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TITLE:

A Stainless Protocol for High Quality RNA Isolation from Laser Capture Microdissected Purkinje Cells in the Human Post-Mortem Cerebellum

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SUMMARY:

This protocol uses a stain-free approach to visualize and isolate Purkinje cells in fresh-frozen tissue from human post-mortem cerebellum via laser capture microdissection. The purpose of this protocol is to generate sufficient amounts of high-quality RNA for RNA-sequencing.

ABSTRACT:

Laser capture microdissection (LCM) is an advantageous tool that allows for the collection of cytologically and/or phenotypically relevant cells or regions from heterogenous tissues. Captured product can be used in a variety of molecular methods for protein, DNA or RNA isolation. However, preservation of RNA from postmortem human brain tissue is especially challenging. Standard visualization techniques for LCM require histologic or immunohistochemical staining procedures that can further degrade RNA. Therefore, we designed a stainless protocol for visualization in LCM with the intended purpose of preserving RNA integrity in post-mortem human brain tissue. The Purkinje cell of the cerebellum is a good candidate for stainless visualization, due to its size and characteristic location. The cerebellar cortex has distinct layers that differ in cell density, making them a good archetype to identify under high magnification microscopy. Purkinje cells are large neurons situated between the granule cell layer, which is a densely cellular network of small neurons, and the molecular layer, which is sparse in cell bodies. Because of this architecture, the use of stainless visualization is feasible. Other organ or cell

systems that mimic this phenotype would also be suitable. The stainless protocol is designed to fix fresh-frozen tissue with ethanol and remove lipids with xylene for improved morphological visualization under high magnification light microscopy. This protocol does not account for other fixation methods and is specifically designed for fresh-frozen tissue samples 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, while preserving RNA quality for subsequent RNA-sequencing. In our hands, this protocol produces exceptional levels of cellular visualization without the need for staining reagents and yields RNA with high RNA integrity numbers (≥8) as needed for transcriptional profiling experiments.

INTRODUCTION:

Laser capture microdissection (LCM) is a valuable research tool that allows the separation of pathologically relevant cells for subsequent molecularly driven evaluation. The use of molecular analyses in these heterogeneous tissue specimens and the correlation with pathological and clinical data is a necessary step in evaluating the translational significance of biological research[1]. When analyzing gene expression data from RNA, the use of frozen tissue sections is highly recommended as it allows for excellent quality of RNA as well as maximized quantity[2]. It has been well established that high quality and quantity of RNA are essential for meaningful data from RNA sequencing[3]. 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[4,5]. Furthermore, RNA degradation is exacerbated when staining techniques are needed to recognize histologic details and cell identification. Specialized staining techniques, such as hematoxylin & eosin, Nissl stain, immunofluorescence and immunohistochemistry are helpful in differentiating cells from surrounding stroma but have been shown to degrade RNA and alter transcript expression profiles[6]. Therefore, our laboratory has created a stainless protocol specifically designed to preserve RNA in post-mortem human cerebellum for the purposes of RNA sequencing after LCM isolation of Purkinje neurons.

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[7]. Ethanol fixation is a better alternative for RNA isolation, as it is a coagulative fixative that does not induce cross-linking[1]. To enhance the 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 the tissues can dry out and become brittle causing tissue fragmentation upon laser capture[7]. 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[8]. Therefore, our protocol centers around the use of 70% ethanol fixation and ethanol dehydration, followed by xylene incubation for morphological clarity.

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 the 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. In either UV-LCM system design, when accumulating cells into the collection cap, the use of a coverslip that enhances cellular clarity during microscopy is not suitable, as the 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. Liquid filled collection caps can be challenging, as the liquid is subject to evaporation and must be replaced frequently while working at the microscope. Time becomes an important factor for RNA stability, as the captured tissue dissolves immediately[7].

Our laboratory studies the postmortem neuropathologic changes in the cerebellum of patients with essential tremor (ET) and related neurodegenerative disorders of the cerebellum. We have demonstrated morphologic changes centered on Purkinje cells that distinguish ET cases versus controls, including a reduced number of Purkinje cells, increased dendritic regression and a variety of axonal changes, leading us to postulate that Purkinje cell degeneration is a core biologic feature in ET pathogenesis[10-13]. Transcriptional profiling has been used in many neurologic diseases to explore the underlying molecular basis of degenerative cellular changes. However, transcriptional profiles from heterogeneous samples, such as brain tissue regions, can effectually mask the expression of low abundance transcripts and/or diminish the detection of molecular changes that occur only in a small population of affected cells, such as Purkinje cells in the cerebellar cortex. For instance, Purkinje cells are vastly outnumbered by the abundant granule cells in the cerebellar cortex by approximately 1:3000; thus, to effectively target their transcriptome requires specific isolation of these neurons. The cerebellar cortex is delineated by distinct layers that differ in cell density and cell size. This cellular architecture is ideal to visualize in a fresh-frozen tissue sample without dye-containing staining reagents. In theory, this protocol could also be applied to other tissue types that have similar distinctive tissue organization.

This protocol was designed to work specifically for Purkinje cell visualization in the human post-mortem cerebellum. 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.

PROTOCOL:

All human samples utilized in this protocol have been obtained with informed consent and have been approved by the Internal Review Board (IRB) at Columbia University and Yale University.

Note: The entirety of this protocol should follow strict RNA handling guidelines, whereby a gloved hand is always used, all surfaces are cleaned with an RNase decontaminator and all working materials are RNA/DNA/Nuclease free.

1.Test RNA Integrity of Tissue prior to Starting LCM

NOTE: Testing RNA quality can be done by many methods. Ensure that RNA from the entire section is tested to provide a representative RNA integrity number (RIN) for the sample.

1.1. Carefully select tissue samples for inclusion that fit within the parameter of the experimental design. If cutting at the cryostat, move to Step 1.2. If not, process the tissue accordingly and move to Step 1.11.

