Video Article

Optimization of Laser-Capture Microdissection for the Isolation of Enteric Ganglia from Fresh-Frozen Human Tissue

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Abstract

The purpose of this method is to obtain high-integrity RNA samples from enteric ganglia collected from unfixed, freshly-resected human intestinal tissue using laser capture microdissection (LCM). We have identified five steps in the workflow that are crucial for obtaining RNA isolates from enteric ganglia with sufficiently high quality and quantity for RNA-seq. First, when preparing intestinal tissue, each sample must have all excess liquid removed by blotting prior to flattening the serosa as much as possible across the bottom of large base molds. Samples are then quickly frozen atop a slurry of dry ice and 2-methylbutane. Second, when sectioning the tissue, it is important to position cryomolds so that intestinal sections parallel the full plane of the myenteric plexus, thereby yielding the greatest surface area of enteric ganglia per slide. Third, during LCM, polyethylene napthalate (PEN)-membrane slides offer the greatest speed and flexibility in outlining the non-uniform shapes of enteric ganglia when collecting enteric ganglia. Fourth, for distinct visualization of enteric ganglia within sections, ethanol-compatible dyes, like Cresyl Violet, offer excellent preservation of RNA integrity relative to aqueous dyes. Finally, for the extraction of RNA from captured ganglia, we observed differences between commercial RNA extraction kits that yielded superior RNA quantity and quality, while eliminating DNA contamination. Optimization of these factors in the current protocol greatly accelerates the workflow and yields enteric ganglia samples with exceptional RNA quality and quantity.

Video Link

The video component of this article can be found at https://www.jove.com/video/57762/

Introduction

This method is designed to obtain high-quality RNA samples of enteric ganglia from human intestinal tissue using laser capture microdissection (LCM). The protocol described here has been optimized to provide sufficient RNA quality and yields for RNA sequencing (RNA-seq) and is intended to be used with freshly-resected, unfixed, flash-frozen human intestinal tissue.

Functional gastrointestinal and gut motility disorders affect one of every four people in the United States. The enteric nervous system (ENS), also referred to as the second brain[1], is often at the center of these disorders, as it plays a crucial role in gut homeostasis and motility. Manipulation of gut motility has generally been restricted to surgical resection of the aganglionic/noncontractile tissue, chronic dietary modification and/or medications. Surprisingly, the full transcriptome of the adult ENS remains to be sequenced, greatly limiting our ability to identify molecules within the ENS that can be targeted pharmaceutically or utilized in stem cell therapies.

There are relatively few methods for isolating RNA from human enteric ganglia. The first approach, cell dissociation[2], requires high incubation temperatures and long incubation times; both of which are known to promote RNA degradation and alter the transcriptome[2,3]. An alternative approach, LCM, more reliably preserves the transcriptome and protects RNA integrity. Although several studies have used LCM to collect ganglia from fresh-frozen human intestinal tissue[4,5,6], these approaches were either hampered by poor RNA quality and quantity, were quite labor-intensive, or needed modification of staining or RNA extraction techniques to work in our hands. Other LCM protocols designed for preserving RNA that were found in LCM product manuals provided additional improvements[7,8], but adaptation was needed when applied to the isolation of enteric ganglia[8,9]. For these reasons, we developed an optimized protocol based on these resources that yields substantial quantities of high-integrity RNA from human enteric ganglia, has a relatively fast workflow, and produces consistent results across a large number of samples.

In this study, we present a synopsis of optimized procedures that facilitate the isolation of high-integrity RNA from enteric ganglia sourced from resected human intestinal tissue. Our method incorporates five important aspects. First, freshly-resected, unfixed human intestinal samples should be trimmed to size, have all excess moisture removed with a laboratory tissue and flattened in a large base mold before flash-freezing atop a slurry of dry ice and 2-methylbutane (2-MB). Second, histologic sections of intestine should be prepared to obtain the full plane of the myenteric plexus on a slide, which offers a large payload of enteric ganglia. Success with this step is largely dependent on the tissue preparation process. Third, the nonuniform structure of ganglia in the ENS requires the use of polyethylene napthalate (PEN) membrane slides[6], which offer the greatest speed and precision during the LCM process. Fourth, ethanol-compatible dyes, such as Cresyl Violet, should be used to preserve

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RNA integrity while staining enteric ganglia. Last, the RNA extraction process is critical for a successful outcome with RNA-Seq. We sought an RNA extraction approach that produces high RNA integrity, maximizes RNA yields when starting with small collections of enteric ganglia, eliminates DNA contamination, and retains as many RNA species as possible.

Taken together, optimization of these factors in the present study greatly accelerates the workflow and yields samples of enteric ganglia with exceptional RNA quantity and quality. Results have been largely consistent among a sizable group of samples, indicating the consistency of this approach. Further, we have used these approaches to successfully sequence dozens of RNA samples from enteric ganglia. The strategies highlighted here can also be broadly adapted for performing LCM of desired ganglia or nuclei of the peripheral and central nervous system and other cases requiring the isolation of high-quality RNA.

Protocol

All protocols described here have been approved by the Vanderbilt University Institutional Review Board (IRB).

1. Preparation Prior to Tissue Arrival

1. Obtain proper IRB approval and coordinate with human organ donation agencies to obtain freshly-resected, unfixed intestinal tissue from donors that meet all research criteria required for the study.
   Note: This protocol can be adapted for use with mice and other rodent models.
   1. Immediately upon resection of each intestinal segment, thoroughly flush the lumen with chilled PBS or tissue-storage medium to remove residual luminal material or feces.
   2. After flushing and discarding waste in an appropriate biohazard receptacle, submerge the tissue in a sufficient volume of tissue-storage medium (such as 3-5 times the volume of intestinal tissue) and store in individually-labeled, water-tight plastic containers, submerged in ice or refrigerated until use).
   3. Process the tissue within 18-26 hours from the time of collection to prevent substantial RNA degradation.
      Note: Depending on the cleanliness of the intestinal segment and storage, it may be possible to extend our suggested time limit.
2. Prepare all materials needed for human tissue collection in advance, as indicated in this protocol (refer to the **Table of Materials** for more-detailed product information). Ensure that all required personal protective equipment is worn at all times.
3. Prepare dry ice buckets along with a dry ice slurry as shown in **Figure 1**.
   1. Crush enough dry ice to fill 4 standard circular ice buckets and one rectangular ice bucket. One of the standard buckets should have small (11 x 21 cm) laboratory tissues placed at the surface of the dry ice. If possible, use new, unopened boxes of laboratory tissues.
   2. Make a dry ice slurry by powderizing 2 L of dry ice in a clean rectangular ice bucket.
   3. Add pre-chilled 2-MB up to the surface of the dry ice. Slightly mix and flatten the slurry using a pipette tip box cover to shape surface of the slurry into a channel surrounded by larger pieces of dry ice along the sides of the rectangular bucket.
   4. Place several thin blocks of dry ice along the edges of the ice bucket to encourage more rapid freezing. There should be room for ≥4 base molds to fit loosely in the dry ice slurry bucket at a given time.
      1. Optionally, stack the ice bucket within another rectangular ice bucket filled ¼ with dry ice. This positioning helps better insulate the dry ice slurry and prevents the need for frequent adjustments of dry ice and 2-MB during the procedure.
   5. Position the dry-ice buckets in an assembly line in the following order: 1) dry ice slurry bucket, 2) laboratory tissue-dry ice bucket, 3 & 4) normal dry ice buckets, 5) rectangular dry ice bucket (**Figure 1A**).

