% muscle tissue (**Figure 4A**).

Post-op, most participants felt discomfort after the procedure that lasted 3-5 days. Participants reported that TA soreness was similar to what would be expected after a day of hiking steep slopes. No mechanical pressure should be put on the incision site for at least 5 days, or it could reopen. Participants were usually left with a small scar, but we have not observed raised or otherwise abnormal changes to the skin. Also, no participants ~~in the studies have~~ developed infections.

The biopsies were permeabilized (i.e., skinned) in a glycerol solution (1:1 mixture of glycerol: rigor solution) for 6 weeks and then prepared for mechanical testing on the day of experiments. Glycerol permeabilization of fibers allows for the diffusion of the bath solution into the fibers, which gives the researcher activation control and also provides an avenue to subject the muscle to pharmaceuticals or other chemicals. Furthermore, glycerol functions as an anti-freezing agent, allowing the muscle to be place in cold temperatures for long term storage, with limited damage. However, some time is needed to allow the glycerol to penetrate the samples, and so initially storing biopsy samples overnight at 4 °C (ideally on a shake plate) is prudent. Muscles can only be stored for so long before their function is compromised. The general guidance on the matter is that muscles will maintain their function within the glycerol solution for at least 3 months in a -20 °C freezer, or one year in a -80 °C freezer.

Muscle samples were visualized under a dissection microscope. Some muscle pieces were small or damaged

(**Figure 4B**) and were removed. Next, groups of fibers were assessed for any structural damage (visually broken or crushed fiber sarcolemma, **Figure 4C**). From these bundles, smaller fiber bundles of 3-10 fibers were dissected away and carefully placed into the experimental chamber of the mechanical test rig (**Figure 4D**). Structurally usable fiber lengths were typically 3-5 mm long. The Bergström needle had a collection window of 7 mm, so the biopsy could only maximally yield ~7 mm long fibers. Thus, the structurally useable fibers we collected were almost as long as possible. Typically, we prepare 5-10 fiber bundle per 50 mg of (collected) tissue. Full details of these procedures can be found elsewhere[14,15,25]. To demonstrate durability of the fibers, we show representative data of a simple mechanical protocol using glycated TA fiber bundles (**Figure 5**). 40 fiber bundles from the biopsies of 10 participants were activated in activation solution[26] (high [Ca^{2+}], pCa < 4.2) at 2.7 µm sarcomere length for 60 seconds and steady-state active stress was measured as 100.71 ± 11 mN mm^{-2} (mean ± SEM).

