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TITLE:

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

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KEYWORDS:

Tibialis anterior, muscle biopsy, ultrasound, human fiber mechanics, biomechanics, modified Bergström technique

SUMMARY:

This technical report describes a variation of the modified Bergström technique for the biopsy of the musculus tibialis anterior that limits fiber damage.

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 adaptations¹, 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 1868². Today, the predominant technique to collect muscle biopsies is the modified Bergström technique³⁻⁵, although other techniques are available including the use of a Weil-Blakesley conchotome⁶ 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 hollow 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^{1,9-11}. Another critical use for muscle biopsies is mechanical experiments such as the measurement of fiber contractile force, muscle fiber stiffness, and history-dependent muscle properties¹²⁻¹⁶. 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 biopsies¹⁷⁻¹⁹. 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 muscle³. 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 group²⁰ 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

1.1. Assess exclusion criteria while taking the participant's detailed medical history during the participant consultation (see below).

1.1.1. 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.

1.2. 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.

1.3. 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.

1.4. 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.

1.4.1. 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

2.1. 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.

2.2. 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.

2.2.1. Give particular attention to the selection of a target area that avoids major veins, arteries, or nerves.

2.2.2. 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.

2.2.3. 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.

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

2.3.1. Plan to make the incision 1-3 cm distal from 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 limits the changes of driving the needle into the central aponeurosis, if the needle is pushed too hard. Furthermore, the needle can be driven distally or proximally, depending on the handedness of the needle operator.

3. Biopsy procedure

3.1. 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.

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

3.2. Shave, clean, and disinfect the selected incision area, as per standard practices²⁴.

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.

3.3. 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).

3.4. Confirm drug effect with skin pinches and gentle pokes with a sterile scalpel.

3.5. 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.

3.6. 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).

3.7. 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).

3.8. 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.

3.9. 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. If this occurs, return the needle to the target location, and reattempt tissue collection.

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

3.11. 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 further 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.

3.12. 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.

3.12.1. If an adequate amount of muscle tissue is collected, continue the protocol. If there is not enough muscle, attempt the biopsy again.

3.12.2. If a second biopsy is needed, carefully monitor the participant, as a second needle push

occasionally makes the participant more uncomfortable than the first.

3.13. Wash muscle samples immediately in a collection solution and prepare for single fiber experiments (see muscle biopsy handling and storage).

3.13.1. 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.

3.14. Close the incision site.

3.14.1. 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.

3.14.2. Place sterile wound dressing (e.g., Leucomed T plus) over the incision site to protect against infection.

3.14.3. Wrap the leg with cohesive elastic bandages (e.g., Unihaft) to limit initial bleeding and protect against external mechanical impact.

3.14.4. Wrap the leg with acrylastic compression bandages to prevent bleeding and protect the deeper bandages from becoming loose or destroyed.

4. Post-biopsy care

4.1. Ask the participant to walk around immediately after the procedure. There will be localized soreness. Instruct the participant to walk as normally as possible.

4.2. 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.

4.2.1. 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

5.1. After tissue extraction, immediately place the tissue into a 5 mL vial containing rigor collection solution (in mM: Tris (50), KCl (2), NaCl (100), MgCl₂ (2), EGTA (1), protease inhibitor tablet (1) pH 7.0) and lightly shake for 4-6 min to wash out blood.

5.2. 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.

5.3. Exchange Rigor solution for overnight rigor (in mM: Tris (50), KCl (2), NaCl (100), MgCl₂ (2), EGTA (1), protease inhibitor tablet (1), 50:50 glycerol, pH 7.0), and store at 4 °C for 12-18 h.

5.4. 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 placed 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 solution²⁶ (high [Ca²⁺], 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 AND TABLE LEGENDS:

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.

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)**.

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.

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).

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 $2.7\ \mu\text{m}$ sarcomere length and held to allow for stress-relaxation. Next, fibers were activated in activation solution²⁶ (shaded area; high $[\text{Ca}^{2+}]$, $\text{pCa} < 4.2$) at $2.7\ \mu\text{m}$ sarcomere length for 60 seconds and steady-state active stress was measured at $100.71 \pm 11\ \text{mN mm}^{-2}$ (mean \pm SEM).

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 biopsy²⁰ 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 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^{32–34}. 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 conducted³⁵. 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 techniques³⁶ 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 $\sim 3.2\ \mu\text{m}$ sarcomere length (measured via laser diffraction), or stretch to $\sim 150\%$ 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 $-80\ ^\circ\text{C}$ 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 pain³⁹. 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 TA⁴⁰, ~6.5-8 cm in vastus lateralis⁴⁰). 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.

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DISCLOSURES:

The authors have nothing to disclose.

