Journal of Visualized Experiments

Tissue Preparation and Immunostaining of Mouse Craniofacial Tissues and Undecalcified Bone

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59113R2
Full Title:	Tissue Preparation and Immunostaining of Mouse Craniofacial Tissues and Undecalcified Bone
Keywords:	Developmental biology, Transgenic mice, BMP signaling, Immunostaining, Craniofacial morphogenesis, Undecalcified hard tissues, Quantification
Corresponding Author:	Yuji Mishina UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	mishina@umich.edu
Order of Authors:	Yuji Mishina
	Jingwen Yang
	Haichun Pan
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	University of Michigan School of Dentistry Dept. of Biologic and Materials Sciences 4222A Dental 1011 N. University Ave. Ann Arbor, MI 48109-1078

TITLE:

Tissue Preparation and Immunostaining of Mouse Craniofacial Tissues and Undecalcified Bone

AUTHORS & AFFILIATIONS:

Jingwen Yang[1,2,*], Haichun Pan[2,*], and Yuji Mishina[2]

[1]The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory for Oral Biomedicine of Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, Hubei, China

[2]Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

[*]These authors contributed equally to this study.

Corresponding Author:

Yuji Mishina (mishina@umich.edu)

Email Addresses of Coauthors:

Jingwen Yang (jingwen.yang@whu.edu.cn, jingweny@umich.edu)

Haichun Pan (haichun@umich.edu)

KEYWORDS:

developmental biology, transgenic mice, BMP signaling, immunostaining, craniofacial morphogenesis, undecalcified hard tissues, quantification

SUMMARY:

Here, we present a detailed protocol to detect and quantify protein levels during craniofacial morphogenesis/pathogenesis by immunostaining using mouse craniofacial tissues as examples. In addition, we describe a method for preparation and cryosectioning of undecalcified hard tissues from young mice for immunostaining.

ABSTRACT:

Tissue immunostaining provides highly specific and reliable detection of proteins of interest within a given tissue. Here we describe a complete and simple protocol to detect protein expression during craniofacial morphogenesis/pathogenesis using mouse craniofacial tissues as examples. The protocol consists of preparation and cryosectioning of tissues, indirect immunofluorescence, image acquisition, and quantification. In addition, a method for preparation and cryosectioning of undecalcified hard tissues for immunostaining is described, using craniofacial tissues and long bones as examples. Those methods are key to determine the protein expression and morphological/anatomical changes in various tissues during craniofacial morphogenesis/pathogenesis. They are also applicable to other tissues with appropriate modifications. Knowledge of the histology and high quality of sections are critical to draw scientific conclusions from experimental outcomes. Potential limitations of this methodology

include but are not limited to specificity of antibodies and difficulties of quantification, which are also discussed here.

INTRODUCTION

The face is a key part of human identity, and is composed of several different types of tissues, such as epithelium, muscle, bone, cartilage, tooth. Those tissues are derived from all three germ layers: ectoderm, endoderm, and mesoderm[1,2]. For proper patterning and development of craniofacial tissues, cell proliferation, death and differentiation need to be highly coordinated and regulated by specific signaling pathways, such as Wnt, Fgf, Hh and Bmp pathways[3–5]. Defects in proliferation, survival or differentiation of cells will lead to craniofacial malformations, which are among the most frequently occurring congenital birth defects. Transgenic mice are useful tools to study mechanisms of craniofacial morphogenesis and pathogenesis[1–5]. Understanding the changes in craniofacial structures during development and pathogenesis will help to clarify key developmental principles as well as the mechanisms of craniofacial malformations[1–5].

The staining of whole mount or sectioned tissues with specific antibodies is an invaluable technique for determining spatial distribution of proteins of interest [6]. Formally, tissue immunostaining can rely either upon immunohistochemistry (IHC) or immunofluorescence (IF). Compared with the opaque reaction product generated with a chromogenic substrate such as 3,3'-Diaminobenzidine (DAB) by IHC, IF involves the use of fluorescent conjugates visible by fluorescence microscopy. Therefore, IF may clearly differentiate positive cells from background noise, and allows images to be quantitatively analyzed and enhanced in a straightforward fashion by software such as ImageJ and Adobe Photoshop[7,8]. The whole mount staining approach works on small blocks of tissue (less than 5 mm thick), which can provide three-dimensional information about the location of proteins/antigens without the need for reconstruction from sections[9,10]. However, compared with tissue sections, whole mount immunostaining is time consuming and requires large volumes of antibody solutions. Not all antibodies are compatible with the basic whole mount approach. In addition, the incomplete penetration of antibodies will result in uneven staining or false negative staining. Here we will focus on the immunofluorescence detection of proteins/antigens on sectioned tissues. For hard tissues (eg, head, tooth, long bone), calcium deposition during development/pathogenesis makes the sample difficult to section and easily rinsed off during immunostaining treatment[11,12]. Most of the currently available protocols decalcify hard tissues before embedding to make sectioning easier, which is time consuming and can destroy morphology and antigens of samples if handled improperly[11,12]. To overcome the issues, we optimized an approach for cryosectioning of hard tissues without decalcification, leading to improved visualization of their morphology and distribution of signaling proteins.

The protocol described here is being used to determine morphometric and histological changes in the craniofacial tissues of BMP transgenic mice. Specifically, the protocol includes (1) harvesting and dissecting head tissues, (2) section and immunostaining of experimental markers (Ki67, pSmad1/5/9) along with TUNEL staining, (3) imaging the sections using fluorescence microscope, and finally (4) analyzing and quantifying the results. The protocol to prepare and cryosection hard tissues without decalcification is also described[13]. Those methods are optimized

for craniofacial tissues. They are also applicable to other tissues from various ages of samples with appropriate modifications.

PROTOCOL:

All mouse experiments were carried out in accordance with University of Michigan guidelines covering the humane care and use of animals in research. All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan (Protocol #PRO00007715).

1. Tissue preparation

1.1. Preparation of embryonic tissues

1.1.1. Prepare one 10 cm dish and several 3.5 cm dishes containing phosphate buffered saline (PBS), and one 12-well culture plate containing 2 mL 4% paraformaldehyde (PFA) in PBS in each well for each pregnant mouse. Place all petri dishes and the plate on ice.

NOTE: Handle 4% PFA in a fume hood.

1.1.2. Dissect embryos from pregnant mice in ice-cold PBS with forceps and scissors as previously described[14].

1.1.2.1. Briefly, euthanize a pregnant mouse with CO_2, grab the skin below the center of the belly with forceps and cut through the skin only, then gently pull at the skin to separate it from the underlying the abdominal muscle wall.

1.1.2.2. Next, cut into the abdominal cavity following the same line of the skin incision. Remove the uterus containing a string of embryos and remove the embryos by gently cutting away the uterine wall. The extraembryonic tissues such as the yolk sac and amnion will be removed.

1.1.2.3. Cut and isolate head from each embryo.

1.1.3. Transfer each head into each well of a 12-well plate containing 4% PFA with a plastic transfer pipette or forceps. Fix samples in 4% PFA at 4 °C for 4 h. Rinse samples in PBS at 4 °C with gentle shaking for 12 h.

NOTE: For embryos younger than embryonic day 16.5 (E16.5), fix embryo heads with 4% PFA directly after isolation. For embryos at E16.5 or later, remove to discard skin and adipose tissue from the heads and rinse several times in ice cold PBS before fixation.

1.1.4. Cryoprotect heads.

1.1.4.1. Transfer each head into a new 12-well plate containing 2 mL of 30% sucrose in PBS using a plastic transfer pipette or forceps. Agitate gently at 4 °C until the head sinks to the bottom of the dish.

1.1.5. Embed heads.

1.1.5.1. Transfer the cryoprotected head into a mold containing Optimal Cutting Temperature (OCT) compound. Equilibrate samples in OCT for several minutes. Adjust the location and direction of samples with forceps.

1.1.5.2. Place the mold on dry ice to freeze. Store resulting cryomolds in a plastic bag at -80 °C until ready for cryosectioning.

NOTE: The trimmed side of the samples must face the bottom of the embedding mold.

