Video Article

Production of RNA for Transcriptomic Analysis from Mouse Spinal Cord Motor Neuron Cell Bodies by Laser Capture Microdissection

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Abstract

Preparation of high-quality RNA from cells of interest is critical to precise and meaningful analysis of transcriptional differences among cell types or between the same cell type in health and disease or following pharmacologic treatments. In the spinal cord, such preparation from motor neurons, the target of interest in many neurologic and neurodegenerative diseases, is complicated by the fact that motor neurons represent <10% of the total cell population. Laser capture microdissection (LMD) has been developed to address this problem. Here, we describe a protocol to quickly recover, freeze, and section mouse spinal cord to avoid RNA damage by endogenous and exogenous RNases, followed by staining with Azure B in 70% ethanol to identify the motor neurons while keeping endogenous RNase inhibited. LMD is then used to capture the stained neurons directly into guanidine thiocyanate lysis buffer, maintaining RNA integrity. Standard techniques are used to recover the total RNA and measure its integrity. This material can then be used for downstream analysis of the transcripts by RNA-seq and qRT-PCR.

Video Link

The video component of this article can be found at https://www.jove.com/video/51168/

Introduction

In mammalian tissues composed of multiple different cell types, the advent of laser capture microdissection (LMD) instrumentation has afforded the possibility to select a specific cell type for analysis at the RNA or protein level. At present, amplification and next-gen sequencing techniques enable use of a pool of total RNA from a few thousand cells to obtain a relatively thorough inventory of the transcriptome, including assessment of relative levels of RNAs and identification of various spliced forms. To date, proteomics analyses of a few thousand cells will reach down through only more abundant species. For example, we have identified <1,000 of the most abundant proteins from 3,000-4,000 motor neuron cell bodies (not shown), and Zhu and coworkers have recently reported identifying 2,665 proteins from ~15,000 tumor cells[1]. With further developments of mass spectrometry, however, it is likely that such analyses will extend to far greater depth.

Here, we present a specific protocol used for LMD of motor neuron cell bodies from spinal cord of mice, followed by preparation of RNA. This protocol was used in the context of comparing RNAs from motor neurons of presymptomatic transgenic mutant superoxide dismutase (SOD)1 amyotrophic lateral sclerosis (ALS) mice with RNAs prepared from a wild-type SOD1 transgenic strain by both RNA-seq and qRT-PCR validation[2]. Notably, motor neurons, in the anterior horn of the gray matter in the spinal cord, comprise <10% of the total cell population, surrounded by a sea of astrocytes, and as such, their transcriptional profile cannot be easily deconvoluted from studies of the entire cord. A laser capture approach is thus ideal for analyzing RNA expression in these cells, with physiology preserved by rapidly excising and freezing the cord following brief intracardiac saline perfusion. Motor neuron somata are large and are thus readily detectable, here using a dye that has strong affinity for neurons[3]. In addition, with such size, these cells provide a relatively large amount of RNA per cell captured. The procedure used, as detailed below, could be readily adjusted to obtain other neuronal cell types, as well as potentially other cell types, specifically identified either by dye staining techniques or by antibody staining.

Protocol

The procedures described here for anesthesia, euthanasia, and cardiac perfusion of mice were performed under a protocol approved by the Yale University Institutional Animal Care and Use Committee.

1. Preparation of RNase-free Instruments and Solutions

1. RNase is a common contaminant of all laboratory surfaces, including bench tops, micropipettes, and the investigator.
    1. Change gloves often or regularly wipe with a RNAse decontaminating solution.

2. Stainless steel dissection tools and glassware can be made RNase-free by heating at >200 °C for 1 hr or more. Note: Steam sterilization does not destroy RNase. Alternatively, wipe with a RNase decontaminating solution, then with RNase-free 70% ethanol and air dry.
3. Wipe bench tops, other lab surfaces and micropipettors with RNase decontaminating solution, then with RNase-free 70% ethanol. Designate a specific lab bench or work area for RNA recovery and analysis. Note: Remove tip ejectors before wiping the shafts of the pipettors and leave them off, if possible.
4. Use tubes, microcentrifuge tubes, pipettes, and micropipette tips that are certified by their manufacturer to be RNase-free. Use of very-low binding tips and microtubes is recommended. If possible, use freshly opened boxes or bags of these components and keep them separate from the general lab supply.
5. Obtain RNase-free solutions [Dulbecco's calcium and magnesium-free PBS (PBS), ethanol, RNase-free 70% ethanol] from commercial sources. Use newly opened bottles for each experiment.