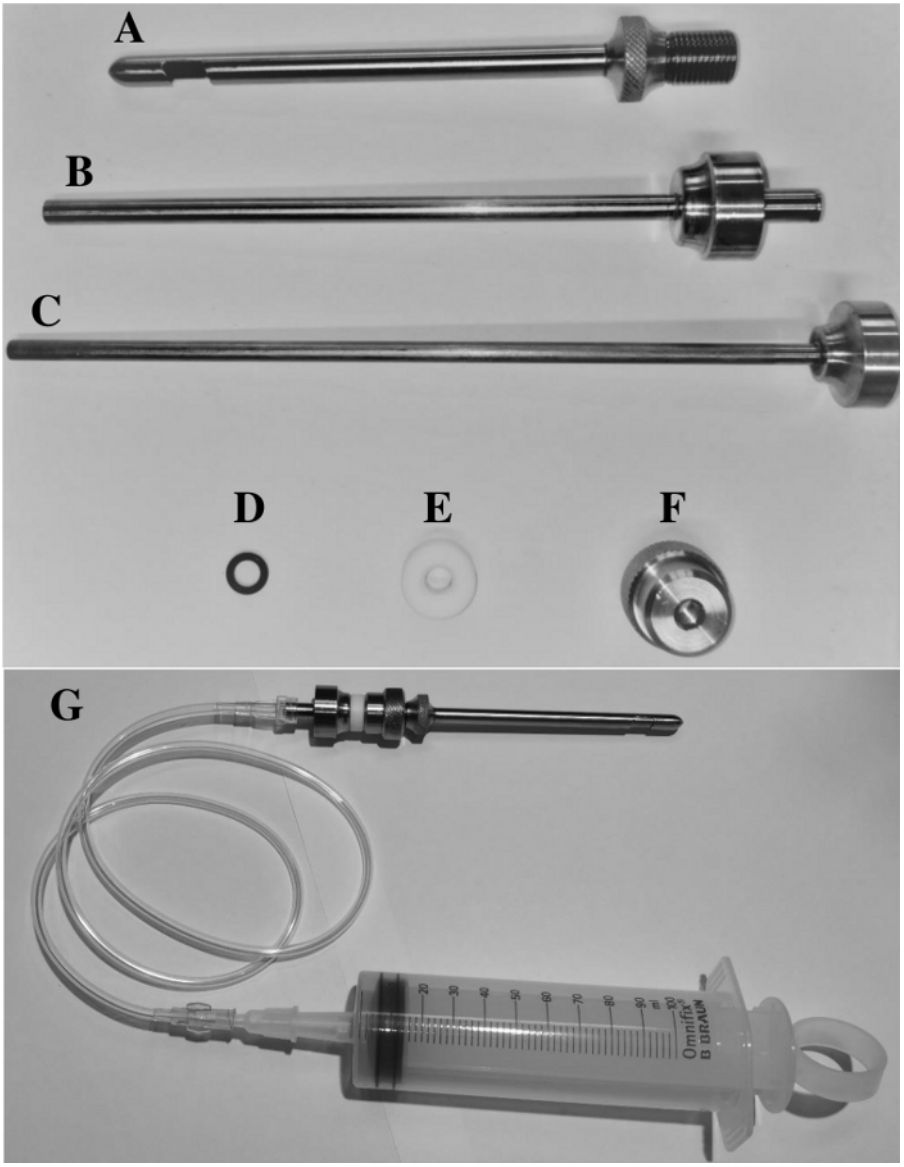

Figure 1: The Bergström needle. The Bergström needle used in this study consists of the needle itself (**A-F**), suction hose (**G**), and syringe (**F**). The Bergström needle consists of an outer needle (**A**) that has a window close to the needle tip, a smaller hollow internal trocar (**B**) that moves up and down the needle and cuts the muscle when passing over the needle window, and a rod (**C**) that moves up and down the trochanter to help remove the muscle from the needle. These pieces are separated by a washer (**D**) that makes the needle airtight, and a spacer (**E**) between the rod and trocar protects against crushing of the muscle biopsy. Finally, a suction hose adaptor is attached. To pull the muscle into the needle window, a suction hose (**G**) is attached to the needle adaptor and syringe. This sucks the air out of the needle and pulls the muscle into the needle window via negative pressure, allowing for sample collection. Please click here to view a larger version of this figure.

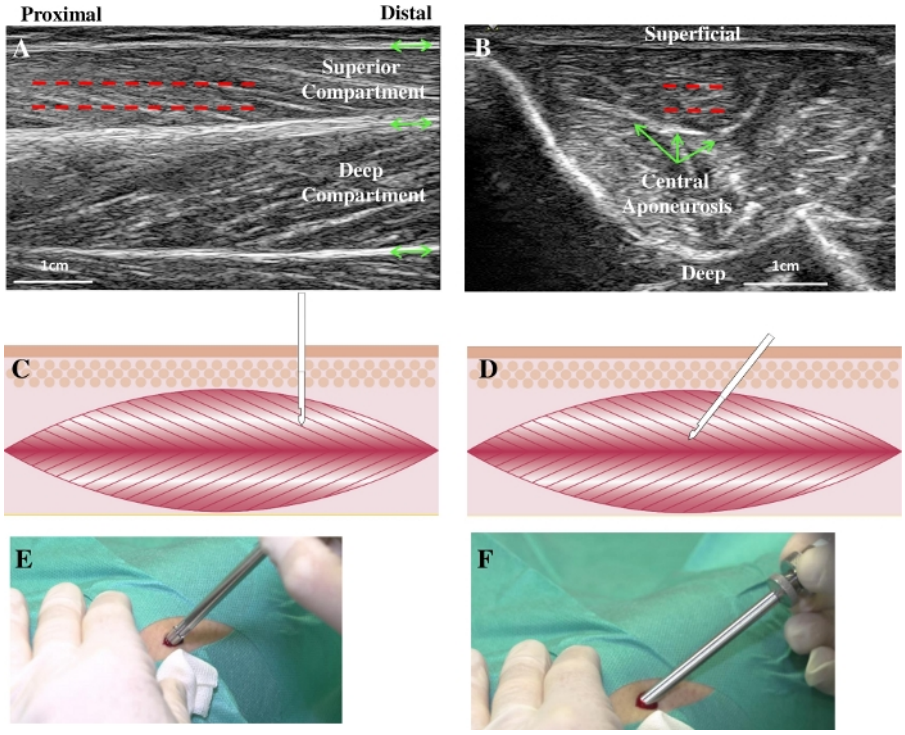

Figure 2: Ultrasound imaging and needle placement. The TA is comprised of superficial and deep compartments that are defined by aponeuroses. The TA is imaged with the ultrasound probe oriented in the distal-proximal (**A**) and medial-lateral (**B**) perspectives so that the 3D shape of the TA can be recognized. Ideal needle depth for collection is between the horizontal dashed lines. A cartoon representation of the needle insertion is shown in panels C and D. After the incision is made, the needle is first positioned perpendicular to the muscle and pushed into the muscle until the needle window is in the muscle (**C**). The needle is then reoriented to a ~45° angle along the long axis of the leg, and pushed into the muscle further, paying careful attention that the needle does not penetrate the deep aponeurosis (**D**). Live pictures (**E, F**) during the procedure are given in reference to the cartoon (**C, D**). Please click here to view a larger version of this figure.

