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TITLE:

3D imaging of PDL collagen fibers during orthodontic tooth movement in mandibular murine model

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KEYWORDS:

periodontal ligament, digital imaging; tissue clearing technique; 3D imaging, micro-CT, orthodontic tooth movement, 3D fiber direction

SUMMARY:

We present a protocol for generating orthodontic tooth movement in mice and methods for 3D visualization of the collagen fibers and blood vessels of periodontal ligament without sectioning.

ABSTRACT:

Orthodontic tooth movement is a complex biological process of altered soft and hard tissue remodeling as a result of external forces. In order to understand these complex remodeling processes, it is critical to study the tooth and periodontal tissues within their 3D context and therefore minimize any sectioning and tissue artefacts. Mouse models are often utilized in developmental and structural biology, as well as in biomechanics due to their small size, high metabolic rate, genetics and ease of handling. In principle this also makes them excellent models for dental related studies. However, a major impediment is their small tooth size, the molars in particular. This paper is aimed at providing a step by step protocol for generating orthodontic tooth movement and two methods for 3D imaging of the periodontal ligament fibrous component of a mouse mandibular molars. The first method presented is based on a micro-CT setup enabling phase enhancement and imaging of fresh collagen tissues. The second method is a bone clearing method using ethyl cinnamate that enables imaging through the bone without sectioning and preserves endogenous fluorescence. Combining this clearing method with reporter mice like *Flk1-Cre*;TdTomato provided a first of its kind opportunity to image the 3D vasculature in the PDL and alveolar bone.

INTRODUCTION:

The basic underlying biological process in orthodontic tooth movement (OTM) is bone remodeling. The trigger for this remodeling process is attributed to changes in the structure of the periodontal ligament (PDL) such as extracellular matrix (ECM) stress, necrosis as well as blood vessel destruction and formation[1-3]. Other possible triggers for alveolar bone remodeling are related to force sensing of osteocytes in the bone, as well as deformation of the alveolar bone itself, however their role in orthodontic movement is still not fully elucidated[4,5].

Despite many studies aimed at revealing structure-function relations of the PDL during OTM, a clear functional mechanism is yet to be defined[6,7]. The major reason for this is the challenge in retrieving data of a soft tissue (PDL) located between two hard tissues (cementum and alveolar bone). The accepted methods to collect structural information usually necessitate fixation and sectioning that disrupt and modify the PDL structure. Moreover, most of these methods yield 2D data that even if not distorted, give only partial and localized information. Since the PDL is not uniform in its structure and function, an approach that addresses the intact 3D structure of the entire tooth-PDL-bone complex is warranted.

This paper will describe a method for generating an OTM in mice and two methods that enable 3D visualization of the collagen fibers in the PDL without any sectioning of the sample.

Murine models are widely used for in vivo experiments in medicine, developmental biology, drug delivery and structural studies. They can be genetically modified to eliminate or enhance specific proteins and function; they provide fast, repeatable and predictable developmental control; they are also easy to image due to their small size[8]. Despite their many advantages, mouse models in dental research are not used frequently, especially when clinical manipulations are warranted, mostly due to the small sized teeth. Animal models such as rats[9-11], dogs[12,13], pigs[14-16] and monkeys[17] are used more often than mice. With the recent development of high-resolution imaging techniques, the advantages of utilizing a mouse model to decipher the convoluted processes in OTM are numerous. This paper presents a method to generate a mesial movement of the molar tooth in the mandible with constant force levels that triggers bone remodeling. Most of the OTM experiments in rodents are done in the maxilla, since the mobility of the mandible and the presence of the tongue add another complexity level. However, the mandible has many advantages when 3D structural integrity is desired. It can be easily dissected as a whole bone; in some species it can be separated into two hemi-mandibles through the fibrous symphysis, it is compact, flat and contains only the teeth without any sinus spaces. In contrast, the maxilla is a part of the skull and closely related to other organs and structures, thus extensive sectioning is needed in order to dissect the alveolar bone with the associated teeth.

Using an in house humidity chamber coupled to a loading system inside a high resolution micro-CT that enables phase enhancement, we developed a method to visualize fresh fibrous tissues in 3D as previously described[9,18-23]. Fresh tissues are scanned immediately after the animal is sacrificed without any staining or fixation, which reduces tissue artefacts as well as biomechanical properties. These 3D data can be utilized for distribution and direction analyses of the fibers as described elsewhere[19].

The second 3D whole tissue imaging method presented here is based on optical clearing of the mandible which enables imaging of the PDL fibers through the bone without any sectioning. Interestingly it also enables visualization of the collagen fibers of the bone itself, however this will not be discussed here. In general, there are two methods for tissue clearing. The first is aqueous-based clearing where the sample is immersed in aqueous solution with refractive index greater than 1.4 either through a simple immersion, hyperhydration or hydrogel embedding. However, this method is limited in the level of transparency as well as the structural preservation of the tissue and therefore necessitates fixation of the tissue. The second method which yields highly transparent samples and does not require fixation is the solvent-based clearing method[24,25]. We generated a modified solvent-based clearing method based on ethyl-3-phenylprop-2-enoate (ethyl cinnamate, ECi) for the mandibular samples. This method has the advantages of using non-toxic food-grade clearing agent, minimal tissue shrinkage, and preservation of fluorescent proteins.

PROTOCOL:

All animal experiments were performed in compliance with NIH's Guide for the Care and Use of Laboratory Animals and guidelines from the Harvard University Institutional Animal Care and Use Committee (Protocol no. 01840).

1. Orthodontic Tooth Movement

1.1. To generate a mouse bed, use a flat plastic platform with a wedge shaped, 45° angled headrest. The headrest can be generated by cutting a plastic box.

1.1.1. Elevate the head end of the platform to generate an approximately 30° angle between the headrest and the table plane. Attach a bent thick paper clip (0.036" in diameter) to the head side end to hold the upper incisors.

1.1.2. On the tail end, generate an elevated surface to which an orthodontic power chain can be attached to hold the lower incisors. See **Figure 1** for an example platform.

1.2. Anesthetize mouse by using intraperitoneal injection of xylazine at 10 mg/kg and ketamine 100mg/kg using 1 mL syringe and a 27 Gauge needle.

1.3. Place anesthetized mouse on custom-made platform and immobilize upper jaw by hooking the upper incisors on the paperclip loop. Open the mouse lower jaw with the orthodontic power chain hooked on the lower incisors. Keep the cheeks retracted with the mini-colibri mouth retractor.

1.4. Place the platform under a surgical microscope or any other stereoscope that can reach to 5-6x magnification.

1.5. Apply 50 µL of saline (roughly 1 drop) on the mouse eyes to prevent corneal dehydration. Replenish saline every 20 min.

1.6. Cut a piece of an aluminum wire (0.08 mm diameter) 1 cm in length. Slide the wire from the buccal side lingually in the interproximal area bellow the contact point between first and second molars using a microsurgical needle holder. Leave 2 mm free edge in front of the first molar in order to be threaded into the spring end.

1.7. Cut a piece of nickel titanium (NiTi) coil, around 7 to 9 threads in length.

NOTE: The elastic properties of the coil will provide constant force for orthodontic movement. The total unstrained length of the coil should be shorter than the gap between the incisor and the molar. Keep in mind that an extra 2 threads are needed on each end to anchor coil to the tooth. Aluminum wire is selected in order to reduce scanning artefact such as beam hardening during the micro-CT scan.

1.8. Insert NiTi coil spring (0.15 mm wire diameter, 0.9 mm inner coil diameter; delivers a force of 10 g) between lower first molar and lower incisor. Use the wire ligature inserted around the first molar in step 1.6, twist the wire tightly around 2 threads of the coil spring to fix the coil on the molar side.

1.9. To ensure uniform force level, use exactly 3 active threads between the first molar and the incisor. Temporarily remove the power chain from the incisor and loop 2 to 3 unstrained threads over the incisor to anchor the coil. Slide the threads down to the incisor free gingival margin.

1.10. Place a layer of flowable composite resin on the incisal border of the coil and cure it with dental curing light. Replace the power chain after curing the resin.

1.11. Using the same curing light, heat up the NiTi coil for 20 s. This will tighten the NiTi coil. The finished placement is shown **Figure 1B**.

1.12. Either leave the contralateral side intact or insert a sham such as the wire between the first and second molars.

1.13. Place the anaesthetized mouse under a heated light to keep the mouse warm until recovery.

1.14. Place the mouse back into an individual cage and monitor daily. No diet change is necessary during orthodontic movement.

NOTE: OTM device on one side causes some discomfort but does not impair feeding. However, inserting devices on both sides are not advised due to the added amount of discomfort. Pain medication is not necessary unless outward signs of pain are seen.

2. Micro-CT scan of PDL fibers in fresh hemi-mandibles

2.1. Mounting the hemi-mandible

2.1.1. After the desired duration of orthodontic movement, sacrifice the mouse via cervical dislocation. Remove the mandible and separate into hemi-mandibles.

NOTE: Since the sample will not be fixed, it is critical to perform the dissection of the jaw and mounting as soon as possible, ideally within 30 min.

2.1.2. Remove the surrounding soft tissue gently with a clean lint-free wipe.

2.1.3. Remove the orthodontic device using microsurgical scissors and tweezers under a stereo microscope with at least 4x magnification.

2.1.4. Keep sample moist in a 1.5 mL volume, micro-centrifuge tube along with a piece of lint-free wipe moistened with water.

2.1.5. Place packable dental composite resin into the sample slot on the stage, then place the fresh hemi-mandible into the composite. Before mounting make sure that the bone surface in contact with the dental composite is free from any soft tissues and dry, otherwise dental composite will not cure properly.

2.1.6. Adjust the position of the hemi-mandible until the first molar is centered at the midline groove of the stage. Make sure the occlusal surface is horizontal. Cure the composite when satisfied with the positioning.

NOTE: Additional small amounts of dental composites can be placed on the sides of the hemi-mandible and/or across the incisor to aid in stabilizing the sample.

2.1.7. Place dampened lint-free wipe inside the humidity pools in the sample stage. Place dental composite on the occlusal surface of the first molar. Before closing the chamber, make sure nothing blocks the x-ray path at the specimen level.

2.1.8. Affix the chamber in the micro-CT. Screw the chamber into the micro-CT sample stage so that movement during imaging is minimized.

2.1.9. Turn on the x-rays and take 2D images while lowering the anvil vertically, until the tip of the anvil is surrounded by the composite but no increase in the force is detected.

2.1.10. Once anvil is embedded in the composite, close the x-ray source. Then, open the micro-CT chamber and cure the composite through the clear Plexiglass window.

2.2. Micro-CT settings (**Figure 2**)

2.2.1. Set the source voltage to 40 kV and current to 200 μA. Using a 10x magnification detector, position the sample within the frame of view. Use binning of 2 for the captured images.

NOTE: Since the PDL is significantly less dense than the bone and tooth, visualizing the PDL requires higher power and exposure time. This protocol will provide settings for visualizing the PDL.

2.2.2. Set single image exposure time to 25 s. Set rotation of the sample stage to a range of 183 degrees or more. Set the scan for 2500 projections. Do not use any x-ray source filter, the resulting scans have a voxel size of 0.76 μm on each side.

2.2.3. Collect a reference scan for proper reconstruction according to the micro-CT guidelines. Use 1/3 number of reference images as the total projections. Reconstruct the volume without additional binning, using a back projection filtered algorithm.