2. Preparation of Flash-Frozen Human Intestinal Tissue

Note: This entire process can take between 45-80 min per intestinal segment, depending on the intestinal tissue quality. We also recommend collecting quality control samples to assess RNA quality and histology prior to proceeding with LCM.

1. Fill a large tray with wet ice and place surgical cutting boards atop the dry ice.
2. In this setup, chill a 500-mL and 100-mL beakers for storing the tissue and trimmed segments in cold storage solution. Pre-chill base molds on the cutting boards so that they are ready to receive tissue.
3. Upon receiving fresh intestinal tissue, pour off the media in which the tissue is transported and retain it in the pre-chilled beakers. Leave some room in these beakers for the temporary storage of the intestinal tissue and trimmed segments, which will be prepared in subsequent steps.
4. Cut the intestinal segment to a length of approximately 20 cm, which provides ample tissue (40-60 cm^2) to generate RNA for downstream applications and quality control samples. Depending on the tissue integrity, it may be feasible to accomplish RNA isolation for downstream processing with a much smaller length (*i.e.,* 5 cm of intestine).
5. Trim away adipose and connective tissue from the intestine using surgical scissors. Residual adipose along the intestinal serosa compromises the ability to fully flatten tissue in subsequent steps. Carefully trim the tissue, so as to avoid nicking the serosa during removal of fat and connective tissue, which can structurally damage the myenteric plexus or make the exterior of the tissue flatten unevenly for sectioning.
6. Make a longitudinal incision along the entire length of the intestinal sample, preferably at the site of mesenteric attachment.
7. Dissect away and discard the tenia coli from the colon, so that it flattens more readily, upon being released from tension.
8. Splay the intestine mucosa-side down onto a chilled cutting board and cut 1.25-cm-wide strips from the full length of the tissue.
   Note: Intestinal tissue often expands after trimming, so this size should be adjusted accordingly.
9. Temporarily store the strips in chilled tissue-storage solution.

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NOTE: We use Belzer UW cold storage solution because it has a long shelf life, is relatively cost-effective and can be stored at room temperature until needed.

10. Remove a tissue strip from the cold storage solution and cut it into segments ~1.5-2 cm long.
11. Quickly blot the intestinal segments on a stack of laboratory wipes, to remove excess liquid and prevent the formation of ice crystals along the intestinal serosa during flash-freezing. If tissue is not sufficiently dried, it will be difficult to obtain high-quality sections from the myenteric plexus.
12. Lay the blotted intestinal pieces mucosa-side up onto a pre-chilled, large, disposable plastic base mold (37 mm x 24 mm x 5 mm).
13. Carefully flatten each segment by lightly stretching the tissue over the surface of the base mold and using curved forceps to gently press down and expel any air bubbles that may be trapped beneath the serosa. Note that the tissue expands while flattening. If needed, trim the segments and cut the remaining samples at a smaller size.
    Note: If the tissue is over-stretched, this will distort the myenteric plexus. After all air bubbles are removed, assess the thickness of the sample prior to freezing. If needed, push the edges of the specimen inward until the intestinal sample approaches its original thickness.
14. Place the loaded base mold onto the surface of the dry ice, 2-MB slurry. The tissue should completely freeze within 30-60 s, depending on the size and thickness of the intestinal segment.
15. Dry the bottom of the base mold to remove residual 2-MB by quickly rubbing the base mold on the laboratory tissues pre-chilled in the second bucket of dry ice.
16. Transfer the dried sample to the third tub of dry ice and bury it under the surface of the dry ice until it is ready to be wrapped.
17. Quickly observe the bottom of the base mold prior to wrapping, to examine the quality of sample preparation. Make note of any samples that do not appear flat or have large air bubbles, as these should be avoided for cryosectioning and LCM.
18. Wrap the sample in pre-labeled aluminum foil that is laid atop dry ice in a bucket so that wrapping of tissue occurs while being kept completely frozen.
19. Wrap the sample with a layer of plastic wrap, to minimize tissue dehydration during storage at -80 °C.
20. Store samples in the rectangular bucket until they are ready to be moved to the freezer.
21. Pre-label and pre-chill freezer boxes at -80 °C for immediate storage of samples after collection.
22. Take a small portion of tissue from each intestinal segment for assessment of RNA integrity prior to conducting LCM.
    1. Pre-label a 2-mL RNase-free tube for each segment, filled with ≥500 µL of lysis buffer. We recommend using RNA Extraction Kit "D", listed in the **Table of Materials**.
    2. Homogenize a small (2 mm x 2 mm) piece of the intestine in a standard tissue micro-homogenizer (20-40 s, until no tissue chunks remain). Then, immediately freeze the RNA lysate on dry ice and store at -80 °C until proceeding with RNA extraction.
    3. Optionally, embed some of the sections in tissue freezing medium (TFM) as a back-up. Prepare tissue sections in the exact same way described in this section, but submerge samples in TFM within the base mold prior to flash-freezing.

3. Cryosectioning

1. Before cryosectioning, prepare all required materials for staining and dehydration and transfer the desired tissue samples from the -80 °C freezer into a bucket of dry ice.
2. Prepare the cryostat for use by setting to the optimal cutting temperature (-18 to -22 °C) and wiping down surfaces with 100% ethanol and treating the blade and brush tray with RNase decontamination solution.
3. To best adhere the sample to the chuck, use the following approach.
    1. Place forceps in dry ice for at least 30 s before unwrapping the intestinal sample and placing it at the surface of the dry ice serosa-side-up.
    2. Pour a 3-5 mm mound of tissue freezing medium (TFM) onto a large cryostat specimen holder and immediately transfer the intestinal sample serosa-side-up onto the TFM.
    3. Quickly transfer the specimen holder to dry ice and cover with powderized dry ice after allowing the TFM to adhere to the sample for 2-5 s at room temperature. Ensure that the TFM remains below the plane of the myenteric plexus.
       NOTE: Ideally, the outer surface of intestinal mucosa will fully adhere to the TFM before the TFM begins to freeze without thawing of the sample.
4. Mount the specimen holder into the cryostat's specimen head and align the specimen with the cryostat blade, such that the plane of the myenteric plexus will be parallel to the cutting blade.
5. Set the sectioning thickness on the cryostat to 8 µm. The purpose of perfectly aligning the specimen is to obtain the greatest possible surface area of myenteric plexus on each slide. This thickness is generally recommended for both human and rodent tissue, but can be adjusted, as needed.
6. Section through the serosa and longitudinal muscle until reaching the myenteric plexus.
    1. To locate the myenteric plexus, mount a sample section onto a slide and stain the section with an aqueous dye, such as 1% Cresyl violet or toluidine blue, *etc.*
    2. View the section under a light microscope at 40-2000X magnification to identify the junction between the longitudinal and circular muscle layers. Microscope parameters are provided in the **Table of Materials**. At the start of sectioning, the serosa will be marked by the presence of connective tissue. Some remaining mesenteric fat may also be present along the serosa. A sheet of the outer longitudinal muscle layer then begins. Once the border between the longitudinal and circular muscle layers is reached, a portion of the enteric ganglia will be observed.
       NOTE: In the next several sections, the myenteric plexus will become very evident, with large swaths of ganglia being present within a single section (**Figure 2C**). If the tissue is not completely flat at the start of sectioning, then only a portion of the ganglia will be present in each section at a given time. Sometimes, the intestinal sample may have an inherently uneven myenteric plexus, despite the tissue appearing perfectly flat. This is especially true for duodenum samples. In our experience, these samples can take much longer to process with LCM, but sufficient RNA can be collected within a single day's session. The exact location of the myenteric plexus can vary considerably between samples, based on the tissue and its preparation prior to freezing.

