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Generation and Characterization of Mouse Models for Skeletal Disease

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Abstract

Our laboratories have used genetically engineered mouse models (GEMMs) to assess genetic contributions to skeletal diseases such as osteoporosis and osteoarthritis. Studies on the genetic contributions to OA are often done by assessing how GEMMs respond to surgical methods that induce symptoms modeling OA. Here, we will describe protocols outlining the induction of experimental OA in mice as well as detailed descriptions of methods for analyzing skeletal phenotypes using micro-computerized tomography and skeletal histomorphometry.

Keywords

microCT; Histomorphometry; Osteoarthritis

1 Introduction

The analysis of skeletal phenotypes of genetically modified mice has contributed to advancements of our knowledge about skeletal development and disease and has provided the foundation for the development of several FDA-approved treatments for bone-related diseases. Our laboratories have used genetically engineered mouse models (GEMMs) to assess genetic contributions to skeletal diseases such as osteoporosis and osteoarthritis. Studies on the genetic contributions to OA are often done by assessing how GEMMs respond to surgical methods that induce symptoms modeling OA. The generation of

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GEMMs is applicable to all tissue and disease types and discussion of methodologies for their generation is available in many other contexts [1, 2]. Here, we focus on several methods specific for generating and analyzing skeletal phenotypes in mice. We first describe the generation of an OA-like model via surgical destabilization of the medial meniscus. We will then describe the evaluation of skeletal phenotypes via micro-computerized tomography (microCT) and histomorphometry.

2 Materials

2.1 Surgical Destabilization of the Medial Meniscus (DMM)

1. Experimental mice of the same strain divided into DMM surgery group (n 5) and sham surgery control group (n 5). Age of mice should be decided according to the goal of experiments, but generally mice at or older than 3 months of age are optimal as their skeletal maturity is reached. All mice should be housed in a gnotobiotic facility, according to the National Research Council's Guide and Institutional Animal Care and Use Committee-approved protocols for the care and use of experimental animals (*see* Note 1).

2. Isoflurane machine: VetEquip Tabletop laboratory animal anesthesia system (VetEquip # 901820).

3. Isoflurane (Piramal # NDC 66794–017-25).

4. Surgical microscope (Leica # M651).

5. Veterinary operating table heated mat (Peco # Mediheat V500).

6. 9 mm Wound forceps and clips (Reflex # RS-9260, RS-9262).

7. Wound clip remover (Reflex # RS-9263).

8. Hair removal lotion.

9. Alcohol antiseptic wipes.

10. Micro-iris scissors.

11. Micro-surgical knife (#3 handle and #10/ #11 blade).

12. Sharppoint 15° 5 mm blade micro-surgical knife.

13. Epinephrine (1:1000 dilution).

14. Amoxicillin (20 mg/kg).

2.2 Osteoarthritis (OA) Histology

1. Histological tissue cassette case.

2. Surgical scissors.

3. Tweezer.

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1. Male mice are more susceptible to developing DMM-induced osteoarthritis [10] thus considered suitable materials for such experiments, but the selection of mouse genders should accommodate the goals of specific studies.

4. Deionized water.

5. Ethanol 70%, 80%, 90%, 95%, 100%.

6. Neutral buffered formalin (NBF) 10%.

7. Formic acid 5%.

8. Xylene.

9. Paraffin (Paraplast X-tra).

10. Oven.

11. Stock solution A: 10 g hematoxylin, 500 mL 80% ethanol.

12. Stock solution B: 20 g ferric chloride, 475 mL distilled water, 5 mL hydrochloric acid (36.5–38%).

13. Modified Weigert's Iron Hematoxylin: equal volume stock solutions A and B (*see* Note 2).

14. Acid-alcohol 1.0%: 5 mL hydrochloric acid (36.5–38%), 495.0 mL 70% ethanol.

15. Acetic acid 1.0%: 5.0 mL glacial acetic acid, 495 mL distilled water.

16. Fast Green 0.08%: 0.20 g Fast Green, 250.0 mL distilled water.

17. Safranin O 1%: 2.5 g Safranin O, 250 mL distilled water.

2.3 Microcomputed Tomography

All standards, equipment, and software for scanning and analysis are produced by Bruker microCT, Kontich, Belgium and Micro Photonics Inc., Allentown, PA.