3. Clearing method (Figure 3)

3.1 Prepare five 1.5 mL micro-centrifuge tubes.

3.2. Prepare 1.4 mL of the following solutions in 1.5 mL micro-centrifuge tubes: 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), 50% ethanol (EtOH) in deionized (DI) water, 70% EtOH in DI water, and two tubes of 100% EtOH.

NOTE: PFA is used for fixation of the sample. ECi clearing will also work on unfixed samples. To clear unfixed samples, simply skip the PFA step.

3.3. Place dissected hemi-mandible in 4% PFA. Cover with aluminum foil and place on the rocker on gentle setting at room temperature for 6 h.

3.4. Move the hemi-mandible to 50% EtOH. Place it on the rocker covered from light for 16 h.

3.5. Move the hemi-mandible to 70% EtOH. Place it on the rocker covered from light for 16 h.

3.6. Move the hemi-mandible to 100% EtOH. Place it on the rocker covered from light for 16 h.

3.7. Repeat 3.6. in the second 100% EtOH tube.

3.8. Prepare 5 mL of ECi in a glass or polypropylene tube.

NOTE: ECi dissolves polystyrene, but not polypropylene. Also, if not using a tissue with fluorescent proteins the sample can be exposed to light during the clearing procedure.

3.9. Move the hemi-mandible to ECi tube. Cover the tube with aluminum foil and place on the rocker on gentle setting for a minimum of 12 h.

NOTE: The protocol can be paused here. Dehydrated sample can be stored in ECi at room temperature. The freezing or melting point of ECi is 6.5 to 8.0 °C. Do not store at 4 °C.

3.10. The hemi-mandible is ready for imaging with fluorescence microscope.

NOTE: During imaging, the sample must be immersed in the ECi to remain optically transparent.

REPRESENTATIVE RESULTS:

This paper presents a method to produce OTM as well as two methods for 3D imaging of collagen fibers inside the PDL without any sectioning. For animal research purposes, when alignment of the teeth is not necessary, a tooth movement is considered orthodontic if it generates remodeling of the alveolar bone at all root levels. Constant force level applied on teeth is required in order to generate a reliable OTM. Here, an activated shape-memory NiTi coil is used to generate a consistent force of 10 g throughout the experimental time of 7 days and beyond if warranted. The coil activation described here (**Figure 1**) generates strain in the NiTi coil within the martensitic phase and bring the coil to the hysteresis state, which delivers constant stress onto the tooth. Warming the coil with the curing light after the coil insertion will also make sure the alloy will not shift to its austenitic form and yield.

Here we show representative results from 9-week old, male mice. The averaged mesiodistal space between the crowns of the first and second molars after 7 days of OTM is 40 μm as measured between the interproximal surfaces of the molars in the micro-CT with 1X magnification (n=12, *st.dev.*= 15 μm) (**Figure 1E**). The averaged space of the PDL in the mesiodistal direction is 80 μm before and after 7 days of OTM (**Figure 4B**). This confirms that the first molar translated mesially and 7 days is an adequate time for generating OTM in a mouse model while generating processes of bone resorption and apposition in nature (**Figure 4**). Mice were fed a standard hard pallet diet. No diet change was made post device insertion.

The first method presented to visualize the changes in the tooth-PDL-bone complex during OTM is phase-enhanced micro-CT of fresh tissue (**Figure 4**) which was described in detail previously[9], [18-20, 22, 23]. In short, provided a phase enhancement capability of either a micro-CT or a synchrotron, mechanical stabilization of the fibrous tissue and humidified environment, fresh collagenous fibers can be visualized without any fixation or contrasting agents. In the PDL the fibers that are seen are those that are connected to both the tooth and the bone, mainly type I collagen [19]. This unique opportunity to visualize in 3D an intact PDL enables analysis of 3D fiber density, fiber orientation as well as analyzing the 3D movement of the tooth as previously described [9, 19]. Specifically, here we present the visualization of the fibrous network in the PDL. At time 0, physiological remodeling in both the bone and the PDL can be observed. Remodeling also occur in the cellular cementum; however, this is not directly related to the presented method and therefore will not be elaborated. The bone-PDL interface is mostly smooth both in the transverse (**Figure 4A**) and sagittal (**Figure 4B**) planes prior to any force application. In the coronal

plane (**Figure 4C**), the bone-PDL interface is rougher especially towards the apical region which might be indicative of the remodeling balance tends towards resorption. At 3 days of OTM (**Figure 4D-F**), upon which the first molar is moved mesially (direction is represented by the dashed arrow), the fiber density in the PDL is reduced (white arrow heads). The bone-PDL interface is rougher than at 0 days due to development of craters in the bone surface which are indicative of osteoclastic activity and bone resorption processes associated with mainly compression forces in the PDL [26], however here seen in tension areas at 3 days. Tissue destruction in tension areas within the PDL was suggested [27, 28] and can be clearly seen using this method. The rough border is seen at different levels of the roots (white arrows) and therefore suggest that the tooth movement is translational in nature and not just tipping of the crown. At 7 days of OTM (**Figure 4G-I**), bone resorption signs, such as craters within the bone lined with rough borders and expansion of the PDL space, are seen at all planes but the PDL space is narrower than at 3 days of OTM (**Figure 4D-F**). The bone-PDL border is smooth in areas mainly at the distal surfaces of the roots, which is a sign for bone apposition. This is expected when translational movement of the tooth through the bone occurs.

Due to the long micro-CT imaging time (~19 h) and the rotation of the stage, mounting of the sample is essential to keep the sample still. Unstable sample will result in blurry scans. **Figure 5** presents how the micro-CT scan looks when the sample has moved during the scan. The tooth and bone are blurry. Neither PDL fibers nor osteocytes are observed. In such incidents there is a silhouette present around the margin of an object. In **Figure 5**, multiple outlines of the tooth crown can be observed (arrows).

Depending on the research goal, the resolution and visualization of the PDL fibers may be sacrificed in trade for shorter scan time when only information on the hard tissues is desired.

A complementary method for 3D visualization of the PDL fibers without any sectioning is via optical microscopy on optically cleared samples. This paper presents a tissue clearing method using ECi (**Figure 3**). This method can be used on a specimen without fixation and preserves any fluorescent signals that exist in the tissue prior to clearing. Hemi-mandibles before and after ECi clearing are shown in **Figure 3B** and **3C**. Adequate sample clearing of the PDL can be confirmed when a grid paper can be seen through the ramus of the mandible. The amount of clearing can be adjusted by lengthening of dehydration process. **Figure 6** shows the second harmonic generation (SHG) signal from collagen fibers in both alveolar bone and the PDL in a cleared mandible. Imaging the collagen fibers of the bone in 3D is a complicated process, which often utilizes electron microscopy methods such as FIB/SEM. However, utilizing the ECi-based clearing method and SHG, the alveolar bone fibers are clearly seen, especially in the horizontal direction. When translating through the sample deep into the PDL from the bone surface, the transition to the PDL fiber level is very clear as the fibers suddenly change their orientation to a vertical one.

Lightsheet microscopy can also be utilized for imaging fluorescent proteins through the bone. In this case of a cleared sample from a transgenic *Flk1-cre*;Tdtomato mouse [19, 29, 30], the fluorescent endothelial cells lining the blood vessels are clearly observed (**Figure 7A, B, C, E**). Proper clearing

is key to generating intelligible images with lightsheet microscopy. When the bone is not completely cleared, blood vessels within the PDL were not be observed (**Figure 7D, F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Orthodontic appliance insertion set up. A. Mouse bed made from lab-supply to support the animal and keep the mouth open. Plastic platform (PP) for the body is on a 30° incline and the headrest (HR) is on a 45° angle from the surface of PP. A 2-tiered tube stand (TS) is used to elevate the end head of PP. The paper clip loop (black arrow) anchors the top incisors, and the bottom orthodontic power chain (white arrow) hooks onto the lower incisors. Red rectangle outlines the zoomed-in area in panel B. 5 mm diameter inspection mirror was used for visual inspection of the molars. **B.** Side view of the mouse bed. Angles between surfaces are marked (green and magenta). **C.** Representative image of the properly placed device. **D.** Molars seen through the inspection mirror prior to device implantation. **E.** Representative image of molars after the orthodontic movement. Dashed lines trace the outline of the first and second molars. **F.** Diagram of the device and its placement. Red line represents the wire ligature around first molar. Orange line represents flowable composite resin used to anchor the coil. NiTi coil is shown in blue and labeled. **G.** Dissected hemi-mandible with the device attached after 7-day orthodontic movement. Note how the 3 coil threads are still open, indicating that the coil is still active after 7 days. Scale bar = 1 mm in E and G.

Figure 2: Hemi-mandible mounted in a custom-made chamber for micro-CT imaging. **A.** Full set-up of the sample chamber within the micro-CT machine. The x-ray source is seen on the left and the detector on the right. Red rectangle outlines the hemi-mandible mounted in the chamber on the sample stage (SS). The example chamber shown here is part of a mechanical testing set-up, including motor (M), anvil (A, outlined by white dashed lines) and anvil shaft (AS) atop the chamber. The full set up is screwed onto the CT stage. Inset image shows close-up of the red outlined region, containing the humidity chamber with sample inside. **B.** Top view of sample mounted on the sample stage. Humidity slots (grey arrow) are built-in on the perimeter to maintain humidity during imaging. On the circular stage in the middle, hemi-mandible can be mounted in the slanted deep groove (black arrow). A thin groove (white arrow) marks the midline of the stage to aid in orienting the sample. **C.** Diagram of the circular stage with the sample groove. The slant of the groove supports the mandible and allows the molars to be mounted along the roots vertical axis. **D.** Representative micro-CT 2D slice combined with a 3D volume image of the hemi-mandible sample. The interproximal gap here is 52 μm. The sample is mounted onto the sample stage below (not shown) and the anvil (A) on top by dental composite (DC). Scale bar = 500 μm.

Figure 3. ECi-based clearing method for dissected mouse hemi-mandibles. A. The dissected hemi-mandible is immersed in 4% PFA, 50% EtOH, 70% EtOH, and 100% EtOH consecutively. After dehydration, the hemi-mandible is stored in ECi for a minimum of 12 h until imaging. **B.** Hemi-mandible immediately after dissection. **C.** Hemi-mandible after completion of clearing. Scale bars = 5 mm

Figure 4: Representative *in-situ* micro-CT scans of the PDL of a fresh sample in the different stages of the orthodontic movement. A-C, No orthodontic movement. **A.** Micro-CT 2D image in the transverse plane of the hemi-mandible showing the mesial (M) and distal (D) roots inside the alveolar bone, B-Buccal, L-Lingual sides of the alveolar bone. In between the tooth roots and the alveolar bone, the PDL space and the fibers within it are clearly observed. **B.** 2D image in the sagittal plane. **C.** 2D image in the coronal plane. **D-E,** 2D images after 3 days of OTM, arrow heads point at areas in the PDL with reduction in collagen fibers density, white arrows point at areas of bone resorption. **G-I,** 2D images after 7 days of OTM, black arrows point at regions of bone apposition. Scale bars = 150 µm.

Figure 5: **2D micro-CT image in the sagittal plane, showing blurry structures of both tooth and bone due to movement of the tooth during the scan.** Arrows point at multiple board lines of the tooth, indicating its movement. Scale bar 150 µm.