2. The source of the Azure B dye is critical to successful recovery of intact RNA. Test each batch of dye before committing precious samples to staining and collection. Collect a few thousand neurons from a nonexperimental animal, prepare RNA, and determine the RNA integrity as described below to find one that consistently produces RIN numbers above 8.5.
    1. Dissolve Azure B dye (1%, wt/vol) in RNase-free 70% ethanol, typically 0.3 g in 30 ml ethanol in a 50 ml centrifuge tube. Mix well on a rocker at RT overnight or until all the particles have dissolved. One tube of this solution can be used to stain multiple slides without affecting the staining intensity.
    2. As a precaution, use a different tube of stain for each biological replicate.

3. Use the cryomolds and O.C.T. for embedding cord sections without further treatment.
4. Maintain the cryostat at -20 to -24 °C. After sectioning, place the slides in a -80 °C freezer until a sufficient number has been prepared. Start RNA preparation as soon as possible after the slides have been made. For long-term storage, keep unsectioned or partially sectioned OCT blocks, rather than slides, at 80 °C.
5. Make the guanidine thiocyanate buffer for RNA preparation from the dissected motor neurons using the solutions provided by the RNA preparation kit manufacturer according to its directions.

2. Preparation of Spinal Cord Sections from Mice (Figure 1A)

1. Clean all the dissection tools, glass slides, razor blades, and dissection platform by baking or wiping with RNase decontaminating solution, followed by RNase-free 70% ethanol. Let dry for 2 min. Keep everything dust-free by covering with lab wipes.
2. Animal handling procedures, including anesthesia, cardiac perfusion, and euthanasia, should be carried out according to the requirements of the local institutional animal care and use committee (IACUC), with the goal of rapidly bringing the animal to the point of spinal cord dissection.
    1. Anesthetize the mouse by intraperitoneal (ip) injection of a lethal dose of Ketamine (450 mg/kg).
    2. Once a deep level of anesthesia is achieved and confirmed by lack of a toe-pinch response, position the mouse ventral side up on a dissection platform (a Styrofoam board, for example), open the chest cavity to expose the heart, and insert the 27 G butterfly needle of an infusion set into the left ventricle (which is on the investigator's right). Nick the right atrium with a sharp forceps, and perfuse with PBS at a rate of 5-7 ml/min for about 2 min using a peristaltic pump. The liquid flowing from the atrium should be largely free of blood at this point.
    3. Remove the perfusion needle, decapitate the mouse, and turn it over on the dissection board. Open the skin to expose the spinal column, use small scissors and forceps to remove the vertebrae, and lift out the spinal cord while severing the nerve roots with a sharp forceps. Speed and practice are essential in moving from the start of the perfusion to removal of the cord; this should take no more than 7 min.
    4. Rinse the cord for 10 sec in RNase-free water to wash off any residual blood. Lay the spinal cord on an RNase-free glass slide with a curved forceps very gently but quickly. Divide the spinal cord transversely using a clean razor blade into 9-10 pieces.
    5. Place the pieces in a cryomold filled with O.C.T. using the same forceps, and align them vertically using a clean needle. Place the mold in a shallow tray containing 2-methylbutane precooled with liquid nitrogen, and then put the tray into a liquid nitrogen bath to fast-freeze the spinal cord pieces to avoid RNA degradation.
    6. Store the OCT-embedded block at -80 °C for up to 6 months before sectioning. The process from retrieval of the cord through freezing should be done within 5 min to maintain RNA integrity.

3. Cryosection the O.C.T.-embedded block at -20 °C with a cryostat to produce 20 µm slices, each containing 9-10 spinal cord cross sections, on RNase-free PEN-membrane 2 µm slides kept initially at room temperature to ensure that the sections adhere to the slide. Immediately refreeze the section on a -20 °C surface inside the cryostat. Keep the slides at -80 °C until used.

