Video Article

A Morphometric and Cellular Analysis Method for the Murine Mandibular Condyle

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Abstract

The temporomandibular joint (TMJ) has the capacity to adapt to external stimuli, and loading changes can affect the position of condyles, as well as the structural and cellular components of the mandibular condylar cartilage (MCC). This manuscript describes methods for analyzing these changes and a method for altering the loading of the TMJ in mice (*i.e.,* compressive static TMJ loading). The structural evaluation illustrated here is a simple morphometric approach that uses the Digimizer software and is performed in radiographs of small bones. In addition, the analysis of cellular changes leading to alterations in collagen expression, bone remodeling, cell division, and proteoglycan distribution in the MCC is described. The quantification of these changes in histological sections - by counting the positive fluorescent pixels using image software and measuring the distance mapping and stained area with Digimizer - is also demonstrated. The methods shown here are not limited to the murine TMJ, but could be used on additional bones of small experimental animals and in other regions of endochondral ossification.

Video Link

The video component of this article can be found at https://www.jove.com/video/55998/

Introduction

The TMJ is a unique load-bearing joint located in the craniofacial region and is formed of fibrocartilage. The MCC of the TMJ is essential for joint function, including unhindered jaw movement while speaking and masticating, but it is commonly affected by degenerative diseases, including osteoarthritis[1]. The TMJ has the capacity to adapt to external stimuli and loading alterations, leading to structural and cellular changes to the components of the MCC[2,3,4,5]. The load-bearing properties of the MCC can be explained by the interactions between its constituents, including water, the collagen network, and densely packed proteoglycans. The MCC has four distinct cellular zones that express different types of collagen and non-collagen proteins: 1) the superficial or articular zone; 2) the proliferative zone, composed of undifferentiated mesenchymal cells and that responds to loading demands; 3) the prehypertrophic zone, composed of mature chondrocytes expressing collagen type 2; and 4) the hypertrophic zone, the region where the hypertrophic chondrocytes expressing collagen type 10 die and undergo calcification. The non-mineralized region is rich in proteoglycans which provide resistance to compressive forces[6].

There is continuous mineralization at the hypertrophic zone of the MCC, where the transition from chondrogenesis to osteogenesis occurs, guaranteeing the robust mineral structure of the subchondral bone of the mandibular condyle[7]. Cellular changes in the unmineralized and mineralized regions ultimately lead to morphological and structural changes in the mandibular condyle and mandible. Maintenance of the homeostasis of all cellular regions of the MCC and the mineralization of the subchondral portion are essential to the health, load-bearing capacity, and integrity of the TMJ.

The multiple collagen transgenic mouse model (as described by Utreja *et al.*)[8] is a great tool to use to understand changes in collagen expression because all transgenes are expressed in the MCC. For an in-depth histological evaluation, histological stains are used to study matrix deposition, mineralization, cell proliferation, and apoptosis, as well as protein expression at the different cell layers of the MCC.

In this manuscript, histological and morphometric analyses are used to evaluate cellular and structural changes in the MCC and subchondral bone of the mandibular condyle of mice. In addition, a cell quantification method, for analyzing fluorescent histological images and for mapping light microscope slides, is described. The compressive static TMJ loading method, which causes cellular and morphological changes at the MCC and subchondral bone[9], is also illustrated to validate our methods.

The methods described here can be used to determine morphometric and histological changes in the mandibular condyle and mandible of rodents or to analyze other regions of endochondral ossification and the morphology of additional mineralized tissues.

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Protocol

The institutional animal care committee of the University of Connecticut Health Center approved all animal procedures.

1. Compressive Static TMJ Loading: Mouth Forced Open

Note: Four-week-old transgenic mice harboring fluorescent reporters for collagen (Col2a1XCol10a1), kindly provided by Dr. David Rowe (University of Connecticut), were used for the experiments described in this manuscript (n = 8; 4 males and 4 females). The Col2a1 cyan (blue) transgene is expressed in cells at the prehypertrophic zone of the MCC, while the Col10a1 cherry (red) cells are present in the hypertrophic region[8] (**Figure 1**). Mice were equally divided into two groups: 1) the loaded group, where mice were subjected to compressive static TMJ loading (described in step 2) and 2) the control group, where mice received no intervention.

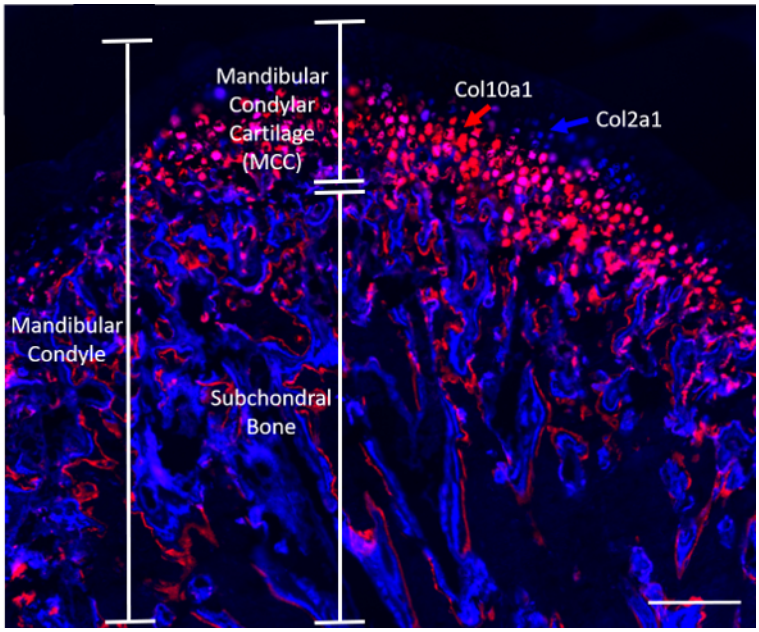

Figure 1.**Representative sagittal of the condyle of a double-collagen fluorescent reporter mouse (Col2a1XCol10a1).** Scale bar = 200 µm.
Please click here to view a larger version of this figure.