Figure 6: ECi cleared mandible showing the first molar imaged with second harmonic generation SHG. White arrow points at a region where collagen fibers of the PDL are seen, note the vertical orientation, black arrows point at a region where both vertical fibers of the PDL as well as horizontal fibers of the alveolar bone are seen. T-tooth, F-furcation, AB-alveolar bone, MR-mesial root, DR-Distal root, scale bar 150 µm. Images were obtained using a 20X multi-immersion lens for solutions with RI of 1.33-1.56. Excitation laser was set at 860nm at 10% power. Pixel dwelling time: 0.51µs; Scan mode: frame; Averaging: 16; Detector Type: nondescanned photomultiplier tube detector; Detector Gain 800V.

Figure 7. Lightsheet microscope images of ECi-cleared *Flk1-Cre;tdTomato* mouse. **A.** Optimally cleared control hemi-mandible. The network of blood vessels within the bone (arrow head) and PDL space (arrow) is visible. **B.** inset of the mesiolingual region of the first molar (red outlined in A) shows the blood vessels. **C.** optimally cleared 7-day OTM hemi-mandible and **D.** sub-optimally cleared hemi-mandible. **E.** 2D image of panel C in the sagittal plane, the image demonstrates well defined blood vessels in bone (grey arrow) and PDL space (white arrows). **F.** Two-dimensional slice image of panel D, same region as images in E, resulting in a blurry image. Scale bars A, C, D = 500 µm, B, E, F = 100 µm Images were taken with a 5X plan objective, using camera as detector. Excitation laser was 561 nm at 4% power.

DISCUSSION:

Generating OTM in mice is highly desired due to the size, genetics and handling advantages. Using the mandible provides an easy handling both in terms of tissue dissection as well as sample preparation and imaging. Here we presented a method to generate OTM with translational movement of the tooth inside the bone within 7 days of OTM. Using this protocol, the overall duration of the tooth movement can be extended, since the coil activation will deliver a constant force for movement of approximately 1 mm. However, the mesial side of the coil is fixed to the incisor, which is constantly erupting. As a result, the force vectors will gradually alter and start generating extrusion forces. This can be avoided if adjustment of the attachment level on the mesial end is performed every 7 days.

The PDL is the initiator for OTM, therefore understanding its structure and function during the different stages of the OTM is of great importance. However, the PDL is not uniform in both its structure and function [19, 22, 31, 32]. As a result, in order to retrieve meaningful data, the PDL needs to be studied in 3D and any tissue sectioning and manipulation should be avoided as much as possible. Yet, investigating a soft tissue situated between two hard tissues makes such requirements challenging to fulfill. Traditional methods of studying the PDL often involve compromising the 3D structure and removing the tissue out of its physiological environment, which consequently alters PDL structural and biomechanical properties. Both structural and biomechanical properties undergo dynamic changes during OTM which justify preserving the tissue 3D context even further. In order to do so we described two methods that enables whole tissue imaging without sectioning which can also be used on the same sample co-localizing both fluorescent signal and morphological and mineralization data.

The provided methodological description directs readers to apply the methods in their fields of study. The micro-CT imaging allows 3D visualization of PDL fibrous network. Images can be analyzed to produce directionality and density analyses and to quantitatively investigate changes in the PDL during OTM. We also described a clearing method that enables visualization with readily available optical microscopic methods such as lightsheet microscopy and confocal imaging. Lightsheet microscopy has the advantage of producing a 3D image of large specimens with relatively fast imaging speed. Confocal microscopy enables high resolution 3D visualization utilizing SHG signal for collagen fibers imaging and fluorescent tags. These methods independently or combined open many possibilities to 3D structural studies with minimal tissue preparation.

Several challenging steps in this protocol require extra attention:

Firstly, during the coil placement, the wire ligature must be placed securely between first and second molars. This process is challenging due to the small dimensions of the mouse teeth. We recommend the use of benchtop stereomicroscope to guide the placement. However, small movements during the procedure may move the mouse and cause the region of interest to go out of the field of view. As an alternative, we suggest using 4-5x magnifying loupes that can be worn on the operator, which could help view the area more dynamically.

Secondly, the clearing results depend on the dehydration process. If the sample is not as transparent as desired, our suggestion is to increase the time spent in the dehydration steps. Most notably, more time in 100% EtOH has been shown to improve the transparency of the final product. However, it should be noted that increased level of dehydration can dramatically reduce the fluorescence levels [24, 25]. The presented ECi-based method was shown to preserve fluorescent signals for longer than 2 weeks [24].

Aspects of this protocol can be modified to study a multitude of other purposes. The chamber we designed inside the micro-CT is coupled with a load cell and a motor and has the ability to perform tension/compression tests on the hemi-mandible samples. Combined with the visualization of the micro-CT, this set up can show changes in the PDL *in-situ* with varying

mechanical loads [21]. The clearing methods are adaptable for other types of tissues as well as for tissues that are not fixed (data not shown), which opens up the possibility to view the structures of unaltered tissue, not limited to the PDL.

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DISCLOSURES:

The authors have nothing to disclose.

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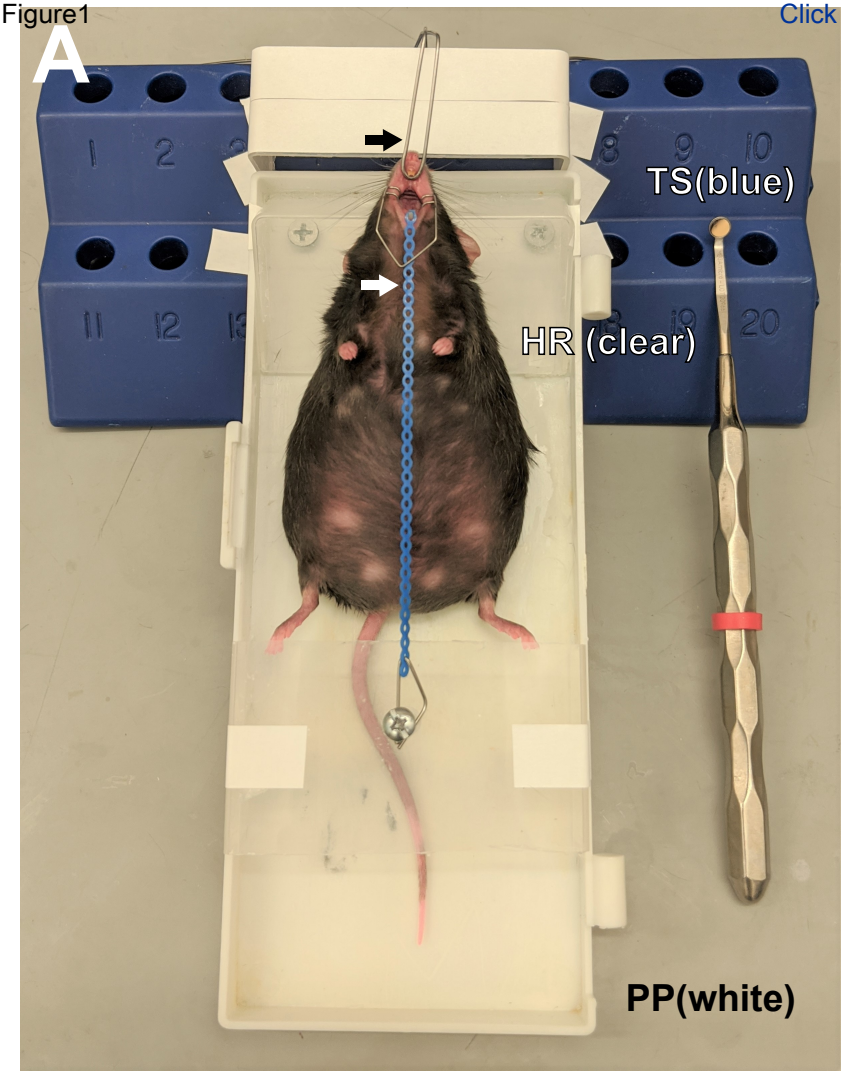
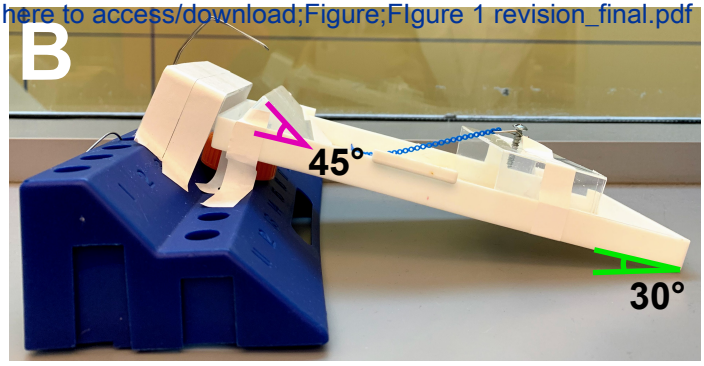
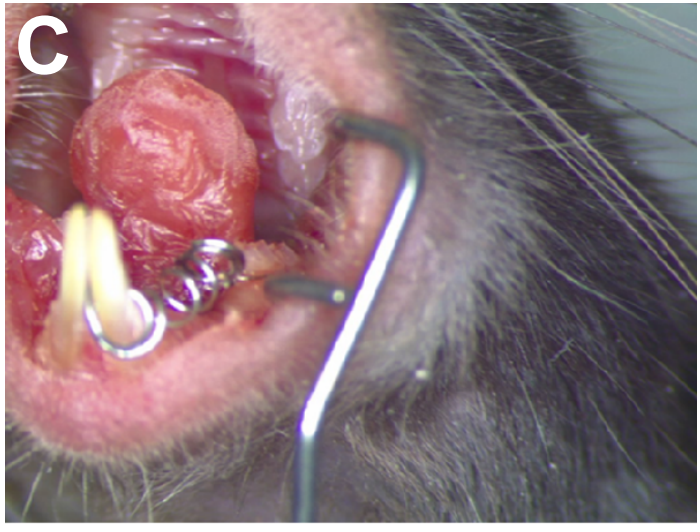
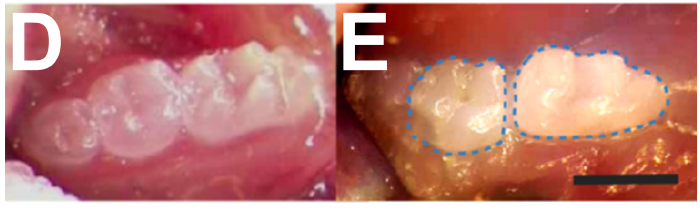
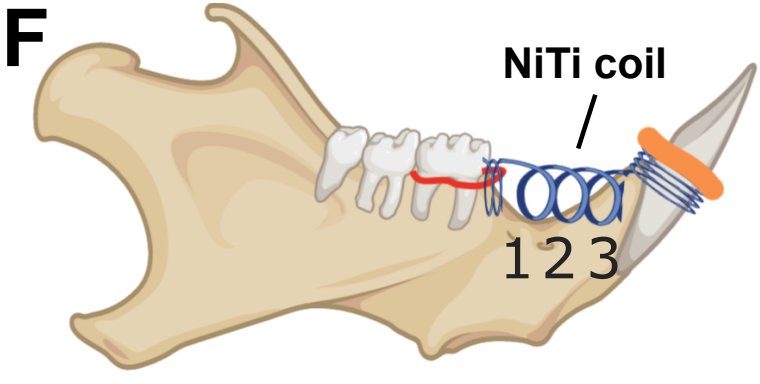
Figure1