3. Staining of Spinal Cord Sections (Figure 1A)

1. On a clean rack, arrange six 50 ml conical tubes. Fill all of them with 25-30 ml of RNase-free 70% ethanol, so that the depth is sufficient to cover all the sections on a slide when the slide is immersed. Make 30-35 ml of 1% Azure B solution as described earlier and keep it on the same rack to minimize time between changing solutions during washing or staining. Keep three or four lab wipes in front of the rack to drain extra solution quickly.
2. Take the slides out of the -80 °C freezer onto dry ice. Thaw one slide by placing it on a gloved palm. Remove most of the moisture and condensation on the slide by wiping it off with a lab wipe, being careful not to touch the sections. Put the slide on fresh silica gel desiccant in a Petri dish for 30-40 sec to dry completely. If lab humidity is high, brief treatment in a vacuum dessicator can be used to dry the slide more quickly.
3. Washing and staining.
    1. Dip the dried slide into the first tube of RNase-free 70% ethanol. Soak the slide for 30 sec, then wash the slide to remove the OCT by dipping it up and down for 45-60 sec . Take the slide out of the solution and drain the excess liquid on lab wipes. Repeat the same

process by dipping the slide in the second tube of 70% ethanol. If the OCT around the spinal cord sections is not completely gone, repeat the washing in the third tube.
2. After draining the excess liquid, submerge the slide in 1% Azure B solution in 70% RNase-free ethanol for 30-45 sec. Drain the excess Azure B on a lab wipe; at this point, the whole slide will be blue.
3. Submerge the slide in the next tube of 70% ethanol, and dip up and down quickly 3-4x to remove excess dye and to make the specific motor neuron staining visible. If the sections look very darkly stained, repeat the destaining step in a fresh tube of 70% ethanol. If the staining seems too light, return the slide to the Azure B solution for another 30 sec and repeat the destaining. Wipe off the excess ethanol and air dry the slide for ~40 sec until all the liquid is gone. The gray matter will be light blue, whereas the motor neurons will be stained deep blue, which is easily visible. Start the dissection immediately.

4. Laser Capture Microdissection (LMD) (Figure 1B)

1. Turn on the microscope and unload the tube holder to attach the cap of a 0.6 ml microfuge tube for sample collection. Put 30 µl guanidine thiocyanate lysis buffer into the cap of a 0.6 ml microfuge tube. Cover the surface of the cap with liquid by spreading the solution using a pipette tip. In this way, all the collected neurons will be dissolved in solubilizing solution as they are dissected. Align the cap with the hole and the objective with the microscope software. Keep the cap in the covered position until starting laser capture.
2. Place the air dried slide on the stage of the LMD microscope upside down, so that the membrane of the PEN slide faces down. The membrane with sections should face the hole, underneath of which is the collection tube.
3. Turn on the laser 10 min before starting slide preparation. Focus on a spinal cord section with the 5X objective. Move to the 20X objective for dissection.
    1. Mark a “dummy” region with the light pen and allow the laser to cut it out without collecting it. This relaxes the PEN membrane and makes it possible to mark and cut multiple regions successively.
    2. Refocus and identify motor neurons in the ventral horn region. These neurons are recognizable by their location, their pyramidal morphology, their large size, and their dark blue staining with Azure B (**Figure 2**).
    3. Using the light pen, select the drawing tool and mark along the edge of the motor neurons, making a complete outline. Try to make the outline as close as possible to the motor neuron to avoid contamination from cells other than the motor neuron. (Test some sections beforehand to see how wide the laser cut-line is and adjust the clearance as necessary.) Mark multiple cells before cutting; the software will remember the positions.
    4. Bring the cap underneath the cutting position by clicking on the cap position. Click the start button to initiate motor neuron dissection with the laser. Collect all the motor neurons from all of the sections on one slide into the cap of one microfuge tube.
    5. After collection is complete, take the microfuge tube out of the holder by unloading the tray and gently dislodging the tube. Completely dissolve the motor neurons in the buffer by pipetting the solution up and down. Move the solution into the body of the tube by centrifuging at 14,000 x g for 2 min at 4 °C. Freeze the sample immediately in dry ice and store it at -80 °C. This process, from slide staining through motor neuron collection, should be done within 30 min for one slide to minimize RNA degradation.