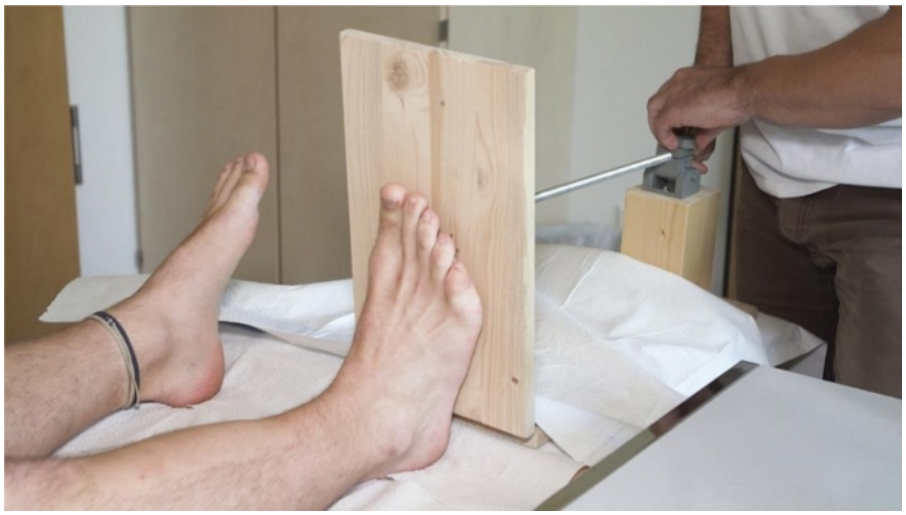

Figure 3: Participant placement. The participant lays in a supine position on the operation table. The head can be elevated for comfort. The right foot is placed in a custom device that keeps the foot slightly dorsiflexed, reducing muscle tension. A curtain is placed in front of the participant so that they cannot watch the procedure. Please click here to view a larger version of this figure.

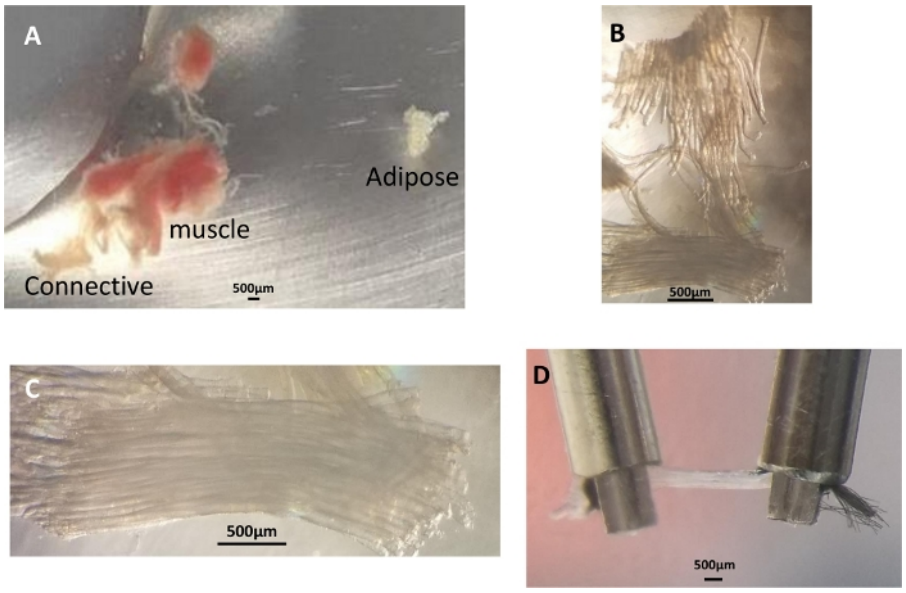

Figure 4: Representative images of muscle tissue. (**A**) Immediately after the biopsy, the muscle sample will be a darker red than other tissues, including adipose tissue and connective tissue (labeled in the panel). (**B**) Dissection of samples with damage/short (top) and viable (below) fiber bundles. (**C**) Magnification of a viable fiber grouping to inspect surface for signs of damage. (**D**) A 6-fiber bundle was dissected away from this fiber bundle (tied on the ends with 6-0 suture for easy movement and attached to the mechanical apparatus. Please click here to view a larger version of this figure.

