Dear JoVE Editors:

Thank you for the editorial and reviewer comments on our renamed manuscript "A Stainless Protocol for High Quality RNA Isolation from Laser Capture Microdissected Purkinje Cells in the Human Post-Mortem Cerebellum." We deeply appreciate the attention to detail and the high-quality comments made by the editorial staff and reviewers. We have attempted to address all comments and provide information for each comment below in our rebuttal. Included in this resubmission are a finalized copy of the manuscript, as well as a marked copy of the revisions we made for ease of viewing additions and eliminations from the text. We thank you for the opportunity to resubmit and look forward to publishing our manuscript with you.

Sincerely,
Regina T. Martuscello, PhD
Elan D. Louis, MD
Phyllis L. Faust, MD, PhD

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The entire document has been proofread and thoroughly checked for spelling and grammatical errors.

2. Please revise lines 116-118 to avoid previously published text.

The original text of lines 116-118 read: "However, transcriptional profiles from brain tissue regions are representative of a heterogeneous mixture of cell types that effectively mask the expression of low abundance transcripts, or molecular changes that occur only in a small population of affected neurons, such as Purkinje cells in cerebellar cortex."

The new text of lines 150-153 reads: "However, transcriptional profiles from heterogeneous samples, such as brain tissue regions, can effectually mask the expression of low abundance transcripts and/or diminish detection of molecular changes that occur only in a small population of affected cells, such as Purkinje cells in the cerebellar cortex."

3. Please revise the title to avoid punctuation.

The title has been changed to "A Stainless Protocol for High Quality RNA Isolation from Laser Capture Microdissected Purkinje Cells in the Human Post-Mortem Cerebellum" to avoid punctuation.

4. Please define all abbreviations (OCT, DEPC, etc.) before use.

All abbreviations have been defined before use.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Examples of commercial sounding language in your manuscript are: RNase away, Qiagen RNeasy, falcon, Zeiss, Agilent PicoChip, etc.
The entire document has been carefully proof read and all mentions of commercial sounding language has been removed.
RNase away has been removed from the 'note' prior to section 1, section 2.1.2, 2.2, 4.7.1, 4.7.2, 4.7.3 and 9.1 in the protocol and replaced with the terminology of RNase decontaminator or RNase cleaner in all sections. Specific product information is only included in the table of materials.
Zeiss has been removed from the entire manuscript. When discussing the Zeiss Opaque Caps, we refer to them only as Opaque caps with specific information in the table of materials.
Qiagen RNeasy has been removed from section 10 in the protocol and instead we indicate to see the Table of Materials for steps 1.11, 10.1.1 and 10.6.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
All personal pronouns have been removed from the protocol text.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
The protocol has been checked and revised to remove all phrasing that is not in the imperative tense. Notes have been carefully examined and removed or consolidated into the protocol where appropriate.

8. 1.11: Please add more details or provide a reference for RNA extraction and testing RNA integrity.
More information regarding RNA extraction and RIN testing are provided in section 1 and section 10 of the protocol along with a reference for bioanalyzer testing of RNA integrity.

Lines 222-225 now read: "1.11. Once all samples are complete, extract RNA using the RNA extraction kit provided (Table of Materials #15). A detailed protocol is provided with the RNA extraction kit. Follow all instructions including the optional step to dry the collection tube membrane and the optional step for DNase digestion (Table of Materials #16)."

Line 227 now reads: "1.12. Test the integrity of extracted RNA via a quality assessment bioanalyzer [14]." A reference has been added here for further information regarding testing RNA integrity.

Lines 463-464 now read: "Dilute 2-Mercaptoethanol in RLT buffer at 1:100 (10µL:1mL, respectively). RLT buffer is provided in Table of Materials kits #14 and #15." This directs readers to the necessary reagents for this step.

Lines 475-476 now read: "10.6. Place the tube containing 100µL of lysis buffer and RNA in -80ºC freezer until all samples are finished and ready for RNA extraction (Table of Materials #14)."

9. 2.3: What is the incubation temperature?

Slides should be incubated at room temperature. This has been updated in 2.3 (line 247).

10. 5.2: Please specify the approximate amount of OCT used.
Additional text was added to sections 1.5 and 5.2. We approximated the height of OCT to be placed on the chuck, which is not measured upon use and its volume would depend on the size of the chuck. We believe this will be a necessary visualization step within the video protocol.