5. RNA Preparation from Motor Neurons

Thaw all of the collected neurons from one animal and pool them together to extract RNA. Samples may look pale blue depending on the number of motor neurons (stained with Azure B) in them. Extract RNA using a suitable kit according to the protocol provided for LMD tissue, eluting in the minimum volume possible. Take 1 µl to check the quantity and integrity of the sample. Fast-freeze the remaining RNA on dry ice and store at -80 °C.

6. Determination of RNA Quality and Quantity

Determine the RNA quality with the 1 µl sample on a capillary electrophoresis microfluidics chip according to the manufacturer’s protocol. Total RNA preparations with an RNA Integrity Number (RIN) above 8.5 can be used for RNA-seq and qRT-PCR. The data output also typically includes the approximate concentration of the sample.

Representative Results

The protocol outlined above and in **Figure 1** should produce 50 ng or more of total RNA with a RIN of >8.5 from 3,000-4,000 spinal cord motor neuron cell bodies dissected from the 9-10 20 µm slices on about 20 PEN slides. Because this number of slides represents less than 10% of an O.C.T.-embedded block of a mouse spinal cord, it is possible to return to the block, which has been stored at -80 °C, and cut more slices to go through another round of RNA preparation. If larger amounts of RNA are needed for an analysis, it is better to make only the number of slides (*e.g.* 20) at one time that can be processed through dissection and RNA preparation in a few days, then make more slides for a second round and combine the products. Be sure to check the RIN of each preparation first to avoid combining a good one with a bad. RNA-seq methods are becoming more sensitive and new PCR technologies are promising higher sensitivity than current ones. Thus, less RNA from fewer neuron cell bodies will be required, allowing greater sequencing depth and more comprehensive validation of interesting hits. On the other hand, full adherence to the MIQE recommendations for qRT-PCR analysis and validation[4] may require larger amounts to allow for the necessary control reactions for low-abundance RNAs.

Figure 2 shows a typical series of images of a portion of a spinal cord ventral horn section after Azure B staining and dissection. In panel A, the large, darkly stained cell bodies are motor neurons, confirmed by anti-Chat antibody staining[2], and are easily differentiated from smaller neighboring cells. Panel B shows the same region with individual cell bodies outlined with the light pen and numbered by the microscope software. The outlines closely follow the margins of the cell bodies, with a small allowance for the cutting width of the laser. This spacing has to be determined for each laser power setting to minimize the inclusion of extraneous material while avoiding loss of motor neuron cytosol. Panels

C and D show the section after the laser has cut and the individual cell bodies have dropped into the collection cap. Notice that the cutting margin does not exactly follow the light pen outline in panel C, but does largely stay outside of it. This irregularity is apparent in panel D, as is the darkened area around each cut, which reflects the heat damage to the tissue caused by the laser. Because of this, if two cell bodies are very close to each other, it is better to outline them for a single cut, rather than trying to cut them separately and losing some RNA to heat damage at their region of near-contact.

Figure 3 shows typical results of an electrophoretic analysis of the RNA integrity of a sample of RNA prepared from ~4,000 mouse motor neuron cell bodies collected by LMD. The upper panel is the electropherogram produced by 1 µl of the total RNA; the inset at the right is an image of the gel. In both, note the prominent ribosomal RNA peaks. The lower panel is the electropherogram of the lane containing RNA size standards. The analysis software calculated an RIN of 9.8 for this sample, with a concentration of 4.9 ng/µl. A total of 13 µl of this solution was available after the analysis, providing 64 ng of RNA for downstream qRT-PCR validation of transcript amounts. For a typical qRT-PCR analysis of moderately abundant transcripts, 0.15-0.20 ng of total RNA is sufficient to produce accurate quantitation[2], so the amount of RNA available from this preparation is sufficient to validate a number of transcripts with multiple primer sets, as recommended[4]. Of course, larger amounts of input RNA can be used to permit validation of rare transcripts. This amount is also more than sufficient to permit library preparation and next-generation RNA-seq.

