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Cryosectioning of Contiguous Regions of a Single Mouse Skeletal Muscle for Gene Expression and Histological Analyses

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Abstract:	<p>With this method, consecutive cryosections are collected to enable both microscopy applications for tissue histology and enrichment of RNA for gene expression using adjacent regions from a single mouse skeletal muscle. Typically, it is challenging to achieve adequate homogenization of small skeletal muscle samples because buffer volumes may be too low for efficient grinding applications, yet without sufficient mechanical disruption, the dense tissue architecture of muscle limits penetration of buffer reagents, ultimately causing low RNA yield. By following the protocol reported here, 30 m sections are collected and pooled allowing cryosectioning and subsequent needle homogenization to mechanically disrupt the muscle, increasing the surface area exposed for buffer penetration. The primary limitations of the technique are that it requires a cryostat, and it is relatively low throughput. However, high-quality RNA can be obtained from small samples of pooled muscle cryosections, making this method accessible for many different skeletal muscles and other tissues. Furthermore, this technique enables matched analyses (e.g. tissue histopathology and gene expression) from adjacent regions of a single skeletal muscle so that measurements can be directly compared across applications to reduce experimental uncertainty and to reduce replicative animal experiments necessary to source a small tissue for multiple applications.</p>
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TITLE:

Cryosectioning of Contiguous Regions of a Single Mouse Skeletal Muscle for Gene Expression and Histological Analyses

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KEYWORDS:

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SHORT ABSTRACT:

Consecutive cryo-sections are collected to enable histological applications and enrichment of RNA for gene expression measurements using adjacent regions from a single mouse skeletal muscle. High-quality RNA is obtained from 20 - 30 mg of pooled cryosections and measurements are directly compared across applications.

LONG ABSTRACT:

With this method, consecutive cryosections are collected to enable both microscopy applications for tissue histology and enrichment of RNA for gene expression using adjacent regions from a single mouse skeletal muscle. Typically, it is challenging to achieve adequate homogenization of small skeletal muscle samples because buffer volumes may be too low for efficient grinding applications, yet without sufficient mechanical disruption, the dense tissue architecture of muscle limits penetration of buffer reagents, ultimately causing low RNA yield. By following the protocol reported here, 30 μ m sections are collected and pooled allowing cryosectioning and subsequent needle homogenization to mechanically disrupt the muscle, increasing the surface area exposed for buffer penetration. The primary limitations of the technique are that it requires a cryostat, and it is relatively low throughput. However, high-quality RNA can be obtained from small samples of pooled muscle cryosections, making this method accessible for many different skeletal muscles and other tissues. Furthermore, this technique enables matched analyses (e.g. tissue histopathology and gene expression) from adjacent regions of a single skeletal muscle so that measurements can be directly compared across applications to reduce experimental uncertainty and to reduce replicative animal experiments necessary to source a small tissue for multiple applications.

INTRODUCTION:

The goal of this technique is to make multiple experimental analyses by different modalities, such as histology and gene expression, accessible from a single small skeletal muscle source tissue. Microscopy applications are the most sensitive to sample preservation methods, which must be carefully controlled to limit the formation of ice crystal artifacts during cryopreservation. Thus, method development is based on the tibialis anterior (TA) muscle frozen partially covered with embedding resin in a -140 °C liquid nitrogen-cooled 2-methylbutane bath as the source material for both immunofluorescence microscopy and gene expression analyses.

The need to use the same source material for diverse technical approaches is particularly important for intramuscular injection-based experiments where the left and right muscles represent different conditions, one experimental and one control. For example, in muscle regeneration studies, one muscle is injected with a toxin to cause widespread tissue damage while the contralateral muscle serves as a vehicle-injected control¹. Similarly, gene therapy studies for muscle disorders typically begin with validation of the gene therapy vector by intramuscular injection to be compared with empty vector, unrelated vector or vehicle control on the contralateral side². Therefore, it is not possible to source each TA muscle to a different application.

Common strategies to deal with this issue are: i) to use a different muscle group for each application, ii) to use additional mice, or iii) to cut off a piece of the muscle for each application. However, substantial differences between muscle groups make it difficult to compare data from separate applications, and additional animals increase expense and are poorly justified if other alternatives exist. Dividing the muscle after dissection to source different applications is the best option in many cases. However, the muscle pieces are often too small to use pulverization under liquid nitrogen or mechanical grinding techniques for homogenization²⁻⁵. As muscle is a highly structural tissue packed with extracellular matrix and contractile proteins, inadequate mechanical homogenization leads to a low yield of subsequent DNA, RNA or protein. The method detailed here allows small quantities of tissue from one source muscle for use in multiple applications, and the inclusion of cryosectioning and needle trituration improves mechanical homogenization for better RNA yield.

PROTOCOL:

All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee under animal use protocol A2013 07-016 (Beedle).

1. Cryopreservation of unfixed skeletal muscle.

1.1) Preparation

1.1.1) Cut cork into small squares (approximately 1 cm x 1 cm) with a razor blade, write on the cork with a fine tip marker that is resistant to 2-methylbutane to identify the source mouse and muscle, and make a very shallow cut (approximately 1 mm) across the top surface. Insert a

plastic coverslip into the cut to use for orienting the tissue. Repeat until a cork is ready for every tissue to be cryopreserved.

1.1.2) Obtain liquid nitrogen, 2-methylbutane, embedding resin, low-temperature thermometer, corks, dissection tools, and study mouse.

CAUTION: Liquid nitrogen is a compressed gas that may explode if heated. Wear a lab coat, low-temperature gloves, and face protection when handling liquid nitrogen; contact with skin or eyes may cause burns or frostbite. **2-Methylbutane** is a flammable, toxic, health and environmental hazard. Wear personal protective equipment (lab coat, gloves, safety glasses), open the stock container in a fume hood, transfer the small amount needed for freezing (typically 200 to 400 mL) to a separate container that can be tightly closed, and avoid inhalation.

1.1.3) Euthanize mice with an approved method of euthanasia under anesthesia. Briefly, insert the mouse into an inhalation chamber with 2.5% isoflurane in oxygen from an isoflurane vaporizer, wait until 20 seconds after the mouse stops moving to check for a pedal reflex. When the pedal reflex is negative, euthanize the study mouse by cervical dislocation⁶.

NOTE: Euthanasia methods require approval by the institutional ethics committee.

1.1.4) Remove skin overlying the distal hindlimb and cut the distal anterior tendons just above the ankle with fine-point dissection scissors. Grasp the distal tendons with fine forceps and gently pull up and towards the knee while cutting lateral fascia to release the muscle. Pull the muscle out perpendicular from the knee and make a final cut to excise the TA muscle⁷.

NOTE: The TA muscle is used here as an example, but any mouse skeletal muscle or the heart can be substituted for the TA in this protocol if appropriate to the user's experimental goals. The only limitation is that a tissue must be small enough to achieve rapid cryopreservation throughout its depth; a maximum tissue size of 1 cm x 1 cm is recommended.

1.1.5) Repeat on the opposite leg, and dissect out any other tissues to be collected. Perform dissection as quickly as possible to limit tissue degradation before cryopreservation.

1.1.6) Orient each muscle for transverse sections on its pre-labeled cork. Stand the muscle perpendicular to the cork with the distal tendon touching the cork and the top of the muscle extending away, held upright by the coverslip.

1.1.7) Cryopreserve all tissues for histological analysis as soon as possible after dissection, preferably within 5 min but definitely not more than 15 min time elapsed since euthanization of the source animal.

1.2) Cryopreservation

1.2.1) Begin cooling the cryopreservation bath five min before the end of the dissection. Pour 2-methylbutane into an open metal beaker to a depth of approximately 3 cm. Pour **liquid nitrogen** into an insulated container to a depth of 2 to 4 cm. Set the beaker of 2-methylbutane

into the liquid nitrogen; the nitrogen will begin to boil. Avoid nitrogen splashing into the 2-methylbutane beaker.

CAUTION: Prepare freezing bath in a fume hood or a well-ventilated area.

1.2.2) Insert a low-temperature thermometer into the 2-methylbutane to monitor temperature and stir frequently with a fork to ensure even cooling. Continue to stir and cool until 2-methylbutane reaches -140 °C, adding new liquid nitrogen to the outer bath as necessary.

NOTE: -140 °C is the optimum temperature to minimize ice crystal artifacts in striated muscle; other tissues may cryopreserve best at different temperatures (e.g. brain, -90 °C).

1.2.3) Apply embedding resin only to the lower 35-50% of the muscle where it meets the cork and immediately drop the cork into the 2-methylbutane at -140 °C. Rapidly repeat for up to 8 tissues per freeze batch. Stir for 30 sec, scraping the bottom of the beaker to ensure that tissues don't freeze into the solidifying 2-methylbutane.

1.2.4) Use a fork, slotted spoon or large forceps to pull each tissue cork from the 2-methylbutane. Quickly remove the coverslip, dab off excess 2-methylbutane into the beaker, and then drop the tissue cork into the outer nitrogen bath. Repeat for all remaining corks in the beaker.

1.2.5) Transfer samples in liquid nitrogen or on dry ice to a -80 °C freezer for storage.