1. Fabricate springs usingbeta titanium alloy archwires (0.017 x 0.025 in) (**Figure 2A**).
2. Anesthetize the experimental animals by an intraperitoneal injection of a mixture of ketamine (90 mg/kg bodyweight) and xylazine (13 mg/kg bodyweight).
3. Gently open the mouth of the mice and insert the springs, engaging the loops in the maxillary and mandibular incisors (**Figure 2B and C**).
4. Induce compressive static TMJ loading by keeping the springs in the incisors of the anesthetized mice for 1 h. Repeat this procedure for 5 days.
5. Inject the mice with 5-ethnyl-2'-deoxyuridine (EdU ; 30 mg/kg bodyweight) intraperitoneally for cell proliferation analysis 2 days and 1 day before euthanasia.

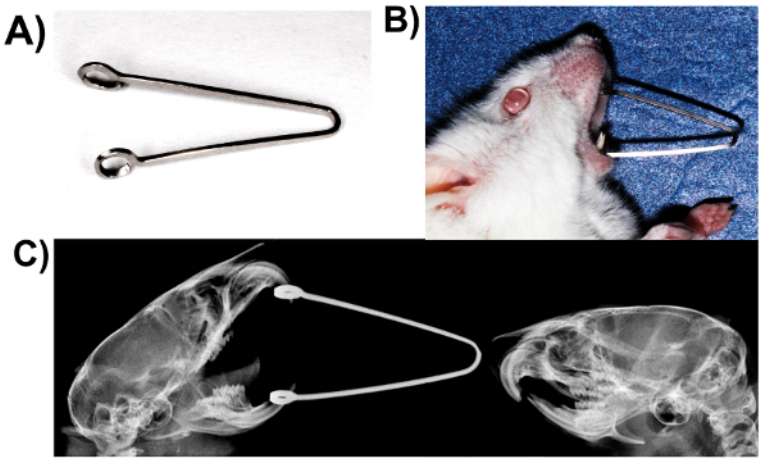

Figure 2. Compressive static TMJ loading: mouth forced open model. (A) Spring fabricated of 0.017 x 0.025 beta titanium alloy archwire. **(B)** Loaded mouse with spring. **(C)** Radiograph of loaded and control mice showing differences in the positioning of the mandible. Please click here to view a larger version of this figure.

2. Mandible Dissection and Fixation

1. At the endpoint of the forced-open mouth procedures, euthanize the experimental animals by an approved method.
2. Dissect the mandibles by cutting the muscular attachment without scraping the cartilage of the condyle.
3. Place the cleaned mandibles in 10% formalin for 24 h for fixation.
   Caution: Formalin is an irritant; wear appropriate personal protective equipment.

3. X-ray Imaging and Morphometric Measurements

1. Place the mandibles in a flat container (*e.g.,* a 55 mm x 16 mm Petri dish) and take radiographs of the samples using a cabinet x-ray system at a 26 kV for 5 s.
   NOTE: Place a scale bar before saving the images.
2. Perform morphometric measurements of the mandibles using an image analysis software (see the **Table of Materials**).
   NOTE: Before performing any measurements, use the scale bar at the radiograph to determine the unit. This step is important to properly measure the anatomical structures. Click on the "Unit" button (**Figure 3A**).
3. Select the anatomical points using the "marker style 2" in the imaging software (**Figure 3B**). To follow the method described in this paper, select the following points (**Figure 3B**):
   1. Select the condylion (point 1), the most posterior point of the mandibular condyle;
   2. Select the incisor process (point 2);
   3. Select the deepest point at the sigmoid notch (point 3);
   4. Select the deepest point in the concavity of the mandibular ramus (point 4);
   5. Select the most anterior point of the condylar articular surface (point 5); and
   6. Select the most posterior point of the condylar articular surface (point 6).

4. After selecting the anatomical points, perform morphometric measurements using the "length" tool, but for the condyle head length, use the "perpendicular line" tool from points (3) and (4) (**Figure 3C**).
   1. Measure the mandibular length, from the condylion (1) to the incisor process (2).
   2. Measure the condyle head length - the perpendicular distance from the condylion (1) to a line traced from the deepest point at the sigmoid notch (3) to the deepest point in the concavity of the mandibular ramus (4).
   3. Measure the condyle head width - the distance from the most anterior to the most posterior point of the condylar articular surface (5 - 6).
   4. Copy the measurements from the "measurement list" on the right side of the screen (**Figure 3C**).

