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Alisha DSouza, Ph.D.
Senior Review Editor, *JoVE*

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Re: Resubmission of manuscript, JoVE60503 "*Laser Capture Microdissection of Mouse Embryonic Cartilage and Bone for Gene Expression Analysis*"

Dear Dr. DSouza,

Thank you for the opportunity to revise our manuscript. We appreciate the careful review and constructive suggestions from the editors and reviewers. We have edited the manuscript according to editorial and reviewer comments. We believe that the manuscript is improved substantially after making the suggested revisions. The changes have been tracked within the manuscript.

As requested, we have addressed each point raised by the editor and reviewers in a separate uploaded file.

Thank you for your consideration.

Sincerely,

Meng Wu, PhD
Instructor
Department of Genetics and Genomic Sciences
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TITLE:

Laser Capture Microdissection of Mouse Embryonic Cartilage and Bone for Gene Expression Analysis

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KEYWORDS:

Laser capture microdissection, Meckel's cartilage, mandibular bone, cresyl violet, RNA isolation, RNA sequencing

SUMMARY:

This protocol describes laser capture microdissection for the isolation of cartilage and bone from fresh frozen sections of the mouse embryo. Cartilage and bone can be rapidly visualized by cresyl violet staining and collected precisely to yield high quality RNA for transcriptomic analysis.

ABSTRACT:

Laser capture microdissection (LCM) is a powerful tool to isolate specific cell types or regions of interest from heterogeneous tissues. The cellular and molecular complexity of skeletal elements increases with development. Tissue heterogeneity, such as at the interface of cartilaginous and osseous elements with each other or with surrounding tissues, is one obstacle to the study of developing cartilage and bone. Our protocol provides a rapid method of tissue processing and isolation of cartilage and bone that yields high quality RNA for gene expression analysis. Fresh frozen tissues of mouse embryos are sectioned and brief cresyl violet staining is used to visualize cartilage and bone with colors distinct from surrounding tissues. Slides are then rapidly dehydrated, and cartilage and bone are isolated subsequently by LCM. The minimization of exposure to aqueous solutions during this process maintains RNA integrity. Mouse Meckel's cartilage and mandibular bone at E16.5 were successfully collected and gene expression analysis showed differential expression of marker genes for osteoblasts, osteocytes, osteoclasts, and chondrocytes. High quality RNA was also isolated from a range of tissues and embryonic ages.

This protocol details sample preparation for LCM including cryoembedding, sectioning, staining and dehydrating fresh frozen tissues, and precise isolation of cartilage and bone by LCM resulting in high quality RNA for transcriptomic analysis.

INTRODUCTION:

The musculoskeletal system is a multicomponent system composed of muscle, connective tissue, tendon, ligament, cartilage and bone, innervated by nerves and vascularized by blood vessels[1]. The skeletal tissues develop with increasing cellular heterogeneity and structural complexity. Cartilage and bone develop from the same osteochondroprogenitor lineage and are highly related. Embryonic cartilage and bone develop in association with muscles, nerves, blood vessels, and undifferentiated mesenchyme. Cartilage may also be surrounded by bone, such as Meckel's cartilage and condylar cartilage within the mandibular bone. These tissues are anatomically associated and interact with each other through extracellular signals during development. In the study of gene expression in the development of cartilage and bone, one obstacle is the heterogeneity of skeletal structures composed of multiple tissue types. Precise isolation of the specific tissue of interest is key for successful transcriptional analysis.

Laser capture microdissection (LCM) is a powerful tool to isolate cell types or regions of interest within heterogeneous tissues, and is reproducible and is sensitive to single cells[2]. It can precisely target and capture cells of interest for a wide range of downstream assays in transcriptomics, genomics, and proteomics[3,4]. The quality of the isolated RNA, DNA, or protein can be assessed with a bioanalyzer or equivalent platform. For example, RNA quality is indicated by the RNA integrity number (RIN)[5].

Here, we provide a protocol for the rapid staining and isolation of cartilage and bone by LCM from fresh frozen tissues. We use the mouse embryo to demonstrate that this protocol yields high quality RNA for subsequent transcriptomic analysis, such as RNA sequencing (RNA-seq).

PROTOCOL:

Tissues from mice were obtained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and study protocols were approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

1. Preparation of fresh frozen specimen

1.1. Dissect the embryo or tissue of interest. Embed the sample in a disposable embedding mold with optimal cutting temperature (OCT) compound. Adjust the orientation of the specimen with a tip or needle.

1.2. Rapidly freeze the samples in a dry ice/methyl-2-butane bath. Continue to the next step or store at -80 °C.

NOTE: The protocol can be paused here.

2. Cryosectioning for Laser Capture Microdissection

2.1. Defrost the cryostat and clean internal surfaces with 70% ethanol. Treat the anti-roll plate and a pair of forceps with RNase decontamination agent. Set the cryostat to the desired cutting temperature (-18 to -22 °C). Prepare a lidded container with dry ice, placing a sheet of aluminum foil on the dry ice for temporary storage of the sectioned sample slides during cryosectioning.

CAUTION: Dry ice is extremely cold. Always handle dry ice with care and wear insulated gloves whenever handling it.

2.2. Transfer the fresh frozen specimen into a bucket of dry ice. Place a layer of OCT compound onto a cryostat specimen holder and immediately place the specimen on top of the OCT with gentle pressure. When OCT is completely frozen, fasten the holder with the specimen into the cryostat cutting arm.

2.3. Leave the sample in the cryostat for 15 min to equilibrate to the cutting temperature.

2.4. Section the tissue and collect the slices on polyethylene naphthalate (PEN) membrane slides at a thickness of 12 µm. Align consecutive sections on the membrane. Once one slide is completed, allow sections to dry for a few minutes and transfer the slide onto the foil in the dry ice container until all the required slides are collected.

2.5. Store the slides at -80 °C in a slide box.

NOTE: Section thickness is chosen to maximize the amount of tissue collected while allowing efficient cutting of the tissue, and may vary depending on tissue of interest. The protocol can be paused here. Keep slides free from RNase contamination. Avoid temperature changes. Although the RINs of RNA from slides stored for up to 6 months may still indicate high quality of the RNA, we recommend using slides as soon as possible.

3. Sample preparation for LCM

3.1. Prepare solutions for staining and dehydration.

3.1.1. Place 45 mL of 80% ethanol in each of three 50 mL centrifuge tubes on ice. Label them as #1, #2, and #3. Dilute ethanol with RNase/DNase free water.

3.1.2. Place 45 mL of 95% ethanol in a 50 mL centrifuge tube on ice.

3.1.3. Place 45 mL of 100% ethanol in a 50 mL centrifuge tube on ice.

3.1.4. Place 45 mL of xylene in a 50 mL centrifuge tube at room temperature.

CAUTION: Xylene should be used in a fume hood.

3.2. Thaw a PEN membrane slide briefly by placing it against a gloved hand, then wash twice for 30 s each in 45 mL of 80% ethanol (#1 and #2) with agitation in 50 mL centrifuge tubes to remove the OCT.

NOTE: It is important to remove OCT before staining, to prevent OCT loosened during staining from obscuring the sections. Forceps can be used to facilitate removal of OCT that is not washed off by agitation.

3.3. Lay slide on a sheet of aluminum foil. Pipette 0.8 mL of 0.1% cresyl violet in 50% ethanol onto the slide and stain for 30 s. Wash the slide in 45 mL of 80% ethanol (#3) for 30 s, and then dehydrate by passage for 30 s each through 45 mL of 95% ethanol, 100% ethanol, and xylene in 50 mL centrifuge tubes.

3.4. Stand slides on a delicate task wiper for 5 min to drain xylene and dry sections.

NOTE: Slides must be dried completely before LCM.

4. Laser capture microdissection

NOTE: Wear powder free nitrile gloves while performing LCM.