1.2.6) If any tissues remain for cryopreservation, repeat steps 1.2.3 to 1.2.4. Add additional 2-methylbutane to the beaker or liquid nitrogen to the outer bath as necessary and re-cool to -140 °C. Dispose of used 2-methylbutane as hazardous waste.

2. Collect cryosections for histology and RNA applications.

2.1) Preparation.

2.1.1) Pre-weigh a DNase/RNase-free autoclaved tube on an analytical balance. Then, move it into the cryostat chamber to precool. Repeat until tubes are ready for all pooled cryosection samples to be collected.

2.1.2) For skeletal muscle sectioning, confirm that the cryostat chamber temperature is -21 to -22 °C by its internal thermostat. Transfer any containers with tissues to be cut into the chamber and allow the container(s) to equilibrate to the cryostat temperature for at least 15 min before opening.

2.1.3) Insert a new disposable cryostat blade. Alternately, remove the existing blade, spray with RNase decontamination solution, rinse with ddH₂O, and reinsert into the cryostat to cool. Spray a clean tissue with 70% ethanol and carefully wipe the blade and blade clamping platform.

2.1.3.1) Spray a clean tissue with an RNase decontamination solution and wipe cryostat

brushes. Spray a tissue with ddH₂O, wipe the brushes again and set them in the cryostat chamber on a clean surface.

CAUTION: The cryostat blade should be covered by the knife guard when not in use. Also, RNase decontamination solution is toxic and will freeze to form a precipitate in the cold cryostat chamber. Therefore, handle with care and avoid its use in the cryostat chamber.

2.1.4) Within the cryostat chamber, place a dime-sized drop (approximately 300 µL) of embedding resin onto a warm specimen chuck, set a tissue cork on top of the resin, press down, and then set the chuck in the freezing rail or onto a fast freeze element if available.

2.1.5) After the embedding resin solidifies (white), add additional embedding resin on top of the cork, around the lower 35% of the tissue and press the heat extractor into the embedding resin for a fast freeze to better stabilize the tissue. Wait 5 min before sectioning to allow the resin to harden fully.

2.1.6) Ensure that the specimen clamp is in its most retracted position, farthest from the blade. Insert the tissue specimen chuck into the specimen clamp.

2.2) Cryosection preparation.

2.2.1) Loosen the blade carrier holder, set the clearance angle of the blade to 10° (or an angle appropriate for the blade carrier used), and re-tighten. Release the brake and turn the hand wheel to lower the muscle towards the blade. Estimate the closest distance between the tissue and blade, then move the tissue away from the blade and engage the hand wheel brake.

2.2.2) Loosen the height adjustment lever and move the blade carrier forward or back, respectively, if the end of the tissue is more than approximately 2 mm from the blade or if the blade strikes the tissue. Tighten and lower the tissue again to check distance from the blade. Repeat adjustments until the blade is 1 to 2 mm from the end of the tissue by visual estimate.

2.2.3) Lower the tissue towards the blade and assess tissue angle for transverse sections. Lock the hand wheel and release the specimen clamp lever. Push the specimen clamp left or right until the horizontal orientation of the tissue is perpendicular to the blade.

2.2.4) Push the specimen clamp up or down to adjust the “y” orientation so that tissue sections will be perpendicular to the long axis of the muscle. Tighten the specimen clamp lever.

2.2.5) Use the course and fine forward feed to advance the specimen until it just touches the blade. If seeking sections from a particular tissue depth, reset the sum of section thicknesses to zero (top of the tissue).

2.2.6) Set the cryostat to the trim function with section thickness at 30 µm. Cut and discard sections until the preselected depth for tissue collection is reached (e.g. 400 µm from the top).

2.3) Collect cryosections for RNA extraction.

2.3.1) Open the tube for collecting sections and place it near the blade carrier. Use a pre-cooled, clean brush to pick up each section as it is cut from the blade and transfer the cryosection into the tube. Repeat until the pooled sections weigh 30 mg or the desired tissue depth is reached.

NOTE: For an adult mouse tibialis anterior, collection from tissue depth of approximately 400 to 4000 μm typically yields 25-40 mg. Using metal forceps to transfer sections to the collection tube is not recommended as the sections tend to stick and clump on the metal surface.

2.3.2) If embedding resin surrounds the muscle cryosection, lock the handbrake and use a razor blade to shave off small pieces of resin until there is only a thin layer around the top of the muscle. Always cut resin with the blade angled away from the muscle.

NOTE: A thin layer of embedding resin does not substantially impair the downstream RNA preparation. If thicker embedding resin is present, use brushes to tease it away from the muscle before moving the cryosection into the collection tube.

2.3.3) Alternatively, pool sections on the blade carrier and transfer in bulk to the collection tube.

NOTE: However, this method tends to be slower, and sections are more likely to stick and clump together, which can reduce the efficiency of needle homogenization in later steps.

2.3.4) Quickly place the pooled cryosection tube into an analytical balance and record tube weight. Immediately return the tube to the cold cryostat chamber to maintain section temperature near $-20\text{ }^{\circ}\text{C}$. Calculate the weight of the pooled sections.

NOTE: If RNA isolation will occur on a different day, store the pooled cryosection tube at $-80\text{ }^{\circ}\text{C}$ until use.

2.4) Collect cryosections for histology.

2.4.1) Press the section thickness button for fine sectioning and use arrows to set the cryostat section thickness to 7 μm (or other appropriate section thickness, typically 6 to 10 μm).

NOTE: Thinner sections (6 to 10 μm) should be used for histological applications to ensure that staining reagents can penetrate the depth of the tissue section. Thin sections can be taken from any depth during the cryosectioning, but deeper sections are preferred because embedding resin, which increases with tissue depth, does not impair histological staining.

2.4.2) Cut and discard 4 to 7 sections to obtain a consistent, even tissue surface. Make note of the tissue depth.

2.4.3) Cut a section and orient it on the surface of the blade carrier. Pick up the section by quickly and gently touching a warm (room temperature) microscope slide to the section on the blade carrier. Return the slide to room temperature. Continue until the number of desired slides is obtained. Make note of the final tissue depth.

NOTE: Collecting a second (duplicate) section for each tissue is recommended.

2.4.4) With sectioning complete, engage the hand wheel brake, return the specimen to the rear-most position, and remove the tissue chuck. Use the cryostat heat element to melt the embedding resin holding the tissue cork on the specimen chuck. Remove tissue cork, dry with tissue, and return to storage.

2.4.5) Repeat from step 2.1.2 for each remaining tissue. Allow slides to dry for 20 min after the last tissue is mounted. Then, use slides for histological or immunofluorescent staining or freeze at -80 °C in a slide box until needed.

3. RNA isolation from pooled cryosections

3.1) RNA extraction

3.1.1) Move tube(s) of pooled cryosections to ice. Immediately add an organic RNA extraction reagent at a ratio of approximately 1 mL reagent per 50 mg cryosection weight, typically 600 µL per tube.

CAUTION: RNA isolation reagents are toxic, corrosive, and irritating. Follow the manufacturer's instructions for safe handling.

3.1.2) Using a 1 mL syringe with an 18 gauge needle, draw the RNA extraction liquid up and rinse the walls of the tube until all tissue is suspended in the solution. Try to minimize air bubbles during needle homogenization.

3.1.3) Use the tip of the needle to mash and disperse any clumped cryosections and pieces adhering to the tube wall. Triturate to homogenize by passing the sample up and down through the needle for five strokes, and then return the sample to ice. Repeat both steps three to five times, or as many times as is necessary to disrupt all clumps and pass the sample easily through the needle.

3.1.4) Remove the 18 gauge needle and replace it with a 22 gauge needle. Carefully triturate the sample for five strokes, and then return the sample to ice. Repeat the trituration three to four times to achieve a very finely dispersed tissue homogenate.

NOTE: If tissue particles start to block the needle when drawing up the sample, expel all sample from the syringe and switch back to the 18 gauge needle for further homogenization. An additional trituration step with a 25 or 26 gauge needle can be added to obtain maximal yield. However, the needle can become clogged so there is a risk of sample spilling.

3.1.5) Move the sample from ice to room temperature. Incubate for 5 mins to disrupt molecular complexes.

3.1.6) In a fume hood, add 0.1 mL of 1-bromo-3-chloropentane (BCP) per 1 mL of RNA isolation reagent used (step 3.1.1), typically 60 µL per tube. Shake the tube vigorously by hand for 15 sec (do not vortex). Incubate the sample at room temperature for 2 to 3 min. Then, centrifuge for

15 min at 12,000 x g and 4 °C.

CAUTION: BCP is flammable and toxic. Handle in a fume hood and wear gloves, a lab coat, and safety glasses.

3.1.7) Carefully collect the upper aqueous phase (colorless) containing RNA and transfer it to a clean tube. Add an equal volume of 70% ethanol (in DEPC water) and vortex to mix. Incubate at room temperature for up to 10 min.

NOTE: the middle interphase and lower phenol-BCP phases can be processed for genomic DNA and protein, respectively, according to the manufacturer's instructions or collected in a phenol-BCP hazardous waste container for appropriate disposal.

3.2) RNA purification.