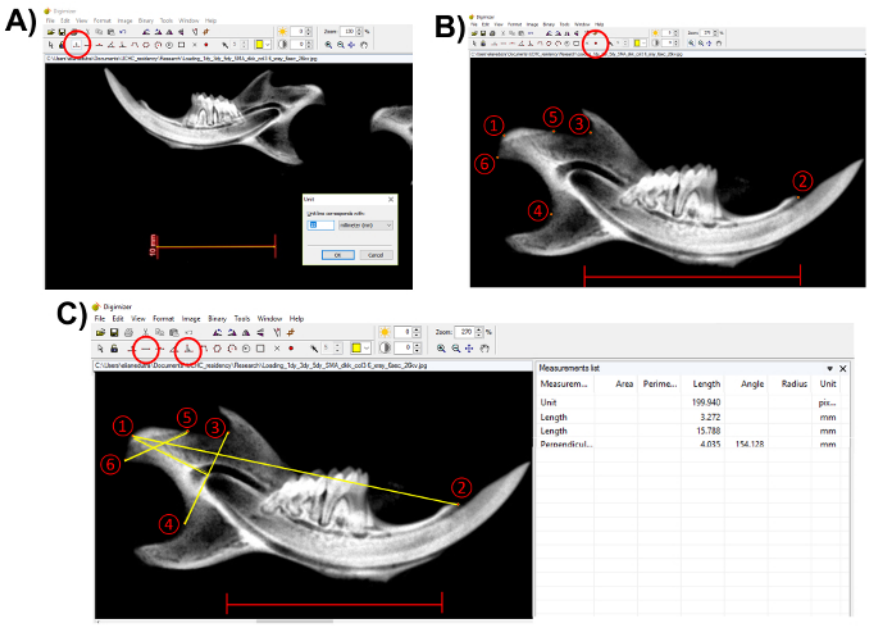

Figure 3. Representation of morphometric measurements of the mandible. (A) Use the scale bar of the radiograph to determine the unit (circled in red, scale bar: 10 mm). **(B)** Select the anatomical points using the "marker style 2" (circled in red). 1) Condylion; 2) Incisor process; 3) Deepest point at the sigmoid notch; 4) Deepest point in the concavity of the mandibular ramus; 5) Most anterior point of the condylar articular surface; 6) Most posterior point of the condylar articular surface. Scale bar: 10 mm.**(C)** Perform measurements with the "length" and "perpendicular" tools (circled in red). Measurements from point 1 to 2: mandibular length; from point 5 to 6: condylar width; perpendicular from

point 1 to 4 - 3: condylar head length. Save measurements from the "measurement list." Scale bar = 10 mm. Please click here to view a larger version of this figure.

4. Condyle Embedding

NOTE: After taking the radiographic images, the mandibles can be embedded and sectioned for histological analysis.

1. Place undecalcified mandibles (that have been fixed in step 2.3) in 30% sucrose in PBS overnight before embedding.
2. Dissect any excess soft tissue and carefully cut the mandibular condyle.
3. Pour some embedding resin (enough to cover the sample) in disposable plastic molds and place the medial surface of the condyle against the base of the mold, positioning the sample parallel to the bottom of the mold.
4. Fix the specimen in the correct place with a piece of dry ice.
5. Fill the mold with embedding resin and place the mold with fixed sample in cold 2-methyl-butane, which can be pre-chilled in a -20 °C or -80 °C freezer or in dry ice.
6. Store the specimens at -20 °C or at -80 °C until sectioning.

5. Condyle Sagittal Sectioning and Slide Preparation

1. Create frozen sagittal sections of the condyles (5 - 7 µm) and transfer the samples to the slide using the tape transfer method[10,11].
2. Adhere the histological sections transferred to the tape using the UV-curable method[10].

6. Histological Staining and Microscopic Imaging

Note: Most of the histological staining is performed as described in the histological section of the paper by Dyment *et al*[10].

1. For baseline scanning, the first step is to image for the Col2a1 and Col10a1 transgenes, as described by Dyment *et al*[10] .
    1. Place coverslips on top of the slides in 30% glycerol and PBS. Perform baseline imaging ofthesectionswitha fluorescent microscope and appropriate filters.

2. Perform fluorescent tartrate-resistant acid phosphatase (TRAP) staining.
   NOTE: TRAP is expressed by hematopoietic cells, including the bone resorbing cells, osteoclasts[12]. The purpose of this stain is to analyze MCC and subchondral bone remodeling.
    1. Remove the coverslip of the slides by soaking them in PBS. Wash the slides in PBS three times for 5 min each.
    2. Prepare TRAP buffer 1 by dissolving sodium acetate anhydrous (9.2 g) and sodium L-tartrate dibasic dihydrate (11.4 g) in 1 L of distilled water. Adjust the pH to 4.2 with acetic acid and store the TRAP buffer 1 at 4 °C.
    3. Prepare TRAP buffer 2 by freshly dissolving sodium nitrite (40 mg) in 1 mL of distilled water.
    4. Prepare TRAP substrate buffer (just before staining) by mixing 7.5 mL of buffer 1 and 150 µL of buffer 2. Apply this solution (200 µL) to each slide for 10 min at room temperature.
    5. Prepare TRAP reaction buffer by mixing 1.840 mL of substrate buffer and 23 µL of fluorescent substrate (see the **Table of Materials**).
       NOTE: The fluorescent substrategeneratesayellowfluorescentsignal for TRAP activity.
    6. Remove excess TRAP substrate buffer by gently pipetting the solution.
    7. Incubate the slides with 200 µL of TRAP reaction buffer for 5 min under a UV source bulb black light.
    8. Wash the slides in PBS three times for 5 min each.
    9. Place coverslips on the slides in 30% glycerol in PBS and perform microscopic imaging[10].