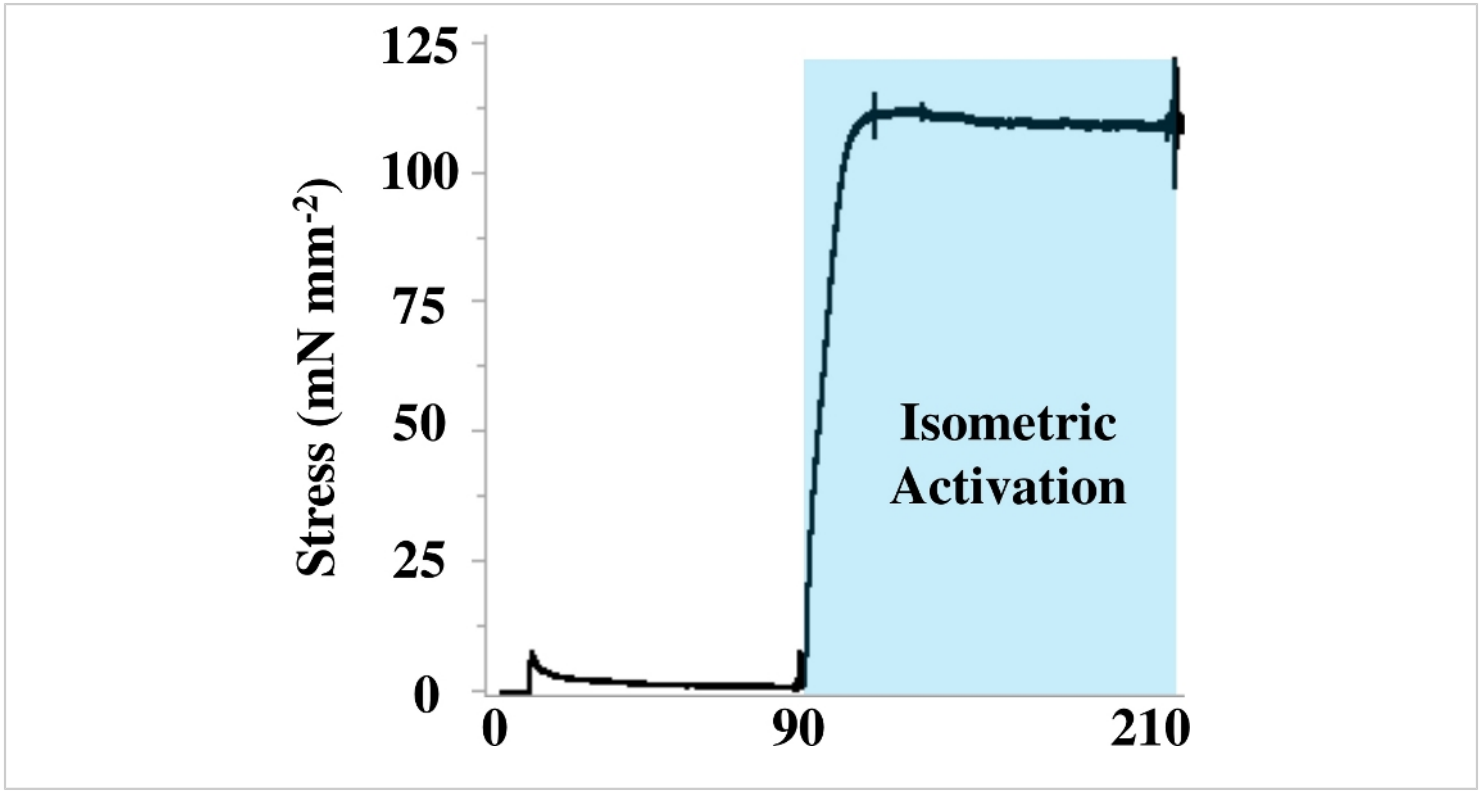

Figure 5: Representative force outputs of a fiber bundle preparation. To demonstrate the durability of the fibers, we show representative stress data of a simple mechanical protocol using glycated TA fiber bundle (3 fibers). In total, 40 fiber bundles from the biopsies of 10 participants were stretched from slack to 2.7 µm sarcomere length and held to allow for stress-relaxation. Next, fibers were activated in activation solution[26] (shaded area; high [Ca^{2+}], pCa < 4.2) at 2.7 µm sarcomere length for 60 seconds and steady-state active stress was measured at 100.71 ± 11 mN mm^{-2} (mean ± SEM).

Please click here to view a larger version of this figure.

Discussion

In this report, we described a technique for the biopsy of structurally undamaged muscle tissue from TA. We found that this procedure yields an acceptable content of usable muscle fibers (5-10 fiber bundle preparations per 50 mg of collected tissue) for mechanical testing. Further, we had enough tissue for follow-up mechanical, genetic, and proteomic experiments.