3.2.1) Follow the manufacturer's instructions for sample binding, washing and elution with minor variations⁸. For example:

3.2.2) Place an aliquot of DNase/RNase-free water into a 37°C bath or incubator to pre-warm for step 3.2.4.

3.2.3) Add the RNA sample from 3.1.8 above to a purification column and centrifuge the sample for 15 sec at 12,000 x g at room temperature. Pour the flow through back into the same column and re-centrifuge. Repeat one additional time to maximize RNA binding, and then discard the final flow through.

3.2.4) Perform wash steps according to the manufacturer's instructions⁸. Apply 700 µL of wash buffer (no ethanol) to the column, spin for 15 sec at 12,000 x g, and discard the flow through.

3.2.5) Add 500 µL of wash buffer (with ethanol) to the column, spin for 15 sec at 12,000 x g, and discard the flow through. Repeat this step one time.

3.2.6) Spin the empty column for 1 min at 12,000 x g to dry the membrane.

3.2.7) Transfer the spin column to a clean RNase-, DNase-free tube. Add 40 µL of pre-warmed DNase/RNase-free water (from 3.2.1) to the center of the column membrane. At room temperature, incubate for 2 min and then centrifuge for 2 min at 12,000 x g.

NOTE: The 40 µL elution volume is approximately 1.3 µL per mg of original tissue for 30 mg of pooled cryosections. Adjust the elution volume as appropriate for different starting tissue weights, but do not reduce below 32 µL. A second elution, using approximately 0.7 µL per mg of original tissue, can be added to increase total RNA yield.

3.2.8) Analyze the RNA (column elution) for concentration and purity by a spectrophotometer, electrophoresis, and/or a bioanalyzer. Store the RNA at -80 °C until needed for downstream applications, such as reverse transcription for quantitative PCR⁴.

NOTE: A DNase treatment is recommended before using the RNA in downstream applications.

This treatment can be performed on some RNA purification columns before the wash step, at this specific point following column elution, or on an aliquot of the RNA as the first step in any downstream application. The RNA should be stable for several years.

4. Histological analysis by immunofluorescent staining of muscle cryosections.

4.1) Simple immunofluorescence protocol.

4.1.1) Use a hydrophobic pen to circle the group of sections on each slide. Gently drop PBS into the circled area (approximately 80 μ L for a small surface area up to 500 μ L for a large surface area), being careful not to touch the tissues. Incubate for 5 min at room temperature.

NOTE: If using frozen slides, remove slides from -80 °C freezer and incubate at room temperature for 20 to 30 min to thaw and dry, then proceed as above. At least two slides are needed: one experimental slide to be incubated in primary and secondary antibody; and one control slide for which primary antibody is excluded, the “secondary only” control.

4.1.2) Tip the slide to pour off PBS. Add 5% donkey serum in PBS (blocking solution) to the circled area; be careful to ensure that the muscle sections do not dry out. Incubate at room temperature for 30 min (or up to several hours in a humidity chamber).

4.1.3) Prepare primary antibody solution to analyze muscle regeneration. Mix blocking solution, with an eMHC antibody (1:40) and a ColVI antibody (1:1000). Prepare approximately 150 μ L, 300 μ L or 500 μ L for small, medium, or large slide areas, respectively. Vortex for 5 sec, and then centrifuge for 2 min at 15,000 x g to pellet any precipitate.

4.1.4) For experimental slides, tip the slide to pour off blocking solution and then add primary antibody solution from 4.1.3. For the secondary control slide, tip the slide to pour off blocking solution and then add fresh blocking solution (no antibody). Incubate slides in a humidity chamber at 4 °C overnight.

NOTE: When pipetting antibody solutions onto slides, always pull liquid from the top of the primary antibody solution tube, do not disrupt any precipitate at the bottom of the tube.

4.1.5) Tip slides to pour off solution. Add PBS dropwise to the circled region of each slide to wash, tip to pour off, then add more PBS and incubate for 3 to 5 min. Repeat for a total of three washes.

NOTE: The wash time is quite flexible and can be lengthened up to 20 min if desired. Generally, the number of solution changes is more important than the time.

4.1.6) Prepare secondary antibody solution for all slides (including the secondary control slide) using a 1:500 dilution of red and green fluorophore-coupled secondary antibodies to detect mouse IgG1 (eMHC) and rabbit IgG (ColVI) and 1:10,000 dilution of DAPI nuclear stain.

4.1.6.1) For example, mix 1 μ L of DAPI with 9 μ L of ddH₂O to make a 1:10 dilution of DAPI. Then, for a 500 μ L final volume, add 1.0 μ L anti-mouse IgG1-red + 1.0 μ L anti-rabbit IgG-green

+ 0.5 μ L of 1:10 DAPI to 447.5 μ L of blocking solution. Vortex to mix and centrifuge for 2 min at 15,000 x g to pellet any precipitate.

NOTE: Ideally, all secondary antibodies should be from the same host species.

4.1.7) Tip the slides to pour off the last PBS wash. Add secondary antibody solution to cover all tissues. Cover the slides to protect them from light and incubate at room temperature for 30 min.

NOTE: All secondary antibodies should be validated to have minimal cross reactivity with other species in dual labelling experiments.

4.1.8) Wash the slides as described in 4.1.5. After the last wash, tip the slide to pour off the PBS and set the slide on a tissue. Add 3 to 4 drops of an aqueous mounting media along one side.

4.1.9) Set the edge of a glass coverslip just to the outer edge of the mounting media. Using forceps, slowly lower the coverslip towards the tissues, then, release the coverslip and gently tap it into position. Finally, gently press each corner of the coverslip to stabilize it.

4.1.10) Protect the slides from light and store at 4 °C until use.

NOTE: For long term storage, apply a thin layer of nail polish along the edge of the coverslip to help prevent the slide from drying.

4.2) Histological evaluation.

4.2.1) Image the slides using an epifluorescent or confocal microscope with appropriate filters to detect eMHC (red); ColVI (green); and DAPI-stained nuclei (blue), typically using a 20X objective^{1,5}.

4.2.2) Confirm that the fluorescent signal from experimental slides is different from the secondary control slide to demonstrate the specificity of the eMHC and ColVI detection⁴.

NOTE: eMHC positive regenerating fibers should be visible from approximately 2 to 7 days after a muscle injury and variably in mice with muscular dystrophy. Collagen VI is present in the extracellular matrix surrounding muscle fibers, blood vessels, and nerves and in the larger connective tissue bundles of peri- and epimysium. DAPI nuclear stain is useful to identify muscle fibers with central nuclei versus peripheral nuclei indicating fiber regeneration, and the presence of infiltrating cells. Necrotic or injured fibers may stain weakly with the mouse IgG secondary antibody due to detection of endogenous IgG that penetrates the fibers through damaged muscle membranes.

4.2.3) To quantify regeneration, take overlapping microscope pictures with each fluorescent filter across the entire image. Merge the eMHC, ColVI and DAPI images for each location and then align the pictures to reconstruct a map of the entire section^{1,5}.

4.2.4) Using analysis software, count the number of eMHC positive fibers and the number of total fibers to calculate recent regeneration ($100 * (\# \text{ eMHC+ fibers} / \# \text{ total fibers})$). Also, count the number of centrally nucleated fibers out of the total fibers to measure regeneration over a

longer period ($100 * (\# \text{ CN+ fibers} / \# \text{ total fibers})$)^{1,5}.

REPRESENTATIVE RESULTS:

Muscle cryosection RNA is high in quality and provides sufficient yield for most applications

Analyses of sixteen skeletal muscle RNA preparations are shown in Table 1 using 19.4 to 41 mg of pooled tibialis anterior (TA) muscle from 8 control mice. Both left (L) and right (R) TA muscles were prepared in regeneration experiments with muscles collected 3 days after longitudinal intramuscular injection of 25 μL of saline or 10 μM cardiotoxin to cause muscle injury using methods previously reported¹. As shown in Table 1, the A260/280 ratios for muscle cryosection RNA are typically close to 2 or higher in these representative samples. As “pure” RNA is considered to have A260/280 of 2.0 and A260/280 of 1.8 to 2.2, the purity of the cryosection RNA samples is excellent⁹. Total RNA recovery was typically over 10 μg per sample with yields of 0.18 to over 1 μg of total RNA per mg of starting tissue, providing sufficient material for most downstream applications. Notably, RNA concentration, total RNA extracted, and RNA yield per mg of starting tissue from TA muscles 3 days post-toxin injury was significantly higher than from saline-injected TAs. This suggests that there is improved homogenization when muscle structure is disrupted by extensive injury and/or that there is an increase in gene transcription and/or RNA stability in 3 day regenerating muscle. The persistence of RNA quality was assessed by simple 1X TAE/1.5% agarose gel electrophoresis of 1 μL of muscle cryosection RNA after samples were stored at -20°C for 18 months. Prominent 18S and 28S rRNA bands are still evident in samples demonstrating high RNA quality, even under suboptimal storage conditions (Figure 1A).