1.2. Preparation of postnatal undecalcified hard tissues

1.2.1. Euthanize at 3-week or 3-month old mouse with CO_2. Remove the skin and adipose tissues. Cut and isolate the head or long bones from the mouse.

1.2.2. Fix and cryoprotect the head or long bone of mice as described in steps 1.1.3–1.1.4.

1.2.3. Embed in 8% gelatin in a similar manner as step 1.1.5. Keep the cryomolds in a plastic bag at -80 °C until cryosectioning.

NOTE: Decalcification is not necessary here. To prepare 8% gelatin, mix 8 g of gelatin with 100 mL of PBS and boil using a microwave. Be aware that the mixture boils over easily.

2. Cryosectioning

2.1. Set cryostat temperature to -18 °C for soft tissues embedded in OCT or -25 °C and lower for undecalcified hard tissues embedded in gelatin. Keep samples in the cryostat chamber for about 30 min to equilibrate to the cryostat temperature.

2.2. Expel the block from the cryomold. Freeze the block onto the specimen chuck (tissue holder) via mounting with an OCT drop. Keep the trimmed side of the sample furthest from the chuck (facing the operator).

2.3. Load the block-mounted chuck onto the cryostat object holder. Adjust the blade holder to make the angle of the blade 3°–5° relative to the sample.

2.4. Collect 10 µm sections onto coated microscope slides. Dry sections completely at RT, then store them at -80 °C.

3. Histological staining and microscopic imaging

3.1. Immunofluorescence staining

3.1.1. Take out slides from -80 °C. Keep slides at RT for 1 h to airdry sections. Rinse slides in 0.1% PBST (0.1% Polyethylene glycol tert-octylphenyl ether in PBS; see **Table of Materials**) three times for 5 min each to wash out OCT and permeabilize sections.

3.1.2. Optionally, perform antigen retrieval (optional).

3.1.2.1. Preheat citrate buffer (10 mM sodium citrate pH 6) in the staining dish with steamer or water bath to 95–100 °C. Immerse slides in the citrate buffer, incubate for 10 min.

3.1.2.2. Take the staining dish out from steamer or water bath to RT. Cool the slides at RT for 20 minutes or longer[15].

NOTE: As alternatives, use Tris-EDTA buffer (10 mM Tris base, 1mM EDTA, 0.05% Tween 20, pH 9.0) or EDTA buffer (1 mM EDTA, 0.05% Tween 20, pH 8.0) for heat-induced antigen retrieval. Use a pressure cooker, microwave, or water bath for heat-induced antigen retrieval, in addition to the hot steamer. An enzyme-induced antigen retrieval using trypsin or pepsin is another alternative. Optimize the concentration and treatment time of enzymatic retrieval to avoid damaging sections. Optimize the antigen retrieval method for each antibody/antigen combination.

3.1.3. Incubate each slide with 200 µL of blocking solution (5% donkey serum diluted in 0.1% PBST) at RT for 30 min, then remove the blocking solution without rinse.

3.1.4. Incubate each slide with 100 µL of primary antibody or antibodies diluted in blocking solution for 1 h at RT or O/N at 4 °C. Rinse slides with PBS three times for 10 min each at RT.

3.1.5. Incubate each slide with 100 µL of secondary antibody diluted in blocking solution for 1 h at RT. Rinse slides in PBS three times for 10 min each at RT. Protect slides from light.

3.1.6. Mount slides.

3.1.6.1. Add two drops of anti-fade medium with DAPI (4', 6-diamidino-2-phenylindole) on the slide. Then cover with a coverslip.

3.1.6.2. Store at 4 °C in dark until ready to image.

NOTE: As an alternative, label the nuclei with DAPI or Hoechst 33324 dye diluted 1:2,000 in PBS at RT first, then mount with glycerol.

3.2. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

NOTE: Double-stranded DNA with 3’-hydroxyl termini (3'OH DNA termini) will form during apoptosis in the cell. Here, we provide a protocol that label the free 3'OH DNA termini in situ via labeling DNA fragments with the digoxigenin-nucleotide utilizing terminal deoxynucleotidyl transferase (TdT) by specific staining using a commercial kit (see **Table of Materials**).

3.2.1. Optionally, stain sections with primary and Alexa Fluor-488 labeled secondary antibodies prior to the TUNEL staining. Rinse the slides in PBS three times for 10 min each.

NOTE: This step is optional for a double staining of a protein and TUNEL in the same slide.

3.2.2. Incubate each slide with 100 µl Proteinase K (10 µg/mL in 10 mM Tris pH 7.5 and 5 mM EDTA) for 5 min at RT. Rinse slides with PBS three times for 10 min each at RT.

NOTE: Adjust the incubation time and temperature of Proteinase K for each tissue type. For 10 µm sections of embryo heads fixed in 4% PFA, incubate for 5 min at RT. In addition to the method using Proteinase K, use alternative treatments as needed, including (1) freshly prepared 0.1% Polyethylene glycol tert-octylphenyl ether, 0.1% sodium citrate, 10 min at 37 °C; (2) 0.25%–0.5% Pepsin in HCl (pH 2) or 0.25% trypsin, 10 min at 37 °C; and (3) microwave irradiation with 0.1 M citrate buffer (pH 6).

3.2.3. Apply 200 µL of blocking solution (5% donkey serum diluted in 0.1% PBST) to each slide, incubate at RT for 30 min, tap off the blocking solution without rinse.

3.2.4. Apply 50 µL of the equilibration buffer supplied by the kit to each slide at RT for at least 10 seconds. Tap off the buffer without rinse.

3.2.5. Prepare reaction mixture (working strength TdT enzyme) by mixing TdT Enzyme with the reaction buffer supplied by the kit at the ratio of 3:7. Apply 50 µL of the reaction mixture to each slide, and incubate at 37 °C for 1 h. Tap off the buffer without rinsing.

3.2.6. Apply 200 µL of the stop buffer (1:30 diluted in ddH_2O) supplied by the kit to each slide, then incubate at RT for 10 min. Rinse slides with PBS three times for 10 min each.

3.2.7. Label with Rhodamine antibody.

3.2.7.1. Apply 50 µL of pre-warmed (RT) anti-digoxigenin conjugate (rhodamine) (1:1 diluted in blocking solution) to each slide. Incubate at RT for 30 minutes in dark.

3.2.7.2. Rinse slides with PBS three times for 10 min each. Mount slides as step 3.1.6.

4. Imaging acquisition

4.1. Use positive controls (tissues positive for the target antigen) to check the signal labeling and negative controls (omit the primary antibody, isotype control, or tissues negative for the target antigen) to evaluate the background of images under the fluorescent microscope.

4.2. Set the equipment and camera conditions (exposures and other general settings) for imaging based on the signal intensity of negative and positive controls.

NOTE: These conditions vary by (1) cameras and microscopes used for imaging, (2) antibodies, and (3) tissues for each experiment. Common conditions used for craniofacial tissues are ISO 200 with an exposure time ranging from 1/100 s to 1 s depend on the quality and specificity of antibodies. Appropriate magnifications vary depending on the size of the samples and purpose of experiments.

4.3. Acquire images with conventional epifluorescence microscope or confocal microscope. Acquire images (including those of corresponding controls) in the same conditions for each color channel. Save images with the same format (tiff is best to preserve information).

5. Fluorescence quantification

NOTE: Statistically comparing the staining between different groups will be more informative in many cases. With the immunofluorescence images, quantify the relative level of the protein by measuring signal density, counting positive cells, or calculating positive areas. For statistical analysis, the minimum number of biologically independent samples is 3. A typical method is to generate at least three sections from each sample and take images for at least three representative areas in each section.

5.1. Quantification of fluorescence intensity using ImageJ

5.1.1. Open the software, and use **Analyze** > **Set Measurements** to check that only **Area** and **Integrated Density** are selected. Use **File** > **Open** to open images to be analyzed.

5.1.2. Use **Toolbar** to select either the square or circle icon on the far left. Select the area to be analyzed on the image using the selection tool. Use **Analyze** > **Measure** to get the readout of the selected area and integrated density in the Results window. Select a region next to a positive cell that has no fluorescence to read out the background.