4.1. Turn on the LCM microscope, laser, and computer. Log on to computer and start the software. Turn the key to the “On” position to start up the laser. When the green light (Laser) is on, press the red button to activate the laser, and then the light (Laser) turns red indicating laser is ready to use.

NOTE: The Laser needs approximately 10 min to warm up.

4.2. Set up the collection tubes.

4.2.1. Insert the cap of a 0.5 mL polymerase chain reaction (PCR) tube into the collector.

NOTE: Choose the matching collector for the desired PCR tubes (0.2 or 0.5 mL).

4.2.2. Pipette 50 µL of extraction buffer (provided by RNA isolation kit listed in the **Table of Materials**) into the cap of the 0.5 mL collection tube.

4.2.3. Insert the collection device into the microscope.

4.2.4. In the **Change Collector Device** window, click on the **Move to Reference Point** button to move the collector to the reference point (RP). Adjust the focus to clearly view the RP. Click the arrows to move the RP to the center of the view.

4.3. Load specimen slides.

4.3.1. Click the left **Unload** button in the toolbar to lower the slide holder.

4.3.2. Place the slide into the holder, with the tissue section facing downwards.

NOTE: This orientation ensures that the captured tissue sections fall into the cap of the collection tube by gravity.

4.3.3. Insert the holder back into the stage.

4.4. Set up the laser parameters.

4.4.1. Select the **Control** option of the **Laser** menu or click on the **Laser** icon.

4.4.2. Adjust these parameters as desired: **Power**, the power of the laser; **Aperture**, the width of the laser; and **Speed**, the speed of cutting in **Draw and Cut** mode.

NOTE: Test the laser in an area out of interest. Higher power or larger aperture makes it easier to cut but causes more damage to cells. Adjust the combination of power, aperture, and speed to obtain efficient cutting and minimal damage of the tissue. For example, Power =n 40, Aperture = 5, and Speed = 7 for cartilage tissue on a 12 µm section.

4.5. Select and cut areas of interest.

4.5.1. Start with 5x objective and find the areas of interest, and then switch to the objective (5x, 10x, or 40x) that best shows the area of interest.

NOTE: After cresyl violet staining, cartilages are stained magenta and mineralized tissues appear brown or black, distinguishable from other tissues. Images can be saved using **Save Image As** from the **File** menu.

4.5.2. Choose the tube for collection (A, B, C, D) by clicking the mark at the collector.

4.5.3. Choose **Move and Cut** or **Draw and Cut**. In **Move and Cut** mode, the specimen is cut manually, using the mouse or the touch-screen pen to draw shapes freehand. In **Draw and Cut** mode, shapes may be drawn freehand with the mouse or touch-screen pen for subsequent cutting. Click the **Start Cut** button to initiate laser cutting.

4.5.4. Once the cut is completed, the target tissue with PEN membrane falls into the cap of the collection tube by gravity. Repeat 4.5 to pool multiple areas of interest if needed.

NOTE: Repeating cuts or increasing Power or Aperture may be necessary if the tissue does not drop into the cap of the collection tube due to incomplete cutting. Mineralized bone (brown or

black) cannot be cut directly by laser.

4.6. Unload the collector and carefully close the PCR tube. Place the microdissected tissues in dry ice. Continue to the next step or store at -80 °C.

NOTE: The protocol can be paused here. Repeat 4.2 to 4.6 for the next sample.

5. Lysis of microdissected tissues and RNA isolation

5.1. Thaw the microdissected tissues at room temperature. Centrifuge briefly and incubate the samples for 30 min at 42 °C.

5.2. After incubation, spin the lysis buffer down into the 0.5 mL PCR tube. Perform DNase treatment and RNA extraction using an RNA isolation kit (see the **Table of Materials**) following the manufacturer's instructions.

REPRESENTATIVE RESULTS:

Coronal sections of fresh frozen mouse tissues at E16.5 were used to demonstrate the isolation and collection of Meckel's cartilage (MC), condylar cartilage, and mandibular bone by LCM. Mouse embryos at E16.5 were dissected and embedded in a cryogenic mold with OCT compound. Samples in molds were rapidly frozen in a dry ice and methyl-2-butane bath and stored at -80 °C.

To demonstrate cresyl violet staining of cartilage and bone, cryosectioning in the coronal plane was performed and samples were collected on microscope slides. Sections were washed, stained, and dehydrated following the protocol above (step 3.2–3.4). Slides were air dried and mounted with permanent mounting medium. All cartilages examined were stained magenta (MC, condylar cartilage, nasal septum cartilage, costal cartilage, cartilage primordium of presphenoid bone, cartilage primordia of radius and ulna) and all mineralized tissues were stained brown or black (**Figure 1A–E**). Both cartilage and bone were easily distinguished from other tissues at multiple anatomical sites.

For LCM, heads of embryos at E16.5 were sectioned in the coronal plane on PEN membrane slides at a thickness of 12 µm, and 6–8 consecutive sections were collected per slide and stored at -80 °C. OCT was removed and sections were stained with cresyl violet and dehydrated following the protocol described above (step 3.2–3.4). MC, condylar cartilage, and the mandibular bone regions were selected and isolated by LCM (**Figure 2**). The selected regions dropped into the lysis buffer in the cap of the collection tube. To obtain RNA for sequencing, we pooled 10 regions of MC, 10 regions of condylar cartilage or 4 regions of mandibular bone into each collection tube as one sample, respectively.

RNA was extracted using an RNA isolation kit following the manufacturer's instructions. Total RNA was analyzed using a bioanalyzer (**Figure 3A–B**). To test the effect of cresyl violet staining on the quality of RNA, we compared RNA from mandibular bone samples stained with cresyl violet and samples without staining (mineralized tissue is visible without staining, but cresyl violet

staining enhances the visibility to distinguish bone from surrounding tissues). No significant difference in RNA integrity was observed between stained samples (n = 4) and samples without staining (n = 4), indicating the quick cresyl violet staining in this protocol has insignificant effect on RNA quality (p = 0.858, two-tailed Welch's t-test, **Figure 3C**). We used our protocol for LCM of various tissues at different developmental stages and RINs were measured, indicating high RNA quality (**Figure 3D**). Average yields of RNA from MC, condylar cartilage, and mandibular bone were 7.50 ± 1.45 ng, 12.55 ± 2.75 ng, and 33.02 ± 7.63 ng (**Figure 3E**) and the yield/area was 19.73 ± 3.82 ng/mm^2, 26.70 ± 5.84 ng/mm^2, and 17.23 ± 3.98 ng/mm^2, respectively (**Figure 3F**), without significant difference among tissues (MC versus condylar cartilage, p = 0.383; condylar cartilage versus mandibular bone, p = 0.260; MC versus mandibular bone, p = 0.674).

Libraries were prepared and sequenced as previously described[6,7]. A representative cDNA size was approximately 500 bp (**Figure 4A**). RNA-seq data was analyzed with MultiQC[8]. We analyzed RNA-seq data from 18 LCM samples (MC1-6, Meckel's cartilage; C1-6, condylar cartilage; M1-6, mandibular bone). The mean quality values across each base position in the reads were generated by FastQC, indicating very good quality calls (**Figure 4B**). Read alignment was analyzed with Picard (**Figure 4C**). The reads showed high aligned percentage and the average percentage of aligned reads was 75%. Gene coverage was analyzed with Picard (**Figure 4D**). Approximately 90% genes in all samples have >1.25x coverage, indicating good quality of the libraries and sequencing data.