Muscle cryosection RNA supports downstream applications

One microgram of RNA per pooled cryosection sample was treated with DNase and reverse transcribed from oligo dT primers. Following RNase treatment, the total volume of the reverse transcribed cDNA was 30 μL . Simple non-quantitative PCR with excess amplification was run to confirm the viability of the cDNA. Myogenic regulatory factor 4 (Mrf4), a transcription factor upregulated with muscle differentiation, was amplified using previously reported mouse primers, sense 5'-CTACATTGAGCGTCTACAGGACC and antisense 5'-CTGAAGACTGCTGGAGGCTG¹⁰, from 2 μL of template using standard PCR. There was robust, specific amplification of the 234 bp Mrf4 fragment from both left and right TA cDNA samples, but not RT- (RNA included, but no reverse transcriptase), RT ddH₂O (reverse transcription with no RNA template), or ddH₂O PCR controls (Figure 1B). The same samples were run in triplicate for Mrf4 and mOaz1 reference control⁴ quantification using relative amplification and passive fluorescence reference on a real-time PCR system. Mrf4 was expressed at 0.097-fold in the toxin-injected right TA compared to the saline-injected left TA, calculated by the $\Delta\Delta\text{Ct}$ method⁴. This is similar to previous reports of low Mrf4 expression 3 days after toxin injury due to a loss of mature muscle fibers¹¹. To compare the consistency of cryosection RNAs for quantitative PCR, Ct values were compared for the mOaz1 reference gene. From six samples, mOaz1 transcript was detected with an average Ct of 17.242 ± 1.483 s.d., whereas the average Ct was 36.332 ± 3.61 s.d. in RT- control samples (n=4). The tight grouping of mOaz1 Ct signals across samples suggests that RNA isolated from TA muscle cryosections performs as expected in

downstream RNA expression analyses.

Histological assessment of adjacent cryosections.

Examples of indirect immunofluorescence staining of 7 μ m tibialis anterior muscle sections from littermate control mice 3 days after toxin injection are shown to detect regenerating fibers (Figure 2). Both sections were collected from muscle less than 150 μ m from the region to be used for RNA analysis at a depth of 4.5 to 4.6 mm from the proximal muscle surface. Embryonic myosin heavy chain (eMHC, red) detects regenerating fibers, collagen VI (ColVI, green) outlines the muscle fibers, and DAPI stains nuclei blue, according to protocol 4. Regions with concentrated nuclear infiltrate (blue signal) indicate sites of toxin injury, as evident by activation of eMHC positive newly regenerating muscle cells. Whole section maps like this example are used for quantification of regeneration by calculating the proportion of embryonic myosin heavy chain-expressing fibers (red, newly regenerated) or centrally nucleated fibers as reported previously¹. Notably, there is surprisingly little damage in the TA muscle in Figure 2A. While there is variation between injections, typical toxin injections affect a much bigger proportion of the muscle compartment¹, as shown in Figure 2B. Therefore, this histological analysis suggests that the toxin injury in Figure 2A was minimal and provides an important tool to interpret gene expression data from the contiguous muscle cryosection RNA sample. If the whole muscle had been used for RNA preparation by standard grinding methods, the unexpectedly small injury area of this sample would make it an outlier that would skew downstream analyses. Instead, pairing histological quantification from the same muscle allows direct measurement of the extent of the injury from adjacent sections. This enables the use of inclusion/exclusion criteria to ensure that all samples included in downstream RNA analyses meet a minimum injury threshold or the normalization of RNA analyses according to injury size.

FIGURE LEGENDS:

Figure 1: Quality assessment of RNA from pooled muscle cryosections. A) 18S and 28S ribosomal RNA bands are prominent in RNA from pooled muscle cryosections 18 months after the RNA preparation. B) Non-quantitative PCR for Mrf4 following reverse transcription. 200 and 300 bp bands of a DNA ladder are indicated. RT-, reverse transcription reaction with RNA template but no reverse transcriptase. RT-H₂O, reverse transcription with ddH₂O, no RNA template. H₂O, no template PCR control with ddH₂O. In these experiments, toxin was injected into the right tibialis anterior (RTA) and saline was injected into the left (LTA).

Figure 2: Sample histological maps for muscle regeneration studies. A,B) Compiled maps of single tibialis anterior muscle sections 3 days after toxin injection showing examples of poor (A) and normal (B) toxin-induced injury. White boxed regions of each section map are shown as inset images for higher magnification viewing, Red, embryonic myosin heavy chain; green, collagen VI extracellular matrix protein; blue, DAPI nuclear stain. Scale bar, 100 μ m.

Table 1: Representative RNA yield and purity measurements from pooled muscle cryosections. Sixteen tibialis anterior (TA) muscles were sectioned and pooled cryosection samples were processed for RNA. Purified RNA (1 μ L) was analyzed with a nanospectrophotometer. Column statistics were performed in a spreadsheet, group

comparisons were performed using statistical software.

DISCUSSION:

To achieve best results with this method, keep embedding resin restricted to the lower third or half of the muscle during tissue cryopreservation because excess resin will slow the collection of the pooled cryosections and may increase embedding resin contamination in the RNA isolation. Also, careful attention during needle homogenization is important to maximize yield and minimize the probability of clogging the needle. The protocol may be modified by using a luer-lok syringe to protect against sample loss if the needle becomes blocked and requires high pressure to dislodge the clog. An additional needle homogenization step with a 25 or 26 gauge needle can also be added to produce a finer tissue suspension to further enhanced RNA yield. While chloroform could be substituted for BCP, this is not recommended as BCP is less toxic and results in lower levels of genomic DNA contamination in the aqueous phase during organic extraction of RNA¹². Increasing the section thickness for pooled cryosections over 30 μm is also not recommended as homogenization will be less efficient.

If RNA yield is below desired levels, various strategies may be employed to increase recovery such as: i) increase the milligram quantity of starting material to increase possible yield; ii) reduce the section thickness below 30 μm to improve mechanical homogenization of the tissue; iii) increase the duration of sample incubation and needle homogenization in the organic extraction reagent to improve mechanical and chemical tissue disruption; and iv) if tissue chunks remain, perform a second extraction step with more rigorous needle homogenization. Alternately, there may be tissue-specific considerations, such as additional phase separation and precipitation steps for samples with high proteoglycan content¹³. During the RNA column purification, a larger elution volume can be used and performing a second elution can maximize total RNA recovery, but at the expense of RNA concentration. A post-column alcohol precipitation can be used to concentrate the RNA if low concentration is a concern with this modification. If RNA degradation is a problem, reducing time to cryopreservation during dissection, more rigorous cleaning of cryostat surfaces and tools to minimize RNase exposure, performing the needle homogenization step in a cold room, addition of an RNase inhibitor reagent to the cryosections¹⁴, and frequent replacement of RNase free solutions may each help to prevent or minimize exposure to RNases and reduce cleavage activity. It is possible that briefly bathing the tissue in an RNase inhibitor reagent after dissection, but before cryopreservation, may further reduce sample degradation. However, preliminary experiments should be performed to ensure that any such treatment does not increase ice crystals or other artifacts during cryopreservation.

While embryonic myosin heavy chain/collagen VI indirect immunofluorescence is used here as an example for muscle analysis of injury, thin cryosections mounted on microscope slides from these experiments can be used for any relevant histological stain that can be conducted on frozen sections, including immunofluorescent techniques with post-fixation and hematoxylin/eosin staining. Indeed, adaptations to the simple immunofluorescent protocol provided here may be necessary. For example, anti-mouse secondary antibodies used to detect a mouse primary antibody (e.g. eMHC) may also detect endogenous mouse immunoglobulins in

the target tissue. Such endogenous antibodies typically accumulate in damaged or necrotic muscle fibers in injured or dystrophic muscle causing background immunofluorescent staining. A secondary control slide (with primary antibody omitted) should always be examined to assess the specificity of staining. If endogenous antibody background is problematic, pre-block steps should be added to the protocol to prevent or minimize detection of endogenous mouse immunoglobulins¹⁵.

The main limitations of the method are that it requires a cryostat and it is time consuming, which makes it relatively low throughput. For example, an expert in the technique was able to process up to 16 muscles for pooled cryosections and microscope slides (8 slides with duplicate sections of all 16 tissues) in approximately 9 to 10 hours. For novices to cryosectioning, collection of pooled cryosections from 2 to 4 samples could be reasonably mastered after cryostat training and one or two practice sessions, instead, obtaining quality cryosections for histology took more experience. Therefore, equipment, time, or training factors may make this method less useful for softer tissues that can be well homogenized with a manual pestle homogenizer.

In comparison with non-cryostat homogenization methods, striated muscle RNA preparations have been reported from muscle biopsies with RNA yields of 0.05 to 0.7 μg RNA per mg of muscle¹⁶ and, more recently 0.27 to 1.08 μg RNA per mg of muscle¹⁷. Therefore, the technique described here provides RNA yields as good as or better than non-cryostat methods with the added advantage of enabling paired histological analyses from a contiguous region of the same sample. Notably, a previous study also used cryosectioning for homogenization in vertebral tissue and similarly found that cryosectioning tissue enhanced homogenization efficiency for RNA isolation¹³. When this technique was tested in bovine skeletal muscle samples, the average RNA yield per sample preparation was $4.09 \pm 0.36 \mu\text{g}$, at the low end of the normal range reported here¹³. Laser capture microdissection is another alternative for collection of tissue for RNA extraction from a cryosection. Laser capture is superior to this pooled cryosection method in that it allows the specificity to collect only a desired subset of cells from the section and it can be performed on a single tissue section up to 50 μm thick¹⁸. However, collection of a micro-dissected sample can be difficult and suitable equipment is not widely available, making pooled cryosection homogenization more accessible to researchers. When both methods are available, a preference to analyze a tissue sub-region for an application needing only small RNA quantities would favor laser capture microdissection while pooled cryosection homogenization is best when sub-region analysis is less important and higher quantities of RNA are needed.