7. Begin collecting serial sections for LCM, with optimal collections providing the greatest number of neurons and glia per slide. Depending on the surface area of the sample, multiple sections can be combined onto a single PEN-membrane slide.
8. Mount the sections onto PEN membrane slides, chilled briefly in the cryostat for 5-10 s. When mounting, the tissue should melt only slightly, maximally preserving RNA integrity.

4. Staining

Note: For an overview of the staining process, refer to **Table 1**. All solutions used are prepared in standard 50-mL conical vials. Treating the outside of the tubes with RNase decontamination solution does not appear to improve results (data not shown).

1. Prepare a saturated solution of 4% Cresyl violet in 95% ethanol at least one day in advance and fully re-suspend the Cresyl violet prior to loading the syringe.
   Note: The concentration of ethanol may need to be lowered for effective staining, as described in the discussion section. We have not tested whether using 50% or 75% ethanol during staining significantly reduces RNA integrity. Cresyl violet is light-sensitive and thus should be protected from light.
2. After mounting sections of myenteric plexus onto the slide, immediately submerge the slide in a conical vial of 95% ethanol (pre-chilled at -20 °C) for 30 s.
   1. If the sections did not fully adhere to the slide in the previous step, slightly warm the bottom of the slide with a gloved hand (a laboratory wipe should be placed in-between the slide and glove), for full adherence before submerging in 95% ethanol. This solution can be re-used for at least 3-6 slides without reducing RNA integrity.
3. Lay the slide face-up on a lab wipe placed atop a pre-treated, RNase-free container[8].
4. Pre-fill a 3-mL syringe with 4% Cresyl violet and attach a 0.2-µm syringe filter.
   Note: We recommend cellulose acetate filters.
5. Apply 6-10 drops of stain directly onto the tissue sections[8] and incubate for 10-30 s, or until sufficient dye is retained when de-stained. While staining, gently rotate the slide container by hand to ensure that the tissue sections are evenly coated with stain.
6. Pour off the stain and immediately de-stain the slide in a vial of 75% ethanol. Repeatedly submerge the slide until the excess dye has mostly seeped off of the sample. This may take between 10-20 s.
7. Finalize de-staining by repeatedly dipping the sample in a vial of 95% ethanol for 10 s. Submerge the sample repeatedly until all excess stain has been removed.
   1. Modify the destaining duration, as needed, to optimize the staining of ganglia. If too much stain is removed, the sample becomes too transparent and it may be difficult to visualize
      NOTE: This staining protocol has been optimized based on several publications[5,8,10,11] which required modification before satisfactory results were obtained. Therefore, the concentration of ethanol used in the dye and during the destaining process should be modified, when necessary, to obtain an optimal result.

5. Dehydration

1. Immediately dip the slide in 100% ethanol 2-3 times, for a total of 10-20 s.
2. Submerge the slide in anhydrous 100% ethanol for at least 30 s. As anhydrous ethanol is very hygroscopic, add molecular sieves (8-12 mesh) to the vial of 100% ethanol prior to the start of the procedure to remove any absorbed water and thus more completely dehydrate the sample[5].
3. Repeatedly dip the slide in xylene for 15-20 s. This step fully removes the layer of ethanol on the slide. Add molecular sieves ahead of time to the tubes of xylene, to fully adsorb excess ethanol and water molecules[5].
   CAUTION: Xylenes are classified as an irritant to the eyes and upper respiratory tract and should be used in a chemical fume hood.
4. Submerge the slide in xylene (with molecular sieves, 8-12 mesh) for at least 10 min.
   Note: A brief break can be taken after this step, if needed. Samples can be left in xylene for 4-5 hours without detrimental effects to staining, LCM or RNA yield/integrity.
5. Optionally, individually prepare several additional slides at this point. If preparing a second round of slides after completing LCM on all prepared slides, the tissue in the cryostat may need to be refaced, due to dehydration of the tissue surface. One to four sections may need to be discarded before usable sections can be collected.

6. Laser Capture Microdissection (LCM)

1. Remove the slide from xylene and air-dry it in a chemical fume hood for at least 1 min. Insert a cartridge of LCM caps into the LCM microscope stage. Load the slide onto the stage and acquire an overview image of the slide.
2. Identify the desired location for LCM and load a cap onto the slide.
3. Align the infrared (IR) laser and adjust its power and duration to make a 20-30 µm diameter capture spot.
4. Locate the ultraviolet (UV) laser and set an appropriate cutting speed and intensity.
5. Outline the desired ganglia to be collected using the LCM software, making sure to stay within the boundary of the collectable area on the cap (depicted in the slide overview of the software program as a green circle).
6. In each of the traces, adjust the IR spots such that there is at least one IR spot every 100-500 µm.
7. Press the IR/UV cut button to proceed with the collection of all marked ganglia.
8. Once collections are completed, either move the cap to a new location and repeat the collection process or examine the LCM cap at the QC station once the cap is sufficiently filled with ganglia (or until the recommended time limit of 60-80 minutes is reached).
9. If debris are present on the cap, wipe it away using a fine-tipped paintbrush that has been pre-treated with RNase decontamination solution, rinsed with nuclease-free water, and completely dried.

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10. Carefully examine the cap by eye or with magnification under a bright field microscope to ensure all debris has been removed. If the debris cannot be removed through use of the pre-treated paintbrush, then use a fine pipette tip (RNase-free) to scrape away the debris.
11. Secure the cap onto a 0.5 mL microfuge tube filled with 230 µL of RNA lysis buffer (Buffer RLT from RNA Extraction Kit "B", with β-mercaptoethanol added at a concentration of 10 µL/mL).
12. Invert the cap, briefly vortex, and incubate at room temperature for 30 min.
13. Centrifuge at ≥ 5,000 x g for 5 min and then transfer the microfuge tube to dry ice.
    Note: The protocol can be paused here. RNA lysates can be stored at -80 °C without a substantial effect on RNA degradation for at least 1 week. If RNA isolation is performed the same day, then chill the samples on ice until they are ready for extraction.
14. Perform all additional collections within the recommended maximal time limit of 80-90 min after removing the slide from xylene. As the dehydrated sections are exposed to ambient humidity, RNases can gradually become reactivated. Restricting the collection period can minimize such effects and maximally preserve RNA integrity.