3. Perform cell proliferation staining.
   NOTE: In this assay, the modified thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) is incorporated into newly synthesized DNA: dividing cells are labeled with a fluorescent dye.
    1. After imaging for TRAP,remove the coverslips and wash the slides in PBS three times for 5 min each. Follow the steps from the 5-ethynyl-2'-deoxyuridine cell proliferation kit to stain the histological sections.
    2. Prepare a reaction cocktail by adding 430 µL of 1X reaction buffer, 20 µL of $CuSO_4$, 1.2 µL of fluorescent dye, and 50 µL of 1X buffer additive (a total volume of 500 µL is enough for one slide).
       NOTE: The manufacturer recommends adding the ingredients in the order described here.
    3. Incubate the slides with the prepared cocktails for 30 min at room temperature, protected from light.
    4. Wash the slides in PBS three times for 5 min each.
    5. Place coverslips on top of the slides in 30% glycerol in PBS with 1:1,000 DAPI.
       NOTE: DAPI (4', 6-diamidino-2-phenylindole) is a blue fluorescent nuclear stain that binds to AT-rich regions in DNA.

4. Perform Toluidine Blue (TB) staining.
   NOTE: TB staining reveals the proteoglycan distribution at the MCC.
    1. Rinse the slides indistilledwater to remove the coverslips.
    2. After removing the coverslips, wash the slides in distilled water three times for 5 min each to completely remove the salts from the PBS.
    3. Prepare TB working buffer by making solution A (dissolve 28.4 g of sodium phosphate dibasic in 1 L of distilled water) and solution B (dissolve 27.6 g of sodium phosphate monobasic in 1 L of distilled water). Mix 94.7 mL of solution A with 5.3 mL of solution B. Dilute this solution 1:1 in distilled water to make 200 mL of 0.1 M working buffer. Correct the pH to 8.0 by adding sodium hydroxide until the pH is fixed.
    4. Prepare 1% stock TB: mix 1 g of TB in 100 mL of distilled water.
    5. Prepare TB working solution by mixing 40 mL of 0.1 M working buffer in 3 mL of 1% stock TB.
    6. Incubate the slides in the TB working solution for 14-17 s.

NOTE: This stain is very time sensitive; the incubation time may need to be adjusted to prevent overstaining.

7. Wash the slides in distilled water three times for 5 min each.
8. Place the coverslips on top of the slides in 30% glycerol in distilled water. Perform light microscope imaging[10].
   NOTE: Do not coverslip the slides stained for TB in glycerol + PBS. PBS washes out this type of stain.

7. Fluorescent Histological Quantification

1. Perform histological quantification of fluorescent images by using an image analysis software (see the **Table of Materials**).
   NOTE: The basic principle of histological quantification in this method is to divide the number of fluorescent pixels of interest by the total number of pixels of the area of interest and multiply the obtained number by 100, resulting in a percentage of positive pixels within that region.
2. Perform the quantification of Col10a1 expression in the MCC of sagittal sections of condyles.
   1. When quantifying all types of cells and stains using this method, select the area to be quantified. Open the histological images in an image analysis software and select the area by using the "Lasso Tool (L)" (**Figure 4A**). For the analysis described here, select the MCC region only; after selecting the area, the total number of pixels will be shown at the "Histogram" box on the screen of the software (**Figure 4A**). Save the number of pixels in the area of interest.
   2. Select the Col10a1 red pixels by using the sampling tool. Click on "Select" on the top panel and then "Color Range." Click on the "eyedropper tool," select the sample color for the pixel of interest in the image, and click "OK." All areas positive for the chosen pixel color will be selected (**Figure 4B**). Copy the number of fluorescent pixels (shown in the "Histogram" box of the screen) and paste them in a spreadsheet or statistical software for analysis.
   3. Divide the number of fluorescent pixels over the number of pixels of the area of interest and multiply this number by 100: fluorescent pixels / pixels in area of interest * 100.
      NOTE: Other type of cells, such as blue Col2a1, can be quantified with this same method, but the blue pixels should be selected instead of the Col10a1 red pixels.

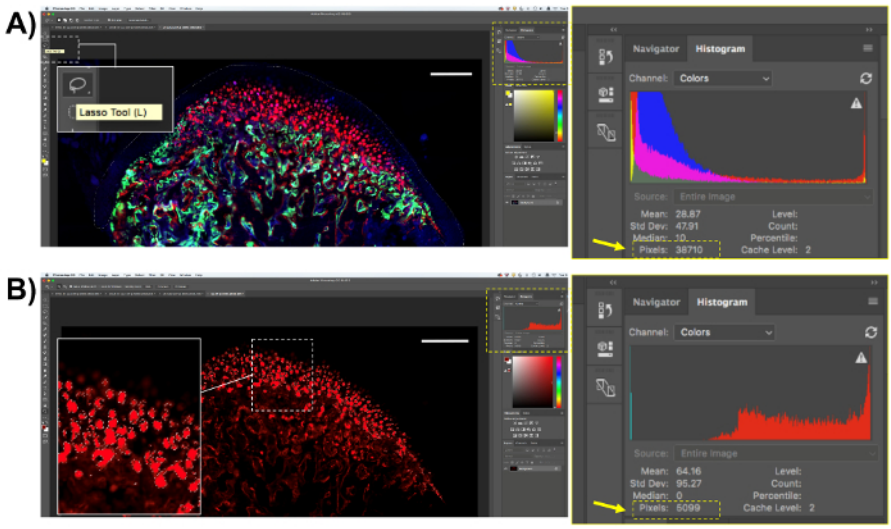

Figure 4. Representation of transgene Col10a1 quantification. (A) Select the area of interest with the "Lasso Tool" (L). For Col10a1-positive cells, select the whole mandibular cartilage. Save the number of pixels from the "histogram" box. **(B)** Select the pixel of interest, in this case, the red fluorescent Col10a1 pixels. Note that only the red pixels within the area of interest will be selected. Save the number of red pixels from the "histogram" box. Scale bar = 200 µm. Please click here to view a larger version of this figure.