5.1.3. Repeat step 5.1.2 to analyze other images. Adjusted the area to be analyzed to match with that of the first image.

5.1.4. Copy all the data in the Results window and paste into a spreadsheet when finished analyzing.

5.1.5. Calculate the corrected fluorescence intensity (CTCF) as Integrated Density - (Area of selected cell x Mean fluorescence of background readings). Compare the difference of the corrected total cell fluorescence between samples and make a graph.

5.2. Quantification of the positive cell number of fluorescent images using ImageJ

5.2.1. Manual cell counting.

5.2.1.1. Use **ImageJ** > **Plugins** > **Analysis** to install the **Cell Counter** plugin.

5.2.1.2. Use **File** > **Open** to open images to be analyzed. Use **Plugins** > **Analysis** > **Cell counter** to open the Counter window and the Results window.

NOTE: Cell counter does not work on stacks. For counting stacks, plugin **Plot Z Axis Profile**, then use **Image** > **Stacks** > **Plot Z Axis Profile** to monitor the intensity of a moving ROI using a particle tracking tool. This tool can be either manual or automatic.

5.2.1.3. Clicking one of buttons at the bottom of the Counter window to initiate counting. Click directly on a cell/object to be count until finishing.

5.2.1.4. Click the **Results** button in the **Count** window. The total number of cells counted will be shown in the **Results** window. Save the result log as spreadsheet and analyze.

5.2.2. Automated cell counting.

5.2.2.1. Use **File** > **Open** to open images to be analyzed. Convert the RGB image into a grey scale image before proceeding.

5.2.2.2. Use **Image** > **Adjust** > **Threshold** to select all the areas that need to be counted.

5.2.2.3. Use **Analyze** > **Analyze Particles** to get the number of cells/particles. Set a range of the valid particle size (e.g., 100-Infinity) instead of the default of 0-Infinity to count cells/particles within a specific range. Save the result log as spreadsheet and analyze.

NOTE: To get other information from the image, besides area, go to **Analyze** > **Set Measurements** and select the box next to the information needed.

REPRESENTATIVE RESULTS

Embryonic craniofacial tissue sections

Following the above steps, heads were dissected from control (*P0-Cre*) or mutant (constitutively activated Bmpr1a in neural crest cells, *P0-Cre; caBmpr1a*) embryos at embryonic day (E) 16.5 or 18.5. After fixing in 4% PFA for 4 h, samples were embedded in OCT and cryosectioned coronally. Resulted sections were immunostained with antibodies against pSmad1/5/9 (downstream BMP

signaling factors) or Ki67 (a cell proliferation marker) without antigen retrieval according to the protocol. As shown, pSmad1/5/9 (**Figure 1A**) and Ki67 (**Figure 1C**) were positive in the frontal bones of control embryos. In mutant embryos, the levels of pSmad1/5/9 was increased (**Figure 1B**), while those of Ki67 was decreased (**Figure 1D**) in the frontal bones. Cell death in those samples were also checked according to the protocol. As shown, more apoptotic cells were observed in the frontal bones of mutant embryos than those of control embryos (**Figure 1E,F**).

Undecalcified craniofacial tissues or long bone sections

Following the above steps for undecalcified hard tissues, heads from 3-week old mice (*P0-Cre; mTmG* (*membrane-tomato and membrane GFP*)) were fixed with 4% PFA and embedded in 8% gelatin. Coronal cryosections were washed with PBST and mounted with anti-fade medium with DAPI. **Figure 2A,B** demonstrate that gelatin does not interfere with fluorescent signals from sectioned tissues.

Heads and femora from 3-week-old or 3-month-old mice were employed to check whether gelatin embedded undecalcified tissues are good for IF. The whole heads and femora were processed and sectioned according to the protocol. The resulted sections were used for SOX9 immunostaining (**Figure 3**) or OSX and E11/Podoplanin double immunostaining (**Figure 4**). As shown, good quality sections were obtained from most of the 3-week hard tissues, including the trabecular and the cortical compartments of the femur (**Figure 3A,B**, **Figure 4A–D**), the frontal bones (**Figure 4E,F**), the incisor (**Figure 3E,F**, **Figure 4I,J**), nasal tissues (**Figure 3C,D**), and the skull including the nasal-premaxilla suture and surrounding bones (**Figure 4G,H**) of the head. While, with 3-month-old samples, good quality sections were only obtained in some of the hard tissues, including the trabecular compartments of the femur (**Figure 3G,H**, **Figure 4K,L**), nasal tissues (**Figure 3I,J**), and the skull including the nasal-premaxilla suture and surrounding bones (**Figure 4M,N**) of the head. As shown in **Figure 3**, SOX9 positive cells were detected specifically in the chondrocytes of the growth plate (**Figure 3B**) and the joint (**Figure 3H**) from the femur, and the nasal septum (**Figure 3D,J**). In the 3-week-old incisor, SOX9 was detected in the mesenchymal cells (**Figure 3F**). OSX and E11 double staining results showed that OSX was detected in osteoblasts, while E11 was detected in osteocytes of bones from the femur and the head (**Figure 4B,D,H,L,N**). In the 3-week incisor, OSX was positive in odontoblasts, while E11 was positive in follicle mesenchymal cells (**Figure 4J**). Those results indicate that undecalcified hard tissues embedded with gelatin well-preserve antigen functions.

FIGURE LEGENDS:

Figure 1: Examples of IF results of pSmad1/5/9, Ki67 or TUNEL in control embryos and mutant embryos with enhanced BMP activity. Constitutively activated *Bmpr1a* (*caBmpr1a*) mice were crossed with *P0-Cre* mice to increase BMP signaling activity in neural crest cells (NCCs). Heads of control (*P0-Cre; caBmpr1a*$^{+/+}$) and mutant (*P0-Cre; caBmpr1a*$^{fx/+}$) embryos were dissected at E16.5 or E18.5, fixed with 4% PFA for 4h, cryoprotected with 30% sucrose for 1 day, embedded in OCT, and cryosectioned at -18 °C. Sections of the frontal bone (similar level with the eye) were used for immunodetection against pSmad1/5/9, Ki67, or TUNEL staining. (**A, B**) pSmad1/5/9 (green) staining patterns in the frontal bones of control (**A**) or mutant (**B**) embryos at E16.5. (**C,**

D) Ki67 (green) staining patterns in the frontal bones of control (**C**) or mutant (**D**) embryos at E18.5. (**E, F**) TUNEL (red) staining patterns in the frontal bones of control (**E**) or mutant (**F**) embryos at E18.5. Nuclei were stained with DAPI (blue). FB = frontal bone, B = brain. Scale bars = 100 µm.

Figure 2: Examples of mTmG reporter signal results of undecalcified tissues in the head. Heads from 3-week old *P0-Cre* mice with *membrane-tomato and membrane GFP* (*mTmG*) reporter were dissected, fixed with 4% PFA for 4h, cryoprotected with 30% sucrose for 2 days, embedded in 8% gelatin, and cryosectioned at -25 °C. Head sections clearly show GFP (green, Cre recombination positive) and Tomato (red, Cre recombination negative) signal in the nasal bone and nasal tissues (**A, B**). Nuclei were stained with DAPI (blue). NB = nasal bone, N = nasal tissues, NS = nasal septum. Scale bars = 250 µm.

Figure 3: Examples of SOX9 immunostaining results of undecalcified tissues in the head and the femora. Heads and femora were dissected from 3-week or 3-month old mice, fixed with 4% PFA for 4h, cryoprotected with 30% sucrose for 2 days, embedded in 8% gelatin, and cryosectioned at -25 °C. Slides were used for immunodetection against SOX9 (red). Nuclei were stained with DAPI (blue) (**B, D, F, H, J**). Adjacent sections of those tissues were used for Hematoxylin & Eosin (H&E) staining (**A, C, E, G, I**). Arrow heads in **A** and **B** indicate growth plate and in **G** and **H**, articular cartilage. Arrows in **A** and **G** indicate trabecular bones and in **C, D, I,** and **J**, nasal septum. DM = dental mesenchyme, DE = dental epithelium, FM = follicle mesenchyme. Scale bars = 50 µm.