7. RNA isolation

1. Prepare an RNase-free workstation for RNA extractions.
2. Prepare all tubes and reagents according to the manual for the RNA Extraction Kit.
   Note: While there are many kits available for RNA isolation following LCM, we have had best success using the RNA Extraction Kit "B", listed in the Materials list. See **Figure 5** for a side-by-side comparison of RNA extraction reagents.
3. Remove samples from the -80 °C freezer and rapidly thaw in a 37 °C water bath.
4. Immediately vortex thawed samples for 2-3 s to evenly distribute the guanidinium thiocyanate salts.
5. Combine each sample with an equal volume of 70% ethanol. Pool 2 or more lysates together, if necessary, to reach a sufficient RNA concentration.
6. Proceed as per the manufacturer's instructions in the manual for the RNA extraction process, using the protocol optimized for microdissected samples.
7. Elute RNA at the final step into the minimum recommended volume of nuclease-free water (14 µL).
8. Following RNA isolation, aliquot 1-2 µL of RNA from each sample into a new pre-labeled RNase-free tube for quality assessment/quality control with a microfluidics device that can visualize small quantities of RNA, such as a Bioanalyzer. Aliquot an additional 1 µL into a separate tube for quantification with a high-sensitivity RNA quantification kit.
    1. Adjust these steps, as needed, to obtain a sufficient amount of RNA for downstream analysis (*i.e.*, pool a larger number of LCM caps prior to RNA extraction).
9. Store RNA at -80 °C until collecting enough samples for downstream analysis.

Representative Results

We have made several improvements to existing protocols that enable the relatively rapid collection of enteric ganglia from human intestinal samples using LCM, meeting the standards for RNA-Seq. First, we optimized the rapid freezing of intestinal tissue segments in large base molds placed at the surface of a slurry of dry ice in 2-MB (**Figure 1B**). The best success during cryosectioning and subsequent LCM was obtained by laying intestinal segments flat within a base mold, facing mucosa-side up (**Figures 1B-1C**). Ensure that the base molds are as flat as possible at the base, as any curvature will make it much more difficult to obtain a large cross-section of myenteric plexus. This approach results in tissue that is easily sectioned at 8-µm thickness (**Figure 1E**) and allows for the complete elimination of TFM from the staining process. We found that TFM interferes with the staining process and thus obscures the identification of enteric ganglia. Positioning the tissue in this orientation also avoids sectioning through the mucosa, which has a high content of RNases[12], although from our experience, it appears that the use of ethanolic stains largely inhibits these RNases. In cases where TFM or Optimal Cutting Temperature (OCT) medium is needed, we found it most reliable to first flatten the sample at the bottom of the base mold and then add the TFM to the mold following this step (**Figure 1D**). This leaves a "window" in which the intestine can be easily visualized, making it easy to align the specimen and produce sections that are completely parallel to the myenteric plexus (**Figure 1E**).

Preparation of cryosections longitudinally down the length of the intestine in an orientation parallel to the myenteric plexus (**Figure 2A**), generates sections in which the greatest area of enteric ganglia per slide are visible upon staining (**Figure 2C**). When approaching the myenteric plexus (**Figure 2C**), at the apposition of the longitudinal and circular muscle layers (**Figure 2B**), 6-12 serial sections can be collected (**Figure 2A**, longitudinal) that contain large swaths of enteric ganglia (**Figure 2C**). LCM caps can be loaded to capacity using a single section (**Figure 4D**). In contrast, when preparing transverse sections of fresh-frozen intestine (**Figures 2A-2B**), there is only a small area of myenteric plexus present on each slide (**Figure 2B**). Very few enteric ganglia can be collected from transverse tissue sections, resulting in greater time invested and higher cost per sample overall. The parallel-sectioning approach uses less than one fifth of the number of LCM caps and slides in comparison with transverse sections and greatly accelerates the collection process.

Before LCM, samples must be stained and fully dehydrated to avoid RNase activity during LCM collection. We designed an experiment to directly compare the effect of aqueous stains vs. ethanolic stains on RNA integrity. In this experiment, two intestinal sections were mounted together on a single slide and were processed in one of four ways, with n=2-3 biological replicates per group. In the first group, fresh intestinal sections were not mounted onto a slide and each was directly transferred into RNA lysis buffer using RNase-free forceps pre-chilled on dry ice (**Figure 3A**). For all remaining groups, sections were adhered to a slide, processed, scraped off from the slide using an RNase decontamination solution-treated razor blade, and transferred to RNA lysis buffer with RNase-free forceps. A standard micro-homogenizer was used to fully lyse the samples in all groups. In the second group, the slides were processed through all steps, but no dye was used during the staining procedure (**Figure 3B**). Group three was processed with the protocol described above, using 4% Cresyl violet dye (**Figure 3C**). In the fourth group, an aqueous stain, toluidine blue, was used while following the protocol for an aqueous-based staining kit using a mixture of toluidine blue and hematoxylin (**Figure 3D**). After extracting RNA, as described, RNA quality was assessed with a Bioanalyzer. The only difference between the aqueous and ethanolic staining protocols was the addition of two water incubations (30 s each), immediately before and after staining, which is required for uptake and retention of the dye. We found that the process of adhering tissue sections to the slide and processing with the dehydration steps led to a slight, unavoidable reduction of RNA quality (**Figures 3A-3B**), but that staining with the ethanolic dye, Cresyl violet, does not further reduce RNA quality (**Figure 3C**). In contrast, the aqueous dye, toluidine blue, led to substantial RNA degradation, likely due to extended aqueous exposure that permits endogenous RNase activity (**Figure 3D**).

The unique shapes of enteric ganglia pose difficulties for standard IR-LCM with conventional glass slides[6] (**Figure 4A**). When using PEN-membrane slides (**Figure 4B**), samples are adhered to a thin sheet that is detached from the majority of the glass slide. The UV laser cuts through both the sample and PEN membrane, resulting in the complete detachment of the ganglia from the slide (**Figure 4C**) and full adherence to the LCM cap (**Figure 4D**). Structures of any desired size and shape (>10-15 µm) can be completely and precisely collected (**Figured 4E-4H**). For some samples with fewer ganglia per section, the cap may be moved ≥ four times before collecting. In this case, mounting two to three sections per slide minimizes the use of PEN-membrane slides.

When isolating RNA from LCM samples for RNA-seq, the goal is to obtain the highest possible RNA quality and quantity, while eliminating all genomic DNA (gDNA). To identify an ideal RNA isolation procedure, we performed a head-to-head comparison with a RNA Extraction Kit "A" (**Figure 5A**), RNA Extraction Kit "B" (**Figure 5B**), or RNA Extraction Method "C" with clean-up of the RNA samples by RNA Extraction Kit "B" (**Figure 5C**). After processing samples with the optimized Cresyl violet staining protocol, LCM was used to collect standardized circular samples (diameter of 500 µm) of intestinal tissue, taken from the longitudinal muscle layer. Each cap was randomly assigned to each of the three RNA isolation groups, being placed atop a 0.5-mL microfuge tube pre-filled with the respective lysis buffer from each kit. The instructions were followed exactly as described for each kit, with RNA Extraction Kit "A" lysis buffer requiring incubation at 42 °C for 30 min. For the other kits, room temperature incubation was used for 30 min. All samples were briefly vortexed upon addition to lysis buffer. Samples were then chilled on ice until extractions were conducted the same day (n=4). We found that RNA Extraction Kit "B" with an on-column DNA digestion step resulted in the highest RNA quality and quantity while effectively eliminating gDNA (**Figures 5A-5C**). Importantly, the RNA lysis buffer provided by RNA Extraction Kit "B" fully lyses the captured ganglia when collecting enteric ganglia (**Figure 5D**).