While histological and RNA isolation methods are the focus here, the pooled cryosection method is easily adapted to prepare protein lysates for Western blot analyses or enzyme activity measurements. For example, pooled cryosections from the heart were solubilized for Western blot analyses⁴ and pooled cryosections from the TA were homogenized for succinate dehydrogenase activity assays of mitochondrial function⁵. Alternatively, genomic DNA and protein fractions can be separated from other phases during the organic extraction after RNA isolation, offering the potential to derive genomic DNA, protein, RNA, and histological measurements from a single tissue after a single cryostat session.

Overall, the main advantage of this method is to increase experimental flexibility by enabling multiple analytical approaches requiring different sample preparation from a single tissue. The method is most appropriate for muscle and other tissues with extensive intra- or extracellular structure that reduces the efficiency of pestle-based tissue homogenization.

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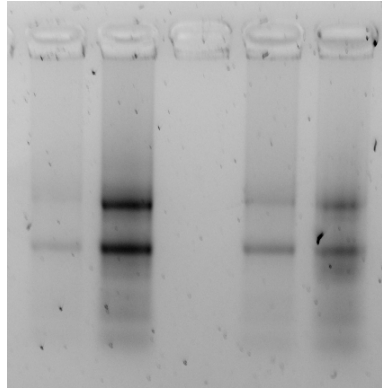
The author declares that she has no competing financial interests.

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¹A



LTA

RTA

LTA

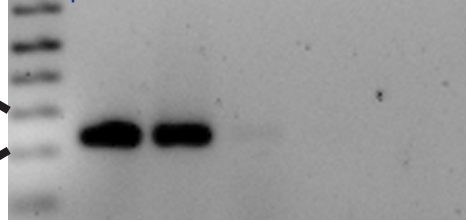
RTA

28S
18S

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B

300
200



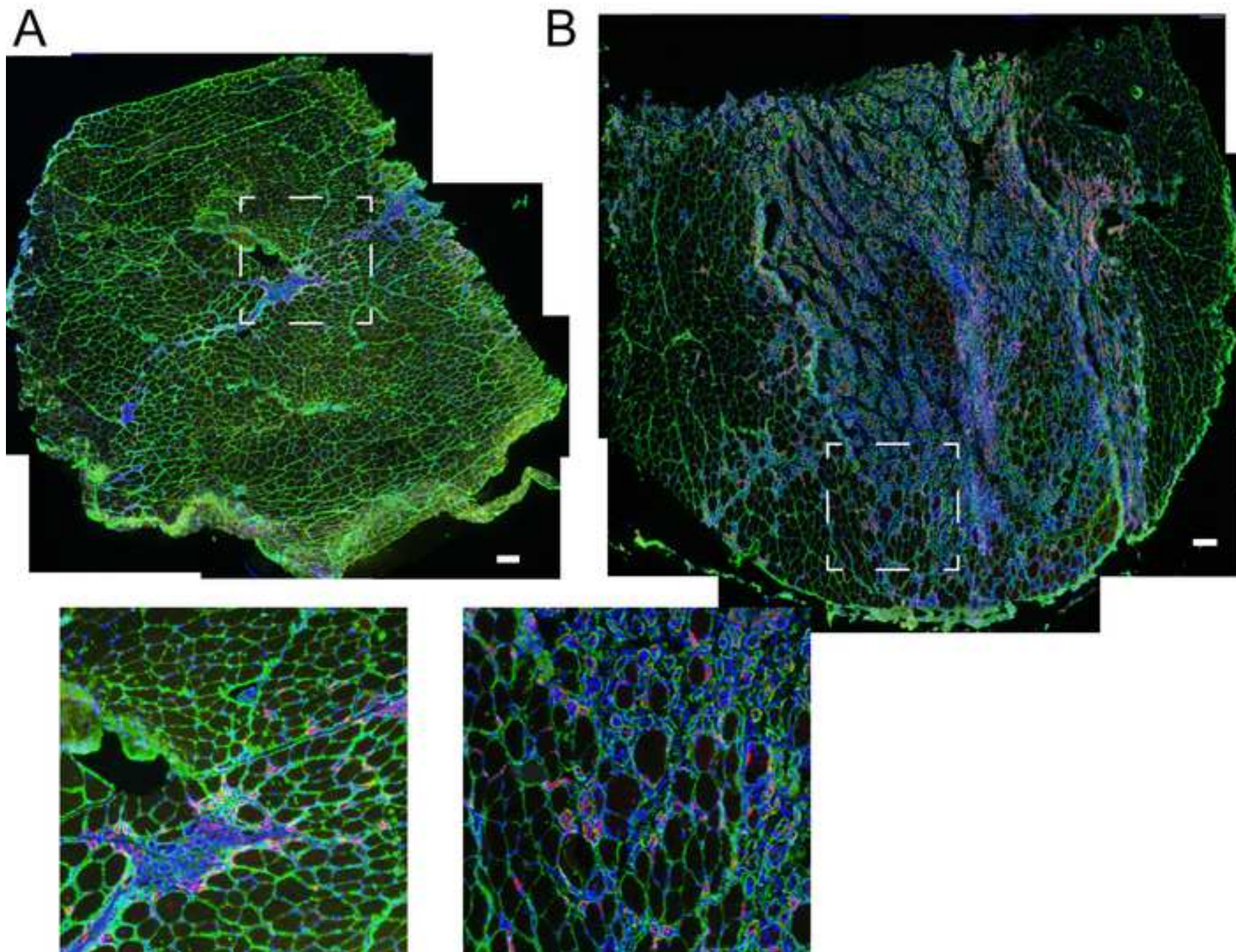
LTA

RTA

RT-

RT-H2O

H2O



Sample	Pooled cryosections from each TA (mg)	Column Elution Volume (μL)	RNA conc. (μg/μL)	RNA total (μg)	Yield μg RNA/ mg tissue	A260/ 280	A260/ 230	Toxin/Saline yield ratio
Sal TA 1 (L)	27.1	40	0.330	13.18	0.49	2.10	1.97	1.86
Tox TA 1 (R)	30.0	40	0.680	27.21	0.91	2.02	2.08	
Sal TA 2 (L)	29.7	40	0.255	10.20	0.34	2.10	2.21	3.32
Tox TA 2 (R)	30.8	40	0.877	35.09	1.14	2.08	2.25	
Sal TA 3 (L)	19.4	35	0.111	3.88	0.20	1.98	1.56	6.07
Tox TA 3 (R)	19.7	35	0.683	23.92	1.22	2.04	2.05	
Sal TA 4 (L)	33.9	44.2	0.211	9.31	0.27	1.93	1.66	4.67
Tox TA 4 (R)	28.5	37.2	0.983	36.57	1.28	2.06	2.23	
Tox TA 5 (L)	23.5	35	0.655	22.92	0.98	2.06	2.15	3.59
Sal TA 5 (R)	27.7	36.2	0.208	7.53	0.27	2	2.15	
Tox TA 6 (L)	31.4	41	1.035	42.44	1.35	2.09	2.08	4.70
Sal TA 6 (R)	41.0	53.4	0.221	11.77	0.29	1.98	1.93	
Tox TA 7 (L)	28.9	37.8	0.729	27.54	0.95	2.08	2.23	3.08
Sal TA 7 (R)	35.2	45.8	0.237	10.87	0.31	2	2.13	
Sal TA 8 (L)	26.0	35	0.132	4.61	0.18	1.86	1.35	2.37
Tox TA 8 (R)	26.2	35	0.315	11.02	0.42	2.02	2.09	

Avg Saline	30.0 ± 6.6 s.d.		0.213 ± 0.069 s.d.	8.92 ± 3.33 s.d.	0.29 ± 0.09 s.d.			3.71 ± 1.38 s.d.
Avg Toxin	27.4 ± 4.0 s.d.		0.745 ± 0.227 s.d.	28.34 ± 9.73 s.d.	1.03 ± 0.29 s.d.			
Mann Whitney test	p=0.4418		p=0.0003	p=0.0006	p=0.0013			