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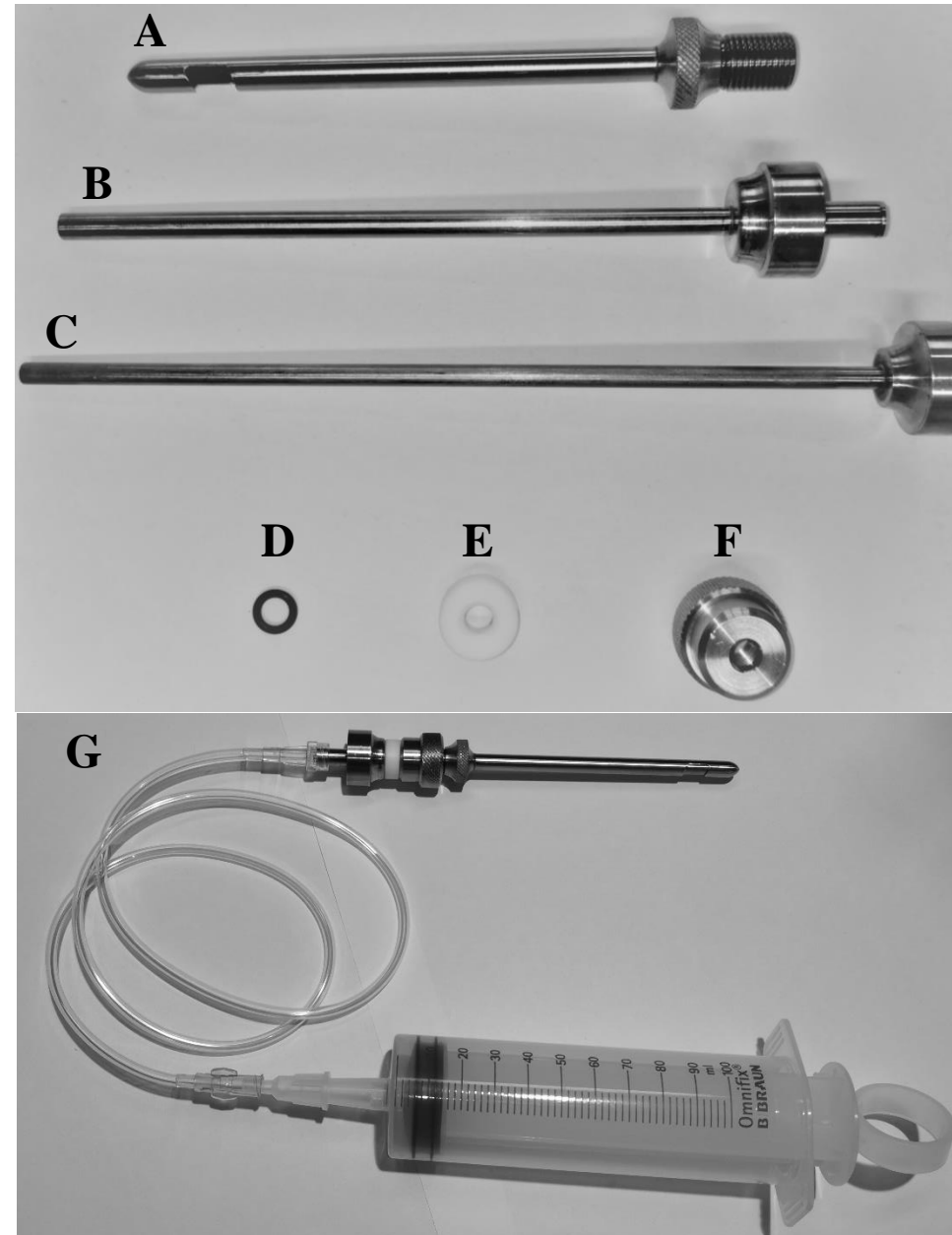
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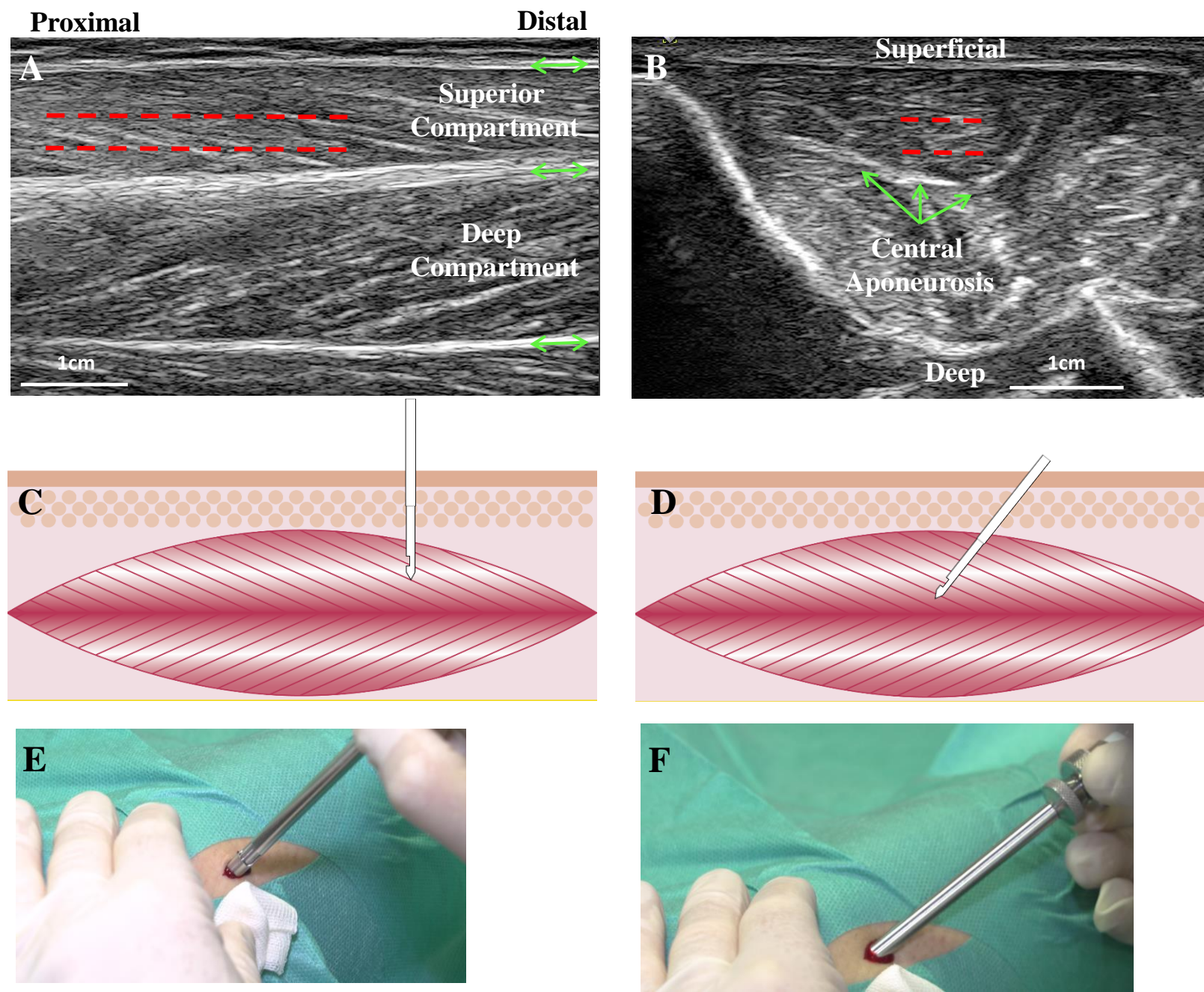
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