## 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.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
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.

**Comment 6:**
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.);
Paragraph 4 and 5: significance with respect to existing methods, future applications.

**Comment 7:**
Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Superfrost Plus, etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
**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."