1.2. Get two containers of dry ice. The size will depend on the number of samples.

1.2.1. In the first container of dry ice, place an empty, labeled 2 mL tube with the tissue code.

NOTE: It takes 10 min for the tubes to freeze. Tubes must be frozen prior to placing the tissue into them; otherwise, the tissue will melt on the tube surface.

1.2.2. In a second container of dry ice, place the tissue from a -80 °C freezer that requires RNA check.

1.3. Clean the cryostat. See Step 4.7 for detailed cleaning procedure.

1.4. Remove the tissue from dry ice and cut a small section of tissue with a RNase decontaminated razor blade. Tissue should be approximately 1 cm x 1 cm in size.

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

1.6. Once OCT hardens, place the chuck with the mounted tissue in the cryostat cutting arm and position in front of the cryostat blade.

1.7. Trim at 30 μm sections until the tissue is even.

1.8. Cut 300 μm of the tissue and place in a frozen 2 mL tube. Place the tube back into dry ice.

1.9. Remove the tissue from the 'chuck' and place back into dry ice until ready to put away.

1.10. Repeat Steps 1.4-1.9 for all tissues.

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).

1.12. Test the integrity of extracted RNA via a quality assessment bioanalyzer[14].

2.Prepare prior to Starting Sectioning for LCM

2.1. Clean the slides holders prior to each round of tissue sectioning for LCM. Perform the cleaning procedure the day before use to allow complete drying overnight.

NOTE: Slide holders are used for tissue section fixation in ethanol and clearing in xylene.

2.1.1. Slide holder cleaning procedure: Rinse with RNase decontaminator followed by diethyl pyrocarbonate treated (DEPC) water. Allow to dry overnight upside down on an RNA/DNA/Nuclease free surface, avoiding any dust or other airborne contamination.

CAUTION: DEPC is highly toxic and should be handled and disposed of using EH&S standard hazardous chemicals protocol.

2.2. Clean the brushes for sectioning with RNase decontaminator and allow to dry overnight. Avoid any dust and other contaminants.

2.3. Place the membrane slides under UV light for 30 min at room temperature. Do not expose the slides to UV more than 1-2 days prior to use. This enhances the binding of the tissue to the membrane slide.

NOTE: Step 2.3 can be combined with Step 4, which allows for slide UV treatment to occur on the same day as tissue sectioning (see Step 4.4).

3.Prepare Fixation Solutions with RNase Free Water and High-Quality Ethanol

NOTE: All solutions are prepared fresh for every experiment. Ensure that the slide holder cleaning procedure from Step 1.1 is completed. All solutions are prepared in slide holders.

3.1. Place 30 mL of 100% ethanol in one slide holder.

3.2. Dilute ethanol with RNase/DNase/Nuclease free water. Place 30 mL of 95% ethanol in one slide holder and put on ice.

3.3. Dilute ethanol with RNase/DNase/Nuclease free water. Place 30 mL of 70% ethanol in one slide holder and put on ice.

3.4. Place 30 mL of 100% xylene in two separate slide holders and label them as #1 and #2.

4. Prepare Cryostat for Tissue Sectioning

4.1. Get a bucket of ice for ethanol solutions and a container with dry ice for the tissue.

CAUTION: Dry ice is extremely cold, so use protective gloves. Do not place ice and dry ice in the same container. Differing temperatures in the ice and dry ice will cause them to form together at an indeterminate temperature.

4.2. Remove the tissue from the -80 °C freezer and place on dry ice for transport to the cryostat.

4.3. Cryostat settings:

4.3.1. Set the section thickness to 14 μm.

4.3.2. Set the trim thickness to 30 μm to preserve tissue quantity.

4.3.3. For cryostats with dual chamber and specimen holding temperatures, set the chamber temperature (CT) to -20 °C. For cryostats with one setting, set the temperature to -18 °C.

4.3.4. For cryostats with dual chamber and specimen holding temperature, set the object temperature (OT) to -18 °C.

NOTE: The OT needs to be warmer than the CT to ensure even cutting. If the tissue is still shredding, the OT can be increased to -15 °C and the CT can be increased to -18 °C.

4.4. Place the tissue in the cryostat for at least 20 min to allow to come to optimal temperature.

NOTE: If the tissue is not at optimal temperature, it will shred upon cutting. Do not place the tissue in -20 °C the night before to preserve RNA integrity and to prevent ice crystal formation. Allow the tissue as much time as necessary to equilibrate to optimal temperature to cut smoothly. Optional implementation of slide incubation under UV may occur here (see Step 1.3).

4.5. Place the ‘chuck’ in the cryostat for at least 20 min to allow to come down to the temperature of the cryostat.

4.6. Place the clean brushes in the cryostat for at least 20 min to allow to come down to the temperature of the cryostat.

4.7. Clean the cryostat

4.7.1. Clean the stage with a 1:1 mixture of RNase decontaminator and 70% ethanol diluted with RNA/DNA/Nuclease free water to prevent freezing on stage.

4.7.2. Clean a new disposable cryostat blade with RNase decontaminator and place in the blade holder on the stage. Allow at least 20 min for the cryostat blade to come to the temperature of the cryostat.

4.7.3. Clean the anti-roll plate with RNase decontaminator and attach to the stage. Allow at least 20 min for the anti-roll plate to come to the temperature of the cryostat.

NOTE: An anti-roll plate is not required for cutting but is highly recommended. If an anti-roll plate is not available, a cleaned brush works as an alternative. If the anti-roll plate is not at the correct temperature, the tissue will melt or stick upon cutting.

5. Sectioning Tissue

5.1. Cut a small piece of the tissue (~1 cm x 1 cm) for sectioning. Return the remaining tissue to dry ice/-80 °C freezer.

NOTE: Ensure that the tissue section is ~95% cerebellar cortex, where Purkinje cells are located.

5.2. Place an approximately 3 mm high mound of OCT to cover the 'chuck'. Start by building the layers on top in a slow, circular motion, until there is a small mound.

5.3. Once OCT is partially frozen, but there is still some liquid in the center, place the tissue on top of OCT. Do not push the tissue deeply into OCT. Allow the tissue to sit on top of OCT until completely frozen. This will take 1-2 min.

