Video Article

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

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## 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 µm 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.

## Video Link

The video component of this article can be found at https://www.jove.com/video/55058/

## 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 control[1]. 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 side[2]. 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 homogenization[2-5]. 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. Preparation
    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.
    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.
    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 sec after the mouse stops moving to check for a pedal reflex. When the pedal reflex is negative, euthanize the study mouse by cervical dislocation[6].
    NOTE: Euthanasia methods require approval by the institutional ethics committee.
    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 muscle[7].
    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.
    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.
    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.
    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.

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

1. Preparation.
    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. 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.
    3. Insert a new disposable cryostat blade. Alternately, remove the existing blade, spray with RNase decontamination solution, rinse with $ddH_2O$, and reinsert into the cryostat to cool. Spray a clean tissue with 70% ethanol and carefully wipe the blade and blade clamping platform.
        1. Spray a clean tissue with an RNase decontamination solution and wipe cryostat brushes. Spray a tissue with $ddH_2O$, 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.

    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.

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.
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. Cryosection preparation.
    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. 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.
    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.
    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.
    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).
    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).

3. Collect cryosections for RNA extraction.
    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 4,000 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. 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.
    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.
    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 °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 °C until use.

4. Collect cryosections for histology.
    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. Cut and discard 4 to 7 sections to obtain a consistent, even tissue surface. Make note of the tissue depth.
    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.
    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.
    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

1. RNA extraction
    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.
    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. 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.

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.
5. Move the sample from ice to room temperature. Incubate for 5 min to disrupt molecular complexes.
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.
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.

2. RNA purification.
   1. Follow the manufacturer's instructions for sample binding, washing and elution with minor variations[8]. For example:
   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. 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.
   4. Perform wash steps according to the manufacturer's instructions[8]. 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.
   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.
   6. Spin the empty column for 1 min at 12,000 x g to dry the membrane.
   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.
   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 PCR[4].
      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

1. Simple immunofluorescence protocol.
   1. Use a hydrophobic pen to circle the group of sections on each slide. Gently drop PBS into the circled area (approximately 80 µl for a small surface area up to 500 µl 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.
   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).
   3. Prepare primary antibody solution to analyze muscle regeneration. Mix blocking solution, with an eMHC antibody (1:40) and a ColVI antibody (1:1,000). Prepare approximately 150 µl, 300 µl or 500 µl 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. 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.
   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.
   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.
      1. For example, mix 1 µl of DAPI with 9 µl of dd$H_2O$ to make a 1:10 dilution of DAPI. Then, for a 500 µl final volume, add 1.0 µl anti-mouse IgG1-red + 1.0 µl anti-rabbit IgG-green + 0.5 µl of 1:10 DAPI to 447.5 µl 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.

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.
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.
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.
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.

2. Histological evaluation.
   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].
   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 detection[4].
      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.
   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. Using analysis software, count the number of eMHC positive fibers and the number of total fibers to calculate recent regeneration (100*(# eMHC+ fibers/# total fibers)). Also, count the number of centrally nucleated fibers out of the total fibers to measure regeneration over a longer period (100*(# CN+ fibers/# 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 reported[1]. 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 excellent[9]. 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'-CTGAAGACTGCTGGAGGCTG[10], 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 dd$H_2O$ (reverse transcription with no RNA template), or dd$H_2O$ PCR controls (**Figure 1B**). The same samples were run in triplicate for Mrf4 and mOaz1 reference control[4] 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 ΔΔCt method[4]. This is similar to previous reports of low Mrf4 expression 3 days after toxin injury due to a loss of mature muscle fibers[11]. 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 µm 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 µm 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 previously[1]. 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 compartment[1], 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.

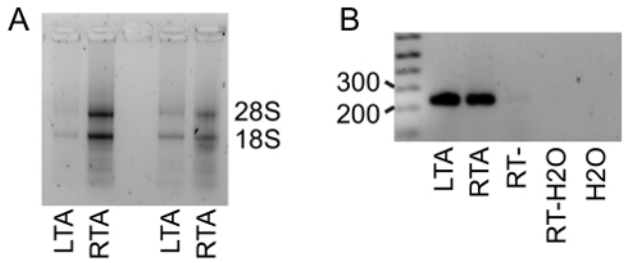


**Figure 1: Quality Assessment of RNA from Pooled Muscle Cryosections. A)** 18S and 28S ribosomal RNA bands are prominent in RNA from pooled muscle cryosections 18 months after the RNA preparation. **B)** Non-quantitative PCR for Mrf4 following reverse transcription. 200 and 300 bp bands of a DNA ladder are indicated. RT-, reverse transcription reaction with RNA template but no reverse transcriptase. RT-$H_2O$, reverse transcription with dd$H_2O$, no RNA template. $H_2O$, no template PCR control with dd$H_2O$. In these experiments, toxin was injected into the right tibialis anterior (RTA) and saline was injected into the left (LTA). Please click here to view a larger version of this figure.

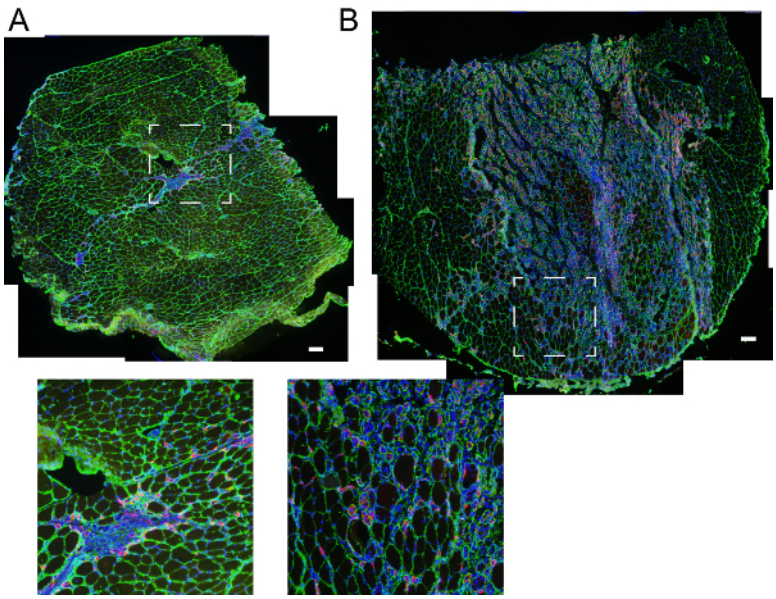


**Figure 2: Sample Histological Maps for Muscle Regeneration Studies. A,B)** Compiled maps of single tibialis anterior muscle sections 3 days after toxin injection showing examples of poor **(A)** and normal **(B)** toxin-induced injury. White boxed regions of each section map are shown as inset images for higher magnification viewing, Red, embryonic myosin heavy chain; green, collagen VI extracellular matrix protein; blue, DAPI nuclear stain. Scale bar, 100 µm. Please click here to view a larger version of this figure.

| 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 | | | |

**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 µl) 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 RNA[12]. 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 content[13]. 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 cryosections[14], 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 immunoglobulins[15].

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 hr. 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 pre mg of muscle[16] and, more recently 0.27 to 1.08 µg RNA per mg of muscle[17]. 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 isolation[13]. When this technique was tested in bovine skeletal muscle samples, the average RNA yield per sample preparation was 4.09 ± 0.36 µg, at the low end of the normal range reported here[13]. 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 thick[18]. 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 analyses[4] and pooled cryosections from the TA were homogenized for succinate dehydrogenase activity assays of mitochondrial function[5]. 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.

## Disclosures

The author declares that she has no competing financial interests.

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