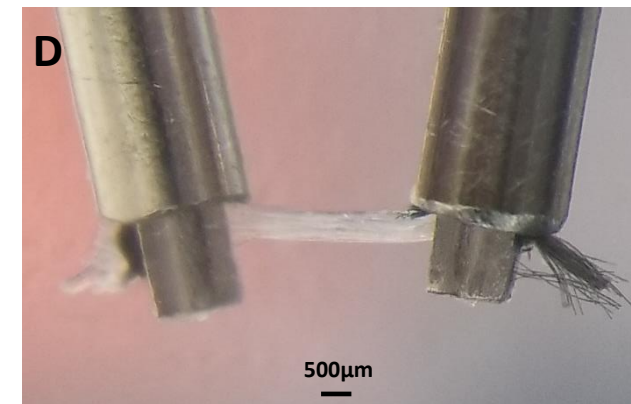
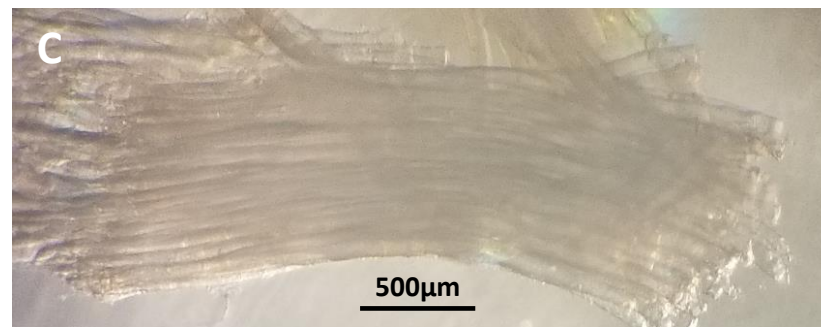
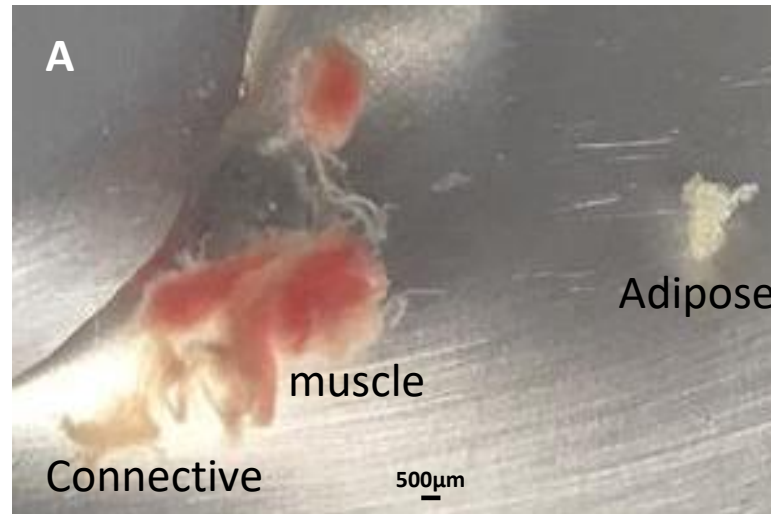
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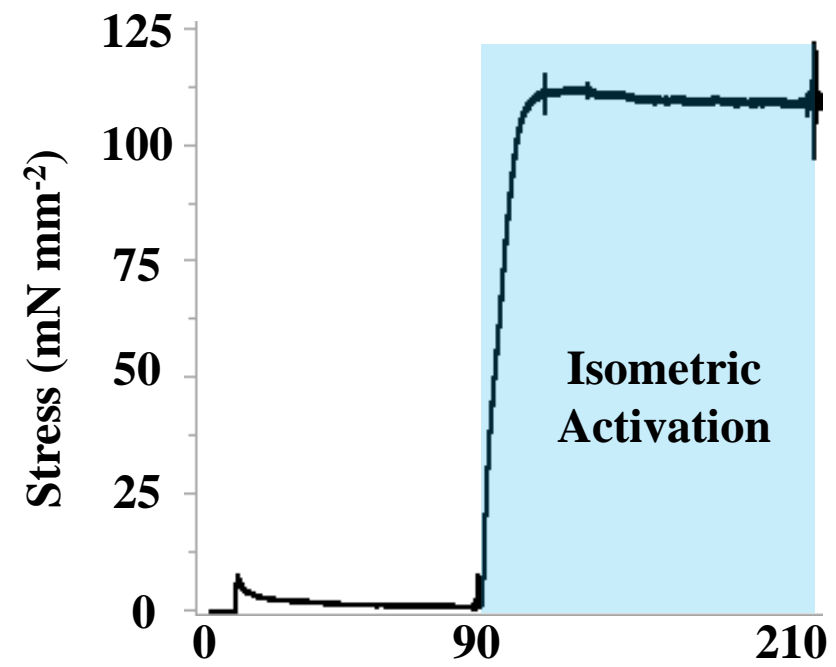
Figure 1