There are several methods typically used for the collection of muscle biopsies[3,4,6,27,28]. The so-called open biopsy[20] produces the highest quality fibers because a surgeon fully exposes the muscle and dissects out the sample. Of course, open surgery is quite an invasive procedure and is not an appropriate procedure to submit healthy participants to, regardless of the research question, because of the potential risks associated with open surgeries. The least invasive biopsy method is the fine needle biopsy[29,30], which uses a relatively smaller needle to collect tissue. The fine needle biopsies are enough to conduct experiments on the genetic/chemical/protein components of fibers[30,31], but often fiber quality is very poor, which makes mechanical testing difficult

or impossible. The Bergström needle technique is a good compromise between the two procedures explained above because the surgery is less invasive than the open biopsy but still collects muscle samples that are larger and (potentially) more structurally intact than fine needle biopsies. Previous reports of the Bergström needle procedure[3,5] are great resources for those learning the technique but only present protocols for the vastus lateralis. ~~The~~ our report demonstrates the technique for the TA that focuses on the collection of high yields of structurally intact fibers for mechanical testing.

To our knowledge there are no detailed publications on the collection of TA biopsies. Nevertheless, the standard practice is to lay the participant supine and have them relax their leg as much as possible. The relaxed foot in this position is naturally plantarflexed, which consequently lengthens the TA and puts it into tension. We find that any muscle tension makes it more difficult to drive muscle into the biopsy needle, even with negative pressure, and so tension should be minimized as much as possible. To accomplish this, the simple, but major modification here was to use a custom-built foot plate that maintained the ankle in a slightly dorsiflexed position (0 - 5° from neutral), keeping the TA slack and improving collection. Clinicians should be careful not to over-dorsiflex the ankle, as the TA will be uncontrollably activated, increasing tension, which is of course counter to the procedure in the first place. The participant can typically feel this muscle activation, so communication is key. From the protocols, the TA yields only ~25% tissue compared with the more commonly used vastus lateralis, ~100 mg and ~400 mg, respectively. Thus, it is important to maximize tissue collection size while also considering if the TA tissue sample will be large enough for the desired research project(s). We have found that taking a second sample immediately after the first does not cause any extra complications or healing time for the participants.

Although the protocol gives some guidance towards other muscle biopsies, the muscle selection will dictate the appropriate procedure. Thus, we strongly suggest to other researchers and clinicians to publish, in full, their biopsy methods. From experience, we identify a few important factors to muscle selection, outside of the research question. First, we suggest considering muscles that are superficial to the skin and have major arteries/nerves that are either deep or easily avoidable. Second, because the participants are awake during the procedure, it is important to consider if the biopsy procedure will be very uncomfortable to the patient, either because of the initial positioning of the patient, or because of the pressure of the biopsy needle, which also pushes on deeper muscles in an uncomfortable way. We have had success with the vastus lateralis and pectoralis. Other potential options are the trapezius, latissimus dorsi, and gastrocnemius (although highly vascularized and prone to bleeding). The hamstring muscles are possible but uncomfortable for the patient, and difficult because they move laterally when collecting the biopsy.

Although Bergström needles can be purchased from manufactures, some laboratories custom-make their own. Small, yet clever, adjustments to the design may increase the yield of long and undamaged muscle fibers. For example, the collection window of the needle used here was 7 mm x 5 mm (length x width). This is appropriate to capture a cube of muscle. However, if the goal is to collect long and undamaged fibers (of the same volume), then the length could be increased, and the width decreased (i.e., 10 mm x 3.5 mm). If the needle is oriented along the fascicle direction, then it is likely that this needle would collect longer fiber sections.

Muscle biopsies are often safely collected without the guidance of an ultrasound image, especially for larger muscles like the vastus lateralis. In this situation, a properly experienced physician can easily palpate the muscle to find the best incision site. However, when the physician is less experienced with the target muscle, or extra care is warranted to avoid major nerves or blood vessels, the ultrasound is a great and simply applied tool. Finally, post-op monitoring of the biopsy area can quickly be accomplished with the aid of an ultrasound.

Pediatric biopsies are certainly possible and commonly carried out[32,33,34]. However, there are typically several changes made to the procedure. A smaller gauge needle and conscious sedation are often required, and the procedure takes place in a hospital environment. In general, the experience could be traumatic for a child and research groups that want to include healthy pediatric participants should carefully weigh this against the potential merits of the study.