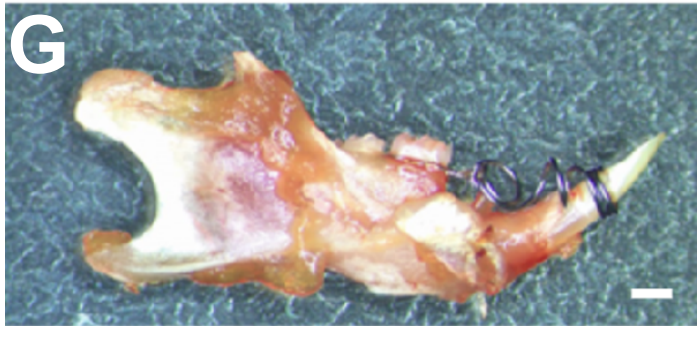
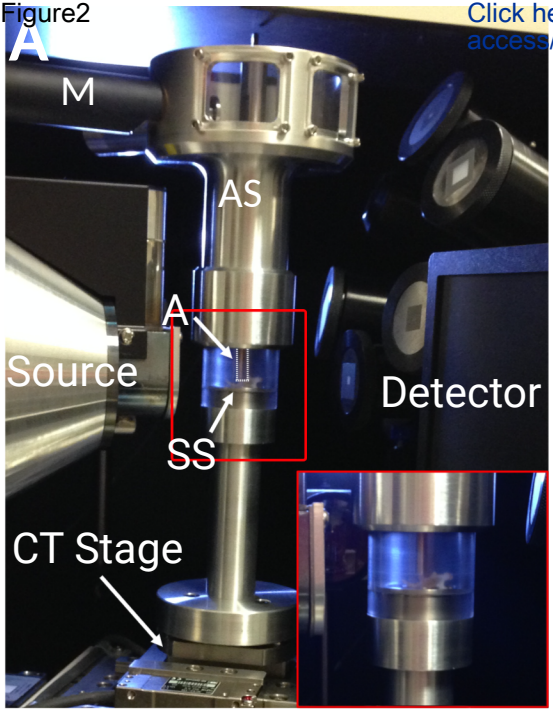
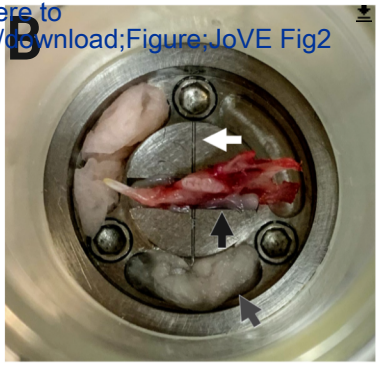
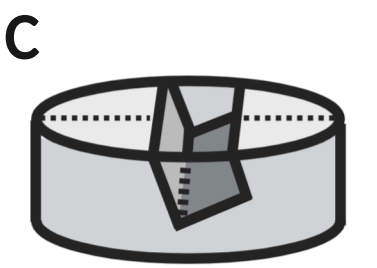