3. Perform the quantification of TRAP activity in the MCC and subchondral bone.
   1. Follow the procedure described in step 8.2, but instead of only selecting the MCC area, select the cartilage and subchondral bone regions (**Figure 5A**).
   2. For TRAP activity analysis, select the yellow pixels generated by the ELF97 substrate, as described in step 8.2.2 (**Figure 5B**); copy the number of positive pixels; and paste them in a spreadsheet or statistical software for analysis.
   3. Obtain the percentage of TRAP-positive pixels by dividing the number of yellow pixels by the total number of pixels in the subchondral bone region and multiplying by 100.

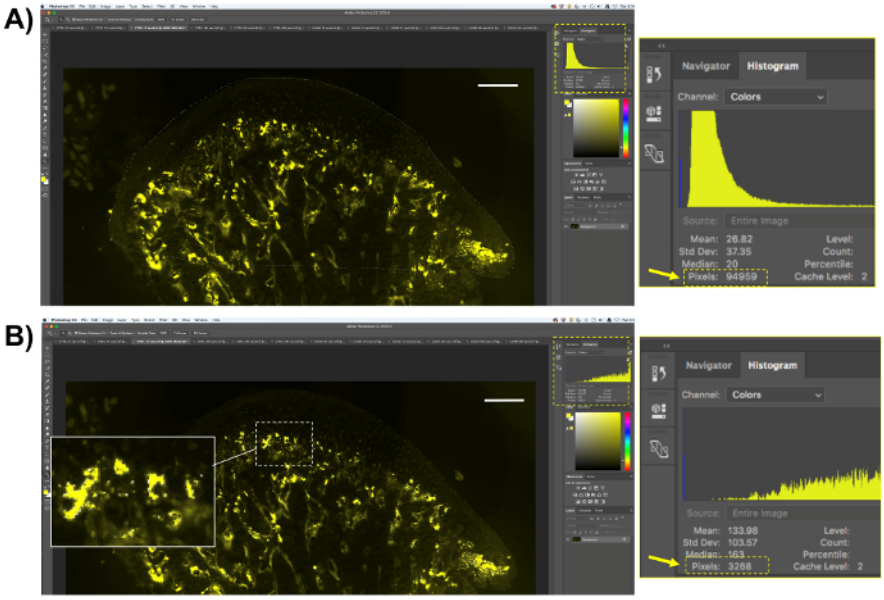

Figure 5. Representation of fluorescent TRAP quantification. (A) Select the area of interest (mandibular cartilage and subchondral bone) and save the number of pixels of this region. **(B)** Select the yellow fluorescent pixels, representing TRAP activity. Note that only TRAP-positive pixels will be select. Save the number of selected pixels. Scale bar = 200 µm. Please click here to view a larger version of this figure.

4. Perform the quantification of EdU-positive cells.
    1. EdU-stained slides are counterstained with DAPI, so instead of counting the total number of pixels in the region of interest, select the DAPI-positive pixels to calculate the percentage of EdU-positive pixels. Select the area of interest as described in step 8.2, but do not save the total number of pixels. Select the DAPI blue pixel as the sampled color, as described in step 8.2.2; copy the number of DAPI-positive pixels (**Figure 6A**); and paste them in a spreadsheet or statistical software for analysis.
    2. Next, select the EdU-positive pixels (yellow fluorescent pixels) and save the number of pixels in the "histogram" box (**Figure 6B**).
    3. Calculate the percentage of EdU pixels by dividing the number of EdU-positive pixels by the number of DAPI-positive pixels and multiplying the obtained number by 100.

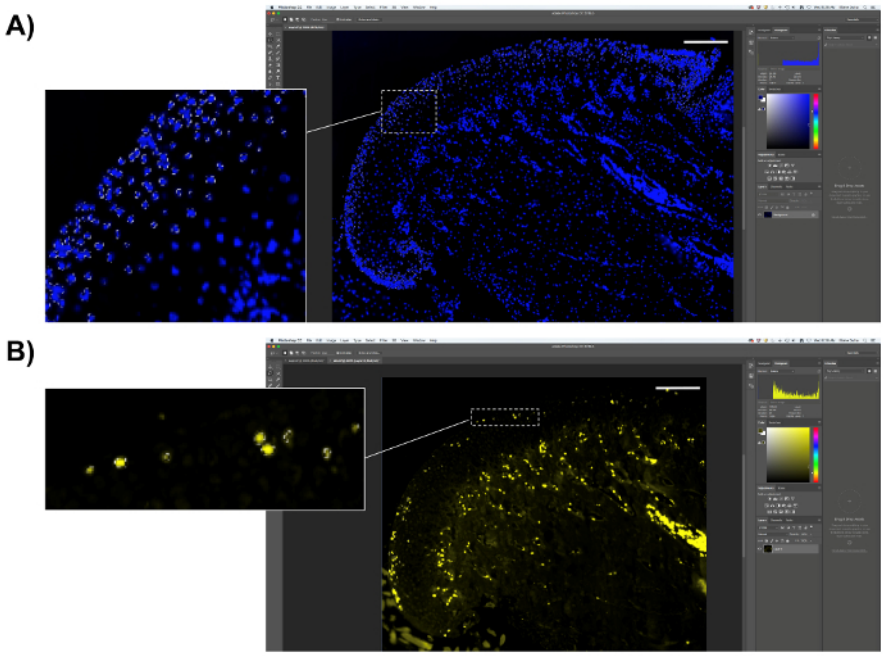

Figure 6. Representation of EdU quantification. (A) Select the proliferative region of the MCC (the outer layer of the cartilage). Select DAPI-positive pixels and save the number of pixels. **(B)** Select EdU-positive pixels (yellow fluorescent) and save the number of pixels. Scale bar = 200 µm. Please click here to view a larger version of this figure.