5.4. Place the 'chuck' with frozen OCT and the tissue into the cryostat cutting arm. Adjust the tissue so it is flush with cutting blade.

5.5. Allow the tissue to sit in the cutting arm for 10-15 min to adjust to new temperature.

NOTE: Depending on the thickness of the tissue, time is needed for temperature acclimation. Allow the tissue to sit as long as required to cut cleanly. Adjust OT and CT to assist with tissue temperature acclimation.

5.6. Slowly move the tissue closer to the blade. Once the tissue has reached the blade, start the trim process. Trim thickness should be set to 30 μm.

5.7. Trim 2-3x until the cortex layers are visible.

5.8. Place and align the anti-roll plate just above the cryostat blade.

NOTE: A silver line will appear from the light in the cryostat at the interface of the blade and anti-roll plate. This indicates the anti-roll plate is directly over the edge of the blade.

5.9. Begin to cut the sections at 14 μm. Properly cut sections will be flat under the anti-roll plate.

NOTE: If the tissue is stuck, ensure that the anti-roll plate is cold enough. If necessary, placing a piece of dry ice on the outside (side not touching the stage) will rapidly chill the anti-roll plate.

5.10. Cut 4-6 sections and align them horizontally across the cryostat stage.

5.11. Angling the slide, pick up all pieces of the tissue at one time.

NOTE: Using the brushes, lift up the ends of the tissue to be more readily available for the slide upon pick up. Do not pick up one section at a time. Exposure to room temperature air will degrade RNA integrity.

CAUTION: Do not let the membrane touch the stage of the cryostat. If the membrane touches the stage, it will begin to detach from the glass slide and will cause errors when attempting LCM.

5.12. Immediately move to Step 6. Do not delay fixation.

6. Fixing Tissue/Stain-less Visualization

6.1. Immediately move to the ethanol fixation protocol

6.2. Place the slide in the slide holder with 70% ethanol for 2 min – on ice.

6.3. Place the slide in the slide holder with 95% ethanol for 45 s – on ice.

6.4. Place the slide in the slide holder with 100% ethanol for 2 min – at RT.

6.5. Dip the slide 3 times in the slide holder with xylene 1 – at RT.

6.6. Place the slide in the slide holder with xylene 2 for 5 min – at RT.

6.7. Allow to dry in a clean area for at least 30 min. Longer dry times are optimal, up to 60 min.

CAUTION: Stand up slides for drying in a fume hood. Xylene is volatile. Laying slides flat will cause xylene to pool in the tissue section, which will cause tissue to be too dark.

7.Optional - Slide Storage

7.1. Place dried slides in individual 50 mL tubes (one slide per tube) and place in the -80 °C freezer for up to 7 days.

NOTE: Slides must be dry prior to placing in the -80 °C freezer. Do not use desiccant inside the tube as particulates will embed in the tissue and membrane. Ensure that the tube cap is tight; if not, condensation will occur on the slide when thawing for use.

CAUTION: RNA integrity decreases after 7 days, as does the quality of tissue visualization.

8. Thawing Stored Slides for Use

8.1. Remove the tube with a single slide from the -80 °C freezer 1 h prior to intended use.

8.2. Do not immediately open the tube. Allow the tube to come to room temperature. Condensation will occur on the outside of the tube only.

8.3. Once the tube has come to room temperature and all condensation is gone (approximately 30-40 min), remove the cap and expose the slide to the room temperature air.

8.4. Allow the slide to acclimate to room temperature for 15-20 min. Leave the slide inside the tube with the cap removed.

NOTE: Slide is now ready for LCM.

9. Laser Capture Microdissection of Purkinje Cells:

NOTE: This section of the protocol will only discuss specifics related to capturing Purkinje cells for subsequent RNA sequencing. This section assumes that the user is familiar with the UV laser capture microscope and the affiliated software.

9.1. Clean the microscope stage and the cap collection arm with RNase decontaminator.

9.2. With gloved hands, place the slide on the microscope and 500 µL opaque cap in the collection arm.

NOTE: Always ensure proper RNA technique by cleaning the work area with RNase decontaminator and use gloved hands while touching the slide or opaque cap. Gloves can be removed once the slide and cap are in place and laser capture has commenced.

9.3. At low magnification, align the opaque cap over the cerebellar tissue, ensuring that the cap covers the entire area visualized in the eye piece.

NOTE: Only the eye piece of the microscope will show if the opaque cap is centered. The camera will not show if the cap is centered over the tissue. Depending on the laser capture scope design, the visualization through the opaque cap will either be in the eye piece only or visualized in both the eye piece and camera.

9.4. Visualize the cerebellar layers with 5X – 10X objective lens and place the cursor over the section where molecular layer and granule cell layer intersect (the Purkinje cell layer).

9.5. Move to 40X and visualize Purkinje cells.

NOTE: See **Figure 1** and **Figure 2** for example visualizations.

9.6. Begin capturing Purkinje cells.

NOTE: To perform RNA-sequencing, at least 5 ng of RNA is required. Approximately 1600-2000 Purkinje cells result in enough RNA material for sequencing. RNA will be stable for up to 8 h at the microscope. Test UV energy and UV focus prior to collection to ensure levels are correct. Over time, the tissue will continue to dry out and the UV energy and UV focus may need to be changed.

10. RNA Collection post LCM

10.1. Prepare the cell lysis buffer (prepare fresh each time)

10.1.1. Dilute 2-mercaptoethanol in lysis buffer at 1:100 (10 µL:1 mL, respectively). Lysis (RLT) buffer is provided in **Table of Materials** (#14 and #15).

10.2.Add 50 µL of cell lysis buffer to the opaque cap, with the cap facing up. Carefully close the tube over the cap.

10.3. Leave the tube upside down for 1 min.

10.4. Vortex the tube for 30 s and quickly spin the lysis buffer to the bottom of the tube.

10.5. Reopen the tube and repeat Steps 10.2-10.4.

10.6. Place the tube containing 100 µL of lysis buffer and RNA from the -80 °C freezer until all samples are finished and ready for RNA extraction (**Table of Materials** #14).