On average, our samples have a RIN of 7.49 ± 0.53 (**Table 2**). RIN scores of greater than 6-7 are deemed of sufficient quality for RNA-seq (depending on the specific RNA profile)[13], giving us confidence that our samples are of sufficient quality for RNA-seq. Considering that the RIN for these samples, upon receipt, has ranged from 7.4 to 9.5, these results indicate that our optimized protocol provides excellent preservation of RNA integrity. Representative RNA quality results from samples submitted for RNA-seq are depicted in **Figures 6A-6C**. Results from RNA-seq demonstrate that our samples were successfully sequenced and have a modest 3'-end bias (**Figure 6D**). Generally, a larger 3'-bias is favored in samples with lower RIN[14]; this effect is moderately reflected in our samples. Despite this, the observed gene biotypes were largely consistent among all samples (**Figure 6E**), indicating the reliability of the data.

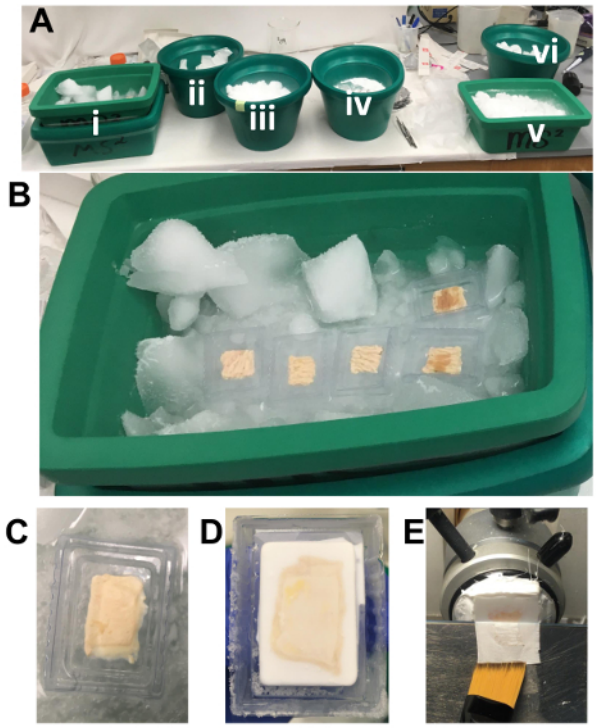

Figure 1. Optimal freezing of intestinal tissue. (**A**) Dry ice buckets are prepared in the order depicted; i) dry ice slurry, ii) Laboratory tissues placed atop dry ice, iii) temporary storage bucket, iv) wrapping bucket, v) pre-freezer storage bucket, vi) pre-freezer storage overflow bucket. (**B**) We optimized rapid freezing of tissue segments in large base molds placed at the surface of a slurry of dry ice and 2-methylbutane. (**C**) A closer-up image of a fully-frozen specimen, observed in the setup shown in panel B. Intestinal segments are laid flat in a base mold facing mucosa-side up. (**D**) This freezing method can be easily adapted for use with TFM by preparing the tissue in the same way, but adding TFM to the base mold before freezing. The resultant sample will have a completely flat myenteric plexus, with a serosa that can be easily visualized. (**E**) Both tissue preparation approaches produce excellent tissue sections at the recommended 8-µm thickness. The procedure using TFM is depicted here. Please click here to view a larger version of this figure.

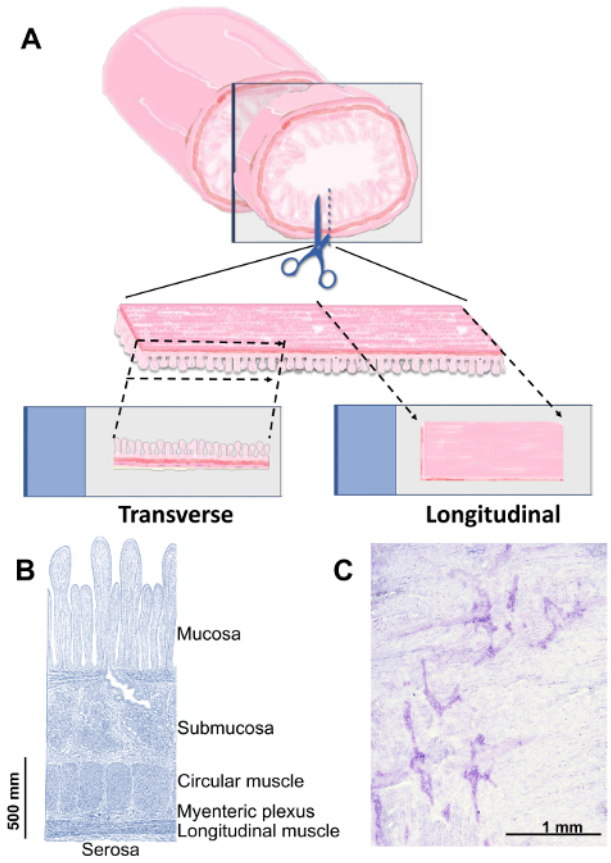

Figure 2. Proper orientation of intestinal tissue provides sections rich in enteric ganglia. When preparing standard transverse cross-sections of fresh-frozen intestine (**A, B**), there is only a small area of myenteric plexus (**B**) present on each slide. Very few enteric ganglia can be collected per slide, which is time-consuming and costly. In contrast, preparing longitudinal sections in the plane of the myenteric plexus (**C**), offers a much larger surface area of ganglia (**C**). Sections of myenteric plexus are obtained by starting from the serosa and sectioning inward through the longitudinal muscle (**B**). When approaching the myenteric plexus, at the junction of the longitudinal and circular muscle layer (**B**), 6-12 serial sections can be collected. Each longitudinal section of myenteric plexus contains large swaths of enteric ganglia (represented in C, using a modified staining procedure, without xylene, to preferentially stain the ganglia). LCM caps can be filled to capacity using a single tissue section. Please click here to view a larger version of this figure.

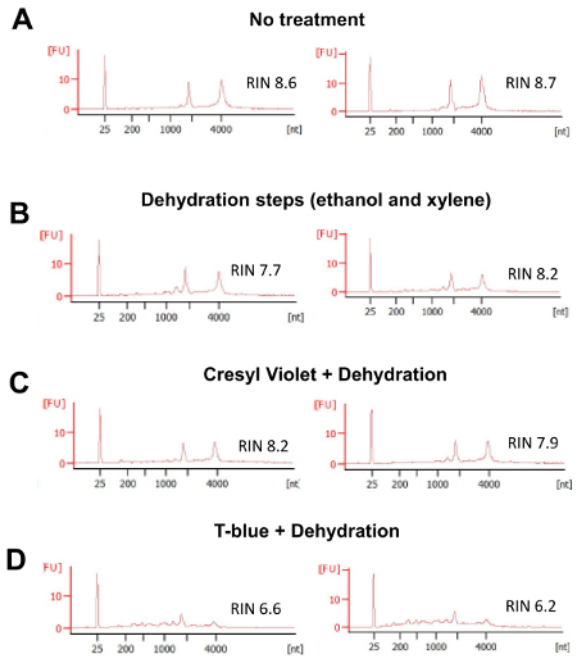

Figure 3. Staining with an ethanol-compatible dye improves RNA quality. RNA quality was measured by the Bioanalyzer and representative results of two samples from each group are displayed. Initial RNA quality measured from fresh, unmounted sections (**A**, No treatment), had a RNA integrity number (RIN) of 8.65 ± 0.07. For sections mounted and processed through all steps, but without stain (**B**), RIN was 7.95 ± 0.35. When staining with 4% Cresyl violet was added to the dehydration steps, there was a negligible effect on RIN, with an average RIN of 7.87 ± 0.35 (**C**). In comparison, the use of the aqueous stain, toluidine blue (T-blue), and the addition of required water rinses to the procedure (**D**) resulted in substantially reduced RNA quality, with a RIN of 6.45 ± 0.21. Each group consisted of n=3 biological replicates except for "No Treatment" and "T-Blue"(n=2). RINs are reported here as mean ± standard deviation. Please click here to view a larger version of this figure.