Line 207 reads: "Place an approximately 3mm high mound of optimal cutting temperature (OCT) compound on a cryostat 'chuck' and allow it to partially freeze. Then place tissue on top of the OCT – do not push into OCT, simply allow it to rest on top."

Line 331 reads: "Place an approximately 3mm high mound of OCT to cover the 'chuck'."

11. 3.1-3.5, 4.3 and sub-steps, 10.1.1-10.1.2: Please write the text in the imperative tense in complete sentences.
All steps have been edited to imperative tense in complete sentences.
Section 3.1-3.4 (lines 259-267) now reads:
3.1. Place 30mL of 100% ethanol in one slide holder.
3.2. Place 30mL of 95% ethanol in one slide holder and put on ice. Dilute ethanol with RNase/DNase/Nuclease free water.
3.3. Place 30mL of 70% ethanol in one slide holder and put on ice. Dilute ethanol with RNase/DNase/Nuclease free water.
3.4. Place 30mL of 100% xylene in two separate slide holders and label them #1 and #2.

Section 4.3 (lines 279-283) now reads:
4.3. Cryostat settings:
4.3.1. Set the section thickness to 14µm.
4.3.2. Set the trim thickness to 30µm to preserve tissue quantity.

Section 10.1 (lines 461-464) now reads:
10.1. Prepare the cell lysis buffer (best to be prepared fresh each time):
10.1.1. Dilute 2-Mercaptoethanol in RLT buffer at 1:100 (10µL:1mL, respectively). RLT buffer is provided in Table of Materials kits #14 and #15.

12. Please remove the titles and Figure Legends from the uploaded figures/table. Please include all the Figure/Table Legends together at the end of the Representative Results in the manuscript text.
Figure legends and titles have been placed together at the end of the representative results in the manuscript text.

13. Figures 1 and 2: Please define the scale bars in the figure legend.
Scale bars definition has been added to figure legends.

14. Figure 2: Please remove commercial language (Zeiss) from the figure.
All commercial language has been removed from the figures.

15. Figure 3: Please change “uL” to “µL” (i.e., use the micro symbol), and include a space between all numbers and their units (i.e., 823 pg/µL, 976 pg/µL, etc.), if possible. In panel C, please add pg after the number.
Figure 3 has been updated to reflect the correct symbol for microliter. Spaces have been added between all numbers and their units in the figure and in the table. Pg has been added into panel C.

16. Table 1: Please include a space between all numbers and their units (i.e., 822 pg/µL, 976 pg/µL, etc.). Please change “mins” to “min”.
The table has been updated to “min”, and spaces have been placed between numbers and their units.

17. Table of Materials: Please use the micro symbol µ instead of u. Please include a space between all numbers and their units.
The symbol for micro has been added to the table of materials.

18. References: Please do not abbreviate journal titles.
All reference journals have been updated to their full titles.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
1. This article describes a methodology to detect cerebellar Purkinje cells in human postmortem brain tissue by laser microdissection without a staining step. The entire text should be carefully revisited - it partly lacks precision and clarity, and has missing, or provides unnecessary or misleading information.
Thank you for thoroughly reading through our proposed protocol manuscript. We objectively reviewed the entirety of the text and attempted to increase the precision and clarity. We have rearranged the introduction to better introduce the rationale behind the protocol as well as reduce extraneous information on ET pathology.
In the discussion, we have added text on other methods for LCM and RNA, removed the discussion on post-mortem intervals and clarified our position on the novelty of this protocol.

Please see the numerous examples provided below that detail these changes to the text and areas of text removal, all of which are intended to increase clarity, precision, and remove misleading information.

2. The authors should clearly state which system they use and preferentially address the three different methods to laser-dissect cells from tissue: Leica- or Zeiss-based laser microdissection (LMD), and Arcturus-based laser-capture microdissection (LCM). The differences between these systems should be clear to the reader. In addition, the authors need to use correct terminology: Other than the IR-based LCM system, the Zeiss PALM system used here is a UV-based laser microdissection system that catapults cells in a collection tube. That should be clear.
We agree that this is an important review point and have clarified this within the manuscript.