Fiber bundles or unused material can be transferred to other experiments before or after fiber mechanics. For example, techniques that assess sarcomeric protein content or classify isoform type can be conducted[35]. However, to limit protein degradation and to improve analysis success, tissue should be flash frozen in liquid nitrogen either after original extraction, immediately after mechanical evaluation, or processed immediately for protein analysis. Fibers can also be prepared for immunohistochemistry or other imaging techniques[36] that allow for the assessment of protein position within the fiber. In this case, fibers can be placed in a fixative solution (e.g., 4% paraformaldehyde/0.25% glutaraldehyde in physiological buffer at pH 7; no glutaraldehyde for immunohistochemistry) while still on the mechanical test apparatus, preserving the sarcomeric structures at a desired sarcomere length. If possible, a small piece of the original biopsy can be harvested, washed vigorously in collection solution for 10 min and then placed into fixative solution. Many groups prefer to immediately flash-freeze freshly excised samples in isopentane, which limits the formation of damaging ice crystals, and improves image quality for visual assessments. This is indeed the gold-standard for flash freezing; however, we find that the ice crystal damage from nitrogen-freezing is only focused on extra-myofibril structures. We have satisfactory structural integrity of sarcomeric components in samples also frozen in liquid nitrogen, and so we think that nitrogen is a possibility, especially if it is more readily available, or the surgical team/local chemical authority is not willing to use isopentane. An important and often unreported problem with preparing samples for viewing is that the sarcomeres are often contracted/short, with the I-band region of the sarcomere short or unobservable. To overcome this, the researcher must manually stretch the fiber samples (by the testing apparatus or by hand using fine tweezers) before fixing. As a general rule, we stretch to ~3.2 µm sarcomere length (measured via laser diffraction), or stretch to ~150% of slack length, in a low-calcium physiological relaxing solution. Finally, if subsamples are wanted for RNA expression analysis, the method of flash-freezing does not affect results, but samples must be frozen immediately after original extraction and placed in a -80 °C freezer, as RNA is very unstable. There are some RNA-protection storage solutions on the market, but we have found mixed results with their use, and only flash freeze fresh samples.

To maximize the amount of information collected during one trial, simultaneous collection of other data can be completed while conducting mechanical tests. For example, the study of sarcomeric structures can be performed during mechanical tests by using low-angle X-ray diffraction imaging, as is done

in other animals[37,38]. For genetic experiments, excised muscle must be immediately processed for that purpose or flash frozen because DNA/RNA are relatively less stable than proteins.

Some limitations are already described above. Here we discuss the procedure itself. A large limitation for most groups is having a team member who is appropriately trained in biopsy collection. Regardless of the person's profession (physician, medical assistant, technician, or otherwise), this procedure is difficult because the investigator drives the needle blindly and must rely on "feel"[3,28] to locate the needle window accurately. Mistakes are not tolerable because consenting human participants for biopsies are sparse, one biopsy is preferable to many, and mistakes could lead to vascular or nerve damage. Therefore, any training possibilities should be completed before a human biopsy is performed. For example, to gain a "feel" for driving the needle, pork meat with skin still attached can be purchased from most grocery stores and used as a proxy for human skin and muscle. Another valuable experience is to shadow a trained research group.

We assessed participants' pain/discomfort more qualitatively, relying on the physician's experience and conversations with the participant to assess perceived pain. However, the assessment of pain and post-biopsy discomfort can be more quantified and comparable across individuals and studies through the use of validated pain/discomfort surveys. These points have surprisingly little treatment in the literature. However, one recent study presented a way to quantify participant pain/discomfort before, during and after biopsies, by utilizing well established surveys of pain[39]. We note that this paper used the vastus lateralis as the target muscle, and so follow up studies are needed to compare pain assessments between muscles.

Regardless of extraction method, the Bergström technique cannot excise the total length of the fiber in the muscle because fibers are too long (~6-8 cm in TA[40], ~6.5-8 cm in vastus lateralis[40]). Therefore, it is unavoidable that for a long piece of collected fiber, the ends are destroyed by the biopsy technique. Often, the usable central portion of a fiber is small and so it is hard to mechanically test. Even though the technique provides reasonably long central regions (3-5 mm), the investigator must carefully check the quality of the fiber bundles during dissection because the use of damaged fibers will alter passive or active force outputs. Visual observation of successful biopsies will show a portion of fibers that are undamaged from the biopsy procedure. When viewed from a traditional dissection light microscope, the surface of fibers will look smooth, with no holes or tears (**Figure 4**). Furthermore, fibers should look cylindrical and have no flattened areas. Although not visible, the muscle itself will degrade over time because of naturally occurring proteases that start to break down the muscle proteins almost immediately after extraction. Thus, it is critical to add protease inhibitors to all solutions used with the fibers. Furthermore, we also suggest extra washes of the biopsies to remove as much blood as possible.

Even with careful preparation, fiber damage can occur and lead to poor fiber activations. There are many reasons for fiber damage because fibers are very sensitive to almost every part of the procedure. For example, during the biopsy, if the trocar is not sharp enough, it can push into the muscle tissue during the extraction instead of cutting through it, which can stretch and destroy the fibers. The collection solution must be appropriately prepared because fibers are sensitive to

osmotic changes, pH, and temperature. When handling the fibers, great care must be taken to completely limit pressure on the fibers. Instead, tweezers should be used to grab the biopsy by its connective tissue. Another alternative is to use a size 0-7 silk suture to wrap an unusable end of the biopsy and then grab this when handling. Finally, the glycerol serves two roles: the first is to keep the muscle from freezing while at -20 °C and the second is to be a mild detergent to the fiber. That is, glycerol permeabilizes the fiber to outside solutions, allowing for the influx of calcium (via an activation solution). For most muscles, this process takes ~10 days. However, depending on the amount of collagen content and size of sample, this could take up to 6 weeks. Fibers must be permeabilized for any high-calcium activation to occur during mechanical experiments. Fibers are generally usable for at least 3 months. To limit fiber waste, a longer permeabilization wait time (4-6 weeks) is suggested for TA muscle fibers.