8. Quantification of Cartilage Thickness and Proteoglycan Distribution

1. Analyze the cartilage thickness (distance mapping) and the toluidine blue-stained area using the Digimizer Image software.
   NOTE: Use the scale bar in the histological image to determine the unit (**Figure 7A**).
2. Perform a distance mapping measurement.
   NOTE: The distance mapping measurement will provide the thickness of the cartilage at the mandibular condyle. In the method described here, five regions of the MCC are selected, giving an average of the overall thickness of the MCC. The researcher may choose to measure three different regions, or even one single region in the middle of the MCC.
   1. Using the "length" tool, measure the length of the cartilage from the outer surface to the end of the toluidine blue-stained area (**Figure 7B**) in five regions, or in as many locations as preferred.
   2. Copy the length measurements from the "measurement list."
      NOTE: The software also provides an average of the measurements (**Figure 7B**).

3. Measure the toluidine blue-stained area.
   NOTE: In the measurement of the toluidine blue-stained area, the proteoglycan area in the MCC will be obtained.
   1. Select the "area" tool and contour the toluidine blue-stained area (**Figure 7C**).
   2. Copy the area measurements from the "measurement list."

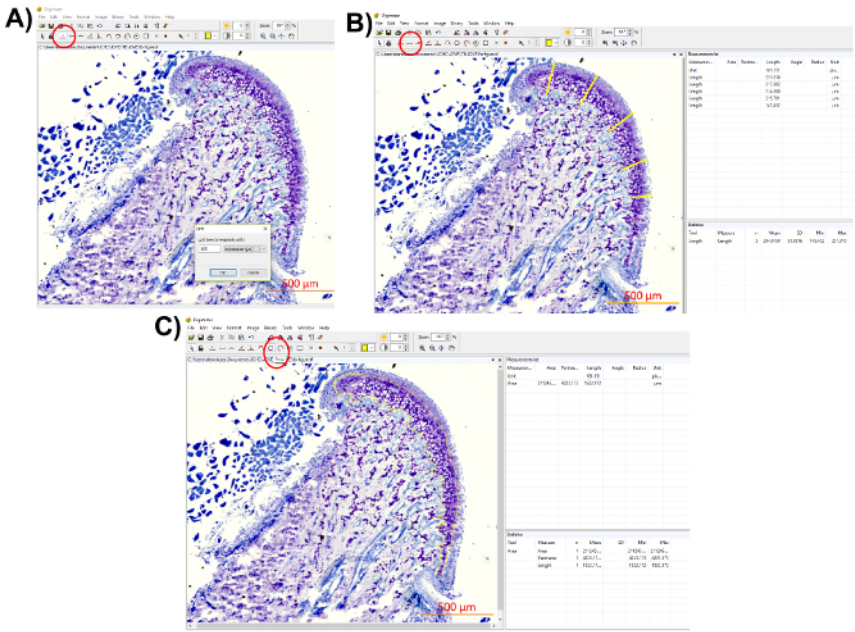

Figure 7: Representation of proteoglycan distribution quantification. (A) Use the scale bar of the histological image to determine the unit by clicking on the "unit" button (circled in red, unit selected: 500 µm). **(B)** Measure thickness of the cartilage in different locations by using the "length" tool (circled in red). Save the measurements from the "measurement list" in the upper right panel. The software also provides "statistics" in the lower right panel, so the mean and SD of the measurements can be obtained directly. **(C)** Measure the toluidine blue-stained area by using the "area" tool (circled in red). Circle the area of interest and save the measurement from the "measurement list." Please click here to view a larger version of this figure.

Representative Results

Descriptive statistics were performed to examine the distribution of morphometric measurements (mandibular length, condylar length, condylar width) and histological analyses. Outcomes were compared between the loaded group (*i.e.,* mice subjected to compressive loading with the beta titanium spring) and the control group (*i.e.,* matching control mice that did not receive any procedure). Statistically significant differences between means were determined by unpaired t-test, and a p-value of <0.05 was deemed to be statistically significant.

Morphometric measurements, as described in step 4 and **Figure 3**, were performed in the mandibles of the loaded and control groups. The loaded group presented with significantly increased mandible length (loaded: 16.62 ± 0.23 mm versus control: 16.21 ± 0.2 mm; $p < 0.05$) and condyle head length (loaded: 4.6 mm, SD 0.08 mm versus control: 4.4 mm; SD 0.06; $p < 0.05$) in comparison to the control group. Nevertheless, there was no significant difference in condyle width between groups (loaded: 3.06 ± 0.12 mm versus control: 2.9 ± 0.11; $p = 0.09$).

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Quantification of the collagen distribution using the method described in step 8 revealed significantly increased Col10a1 expression in the MCC of the condyles of the loaded group in comparison to the control (loaded: 21 % ± 4.46% versus control: 7.50% ± 2.03%; $p < 0.005$; **Figure 8A and D**). On the other hand, there was no statistically difference in Col2a1 expression between the loaded and control groups (loaded: 4.85% ± 1.95% versus control: 2.92% ± 1.89%; $p = 0.13$; **Figure 8B and E**). In addition, the analysis of TRAP activity showed increased TRAP-positive regions in the subchondral region of the condyles of loaded mice (loaded: 5.28% ± 1.45% versus control: 2.41% ± 1.39%; $p < 0.05$; **Figure 8C and F**). Similarly, we observed increased cell proliferation, as indicated by increased EdU-positive cells in the MCC of the loaded group in comparison to control mice (loaded: 6.23% ± 1.89% versus control: 1.90% ± 1.03%; $p < 0.05$; **Figure 9A and C**).