Text has been added (highlighted) to the abstract lines 59-64: “This protocol does not account for other fixation methods and is specifically designed for fresh-frozen tissue samples only captured using an Ultraviolet (UV)-LCM system. Here we present a full protocol for sectioning and fixing fresh frozen post-mortem human cerebellar tissue and purification of RNA from Purkinje cells isolated by UV-LCM, with the intent of preserving RNA quality for subsequent RNA-sequencing.”

A new paragraph has been added in the introduction lines 120-134: “It is important to note the different types of laser-based microdissection systems, as they have been shown to differ in speed, precision, and RNA quality. The infrared (IR) laser capture microdissection and the ultraviolet (UV) laser microbeam microdissection systems were both novel LCM platforms that emerged almost concurrently [8]. The IR-LCM system employs a “contact system” using a transparent thermoplastic film placed directly on the tissue section, and cells of interest selectively adhere to the film by focused pulses from an IR laser. Alternatively, the UV-LCM system is a “non-contact system” whereby a focused laser beam cuts away cells or regions of interest in the tissue; depending on the configuration in two currently available commercial platforms that use either an inverted or upright microscope design, tissue is acquired into a collection device by a laser-induced pressure wave that catapults it against gravity or tissue is collected by gravity, respectively. Significant advantages of the UV-LCM system include faster cell acquisition, contamination-free collection with the non-contact approach and more precise dissection due to a much smaller laser beam diameter [9]. This protocol was specifically designed for a UV-LCM system and has not been tested in an IR-LCM system.

Text has been added in the protocol lines 422-424: “Note: This section of the protocol will only discuss specifics related to capturing Purkinje cells for subsequent RNA sequencing. This section assumes the user is familiar with the UV laser capture microscope and the affiliated software.”

Text has been added in the representative results lines 485-486: “This protocol details the steps for preparing fresh frozen post-mortem human brain tissue for UV-LCM.”

Text has been added in the discussion lines 573-574: “The protocol presented here is specifically modified to be a stainless approach in visualizing morphologically distinct tissues for UV-LCM.” As well in lines 639-640: “We have presented here a complete method for stainless visualization of Purkinje cells in the post-mortem human cerebellum for the purposes of UV-LCM.”

All of these additions address the need to describe the different LCM systems, how they relate to this method and to ensure users know this is a UV-LCM protocol. The protocol is applicable to either inverted or upright microscope designs of UV-LCM systems.

3. It is misleading to imply that a novel/unique methodology is presented. The method/principal described here (sectioning, fixation, laser capture, quality assessment, etc.) is not new. In fact, everything that is described (all steps, methods, material used, etc.) is standard procedure and has been used with modifications in many studies to dissect postmortem tissue or cells. The authors are way over the top to imply that they have created something novel (first of its kind???, unique???, etc.) and should substantially tone down their text and discuss and cite the relevant literature - there are many (methodology) papers on this that have been ignored by the authors.

It is accurate that each of the steps provided here is not novel and when taken apart each qualifies as a standard procedure with modifications. We have edited the document to remove all mentions of novel or unique, and instead describe this protocol as "combining many aspects of different LCM protocols to provide an enhanced method for visualizing Purkinje cells without the need of dye containing staining reagents to prepare high-quality RNA for transcriptome sequencing." [Lines 165-168]

However, we did an extensive literature search and have not found any protocols that are specifically designed for excision of Purkinje cells from the post-mortem human cerebellum intently designed for purifying high quality RNA. We have found one protocol paper looking at such methods within the context of the mouse cerebellum and one paper looking within the human cerebellum utilizing a cresyl violet staining protocol with IR-LCM producing low quality RNA for microarrays. Therefore, we have added text to lines 589-595 to read: "Importantly, we identified only one LCM study in the literature that described collection of human Purkinje cells, which are of considerable interest to the study of cerebellar degenerative and developmental disorders. This study stained tissue frozen tissues with cresyl violet and isolated Purkinje cells with an IR-LCM system; sample RINs as low as 5 were used, but deemed acceptable for microarray analysis [22]. Therefore, this is the first protocol study we have found that is designed specifically for high-quality RNA from Purkinje cells in the post-mortem human cerebellum excised via UV-LCM." This is the only location where we discuss the novelty of this protocol.