Differential gene expression analysis was performed[6,7]. There were 4,006 genes significantly differentially expressed (p < 0.05) between the mandibular bone and MC (**Figure 5A**). Genes specific to osteoblasts or osteocytes (*Col1a1*[9], *Col1a2*[10], *Dkk1*[11], *Dmp1*[12], *Dstn*[13], *Runx2*[14], *Sp7*[15], and *Sparc*[16]) were more highly expressed in the mandibular bone compared to MC, while chondrocyte-specific genes (*Acan*[17], *Col2a1*[18], *Col9a1*[19], *Col9a2*[20], *Col9a3*[21], *Comp*[22], *Lect1*[23], and *Sox5*[24]) were more highly expressed in MC compared to the mandibular bone (**Figure 5B** and **Table 1**). In addition, osteoclast markers such as *Acp5*[25], *Csf1r*[26], *Ctsk*[27], *Itgb3*[28], and *Oscar*[29] were also identified as more highly expressed in the mandibular bone compared to MC (**Figure 5B** and **Table 1**), indicating successful isolation of targeted tissues.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative cresyl violet staining of cartilage and bone in coronal sections of the mouse embryo at E16.5. (**A**) Meckel's cartilage and hemimandible. (**B**) Meckel's cartilage, condylar cartilage, and hemimandible. (**C**) Nasal septum and maxillae. (**D**) Cartilage primordium of presphenoid bone and maxillae. (**E**) Cartilage primordium of radius, cartilage primordium of ulna, and costal cartilage. C = condylar cartilage; CC = costal cartilage; CP = cartilage primordium of presphenoid bone; M = hemimandible; MC = Meckel's cartilage; MX = maxilla; NS = nasal septum; R = cartilage primordium of radius; U = cartilage primordium of ulna. Scale bar = 200 µm.

Figure 2: Representative regions isolated by LCM and collected for RNA-seq. (**A–C**) Representative stained MC (A), condylar cartilage (B) and hemimandible (C; MC already isolated) in cryosection before LCM. (**D–F**) The regions in A, B, and C after targeted tissues were isolated

by LCM. Scale bar = 200 μm.

Figure 3: RNA quality and quantity of LCM samples. (**A–B**) Representative electropherogram (A) and the associated gel image (B) from a bioanalyzer for a mandibular bone sample. (**C**) RINs of total RNA from mandibular bone samples stained with cresyl violet (n = 4) or without staining (n = 4). (**D**) RINs of total RNA from different tissues isolated by LCM. Meckel's cartilage at E16.5 (n = 6); Condylar cartilage at E16.5 (n = 6); Mandibular bone at E16.5 (n = 6); Nasal septum cartilage at 14.5 (n = 6); Brain at E14.5 (n = 6); Brain at E16.5 (n = 7); Brain at E18.5 (n = 4). (**E**) Yield of total RNA from three tissues. Each MC or condylar cartilage sample was a pool of 10 microdissected regions of cartilage and each sample of mandibular bone was a pool of 4 microdissected regions of hemimandible. (**F**) The yield per unit area (ng/mm^2) in each tissue. Data are mean ± s.e.m.

Figure 4: The quality of libraries and RNA-seq data generated from LCM samples. (**A**) Representative cDNA sizes of a library from a mandibular bone sample determined by a bioanalyzer. (**B–D**) Quality control analysis of RNA-seq from 18 LCM samples (MC1-6, Meckel's cartilage; C1-6, condylar cartilage; M1-6, mandibular bone) by MultiQC. (**B**) The mean quality values across each base position in the reads were generated by FastQC. The background of the graph divides the y axis into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). (**C**) Alignment of reads was analyzed by Picard. The summary is shown as the percentages of aligned reads. (**D**) Normalized gene coverage analyzed with Picard.

Figure 5: Differential expression analysis of RNA-seq data from mandibular bone and MC isolated by LCM. (**A**) Hierarchical clustering of 4,006 genes significantly differentially expressed ($P < 0.05$) between the mandibular bone and MC. Three biological replicates were used for each tissue. M1-3, mandibular bone; MC1-3, MC. (**B**) Volcano plot showing fold changes and *p*-values of differentially expressed genes between mandibular bone and MC. Examples of highly differentially expressed cell-specific genes are shown: osteoblast and osteocyte markers in blue, osteoclast markers in green, and chondrocyte markers in red.

Table 1: Differential expression of known genes in various cell types of bone and cartilage (mandibular bone versus MC).

DISCUSSION:

LCM enables the isolation of enriched or homogenous cell populations from heterogeneous tissues. Its advantages include rapid and precise capture of cells in their in vivo context, while potential disadvantages include it being time consuming, expensive, and limited by the need for the user to recognize distinct subpopulations within a specified sample[30]. This protocol provides details of LCM of mouse embryonic cartilage and bone, highlighting the use of cresyl violet staining in a rapid procedure to visualize cartilage and bone for precise tissue collection while maintaining high RNA integrity for subsequent analysis by RNA-seq. One limitation of this protocol is that the LCM system used here is not able to directly cut across mineralized tissues (e.g., the mandibular tissue in black in **Figure 2C**); as a result, the whole bone area needs to be dissected. Notably, the microdissected ossified regions isolated by LCM are not homogeneous, and include at least the subpopulations of osteoblasts, osteocytes, and osteoclasts (**Table 1**).

Nevertheless, the enrichment by LCM is valuable as our previous study has shown that transcriptional changes in specific subpopulations such as osteoclasts in the microdissected bone tissue can still be detected by RNA-seq[6].

For gene expression analysis, we have optimized the sample preparation and LCM procedure for high RNA quality and yield. Our protocol starts with fresh frozen tissues. Fresh frozen tissue sections allow for excellent quantity and quality of extracted RNA[3], as mRNA is sensitive to standard methods of fixation[31]. RNA is quickly degraded by RNase contamination, and maintenance of an RNase-free environment throughout sample preparation, LCM, and RNA isolation is critical for successful application. Exposure to water during section processing is detrimental to RNA quality[31,32]. Our protocol limits such exposure, with the highest level of aqueous exposure being 50% during cresyl violet staining. We have tested that a quick staining (30 s) with 0.1% cresyl violet in 50% ethanol gives a distinguishable color to cartilage and does not lower the RNA integrity for downstream analysis such as low input RNA-seq (**Figure 3C** and **Figure 4**). The yield/area (ng/mm^2) is approximately 20 ng/mm^2 (**Figure 3F**), similar to or higher than previous optimized methods[33]. According to the cell densities in MC and mandibular bone[6], we estimate that with this protocol the average yield from one cell is approximately 5 pg RNA per cell, and 1–5 ng of total RNA can be extracted from 200–1,000 cells, which can be used for low-input RNA-seq[6,7,33]. This yield efficiency is much higher than established LCM methods[34], and also superior or similar to recent optimized protocols[33,35].

The ability to distinguish different cell types in histological sections is essential for precise collection of specific tissues of interest. Cresyl violet is a hydrophilic, basic stain that binds to negatively charged nucleic acids[36]. This property makes it useful for counterstaining with cell-type selectivity[37], and it is a standard histological stain for neurons[38]. Cresyl violet has been used in LCM for a variety of tissues, providing good tissue morphology and high RNA quality[33,36,39,40]. Compared with other staining methods, cresyl violet staining provides cytoplasmic and nuclear details, and a low RNA degradation rate[32]. We demonstrate here that cresyl violet staining is an easy and quick method to visualize cartilage and bone and that tissue stained with this method conserves high RNA integrity. Xylene treatment is commonly used for dehydration of the tissues before LCM[35,36,41,42]. We use xylene to enhance the visualization of tissue morphology[35] which is essential to distinguish targeted tissues, especially on PEN membrane slides that are not as optically clear as plain glass slides. We found no significant occurrence of section loss from PEN slides during staining and washing steps, even with agitation to remove OCT.