REPRESENTATIVE RESULTS:

This protocol details the steps for preparing fresh frozen post-mortem human brain tissue for UV-LCM. Thorough specifications and annotations are given for cryostat cutting, as it can be difficult to cut fresh frozen brain tissue with a high degree of precision. The most important point to consider is that fresh frozen tissue is extremely cold and requires a significant amount of time

to acclimate to the warmer temperature of a cryostat. This step cannot be rushed, and it cannot be overstated how central this step is in the success or failure of this protocol. If the tissue is not prepared properly at the cryostat, all subsequent attempts at identifying cells or regions of interest will be very difficult, if not impossible.

Following cryostat sectioning and allotted drying time, the tissue is ready for LCM. When visualizing the tissue under the microscope, the cellular layers of the cerebellum are easily visible with 5X and 10X objective lenses. **Figure 1** shows representative images of tissue fixed in ethanol only (**Figure 1A**) and tissue incubated in xylene following ethanol fixation (**Figure 1B**). We rigorously tested various ethanol and xylene incubation times/temperatures on the ability to both fix and visualize the tissue. If the tissue is not incubated long enough in xylene, the resulting image will more closely resemble **Figure 1A**, rather than **Figure 1B**. Xylene incubation causes the tissue to become darker and better delineates cellular layers than does ethanol alone. When cutting at the laser capture microscope, the 40X objective lens is required to ensure capturing only Purkinje cells, and not the surrounding tissue. Incubation in xylene produces a high quality, morphologically intact image (**Figure 1D**) compared to ethanol fixation alone (**Figure 1C).**

To further enhance the visualization of our tissue, we tested the coverslip ability of an Opaque cap. Other UV-LCM protocols utilize a liquid filled cap of a 200-500μL tube that dissolves the tissue on contact. Liquid filled caps can reduce tissue visualization, causing the resulting image to be granular and iridescent under the microscope (**Figure 2A**). Tissue visualization through the Opaque cap (**Figure 2B**) results in a smoothened tissue appearance that is softer and sharper in form. Notably, the Opaque cap allows for collection up to 8 h without the need for replacement. Representative images of excised Purkinje cells at low power (**Figure 2C**) and at high power (**Figure 2D, E**) show precise removal of just the Purkinje cell body. For reference, **Figure 2F** shows a Luxol Fast Blue/ Hematoxylin and Eosin (LH&E) stained cerebellar cortex, which specifically highlights the different layers of the cerebellum and the placement of the Purkinje cells at the molecular layer and granule cell layer juxtaposition.

The purpose of this protocol is to obtain high quality RNA for subsequent RNA sequencing. We tested our protocol on six different post-mortem human brain samples, which each underwent three days of LCM to collect 1500-2000 cells for RNA extraction. Approximately 6-8 h at the microscope produced 500-700 cells, which were then combined with the previous days LCM products prior to RNA extraction. Samples underwent RNA extraction and were resuspended in 14 μL of RNAse-free water (**Table of Materials** #14). All six samples produced high quality RNA, with RNA integrity numbers (RINs) ≥8 (**Figure 3 A-F**). This procedure resulted in RNA quantities of 11.5 ng (**Figure 1A**), 8.3 ng (**Figure 1B**), 13.6 ng (**Figure 1C**), 11.5 ng (**Figure 1D**), 14.9 ng (**Figure 1E**) and 26.9 ng (**Figure 1F**). Of note is that all post-mortem human brain samples were tested for RNA quality prior to LCM (**Table 1**). If the sample of origin has RNA degradation, LCM will only further degrade its integrity.

FIGURE LEGENDS:

Figure 1: Cerebellar layers and Purkinje Cell visualization with and without xylene treatment. Representative images with and without xylene following ethanol fixation and dehydration. The

molecular layer (ML) and granule cell layer (GCL) are labeled. Purkinje cells are marked with arrows. (**A**) 5X representation of cerebellar layer visualization without xylene. Scale bar: 10 µm. (**B**) 5X representation of cerebellar layer visualization with xylene. Scale bar: 10 µm. (**C**) 40X representation of cerebellar layer visualization without xylene. Scale bar: 1 µm. (**D**) 40X representation of cerebellar layer visualization with xylene. Scale bar: 1 µm.

Figure 2: Purkinje Cell Visualization before and after LCM. Representative images at 40X with xylene following ethanol fixation and dehydration. The ML and GCL are labeled. Purkinje cells are marked with arrows. **(A)** Visualization at the microscope under liquid filled cap at 40X. Scale bar: 1 µm. (**B**) Visualization at the microscope under Opaque collection cap at 40X. Scale bar: 1 µm. (**C**) 5X visualization of the cerebellar cortex showing the excised Purkinje cells between the ML and GCL. Scale bar: 10 µm. (**D**) 40X visualization of a Purkinje cell prior to excision. Scale bar: 1 µm. (**E**) 40X visualization of successful Purkinje cell capture. Scale bar: 1 µm. **(F)** 20X representative LH&E stained cerebellum showing Purkinje cell bodies with marked arrows. Scale bar: 5 µm.

Figure 3: RNA Quality Control Bioanalyzer Results of LCM samples. Panels **A-F** show representative RNA quality control readouts. Each panel is a different sample that underwent the same LCM process of fixation and visualization with ethanol and xylene only. RNA integrity numbers (RINs) are all > 8.0. Representative concentrations [ ] result in a yield of at least 5 ng of total RNA. All samples were eluted in 14 µL of RNAse-free water.

Table 1: **Summary of RNA Integrity**. Sample numbers correspond to the bioanalyzer results presented in **Figure 3**. Section preparations 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.