Last, we again mention that cresyl violet staining, a popular dye used in many LCM protocols is not effective for Purkinje cell isolation, which may not be a known fact by many: "Cresyl violet (Nissl) staining is a popular dye containing reagent used in many protocols, as it stains nuclei in neurons in the brain and causes the least amount of RNA degradation [6]. However, the larger nucleus of the Purkinje cell is not well stained by cresyl violet, providing no significant benefit for visualizing Purkinje cells." [Lines 586-589].

We recognize that there are numerous methodology papers that describe various methods for preparing tissue for LCM. In order to be more inclusive, we have added 9 additional references of LCM protocols and have expanded the discussion on proper method selection for tissue and cell types. Lines 582-585: "Many other protocols exist that also utilize alternative staining methods specifically for UV- and IR-LCM with the intention of preserving RNA. However, most other methods contain at least one staining or antigen specific reagent [6,16,17], are designed to one cell or organ type [18-20], or require specialized RNA-seq kits to enhance integrity [21]." Further in the discussion we address the issues with using these types of kits. Please see response under #6 for specific text details.

4. There is text that doesn't really relate to and distract from the purpose of this article. For example, why discussing formalin fixation and Purkinje cell pathology in context of ET? In addition, some of the language is odd, imprecise, and misleading. For example, why is the lack of coverslips a pitfall of LCM, and what are the significant limitations and challenges when using RNA from frozen tissue? There is a wealth of information on new technologies that can generate, e.g., RNA-Seq data from lower quality RNA. Overall, the authors should be more careful in their statements.

We agree that the text of the document should directly relate to the overarching goal of the protocol with minimal deviation. As this protocol is specifically designed for Purkinje cell excision from the cerebellar cortex, we included extra information about Purkinje cell pathology in the context of ET, specifically for

individuals who would like to replicate this work. However, the discussion on formalin fixation is relevant for RNA processing and not Purkinje cell pathology in ET. Therefore, we rearranged the introduction to discuss ET pathology at the end to avoid misappropriating or misleading facts.

The paragraph discussing cross-linking and its effects on RNA integrity have been moved to lines 108-118 and edited for clarity: "In processing fresh frozen tissue for LCM, the fixation method can variably affect both RNA and tissue integrity. Formalin fixation is standard for morphological preservation, but causes cross-linking that may fragment RNA and interfere with RNA amplification [6]. Ethanol fixation is a better alternative for RNA isolation, as it is a coagulative fixative that does not induce cross-linking [1]. To enhance visualization of tissue morphology, xylene is the best choice, as it removes lipids from the tissue. However, there are known limitations when utilizing xylene in LCM, as tissues can dry out and become brittle causing tissue fragmentation upon laser capture [6]. Xylene is also a volatile toxin, and must be handled properly in a fume hood. Nevertheless, xylene has been shown to enhance tissue visualization while also preserving RNA integrity [7]. Therefore, our protocol centers around the use of 70% ethanol fixation and ethanol dehydration, followed by xylene incubation for morphological clarity."

The example of the coverslip pitfall is an important note in histology. The lack of a coverslip is significantly detrimental to the overall visualization of the tissue and cellular identification. The rationale as to why a coverslip is not suitable for LCM and a pitfall is included in lines 134-138: "In either UV-LCM system design, when accumulating cells into the collection cap, use of a coverslip that enhances cellular clarity during microscopy is not suitable, as cells would be unable to enter into the collection cap. Therefore, to enhance tissue visualization, we tested the use of Opaque collection caps, which are designed to act as a coverslip for microscopic visualization in UV-LCM systems, against liquid filled collection caps." We hope this clarifies why the opaque caps can be helpful when performing LCM.

The limitations and challenges when working with RNA from post-mortem tissues is wholly centered around the quality of RNA. For clarity, we have edited the original text of "Currently, there are significant limitations and challenges when using RNA from fresh frozen tissue for LCM. Specifically, RNA degradation is among the biggest challenges, as it occurs immediately upon death and its extent is mediated by various factors associated with the tissue collection method."
The text in lines 97-99 now reads: "However, when using RNA from fresh frozen post-mortem tissue for LCM, RNA degradation is a major challenge, as it occurs immediately upon death and its extent is mediated by various factors associated with the tissue collection method." We hope this clarifies the specific challenge of RNA degradation in post-mortem human tissues for the reader.