Microdroplet or microfluidics-based single-cell RNA-seq (scRNA-seq) is a high-throughput method to analyze transcriptomes of tissues with heterogeneous cell types and has begun to be used in skeletal biology[43]. In contrast to LCM, scRNA-seq involves enzymatic and/or mechanical disaggregation of tissue into single cells. Most protocols for single cell preparation require tissues to be incubated at room temperature or 37 °C for extended periods, which alters the transcriptome[44,45]. In addition, isolation of single cells in cartilage and bone may be physically limited by skeletal element complexity of trabecularization and mineralization. It is still a technical challenge to isolate a sufficient number of viable cells from skeletal tissues that are accurately representative of the cellular diversity of tissues *in vivo*, and incomplete dissociation

of the cells may cause bias in the detection of cell types[43]. While scRNA-seq allows identification of distinct cell types, the sequencing depth is low, and typical sequencing approaches capture 3' transcript ends and do not allow alternative splicing analysis. Bulk RNA-seq of LCM-derived tissue homogenizes cell differences, but allows comprehensive transcript detection and alternative splicing analysis. LCM is therefore especially useful for skeletal tissues that are not easily separable from surrounding tissues and internally complex, and fresh frozen preparation of the tissue can preserve both transcriptional profiles and RNA integrity for transcriptional analysis. Another weakness of scRNA-seq is that after single cell preparation, the spatial information of the cells is lost. A combination of LCM and scRNA-seq has been developed to permit the study of the transcriptome of a small sample from defined geographical locations (Geo-seq)[46], which is another approach of utilizing LCM to study regionalized gene expression.

In summary, this protocol provides details of optimized LCM of cartilage and bone, highlighting the use of cresyl violet staining in a rapid procedure to visualize cartilage and bone for precise tissue collection while maintaining high RNA integrity for subsequent analysis by RNA-seq. This protocol has been used successfully for LCM of cartilage and bone at different developmental stages for gene expression analysis[6,7], and also can be used for other tissues.

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DISCLOSURES:

The authors have nothing to disclose.

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537

Figure 1

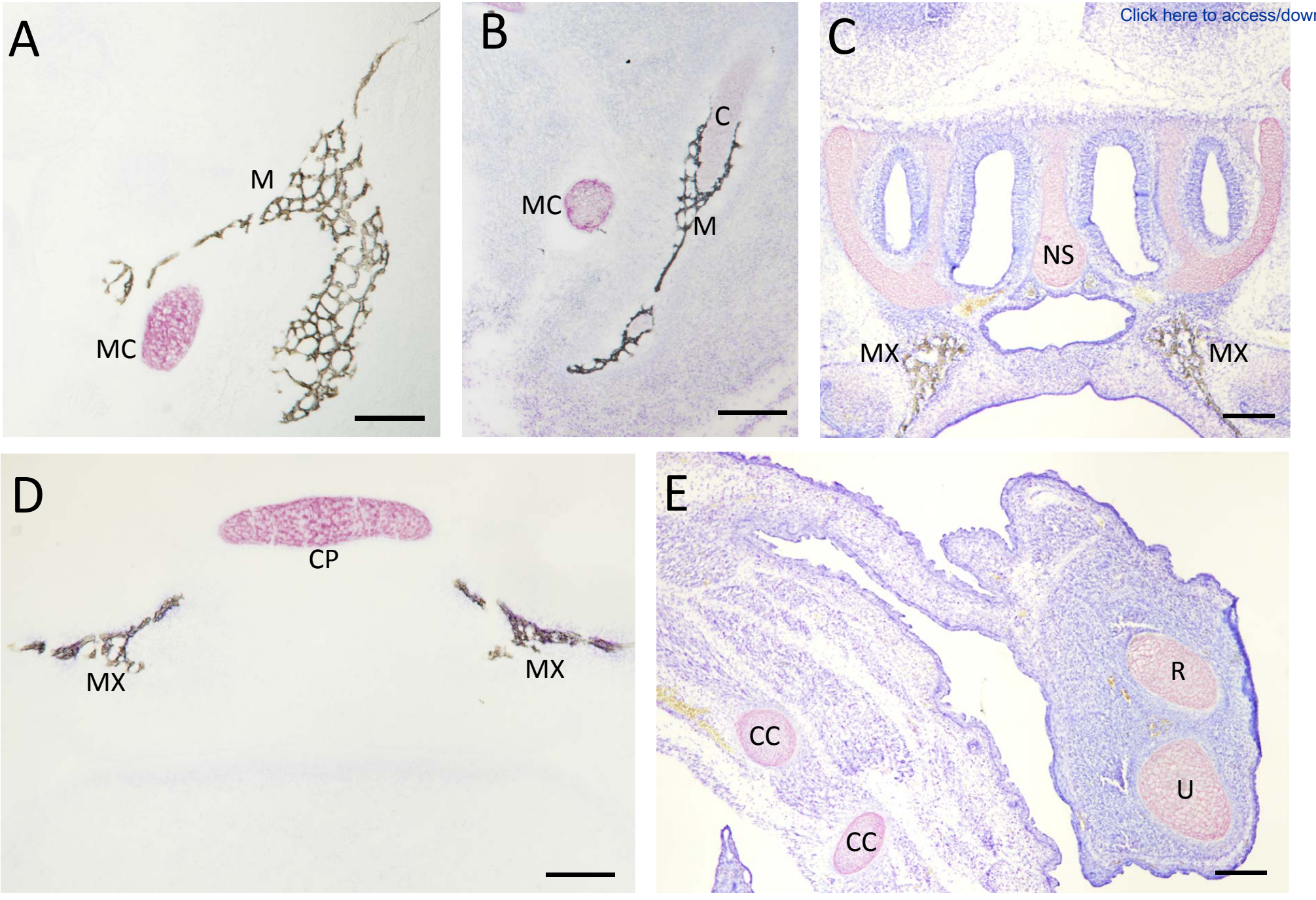

Figure 2

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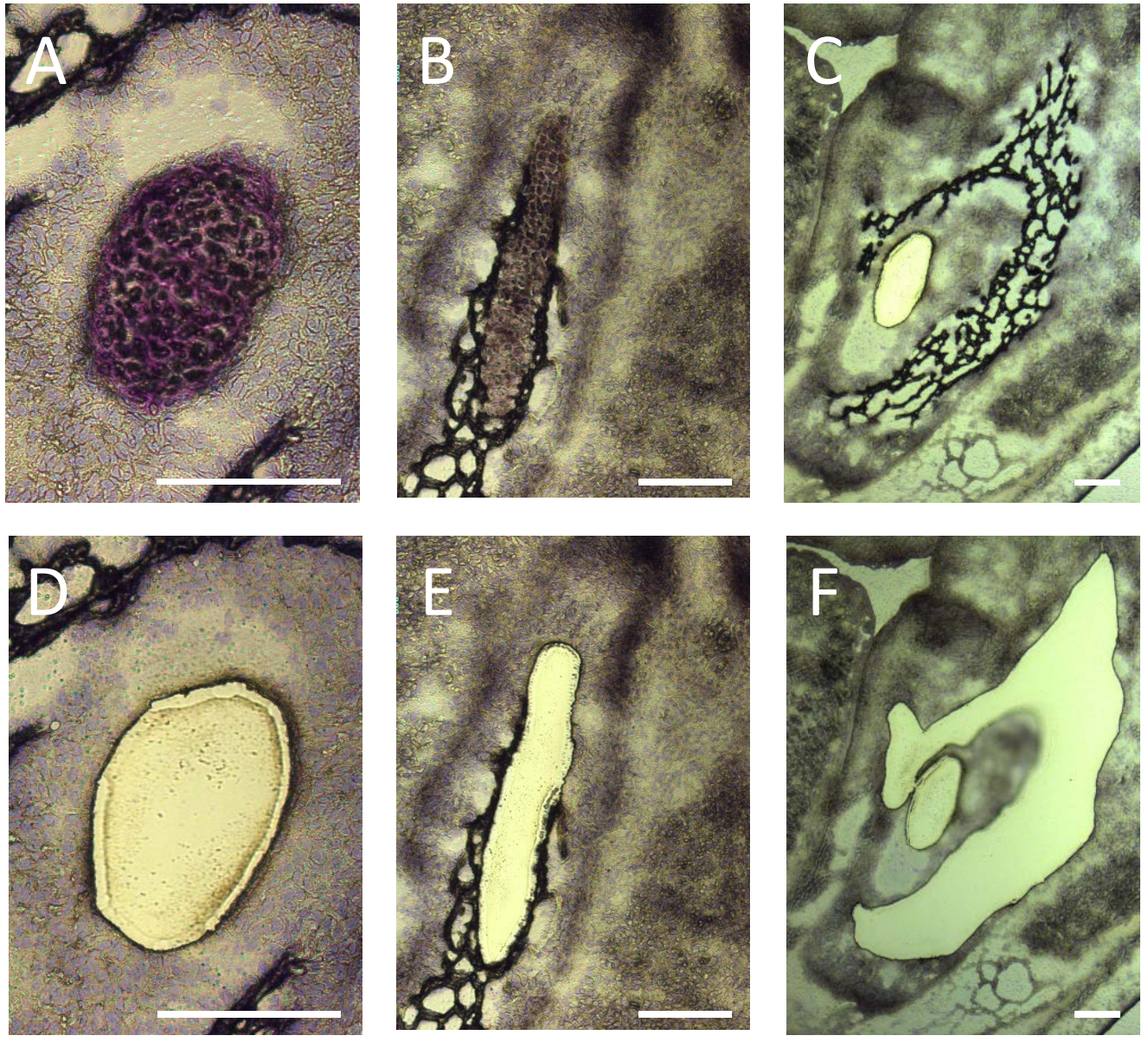