Figure2

Click here to access/download;Figure;JoVE Fig2







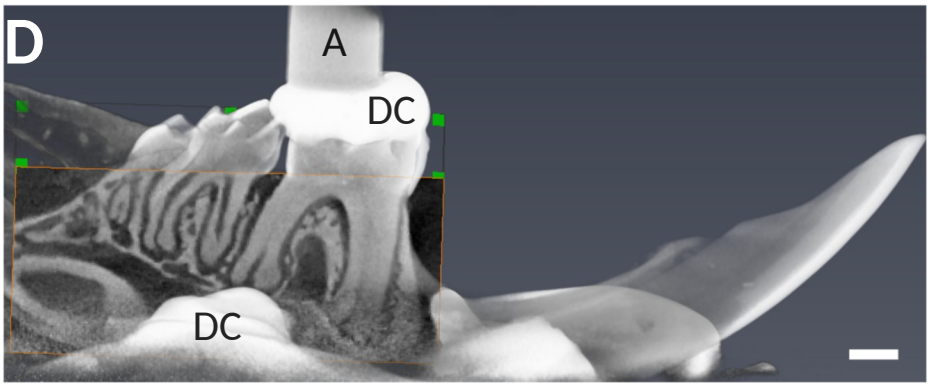


Figure3

Click here to access/download;Figure;JoVE Fig 3

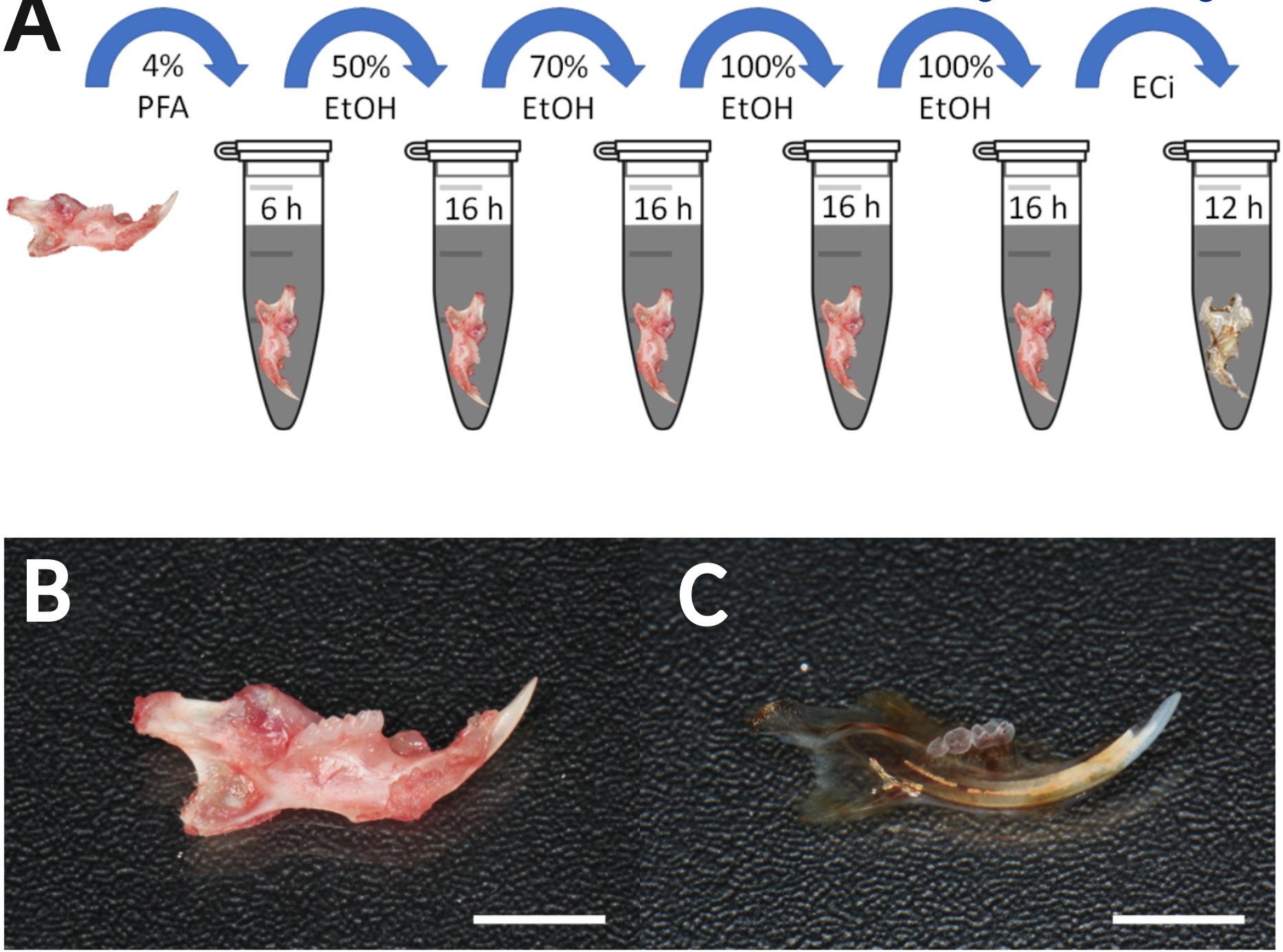


Figure4

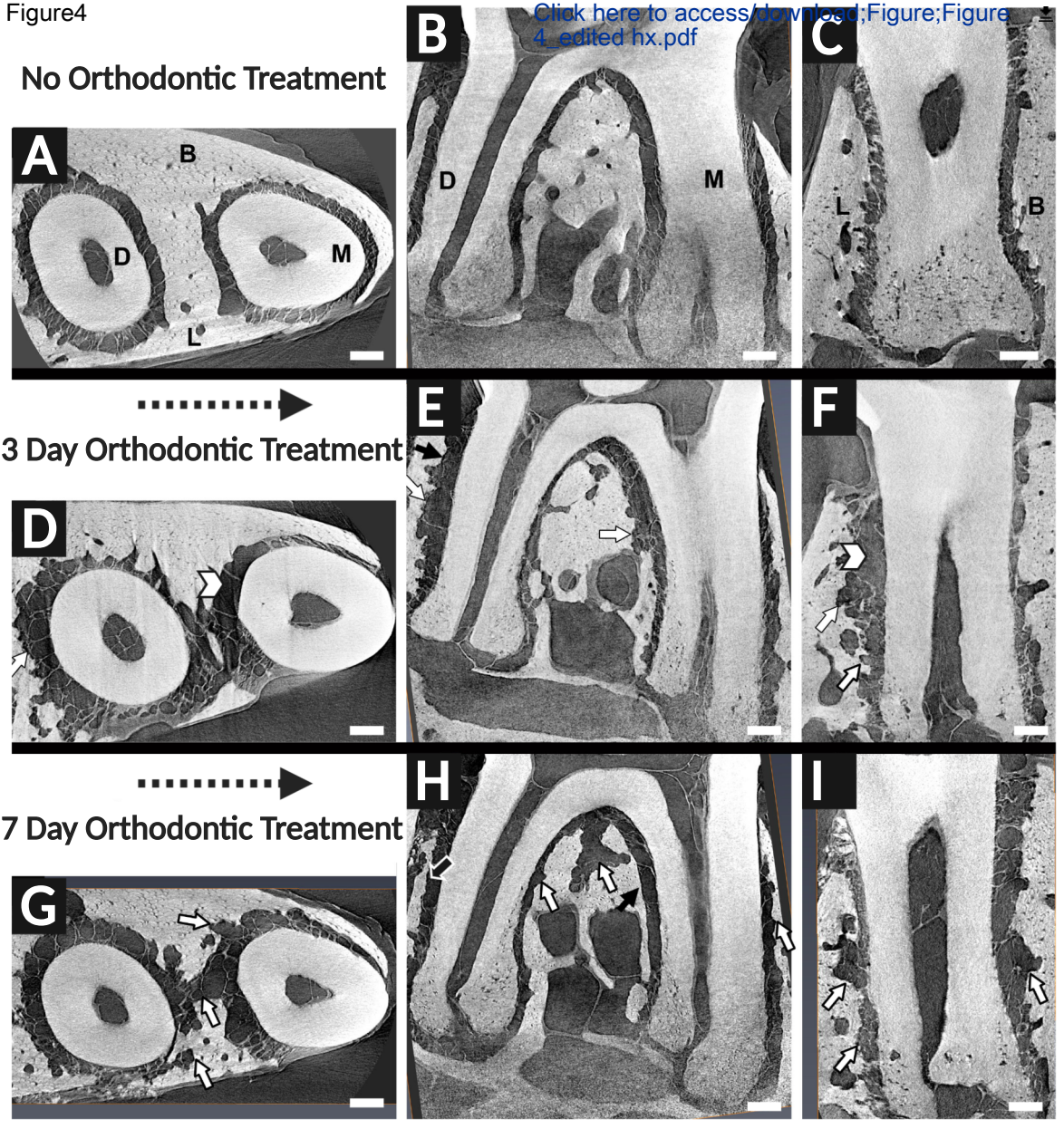


Figure5

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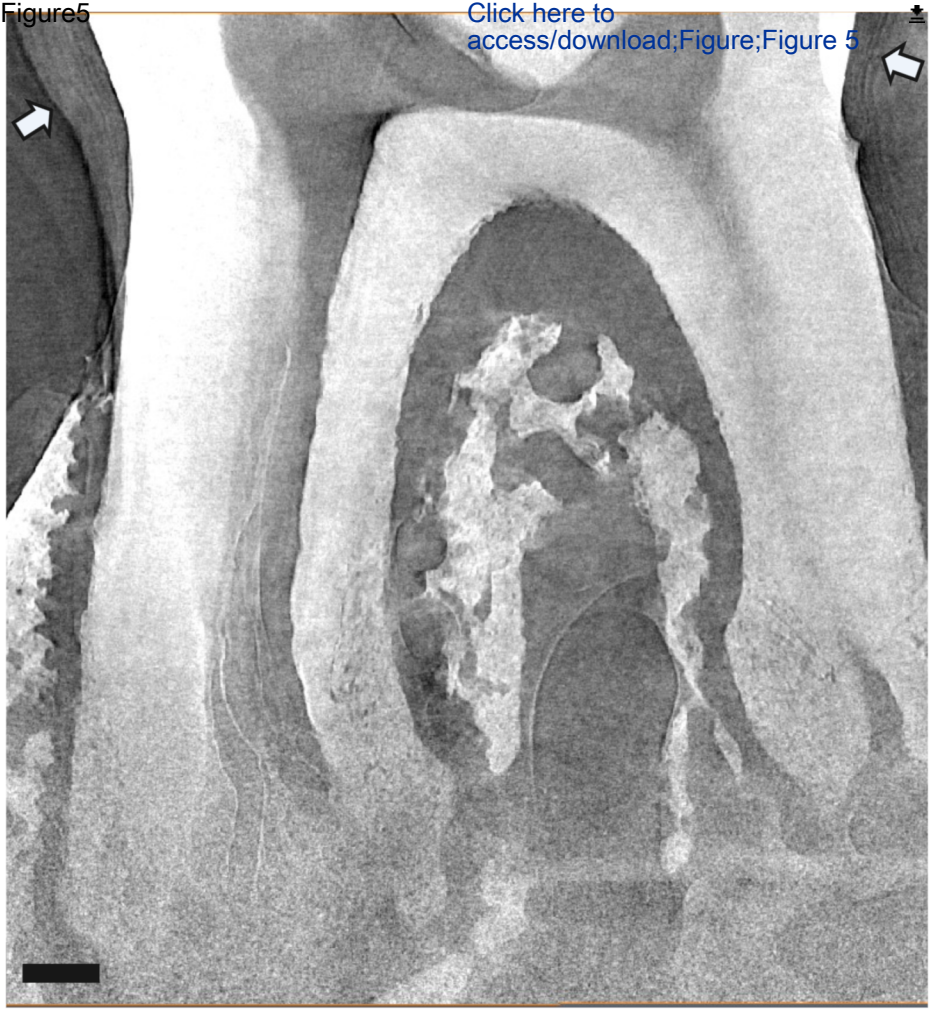


Figure6

Click here to access/download;Figure;JoVE

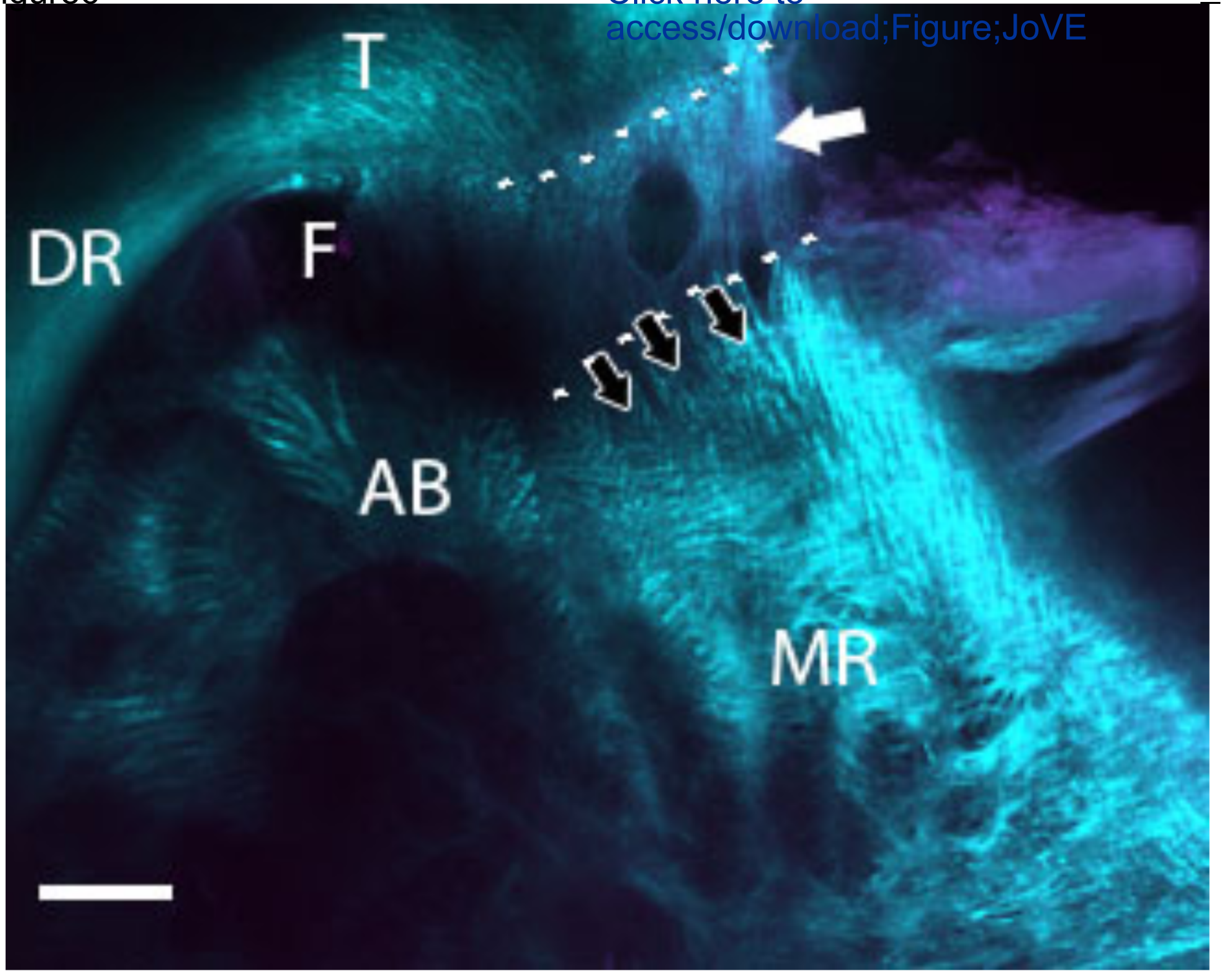
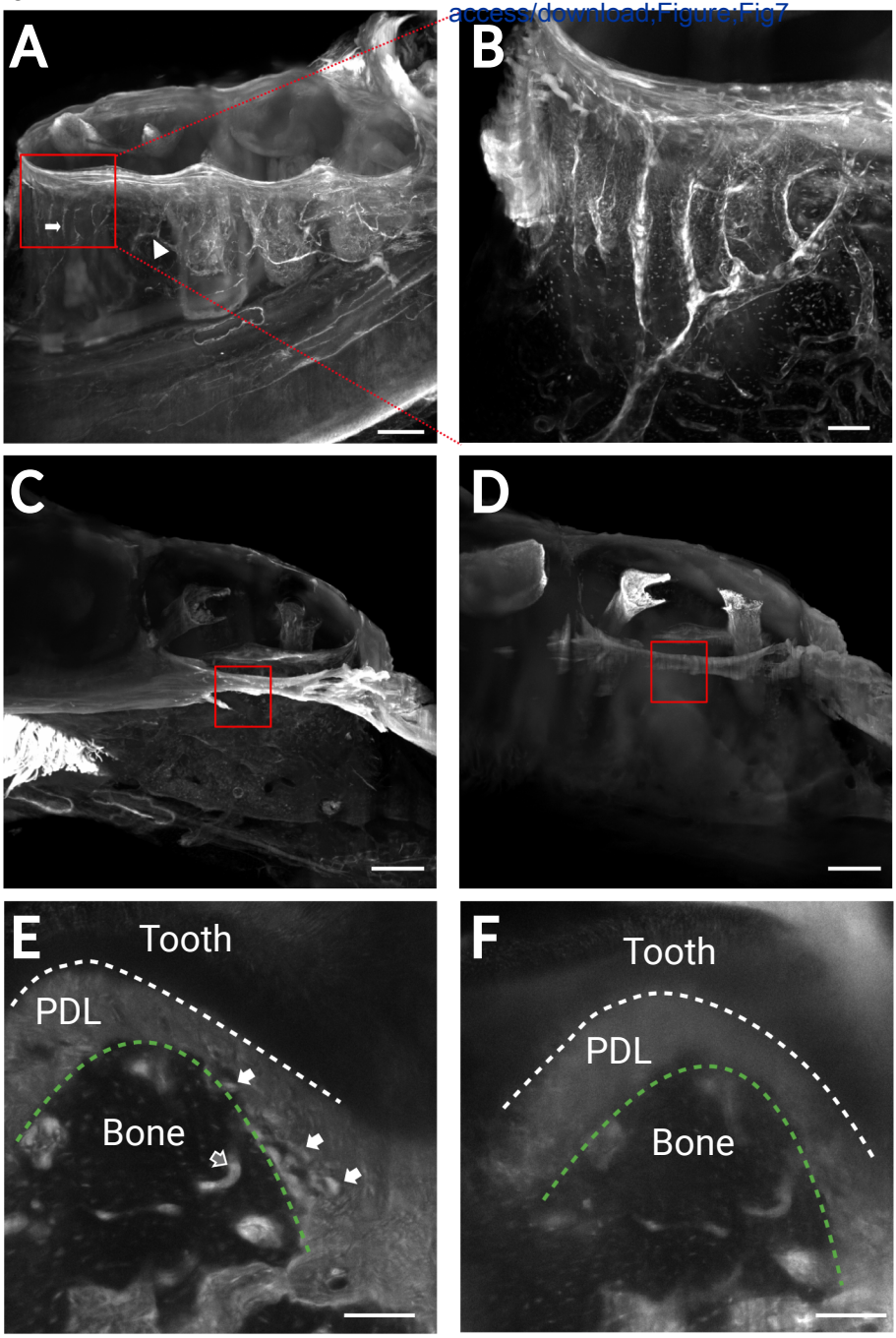