Disclosures

The authors have nothing to disclose.

Acknowledgments

We thank Michaela Rau, Lea-Fedia Rissmann, Michael Marsh, Janina-Sophie Tennler, Kilian Kimmeskamp, and Wolfgang Linke for assisting with the project. Funding for this project was provided by the MERCUR Foundation (ID: An-2016-0050) to DH.

References

1. Franchi, M. et al. Architectural, functional and molecular responses to concentric and eccentric loading in human skeletal muscle. *Acta Physiologica.* **210** (3), 642-654 (2014).
2. Duchene, G. B. A. De la paralysie musculaire pseudo-hypertrophique, ou paralysie myo-sclérosique / par le Dr Duchenne (de Boulogne). *Archives of General Internal Medicine.* **11** (30) (1868).
3. Shanely, R. A. et al. Human skeletal muscle biopsy procedures using the modified Bergström technique. *Journal of Visualized Experiments.* (91), 51812 (2014).
4. Evans, W. J., Phinney, S. D., Young, V. R. Suction applied to a muscle biopsy maximizes sample size. *Medicine and Science in Sports and Exercise.* **14** (1), 101-102 (1982).
5. Bergstrom, J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scandinavian Journal of Clinical and Laboratory Investigation.* **35** (7), 609-616 (1975).
6. Baczynska, A. M. et al. Human Vastus Lateralis Skeletal Muscle Biopsy Using the Weil-Blakesley Conchotome. *Journal of Visualized Experiments.* (109), e53075 (2016).
7. Pesta, D., Gnaiger, E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods in Molecular Biology.* **810**, 25-58 (2012).
8. Buck, E. et al. High-resolution respirometry of fine-needle muscle biopsies in pre-manifest Huntington's disease expansion mutation carriers shows normal mitochondrial respiratory function. *Plos One.* **12** (4), e0175248 (2017).
9. Murgia, M. et al. Single Muscle Fiber Proteomics Reveals Fiber-Type-Specific Features of Human Muscle Aging. *Cell Reports.* **19** (11), 2396-2409 (2017).
10. Friedmann-Bette, B. et al. Effects of strength training with eccentric overload on muscle adaptation in male athletes. *European Journal of Applied Physiology.* **108** (4), 821-836 (2010).

11. McPhee, J. S. et al. The contributions of fibre atrophy, fibre loss, in situ specific force and voluntary activation to weakness in sarcopenia. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences.* **73** (10), 1287-1294 (2018).
12. Nocella, M., Cecchi, G., Bagni, M. A., Colombini, B. Force enhancement after stretch in mammalian muscle fiber: no evidence of cross-bridge involvement. *American Journal of Physiology. Cell Physiology.* **307** (12), C1123-9 (2014).
13. Patel, J. R., McDonald, K. S., Wolff, M. R., Moss, R. L. Ca2+ binding to troponin C in skinned skeletal muscle fibers assessed with caged Ca2+ and a Ca2+ fluorophore. Invariance of Ca2+ binding as a function of sarcomere length. *The Journal of Biological Chemistry.* **272** (9), 6018-6027 (1997).
14. Hessel, A. L., Joumaa, V., Eck, S., Herzog, W., Nishikawa, K. C. Optimal length, calcium sensitivity and twitch characteristics of skeletal muscles from mdm mice with a deletion in N2A titin. *The Journal of Experimental Biology.* **222** (Pt 12) (2019).
15. Joumaa, V., Herzog, W. Calcium sensitivity of residual force enhancement in rabbit skinned fibers. *American Journal of Physiology. Cell Physiology.* **307** (4), C395-401 (2014).
16. Joumaa, V., Rassier, D. E., Leonard, T. R., Herzog, W. The origin of passive force enhancement in skeletal muscle. *American Journal of Physiology. Cell Physiology.* **294** (1), C74-8 (2008).
17. Hilber, K., Galler, S. Mechanical properties and myosin heavy chain isoform composition of skinned skeletal muscle fibres from a human biopsy sample. *Pflugers Archiv: European Journal of Physiology.* **434** (5), 551-558 (1997).
18. Miller, M. S. et al. Chronic heart failure decreases cross-bridge kinetics in single skeletal muscle fibres from humans. *The Journal of Physiology.* **588** (Pt 20), 4039-4053 (2010).
19. Pinnell, R. A. M. et al. A. Residual force enhancement and force depression in human single muscle fibres. *Journal of Biomechanics.* **91**, 164-169 (2019).
20. Einarsson, F., Runesson, E., Fridén, J. Passive mechanical features of single fibers from human muscle biopsies--effects of storage. *Journal of Orthopaedic Surgery and Research.* **3**, 22 (2008).
21. Flann, K. L., LaStayo, P. C., McClain, D. A., Hazel, M., Lindstedt, S. L. Muscle damage and muscle remodeling: no pain, no gain? *The Journal of Experimental Biology.* **214** (Pt 4), 674-679 (2011).
22. Commission for Hospital Hygiene and Infection Prevention (KRINKO), Federal Institute for Drugs and Medical Devices (BfArM) Anforderungen an die Hygiene bei der Aufbereitung von Medizinprodukten [Hygiene requirements for the reprocessing of medical devices]. *Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz.* **55** (10), 1244-1310 (2012).
23. Koch-Institut, R. Ergänzung zur Empfehlung „Anforderungen an die Hygiene bei der Aufbereitung von Medizinprodukten. *RKI-Bib1 (Robert Koch-Institut).* (2018).
24. Rutala, W. A., Weber, D. J. Disinfection and sterilization in healthcare facilities. *Practical Healthcare Epidemiology.* 58-81 (2018).