DISCUSSION:

The protocol presented here is specifically modified to be a stainless approach in visualizing morphologically distinct tissues for UV-LCM. This method is designed to maximize RNA integrity for subsequent direct RNA sequencing, while maintaining an enhanced level of tissue visualization. The ability to distinguish different cell types for capture, to create the purest population of cells possible, is essential for understanding different molecular profiles in human tissues[15]. 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]. 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. Importantly, we identified only one LCM study in the literature that described the collection of human Purkinje cells, which are of considerable interest to the study of cerebellar degenerative and developmental disorders. This study stained 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 that is designed specifically for high-quality RNA from Purkinje cells in the post-mortem human cerebellum excised via UV-LCM.

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[23-25]. 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. If the RNA integrity of the starting sample is of low quality, the resulting RNA from an 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[26,27]. Therefore, utilization of high-quality RNA from LCM samples for sequencing is highly advantageous.

The visualization method employed in this protocol heavily relies on the user's expertise when cutting fresh frozen tissue on a cryostat. The protocol goes into extensive detail on how to best prepare the tissue for sectioning and place the tissue on the slide. These are undoubtedly the two most critical steps of this protocol. If the tissue is not acclimated to the cryostat temperature, shredding will occur, which significantly hinders the morphological quality of the tissue. Shredding is the result of a few potential issues; troubleshooting possibilities include waiting longer for the tissue to come to temperature or altering the cryostat OT and/or CT to a warmer range. Once the tissue has been successfully cut and placed on the stage, it is important to orient the tissue and the slide properly to ensure all tissue fits within the membrane and can be accurately picked up from the stage. When attempting to pick up the tissue from the cryostat stage, the tissue must be cold, and the slide should be warm. Alternative protocols exist that recommend to chill the slide prior to picking up the tissue and warmed with a finger over the area of tissue placement[28]; however, in our hands, this does not provide any benefit, and often causes difficulty in picking up the tissue and excessive tissue folds. With a slide at room temperature, the tissue melts immediately upon contact with the slide. To ensure a clean pickup, it is necessary to angle the slide so that it comes into contact with the tissue at the membrane area but does not go so far as to touch the stage itself. The membrane will begin to detach from the glass slide if it touches the stage, which damages the membrane and makes laser capture difficult. If this occurs, it will be noticeable once LCM has begun and cannot be undone.

Troubleshooting options are limited; if at any time it is thought the membrane or slide have been compromised, it is wise to discard. It is recommended to practice fresh frozen tissue sectioning prior to starting a project or using a pathology core that can produce high quality tissue sectioning. However, if using a core service, ensure that proper RNA technique is followed to prevent RNase contamination. RNA degradation can readily occur when improper technique is used.

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. We have also included proper RNA preservation methods and techniques to ensure high levels of RNA integrity. This protocol is not necessarily specific to any one cell or tissue type but does maintain the requirement that the region/cell of interest is morphologically distinguishable from the surrounding stroma without the need for antigen or dye specific recognition.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