Proteoglycan distribution in the MCC of loaded and control mice was quantified by evaluating the toluidine blue-stained area and the distance map, as described in step 9 and **Figure 7**. We found significantly increased distance mapping in the MCC of condyles of the loaded group in comparison to control (loaded: 210.22 µm ± 4.11 µm versus control: 187.36 µm ± 8.64 µm; $p < 0.005$; **Figure 9B and D**). However, the proteoglycan-stained area was not statistically different between the loaded and control groups (loaded: 203,897.93 μm^2 ± 10,171.00 μm^2 versus control: 202,875.09 μm^2 ± 33,419.09 μm^2; $p = 0.94$; **Figure 9B and E**)

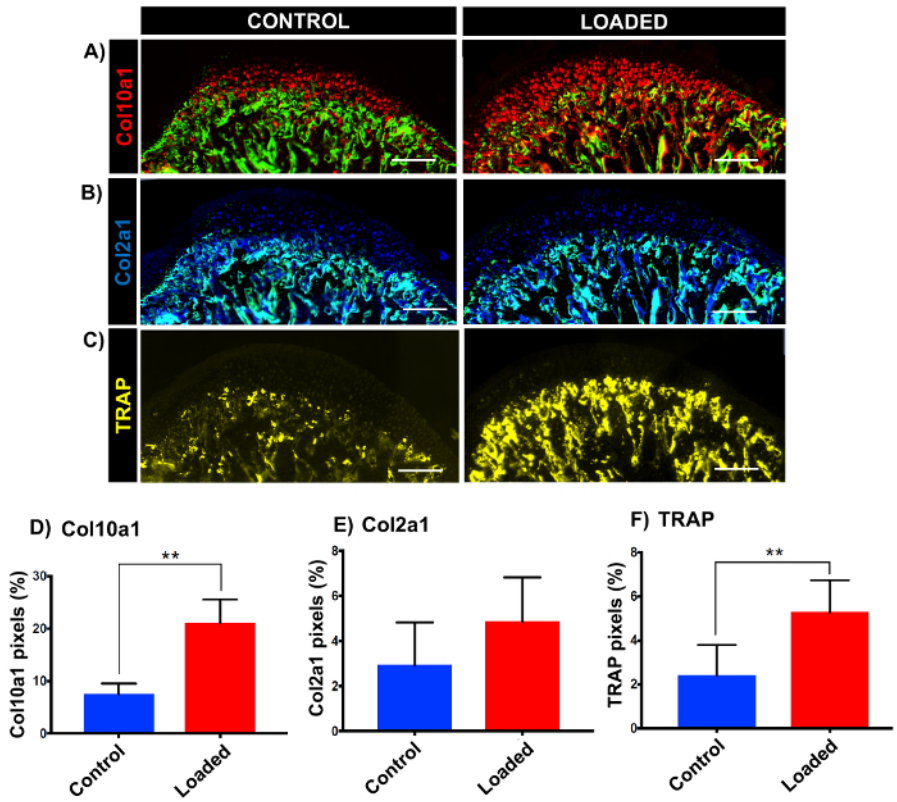

Figure 8: Representative results: (A) Col10a1-positive cells in the sagittal sections of the MCC of condyles from control and loaded mice. There are increased numbers Col10a1-positive cells in the loaded group **(D)**. **(B)** Col2a1-positive cells in control and loaded mice. There is no difference in Col2a1-positive cells between groups **(E)**. **(C)** TRAP staining in condyles of control and loaded mice. Increased TRAP activity in the loaded group in comparison to control **(F)**. The histograms **(D-F)** represent the means ± SD for n = 4 *per group*. ** Significant difference between the control and loaded groups ($p < 0.005$). Scale bar = 200µm. Please click here to view a larger version of this figure.