Name of Material/ Equipment	Company	Catalog Number	Comments/Description
26 guage subcutaneous needle with 2 ml glass syringe	B. Braun Melsungen AG Carl-Braun-Straße 1 34212 Melsungen, Hessen Germany	4606027V	Drug administration
5mm Berstöm needle	homemade	N/A	Tissue collection. Similar to other Berstöm needles
Acrylastic	BSN medical GmbH 22771 Hamburg	269700	elastic compression bandage
Complete protease inhibitor cocktail	Roche Diagnostics, Mannheim, Germany	11836145001	Protease inhibitor tabeletes added to all solutions that hold muscle tissue.
Cutasept	PAUL HARTMANN AG Paul-Hartmann-Straße 12 89522 Heidenheim Germany	9805630	Disenfectant spray for the skin
Leucomed T plus	BSN medical GmbH 22771 Hamburg	7238201	Transparent wound dressing with wound pad to seal the wound and protect against infection
Leukostrip	Smith and Nephew medical Linitied 101 Hessle road, Hull Great Britain	66002876	wound closure
Surgical disposable scalpels	Aesculap AG Am Aesculap-Platz 78532 Tuttlingen Germany	BA200 series	Incision

Unihaft cohesive elastic bandage	BSN medical GmbH 22771 Hamburg	4589600	cohesive elastic bandage that protects against mechanical impact
Xylocitin 2% with Epinephrin	Milbe GmbH Münchner Straße 15 06796 Brehna Germany	N/A	<u>Controlled substance</u> anesthesia, vasoconstriction

Dear Editor or handling manager,

We submit our revised manuscript based on the reviewers' and editor's suggestions. The comments were very thoughtful and we used them to improve the manuscript. The modifications to the manuscript are outlined below, with the changes to the resubmitted main text indicated with red font for easy identification. We copied the decision letter into this document and responded to each comment (red font). Our video was updated as suggested, and a new high-resolution version submitted. Finally, all authors agreed to this revised manuscript. If you have any questions, please do not hesitate to contact me.

Sincerely,

Anthony Hessel

Editorial Comments:

- The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.
We reviewed the revised manuscript for grammatical errors.

- Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) Some examples NOT in the imperative: 1.1, 1.1.1, 2.1,6.4, etc.

We have reviewed all protocol language for use of the imperative case. When not possible, we either use "notes" or moved the discussion to the results or discussion. We highlight a few of the changes in the protocol in red font.

- Protocol Detail:

1) Please ensure homogeneity between the protocol text and video. All specific details mentioned in the video must also be present in the protocol text.

2) The text should be able to serve as a stand-alone document.

3) Section 2 should be merged into another section or simply be made into a note (without the subheading). Each section of the protocol must have at least 2 steps under it.

Our resubmission meets these requirements. Section 2 pertained to the biopsy team composition. One reviewer asked for more details on section 2. To accommodate both comments, we moved this section out of the protocol and to the "important notes" section. The exact words are shown below in response to that reviewer's comment. This change does not affect continuity with the video.