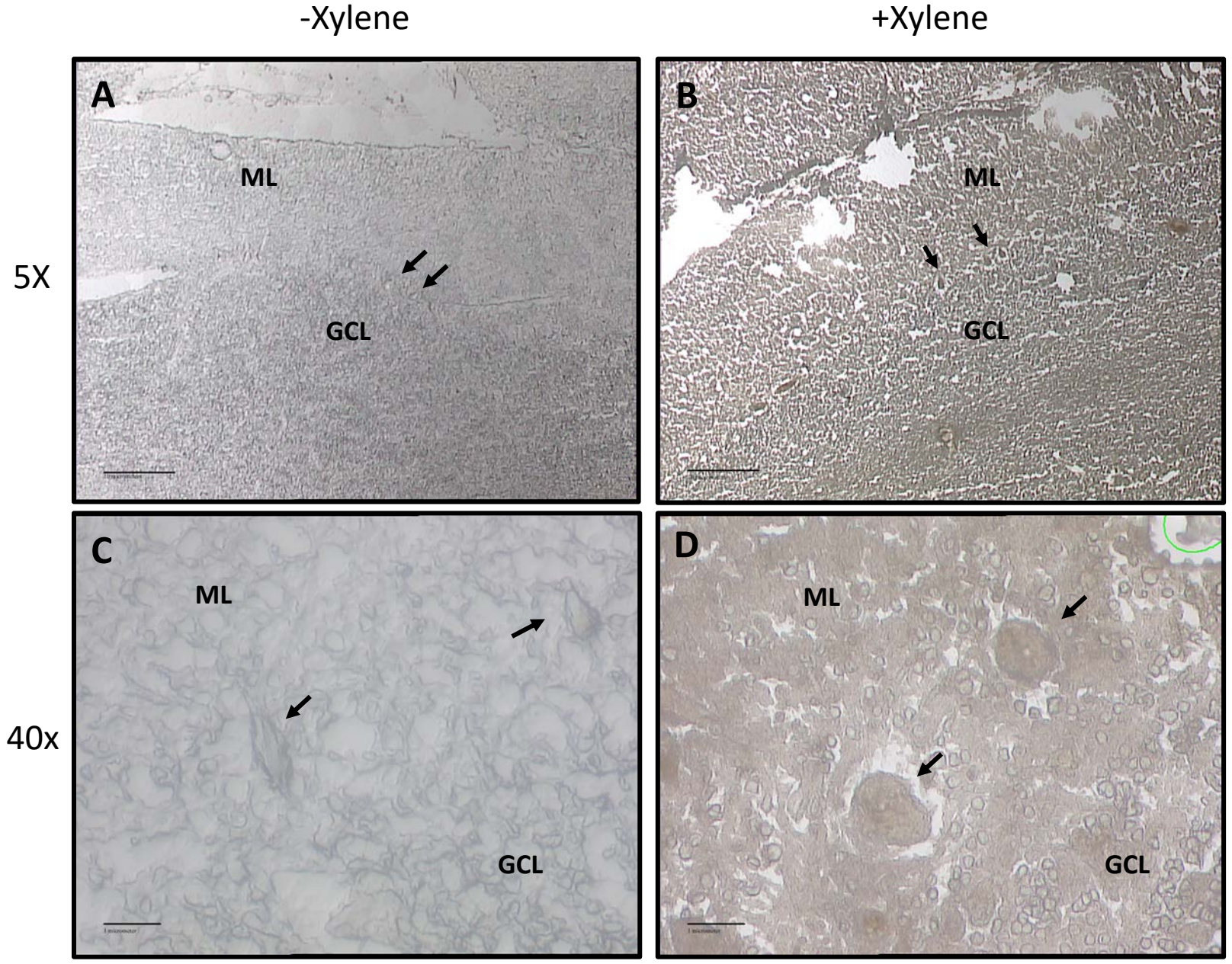

Figure 2

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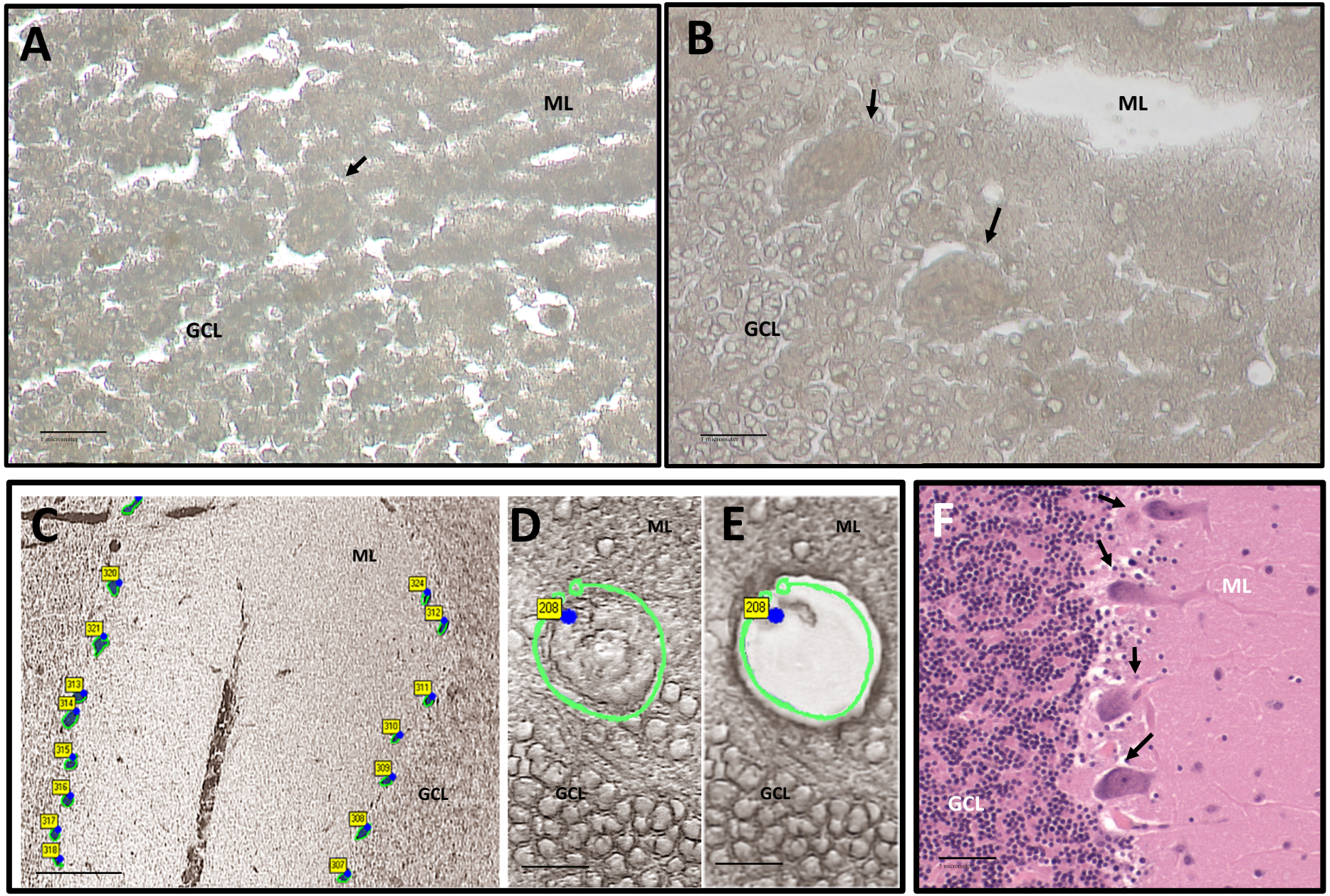