Figure 4: Examples of OSX and E11 double immunostaining results of undecalcified tissues in the head and the femora. Heads and femora were dissected from 3-week-old or 3-month-old mice, fixed with 4% PFA for 4h, cryoprotected with 30% sucrose for 2 days, embedded in 8% gelatin, and cryosectioned at -25 °C. Sections were used for double immunostaining with antibodies against OSX (Red) and E11/Podoplanin (Green). Nuclei were stained with DAPI (blue) (**B, D, F, H, J, L, N**). Adjacent sections of those tissues were used for H&E staining (**A, C, E, G, I, K, M**). Arrows in **A, B, K,** and **L** indicate trabecular compartments of the femur; **C** and **D**, cortical compartments of the femur; and in **E** and **F**, the frontal bones. Arrowheads in **A** and **B** indicate growth plate. BM = bone marrow, N = nasal tissues, DM = dental mesenchyme, DE = dental epithelium, FM = follicle mesenchyme, NPS = nasal premaxilla suture. The frontal bones (**E, F**) and the nasal-premaxilla suture and surrounding bones (**G, H, M, N**) are also shown. Scale bars = 50 µm.

DISCUSSION:

Here we provide a detailed protocol for preparation of mouse head and undecalcified bone tissues, and cryosectioning for immunostaining of cell proliferation, cell death, and BMP signaling markers. We also detail the strategy for obtaining quantitative data from immunofluorescent images. Those methods can also be applicable to other tissues with appropriate modifications.

Conditions for tissue preparation vary by the size and type of tissues. The fixation and cryoprotection time usually need several hours to overnight. After fixation, the tissue can also be embedded in paraffin and sectioned with a microtome[16]. Though both paraffin and OCT work

well for immunostaining, there are some differences between them. Paraffin blocks can be kept for multiple years at RT, while OCT blocks are for 1 year at -80 °C. Paraffin preserves tissue morphology, while ice crystal formed during OCT embedding may negatively affect tissue structures. Paraffin sometimes masks epitopes of antigens, while OCT preserves enzyme activities and antigen epitopes. Therefore, there is no need of antigen retrieval for most of the antibodies if fixed in 4% PFA for only 4 h or less and embedded in OCT. It is, however, still possible to get better results by antigen retrieval, if positive controls did not show good staining results in cryosections.

Both Hoechst dye and DAPI can be used for nuclear counter-staining. They have similarities, as both (1) are UV-excited, minor groove-binding chemicals to emit signals proportional to total DNA content, and (2) are subjected to photo-bleaching after a long exposure. However, Hoechst dyes are typically used for staining DNA content in live cells due to their high permeability. DAPI is typically used for staining DNA in fixed cells due to its low membrane permeability. In addition, DAPI generates a stronger and more stable signal than Hoechst.

Proper controls are essential for IF. The specificity of every new antibody should be confirmed by Western blot analysis, if applicable. The optimal working concentration of a particular primary antibody should be determined through the use of serial dilutions. A positive control (tissue or cell which is proved to express the protein/antigen) should be included to check the IF process and specificity of antibody. A negative control should also be included, e.g., absence of the primary antibody, or the substitution of normal IgG from the same species for the primary antibody, or tissues negative for the target antigen. When taking pictures, the sample without secondary antibodies (background control) should be examined independently with each channel to set the limits of signal gain and offset to be adapted for the final imaging. For detection of multiple labels, background control and single-labeled controls need to be prepared to avoid spectral overlap artifacts. All channels that will be used to obtain an image of a multiple-label sample must be subjected to independent background correction, because the level of autofluorescence in each channel varies substantially.

We also provide the protocol for preparation and cryosectioning of undecalcified hard tissues embedded in gelatin. For the OCT embedded decalcified hard tissues, most of the hard tissue sections will be detached from slide glasses during immunostaining procedures, because of their low adhesion character on the slide. The adhesive tape designed to facilitate cryosectioning helps to generate good quality sections. But, those sections are easily damaged when the tape is peeling off. For gelatin embedded tissues, there is no need for a tape-transfer system to generate good quality sections. As an embedding medium, gelatin can infiltrate the sample well, although it has a lower viscosity compared with OCT. Gelatin has been used in other histological applications, such as brain tissue[17,18] and ultrathin sections of cells for immunocytochemistry[19]. Here, gelatin was used to embed undecalcified bone, which generates blocks easier to cryosection than OCT. There are several small tips to get good sections of gelatin embedded undecalcified hard tissues. The critical step is to embed with gelatin instead of OCT. To get better penetration, keep samples in 30% sucrose one more day after samples sink to the bottom. It is equally important to set the temperature lower than usual at about -25 °C. An ultra-sharp blade

is not necessary. Although a lower cryo temperature (-25 °C) makes some improvements for cryosectioning of OCT embedded undecalcified hard tissues, it is still difficult to get a good integrity of tissue structures. As shown in **Figure 2**, **Figure 3**, and **Figure 4**, good quality sections applicable for immunostaining were obtained from gelatin embedded hard tissues (eg, trabecular bones, cortical bones, skull bones, nasal tissues, and incisor). Those results proved that gelatin embedding significantly improves the specimen integrity of hard tissue sections, but also enhances adhesion of the sections to slide glasses. In addition, gelatin preserves antigen functions, and exhibits compatibility with fluorescent signals and immunostaining. However, this technique only works well for up to 3-month-old samples. Potential improvements of this method are (1) to dissect the samples further to separate the target tissue from other parts to make the structure of the tissue simple (in the case of teeth, the mandible or maxilla should be dissected and fixed instead of the whole head) and (2) to use 10% EDTA to decalcify tissues for only 2–3 days before cryoprotection. This short time of decalcification will not compromise the immunostaining results. Another concern is that, as a non-aqueous embedding media, gelatin cannot be easily removed from slides, which may lead to higher background depending on staining methods (e.g., H&E staining).

Immunostaining results are not easy to quantify, so they are usually used semi-quantitatively. Difficulties and limitations of the quantification of immunostaining of craniofacial tissues include but are not limited to the following: (1) it is difficult to define the area to be counted due to the complexity of the structure of craniofacial tissues; (2) it is difficult to define the labeled area or labeled cells due to the non-linear nature of immunostaining; (3) there is limited information for the dynamic range of the signal; (4) it is hard to compare the intensity of signals between images or groups due to the fading of fluorescent signal during image acquisition; and (5) the signal background may change significantly among antibodies, slides, and samples. To increase the reliability of the quantification results, the experiment should be carefully and strictly performed. All samples should be processed under the same conditions. During immunostaining, various controls are required to evaluate signal background and define the positive area or cells for the signal. Take convincing and representative images to clearly show the area to be counted and labeled cells with good contrast. In addition, during image acquisition, the camera setting and equipment setting must be kept consistent.

Taken together, we present a simple standard protocol for immunofluorescence on mouse craniofacial tissues, especially for undecalcified hard tissues. Immunostaining analysis of craniofacial tissues will not only help to understand the mechanism of morphogenesis during development, but also illustrate the changes during pathogenesis. In addition, immunostaining can also be used to study the expression pattern of other signaling pathway ligands, receptors, or other phenotypic markers, besides cell proliferation, cell death, and the BMP signaling pathway. However, the critical steps of an immunostaining experiment must be modified appropriately for each antigen/antibody or tissue to get specific staining and minimized non-specific background signals.

ACKNOWLEDGMENTS:

This work was supported by the National Institutes of Health (R01DE020843 to Y.M.), the International FOP Association (Y.M.), and a grant-in-aid from the National Natural Science Foundation of China (31500788 to J.Y.).

DISCLOSURE:

The authors have nothing to disclose.