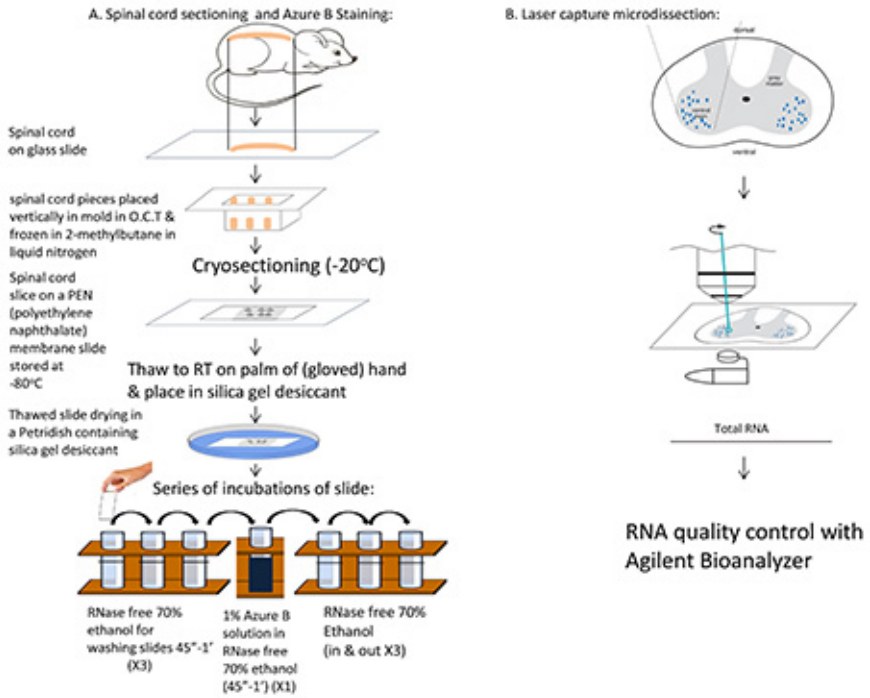

Figure 1. Overview of spinal cord slice production and laser capture microdissection of spinal cord motor neuron cell bodies. A) Major steps in slice production and staining. **B**) Cartoon view of spinal cord section and laser dissection. Click here to view larger image.