Figure 3

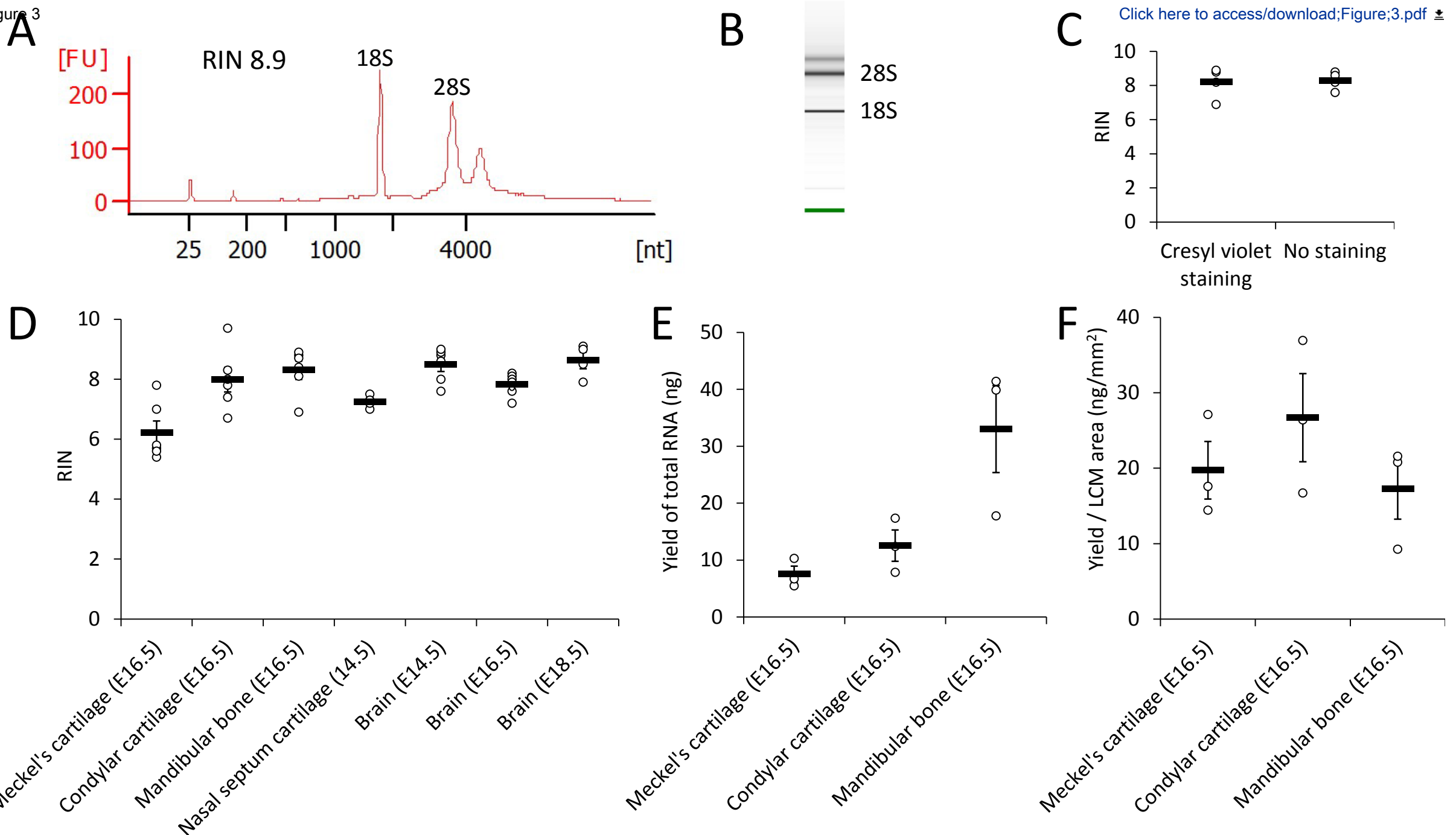

Figure 4

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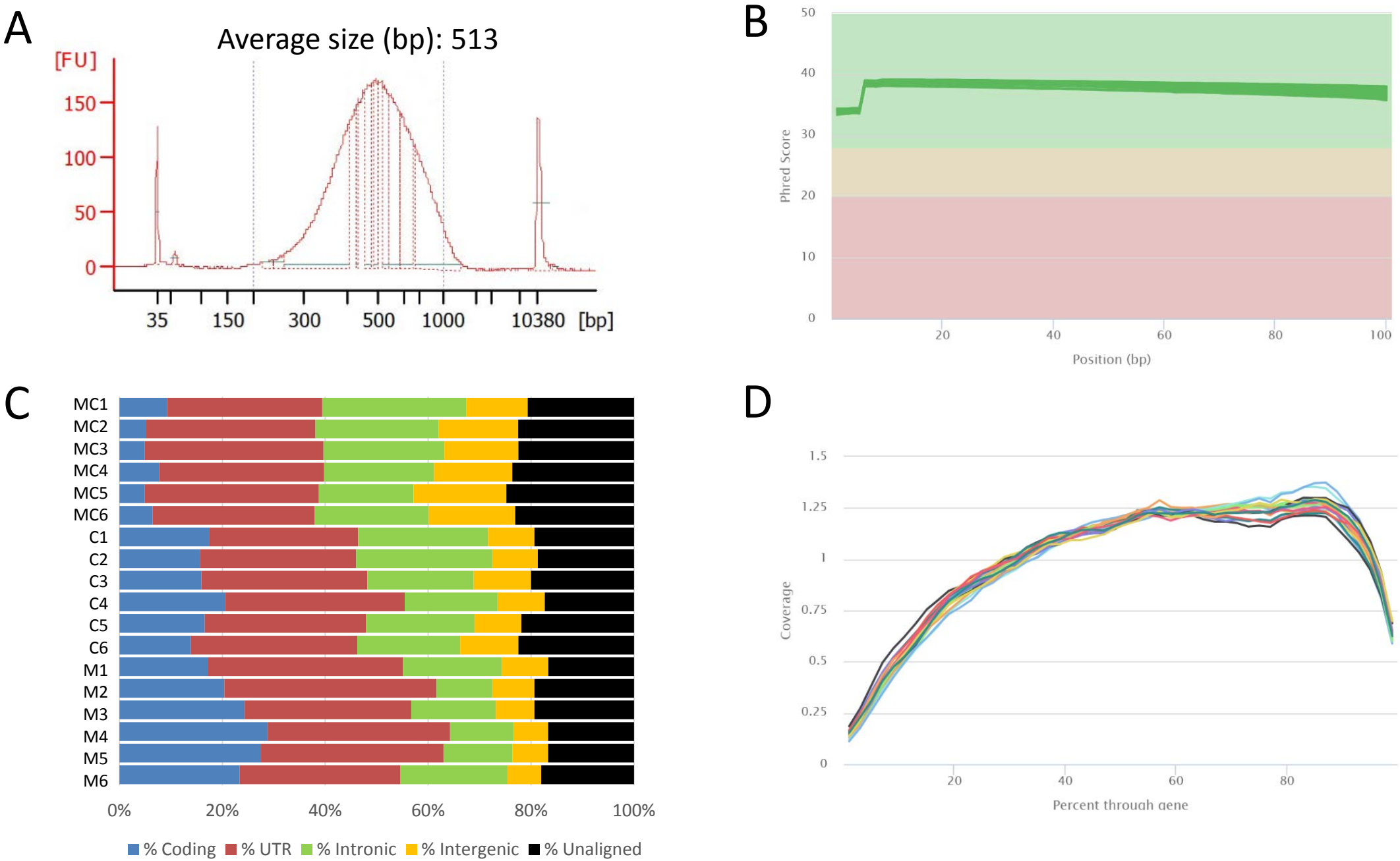