REFERENCES:

1. Trinh, Le A., Fraser, S.E. Imaging the cell and molecular dynamics of craniofacial development: challenges and new opportunities in imaging developmental tissue patterning. *Current Topics in Developmental Biology.* **115**, 599–629 (2015).
2. Marcucio, R. et al. Facial morphogenesis: physical and molecular interactions between the brain and the face. *Current Topics in Developmental Biology.* **115**, 299–320 (2015).
3. Graf, D. et al. Common mechanisms in development and disease: BMP signaling in craniofacial development. *Cytokine & Growth Factor Reviews.* **27**, 129–139 (2016).
4. Snider, T. N., Mishina, Y. Cranial neural crest cell contribution to craniofacial formation, pathology, and future directions in tissue engineering. *Birth Defects Research Part C: Embryo Today.* **102** (3), 324–332 (2014).
5. Mishina, Y., Snider, T. N. Neural crest cell signaling pathways critical to cranial bone development and pathology. *Experimental Cell Research.* **325** (2), 138–147 (2014).
6. Van Hecke, D. Routine Immunohistochemical Staining Today: Choices to Make, Challenges to Take. *Journal of Histotechnology.* **1**, 45–54 (2002).
7. Xiao, C., Dan-Bi, C. Double staining immunohistochemistry. *North American Journal of Medical Sciences.* **2**, (5), 241–245 (2010).
8. Zongli, Q. et al. Comparison of immunofluorescence and immunohistochemical staining with anti-insulin antibodies on formalin-fixed paraffin-embedded human pancreatic tissue microarray sections. *International Journal of Clinical and Experimental Pathology.* **10**, (3), 3671–3676 (2017).
9. Montgomery, S. C., Cox, B. C. Whole mount dissection and immunofluorescence of the adult mouse cochlea. *Journal of Visualized Experiments.* (107), e53561 (2016).
10. Dun, X. P., Parkinson, D. B. Whole mount immunostaining on mouse sciatic nerves to visualize events of peripheral nerve regeneration. *Methods in Molecular Biology.* **1739**, 339–348 (2018).
11. Akkiraju, H. et al. An Improved Immunostaining and Imaging Methodology to Determine Cell and Protein Distributions within the Bone Environment. *Journal of Histochemistry & Cytochemistry.* **64**, (3), 168–178 (2016).
12. González-Chávez, S. A. et al. Assessment of different decalcifying protocols on Osteopontin and Osteocalcin immunostaining in whole bone specimens of arthritis rat model by confocal immunofluorescence. *International Journal of Clinical and Experimental Pathology.* **6** (10), 1972–1983 (2013).
13. Kapelsohn, K. Improved Methods for Cutting, Mounting, and Staining Tissue for Neural Histology. *Protocol Exchange.* (2015).
14. Kalaskar, V. K., Lauderdale, J. D. Mouse embryonic development in a serum-free whole embryo culture system. *Journal of Visualized Experiments.* (85), e50803 (2014).

15. Shi, S. R. et al. Antigen retrieval techniques: current perspectives. *Journal of Histochemistry & Cytochemistry.* **49**, (8), 931–937 (2001).
16. Adell, T. et al. Immunohistochemistry on paraffin-embedded planarian tissue sections. *Methods in Molecular Biology.* **1774**, 367–378 (2018).
17. Griffioen, H. A. et al. Gelatin embedding to preserve lesion-damaged hypothalami and intracerebroventricular grafts for vibratome slicing and immunocytochemistry. *Journal of Neuroscience Methods.* **43**, 43–47 (1992).
18. Sarkar, S. et al. In situ demonstration of Fluoro-Turquoise conjugated gelatin for visualizing brain vasculature and endothelial cells and their characterization in normal and kainic acid exposed animals. *Journal of Neuroscience Methods.* **219** (2), 276–284 (2013).
19. Oorschot, V. et al. A novel flat-embedding method to prepare ultrathin cryosections from cultured cells in their *in situ* orientation. *Journal of Histochemistry & Cytochemistry.* **50**, 1067–1080 (2002).