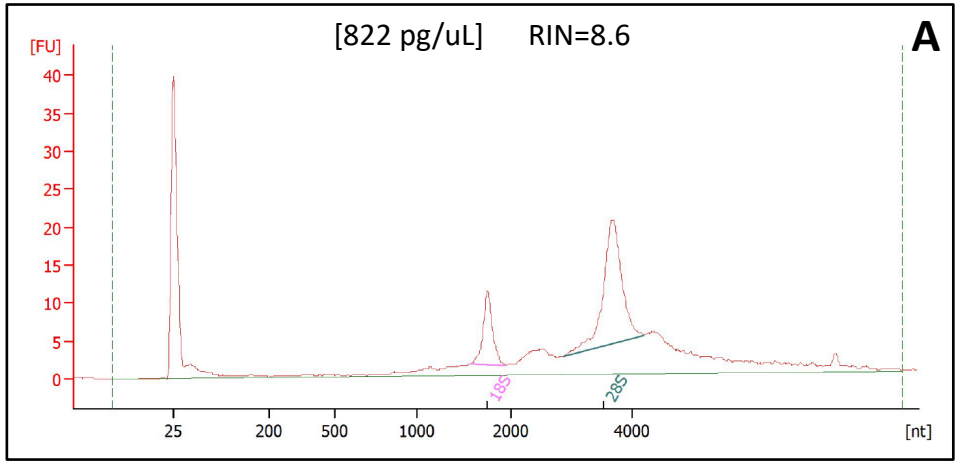
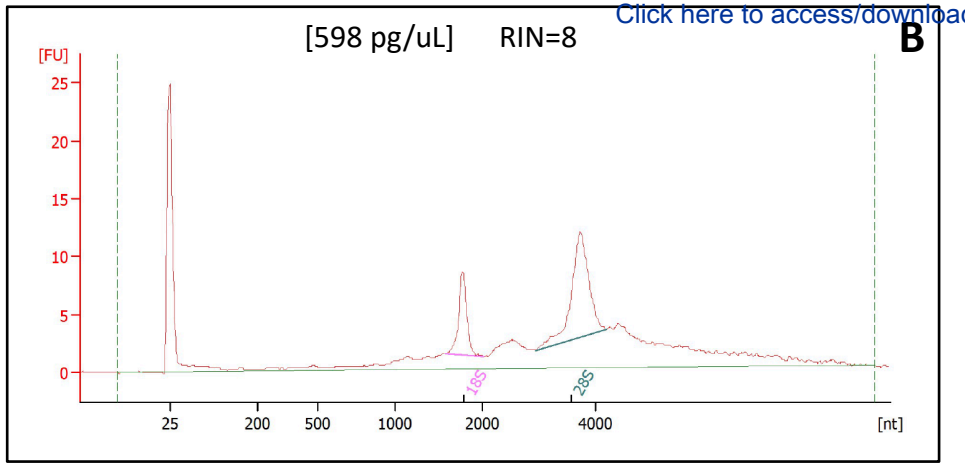
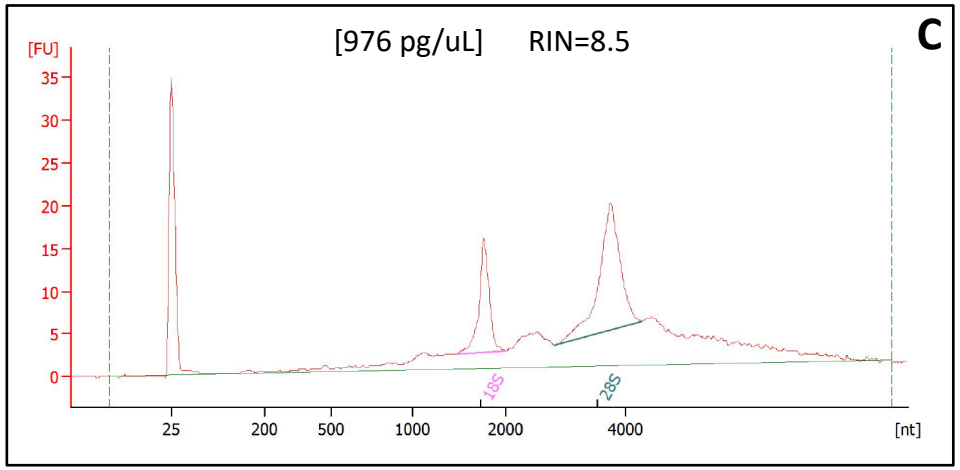
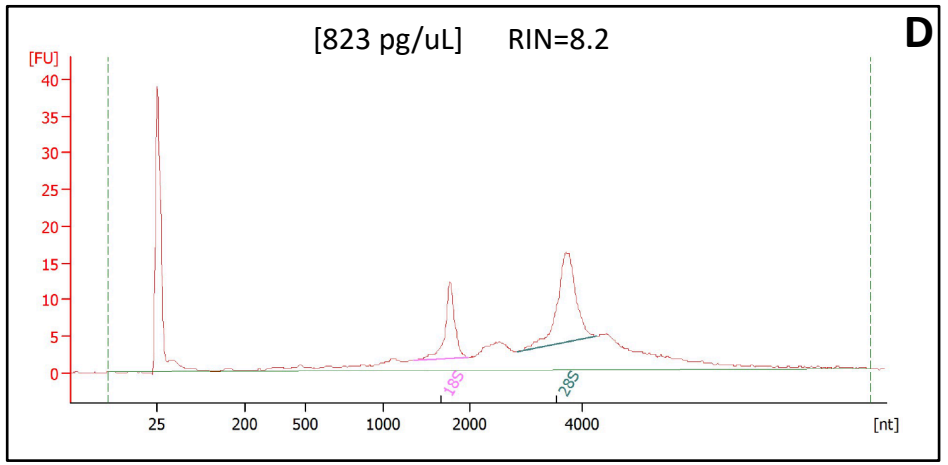