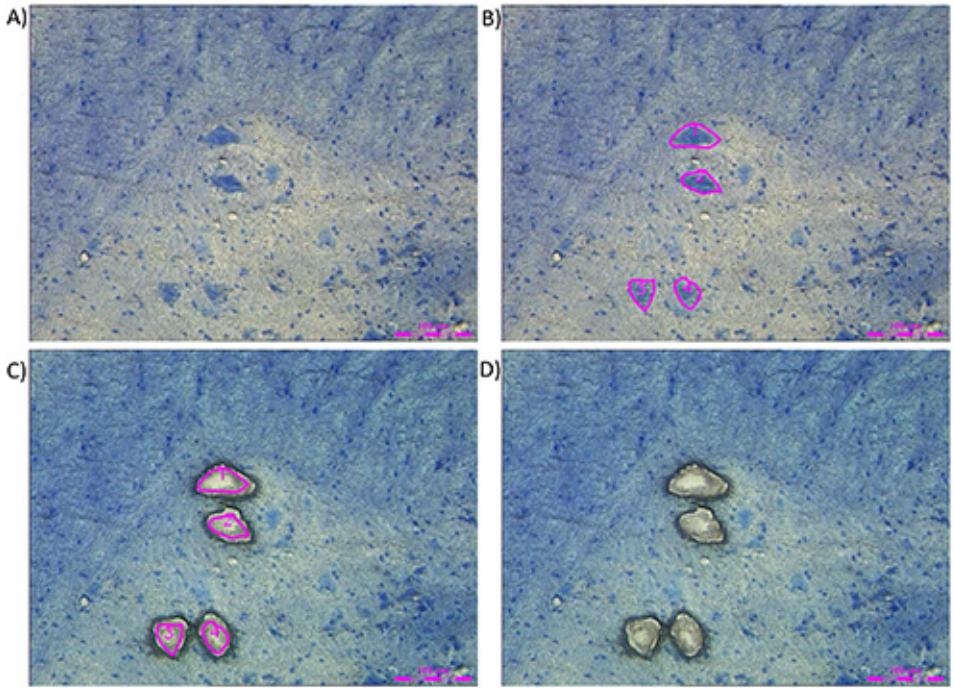

Figure 2. Before-and-after images of a laser dissection of Azure B stained motor neuron cell bodies. A) A portion of the ventral horn of a stained section. Note the four large, darkly stained cells. There are also a few lightly stained and somewhat smaller cells, which are not motor neurons (confirmed by anti-Chat antibody staining; not shown, but see Bandyopadhyay[2]) and which will not be selected for dissection. The many small, dark spots are probably neuronal processes (dendrites and axons), but this has not been confirmed. **B**) The four cell bodies outlined with the light pen. Note that the outlines closely follow the margins of the cells, with a minimum distance between them. This spacing should be established empirically for each microscope and laser. The software numbers the individual neurons, which simplifies keeping track of how many have been collected. **C** and **D**) After the laser has cut and the pieces containing the cell bodies have dropped into the collection cap. Note that the hole left behind is somewhat larger than the outline and is irregular. This reflects the width of the laser beam and its slightly variable cutting efficiency depending on the local composition of the tissue. Also apparent is the rim of darkened tissue surrounding each hole due to local heating damage caused by the laser. Click here to view larger image.

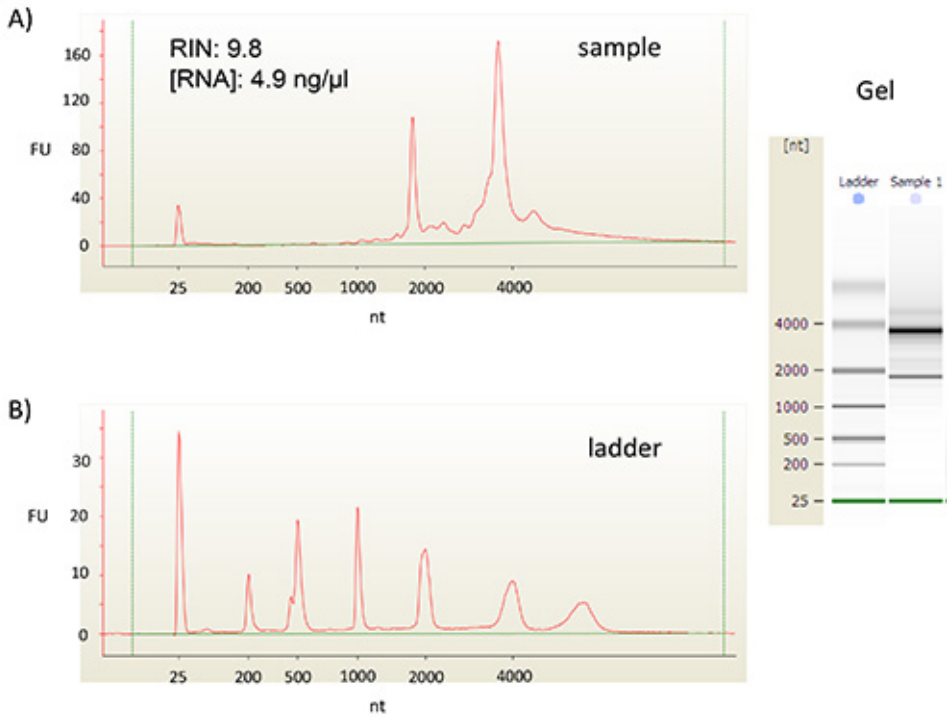

Figure 3. Electrophoretic analysis of the integrity of RNA prepared from LMD samples. A) A 1 µl aliquot of the RNA prepared from ~4,000 motor neuron cell bodies by the above protocol was analyzed by microcapillary electrophoresis on a microfluidics chip. The electropherogram is shown, with prominent ribosomal RNA peaks. On the right is an image of the gel itself. **B**) The electropherogram of the size standards is shown, with the corresponding gel lane to the right. The instrument software calculated a RIN of 9.8 for this sample and a concentration of 4.9 ng/µl. Click here to view larger image.