25. Rassier, D. E., MacIntosh, B. R. Sarcomere length-dependence of activity-dependent twitch potentiation in mouse skeletal muscle. *BMC Physiology.* **2**, 19 (2002).

26. Mounier, Y., Holy, X., Stevens, L. Compared properties of the contractile system of skinned slow and fast rat muscle fibres. *Pflugers Archiv: European Journal of Physiology.* **415** (2), 136-141 (1989).

27. Henriksson, K. G. Semi-open" muscle biopsy technique. A simple outpatient procedure. *Acta Neurologica Scandinavica.* **59** (6), 317-323 (1979).

28. Dietrichson, P. et al. Conchotome and needle percutaneous biopsy of skeletal muscle. *Journal of Neurology, Neurosurgery, and Psychiatry.* **50** (11), 1461-1467 (1987).

29. Iachettini, S. et al. Tibialis anterior muscle needle biopsy and sensitive biomolecular methods: a useful tool in myotonic dystrophy type 1. *European Journal of Histochemistry.* **59** (4), 2562 (2015).

30. Cotter, J. A. et al. Suction-modified needle biopsy technique for the human soleus muscle. *Aviation, Space, and Environmental Medicine.* **84** (10), 1066-1073 (2013).

31. Edwards, R. H., Round, J. M., Jones, D. A. Needle biopsy of skeletal muscle: a review of 10 years experience. *Muscle & Nerve.* **6** (9), 676-683 (1983).

32. Gibreel, W. O. et al. Safety and yield of muscle biopsy in pediatric patients in the modern era. *Journal of Pediatric Surgery.* **49** (9), 1429-1432 (2014).

33. Cuisset, J. M. et al. [Muscle biopsy in children: Usefulness in 2012]. *Revue Neurologique.* **169** (8-9), 632-639 (2013).

34. Nilipor, Y. et al. Evaluation of one hundred pediatric muscle biopsies during a 2-year period in mofid children and toos hospitals. *Iranian Journal of Child Neurology.* **7** (2), 17-21 (2013).

35. Schiaffino, S., Reggiani, C. Fiber types in mammalian skeletal muscles. *Physiological Reviews.* **91** (4), 1447-1531 (2011).

36. Wang, K., Wright, J. Architecture of the sarcomere matrix of skeletal muscle: immunoelectron microscopic evidence that suggests a set of parallel inextensible nebulin filaments anchored at the Z line. *The Journal of Cell Biology.* **107** (6 Pt 1), 2199-2212 (1988).

37. Ma, W., Gong, H., Irving, T. Myosin head configurations in resting and contracting murine skeletal muscle. *International Journal of Molecular Sciences.* **19** (9) (2018).

38. Ma, W., Gong, H., Kiss, B., Lee, E.J., Granzier, H., Irving, T. Thick-Filament Extensibility in Intact Skeletal Muscle. *Biophysical Journal.* **115** (8), 1580-1588 (2018).

39. Bonafiglia, J. T. et al. A comparison of pain responses, hemodynamic reactivity and fibre type composition between Bergström and microbiopsy skeletal muscle biopsies. *Current Research in Physiology.* **3**, 1-10 (2020).

40. Wickiewicz, T. L., Roy, R. R., Powell, P. L., Edgerton, V. R. Muscle architecture of the human lower limb. *Clinical Orthopaedics and Related Research.* (179), 275-283 (1983).