- Protocol Numbering:

1) All steps should be lined up at the left margin with no indentations.

We removed all indentations from the protocol section. Please note that the downloaded template automatically indented the sub-steps of the protocol.

2) Please add a one-line space after each protocol step.

Done as requested.

- Figures: Remove the text “Figure #” from each figure.

Done as requested.

- Table of Materials: Please sort in alphabetical order.

Done as requested.

- If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All materials are original.

Comments on the Video:

Delivery & A/V Specifications

- Please adjust the volume by -4.5 dB (reduce the volume slightly.)

Format & Content Standards

- Please create and insert chapter title cards for the sections of the protocol, the results, and the conclusion. I've included the time codes for the start of sections below.

- 00:41 Protocol:

For your protocol, it could be divided into the ultrasound segment, the biopsy segment, and the removal / storage / processing segment, all with their own title card.

- 03:57 Results

- 04:25 Conclusion

Ethics and Academic Integrity

All protocols with human subjects must include a title card demonstrating approval from your institution's human use ethics committee. This title card should appear between the end of the introduction and the beginning of the protocol. In this video, this would appear after the introduction of the research team at 00:41.

All of these points were successfully incorporated into the revised video.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors provide a great overview of the Bergstrom technique applied to the tibialis anterior,

with a modification that may yield more tissue and many small tips that will be helpful to the physician or others who perform muscle biopsies.

We thank the reviewer for their time commitment to review our manuscript. We found their comments very thoughtful, and we were able to incorporate each of them into our text. Full details are outlined below.

Major Concerns:

The authors provide a very comprehensive overview of the biopsy technique, with a short section on storage and biopsy handling for muscle fiber studies. It would be good to mention other ways to handle/store the tissue, for example to freeze it in isopentane for morphological studies or in liquid nitrogen for protein studies, so that the reader doesn't become confused about the best way to handle the samples, especially because mechanical studies of skinned muscle fibers are awesome but relatively niche.

Tissue freezing and storage techniques are certainly important. It is true that many prefer to freeze samples in liquid-nitrogen cooled isopentane for imaging studies. This is supposed to limit the formation of structure-damaging ice crystals. However, in our hands, we do not observe this problem. For example, we often receive heart biopsies that are taken during surgery and hastily frozen in liquid nitrogen. Although the muscles are often contracted, structural damage is not typically present. Generally, we prefer to fix our samples, rather than freeze them. This also makes storage and long-term transport simple, as fixed muscle can sit in a box for years and still be usable. This is a valid discussion for the readers, and so we expanded our discussion of this in the discussion section.

Starting at line 535:

“Recycling use of leftover tissue content. — 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 conducted³⁵. 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 techniques³⁶ 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 $\sim 150\%$ 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.”

Exclusion criteria are not discussed and are very important to prevent complications. It would be good to add a section on contra-indications for muscle biopsy, especially anticoagulation.

We thank the reviewer for pointing out this oversight. Our ideas on this were hidden in the “take a detailed medical history” line of the protocol. We now add a full description in the “important notes” section.

Starting at line 144:

“Exclusion Criteria. – Exclusion criteria were assessed while taking the participant’s detailed medical history during the participant consultation (see below). Participants were excluded if they suffered an injury to the muscle during the 6 weeks leading up to the biopsy. Participants had to be generally healthy, be aware of no muscle or coagulation disorders, and not currently be on medication that causes blood thinning (e.g. Aspirin). For our study, 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.”

Within the protocol, starting at line 170:

“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.”

Minor Concerns:

Introduction, line 60 - the predominant technique ...

Many centers still use open muscle biopsy, which is not mentioned here at all. It would be good to mention open biopsy techniques and their advantages (e.g. more muscle tissue) and disadvantages (e.g. bigger scar, surgical consult needed). After reading the entire paper I see that this is mentioned in the discussion, perhaps good to have a short mention in the introduction as well.

As the reviewer indicates, open biopsies are perhaps the “best” way to collect nice, healthy tissue samples of exactly what is desired. However, because of the risks of open surgery, they are typically only preferred if they can be added into a surgery for something more necessary (e.g., during knee reconstruction surgery, a small sample of VL can be acquired with very little extra danger to the patient). As requested, we give them some further thought in the introduction (and maintain the original thoughts in the discussion).

Starting at line 91:

“More advanced tissue extraction techniques can be used to limit fiber damage. For example, one group²⁰ 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.”

Introduction, line 92 - of course, every muscle has its own difficulties

This assumes that the reader knows what these difficulties are, would be better to explain (e.g. risk to nerves and blood vessels, muscle depth / anatomy). Also, the section on 'reinventing the wheel' suggests that the physician has to do this by him/herself. However, anyone doing invasive procedure should their limits and consult a more experienced colleague when out of their depth, also when applying a known technique to a different muscle. That said, I agree that it's good to have teaching material available for the tibialis anterior.