We agree that there are numerous new technologies available that can generate RNA-seq data from low quality samples. This is important for many disease studies as quality is not always an option when working with human tissues. To include this information, we added the text to lines 162-168: "Numerous protocols exist for the fixation, staining visualization and RNA preservation of many types of tissues for the purposes of both IR- and UV-LCM. When contemplating an experimental design for UV-LCM, individuals should tailor their protocol to best fit the needs and requirements of starting and ending materials. Here, we combine many aspects of different LCM protocols to provide an enhanced method for visualizing Purkinje cells in the post-mortem human cerebellum without the need of dye containing staining reagents to prepare high-quality RNA for transcriptome sequencing."

Additionally, we have added text in the discussion lines 582-583: “Many other protocols exist that also utilize alternative staining methods specifically for UV- and IR-LCM with the intention of preserving RNA.”
Additionally, we have expanded references and included more information in lines 584-590: “least one staining or antigen specific reagent [6,16,17], are designed to one cell or organ type [18-20], or require specialized RNA-seq kits to enhance integrity [21]. Cresyl violet (Nissl) staining is a popular dye containing reagent used in many protocols, as it stains nuclei in neurons in the brain and shows the least amount of RNA degradation [6]. However, the larger nucleus of the Purkinje cell is not well stained by cresyl violet, providing no significant benefit for visualizing Purkinje cells. This highlights the importance of tailoring a tissue visualization system that is appropriate for the tissue and cell type of interest.”

We hope these edits clarify our statements and intentions for this manuscript, as well as is more inclusive of the literature surrounding different LCM protocols.

5. It is common standard to demonstrate quality of the dissections by showing images pre- and post-capture. That should be provided. In addition, the authors should provide information on how they confirm that the captured cells are indeed Purkinje neurons, i.e., in addition to showing cellular visualization other data should be presented to confirm correct phenotype.
Thank you for this comment, it is an excellent point. We have added representative images of Purkinje cells and cerebellar morphology, as well as pre- and post-capture images to Figure 2 (C-F). The images show clear, precise dissection of readily visualized Purkinje cells, which would clearly markedly enrich the dissected tissue for these neurons over that obtainable in a total cortex lysate. Adding molecular data to show the enrichment of Purkinje cell markers is beyond the scope of this methodology paper, as we have demonstrated it through morphology.

6. In the Discussion, the authors should more clearly address the downsides of dissecting cells without staining. For example, to detect neuronal subtypes, in most cases specific staining is necessary. The here described method is suitable for just a few neuronal types in the brain. There should also be should more detailed discussion on RNA quality in context of producing downstream data. Many studies have generated high quality transcriptome data on captured cells from both unstained and stained postmortem brain tissue - again, there is a literature on that. The entire discussion on RNA integrity and brain tissue quality is rudimentary and misleading. For example, there is way too much emphasis on PMI - many other factors are involved in brain tissue and RNA integrity. Plus, PMI can't be monitored or taken into account by the majority of researchers and, as the authors state, there is no correlation of PMI with RNA integrity. So why going into such great detail into this? Altogether, the Discussion doesn't provide a complete, yet concise, and informative overview of the subject of this article. In addition, everything that has been discussed here has been published before, and part of this literature has been ignored by the authors.
We agree with your points on RNA quality and PMI. We have altered the discussion to include information regarding the inclusion criteria for this protocol and how other protocols may suit the user better, depending on their experimental design. We have also reduced the discussion on PMI and integrated it with a more detailed discussion on how to decide if this protocol is relevant and applicable to the user’s study. We also go further into detail about what low RNA integrity can mean.

Discussion lines 597-604 reads: “The stability of RNA in post-mortem human tissue is a well-known obstacle, as RNA molecules within the cells are quite subject to natural decay after death. Specifically, mRNA has been shown to be the most susceptible to nucleolytic degradation [5]. Monitoring of the post-mortem interval (PMI) time to freezing (PMI-frozen) is one metric that has shown some correlation to RNA degradation in some studies [22-24]. However, **Table 1** shows our PMI-frozen intervals for the six samples shown in **Figure 3**, which indicates no relative correlation with PMI values and RNA quality. Therefore, it is necessary to perform due diligence in checking RNA integrity prior to starting a laser capture project.”