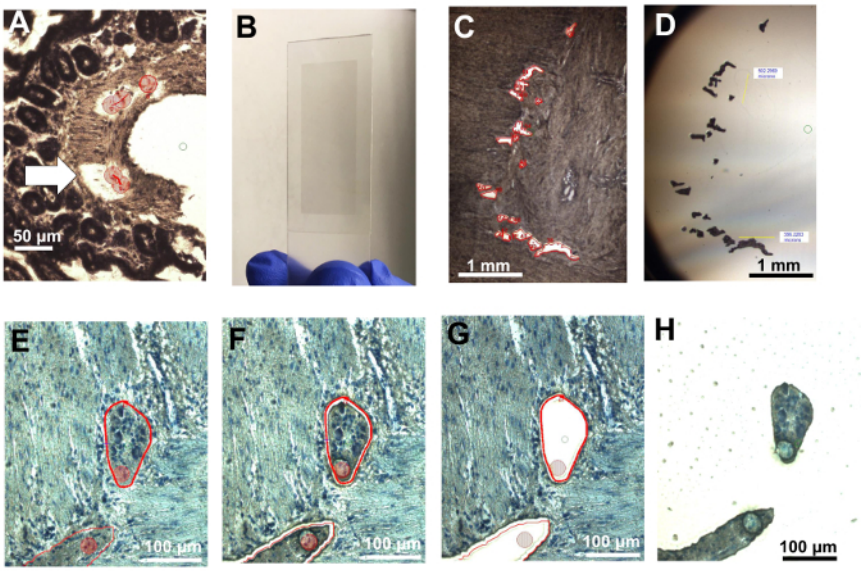

Figure 4. PEN membrane LCM slides enable precise collections of enteric ganglia. Using regular glass microscope slides to collect enteric ganglia via LCM tends to result in unwanted tissue pickup (**A**). The red circles (30 µm in diameter) indicate the size of the IR LCM spots used during the collection process. The white arrow indicates a ganglion collection that pulled up a piece of adjacent muscularis tissue. With PEN-membrane slides (**B**), samples are adhered to a thin sheet that is detached from the majority of the glass slide. When cutting with the UV-LCM laser, both the sample and PEN membrane are sliced, resulting in the complete detachment of the ganglia from the slide, regardless of ganglion shape (**C**), and complete adherence to the LCM cap when removed from the sample (**D**). Structures of any desired size and shape ≥10-15 µm can completely and precisely collected [(**E**), initial tissue mark-up; (**F**) IR and UV laser cutting completed; (**G**) LCM cap removed from section], as visualized on the LCM cap (**H**). The speckled background in panel H is inherent to the cap and is not undesired debris. The green circles blue crosshairs in panels **E-H** are part of the LCM program and are placeholders for the UV and IR lasers, respectively. Please click here to view a larger version of this figure.

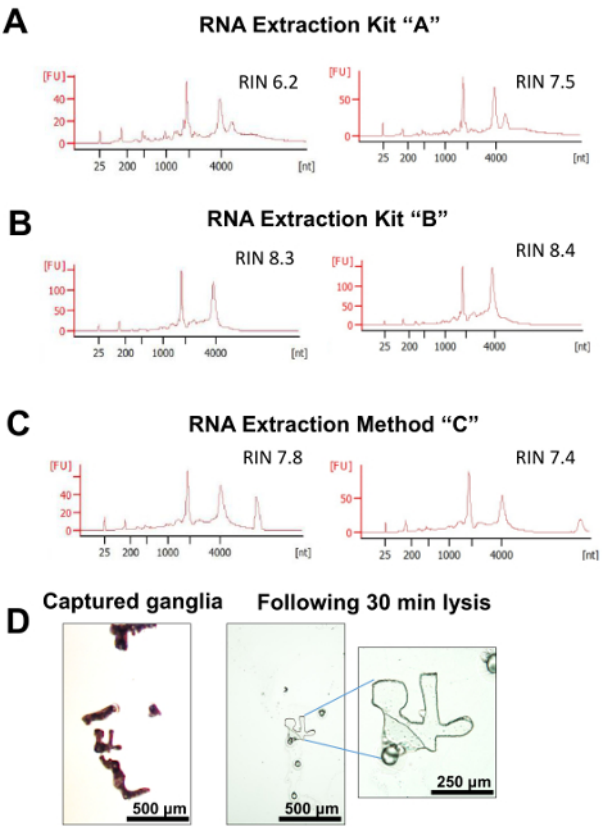

Figure 5. Selecting an optimal RNA isolation kit. RNA integrity results are shown after a concurrent comparison of three different RNA isolation procedures. Two representative plots from each group are displayed to illustrate the variability that can occur between samples processed in parallel. RNA Extraction Kit "A" (**A**) produced samples with a RIN of 6.83 ± 0.65 and provided moderate RNA yields, and tended to have DNA contamination, despite performing an on-column DNase digestion. RNA Extraction Kit "B" (**B**) resulted in the highest measured RIN, at 8.3 ± 0.1. While the phenol-based RNA Extraction Method "C" (followed by clean-up of the RNA samples with RNA Extraction Kit "B") (**C**) appeared to eliminate gDNA and had a RIN of 7.5 ± 0.26, there were large contaminating bands, which may have resulted from melting of the adhesive polymer from the LCM cap during the RNA lysis step. Further, RNA yields from RNA Extraction Kit "A" and Extraction Method "C" were consistently lower than those obtained by RNA Extraction Kit "B". Importantly, the RNA lysis buffer provided by RNA Extraction Kit "B". (with added β-mercaptoethanol) fully lyses the captured ganglia (**D**). Each group had n=3 biological replicates and is presented here as mean ± standard deviation. Please click here to view a larger version of this figure.