We rewrote the section to include some of the important decisions that need to be made on a muscle-to-muscle basis. We certainly hope that a physician would seek outside guidance from other experiences colleagues. We find that this information is often passed down through word of mouth, and not written down, especially for muscles that are less traditionally used for muscle collection. To discuss both concepts more, we modified the section to read:

Starting at line 102:

“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.”

Introduction, line 113 - the tibialis anterior is

The TA is also used in jumping and running, so I personally think that it would be similarly affected as the vastus lateralis. Are there any references that support your statement?

The TA does present some activity during activities like running and jumping, but it is not the primary force generating/bearing muscle. For example, jumping into the air requires ankle plantarflexion, not dorsiflexion. Running loads the calf muscles during the stance phase, where TA activity is more used for ankle stability. Generally, this leads to less wear and tear on the TA compared to the other (primary) muscles. This moves away from our more immediate point: the TA is often not the focus of resistance training, and so it is easier to recruit healthy/athletic

participants who are typically active and have no interest in stopping their activities for our study.

Protocol line 148 - Team composition

In my experience (obtained muscle biopsies both for diagnostic and research purposes), a team of 2 is usually sufficient. The physician performing the biopsy can also interact with the patient and put the acquired material in the required solutions or in liquid nitrogen, while the assistant puts some pressure on the incision site. A bigger team can be helpful especially to quickly handle the tissue, but is not required.

We agree that the procedure can typically be done with just the physician and technician. However, we typically play it safe, and try to have at least one other person on hand, in the unlikely chance that a medical emergency occurs (e.g., patient passes out, or heart attack). In our experience, the participant is much more comfortable and tolerable of the procedure when they are kept mentally removed from the procedure. We accomplish this by having one extra person sit and talk with them about other enjoyable things. These are all important considerations that we did not fully explain, but we now update the main text in the “important notes” section.

Starting on line 160:

“Biopsy team composition. – 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.”

Protocol line 154 - Ultrasound

It's great that such clear instructions for the use of ultrasound are provided, which can also be applied to other muscles. Perhaps its good to mention that ultrasound isn't required for a TA biopsy, its usually quite easy to palpate the muscle to determine the best incision site.

We agree that an experienced physician can perform safe biopsies on big muscles without the aid of an ultrasound. The benefit of the ultrasound system is that it can help locate nerves and blood vessels, and partially assess muscle quality. Even though there is some variation between participants, a physician who has worked on the muscle before can quickly get a feel for what they need to do without the aid of an ultrasound. However, when a biopsy is conducted on a muscle that the physician has less experience with, we think that it is better to spend a few minutes assessing the muscle with the ultrasound. Further, post-op assessment of the biopsy area can be readily monitored with US. To bring this conversation to the reader, we added the following section to the “modification of these methods” section of the discussion.

Starting at line 523:

“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.”

Protocol line 186 - muscle biopsy

It's very interesting that you have a better yield with the foot in this position, I guess this makes sense as the fibers are shortened and more slack fiber length is obtained this way. I am curious whether the patient is able to relax fully in this position and whether it's more painful than in a plantarflexed muscle? I'm going to try this out!

The reviewer is right to think that the person will have trouble relaxing when in a highly dorsiflexed position. However, slight dorsiflexion (0-5 degrees from neutral) is all that is needed to slack the TA while still allowing the participant to keep the muscle deactivated. We did not indicate a target dorsiflexion range, and do so now

Starting at line 225:

“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; Fig. 3). Ask the patient if they can still relax their muscle, as too much dorsiflexion can potentially make it difficult to relax.”

Protocol line 207 - pain receptors

I would like to see a reference here, the biopsy itself can be very painful so I think most participants would disagree that there are no relevant pain receptors in the muscle itself!

This statement was originally too vague and misleading and is now completely removed. The pressure sensors in the muscles can certainly give the perception of discomfort/pain to the participant. Furthermore, accidentally hitting a nerve is definitely an unwelcome feeling for the patient. The definition of “pain” is different for different research groups, and we would prefer to stay out of that argument. To quantitatively assess pain/discomfort in participants, established surveys can be used. Another reviewer (below) pointed out that we do not use these measures, and to add this to our limitation section. They also provide a reference to a new study that quantifies pain in Bergstrom/needle biopsies that was very helpful.

Protocol line 238 - push the tissue

We use the syringe to drive air through the hollow needle to push out the tissue, my experience is that this cause less tissue damage. To do this, place the needle over an empty container and cover this with your gloved hand to prevent splatters. The assistant pushes air through the needle using the syringe, and the tissue comes out. Your method is also perfectly valid, just a different way of doing things.

Great recommendation. We will certainly try this ourselves. We add such a recommendation into our notes from this section.

Starting at line 293:

“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.”

Protocol line 260 and video

The leukoplast is placed in a starshaped pattern, I would recommend to place the strips perpendicular to the incision with slight overlap. This provides better joining / alignment of the incision and better wound healing. I assume the leukostrip is sterile? Please mention this. Also, it would be good to mention in the test that the incision needs to be made in a proximal-distal direction to get less scarring.