To further discuss the downstream necessity of high-quality RNA, text has been added to lines 604-611 in the discussion: “If the RNA integrity of the starting sample is of low quality, the resulting RNA from a LCM product will be of even lower quality. When low RNA integrity cannot be avoided, other methods for enhancing RNA for sequencing could be employed in addition to this protocol [21]. Notably, low-input or degraded RNA can lead to muted complexity and suboptimal results that often necessitate additional amplification steps. The addition of PCR cycles for amplification has been shown to amplify sequences unequally, as well as create read duplicates upon sequencing [25,26]. Therefore, utilization of high-quality RNA from LCM samples for sequencing is highly advantageous.”

Last, the authors fully understand that this protocol is specific to a limited number of cell types and is well suited for the excision of Purkinje cells in the cerebellum. Therefore, we have edited lines 578-585 to read: “Within the context of this protocol, tissue selection is paramount, as the use of antigen or dye specific reagents are not utilized and therefore is not suitable for studies that require such differentiation. While this is a limitation of this protocol, the resulting tissue visualization and RNA integrity are quite superior and maintain relevance in other experimental designs. Many other protocols exist that also utilize alternative staining methods specifically for UV- and IR-LCM with the intention of preserving RNA. However, most other methods contain at least one staining or antigen specific reagent [6,16,17], are designed to one cell or organ type (that are not human autopsy tissue) [18-20], or require specialized RNA-seq kits to enhance integrity [21].”
We hope these edits and additions further clarify the importance of tailoring a protocol to the system and cell type of interest.

7. Others:
- In list of material: Fisher 50-363-579 is Tissue TEK OCT compound, not 'optical cutting temperature'
Thank you for picking up this error, the catalog number and reagent have been updated.
- It is unclear why the authors provide a supplement table showing PCR primers.
We apologize for the confusion on the table 1 included with the manuscript. This table is meant to show RNA integrity and PMI with the associated RNA bioanalyzer readouts from Figure 3. We have edited the title of the table and the table legend in hopes of alleviating this confusion.
Lines 526-527 read: “Of note is that all post-mortem human brain samples were tested for RNA quality prior to LCM (**Table 1**).”
Table legend reads: “Summary of RNA integrity: Sample numbers correspond to the bioanalyzer results presented in Figure 3. Section preps are performed prior to LCM to ensure that starting tissue is of good quality. Shown are the original tissue RINs from section preps, LCM RINs and concentration of RNA from LCM, as well as the post-mortem intervals to frozen (PMI-Frozen) for the tissues.”

Reviewer #2:

Manuscript Summary:
This JoVE article by Martuscello et al., "Laser Capture Microdissection of Purkinje Cells in Post-Mortem Human Cerebellum: A Stainless Protocol for RNA isolation and RNA-sequencing" describes a method to capture a specific cell type in the cerebellum, the purkinje cell for transcriptional profiling. The authors are investigating neuropathological changes in the cerebellum of human patients diagnosed with essential tremor. Given the heterogeneity of the mammalian brain, transcriptional studies of human post-mortem brain tissue necessitates use of precise microdissection techniques such as those described herein. The JoVE manuscript is clearly written with understandable instructions. Clarification of the following points below would improve the narrative but overall, this will make for a nice JoVE video which addresses an important new application of LCM techniques, a stainless method, to genomic studies of human post-mortem brain tissue.