Figure 1

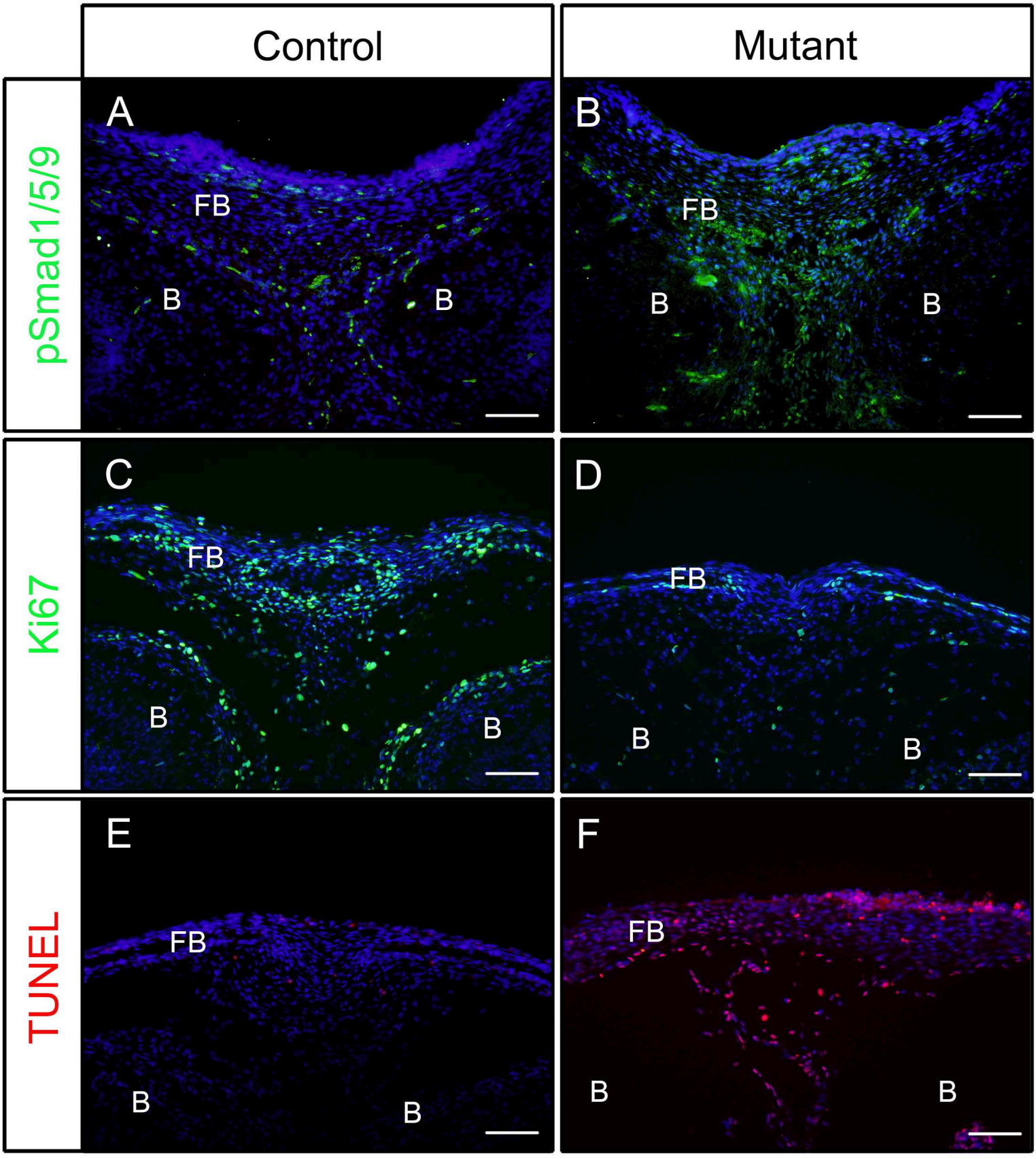

Figure 2

Click here to access/download;Figure;Revised Figure 2_Final.eps

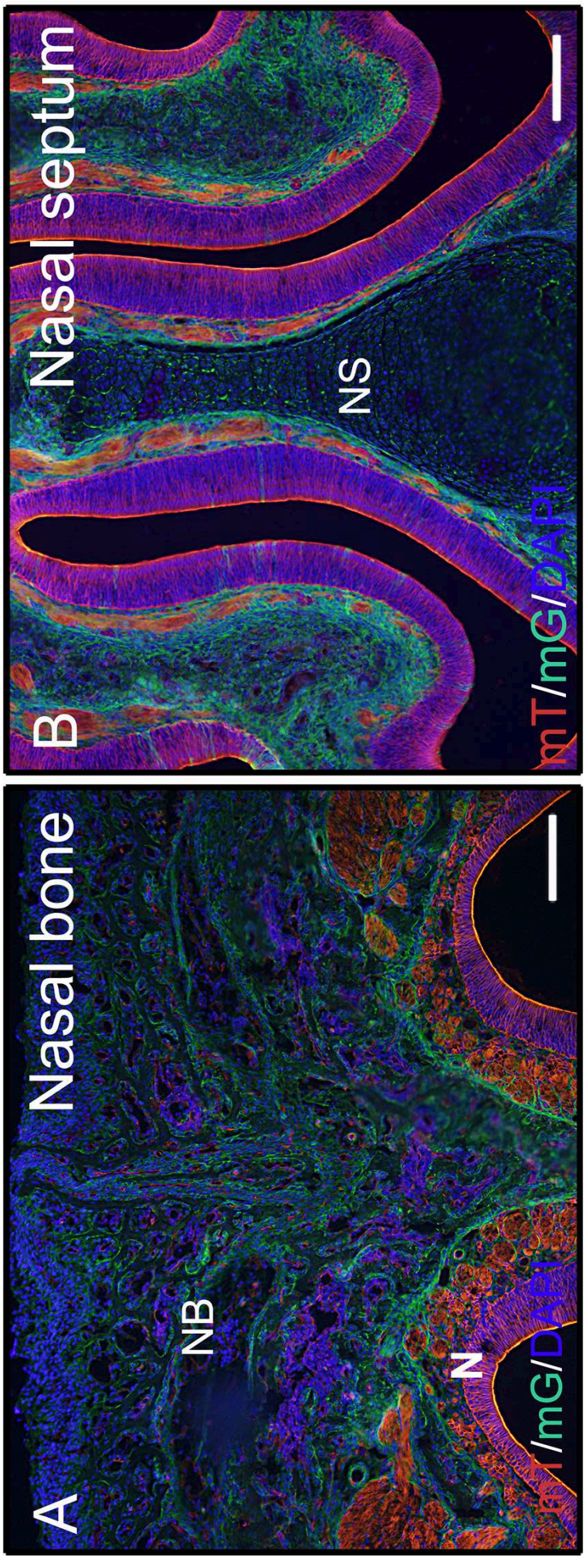

Figure 3

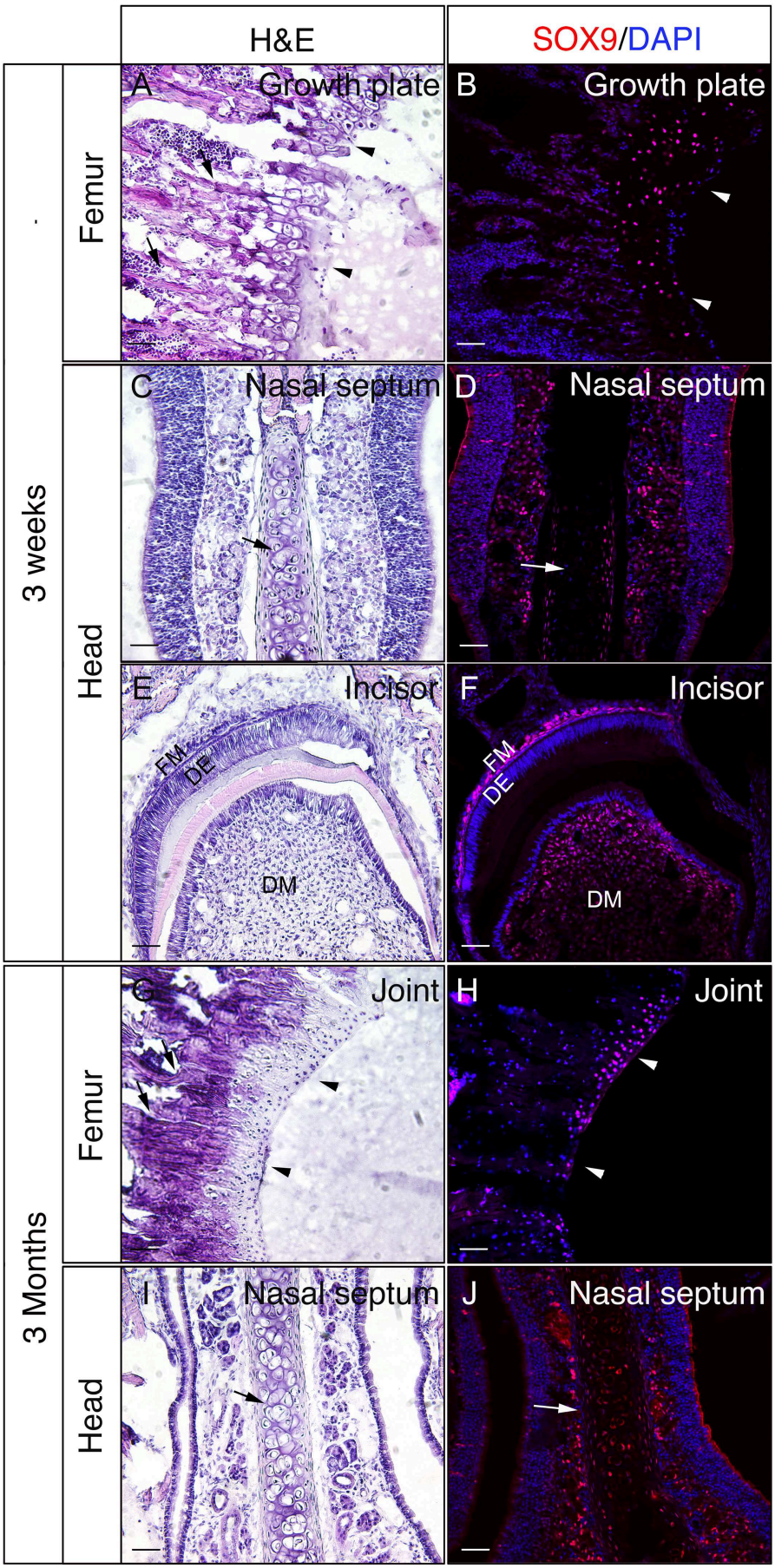

Figure 4

Click here to access/download;Figure;Revised Figure 4_Final-s.eps

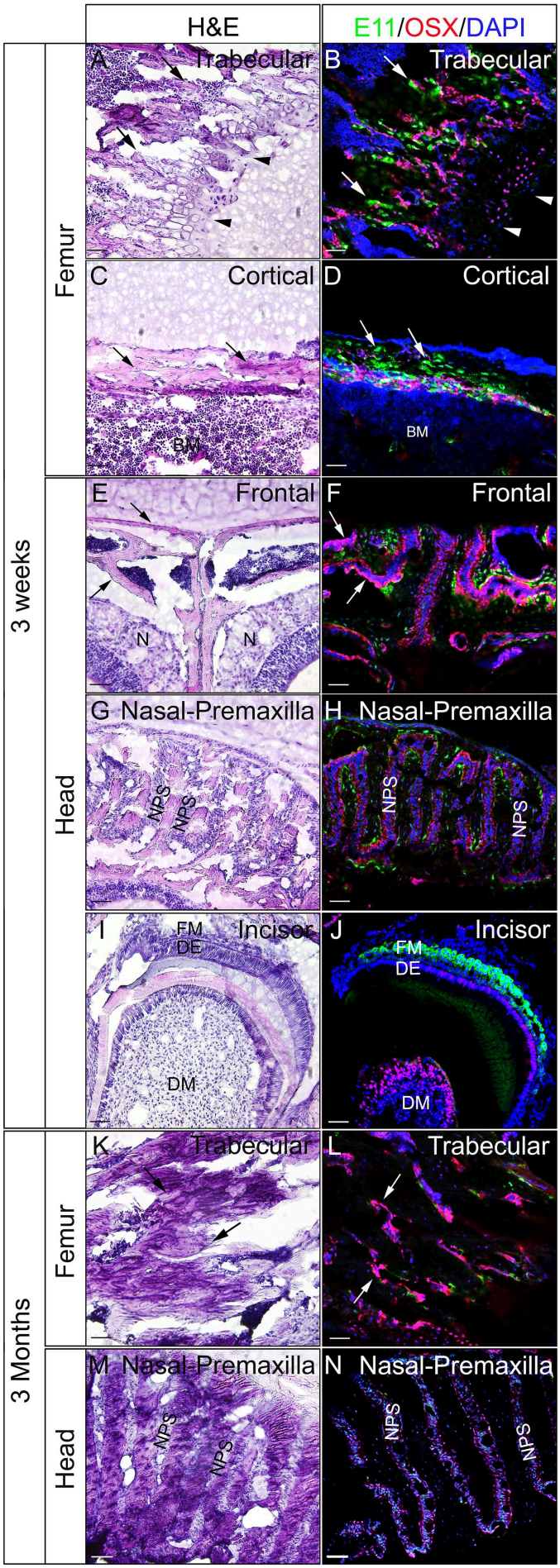

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_Yang_Mishina_012219.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Adhesive tape	Leica	#39475214	
Alexa fluor 488-goat anti-Rabbit secondary antibody	Invitrogen	A-11034	
Antifade Mountant with DAPI	Invitrogen	P36931	
Bovine serum albumin	Sigma	A2153	
Coverslips	Fisher Brand	12-545-E	
Cryostat	Leica	CM1850	
EDTA	Sigma	E6758	
Fluorescence microscope	Olympus	BX51	
Gelatin	Sigma	G1890	
In Situ Cell Death Detection Kit	Millipore	S7165	
Microscope slides	Fisher Brand	12-550-15	
OCT Compound	Fisher Healthcare	23-730-571	
Paraformaldehyde (PFA)	Sigma	P6148	
Phosphate buffered saline (PBS)	Sigma	P4417	
Polyethylene glycol tert-octylphenyl ether	Sigma	T9284	Triton X-100
Proteinase K	Invitrogen	AM2542	
Rabbit anti-Ki67 antibody	Cell Signaling Technology	9129	Lot#:3; RRID:AB_2687446
Rabbit anti-pSmad1/5/9 antibody	Cell Signaling Technology	13820	Lot#:3; RRID:AB_2493181
Sodium citrate	Sigma	1613859	
Sucrose	Sigma	S9378	
Tris	Sigma	10708976001	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	**Tissue Preparation and Immunostaining of mouse craniofacial tissues and undecalcifined Bone**
Author(s):	**Jingwen Yang, Haichun Pan, and Yuji Mishina**

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[ ] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[X] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	**Yuji Mishina**
Department:	**Department of Biologic and Materials Sciences**
Institution:	**The University of Michigan School of Dentistry**
Title:	**Professor**

Signature:  Date: **Sept. 15, 2018**

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Phillip Steindel, Ph.D.
Review Editor
JoVE

Dear Dr. Steindel,

Thanks for your letter on September 18, 2018 about our manuscript, JoVE59113R1 "Tissue Preparation and Immunostaining of Mouse Craniofacial Tissues and Undecalcified Bone," for providing us an opportunity to revise. We are deeply grateful for the supportive responses and insightful suggestions from you and the four reviewers. According to the reviewers' comments, we have revised the manuscript to address the suggestions and concerns. We also added the following data according to their suggestions: (1) histologic and immunologic assessments of undecalcified cryosections from 3-week old mineralized tissues, and (2) those from 3-month old mineralized tissues. Each specific comment from the reviewers has been addressed below.

Thank you very much for providing us the opportunity to revise our manuscript. We hope it is now considered suitable for publication in JoVE.

_____

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you.

2. Please provide an email address for each author.

The email address for each author is provided (Line 12, 13). Thank you.

3. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The summary section is added before the Abstract (Line 15). Thank you.

4. Abstract: Please expand the Abstract to provide a summary of the advantages and limitations of the technique.

A summary of advantages and limitations of the technique is provided in the Abstract (Line 28-34). Thank you.

5. Keywords: Please provide at least 6 keywords or phrases.

Key words are added and increased to 7. Thank you.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ApopTag, Millipore, Triton X–100, etc.

Commercial language has been changed. Thank you.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement has been added (Line 86). Thank you.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

The numbering has been changed as suggested. Thank you.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as

“could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

12. Line 79: Please add more specific details which are required for filming. (Line 101 in the revised manuscript)

13. Please specify all surgical instruments used in the protocol.

14. Line 103: Please specify the age of postnatal mice. (Line 128 in the revised manuscript)

15. Line 206: What are the positive and negative controls? (Line 232 in the revised manuscript)

16. Line 208: Please specify the conditions used. (Line 237 in the revised manuscript)

17. Line 213: Please specify the image format. (Line 246 in the revised manuscript)

18. Line 232: Please specify how to calculate the corrected fluorescence intensity. (Line 275 in the revised manuscript)

19. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

20. Please include single-line spaces between all paragraphs, headings, steps, etc.

The protocol was changed as suggested 9-20. Thank you.

21. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

22. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

23. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

The essential steps of the protocol for the video were highlighted in yellow as suggested. Thank you.

24. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Critical steps within the protocol were discussed. Thank you.

25. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

A Disclosure section was included (Line 462). Thank you.

26. References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then "et al.".

References were modified as suggested. Thank you.

27. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Table was changed as suggested. Thank you.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes a step-by-step methodological instruction for mouse craniofacial tissue preparation and immunostaining. Histological sample preparation in craniofacial tissues is very challenging as they contain both soft and hard tissues together, thus this manuscript is an important and interesting area of study.
The manuscript is well organized and thoroughly written.

Thank you for providing the opportunity to review your interesting work.

Major Concerns: None.

Minor Concerns: None.

Reviewer #2:
Manuscript Summary:
Not very novel but in general a well explained method that is widely used in developmental biology labs.

Major Concerns:
The title is misleading and needs to be changed. The protocol is for developing tissues (embryonic and postnatal). I think it very unlikely that this method would work well on adult specimens without decalcification. The authors emphasise the use of this method on postnatal samples in the text (P21). Have they tried heads from later stages, 6-8 weeks for example? If not the title should be changed to emphasise that this method is for developing tissues.

Thank you for the comments. The protocol works for mouse heads and long bones up to 3 months. We provided additional figures (Figure 3 and 4) to show examples.

In figure 2 mTmG mice are shown. These would be already fluorescent and would not use the immuno techniques outlined in the methods therefore they are not appropriate to be used. They are pretty but do not support the point of the paper. The tibia sections are fine.
The authors should show some cranial structures at postnatal stages, ideally hard tissues such as teeth, using immunos to confirm their technique works.

Thank you for the comments. The intention of the mTmG images is to clearly show that (1) undecalcified hard tissues embed with gelatin can generate good quality sections, and (2) gelatin does not interfere fluorescent signals from sectioned tissues. Therefore, we would like to keep those images as Figure 2.

For 3-week old samples, this technique works well for hard tissues, such as femora, skull bones, teeth, and nasal tissues, except the molar. For 3-month old samples, this technique works well for some of hard tissues, such as trabecular compartments of the femur, bones around the nasal-premaxilla suture, and the nasal area of the head. Results were shown in Figure 3 and Figure 4. We clearly stated this information in the Representative results part (Line 342-349).

This technique does not work well on molars, and other hard tissues from 3-month old mice. Potential improvements of this method are (1) to dissect the samples further to separate the target tissue from other parts to make the structure of the tissue simple. For the case of teeth, the mandible or maxilla should be dissected and fixed instead of the whole head, (2) to use 10% EDTA to decalcify tissues for only 2-3 days before cryoprotection. This short time decalcification will not affect the immunostaining results. We clearly stated this information in the Discussion part (Line 425-431).

Minor Concerns:
Is the lower cryo temperature for both gelatin and OCT embedded undecalcified hard tissue? section 2.1.

Lower cryo temperature only works good for gelatin embedded undecalcified hard tissue. We modified the description to make this point clear (line 417). Although lower temperature (-25°C) makes some improvements for cryosectioning of OCT embedded undecalcified hard tissues compared with the case of higher temperature (-18°C), it is still difficult to get a good integrity of tissue structures.

No information is provided regarding blocking solution composition. Section 3.

Blocking solution composition (5% donkey serum diluted in 0.1%PBST) is added. Thank you.