This is a good suggestion. We typically add an initial strip perpendicular to the middle of the incision site, with the goal of bringing the edges together, and then use the star shape patten. We believe that the reviewer suggests placing all strips in the perpendicular orientation, along the whole length of the incision. After we lay these initial strips, a starshaped pattern is still helpful because it guards against multi-directional loading of healing site. With the goal to limit scarring as much as possible, we now incorporate these ideas into the protocol. Finally, we added the word “sterile” into our description of the product.

Starting at line 318:

“3.14. Close the incision site.

3.14.1. 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.

3.14.1.1. Notes: proper handling of this step will reduce scarring. Suturing the wound can be done but is not necessary. Other options include wound glue.”

.....

Protocol line 283 - biopsy handling and storage

This is specifically for force measurements; of course other investigations require other storage methods. It would be good to mention this.

We give this comment full treatment above (major comments section). We now discuss other options much more thoroughly (updated section shown above).

General

Please mention Bergstrom needle size somewhere (4 or 5 mm)

We used 5 mm. We now give this information in the paper.

Discussion

It would be nice to briefly discuss other muscles that are suitable for muscle biopsy (e.g. deltoid, vastus) and those who are less suited due to vascularisation or nerves (e.g. gastrocnemius). This would give the reader a starting point to search additional literature on biopsies in other muscles and an overview of important things to consider before undertaking a biopsy in a different muscle.

We understand how our section titles mislead the reader, and so we rewrote them to “comparisons to other methods”, and “potential adaptations of this protocol”. We then added a few sentences to our new section called “potential adaptations of this protocol”, to discuss the selection of other muscles.

Starting at line 503:

“Potential adaptations of this protocol. — Although our 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 our experiences, 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.”

Your modification isn't necessarily to the biopsy technique but to the position of the foot. The proposed modification isn't explicitly mentioned in the discussion (warrants it's own paragraph) and it is confusion that part of the discussion is about modifications but not about the modification you propose in the text.

We now organize this section differently and give an appropriate discussion to the positioning of the foot. We now have two sections, “comparisons to other methods”, and “potential adaptations of this protocol”. We discuss the use of the foot plate in the “comparisons to other methods” section.

Starting at line 486:

“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, our simple, but major modification 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 our 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.”

Video

There is a spelling mistake: aponerosis > aponeurosis

This is now corrected.

The name and signature of the volunteer appear to be visible in the video, please blur or make it clear if a pseudonym was used for the video.

We have blurred it out.

The manufacturer of the setup (Aurora Scientific) is visible in the video, consider removing this to prevent any suggestion of a conflict of interest / promotional purposes.

We decided to leave this, as we declare no conflict of interest. There are very few commercially available systems, which we have tried most. We could discuss their merits if the editor thinks this not a tangential idea.

Reviewer #2:

Manuscript Summary:

This is a technical report on how to perform a muscle biopsy from the tibialis anterior muscle. Incorporating ultrasound measures to ensure/minimize any damage to non muscle tissue is an important consideration which the authors clearly articulate. The information provided in the text, and video is extremely helpful to anyone considering this procedure. The care and detail which went into this report and video is exceptional.

I thank, and congratulate the authors for this fine service and contribution to the field. I recommend publication as is.

We thank the reviewer for carefully assessing our manuscript.

Reviewer #3:

Manuscript Summary:

The present manuscript describes a protocol for muscle sampling of the tibialis anterior (TA) in preparation for mechanical testing. Overall, the report is well-written and the protocol is detailed, very clear, and effectively describes the video presentation. The authors are to be commended for the level of reporting detail, and I believe future researchers can easily adopt and replicate this protocol based on the written manuscript and accompanying video. I have some considerations and comments below that mostly pertain to the introduction and discussion sections. In particular, the authors should address limitations surrounding their evaluation of pain and biopsy-related discomfort.

We thank the reviewer for their careful assessment of our manuscript. We incorporated their ideas into the manuscript. We provide details below.

Major Concerns:

1. Lines 104-113: I believe the rationale for using the TA over the vastus lateralis is incomplete. As the authors suggest, the TA is not activated - or at least as much as the vastus lateralis - in activities external to prescribed exercise intervention. While sampling the TA may therefore better eliminate the confound of external physical activity, this advantage only occurs if the prescribed exercise intervention effectively targets/activates the TA (e.g. dorsiflexion) and this point should be made more clear in the introduction.

The reviewer brings up a good point about the TA. For the intro, we focused on the point that the TA is typically not directly trained. However, we appreciate that some researchers will want to see changes to muscle function after training (e.g. a longitudinal study of some form). To do this,

a controlled approach to TA training would be needed, and could be accomplished with the use of a dynamometer. We add a little into the intro to discuss this point.

Starting at line 124:

“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.”

2. Related to point 1, while a resistance-type training program can ostensibly be prescribed based off dorsiflexion, it is likely very challenging to prescribe an aerobic exercise that targets the TA. Thus, can TA biopsies be used to examine adaptations to aerobic training? The authors should consider acknowledging this as limitation of TA biopsies in the discussion.