1. Neutral buffered formalin (NBF) 10%.

2. Deionized (DI) water.

3. Ethanol (EtOH) 70%.

4. Gauze cut to length of the sample.

5. Plastic conical.

6. Bruker Skyscan 1172.

7. Skyscan 1172 (Scanning software) Version 1.5.26.0.

8. Software NRecon Version 1.7.4.6.

9. GPUReconServer Version 1.7.4.2.

10. Data Viewer Version 1.5.6.3 software.

[2.]Mix equal volume of stock solutions A and B to make the Modified Weigert's Iron Hematoxylin working solution right before use. The working solution has a shelf life of about 1 week when stored in dark.

2.4 Bone Mineral Density Calibration

    1. Bone mineral density standards 0.25 and 0.75.

    2. CT Analyzer Version 1.18.8.0 software.

2.5 Trabecular Analysis of Microcomputed Tomography Images

    1. CT Analyzer Version 1.18.8.0 software.

2.6 Cortical Analysis of Microcomputed Tomography Images

    1. CT Analyzer Version 1.18.8.0 software.

2.7 Bone Modeling

    1. CTvol Version 2.3.2.0 software.

2.8 Fluorochrome Labeling of Bone

    1. Calcein Green 1.0% stock solution: In a 50 mL conical tube, dissolve 400 mg NaHCO3 in 20 mL of saline (0.9% NaCl). Slowly add 200 mg calcein (Sigma-Aldrich # C0875). Calcein will foam if added quickly. pH to 7.4 using 1 N NaOH or 1 N HCl and filter sterilize into evacuated 20 mL glass vial. Wrap in foil and store at 4 °C (*see* Note 3).

    2. Calcein Green 0.10% working solution: Dilute the Calcein Green 1.0% stock solution in saline. Pipette 2.0 mL of 1% calcein stock and 18.0 mL of saline. Wrap in foil and store at 4 °C (*see* Note 4).

    3. Alizarin Complexone 2.5% stock solution: In a 50 mL conical tube, dissolve 400 mg $NaHCO_3$ in 20 mL of saline (0.9% NaCl). Slowly add 500 mg alizarin complexone (Sigma-Aldrich # A3882). Alizarin will foam if added quickly. pH to 7.4 using 1 N NaOH or 1 N HCl and filter sterilize into evacuated 20 mL glass vile. Wrap in foil and store at 4 °C.

    4. Alizarin Complexone 0.20% working solution: Dilute 2.5% stock solution with saline to 0.2%. Pipette 0.8 mL of 2.5% alizarin stock and 9.2 mL of saline.

    5. Demeclocycline HCL 0.75% working solution: In a sterile 200 mL bottle, dissolve 1 g demeclocycline hydrochloride (Sigma-Aldrich # D6140) in 133 mL of sterile saline (0.75% solution). Place on a rocker plate and rock gently for 20 min. Aliquot into light-proof 1.5 mL tubes and store at −20 °C.

2.9 Fixation

    1. DI water.

    2. Ethanol 70%.

    3. Neutral Buffered Formalin (NBF) 10%.

[3.]Wrap in foil to prevent exposure as the solution is light sensitive.
[4.]Stock solution is diluted to the 10 mg/kg recommended dose for injecting mice.

2.10 Infiltration

1. Dremel hand tool.

2. Automated tissue processor.

3. Ethanol 70%, 95%, 100%.

4. Fisherbrand Histocassette Tissue Cassette.

5. Infiltration solution: 85% Methyl methacrylate (MMA) and 15% dibutyl phthalate (DBP).

6. Methyl methacrylate (MMA) 100%.

7. NBF 10%.

8. Rapid Infiltration Processor (RIP).

9. Xylene.

10. Embedding molds.

11. Glass culture tubes.

2.11 Embedding and Cross-Sectioning

1. 600-grit Sandpaper.