Can the authors clarify when to use DAPI and when to use Hoechst. Section 3.9.

Thank you. The following sentences are added in the Discussion in red (line 379-385).

Both the Hoechst dye and DAPI can be used for nuclear counter-staining. They have similarities, (1) are UV-excited, minor groove-binding chemicals to emit signals proportional to total DNA content, and (2) are subjected to photo-bleaching after long exposure. However, Hoechst dyes are typically used for staining DNA content in live cells due to its high permeability. DAPI is typically used for staining DNA in fixed cells due to its low membrane permeability. In addition, DAPI generates a stronger and more stable signal than Hoechst.

In the quantification section no N numbers are mentioned. As use of statistics is important for any quantification, I think it would be useful to state a minimum number of independent samples to analyse in order to gain meaningful results. Information could also be added on how equivalent representative areas are selected for analysis.

Thank you for the comment. The minimum number of independent samples to analyze is 3. The corresponding sentences are added in the Protocol in red (line 253).

Reviewer #3:

Manuscript Summary:

The manuscript entitled "Tissue Preparation and Immunostaining of Mouse Craniofacial Tissues and Undecalcified Bone" describe the protocol for immunostaining for craniofacial bone and following imaging and analysis. Because of the complexity of the structure of craniofacial tissues, we have frequently met the problem for the staining. This manuscript provides the detail not only for the immunostaining but also fixation, embedding, and sectioning, which are highly informative and valuable in the field. Especially, as we always have the problem of undecalcified tissue sectioning and staining, such as poor integrity of tissue structure and detachment problems, that should be highly valuable in the field. Importance to prepare negative/positive controls is mentioned, which would be appreciated especially by the researcher who is not familiar with immunostaining. Presented staining data in Figure 1 and 2 are specific, showing the usefulness of the described method. Compare to the preparation and staining sections, imaging and analysis parts lack the detail and less informative. However, my enthusiasm to support this manuscript is still very high. Detail comments are listed below.

Major Concerns:

1. In the section describing quantification, it is helpful to mention the difficulty and limitation of the quantification of immunostaining. Non-linear nature of immunostaining and fading of fluorescent signal during image acquisition always raise the question about the reliability of their quantification.

Thank you for the comment. The following sentences are added in the Discussion in red according to the suggestion (Line 435-443).

Immunostaining results are not easy to quantify, which usually used as semi-quantification. The difficulty and limitation of the quantification of immunostaining of craniofacial tissues including but not limited to the following:

1. difficult to define the area to be counted due to the complexity of the structure of craniofacial tissues,
2. difficult to define the labeled area or labeled cells due to the non-linear nature of immunostaining,
3. limited information for dynamic range of the signal is available,
4. hard to compare the intensity of signals between images or groups due to the fading of fluorescent signal during image acquisition,
5. signal background may change significantly between antibodies, slides, or samples.

2. Based on the difficulty, it may be nice to describe the strategies to acquire the reliable quantifiable data.

Thank you for the comment. Following descriptions are added to the discussion (line 443-449).

To increase the reliability of the quantification results, the experiment should be carefully and strictly performed. All samples should be processed under the same condition. During immunostaining, various controls are necessary to evaluate signal background and define the positive area or cells. Take convincing and representative images to clearly show the area to be counted and labeled cells with good contrast. In addition, during image acquisition, the camera setting and equipment setting should be kept consistent.

3. Quantification process for stack images acquired by the confocal microscope is not clear.
Thank you. The following sentences are added in the Protocol (Line 289).

For counting stacks, plugin “Plot Z Axis Profile”, then use the “Image” - “Stacks” - “Plot Z Axis Profile” to monitor the intensity of a moving ROI using a particle tracking tool. This tool can be either manual or automatic.

Reviewer #4:

Manuscript Summary:

The manuscript by Yang et al describes methods for preparation of mouse craniofacial tissues that avoid the time and expense normally needed to render calcified tissues, such as bone, easier to section. If the technique described here is effective and can be shown so in the accompanying video, then I think it would be a valuable addition to the literature.

Major Concerns:

1. Although this seems a simple and straightforward technique, it would be very useful to know what aspects of the manipulations are critical to allow them to section mature bone without decalcification. Normally, sectioning mature bone is a problem as the blade often fails to cut through the bone and instead causes this skeletal element to shift and damage the sample morphology. How does the rather simple fixation protocol avoid this issue for the frozen and paraffin embedded tissues? Is it critical to use an ultra sharp blade too? What are the critical parameters for someone to get this to work and avoid failure? Temperature, length of fixation etc etc?

There are a couple of tips for cryosectioning of undecalcified hard tissues.

Samples were processed as usual. To get a better penetration, keep samples in 30 % sucrose one more day after samples sink to the bottom. The critical step for this protocol is embed with gelatin instead of OCT. It is equally important to set the temperature lower than usual at about -25°C. An ultra-sharp blade is not necessary. We clearly stated this information in the revised text (line 413-417).

2. One confusing issue with the manuscript is the use of "undecalcified hard tissues" alongside "uncalcified hard tissues" e.g. page 3, line 123. Here, I am not sure whether uncalcified is a typographical error and it should be undecalcified, or whether they are referring to cartilage which can also be considered a hard tissue but is not calcified, or whether they are referring to immature bone that has not mineralized yet. This confusion also occurs in Section 2 "Undecalcified bone tissue sections" as well as in the Discussion. If undecalcified and uncalcified are meant to be separate terms it would be valuable to list their respective tissues.

The “uncalcified hard tissues” should be “undecalcified hard tissues”. Sorry for the mistake.

3. In the protocol, section 1.1.3 they state procedures to study samples at E16.5 or later. For completeness, they should mention how they treat samples before this stage.

Thank you for the comment. For samples before E16.5, fix the embryo head with 4% PFA directly after dissection. This information was added to line 112-114.

4. In the protocol, section 1.2 they discuss postnatal undecalcified hard tissue samples. It would be useful to know the age range when they have been able to get good histology on the skulls. I would imagine at some point, for example as the mice reach 6 months of age or older, that the skulls get too big and hard even for this approach.

Thank you for the comment. This protocol works up to 3 months. It works good for most of the hard tissues from the femora and heads at 3 weeks, but only works for some of them (such as trabecular bone, nasal-premaxilla bone, and nose) at 3 months. This technique does not work well on the molar at 3 weeks, and other hard tissue at 3 months. There are a couple of techniques to improve the results, which we also stated in the Discussion part (Line 342-349).

5. Section 3.2.2. In this section they discuss alternatives to proteinase K. For each of the alternatives, the concentration and length of treatment should be given - it is for some, but not all.

Thank you for the comment. The concentration and length of treatment for the alternatives were added.

Minor Concerns:
The manuscript has a number of textual errors and would benefit from editorial assistance. Here are a few examples:
Page 1. Introduction line 27. "Face is our identity, which are composed of" Maybe "and is composed of".
Page 1. Line 28. "tooth et al" should be "tooth etc"
Page 1. Line 32. "Malformation" should be "malformations" as the next part of the sentence refers to them in the plural.
Page 1. Line 36 "principals" should be "principles"
Page 1. Line 44. "IF will clearly differentiated" - Two things here - "will" I feel is too strong and a typo with a "d" at the end of differentiated. Maybe "IF may clearly differentiate"
Page 2. Line 55. ""and easily be rinsed off" should be "and easily rinsed off".
Page 2. Line 57 "which are time consuming" should be "which is time consuming"
Page 3. Line 100 "Store resulted cryomolds" should be "store resulting cryomolds"
Page 3. Line 108. "as described in 1.1.8 -1.1.9" There is no 1.1.9
Page 4. Line 164. "of fluorescent IF results" should be "of IF results"
Page 5. Line 180. Should be "Tris pH 7.5"
Page 5. Line 203. "Mount slides as step 3.1.10" should be 3.1.9
Figure legends. Lines 396-7 "The head of control … and mutant ….embryos was" should be "The head of control …. and mutant …. embryos were"
Line 410 "(A, B) Nucleus were" should be "(A, B) Nuclei were"

Those errors have been corrected as suggested. Thank you!