This is an important point to consider when designing longitudinal studies on muscle-adaptations to exercise. Indeed, we would agree that this muscle would not be perfect for aerobic exercises (at least not as the primary functioning muscle). However, these concepts, which are already more detailed after the additions in the intro from the previous comment (shown above), move out of the scope of this paper’s purpose and would be a bit out of place. We already indicate that the selection of the target muscle must fall in line with the research questions under study, and we think that this is sufficient. If the editor would like some sentences incorporated for this point, we would be happy to do so.

3. A limitation of the current study is the methods for assessing pain and post-biopsy discomfort (lines 300-321). It is unclear how these assessments were made, but it seems that validated pain/discomfort surveys were not used. Using several validated surveys, a recent study evaluated pain and the impact of biopsies (Bergstrom and microbiopsy needles) on discomfort and activities of daily living during the recovery period

(<https://www.sciencedirect.com/science/article/pii/S2665944120300067>). The authors should acknowledge the limitation(s) associated with not using validated surveys to ensure valid assessments of pain and discomfort. Further, the authors could allude to the need for future work to use validated surveys - similar to the ones used in the above-mentioned study - in order to accurately evaluate pain and discomfort during/following this TA sampling technique.

This is a timely and helpful paper, thank you! We did not quantify the pain or discomfort of the patient, and instead relied on the experiences of the physician. However, quantifying pain and discomfort would be very helpful, especially if we want to compare pain scores between individuals or treatments. In the limitations section, we now discuss this point in detail.

Starting on line 581:

“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 pain¹. 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.”

Minor Concerns:

1. Introduction: Given that few studies conduct mechanical tests on skinned fibers (mentioned on line 74), the average reader may be unfamiliar with this analysis. Adding a brief definition/example - even in lay terms - of mechanical testing in the introduction could help a naïve reader better understand this analysis and appreciate the importance of collecting long, undamaged fibers.

This is a great point. We have added some lay-term descriptions of mechanical testing.

Starting at line 71:

“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^{1,9-11}. Another critical use for muscle biopsies is mechanical experiments such as the measurement of fiber contractile force, muscle fiber stiffness, and history-dependent muscle properties¹²⁻¹⁶. 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.

2. Line 106 - should say refrain instead of reframe?

Fixed.

3. Lines 287-292: Could the authors briefly explain the rationale for storing at 4C before transferring to -20? E.g., what is the Rigor collection solution doing to the tissue during this stage?

The role of the collection solution is to remove the blood (and any proteomes, or autophagy precursors) from the tissue sample in exchange for the protease-inhibitor rigor solution. The glycerol solution will prevent the muscle from freezing in subzero temperatures (destroying its mechanical properties), but a waiting period is needed for the glycerol to diffuse into the sample, or it will still freeze. Thus, we usually allow the muscle to sit in the glycerol solution overnight in the fridge (often on a shaker). We now quickly refer to this in the protocol section of the paper, but also give it a full treatment within the results section. That section can be seen in our response to a similar light of thought below.

Starting at line 357:

“Exchange the Rigor solution for fresh rigor, lightly shake for 4-6 mins, and then store at 4 °C for 4-6 hrs to allow the exchange of protease-inhibitor storage solution and blood.”

4. Line 293: Could the authors provide justification for the three-month storage maximum? I.e., what happens, or may happen, to the fibers after three months?

This is a great point. In our results, we now expand on the permeabilization procedure, and important points to consider. More information was also asked from another reviewer.

Starting at line 395:

“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 placed 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. Our 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.”

Author Biographies

Dr. Anthony Hessel is a research scientist at the Institute for Physiology II, University of Muenster. He received his BSc (Allegheny College), MSc (Northern Arizona University), and PhD (Northern Arizona University) in biology with a focus on skeletal muscle physiology and biomechanics. His first postdoctoral position was at the Department of Movement Science, Ruhr University Bochum, which is where this project was conducted. His current research interests include identifying the role of the titin protein in force production and understanding the effect of exoskeletons on low limb muscle biomechanics.

Dr. Markus de Marées is a physician and research scientist at the department of Sports Medicine and Sports Nutrition, Ruhr University Bochum. He received his PhD (Sports University Cologne) in medicine with the focus on effects of hypoxia on the human body and MSC in particular. His current research encompasses the effects of hypoxia on people with congenital heart defects and the importance of collagen in connection with muscle physiology.

Dr. Daniel Hahn is an Associate Professor for Human Movement Science at the Faculty of Sport Science at Ruhr University Bochum (Germany). He received his Diploma (Technical University of Munich, Germany) in Sport Science, his Doctorate in natural science (Technical University of Munich, Germany) with a focus on human muscle function and biomechanics, and he did postdoctoral training in Sensorimotor Neuroscience at the University of Queensland, Australia. Current research in his laboratory seeks to understand in vivo neuromuscular function of humans. A major focus of this research is how the intact muscle-tendon complex and the nervous system interact to produce force under varying contraction conditions and task demands. Within this context he is particularly interested in eccentric muscle action as well as the history dependence of muscle action and how this contributes to economic human locomotion.

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Author(s):	Anthony L. Hessel; Daniel Hahn; Markus de Marées

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12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

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