Comments:
1. It is important to keep in mind that JoVE articles are visual teaching tools to help those investigators who are not familiar with these techniques and/or the histology of the specific brain region being studied. Although this is a demonstration of a stainless LCM technique, I think it would be very helpful to show an image of a nissl or H&E stained section of the cerebellum such as shown in the links below (the authors can provide their own stained section) which is clearly labeled with the granule and molecular cell layers and the purkinje cells, next to the unstained images to clearly demonstrate the purkinje cell type that is being laser captured. Being relatively unfamiliar with cerebellar histology, I had to look up several examples as seen in the links below to be convinced that the cells in figures 1 and 2 were indeed purkinje cells.
https://www.google.com/search?q=images+of+purkinje+cells+in+cerebellum&rlz=1C1CHBF_enUS793US794&tbm=isch&tbo=u&source=univ&sa=X&ved=2ahUKEwisspT43LHdAhUKRa0KHd5mA3sQsAR6BAgGEAE&biw=1500&bih=895#imgrc=uGXa8rC6Wd6K8M:
https://www.google.com/search?q=images+of+purkinje+cells+in+cerebellum&rlz=1C1CHBF_enUS793US794&tbm=isch&tbo=u&source=univ&sa=X&ved=2ahUKEwisspT43LHdAhUKRa0KHd5mA3sQsAR6BAgGEAE&biw=1500&bih=895#imgdii=jtsBFUoxWOucFM:&imgrc=uGXa8rC6Wd6K8M:
We agree that a representative stained image of the cerebellum identifying the layers and Purkinje cells would be very helpful to a reader not familiar with cerebellar morphology. Therefore, we have added the representative image to Figure 2 (F).

2. This is a minor criticism but in LCM manuscripts/videos, it is very helpful to the reader/viewer to see an image of a successful laser capture, i.e. a before LCM, and after LCM image. Also, an image of the holes in the section after successful laser capture of the cell of interest may be helpful. An additional figure of a several laser captured purkinje cells and the original section would be helpful.
Thank you for this comment, it is an excellent point. Therefore, we have added three images showing the representative capture of Purkinje cells along the Purkinje cell layer as well as a high magnification picture of 'before' and 'after' capture in Figure 2 (C-E).

3. Given that different LCM systems have different modes of operation, it should be made clear in the

manuscript that the Zeiss LCM system in this video utilizes a laser-induced pressure wave to catapult the UV cut cells of interest into the collection tube whereas others such as the Arcturus LCM system captures cells onto a photoreactive membrane with an IR laser which is then transferred to lysis buffer in a collection tube. Therefore, the LCM section is specific for the Zeiss system only.
We agree that this is an important review point and have clarified this within the manuscript.

Text has been added (highlighted) to the abstract lines 59-64: "This protocol does not account for other fixation methods and is specifically designed for fresh-frozen tissue samples only captured using an Ultraviolet (UV)-LCM system. Here we present a full protocol for sectioning and fixing fresh frozen post-mortem human cerebellar tissue and purification of RNA from Purkinje cells isolated by UV-LCM, with the intent of preserving RNA quality for subsequent RNA-sequencing."

A new paragraph has been added in the introduction lines 120-134: "It is important to note the different types of laser-based microdissection systems, as they have been shown to differ in speed, precision, and RNA quality. The infrared (IR) laser capture microdissection and the ultraviolet (UV) laser microbeam microdissection systems were both novel LCM platforms that emerged almost concurrently [8]. The IR-LCM system employs a "contact system" using a transparent thermoplastic film placed directly on the tissue section, and cells of interest selectively adhere to the film by focused pulses from an IR laser. Alternatively, the UV-LCM system is a "non-contact system" whereby a focused laser beam cuts away cells or regions of interest in the tissue; depending on the configuration in two currently available commercial platforms that use either an inverted or upright microscope design, tissue is acquired into a collection device by a laser-induced pressure wave that catapults it against gravity or tissue is collected by gravity, respectively. Significant advantages of the UV-LCM system include faster cell acquisition, contamination-free collection with the non-contact approach and more precise dissection due to a much smaller laser beam diameter [9]. This protocol was specifically designed for a UV-LCM system and has not been tested in an IR-LCM system.

Text has been added in the protocol lines 422-424: "Note: This section of the protocol will only discuss specifics related to capturing Purkinje cells for subsequent RNA sequencing. This section assumes the user is familiar with the UV laser capture microscope and the affiliated software."

Text has been added in the representative results lines 485-486: "This protocol details the steps for preparing fresh frozen post-mortem human brain tissue for UV-LCM."

Text has been added in the discussion lines 573-574: "The protocol presented here is specifically modified to be a stainless approach in visualizing morphologically distinct tissues for UV-LCM." As well in lines 639-640: "We have presented here a complete method for stainless visualization of Purkinje cells in the post-mortem human cerebellum for the purposes of UV-LCM."

All of these additions address the need to describe the different LCM systems, how they relate to this method and to ensure users know this is a UV-LCM protocol. The protocol is applicable to either inverted or upright microscope designs of UV-LCM systems.