Name of Reagent/ Equipment (in order of use)	Company	Catalog Number	Comments/Description
Cork	VWR Scientific	23420-708	Cut into small squares with a sharp blade.
Plastic coverslip	Fisher Scientific	12-547	Used to orient the muscle during freezing.
Low temperature thermometer	VWR Scientific	89370-158	
2-methylbutane	Sigma	M32631-4L	Caution: hazardous chemical. Store in flammable cabinet.
Embedding resin: "cryomatrix"	Thermo Fisher Scientific	6769006	Other embedding resins can be substituted for cryomatrix.
		microm HM550	
Cryostat	Thermo Fisher Scientific	with disposable blade carrier	Any working cryostat should be sufficient for the protocol.
Disposable cryostat blade	Thermo Fisher Scientific	3052835	Use an appropriate blade or knife for the cryostat to be used.
RNAse decontamination solution: "RNase Zap"	Thermo Fisher Scientific	AM9780	
Analytical balance	Mettler Toledo	XS64	
Paint brush	Daler Rowney	214900920	Use to handle cryosections. Can be found with in stores with simple art supplies.
Razor blade	VWR Scientific	55411-050	
Microscope slide	VWR Scientific	48311600	
RNA organic extraction reagent: TRIzol	Thermo Fisher Scientific	15596026	Caution: TRIzol is a hazardous chemical. Note: Only organic extraction reagents are recommended for RNA extraction from skeletal muscle.
18 gauge needle	VWR Scientific	BD305185	
22 gauge needle	VWR Scientific	BD305155	
26 gauge needle	VWR Scientific	BD305115	Optional. Can be used for a third round of sample trituration in the RNA extraction protocol.
1 mL syringe	VWR Scientific	BD309659	For very high value samples, a luer-lok syringe is recommended (e.g. VWR BD309628).
1-bromo-3-chloropentane (BCP)	Sigma	B9673	
For 70% ethanol in DEPC water: 200 proof alcohol	Decon Laboratories, Inc.	+M18027161M	Mix 35 ml 200 proof alcohol + 15 mL DEPC water.

For 70% ethanol in DEPC water: DEPC-treated water	Thermo Fisher Scientific	AM9922	Mix 35 ml 200 proof alcohol + 15 mL DEPC water.
RNA purification kit: PureLink RNA minikit DNase/Rnase-free water	Thermo Fisher Scientific	12183018A	Final steps of RNA preparation.
Spectrophotometer: Nanodrop 2000	Gibco	10977	DEPC-treated water can also be used.
	Thermo Fisher Scientific	NanoDrop 2000	
Dnase I	Thermo Fisher Scientific	AM2222	Treat purified RNA to remove any DNA contamination before downstream applications.
Hydrophobic pen	Thermo Fisher Scientific	8899	
Dulbecco's PBS	Gibco	14190	PBS for immunofluorescence protocol.
Donkey serum	Jackson ImmunoResearch Laboratories, Inc	017-000-121	Rehydrate normal donkey serum stock according to the manufacturer's instructions, then dilute an aliquot to 5% for immunofluorescence. Normal goat serum can also be used.
eMHC antibody	University of Iowa Developmental Studies Hybridoma Bank	F1.652	
Collagen VI antibody	Fitzgerald Industries	#70R-CR009x	
Donkey anti-rabbit AlexaFluor488	Thermo Fisher Scientific	A21206	
Goat anti-mouse IgG1 AlexaFluor546	Thermo Fisher Scientific	A21123	
DAPI (4',6-diamidino-2-phenylindole)	Thermo Fisher Scientific	D1306	
Aqueous mounting media: Permafluor	Thermo Fisher Scientific	TA-030-FM	Only use mounting media designed for fluorescent applications with anti-fade properties.
Glass coverslip	VWR Scientific	16004-314	Use for mounting slides at the end of immunofluorescence protocol
Image analysis software: ImageProExpress	Media Cybernetics, Inc.	Image-Pro Express, or more advanced products	Freeware ImageJ should also work for manual counting. More advanced software with segmentation abilities may allow partial automation of the process. E.g. ImageProPremier.

Merge and map
section images:

Photoshop	Adobe	Photoshop
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Sigma C9659 has been discontinued.
Other options for cardiotoxin are EMD
Millipore #217503; American Custom
Chemicals Corp. # BIO0000618; or Ge
Script # RP17303; but these have not
been validated.

Cardiotoxin	Sigma	C9659
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reverse transcription

kit: Superscript III

First-strand synthesis system	Thermo Fisher Scientific
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18080051

Any validated, high quality reverse
transcription reagents can be used.
Any validated, high quality Taq
polymerase system can be used. If DNA
sequencing is to be used for any
application downstream of the PCR, then
a high fidelity PCR system should be used
instead.

Standard PCR: GoTaq	Promega
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M8298

For use in qPCR when not using a
dedicated qPCR master mix. Use with
SuperROX (for Applied Biosystems
instruments) and GoTaq Flexi polymerase
and buffers.

SYBR green	Thermo Fisher Scientific
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S7585

SuperROX is more stable in the PCR
reaction, so it is preferred for use as a
qPCR passive reference dye over ROX
(carboxy-X-rhodamine). For qPCR with
Applied Biosystems instruments

ROX: SuperROX, 15 μM	BioResearch Technologies, Inc. Novato CA
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SR-1000-10

Real-time PCR	Applied Biosystems
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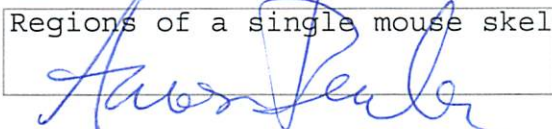
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CORRESPONDING AUTHOR:

Name: Aaron M. Beedle, Ph.D.
Department: Pharmaceutical & Biomedical Sciences
Institution: University of Georgia
Article Title: Histological and Gene Expression Analyses from Contiguous Regions of a single mouse skeletal muscle.
Signature:  Date: 5/29/2016

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July 22, 2016

Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140
Ph: 617-401-9938

ATTN: Jaydev Upponi, PhD., Science Editor
ATTN: Indrani Mukherjee, PhD., Science Editor

Dear Journal of Visualized Experiments,

Thank you for providing additional comments on my manuscript JoVE55058R1 "Cryosectioning of Contiguous Regions of a Single Mouse Skeletal Muscle for Gene Expression and Histological Analyses."

Please find below the point-by-point changes to address questions or concerns arising from Editorial and Peer review.

Please contact me if any questions arise.

Sincerely,



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Editorial comments:

E1. Formatting:

- a. Because the focus of the video will be on sectioning rather than the downstream analyses, please re-write the title as follows: Cryosectioning of Contiguous Regions of a Single Mouse Skeletal Muscle for Gene Expression and Histological Analyses

Response: DONE

- b. Please use subscripts in chemical names (ie dH₂O, see 2.1.3.1). **Response: DONE**

- c. 3.2 can be a heading or a step, not both. Please move the statement following RNA purification to its own step or rephrase as "For RNA purification, follow...".

Response: DONE

- d. 4.1.7 should be a substep of 4.1.6. **Response: DONE**

E2. Grammar:

- a. 1.2.5 – Please use complete sentences in imperative tense. **Response: DONE**
- b. 2.1.3.1 – Please correct the run-on sentence. **Response: DONE**
- c. 3.2.3 – Please use imperative tense or convert to a note. **Response: the original 3.2.3 (now 3.2.8 due to a typo). The first sentence “The column flow through is the purified total RNA” was deleted and “(column elution)” was added to the next phrase. “Analyze the RNA (column elution) for...”**
- d. All sentences should begin with capital letters, including those in notes. **Response: “2-methylbutane” is now “2-Methylbutane”. Table 1 legend “1 µL of each...” is now “Purified RNA (1 µL) was analyzed with a nanospectrophotometer.”**

E3. Additional detail is required:

- a. 1.1.4 – What tools are used for dissection? **Response: the text is revised to include “fine-point dissections scissors” and “fine forceps”.**
- b. 1.2.3 – How much resin should be applied? Should it completely cover the muscle? **Response: No, only the lower approximate half of the muscle should be embedded. The text was changed to: “Apply embedding resin **only** to the lower 35-50% of the muscle...”**
- c. 3.2.3 – Please provide a citation for downstream analyses. **Response: Now 3.2.8. Citation added. Note, “The RNA should be stable for several years.” Was deleted (not imperative tense).**
- d. Branding should be removed from Line 451 – SYBR, ROX. **Response: “SYBR green and Rox [for]...” has been deleted from the text.**
- e. Results: Figure 2 – The choppy outline of the map makes the scale bar difficult to see. Please place it in the lower right corner or otherwise make it more obvious. **Response: DONE**
- f. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes. **Response: Not applicable.**
- g. JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information. **Response: DOIs are included for all citations except the American Veterinary Care Euthanasia Manual (citation 6), for which no DOI is available.**

Reviewers' comments:

Reviewer #1: *No concerns to address.*

Reviewer #2:

Major Concerns:

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R2.1. My only somewhat major/minor criticism is the analysis of the negative aspects of the protocol. While sectioning larger/thicker sections at 30 μm may work fine for H&E histochemistry and muscle fiber typing, some important muscle markers (e.g. Pax7 and other muscle satellite/stem cell markers) may require antigen-retrieval for immunofluorescence in thick sections. The author should clarify whether or not this application of the protocol could be applied for muscle satellite cell/nuclear proteins as well. If the author makes these text changes, I believe the manuscript to be suitable for publication.