Figure 5

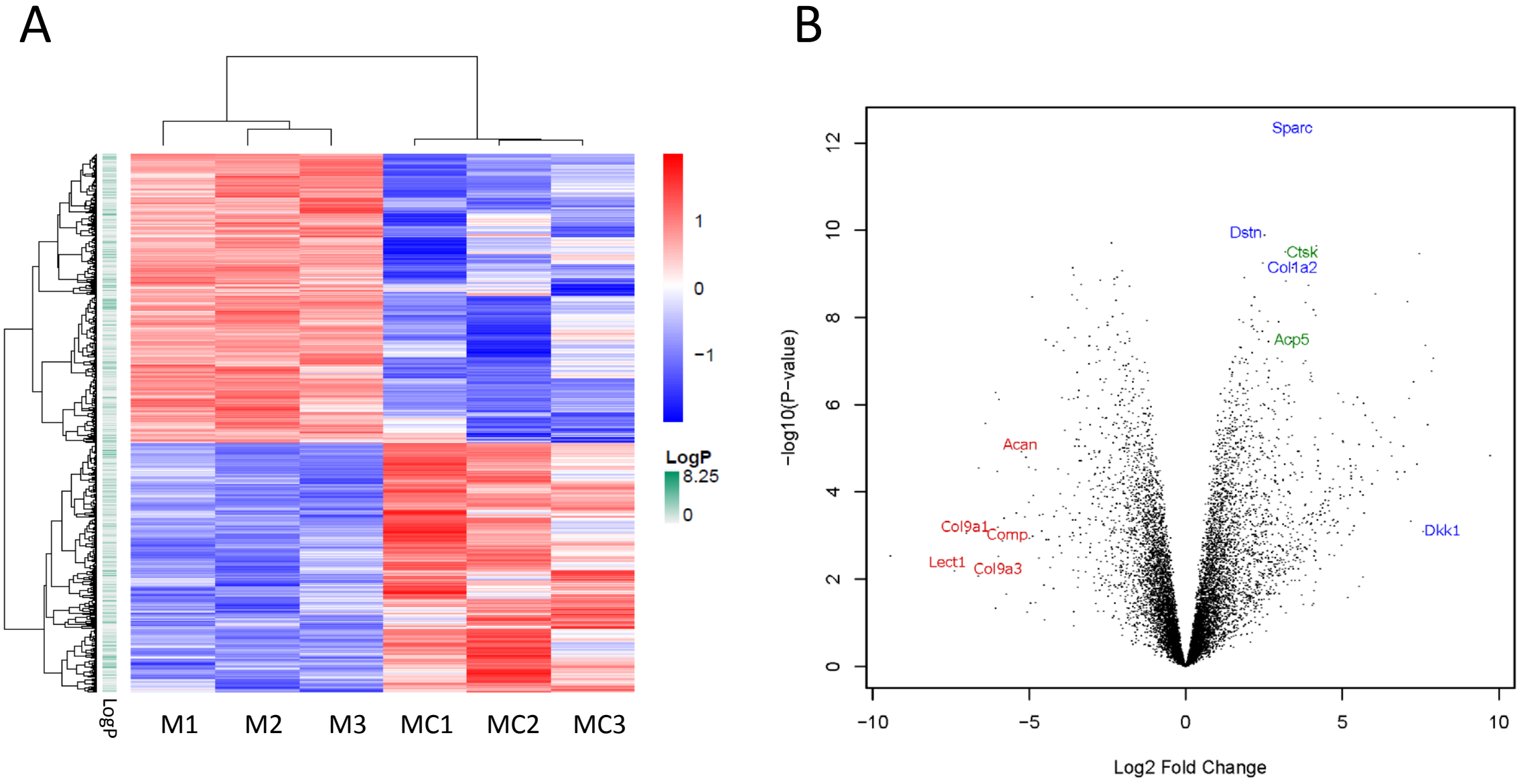

Table 1

Gene	Cell type	log2FoldChange	Average Expression	Adjusted P Value
Col1a1	Osteoblast/Osteocyte	2.64	12.94	2.22E-05
Col1a2	Osteoblast/Osteocyte	3.41	12.87	8.27E-07
Dkk1	Osteoblast/Osteocyte	7.59	2.01	5.21E-03
Dmp1	Osteoblast/Osteocyte	9.73	3.42	3.19E-04
Dstn	Osteoblast/Osteocyte	2.51	6.87	5.73E-07
Runx2	Osteoblast/Osteocyte	1.24	8.62	4.08E-02
Sp7	Osteoblast/Osteocyte	2.24	6.95	5.81E-03
Sparc	Osteoblast/Osteocyte	3.40	12.26	5.56E-09
Acp5	Osteoclast	3.38	5.74	6.46E-06
Csf1r	Osteoclast	2.01	5.80	1.17E-04
Ctsk	Osteoclast	3.19	7.56	5.73E-07
Itgb3	Osteoclast	2.88	5.37	2.43E-04
Oscar	Osteoclast	3.41	2.67	1.63E-04
Acan	Chondrocyte	-5.23	5.01	2.76E-04
Col2a1	Chondrocyte	-3.61	9.47	3.29E-03
Col9a1	Chondrocyte	-7.01	3.58	5.51E-03
Col9a2	Chondrocyte	-5.40	3.76	2.60E-03
Col9a3	Chondrocyte	-6.63	3.80	2.90E-02
Comp	Chondrocyte	-5.86	1.54	7.51E-03
Lect1	Chondrocyte	-7.37	1.34	2.35E-02
Sox5	Chondrocyte	-2.88	4.43	6.06E-04

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2-Methylbutane	ThermoFisher Scientific	O3551-4	
Bioanalyzer	Agilent	G2939BA	
Centrifuge tube	ThermoFisher Scientific	339653	e polypropylene centrifuge tubes, 50 mL
Cresyl violet acetate	Sigma-Aldrich	C5042	
Cryostat	Leica Biosystems	CM3050 S	
Delicate task wiper	ThermoFisher Scientific	06-666	
Disposable embedding mold	ThermoFisher Scientific	1220	
Distilled water	Invitrogen	10977-015	DNase/RNase-Free
Ethanol, absolute (200 proof)	ThermoFisher Scientific	BP2818	Molecular biology grade
Glass PEN membrane slide	Leica Microsystems	11505158	
LCM system	Leica Microsystems	Leica LMD6500	
Microscope cover glass	ThermoFisher Scientific	12-545FP	
Microscope slides	ThermoFisher Scientific	12-550-15	
OCT compound	Electron Microscopy Sciences	102094-106	
PCR tube with flat cap, 0.5 mL	Axygen	PCR-05-C	LCM collection tubes
Permanent mounting medium	Vector Laboratories	H-5000	
RNA isolation kit	ThermoFisher Scientific	KIT0204	
RNase decontamination agent	Sigma-Aldrich	R2020	ntamination agent for cleaning surfaces
Xylene	Sigma-Aldrich	214736	

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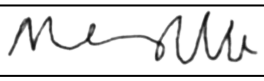
Signature:	
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Response to Comments

We thank the editors and reviewers for their constructive and supportive comments. We have revised our manuscript in response to their comments.