2. Allen wrench.

3. Binder clips.

4. DI water.

5. Embedding solution: 85% MMA, 15% DBP, and 0.5% Perdadox 16.

6. Eukitt Mounting Medium Electron Microscopy Sciences # 15320.

7. Extech Labcut 150.

8. 3"×.006"× ½ Diamond Wafering Blade.

9. Glass culture tubes with rubber stoppers.

10. Glass slides.

11. Lab Armor Bead Bath.

12. Micrometer.

13. Paper towel.

14. Sonicator.

15. Small weight.

2.12 MMA Embedding and Coronal Sectioning

1. Automatic rotary microtome.

2. Bead bath.

3. Clamps.

4. Coating solution: 85 mL DI water, 1 g gelatin powder, and 15 mL glycerol incubate the solution at 42 °C in a water bath until dissolved or visible particles are no longer visible (*see* Note 5).

5. Cutting solution: 10 mL 70% ethanol, 90 mL distilled water, and 2 drops of soap (*see* Note 6).

6. Embedding solution: 85% MMA, 15% DBP, and 0.5% Perdadox 16.

7. Ethanol 70%, 75%.

8. Forceps.

9. Fine tip paint brush.

10. Glass slides.

11. Kimwipes.

12. MetaServ 250 Single Grinder/Polisher.

13. Paperclips.

14. Paper towels.

15. Petri dish.

16. Plastic film.

17. Rubber roller.

18. Silicone stopper.

19. Oven.

20. Prefabricated mold for embedding.

21. Acetone.

22. Cellusolve.

23. DI water.

2.13 Golder's Trichrome Stain

1. Wiegert's Iron Hematoxylin: Prepare fresh by combining 50 mL Wiegert's Solution A and 50 mL Wiegert's Solution B.

2. Ponceau Fuchsin solution A: 1 g Ponceau, 100 mL DI water.

3. Ponceau Fuchsin solution B: 1 g Acid fuchsin, 100 mL DI water.

4. Ponceau Fuchsin stock solution: 30 mL Ponceau fuchsin solution A, 10 mL ponceau fuchsin solution B.

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[5]·Once prepared, the coating solution is stable at room temperature for 1 week.
[6]·Once prepared, the solution is stable at room temperature until expiration date of the ethanol lot.

5. Acetic acid 1%.

6. Ponceau Fuchsin working solution: Dilute 10 mL ponceau fuchsin stock solution in 150 mL 0.2% acetic acid.

7. Phosphotungstic Acid (PTA) Orange G: Combine and filter 2 g Orange G, 4 g phosphotungstic acid, and 100 mL DI water.

8. Light Green: combine and filter 0.2 g Light Green, 0.2 mL acetic acid, and 100 mL DI water.

9. DI water.

10. Ventana Symphony tissue processor.

2.14 Paraffin Embedding and Sectioning

1. DI water.

2. Embedding center (Leica EG1150H).

3. Ethylenediaminetetraacetic acid (EDTA) 10%: pH 7.4.

4. Ethanol 70%, 80%, 95%, 100%.

5. Fisherbrand Histocassette Tissue Cassette.

6. Forceps.

7. Glass slides.

8. Kimwipes.

9. NBF 10%.

10. Oven.

11. Paraffin/tissue infiltration media.

12. Steel microtome knife.

13. Xylene.

14. Tissue processor.

15. Water bath.

2.15 TRAP Stain

1. Sigma-Aldrich product 387-A: Acid Phosphatase, Leukocyte (TRAP) kit.

2. Aqueous mounting media.

3. Glass slides.

4. Glass coverslips.

2.16 Slide Imaging

1. ET-DAPI/FITC/Texas Red filter set (Chroma product # 69002).

2.   Leica Aperio AT2 High Volume, Digital Whole Slide Scanning.

3.   Lumen Dynamics X-Cite Series 120 Q.

4.   QImaging QIClick Digital CCD Camera.

5.   Zeiss Axio Imager.A2.

6.   BIOQUANT OSTEO 19.2.6. software.

2.17 Histomorphometry

1.   BIOQUANT OSTEO 19.2.6 software.

3 Methods

3.1 DMM Surgery

The methods described below that we utilize in the laboratory have been developed and optimized based on several previous studies [3-6]. Operate the anesthesia machine according to the VetEquip user guide and operating manual (http://www.vetequip.com/pdfs/LAAS%20

Manual.pdf). DMM surgery should be performed on both knees of each individual mice of the DMM group, and sham surgery will be performed to another group of mice for controls. Surgery should be carried out with standard sterile techniques, i.e., preoperative instrument sterilization and disinfection, and cleansing of incision area.