Response: Thick sections (30 μm) are pooled to use for RNA (or other molecule) extraction. We agree that 30 μm sections are not suitable for many histological analyses. Therefore, this protocol does not recommend the use of 30 μm tissue sections for any histological staining or measurement. Rather, we include instructions to switch to thin cryosections (7 μm) for histological analyses in step 2.4.1. Such thin sections are appropriate for all of the applications mentioned by the reviewer, including detection of satellite cells and nuclear proteins. For example, we have used these 7 μm sections for myogenin and Pax7 staining.

“2.4) Collect cryosections for histology.

2.4.1) Press the section thickness button for fine sectioning and use arrows to set the cryostat section thickness to 7 μm (or other appropriate section thickness, typically 6 to 10 μm).”

We edited the results text to further emphasize the section thickness for histology “...7 μm tibialis anterior muscle **section**...”.

Minor Concerns:

R2.2. Long Abstract: page 1. "By the protocol here" This sentence sounds awkward. Consider changing to "By following the protocol here" or something similar.

Response: The suggested change is made in the text.

R2.3. Perhaps a minor note that this freezing technique is performed on unfixed muscle biopsies would be important to mention.

Response: The title of the first procedure section was changed to “1. Cryopreservation of **unfixed** skeletal muscle.”

R2.4. Page 5. 2.4.1). Please clarify the purpose of switching from a 30 micron section to a thinner 6 or 7 μm section? I am assuming this is for a quick examination of the histology/quality of the muscle preparation?

Response: The purpose of switching to the thin 7 μm section is to allow for any desired histological or fluorescent stain of interest. In step 2.4.5, instructions are provided to use the slides fresh or to store slides at -80C until further use. We cut slides for multiple experiments at the same time and freeze all of the slides. This allows distinct histological analyses to be performed on close serial sections without restricting the staining to the same day as the cryosectioning takes place. Essentially, the purpose is to allow any/all of the different types of histological analysis that the reviewer mentions in the major comment using techniques with or without post-fixation, with or without antigen retrieval, etc. A note has been added to step 2.4.1 to address this issue:

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“NOTE: Thinner sections (6 to 10 μm) should be used for histological applications to ensure that staining reagents can penetrate the depth of the tissue section.”

R2.5. Pages 7-8 3.2.3) A note about quality of the RNA for other applications (e.g. RNA-sequencing) might be warranted here. I will leave this to the discretion of the author.

Response: Unfortunately we have no current projects involving RNA-Seq. Therefore, we have not yet been able to validate mRNAs derived from this protocol for next generation sequencing applications.

Reviewer #3:

Minor Concerns:

R3.1. Line 85: "fine tip marker". While this is a standard piece of laboratory equipment and not necessary to include in the equipment list with a manufacturer, it might be helpful to include here that care should be taken in the use of some markers as their ink may be soluble under certain conditions, including exposure to 2-methylbutane.

Response: the text has been edited to "...fine tip marker that is resistant to 2-methylbutane..."

R3.2. Line 113 (1.1.6): It is not clear how the muscle is oriented here, however it seems likely that this video portion will effectively present this. Even so, some additional information here would be helpful.

Response: the text has been edited to "1.1.6) Orient each muscle **for transverse sections** on its prelabeled cork. **Stand the muscle perpendicular to the cork with the** distal tendon touching the cork and the top of the muscle extending away, held upright by the coverslip."

R3.3. Line 143 (1.2.6): Is the 2-methylbutane discarded at the end of the experiment or stored for reuse in subsequent freezing sessions?

Response: In theory, 2-methylbutane can be reused. However, I don't recommend it's reuse because water content will rise during each use, which may increase the risk of ice crystals forming in the tissue during cryopreservation. The protocol item has been edited to read "Dispose of used 2-methylbutane as hazardous waste."

R3.4. Line 234 (2.4) It is not clear why RNA sections are collected first and then the histological section is collected second. Can this be reversed by people who use this protocol or are there reasons to maintain this particular order? It might prove to be more valuable to have a histological section from the upper levels of the block.

Response: Thin sections for histology can be collected before, after or intermingled with the thick sections for RNA extraction. However, the top of the muscle, which is sectioned first, is free of embedding resin while deeper sections will be surrounded by embedding resin. Extraction procedures from thick sections are expected to be impaired by increasing contamination with the resin, but histological applications are unaffected. Therefore, as a practical consideration, it is faster to take thick sections from the top of the muscle than from deeper in the muscle where more time must be spent trimming embedding resin away from the muscle.

The manuscript text has been changed to: "Thin sections can be taken from any depth

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during the cryosectioning, but deeper sections are preferred because embedding resin, which increases with tissue depth, does not impair histological staining.”

R3.5. Line 256 (3.0) Is it necessary to avoid the formation of air bubbles during needle trituration or does that not affect the results of the RNA isolation?

Response: With needle homogenization, some air bubbles will always be introduced. We have no clear data to suggest air bubbles are specifically detrimental to the quality of RNA extracted, but we recommend minimizing bubbles as a precaution. The text of 3.1.2 now reads:

“3.1.2) Using a 1 mL syringe with an 18 gauge needle, draw the RNA extraction liquid up and rinse the walls of the tube until all tissue is suspended in the solution. Try to minimize air bubbles during needle homogenization.”

R3.6. Line 409: "Necrotic fibers may stain weakly with the mouse IgG1 secondary antibody." It is also possible that viable muscle fibers can show elevated IgG staining in the cytosol if that muscle fiber was injured and survived the initial injury (much like with Evan's blue staining). This should be mentioned here to avoid confusion in those using this protocol.

Response: Yes, this is true. We have edited the text to read:

“Necrotic or injured fibers may stain weakly with the mouse IgG secondary antibody due to detection of endogenous IgG that penetrates the fibers through damaged muscle membranes.”

R3.7. Line 469: "Typically toxin injections affect a much bigger proportion of the muscle compartment". It appears that the results provided in Figure 2 are not typical for these experiments. It would be helpful to include an additional example image in Figure 2 (or a new figure) that will indicate a more typical example. This would be helpful for users to see what their expected result will look like.

Response: A second section with widespread toxin damage is now included in Fig. 2B and discussed in the text.

R3.8. Line 521-526: This is a good assessment of this approach versus other methodology. It might be useful to add a few additional sentences that compare this approach to the advantages and disadvantages associated with laser capture microdissection (LCM).

Response: The following section has been added. “Laser capture microdissection is another alternative for collection of tissue for RNA extraction from a cryosection. Laser capture is superior to this pooled cryosection method in that it allows the specificity to collect only a desired subset of cells from the section and it can be performed on a single tissue section up to 50 μm thick¹⁵. However, collection of a microdissected sample can be difficult and suitable equipment is not widely available, making pooled cryosection homogenization more accessible to researchers. When both methods are available, a preference to analyze a tissue subregion for an application needing only small RNA quantities would favor laser capture microdissection while pooled cryosection homogenization is best when subregion analysis is less important and higher quantities of RNA are needed.

Reviewer #4:

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Major Concerns:

R4.1. Table 1. Its not clear with a n=2 if the 2-3 fold variation in yield is toxin injection specific or just random sample variability? Does the investigator have a larger data set or previous published data to show this is statistically valid conclusion?

Response: Additional samples have been added for an n=8 mice, each with paired saline and toxin injected limbs. With these additional data, the higher RNA yield in toxin-injected muscle is confirmed. On average, the RNA yield from 3 day post-toxin injected muscle is 3.71 fold higher than the contralateral saline injected muscle. RNA yields are significantly higher in toxin compared to saline-injected muscle (p=0.0013).

R4.2. Table 1. should say pooled cryosections "from each TA".

Response: Done.

R4.3. Figure 1. If a larger data set were shown with quantitative data of qRT-PCR of housekeeping genes from multiple mice, conclusions of high quality RNA and quantitative reproducibility by this technique would be more strongly supported.

Response: Yes, a larger dataset will provide greater confidence in the fidelity of the cryosection RNA method. Therefore, we added additional data of qPCR detection of housekeeping gene mOaz1 across additional samples to the results: **"To compare the consistency of cryosection RNAs for quantitative PCR, Ct values were compared for the mOaz1 reference gene. From six samples, mOaz1 transcript was detected with an average Ct of 17.242 ± 1.483 s.d., whereas the average Ct was 36.332 ± 3.61 s.d. in RT- control samples (n=4). The tight grouping of mOaz1 Ct signals across samples suggests that RNA isolated from TA muscle cryosections performs as expected in downstream RNA expression analyses."**

R4.4. Figure 1. Legend should probably indicate RTAs were toxin injected for clarity.

Response: Done.

R4.5. Figure 2. Show a higher powered inset of the regenerating fiber staining. Hard to see in provided image.

Response: A higher power inset is now shown and a second section with greater toxin injury is also included for comparison (RE: reviewer 3, comment 7).