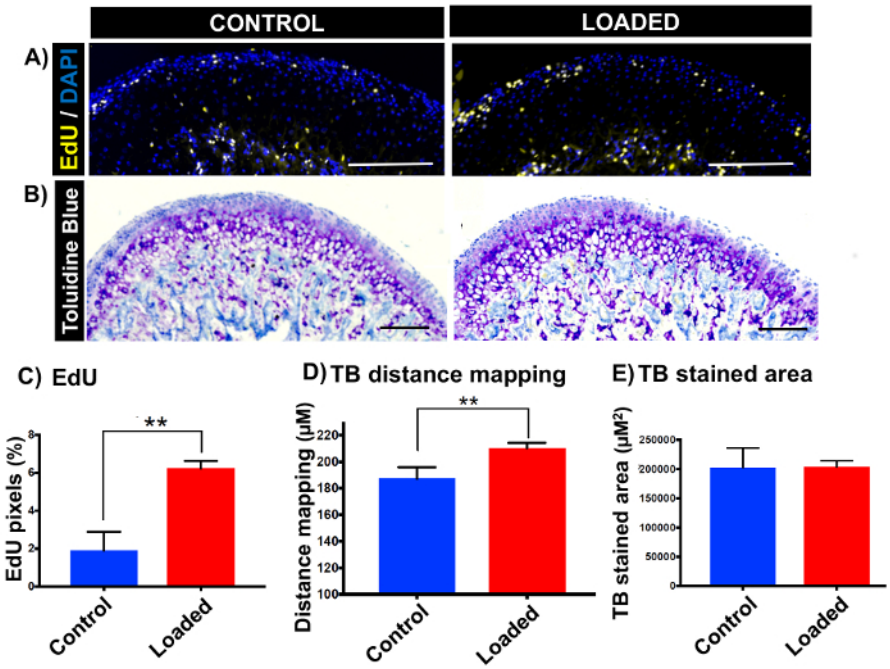

Figure 9:**Representative results: (A)** EdU staining in condyles of control and loaded mice. Increased cell proliferation in the loaded group, as represented by increased EdU-positive cells **(C)**. **(B)** Toluidine blue staining in the condyles of the control and loaded mice. Increased cartilage thickness in the loaded mice, as shown by increased distance mapping in the experimental group **(D)**. No difference in proteoglycan-stained area between the control and loaded groups **(E)**. Histograms **(C-E)** represent the means ± SD for n = 4 *per group*. ** Significant difference between the control and loaded groups ($p < 0.005$). Scale bar = 200 µm. Please click here to view a larger version of this figure.

Discussion

This manuscript described methods for the morphometric measurement and cellular analysis of murine mandibular condyles and mandibles. The radiographic morphometric measurements can also be used to analyze other bones from small experimental animals. In addition, the cellular analysis (cell quantification and cartilage distance mapping) are not limited to the rodent mandibular condyle, but can be used to quantify histological sections of numerous tissues.

Transgenic mouse models expressing fluorescent reporters are excellent tools to visually understand changes in gene expression. The double-collagen fluorescent reporter mouse model (Col2a1XCol10a1) used in this report was especially suitable for the study of the MCC, since the transgenes are expressed in the prehypertrophic and hypertrophic region of the MCC so that collagen distribution could be evaluated. If the researcher does not have access to a transgenic mouse model expressing fluorescent reporters, other methods of histological protein expression analysis, such as immunofluorescence, can be used.

For the morphometric measurements, the critical step before taking the cabinet radiograph images is to remove all soft tissues of the mandible or of the bones to be analyzed. Excessive muscle or soft tissue attached to the bones could mask the real measurements of the structures. It is important to be aware of the limitations of the cabinet radiograph system. Like every x-ray, it is a 2-dimensional representation of a 3-dimensional structure, and overlap of structures and artifacts should be carefully interpreted. In addition, the positioning of the bones in the radiographic cabinet should be consistent.

The cellular quantification by means of counting the number of positive pixels is an efficient method for histological quantification in a cell-rich region such as the MCC, but the limitation of this approach is that it does not provide the precise number of cells. In addition, since it quantifies only the pixels selected, it is recommended to quantify all images of interest using the same "sampled" pixel to avoid the quantification of pixels of different intensities when analyzing different images.

A general piece of advice when quantifying histological sections is to analyze multiple serial sections of each sample (in this manuscript, three serial sections were analyzed for each condyle), since variations within each sample are usually observed.

The methodology mentioned in our experiments is simple, easy to use, and can be used in any mineralized tissue study in which reporter mice are being used. With the existing methodology, it is possible to visualize the cells that are stained for TRAP (Col2a1 or Col10a1) or EdU (Col1a1 or Col2a1) by visualizing the co-localization of cells.

Disclosures

The authors have no competing financial interests.

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References

1. LeResche, L. Epidemiology of Temporomandibular Disorders: Implications for the Investigation of Etiologic Factors. *Crit Rev Oral Biol Med.* **8** (3), 291-305 (1997).
2. Chen, J. *et al.* Altered functional loading causes differential effects in the subchondral bone and condylar cartilage in the temporomandibular joint from young mice. *Osteoarthr Cartil.* **17** (3), 354-361 (2009).
3. Pirttiniemi, P., Kantomaa, T., Sorsa, T. Effect of decreased loading on the metabolic activity of the mandibular condylar cartilage in the rat. *Eur J Orthod.* **26** (1), 1-5 (2004).
4. Chavan, S. J., Bhad, W. A., Doshi, U. H. Comparison of temporomandibular joint changes in Twin Block and Bionator appliance therapy: a magnetic resonance imaging study. *Prog Orthod.* **15** (57) (2014).
5. Dutra, E. H. *et al.* Cellular and Matrix Response of the Mandibular Condylar Cartilage to Botulinum Toxin. *PLoS ONE.* **11** (10), e0164599 (2016).
6. Benjamin, M., Ralphs, J. R. Biology of fibrocartilage cells. *Int Rev Cytol.* **233**, 1-45 (2004).
7. Shen, G., Darendeliler, M. A. The Adaptive Remodeling of Condylar Cartilage- A Transition from Chondrogenesis to Osteogenesis. *J Dent Res.* **84** (8), 691-699 (2005).
8. Utreja, A. *et al.* Cell and matrix response of temporomandibular cartilage to mechanical loading. *Osteoarthr Cartil.* **24** (2), 335-344 (2016).
9. Kaul, R. *et al.* The Effect of Altered Loading on Mandibular Condylar Cartilage. *PLoS ONE.* **11** (7), e0160121 (2016).
10. Dyment, N. A. *et al.* High-Throughput, Multi-Image Cryohistology of Mineralized Tissues. *J Vis Exp.* **115**, e54468 (2016).
11. Kawamoto, T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch Histol Cytol.* **66** (2), 123-143 (2003).
12. Hayman, A. R. Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy. *Autoimmunity.* **41** (3), 218-223 (2008).

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