Figure 7

Click here to access/download;Figure;Fig7



Name of Material/ Equipment	Company	Catalog Number
1-mL BD Luer-Lok syringe	BD	309628
1X phosphate buffered saline	VWR Life Sciences	0780-10L
200 proof ethanol	VWR Life Sciences	V1016
Aluminum alloy 5019 wire	Sigma-aldrich	GF15828813
Avizo 9.7	Thermo Fisher Scientific	N/A
Castroviejo Micro Needle Holders	Fine Science Tools	12060-01
Clr Plan-Apochromat 20x/1.0,CorrV	Zeiss	N/A
Cone socket handle, single ended, hand-form	G.Hartzell and son	126-CSH3
EC Plan-Neofluar 5x/0.16	Zeiss	440321-9902
Elipar DeepCure-S LED curing light	3M ESPE	76985
Eppendorf safe-lock tubes, 1.5mL	Eppendorf	22363204
Ethyl cinnamate, >= 98%	Sigma-aldrich	W243000-1KG-K
Hypodermic Needle, 27G x 1/2''	BD	305109
Ketathesia 100mg/ml	Henry Schein Animal Health	NDC:11695-0702-1
KIMWIPES delicate task wipers	Kimberly-Clark	21905-026 (VWR Catalog number)
LightSheet Z.1 dual illumination microscope system	Zeiss	LightSheet Z.1/LightSheet 7

LSM 880 NLO multi-photon microscope	Zeiss	LSM 880 NLO	
MEGAmicro, plane, 5mm dia, SS-Thread	Hahnenkratt		6220
MicroCT machine, MicroXCT-200	Xradia	MICRO XCT-200	
Mini-Colibri	Fine Science Tools	17000-01	
PermaFlo Flowable Composite	Ultradent		948
Procedure platform	N/A	N/A	
Routine stereo micscope M80	Leica Micosystems	M80	
Sentalloy NiTi open coil spring	TOMY Inc.		
T-304 stainless steel ligature wire, 0.009'' diameter	Orthodontics	SBLW109	
X-Ject E (Xylazine) 100mg/ml	Henry Schein Animal Health	NDC:11695-7085-1	
Z100 Restorative, A2 shade	3M ESPE	5904A2	

	Comments/Description
	0.08 mm diameter wire, length 100th, temper hard. Used as wire ligature around molar.
	Used to analyze microCT scans
	Used for second harmonic generation imaging
	Handle of the inspection mirror Used for light-sheet imaging
	Purchased from VWR
	Used for lightsheet imaging

Used for two-photon imaging

Front surface inspectrio mirror

Custom-made from lab materials

A 0.15mm diameter closed NiTi coil with an inner coil diameter of 0.9mm delivers a force of 10g.
Similar products can be purchased from Dentsply Sirona.

0.009''(.23mm) diameter, Soft temper

We would like to thank all the reviewers for their thorough review and comments. We addressed the all of the remarks and hope the reviewers will find the manuscript ready for publication.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
3. 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.

For example: Kim wipe, Eppendorf, etc.

Thank you for the reminder! We have gone through the manuscript and addressed any issues related to comments 1-3 from.

4. Line115: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.

We have corrected this.

5. Line 116: Please specify how the upper jaw was immobilized.

Specific instructions has been added to step 1.3.

6. Line 119: Please specify the volume of saline used.

We specified volume of 50µL (roughly 1 drop) in step 1.5.

7. Line 124-127: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have shortened this section and modified it to a note after step 1.7.

8. Please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral for time units. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Line: 138, 154,198,215-219, 223, etc.).

These instances have all been corrected.

9. Line 150-151: Please specify the method of euthanasia used to sacrifice the animal.

Method of euthanasia has been specified in step 2.1.1.

10. Please include a one-line space between each protocol step and highlight up to 3 pages

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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have corrected the line space and highlighted the essential steps.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

We have corrected the format of the references.

12. Please sort the Table of Materials in alphabetical order.

It has been sorted.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This work describes the technical steps of three protocols under one umbrella: induction of orthodontic tooth movement in a mouse model; phase-contrast microCT imaging of the PDL, dental roots and alveolar bone; and a novel 3D histology method that combines attaining optical transparency by leveling refraction coefficients of the sample component and fluorescent optical microscopy. These methods can be used together, as well as separately in the context of related studies.

This is a very interesting, thought-through and validated triple protocol. The publication is timely and will be of great utility for researchers working at the interphase of clinical and basic science, for anatomists, zoologists, physiologists, and of course dentists.

Rich illustration of the steps is clear and self-explanatory for reproducing these methods. This reviewer is very happy to see some illustrations of mishaps (like Fig. 5) - this is the way we learn, as well as practical tips (like, use at least 3 coils of the NiTi spring not engaged in the attachment; or extend the dehydration stage if the optical transparency is insufficient). These practical tips illustrate that the authors have tried multiple variants of the protocol, and present the most consistent one.

Major Concerns:

Not so much here: why the page footer say November 2017? Is it a typo, or the work is 3-years old? If it is that old, has the content been updated?

This is a typo that has been corrected.

as well, the paper would benefit from proof-reading by a native English speaker. That is exclusively for style and language precision, because the content and logic of the

manuscript are very good. A more appropriate selection of synonyms, and better grammar will make this paper more impactful.
Thank you for this suggestion, we edited the manuscript and hope it is read better.
Minor Concerns:
In the order of their appearance.
Line 27 "complex biological remodeling process" - did the authors mean "complex biological process" because remodeling comes later in the same sentence.
Yes. It has been corrected.

Line 31 "high remodeling rate" - did the authors mean "high metabolic rate" or "high growth rate"? because mice don't remodel bone that much in a sense we apply to large animal bone remodeling (e.g., osteonal remodeling, or hemiosteonal remodeling)
We agree with the reviewer that the word metabolic will be more appropriate - We changed it to metabolic.

Line 51 "fast-paced" movement. relative to what? For professional orthodontists - fast indeed, but for other folk, especially for patients maybe not. Please specify - fast with respect to what?
We removed fast-paced.

Line 118, mouth retractor. Is it a custom-made cheek and lip retractor? Is it possible to specify in one sentence?
It is a commercially available product. We have specific the generic product name more in text (Step 1.3), and we will list it in the material list.

Line 138 - heating the coil to facilitate austenite to martensite phase transition. wouldn't that occur naturally when the subject closes their mouth and the coil temperature equilibrates at 37 degrees?
We recommend artificially heating the coil in order to immediately check the placement of the coil, instead of waiting for temperature equilibrium which may take a longer time and anesthesia may lapse.

Line 154 - what is special about 30 minutes after sacrifice? Doesn't decomposition commence immediately? Maybe it is worth to say "as soon as possible" instead of 30 minutes?
30 minutes is our suggestion for a time goal that is achievable based on our experience. We

modified the text to reflect the sentiment of "as soon as possible" as well. (Note after Step 2.1.1)

Line 184 - until embedded. Did the authors mean until it touches? until the anvil is in contact? Embedding implies somewhat tight 3D interaction.
Indeed, anvil should be embedded in the composite.

Line 227 - to remain "optically transparent"?
Yes. The text has been modified.

Line 232 - "dental alignment is not the goal of the experiment"?
Dental alignment is not the goal here as well as not generating a functional occlusion. Any movement of the tooth that generates remodeling of the alveolar bone around the roots is considered useful for orthodontic research purposes.

Line 238 - "light-curing bulb" - is it seriously a bulb? a dental curing lamp has a fiber light transducer on the outside, and a diode inside. or there's a bulb indeed?
Light sources vary in form. We modified the text (line 147) to accommodate for that variation. In reality, as long as the source provides the right wavelength and intensity for curing the composite, it is an acceptable tool.

Line 251 - "pdl-bone border" is it an interface? thre is also a typo in "border" in the next line
The border is an interface. We have corrected the typo in the next line.

Figure 1 D - the gap between two molars doesn't look like 0.4 mm. Please check.
Thank you for pointing this, we had a typo 0.04mm (40 μm) (line 272) is the correct number.

Figure 4 H, black arrow - how do you identify this site as the site of bone apposition?
This is a good question, we cannot verify this through histological markers since this is not the scope of the paper however we see changes in the bone that indicate bone apposition such as a smooth surface in areas where resorption craters were seen at 3 days and a uniform and narrower PDL relative to 3 days.

Line 329 - have you already explained that SHG is second harmonic generation? Why not spelling it out?
We have the full name in text, first appearing in representative results in line 322. We defined the abbreviation there.

Line 371 - did you mean "transparency of the final product"?
Yes. We have changed the wording here. Thank you for the suggestion!

Reviewer #2:
In reviewing the manuscript, there are some areas of clarification:
Title: 3D imaging of PDL collagen fibers during orthodontic tooth movement in a mouse model - The word DURING in radiology is based that we are looking at the moment of the movement. In this case, I would suggest replacing this word that can suit better your approach.

We believe that this is indeed a proper description that is within the word limit of the journal, we have not stated that this is real time nor time lapse imaging.

Abstract: The objective of your study was to evaluate the PDL during orthodontic movement, but in this case, I think that you should focus more on the methods you used to evaluate this. At the end of your abstract, instead talk about the murine model in orthodontic, I would suggest concluding with something about the method you used.

We edited the text of the abstract.
I would recommend reviewing if the keywords are according to Index Medicus;
In the introduction, I would suggest adding information about 3D analysis and the kind of images you used in the protocol.
We modified the keywords, the detailed 3D analysis is not the scope of the paper and is elaborated in our previous papers.
Protocol:
1.2. Move this sentence after 1.4. Since the microscope is used to see more details inside the mouse mouth.
Thank you! We changed it per reviewer suggestion.

Specify how you exactly measured the force you applied. – NiTi coils produce a constant force upon their activation this is due to the superelastic property of the alloy. Force levels are calibrated and determined by the manufacturer.

Add information about the diet the mouse eats during the movement.
We added this to the manuscript at the end of the second paragraph of results (line 276)

If it is difficult to do the imaging in a fresh sample, how did you handle to do the images? Did you divided groups per day?
The most challenging part of imaging fresh sample is to maintain humidity. We have included suggestions for constructing a humidity chamber (Step 2.1.7). We also suggested that the preparation (dissection and mounting) should be done in a speedy manner (line 169-170). Even though the tissue is fresh, there is not a high risk of damage, since the PDL is enclosed by bone.
Additionally, we suggest only processing one mouse at a time for microCT scans of fresh tissue. This can be achieved by staggering the dates of imaging and experiments.

3.1.5.; 3.1.6. and 3.1.7. Improve your description, looks that these sentences can be together.
We have made the text here more concise.

You can use the micro position of the MicroCT to improve the position of your sample as well.
No, this cannot be done since the loading system is screwed into the stage – this method was published in detail in few publications before. Therefore, we are not elaborating on the technique and system in this manuscript. We do show in the video how the mounting is performed, since this was not done before and would be of value to the readers.