1.   Anesthetize the mice with 4% isoflurane and 1 L/min oxygen in the VetEquip tabletop laboratory animal anesthesia chamber.

2.   Once the mice are anesthetized, transfer them to a mask and maintain with 3% isoflurane and 1 L/min oxygen.

3.   Place the mice on a veterinary operating table heated mat to maintain body temperature during surgery.

4.   To bare the incision area, apply hair removal lotion on the medial side of knee for 3 min. Then wipe with alcohol antiseptic wipes.

5.   In order to expose the medial side of stifle joint capsule, use a micro-iris scissor or a micro-surgical knife to open a 3 mm longitudinal incision through the skin and subcutaneous tissue over the distal patella to proximal tibial plateau.

6.   Expose medial meniscotibial ligament (MMTL), which anchors medial meniscus (MM). During the process, avoid destabilizing patellar tendon, medial collateral ligament, and articular cartilage.

7.   To prevent bleeding that may block the vision for performing transection, one drop of 0.1% epinephrine can be applied at the incision site (optional).

8.   Under a surgical microscope can assist accurate transection, transect the MMTL with a micro-surgical knife (sharppoint 15° 5 mm blade) to release the MM. Locating the patellar ligament first, this can assist the identification of the incision region.

9.   The sham group mice will be operated through the same approach without MMTL transection.

10.   Close the incision together with a 9 mm wound forceps and clips.

11.   To prevent joint infection, Amoxicillin (20 mg/kg) can be injected after surgery (optional).

12.   Remove the clips with a wound clip remover after wound has healed.

3.2   Osteoarthritis (OA) Histological Grading

At 4 to 6 weeks (or at specific time point according to the experimental design) post DMM surgery, OA phenotypes can be graded or assessed by histological analysis, gene expression assay, microCT, pain behaviors, or gait analysis. Here we focus on the histological grading.

1.   Collect knees from mice and arrange in a histological tissue cassette case (*see* Note 7).

2.   To fix the tissue, place the tissue cassettes with mouse knees in 10% NBF for 48 h at 4 °C. Rinse in DI water 3× for 10 min each time and store in 70% ethanol at 4 °C.

3.   To decalcify the samples, place the fixed tissues with cassettes in a beaker containing at least 15 volumes of 5% formic acid on a swirling/rocking platform for 7 days (*see* Note 8).

4.   Rinse the tissues in DI water 4× for 10 min each time.

5.   Dehydrate the tissues in 70%, 95%, and 100% ethanol for multiple rounds 1–2 h each (*see* Note 9).

6.   Clear tissues in xylene for 1 h 2×.

7.   Infiltrate tissues with paraffin (Paraplast X-tra) at 58 °C for 1 h 2×.

8.   Embed tissues into wax blocks at 62 °C.

9.   Rehydrate tissues for histology using xylene, absolute alcohol, 95% ethanol, 80% ethanol, then rinse in distilled water (*see* Note 10).

10.   Cut 5 mm sections of the tissues embedded in the blocks (*see* Note 11).

11.   Bake the slides at 60 °C overnight and cool at room temperature for 20–30 min.

[7.]Sacrifice the mice in a CO_2 chamber. Dissect both hind limbs from the hip of the mice using surgical scissors and tweezers. Remove about 1/3 of femur from the proximal end and 1/3 of the tibia from the distal end using surgical scissors. Straighten each knee and place in a labeled histological tissue cassette with the patella facing up. This will help the following embedding and sectioning in the frontal orientation.