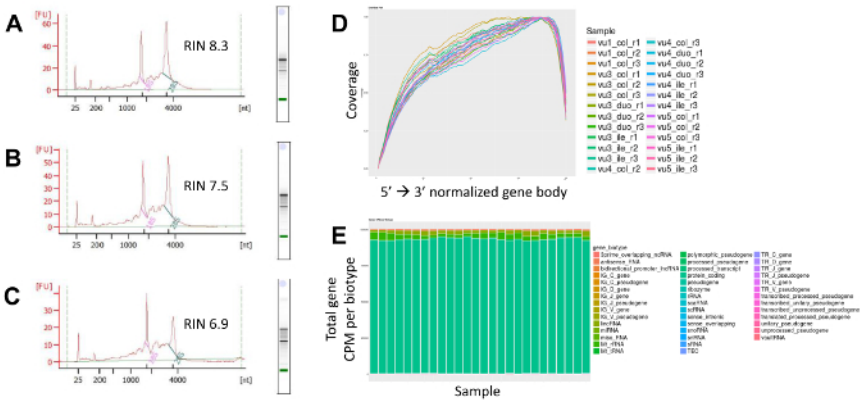

Figure 6. Representative results. When pooling two caps of enteric ganglia per sample, we obtain sufficient quantities of RNA for RNA-seq (>1 ng) along with excellent RNA quality. Representative RNA plots are shown for a sample with a RIN of 8.3 (**A**), 7.5 (**B**), and 6.9 (**C**). RNA-seq data were run through a quality-assessment programs, run in R. (**D**) The end-bias plot indicates that the samples were successfully sequenced and have a modest 3'-end bias. (**E**) The gene biotype plot also represents that sequencing results are largely consistent among the samples. In total, we have had a >95% success rate in generating samples usable for RNA-Seq Please click here to view a larger version of this figure.

Step	Duration	Purpose
95% Ethanol (-20 ^{o}C)	30 s	Fixation
4% Cresyl Violet in 95% Ethanol	10-30 s	Stain
75% Ethanol (with repeated dipping)	15-25 s	De-stain
95% Ethanol (with repeated dipping)	5-15 s	De-stain
100% Ethanol	15 s	Dehydration
100% Ethanol (Anhydrous)	30 s	Dehydration
Xylene (with repeated dipping)	15-20 s	Dehydration
Xylene	>10 min	Dehydration
Air-dry	1-3 min	Evaporation of xylene

Table 1. Staining and Dehydration Protocol Overview. This table outlines our optimized staining protocol. Note that any ethanol-compatible stain can be used to replace Cresyl violet, if it offers better results. In some cases, the durations of staining and destaining will need to be extended or shortened. Generally, the longer the fixation time, the faster the tissue will be fully stained. We have not noticed any reduction in RNA integrity after re-using vials up to 3 times when preparing slides from the same tissue segment.

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Donor	Tissue	RIN
F	Colon	7.1, 7.4, 7.2
M	Ileum	7.2, 6.9, 7.8
M	Duodenum	8.2, 7.4, 7.7
	Ileum	7.4, 7.6, 7.3
	Colon	7.7, 7.8, 7.3
F	Duodenum	7.1, 7.3, 6.9
	Ileum	7.8, 7.9, 7.6
	Colon	7.3, 8.0, 7.5
M	Ileum	6.9, 6.7, 6.9
	Colon	6.8, 6.4, 6.6
M	Ileum	7.2, 7.3, 7.6
	Colon	6.7, 7.6, 7.7
M	Ileum	8.3, 7.8, 7.4
	Colon	7.0, 7.4, 7.0
F	Duodenum	8.3, 8.8, 8.0
	Ileum	8.1, 8.0, 7.8
	Colon	8.4, 8.6, 7.1

Table 2. Representative RNA integrity results. The samples displayed in this table were all obtained from human enteric ganglia. All selected samples have sufficient RNA quality and quantity for RNA-seq analysis. The average RIN of all samples listed in this table is 7.49 ± 0.53. RNA concentration was also measured using the RiboGreen Quant-iT assay and all samples displayed in in this table have met the minimal quantity required for our RNA-seq (>1 ng) experiments. Smaller quantities can be used, depending on the pre-amplification procedure. However, such procedures are more reliable when one expects there to be large differences in gene expression between samples or groups being compared; otherwise these outcomes can mask true differences in some RNA-seq applications. It is therefore generally recommended to start with more material when possible.

Discussion

This procedure enables the efficient collection of numerous enteric ganglia as a source to derive RNA for RNA-seq. Here, we have accelerated the processes outlined in existing protocols while maximally preserving RNA integrity. As all steps in this procedure are interdependent, it is important that all issues be eliminated from the onset of the study and that all samples are prepared as similarly to one another as possible, to obtain reliable RNA-seq.

From the onset of the procedure, RNA integrity can be negatively impacted if the time interval (cold ischemic time) between collection of tissue at the time of organ donation and receipt of tissue for processing in the laboratory is too long. Given this concern, we were surprised that high-quality RNA can still be extracted from intestinal samples even after 24 hours of cold ischemic time. When the intestinal samples are chilled and stored properly in cold storage solution, there is superb protection of tissue integrity and RNA quality. We require that the intestinal tissue is flushed upon collection by the organ collection team, to limit the exposure of the intestine to digestive enzymes, RNases and other molecules that could impact RNA quality during this period. We have found that when the intestinal specimen is not completely flushed, this can significantly reduce the sample's RIN.

Using our optimized procedure for preparing intestinal tissue samples, we are able to avoid embedding samples in TFM. Because TFM interacts with ethanol and xylene to form a dark brown precipitate that interferes with the visualization of enteric ganglia, we prefer excluding it from our samples. However, when working with intestinal tissue from mice or other species, the exclusion of TFM may not be possible. In this case, we have experimented with removing TFM from an adhered section on a slide. If the tissue has a sufficient surface area, ethanol (chilled at -20 °C) can be dripped onto the slide with a transfer pipette to quickly fix the TFM, allowing it to be removed with forceps. Dripping ethanol onto the slides avoids the accumulation of TFM within the vial of ethanol, which can exacerbate the browning effect when multiple slides are being processed.

During flash-freezing, we favor using a dry ice slurry with 2-MB, rather than 100% ethanol, because 2-MB readily evaporates when landing on the surface of the tissue. Ethanol tends to evaporate more slowly, especially when combined with TFM, forming a chemical sludge on the tissue block that cannot be easily removed. Both ethanol and 2-MB tend to fizz and splatter vigorously when freezing in small freezing containers, because the small volume of the dry ice slurry has a low specific heat capacity. Instead, using a large rectangular bucket filled with powderized dry ice provides a large cooling mass and prevents temperature fluctuations while the tissue is being frozen.

As mentioned, it is sometimes easier to obtain continuous sheets of enteric ganglia from some tissue samples relative to others. Understandably, the overall speed of LCM is subject to variability depending on the characteristics of the source tissue or the intestinal region being sampled. For this reason, it is often best to prepare many fresh-frozen samples at the onset. We have generally found that a total of 20 samples per segment of intestine (~2 cm x 1.5 cm, each) is far more than sufficient to provide ample RNA for downstream applications. However, if only a small length

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of intestinal tissue is available, we recommend a minimum length of 5 cm. With our approach, the lowest yield that would be obtained would range from 18-30 ng of RNA, as further discussed below. The minimum 5 cm length can potentially be reduced, depending on the application, especially if higher amounts of cDNA preamplification are used. These parameters were not tested in the current study. For example, enteric ganglia have been collected from full-thickness intestinal biopsy samples (2 cm x 2 cm) for use with qPCR[4].