3.1.10. Improve your description of the treatment you did in your samples before the scan. Did you use any enhanced solution for soft tissue?
No extra treatment was done to the sample before scan. No enhanced solution was used. We did exactly as described. For clarity, we have emphasized that the tissue is fresh for micro-CT scans in text at multiple times. (Header of step 2, Step 2.1.5, and in third paragraph of representative results)

MicroCT setting - You need to improve your description of the set up using the MicroCT scan. For example: it is missing that you need to calibrate the machine before performing the scan, organize all the parameters used, and then also explain how you did the reconstruction.
As previously mentioned, the imaging technique and image analysis were thoroughly described in previous publications which are referenced in our manuscript as well.

There are two 3.1, and check if the order you wrote is correct.
We have updated the numbering.

What are the steps you did to analyze in the fluorescence microscope? Please add the parameters as well.

We added the parameters in figure legends of Figure 6 and 7.

Representative results:
In your results, you show some measurements you did in your samples that is not explain in your protocol.
I believe the reviewer refers to the measurement of the size of the gap and the PDL space. We have added brief description on how the gap is measured (interproximal space between crowns of first and second molar) in representative results (line 271) and again in legend of figure 2.

Moreover, I would suggest also in your protocol add all the steps you follow to get your results (software, etc).
We added the name of the softwares to material list and references (references number 18 and 20) for further details.

The focus of this manuscript is the challenging orthodontic manipulation and the new clearing method we think that adding detailed information on the image analysis process that is referenced will complicate the manuscript.

Discussion:
You need to talk about MicroCT and fluorescence microscope, why did you use these methods? Discuss with other papers that use these methods, etc.
We added a paragraph in Discussions (3rd paragraph) to address this. Thank you for your suggestion!

The manuscript is clearer when short, concise statements are used rather than longer sentences or sentence fragments. I would also suggest review your grammar.
Thank you! We agree. We edited the manuscript and improved the language.

Reviewer #3:
Manuscript Summary:
This manuscript clearly shows the effective method for 3D imaging of mouse periodontal ligament (PDL) using micro-CT and clearing. Other 3D imaging methods of PDL fiber consisted collagen bundle require usually fixation and numerous serial sectioning: Light and electron microscopy. Additionally, Author's method can observe more wider area than

conventional observation method for PDL analysis. However, I have some comments about the manuscript, which I hope, may help them to improve your manuscript.

Major Concerns:
This manuscript describes a method of generating OTM and two imaging methods that enables 3D visualization of PDL fiber: micro-CT imaging and clearing method. Both imaging methods are interesting and important for the researchers working in tooth biology. However, the structure of the manuscript is complicated. I suggest the manuscript is divided into two parts to make them readable. The one is about OTM and micro-CT imaging for submission to Jove. The other is about clearing method, which might be the suitable for Microscopy. Please consider this point.
Thank you very much for raising this point. We discussed this option in depth, however we think that focusing on the microCT method which was published few times before in detail by one of the authors will reduce the value of this manuscript. The clearing method is new especially for this purpose and therefore we think it is complimentary and of great importance for this method paper. We are indeed working on another manuscript focused on the imaging of the cleared mandible that will be submitted to another journal soon.

Minor Concerns:
1. The authors need to explain briefly why micro-CT in this protocol can detect and analyse collagen bundles. The submitted method is powerful and useful method for PDL biology. Using this method, the authors have achieved great work. Conventional micro-CT cannot observe and analyse detail morphology of collagen bundles.

It should be stated whether this protocol can only be used for Xradia that is high power and resolution micro-CT.
We added information about the imaging technique and required microscopic abilities. Synchrotron as well as any phase enhancement/contrast microCT will also produce such images.

PDL fiber is dense and closely arrange in the observation light and electron microscopy (Beertsen, et al. Periodontal 2000, doi.org/10.1111/j.1600-0757.1997.tb00094.x and Hirashima, et al. Anat sci int, doi: 10.1007/s12565-019-00502-5). In figure 4 of this manuscript, PDL fibers are sparse. The difference between Xradia observation and other microscopy, i.e. light and electron microscopy, need to be described.
We added relevant information to the introduction line 78.

2. Page 2, Line 71~75: The authors describe OTM model in maxilla and mandible. Please describe why the authors chose a mandible model, and what is the advantage of the

mandible model over the maxilla model.
We added this to the introduction, starting at line 69.

3.Page 2, Line 74 and 80: The Authors use same expression "Here we will describe how to~"S. Could you avoid the same phrase?
The wording has been changed.

4. Page 2, Line 81 and page 5, Line 200: The resolution (pixel size) of 3D scan using micro-CT is mentioned. "Pixel size" is only used for 2D imaging. For 3D imaging, "Voxel size" is used. The authors should mention voxel size.
The terminology has been corrected.

5. Page 2, Line 107: "upper incisors On the tail" --> " upper incisors. On the tail "?
Grammar has been corrected.

6. Page 2 and Figure 1A: The picture is only front view of custom-made procedure platform. Adding the picture of side view, the manuscript is more accessible.
We added a figure of a side view in Figure 1B.

7. Page 3, Line 108 and 109: "See Figure 1A for an example platform" --> "Figure 1"
Numbering has been corrected.

8. "Dental composite or the composite or flowable composite" are used frequently in the manuscript. Dose these phrases mean dental composite resin? These phrases should be unified with dental composite resin.

We use two different kinds of dental composite resin in the protocol. Flowable composite resin is used to anchor the coil to the incisor during implantation of the device; packable composite resin is used to mount hemi-mandible to the sample stage. We will be more specific in text. Thank you for the suggestion!

9. Judging from figure 2D and figure 4, Amira or Avizo software (FEI) may be used for imaging analysis and figures. The authors fail to provide software information.
Yes, Avizo is used to analyze the micro-CT scans. We added the software information in the material list. Per journal rule, we cannot include that information in the main text.

10. Page6, Line 245~260: The authors mention that rough surface of alveolar bone indicates bone resorption because the boarders between PDL and alveolar bone is rougher relative to other regions. The sentence about bone resorption in line 252 and 253 is not convincing.

Micro-CT imaging cannot detect cells. In order to morphologically define an area of rough bone surface as a bone resorption area, osteoclasts must be present in the same spot. Or same individual mouse must be imaged and compared the same area using micro-CT. The authors should change contents of this paragraph.
The reviewer is right we changed the description with more accurate word selection.

Reviewer #4:
Manuscript Summary:
Study title: 3D imaging of PDL collagen fibers during orthodontic tooth movement.

This paper describes a step-by-step protocol for generating an orthodontic tooth movement (OTM) of the first mandibular molar and for 3D acquisition and visualization of a tooth-PDL-complex in the mouse model without the need for 2D sectioning. This methodology could be used to study the role of periodontal ligaments (PDL) during OTM, and to visualize associated vasculature in a 3D context.

Major Concerns:
To make useful use of this protocol, I recommend that the following issues should be addressed

1- This protocol describes orthodontic tooth movement of the first mandibular molar in a mouse model. The author states "since the mobility of the mandible and the presence of the tongue add another complexity level. Here we will describe how to overcome these difficulties and generate OTM in the mandible".
* The protocol does not clearly explain how to overcome these difficulties. e.g mandible stabilization or tongue retraction.
Our animal platform provides the necessary as well as the way the ligature is threaded from one side of the tooth to the other, as described in the text (Step 1.1 and step 1.6). We added another picture of a side view of the platform in Figure 1B.
* What are the advantages of using the mandible vs. maxilla to model the orthodontic tooth movement?
Thanks you for bringing this up we added this to the introduction, starting at line 69.
2- There are some places in the protocol that need clarification:
* Page 5 Step 1.9: How the coil threads were activated?
“Non-active” meant the coil was not strained. However, we understand the confusion it may cause, and have corrected the wording.
* Not clear how to manage to insert coil threads into the incisor while the orthodontic power chain is in place?
Good point. Power chain needs to be temporarily removed. We have added description to

clarify (Step 1.9 and 1.10).

* Page 5 Step 1.5: How often saline was applied to the mouse eyes? Saline is a solution that does not stay in place.

The surface tension will hold the droplet of saline on the eyes. We have also added instructions for replenishing the saline (Step 1.5).

* Page 6 Line 151: "Remove the mandible from.." (missing words or not complete).

Wording has been corrected.

* Page 8 Para2: What are the anatomical landmarks used to measure the mesiodistal space between 1st and 2nd molars and how was the PDL space measured?

We measured gap size at the Interproximal regions of the crowns. We have added description of this in representative results (line 272)

* It would be informative if this protocol recommends how many mice to include to perform OTM under these experimental conditions.

We have added samples size for the gap size measurements in results (line 272). For other measurements of interest, the exact number of mice needed depends on the specific goal of experiments and the desired statistical power.

* 19hr scanning is quite a long time. Was chamber humidity maintained throughout the scanning?

Yes. The chamber is air tight. Humidity is maintained throughout detailed information about the microCT method can be found in our previous publications as mentioned in the text (Step 2.1.7).

* The use of samples for additional experiments: After finishing microCT scanning, were the samples retrieved without composite attached. Is there any recommendation for the use of the mandible for different experiments?

We do not recommend using the unfixed sample after a 19h scan, due to decomposition.

Additional comments:

Ligation is a technique that is used to induce periodontal diseases in a murine model.

The ligation technique for generating periodontal disease utilizes a thread such as silk that can accumulate bacteria. We are employing this technique in our lab for studies on periodontitis. The metal wire is smooth and is not a surface for bacterial accumulation, we do not see the same bone resorption features as using a silk thread. On that note the other alternative to move murine teeth that is described in the literature is by impacting a piece of rubber in between the teeth which is very traumatic and generates tipping movement.

During the study period, did the wire ligation cause any periodontal diseases: inflammation, bone destruction, and increased osteoclast activity? Including contralateral shame (the wire inserted between molars) will be informative. This could be easily demonstrated by

histology to rule out this possibility.
This indeed is a great idea and was done previously by other groups, however it is not the scope of this work.

In Figure 4, How could you confirm that the structure seen in PDL space is indeed PDL collagen bundles? Because this also appears in the dental pulp. Using this method, is there a way to differentiate between PDL and blood vessels. Both are soft tissues.
Collagen fibers do exist in the pulp and that is what seen in our scans. Indeed, not all collagen fibers can be seen using the microCT method – only the principal ones stretched between the tooth and the bone, which are type I. This was discussed in details in our previous publications, as mentioned in the manuscript (References number 18 and 20). In regarding to the blood vessels – this is the reason we present here a second clearing technique with endothelial cells reporter mouse.

This protocol describes mesial OTM within 7 days ~ 0.4mm as demonstrated by the distance between the 1st and 2nd molars. Is there a way to confirm the mesial tooth movement (Vs. tooth tipping)?
We show that the PDL space is increased along the entire length of the PDL (Figure 4) which provides evidence for translational movement.

Minor Concerns:
Some figures need more attention:
* It would be useful if figure 1 was labeled: plastic platform, table, headrest, mouth retractor, the chain.. etc. (Easy to visualize while reading).
We have added labels to major parts of the mouse bed to Figure 1A. Figure caption has been updated to give more detailed description of the parts. To avoid crowding the figure, we respectfully elected not to label all the parts suggested by the reviewer.

* Figure 1B is not a true magnified image of Figure 1A.
That is true. While it is the same area, it is not from the same stage of the procedure. We will remove the red outline to avoid any confusion.

* While annotated in the figure legend; labeling the components of orthodontic apparatus in Figure 1E or F would be helpful for the readers who are not orthodontists.
Color-coded components shown in Figure 1F are described in detail in the caption. We have also added label for NiTi coil in Figure 2.