Discussion

The most significant measure of success of this protocol for producing total RNA by laser microdissection of spinal cord slices is the RIN value[5]. For RNA samples from higher eukaryotes, values above 8.5 regularly yield high quality sequencing and qRT-PCR data. If yield is low but quality is high, repeat the preparation. If the integrity is lower (even 7.5-8), however, it is better to find the source of the problem and try again. There are many steps where something can go wrong, but really only two sources of the problem - endogenous RNase contamination or exogenous RNase contamination. The first can be addressed by practice, so that the time from sacrifice of the mouse to freezing its O.C.T-embedded cord is minimized. The other point in the protocol where endogenous RNase can contribute to a poor result is during the preparation of the slices. Each slice should adhere to a room temperature PEN slide quickly, but it will melt (the O.C.T becomes clear). The faster the slice can be refrozen the better. The cryostat we use has flat surfaces inside the chamber that are at -20 °C, which we use to quickly refreeze the slice. Find a similar spot in the cryostat used and make sure the slice becomes opaque again rapidly.

Tracking down sources of exogenous RNase can be difficult, because there are so many possibilities. The only reagents used which are not expressly RNase-free are O.C.T. and the Azure B. If the Azure B has performed satisfactorily before, try a fresh bottle of O.C.T., although this reagent has never been a problem. RNase-free reagents can also be replaced, even from a different supplier. A much more likely source is the investigator. In addition to a thorough cleanup of the working area, it is often useful to have another lab member follow along and observe all the steps in the procedure. Even if this is someone who does not routinely perform this procedure, a fresh set of eyes may pick up an otherwise unnoticed source of contamination.

Extending this protocol to recovering RNA from other spinal cord cells, from spinal cord cells from other species, or from specific cell types in other organs will require modifications in several areas directed at achieving clear identification of the cells of interest while obviating, or at least minimizing, the impact of endogenous RNase. In the protocol here, rapid removal and freezing of the cord, coupled with Azure B staining in 70% ethanol, allowed both minimization of RNA degradation and easy identification of motor neuron cell bodies. Azure B is a well-established histochemical stain for neurons that appears to bind to RNA[3] and has been used to evaluate RNA content of neurons in autopsy samples of brain tissue from patients with neurodegenerative disease[6]. Notably, Azure B staining did not affect either the integrity of the purified RNA or its ability to be reverse-transcribed and analyzed. Other dyes, such as cresyl violet[7] and toluidine blue[8] have been used in similar LMD protocols. Collecting the cell bodies directly into guanidine thiocyanate solution as they were dissected provided the final step that resulted in the routine recovery of intact RNA.

Similar approaches can be envisioned for other cells and organs, recognizing that identifying the cells of interest while minimizing or, preferably, preventing RNA degradation by endogenous RNases is the critical requirement for successful analysis. In tissues that have high levels of RNase, such as pancreas, rapid handling and low temperature have been combined to permit recovery of RNA from β-cells, taking advantage of their natural autofluorescence for visualization[9]. Procedures using antibody staining for identification have also been reported[10], but have not been fully successful in our hands. Finally, many mouse models are available that express fluorescent fusion proteins (*i.e.* GFP, YFP, RFP) in cells of interest, which could be used to identify them in tissue sections on the LMD microscope. In fact, the mouse strains we have analyzed

with this protocol express a fusion protein between SOD1 and YFP[11], and their spinal cord motor neurons are highly fluorescent. We were not able, however, to find a set of ethanol washes and/or dehydration conditions that would simultaneously remove the O.C.T., inhibit RNase activity, and consistently maintain sufficient YFP fluorescence to allow visualization of the motor neurons and their recovery by LMD. Perhaps a new fluorescent protein or a variant of the available ones that maintains fluorescence in organic solvents can be found to overcome this limitation.

Disclosures

The authors declare they have no competing financial interests.

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