[8.]Another common method for decalcification is incubation in 14% EDTA solution for 10 days. EDTA-decalcified tissues generally have better result for immunohistochemistry than formic-acid-decalcified ones. However, the Safranin O stain shows better color intensity on the formic-acid-decalcified sections.

[9.]Dehydrate the tissues in the following order: 70% ethanol for 1 h, 95% ethanol for 1 h, absolute ethanol for 1 h 2×, absolute ethanol for 1.5 h, and absolute ethanol for 2 h.

[10.]Deparaffinize sections, 2 changes of xylene, 3 min each, then rehydrate in 2 changes of absolute alcohol, 2 min each. Incubate in 95% ethanol for 2 min followed by 80% ethanol for 2 min. Wash sections briefly in distilled water.

[11.]According to the recommendation of Osteoarthritis Research Society International (OARSI) [11], the knee joints should be paraffin-embedded in frontal orientation and sectioned through the entire joint at 80 mm interval. Each section should be 5 mm.

12. Deparaffinize and hydrate in 70% ethanol and air dry.

13. Incubate the slides with Weigert's Iron Hematoxylin for 30 min.

14. Wash the slides gently in running tap water for 10 min.

15. Differentiate by single dip in 1% acid-alcohol.

16. Rinse the slides in DI water 3×.

17. Incubate the slides with 0.08% Fast Green for 5 min.

18. Rinse the slides in 1.0% acetic acid for 10 s (blot, no rinse).

19. Incubate the slides in 1% Safranin O for 30 min (blot, no rinse).

20. Dehydrate the sections with 1 change of 95% ethanol and two changes of 100% ethanol by 1 dip each and air dry.

21. Clear the slides in xylene for 1 min, 2× and coverslip.

22. Score histologically to evaluate OA progression in a blinded manner following the semiquantitative scoring system established by Sonya S. Glasson et al. The OA phenotype of all four quadrants of joint, including the medial femoral condyle, medial tibial plateau, lateral femoral condyle, and lateral tibial plateau should be scored (*see* Note 12).

3.3 Imaging by Microcomputed Tomography

These methods have been adapted and optimized for use in our laboratories from several sources [7-9]. Methodology is specific to femurs from mice at 3–6 months of age. All microCT methods were performed under the recommended guidelines of the SkyScan software Standard Operating Procedures by Bruker MicroCT (Kontich, Belgium).

1. Fix limbs in 10% NBF for 48 h. Rinse limbs in deionized (DI) water and store in 70% ethanol.

2. Prepare the limbs by wrapping them in gauze saturated in 70% ethanol. Load the limbs into a conical submerged in 70% ethanol while preventing air bubbles from entering. Samples should not be overlapping and should be snug in the container.

3. Turn the Skyscan 1172 on by turning key to "START" position. The key will automatically snap back into the "ON" position.

4. Open Skyscan 1172 software and start the X-ray tube by pushing the X-ray icon. A pop-up window will open with a progress bar as the tube ages. Once the X-ray is on, an indicator window will open.

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12. The mouse OA knee damage categories followed by scores in parentheses include normal (0), loss of Safranin O with no structural changes (0.5), small fibrillations with no loss of cartilage (1), vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina (2), vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface (3), vertical clefts/erosion to the calcified cartilage extending to 25–50% of the articular surface (4), vertical clefts/erosion to the calcified cartilage extending to 50–75% of the articular surface (5), vertical clefts/erosion to the calcified cartilage extending >75% of the articular surface (6). Use mean value of the scores from the 4 quadrants of an individual knee joint for statistical analyses.

5. Press the "grab image" icon to obtain a continuous preview from the X-ray camera and establish scan settings (*see* Note 13).

6. Perform a flat field correction (*see* Note 14).

7. Open the chamber on the scanner by pressing the "specimen door" icon, fasten the sample to a stage, and secure the stage on the rotation axis by turning the brass ring to tighten it.

8. Close the chamber by pressing the "specimen door" icon, turn the X-ray back on, and restore the settings established in **step 5**.

9. Set an oversize scan (*see* Note 15).

10. Select the "Start scanning" button in the Scout Scan window to open the settings dialog window and complete scan settings (*see* Note 16).