Response to Editorial Comments:

Comment 1:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response

We have proofread the manuscript and corrected errors.

Changes in Manuscript:

Line 64, "transcriptomics".

Corrected grammatical errors.

Corrected format errors in references.

Comment 2:

Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines 45- 47, 180-184 to avoid this overlap.

Response

We have deleted unnecessary background and details of previous published work to avoid the overlap.

Changes in Manuscript:

Line 45, the previously published work was deleted as it is not relevant to the protocol: "There are two distinct osteogenic processes: endochondral ossification and intramembranous ossification. Endochondral ossification gives rise to long bones that comprise the appendicular skeleton, the skull base, vertebrae, and the lateral medial clavicles. Intramembranous ossification gives rise to the flat bones that comprise the cranium, many of the facial bones, and medial clavicles[2]."

Line 238, the description has been shortened to avoid overlap.

Comment 3:

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 1.2: What is the source of the frozen sections? How were the sections frozen prior to use? Mention briefly.

Response

We have added the step "Preparation of Fresh Frozen Specimen".

Changes in Manuscript:

Line 72-81, added "Preparation of Fresh Frozen Specimen".

Comment 4:

Protocol Numbering: Please add a one-line space after each protocol step.

Response

One-line space has been added after each protocol step.

Comment 5:
Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
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3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
Response
The protocol steps for filming have been highlighted.

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Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
Response
We have edited the discussion:
Paragraph 1: advantages and limitations of the technique;
Paragraph 2 and 3: critical steps within the protocol (RNase-free environment, reduced exposure to water, etc.), modifications and troubleshooting (cresyl violet staining, xylene treatment, washing steps, etc.);
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Response
We replaced all commercial names.
Changes in Manuscript:
Line 141, replace "Kimwipe" with "delicate task wiper"; **Line 227**, deleted "Arcturus Picopure"; **Line 238**, deleted "Superfrost Plus"; **Line 256**, deleted "Agilent"; **TABLE OF MATERIALS**, remove commercial names for RNA isolation kit, OCT compound, RNase decontamination agent and distilled water.

Comment 8:
Please define all abbreviations at first use.
Response
We have checked the manuscript and defined all abbreviations at first use.
Changes in Manuscript:
Line 65, defined "RNA sequencing"; **Line 150**, deleted "LED".

Comment 9:
If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
Response
All figures and tables are original.

Response to Comments from Peer-Reviewers:
Reviewer #1:
Manuscript Summary:
This manuscript described a protocol to laser capture microdissect merkel's cartilage and mandibular bone from mouse embryo E16.5 for RNA extraction and RNA-Seq.

Comment 1:
Major Concerns:
The only real problem with this manuscript is that their protocol is limited to LCM of mandibular bone and MC in E16.5 mice. Could the same protocol be used for LCM of other tissues? Or maybe the same tissue at a different ages (how about E19 or Newborn)? Without such the protocol is only useful if you are performing EXACTLY the same procedures. A protocol that can generalize is a lot more useful. Maybe the authors should at least comment on that?
Response
We have used the same protocol for different tissues at different ages. New data have been added demonstrating LCM of condylar cartilage at E16.5, nasal septum at E14.5, and brain tissues at E14.5, E16.5 and E18.5, in addition to MC and mandibular bone.
Changes in Manuscript:
Figure 1B; **Figure 2B,E**; **Figure 3D,E,F**.

Minor Concerns:
Comment 2:
Why did the authors used cresyl violet for staining? Did the authors compare it with other staining method? Maybe it is at least worth a discussion.

Response

The advantage of cresyl violet staining is that it can distinguish cartilage clearly from surrounding tissue and provides cytoplasmic and nuclear details, and maintains RNA integrity compared to other stains such as hematoxylin and eosin. We add more information about cresyl violet, with references, to the discussion.

Changes in Manuscript:

Line 378, "Compared with other staining methods, cresyl violet staining provides cytoplasmic and nuclear details, and a low RNA degradation rate[32]."

Comment 3:

Did the authors performed DNase treatment for the RNA samples?

Response

Yes, we performed DNase treatment following the manufacturer's instructions, recommended in the manual of the RNA isolation kit.

Changes in Manuscript:

Line 226, step 5.2, "Perform DNase treatment and RNA extraction using an RNA isolation kit (see Table of Materials) following the manufacturer's instructions."

Comment 4:

Could the author comment on how much cells were being dissected out from each slide, for both the MC and mandible? It would be useful to provide a mathematical conversion of the yield. That is, how much RNA (ng) was being extracted from how many cells total, and are the [RNA]/cell comparable between the two tissues?

Response

We have included this result in **Figure 3E and F**. Average yields of RNA from MC, condylar cartilage and mandibular bone were 7.50±1.45 ng, 12.55±2.75 ng and 33.02±7.63 ng (**Figure 3E**) and the yield/area was 19.73±3.82 ng/mm^2, 26.70±5.84 ng/mm^2 and 17.23±3.98 ng/mm^2, respectively (**Figure 3F**), without significant difference among tissues. We estimate that with this protocol the average yield from one cell is approximately 5 pg RNA per cell, and 1-5 ng of total RNA can be extracted from 200-1,000 cells, which can be used for low-input RNA-seq.

Changes in Manuscript:

Line 264, "Average yields of RNA from MC, condylar cartilage, and mandibular bone were 7.50±1.45 ng, 12.55±2.75 ng, and 33.02±7.63 ng (**Figure 3E**) and the yield/area was 19.73±3.82 ng/mm^2, 26.70±5.84 ng/mm^2, and 17.23±3.98 ng/mm^2, respectively (**Figure 3F**), without significant difference among tissues (MC versus condylar cartilage, P = 0.383; condylar cartilage versus mandibular bone, P = 0.260; MC versus mandibular bone, P = 0.674)."

Line 366, "The yield/area (ng/mm^2) is approximately 20 ng/mm^2 (**Figure 3F**), similar to or higher than previous optimized methods[33]. According to the cell densities in MC and mandibular bone[6], we estimate that with this protocol the average yield from one cell is approximately 5 pg RNA per cell, and 1-5 ng of total RNA can be extracted from 200-1,000 cells, which can be used for low-input RNA-seq[6,7,33]. This yield efficiency is much higher than established LCM methods[34], and also superior or similar to recent optimized protocols[33,35]."

Reviewer #2:

Comment 1:

The manuscript "Laser Capture Microdissection of Mouse Embryonic Cartilage and Bone for Gene Expression Analysis" presents a review of rapid method of tissue processing and isolation of cartilage and bone based on laser capture microdissection (LCM). This provides a detail protocol for mouse bone and cartilage tissue sectioning, staining and sampling, highlighting the use of cresyl violet staining in a rapid procedure to visualize cartilage and bone for precise tissue collection. However, the weaknesses of this protocol are lack of novelty and the latest researches are not included. For example, some optimized methods using LCM to get transcriptome, such as Farris (Farris et al., 2017) and Chen (Chen et al., 2017), need to be mentioned, and making comparisons with those methods and highlighting the uniqueness of this protocol should be done.

Response

We have included the latest research and compared our protocol with current methods listed. Our representative results show that the yield efficiency is superior or similar to recent optimized protocols and this protocol includes a rapid procedure to visualize cartilage and bone for precise tissue collection which has not been reported before. It is a valuable method especially for bone and cartilage research. Comparisons were made with the following references:

30. Mahalingam, M. Laser capture microdissection: Insights into methods and applications. Methods in Molecular Biology. doi: 10.1007/978-1-4939-7558-7_1 (2018).