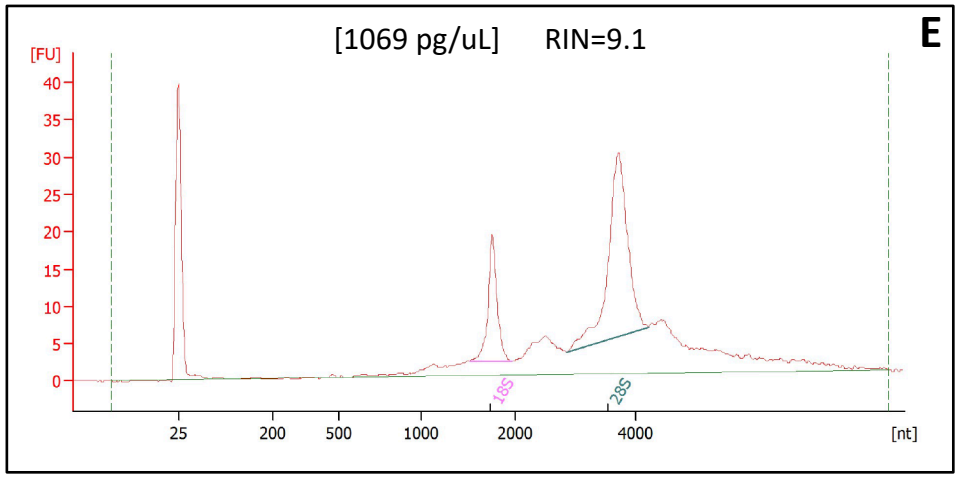
Figure 3











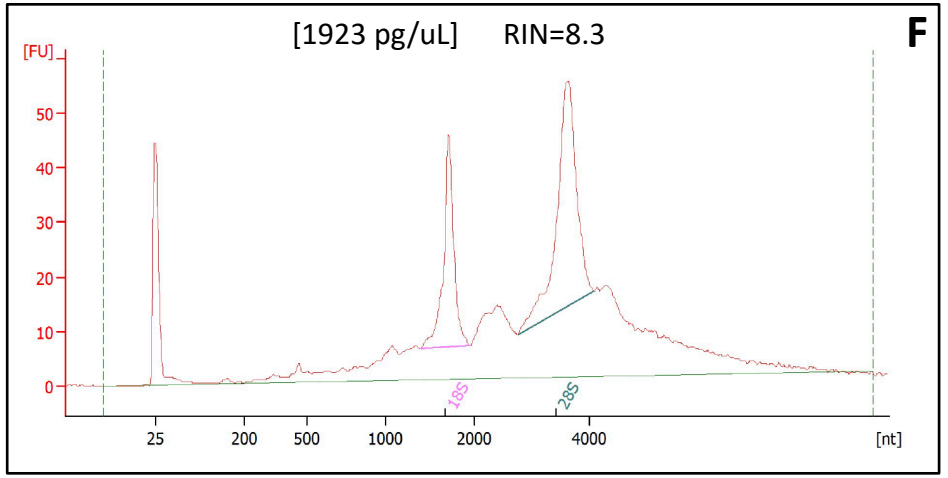


Table 1. Summary of RNA Integrity				
Sample	*RIN - Section Prep*	*RIN - LCM*	*[RNA] - LCM*	*PMI-Frozen*
A	9.2	8.6	822 pg/µL	450 min
B	9.8	8	598 pg/µL	550 min
C	9.1	8.5	976 pg/µL	455 min
D	9.8	8.2	823 pg/µL	463 min
E	9.8	9.1	1069 pg/µL	1139 min
F	9.6	8.3	1923 pg/µL	1080 min

Table of Materials

	Name of Material/ Equipment	Company	Catalog Number
1	MembraneSlide NF 1.0 PEN	Zeiss	415190-9081-000
2	AdhesiveCap 500 Opaque	Zeiss	415190-9201-000
3	RNase Away	Molecular BioProducts	7005-11
4	200 Proof Ethanol	Decon Laboratories	2701
5	Xylenes (Certified ACS)	Fisher Scientific	X5P-1GAL
6	UltraPure Distilled Water	Invitrogen	10977-015
7	Slide-Fix Slide Jars	Evergreen	240-5440-G8K
8	Anti-Roll Plate, Assy. 70mm 100um	Leica Biosystems	14041933980
9	Diethyl pyrocarbonate (DEPC)	Sigma Aldrich	D5758-50mL
10	Kimwipes	Fisher Scientific	06-666A
11	Tissue-Plus O.C.T. Compound	Fisher Scientific	23-730-571
12	Edge-Rite Low-Profile Microtome Blades	Thermo Scientific	4280L
13	Leica Microsystems 3P 25 + 30MM CRYOSTAT CHUCKS	Fisher Scientific	NC0558768
14	RNeasy Micro Kit (50)	Qiagen	74004
15	RNeasy Mini Kit (50)	Qiagen	74104
16	RNase-Free DNase Set	Qiagen	79254

17	2-Mercaptoethanol	Sigma Aldrich	M6250-100ML
18	Globe Scientific Lot Certified Graduated Microcentrifuge Tube (2 mL)	Fisher Scientific	22-010-092

Comments/Description
Membrane slides for tissue. We do not recommend using glass slides.
Opaque caps are used to enhance visualization on inverted scopes only
Other RNase decontamination products are also suitable. Use to clean all surfaces prior to work.
If using alternative Ethanol, ensure high quality. Essential for tissue fixation.
If using alternative xylene, ensure high quality. Essential for tissue visualization.
Other RNA/DNA/Nuclease free water also suitable. Utilized in ethanol dilution only.
Any slide holder/jar is also suitable. Must be cleaned prior to use. Used for fixation following sectioning.
Anti-roll plate type is determined by type of cryostat and attachment location on stage. All cryostats have differing requirements.
DEPC from any vendor will do. Follow directions for water treatment.
Kimwipes can be ordered from any vendor and used at any size. Kimwipes are to be used to clean microscope and cryostat.
Any brand of OCT is acceptable. No tissue will be embedded in the OCT, it is strickly to attach tissue to 'chuck'.
Blade type is determined by type of cryostat. All cryostats have differing requirements.
Chuck type is determined by type of cryostat. All cryostats have different requirements. Either size is suitable.
Required for RNA extraction post LCM. RLT Lysis buffer essential to gather captured cells from opaque cap.
Required for RNA extraction of section preps, which are performed prior to LCM to test RNA integrity of starting tissues.
Dnase will remove any DNA to allow for a pure RNA population. Ensure Dnase is made fresh.

Purchased volume at user discretion. Necessary for addition to RLT buffer for cell lysis. Prevents RNase activity.

Any 2 mL tube that is RNase free, DNase free, human DNA free, and Pyrogen free will do.

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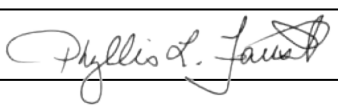
Signature:	
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Editorial comments:

The manuscript has been modified and the updated manuscript, **58953_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

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 thoroughly edited and is free of spelling and grammatical errors to our best effort.

2. Please use a single space between numerical values and their units.

A space has been placed between all numerical values and their units, including temperatures.

3. Please do not highlight note/caution for filming.

Notes and cautions have been removed from the highlighted text for filming.

4. Step 2.3: What's the temperature for incubation?

Section 2.3 states: Place membrane slides under UV light for 30 min at **room temperature**.

5. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next.

Highlighted steps for filming have been re-evaluated and altered to create a narrative with a logical flow from one step to the next. The highlighted steps now start with setting up and cleaning the cryostat prior to cutting and include a few extra steps afterwards to complete the process. If the film editing believes some other steps should be included or removed we are open to altering the flow of the video.

In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.

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.