* Figure 4C: Did the author see evidence of resorption in both molar roots, which one is shown in the figure.?

We agree with the reviewer and the wording was changed to better convey what is evidently seen from the images.

* Figure 4: "black arrows point at regions of bone apposition": How did the author identify bone apposition from microCT data?
See previous answer – we have modified the wording.
* Figure 7: Adjust the red box size to accommodate the whole magnified region shown in Figure 7B
We believe the size is correct
* Figure 7A: Looking at the figure, it is difficult to identify where the bone is and where the PDL space. A dotted line to outline the regions will be useful.
The tooth is seen through the bone, thus demarcating this will be impossible.
* What is the difference between Figure 7A and C.
We have clarified that 7A is the image of control hemi-mandible (no OTM), while C is after 7-day OTM. (Legend of Figure 7)

Reviewer #5:
Manuscript Summary:
The manuscript entitled "3D imaging of PDL collagen fibers during orthodontic tooth movement in a mouse model" by Naveh et al 2020. aims to describe a protocol used for the 3D visualization of the periodontal ligament (PDL) of the mandibular molar teeth in a mouse model. To do this, the authors performed an experiment using orthodontic movements (OTM) of the mandibular mola teeth in an animal model using a NiTi coil for a period of 7 days (https://pubmed.ncbi.nlm.nih.gov/32699355/). In addition to the animal model (reporter mice Flk1-Cre; TdTomato), this is indeed one of the few in vivo reported approaches that use the OTM in the mouse mandible. There are previous publications regarding the mouse mandible, a reporter mouse model, and the use of OTM, but approaching the bone cells effect in an organ culture model (https://pubmed.ncbi.nlm.nih.gov/21208081/). It is also relevant the use of a validated reporter mouse model for the detection of the endothelial cover of the vasculature (https://pubmed.ncbi.nlm.nih.gov/21208081/). The authors used two techniques: The first technique is the microCT with phase enhancement analysis of the fresh mouse mandibles under OTM at the molar teeth; the second technique corresponds with the use of fluorescence microscopy of the mandibular samples using (previously to the imaging analysis) a clearing technique with a solvent-based material (Ethyl cinnamate or Eci) which is known clearing technique for biomaterial visualization without the loss of the tissue fluorescence (https://www.uni-due.de/experimental-immunology/eci.php9). This protocol

represents a novel technique for PDL imaging in an animal model not entirely prefer for OTM approaches. Despite the novelty of this report, as a written protocol presents some issues that I suggest considering in order to improve its reading, reproducibility and replicability by other researchers.

Major Concerns:

1. At the beginning of the protocol one of its most interesting features is the avoidance of the use of sample sectioning for 3D visualization of both the PDL collagen fibers and the associated vasculature. However, in order to validate this approach, I recommend performing control experiments. It is understandable the premise regarding tissue distortion and loss under histological techniques, but it is important to validate the 3D imaging herein exposed with a proxy evaluation, or perhaps use the FIB/SEM approach to contrast the findings. Unfortunately, it is not possible to validate these results by themselves only.
The micro-CT methods were published previously and thoroughly discussed and validated with other methods. It is not clear to us what the reviewer is looking for in terms of validating the clearing method other that indeed observing the PDL through the bone. Flk1cre mice were used in other publications and are known reporter mice for endothelial cells. They are used here to show the preservation of the fluorescent proteins through the clearing process not provide an analysis of the blood vessels network. This will be a paper on its own. We will be happy to image and analyze the tooth-PDL-bone complex during orthodontics using FIB/SEM although this method necessitates extensive tissue preparation unless used in a cryo mode, this would be a paper on its own as well.

2. In the first paragraph of the introduction (lines 47-52), please add/consider the role of bone cells such as the osteocytes as trigger of the alveolar bone remodeling during OTM.
We added this

3. The authors indicated a pixel size of 0.76 microns in several parts of the manuscript. Since this protocol intends to describe a 3D technique, I recommend clarifying this measurement as voxel instead pixel.
This has been corrected.

4. The protocol initiates with the description of the ethical considerations for this research. However, there is a lack of the description regarding the general health of the mice, including the effect of OTM in vital functions such as feeding (considering the poorly explored mandibular model for OTM), incisor teeth eruption, among others. In addition, it is relevant to adhere to the 3Rs statements and a short description of the age, sex and sample size of the animals would help to understand the impact of this protocol in the animal

research protective strategies

We have added some text to clarify the effect of the OTM device on the health of mice and diet (Representative results, line 270 and 276; and in step 1.14). OTM device on one side (assuming it is corrected inserted) does not affect feeding. It does cause some discomfort to the mouse for the first 24-48 h post procedure, which is why we advise daily monitoring for signs of pain or excessive discomfort. Pain medication may be given if outward signs of pain are seen. Incisor eruption is not affected by the device either. However, eruption moves the anchor point of the coil and should be taken into account when deciding the duration of OTM. The effect can be minimized by periodically detaching and re-anchoring the coil at a lower spot on the incisor. We have added this to the text for clarity (Discussion, line 411).

We have also included description of the age, sex and samples size we used in representative results (line 270-272).

We are making these recommendations based on our approved animal protocol. While we agree with the reviewer that the 3Rs of animal research and other ethical guidelines should be adhered to, we do not think it is the scope of this protocol to present those details. It would not be instructional to the readers since these details would vary considerably between studies.

5. The main sections of the protocol as follows: 1. Orthodontic movement; 2. MicroCT scan for 3D imaging of fresh PDL collagen fibers, using microCT´s with phase enhancement ability, here we used Zeiss Xradia microCT; 3. Clearing method. Considering this order, the subsections of the section number 2 are mislabeled. In addition, there are repeated subsections in the section number 3 with similar (but not equal) information and a mislabeling of the three final subsections. Please review the order of the numbering and the corresponded information.

Mislabeling has been corrected.

6. The reading of the whole steps is difficult without proper orientation of the associated figures. Perhaps the use of an initial indication of the relevant figures at the beginning of each main section would help to catch up the order in advance.

We are following the journal guidelines.

7. In line 119: The use of artificial tear in gel presentation would be advisable to prevent eye damage in the animal during anesthesia state.

Thanks for the advice! We report here of our method and experience. We use saline to wet the eyes of the mouse during procedure.

8. In line 141: Considering the use of the contralateral side as sham or negative control, the results should be presented using this comparative analysis.

The mouse without orthodontic tooth movement is our control (figure 4 A-C). Others have investigated the validity of OTM methods both with a coil and a rubber band, these are the

current methods for moving murine teeth orthodontically. We are providing information on generating such OTM in the mandibular teeth and the imaging methods.

9. In line 150: What was the euthanasia method? Please describe the advantages of the used euthanasia method, considering the wide range of options and the interest in maintaining intact the entire mandible.

Euthanasia method is now described in text (Step 2.1.1). Cervical spinal dislocation does not pose any risk for damaging the mandibles.

10. In lines 153-154: What is the rationale behind a 30-minutes post-dissection approach to consider the mandibular sample as fresh? There are some organ cultures studies that may help to discuss this item.

The 30-minute reference timeframe is a suggestion based on our experience as cited in the text (line 169-170). We don't think that organ culture is the right comparison here.

11. In line 174: The use of resin composite in the teeth surfaces is advisable for the OTM. However, the radiopacity of the resin composites may add some undesirable noise specially during microCT analysis. To overcome this problem, please consider the use of non-radiopaque dental biomaterials of rapid auto-curing properties and low dimensional changes such as Resin Pattern from GC America in order to stabilize the mandible.

The dental composite does not generate noise artefacts and the x-rays easily go through it. It is very easy to identify it. There is also a great advantage for the bonding material being radiopaque since we can verify the embedding of the tooth and anvil as well as its borders to rule out bonding of adjacent teeth and bone.

12. In line 202: Which microCT guidelines? Please add the reference.

Reference images required for reconstruction differ based on the type of reconstruction software and micro-CT equipment used. Per journal guideline, we are not advising which commercial software or micro-CT machine to use. We do describe our guideline in the same protocol step 2.2.3.

13. In lines 208-209 and 211-213: There is similar but not equal information. Which one is the correct?

This was a typo. We have deleted the wrong instruction line.

14. In line 214: Fixation vs clearing method?

We further clarified that the first step is for fixation purposes (step 3.2 and note).

15. In line 225: What happens at 4 Cº?

As noted in text, 4 degrees Celsius is below the freezing point of ECi (step 3.9 note). As a result, the solution would turn solid, which is not desirable for clearing or tissue structural preservation.

16. In line 230: Predictable OTM based on what rationale/contrasting previous results.

We changed the wording here. We also now present our measurement results in line 272.

17. In lines 240-242: There is a mention of average measurements but there is no a

statistical analysis and sample size data to understand the dispersion of these measurements.

We corrected this. See previous question.

18. In lines 252-260: The terms bone resorption and bone apposition should not be used since the techniques here reported are not validated to determine this cellular response in comparison to others (e.g., histological analysis). Instead, it is advisable to consider the identification of "signs" that suggest a bone remodeling process either resorptive or appositive in nature.

We changed the wording as recommended.

19. In line 261: Please describe if the used microCT equipment presents a moveable beam ray with a fixed position of the sample or, in contrast, the beam is fixed as the sample moves during scanning.

micro-CTs are built such that the x-ray source and detector are in line and the stage is rotating. We believe it is not necessary to add this.

20. In line 262: Figure 5 should be presented as a good scan versus a bad scan, not only the bad one.

Examples of good scans are found in Figure 4.

21. In line 270: The method of clearing can be used in a non-fixed sample, but in line 214 there is a fixation step. Please clarify which approach is more suitable (Please revew point 14).

Thank you for pointing this out. It is now noted in the protocol that it can also be used on fresh samples (Step 3.2 and note).

22. In lines 273-282: There is not a clear pathway to understand a cutoff point to consider the bone "correctly" cleared. Please clarify the parameters to overcome this relevant decision in a mouse mandible sample.

We added this to the text in line 321 in representative results.

23. Figure 2C: Please add a graphic of the mandible in position.

Respectfully, we believe this is not necessary. Figure 2C is meant to show to the readers the design of the slanted groove. A mandible in the groove can be seen in Figure 2B.

24. Figure 3C: What is the cutoff (objectively) point to consider the mouse mandible correctly cleared? Perhaps a transparency analysis with a standardized background (frequently used in dental clinical photography) would help to report this item.

Please see response for 22.

25. Figures 4: All composite figures should be labeled with the plane coordinates. Also, it should be 3 days instead 3 day (same for 7 day).

We corrected the labeling, orientation and plane labeling is mentioned in all images and captions (Figure 4).

In general, considering the attached figures, the general concern about the external validity of the obtained results is difficult to determine. The 3D visualization of the PDL and the associated vasculature is very important for dental research. The approach, however, may remain as a subjective evaluation. I recommend making figures with defined planes in the 3D environment that will help to understand how this protocol may add tangible data during a defined intervention.

The image analysis process can be done in many ways depends on the combined methods and co-localization chosen for a particular experiment. This is not the scope of the paper. All figures include details regarding the captured planes and orientation.

Minor Concerns:

Since the protocol of this in vivo experiment was performed on the mouse mandible, the authors may consider add this to the tittle (…during mandibular orthodontic…).

Thank you for the suggestion! We added it to our title.