R4.6. Please provide troubleshooting steps for common problems like low yield, RNA degradation. For example previous studies suggest RNAlater improves yield and degradation on cryosections (BioTechniques 34:48-50, 2003)

Response: The following section is now added to the discussion: "If RNA yield is below desired levels, various strategies may be employed to increase recovery such as: i) increase the milligram quantity of starting material to increase possible yield; ii) reduce the section thickness below 30 μ m to improve mechanical homogenization of the tissue; iii) increase the duration of sample incubation and needle homogenization in the organic extraction reagent to improve mechanical and chemical tissue disruption; and iv) if tissue chunks remain, perform a second extraction step with more rigorous needle homogenization. Alternately, there may be tissue-

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specific considerations, such as additional phase separation and precipitation steps for samples with high proteoglycan content^{refb}. During the RNA column purification, a larger elution volume can be used and performing a second elution can maximize total RNA recovery, but at the expense of RNA concentration. A post-column alcohol precipitation can be used to concentrate the RNA if low concentration is a concern with this modification. If RNA degradation is a problem, reducing time to cryopreservation during dissection, more rigorous cleaning of cryostat surfaces and tools to minimize RNase exposure, addition of an RNase inhibitor reagent to the cryosections, frequent replacement of RNase free solutions, and performing the needle homogenization step in a cold room may each help to prevent or minimize exposure to RNases and reduce cleavage activity. It is possible that briefly bathing the tissue in a RNase inhibitor reagent after dissection, but before cryopreservation may further reduce sample degradation. However, preliminary experiments should be performed to ensure that any such treatment does not increase ice crystals or other artifacts during cryopreservation.”

Note, due to JoVE restrictions, I cannot use the brand name “RNAlater” in the manuscript text, Rather, I refer to it as an RNase inhibitor reagent.

Minor Concerns:

R4.7. Step 1.1.2 The protocol indicates that protective wear should be used when drawing liquid nitrogen from a cylinder. Should include full face protection. Should be extended to indicate PPE should be worn in all appropriate steps with cryogenic liquid except where rapid handling/dissection precludes wearing the gloves.

Response: The PPE statement has been broadened to include all liquid nitrogen handling and full face protection is added. The revised statement is: “**Liquid nitrogen** is a compressed gas that may explode if heated. Wear a lab coat, low-temperature gloves, and face protection when handling liquid nitrogen; contact with skin or eyes may cause burns or frostbite.”

R4.8. Step 1.1.4 Its not clear if or why the technique is limited to TA muscle, as it seems it could just be applied generally for any quickly frozen muscle. A description of limitations of the size of the muscle the technique could be applied to should be indicated if there are some.

Response: Correct, the technique is not limited to TA; it can be applied to any mouse muscle (or tissue) of sufficient size. The following note is added to the protocol:

“NOTE: The TA muscle is used here as an example, but any mouse skeletal muscle or the heart can be substituted for the TA in this protocol if appropriate to the user’s experimental goals. The only limitation is that a tissue must be small enough to achieve rapid cryopreservation throughout its depth; a maximum tissue size of 1 cm x 1 cm is recommended.”

R4.9. Step 1.1.3 Does not include an appropriate method of assessing anesthesia. Stops moving is not anesthesia. Cervical dislocation is not uniformly accepted as a method of confirmation of euthanasia. Also some may not have access to isoflurane vaporizer. It might be better to just say an "Euthanize mice with an approved method of euthanasia under anesthesia. For the experiments shown, 2.5% isoflurane....."

Response: The text has been edited as suggested. Cervical dislocation is accepted for

euthanasia at the University of Georgia, and is the IACUC-approved method for these experiments. “1.1.3) Euthanize mice with an approved method of euthanasia under anesthesia. The protocol for the experiments shown is: insert the mouse into an inhalation chamber with 2.5% isoflurane in oxygen from an isoflurane vaporizer, wait until 20 seconds after the mouse stops moving to check for a pedal reflex. When the pedal reflex is negative, euthanize the study mouse by cervical dislocation⁶.”

R4.10. Step 1.2. The cryopreservation bath should be prepared before dissecting the tissue. The order implies it is not. Related to this point is the time from dissection to freezing important? If so, what that time range should be less than, should be clearly stated.

Response: The optimal timing to set up the freezing bath is dependent on the number of mice/number of tissues being dissected and the skill of the dissector. 2-methylbutane in the freezing chamber will begin to solidify at -150°C. Therefore, the longer the cryopreservation chamber is maintained, the less 2-methylbutane will be in liquid form due to freezing and evaporation. There must be sufficient 2-methylbutane liquid in the freezing bath to cover the tissue corks or else the cryopreservation will be uneven or incomplete ruining the tissue(s) for histology. If only a few tissues are taken from a single mouse, the cryopreservation bath should be prepared before starting the dissection. But, if the dissection will take 10 min or longer, then setting up the cryopreservation bath should be delayed until after the dissection starts so that there is sufficient 2-methylbutane liquid at -140°C for the freezing. Alternately, a much greater starting volume of 2-methylbutane could be used, but this will take much longer (and much more liquid nitrogen) to get the bath to cryopreservation temperature. To address this issue, we now instruct for the cryopreservation bath to be started approximately 5 minutes before the end of the dissection and to aim to have tissues frozen within an absolute maximum of 15 minutes after euthanization of the source mouse, with less time always being preferred.

“1.1.7) Cryopreserve all tissues for histological analysis as soon as possible after dissection, preferably within 5 minutes but definitely not more than 15 minutes time elapsed since euthanization of the source animal

1.2) Cryopreservation

1.2.1) Begin cooling the cryopreservation bath five minutes before the end of the dissection. Pour 2-methylbutane into an open metal beaker to a depth of approximately 3 cm...”

R4.11. Step 1.2.5 "container in liquid nitrogen" What is container, How is this kept submerged, or is it just in vapor phase etc. Maybe just use a cooler with dry ice to store the samples before moving to -80?

Response: we have simplified the instructions to read “1.2.5) Transfer samples in liquid nitrogen or on dry ice to a -80 °C freezer for storage.”

R4.12. Step 2.1.3. If not using a disposable blade, does the cryostat blade, and or the knife guard need to be prepared to remove RNAases?

Response: The following text has been added – “2.1.3) Insert a new disposable cryostat blade. Alternately, remove the existing blade, spray with RNase decontamination solution, rinse with ddH₂O, and reinsert into the cryostat to cool.”

R4.13. Step 2.1.4. Add "Within the cryostat chamber..."

Response: Done.

R4.14. Step 2.2.4 number is repeated, Step 3.1.3 is missing, Step 3.2.3 numbering is repeated after 3.2.6

Response: Thank you, these errors are now corrected.

R4.15. Step 4.1 Perhaps should include optional post-fixation of sections protocol for other antibodies, in addition to fresh frozen method for the antibodies here.

Response: We considered providing additional detailed immunofluorescence protocols. However, the immunofluorescent technique is not the primary focus of the manuscript. Rather, it is generally expected that most readers interested in this technique will already have established immunofluorescent targets and protocols. Instead, the focus of this article is to enable the user to couple those methods to other molecular analysis (eg. RNA or protein) from the same source material. With these considerations and the problem of selecting an appropriate target/protocol from the wide array of different post-fixation methods, the simple eMHC/collagen VI/DAPI stain was selected as the immunofluorescent protocol example because it provides the widest analysis options (fibrosis, recent regeneration, historical regeneration).

To further address this issue, the following statement is added to the discussion: “While embryonic myosin heavy chain/collagen VI indirect immunofluorescence is used here as an example for muscle analysis of injury, thin cryosections mounted on microscope slides from these experiments can be used for any relevant histological stain that can be conducted on frozen sections, including immunofluorescent techniques with post-fixation and hematoxylin/eosin staining.”

R4.16. Step 4.1.6. How is non-specific anti-mouse IgG labelling prevented? Dystrophic fibers with membrane damage often have cytoplasmic IgG which could be misinterpreted.

Response: This statement has been added to the discussion: “Indeed, adaptations to the simple immunofluorescent protocol provided here may be necessary. For example, anti-mouse secondary antibodies used to detect a mouse primary antibody (e.g. eMHC) may also detect endogenous mouse immunoglobulins in the target tissue. Such endogenous antibodies typically accumulate in damaged or necrotic muscle fibers in injured or dystrophic muscle causing background immunofluorescent staining. A secondary control slide (with primary antibody omitted) should always be examined to assess the specificity of staining. If endogenous antibody background is problematic, pre-block steps should be added to the protocol to prevent or minimize detection of endogenous mouse immunoglobulins¹⁵.”

R4.17. Step 4.1.7 add to the note that all secondary antibodies should be validated to have

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minimal cross reactivity with other species in dual labelling experiments.

Response: Note is added as directed.

R4.18. The recent methods paper by Lee JYT et al J Biol Methods 2015;2(2):e20. doi: 10.14440/jbm.2015.40 should be cited that described RNA preparation methods from cryosections of bovine and mouse muscle and perhaps discussed if there are significant differences.

Response: Thank you for bringing this paper to my attention, it is very relevant. We have modified the discussion with the following text: 'Notably, a previous study also used cryosectioning for homogenization in vertebral tissue and similarly found that cryosectioning tissue enhanced homogenization efficiency for RNA isolation^{refb}. When this technique was tested in bovine skeletal muscle samples, the average RNA yield per sample preparation was $4.09 \pm 0.36 \mu\text{g}$, at the low end of the normal range reported here. Laser capture microdissection is another alternative for collection of tissue for RNA extraction from a cryosection.'