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,



Aaron M. Beedle, Ph.D.

Assistant Professor

Pharmaceutical & Biomedical Sciences

University of Georgia College of Pharmacy

Athens, GA 30605

Ph: 706-542-6422

[beedlea@uga.edu](mailto:beedlea@uga.edu)

**Editorial comments:**

E1. Formatting:

1. 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**
2. Please use subscripts in chemical names (ie dH2O, see 2.1.3.1). **Response: DONE**
3. 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**
4. 4.1.7 should be a substep of 4.1.6. **Response: DONE**

E2. Grammar:

1. 1.2.5 – Please use complete sentences in imperative tense. **Response: DONE**
2. 2.1.3.1 – Please correct the run-on sentence. **Response: DONE**
3. 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…”
4. 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:

1. 1.1.4 – What tools are used for dissection? Response: the text is revised to include “fine-point dissections scissors” and “fine forceps”.
2. 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 test was change to: “Apply embedding resin **only** to the lower 35-50% of the muscle…”
3. 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).
4. Branding should be removed from Line 451 – SYBR, ROX. Response: “SYBR green and Rox [for]…” has been deleted from the text.
5. 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

1. 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.
2. 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:*

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 um 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 7μm 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 um 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:

“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 t**he 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 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 disadvantaged 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:**

*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 cyrosections "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 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 contentrefb. 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 crypreservation 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 -140C 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 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 isolationrefb. 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. Laser capture microdissection is another alternative for collection of tissue for RNA extraction from a cryosection.”