As we demonstrate in this study, the use of the ethanol-compatible dye, Cresyl violet, offers much better protection of RNA integrity than the aqueous stain, toluidine blue. When submerged in ethanol, RNases become inactive[5,15]. However, RNases can still regain function once exposed to water[7,15]. When staining with aqueous dyes, the tissue must be exposed to nuclease-free water for nearly 1 min[9], which leads to substantial RNA degradation. When using toluidine blue tissue staining, we were unable to reduce the exposure to water before and after staining, because such modifications also reduced the uptake and retention of toluidine blue, respectively. A similar problem was also encountered with a hematoxylin-based dye[9]. In the modified protocol described here, staining with Cresyl violet avoids direct exposure of the intestinal samples to water, thereby maximally preserving RNA integrity. These results eliminate the need for additional RNase inhibitors in the staining solution. We found that such inhibitors are ineffective at enabling the use of the aqueous dye, toluidine blue, as it interfered with both the uptake and retention of the dye.

When initially optimizing our staining protocol, we were unable to reproduce the results obtained in other protocols and reports[5,7,8,11]. While the reason for this is unclear, we found several factors that led to inconsistent dye-uptake and retention. For example, although certain protocols have successfully stained tissue using Cresyl violet in 100% ethanol[8] or in 75% ethanol[5], the dye only faintly labeled ganglia or had high background, compared to using Cresyl violet in 95% ethanol. Last, the percentage of Cresyl violet used in the staining solution is also very important. Most protocols recommend a 1% Cresyl violet solution[7,8], but this concentration did not provide reliable staining in our hands, even after staining sections for up to 2 min. We had best success staining with a clarified saturated staining solution of 4% Cresyl violet[11] applied to the sample through a sterile syringe filter[8]. This saturated solution enables much more rapid staining of the sample, in as little as 10-15 s. Pre-fixation of the sample in chilled 95% ethanol (at -20 °C) enables a more rapid uptake of the stain.

Another report found that enteric ganglia were most distinctly stained in fresh-frozen human intestinal sections when combining Cresyl violet with eosin in a 75% ethanol solution[5]. However, we found that eosin made it more difficult to identify ganglia and that Cresyl violet, alone, offers superior detection. In a different study, it was found that a buffered Cresyl violet solution provided consistent staining results for endometrial cancer tissue[10]. However, we found this did not offer any improvement for human intestinal tissue and also could not be implemented when using 95% ethanol for the staining solution.

We have tested a number of conditions in which the protocol can be paused and resumed at a later time. Many protocols suggest sectioning slides ahead of time and re-freezing them at -80 °C immediately after mounting onto the slide[5,9]. With this approach, staining and LCM can then be resumed within the next week, offering great flexibility. However, not only did this greatly interfere with staining; it also reduced RNA integrity in our hands (data not shown). To circumvent this issue, Grover *et al.* recommend staining and dehydrating the sections prior to storage in the freezer[11]. In this approach, slides are stained and dehydrated (as in **Table 1**) up to the 100% anhydrous ethanol step (protocol section 5.2). Then, samples are incubated in a second vial of 100% anhydrous ethanol for 10 min and are transferred immediately to a conical tube with desiccant gel, which is then chilled on dry ice and transferred to the -80 °C freezer. When removing samples from the freezer, slides are immediately submerged in 100% anhydrous ethanol for 30 s and air-dried prior to performing LCM[11]. Unfortunately, in our hands, this approach reduced RIN by 0.6-0.8 (data not shown). We therefore resorted to performing all sectioning, staining, and LCM in the same day in order to obtain maximal RNA integrity in our conditions. This proves to be a time-consuming process, but 10-14 LCM caps can be filled during a full day's work.

The most reliable point at which our protocol can be paused is at the xylene incubation step. Slides have been left in xylene (with molecular sieves) for up to 4-5 h without any apparent effect on RNA integrity. We have found that when three slides are prepared at a time, all can be processed with LCM within this timeframe, even if an hour break is needed. An alternative option is to desiccate slides after staining and dehydration steps within a vacuum-sealed exicator[16], but we have not yet attempted this approach.

RNA isolation is yet another crucial step to the procedure, with the most important factors being: obtaining the maximal possible yield of RNA, retaining the full spectrum of RNA (without loss of smaller RNA species)[17,18], and completely eliminating genomic DNA from the sample[17]. In our hands, RNA Extraction Kit "B" provided the best outcome with our samples, as it offered greater yields and was more efficient at genomic DNA elimination. Our observations regarding differences in yields between RNA extraction reagents are consistent with prior reports[17,18]. However, there are also many LCM studies that have reported success with other RNA extraction reagents[19,20]. Prior reports also indicate that the column-based RNA extraction processes offer better retention of small RNA molecules than the phenol-based RNA Extraction methods[18] as small RNA molecules can be lost in the supernatant during RNA precipitation steps.

We observed that RNA lysis buffers that use guanidinium thiocyanate with added β-mercaptoethanol is highly effective at removing all captured ganglia from the LCM cap (**Figure 5D**). While most LCM protocols do not suggest vortexing the lysate, we have found that this produces excellent results and does not result in RNA degradation. It also provides an even greater likelihood that all captured ganglia will be released from the cap for RNA extraction. Using these methods, we generally obtain RNA yields from LCM of enteric ganglia in the range of 1.8 to 18 ng/cm^2 of intestinal tissue, depending on how many ganglia are present on a given slide. Our RNA-seq procedure requires a minimum input of 10 ng RNA, but other procedures can have much lower RNA input if a sequencing processing includes a greater number of pre-amplification cycles. The total surface area of tissue stored from the preparation and flash-freezing process is 40-60 cm^2, which provides ample RNA for cDNA library prep and low-input RNA-Seq applications.

While our procedure is designed for the collection of whole enteric ganglia, the approach can be readily modified for the collection of single neurons, or glia, if desired[5]. However, because glial processes densely ensheath neurons of the ENS, glial RNA will be included along with the RNA of captured neurons, albeit at lower counts. This raises issues when trying to identify neuronal transcripts expressed at a low copy number. Single-cell RNA-seq offers the best precision and detection in this regard, but requires neurons to be dissociated from the intestinal tissue, stained with pan-neuronal markers, and isolated with flow-cytometry[21] or identified from whole-intestinal dissociations after sorting with bioinformatics approaches. The downside of most dissociation studies is that most protocols require that the sample be incubated at room temperature or 37 °C for extended periods, which is known to alter the transcriptome[3]. Recently, the cold-active protease from *Bacillus*

licheniformis has been used for generation of single cell suspensions for mouse tissues and it may be possible to apply this enzyme for dissociation of human tissue at 4 °C[22]. Until such optimization is accomplished, LCM of fresh frozen human tissue is a robust means to capture RNA for expression profiling of peripheral nervous system elements like enteric ganglia.

In summary, we have developed a reliable and consistent protocol for the collection of RNA from human enteric ganglia. We have optimized the preparation of human intestinal tissue, staining, LCM process, and RNA extraction based on numerous publications and protocols. We have also demonstrated the reliability of this approach in collecting RNA samples of sufficient RNA quality for RNA-Seq. This protocol can be applied broadly to tissues collected from patients with a number of gastrointestinal diseases and can be readily adapted for use with rodent models, thereby offering a reliable strategy for the development of novel therapeutics.

Disclosures

The authors have no conflicts of interest to disclose.

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