33. Farris, S., Wang, Y., Ward, J.M., Dudek, S.M. Optimized Method for Robust Transcriptome Profiling of Minute Tissues Using Laser Capture Microdissection and Low-Input RNA-Seq. *Frontiers in Molecular Neuroscience*. **10**, 185 (2017).

35. Martuscello, R.T., Louis, E.D., Faust, P.L. A Stainless Protocol for High Quality RNA Isolation from Laser Capture Microdissected Purkinje Cells in the Human Post-Mortem Cerebellum. Journal of Visualized Experiments. (143) (2019).

We have also discussed this latest application:

46. Chen, J., Suo, S., Tam, P.P., Han, J.D.J., Peng, G., Jing, N. Spatial transcriptomic analysis of cryosectioned tissue samples with Geo-seq. Nature Protocols. 12 (3), 566–580 (2017).

Changes in Manuscript:

Line 366, "The yield/area (ng/mm^2) is approximately 20 ng/mm^2 (**Figure 3F**), similar to or higher than previous optimized methods[33]. According to the cell densities in MC and mandibular bone[6], we estimate that with this protocol the average yield from one cell is approximately 5 pg RNA per cell, and 1-5 ng of total RNA can be extracted from 200-1,000 cells, which can be used for low-input RNA-seq[6,7,33]. This yield efficiency is much higher than established LCM methods[34], and also superior or similar to recent optimized protocols[33,35]."

Line 403, "Another weakness of scRNA-seq is that after single cell preparation, the spatial information of the cells is lost. A combination of LCM and scRNA-seq has been developed to permit the study of the transcriptome of a small sample from defined geographical locations (Geo-seq)[46], which is another approach of utilizing LCM to study regionalized gene expression."

Comment 2:

This protocol claim that it yields high quality RNA for subsequent transcriptomic analysis. However, besides the quality control of total RNA, it would be better to show the quality of RNA-seq data, like the mapping ratio, the number of detected genes.

Response

We have added a new figure (**Figure 4**) to demonstrate the quality analysis of library and RNA-seq data.

Changes in Manuscript:

Figure 4: The quality of libraries and RNA-seq data generated from LCM samples.

Line 270, "A representative cDNA size was approximately 500 bp (**Figure 4A**). RNA-seq data was analyzed with MultiQC[8]. We analyzed RNA-seq data from 18 LCM samples (MC1-6, Meckel's cartilage; C1-6, condylar cartilage; M1-6, mandibular bone). The mean quality values across each base position in the reads were generated by FastQC, indicating very good quality calls (**Figure 4B**). Read alignment was analyzed with Picard (**Figure 4C**). The reads showed high aligned percentage and the average percentage of aligned reads was 75%. Gene coverage was analyzed with Picard (**Figure 4D**). Approximately 90% genes in all samples have >1.25x coverage, indicating good quality of the libraries and sequencing data."

Line 315, "**Figure 4: The quality of libraries and RNA-seq data generated from LCM samples. (A)** Representative cDNA sizes of a library from a mandibular bone sample determined by a bioanalyzer. **(B-D)** Quality control analysis of RNA-seq from 18 LCM samples (MC1-6, Meckel's cartilage; C1-6, condylar cartilage; M1-6, mandibular bone) by MultiQC. **(B)** The mean quality values across each base position in the reads were generated by FastQC. The background of the graph divides the y axis into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). **(C)** Alignment of reads was analyzed by Picard. The summary is shown as the percentages of aligned reads. **(D)** Normalized gene coverage analyzed with Picard."

Comment 3:

For store the slides at -80 °C can be up to 6 months, are there some data to support this conclusion? The RNA is sensitive to degraded, and the tissue maybe stored for up to 6 months. However, after sectioning tissue, it would be better showing results to support this.

Response

We previously tested slides stored longer than 6 months at 80 °C. The RIN could be still good, but we think our original description was not substantiated sufficiently, so the note has been changed and we recommend using slides as soon as possible.

Changes in Manuscript:

Line 109, "Although the RINs of RNA from slides stored for up to 6 months may still indicate high quality of the RNA, we recommend using slides as soon as possible."

Comment 4:

What's the function of xylene in staining? Is it necessary for this step? It's harmful to the body. If remove the step, what's the result?

Response

We use xylene to enhance the visualization of tissue morphology. Without xylene treatment, it is more difficult to distinguish cartilage from adjacent tissues on PEN membrane slides.

Changes in Manuscript:

Line 381, "Xylene treatment is commonly used for dehydration of the tissues before LCM[35,36,41,42]. We use xylene to enhance the visualization of tissue morphology[35] which is essential to distinguish targeted tissues, especially on PEN membrane slides that are not as optically clear as plain glass slides."

Comment 5:
For the step of wash the slide in 50 mL centrifuge tubes, what's the unfalling rate? And some suggestions for prevent falling off will be helpful.
Response
The sections are stable on PEN membrane slides during staining and washing steps. No loss of tissue from PEN slides has been observed.
Changes in Manuscript:
Line 384, "We found no significant occurrence of section loss from PEN slides during staining and washing steps, even with agitation to remove OCT."

Comment 6:
Exposure to water is detrimental to RNA quality, and the highest level of aqueous exposure of this protocol is 50% during cresyl violet staining. Some methods use cresyl violet in 70% ethanol, would it be better? And is there assay to test the concentration of ethanol?
Response
We have tested that a quick staining (30 s) with cresyl violet in 50% ethanol gives a distinguishable color to cartilage and does not lower the RNA integrity for downstream analysis such as low input RNA-seq, similar to a previous study (Kolijn, K. & Van Leenders, G. J. L. H, 2016, Reference 39). We added Figure 3C to demonstrate this.
Changes in Manuscript:
Figure 3C, "RINs of total RNA from mandibular bone samples stained with cresyl violet (n=4) or without staining (n=4)."
Line 363, "We have tested that a quick staining (30 s) with 0.1% cresyl violet in 50% ethanol gives a distinguishable color to cartilage and does not lower the RNA integrity for downstream analysis such as low input RNA-seq (**Figure 3C** and **Figure 4**)."

Comment 7:
What's the limited amounts of cells for RNA extraction of this method?
Response
The average yield from one cell is approximately 5 pg RNA per cell, and 1-5 ng of total RNA can be extracted from 200-1,000 cells, which can be used for low-input RNA-seq. This has been added to the discussion.
Changes in Manuscript:
Line 366, "The yield/area (ng/mm^2) is approximately 20 ng/mm^2 (**Figure 3F**), similar to or higher than previous optimized methods[33]. According to the cell densities in MC and mandibular bone[6], we estimate that with this protocol the average yield from one cell is approximately 5 pg RNA per cell, and 1-5 ng of total RNA can be extracted from 200-1,000 cells, which can be used for low-input RNA-seq[6,7,33]."

Comment 8:
To improve the usability of the protocol, describing the new finding of RNA-seq analysis and highlighting the advantages of this protocol would be necessary.
Response

We have referred to two of our previous studies using the protocol for different tissues and ages, and discussed the advantages of this protocol:

6. Motch Perrine, S. M., Wu, M., et al. Mandibular dysmorphology due to abnormal embryonic osteogenesis in FGFR2-related craniosynostosis mice. Disease Models & Mechanisms 12 (5), dmm038513 (2019).
7. Holmes, G., O'Rourke, C., et al. Midface and upper airway dysgenesis in FGFR2-craniosynostosis involves multiple tissue-specific and cell cycle effects. Development 145 (19), dev.166488 (2018).

Changes in Manuscript:

Line 408, "this protocol provides details of optimized LCM of cartilages and bones, highlighting the use of cresyl violet staining in a rapid procedure to visualize cartilage and bone for precise tissue collection while maintaining high RNA integrity for subsequent analysis by RNA-seq. This protocol has been successfully used for LCM of cartilage and bone at different stages for gene expression analysis[6, 7], and also can be used for other tissues."