11. Click "OK" to start the scan.

12. After scanning is complete, open NRecon and load a dataset by choosing "Open Dataset" in the "Actions" tab and choosing one of the images from a scan. Choosing one image will open the entire dataset associated with the sample.

13. Perform a thermal correction (*see* Note 17).

14. Choose a dynamic range based on the histogram of the sample (*see* Note 18).

15. Correct beam-hardening (*see* Note 19).

16. Correct misaligned segments (*see* Note 20).

[13.]To establish settings, double-click the indicator window to open the X-ray energy settings. Set the voltage to 60 kV and current to 167 μA. The "always maximize power" box should be checked, which will automatically adjust the power to 10 W and thus adjust voltage or current accordingly. Set the pixel resolution to 2000 × 1200 and the X-ray camera image pixel size to 6 μm. Apply a 0.5 mm aluminum filter.

[14.]Open "Preferences" in the "Options" tab. Un-check the two flat-field correction boxes and click "OK." Select "Acquisition Modes" in the "Options" tab. Locate the window with the arrow which corresponds to the filter, camera position, and pixel size. Set the exposure to 360 and adjust the voltage and current windows if needed. Check the option, "Acquire bright + dark for current mode." Grab image to obtain a live preview and right click to view the intensity profile. The average intensity should be ~60%. Note: If the average intensity is not ~60%, allow the X-ray to stabilize and adjust the exposure until it reaches ~60%. Open "Preferences" in the "Options" tab. Check the two flat-field correction boxes and click "OK." The average intensity should be 88% (±2%).

[15.]Select "Set Oversized Scan" in the "Actions" tab. Click "Scout Scan" and allow the scan to run to the bottom of the stage. Enter the sample name in the "Prefix" window. Select the "Top" button and click the top of the sample, followed by the "Bottom" button and click the bottom of the sample. Repeat this process for each sample in the scout scan preview window.

[16.]Designate a file location by choosing "Browse" and select a location. Set rotation step (deg.) to 0.4, averaging (frames) to 5, random movement to 10. Un-check the following: use 360-degree rotation, open chamber after scanning, camera offset and partial width. Check "X-Ray off after scanning."

[17.]Select "X/Y alignment with a reference scan" under the "Actions" tab. Move the adjustable box to cover the sample. Adjust the "Max. x-shift from start point (+−)" and "Max. y-shift from start point (+−)" to 20. Change the "Matching criteria and method" to "Least-Square." Change the "Projection pairs to match" to "All." Click "Match," and after the application is complete, click "Accept."

[18.]In the "Start" tab, select the area in the sample to preview. For a mouse femur, there will be multiple segments. Select "Preview." In the "Output" tab, change the graph to log view. To toggle between graphs, double-click the graph. Click the tab underneath the histogram and set the minimal value to 0. Adjust the maximal value, indicated by the red line, by dragging the line and placing it just after the peaks from the dataset end. Use this dynamic range for the rest of the samples in the project.

[19.]In the "Output" tab, right click and drag over a nonporous part of a sample. This will open an absorbance vs. distance graph. This works best when using cortical bone. Record the shape of the graph (cupping indicates beam-hardening, arching indicates over-correction of beam-hardening, and flat indicates little to no beam hardening). Repeat two or three times in different proximal and distal regions of the sample. Click on the "Settings" tab and perform the necessary corrections for each graph type (increase the beam-hardening correction percentage for cupping, decrease the beam-hardening correction percentage for arching, and no further correction needed for flat graphs). Adjust beam-hardening settings until the graphs become flat. The same beam-hardening settings are used for the rest of the samples in a project. For a mouse femur, this number typically ranges from 20% to 40%.

17. Reduce ring artifacts (*see* Note 21).

18. Smooth the images (*see* Note 22).

19. Choose a region of interest (ROI) to save (*see* Note 23).

20. Choose a volume of interest (VOI) to save (*see* Note 24).

21. Reconstruct and save the images (*see* Note 25).

22. After the images have been reconstructed, open Data Viewer and load a reconstructed dataset from a "Rec" folder of a sample.

23. After the sample is open, click the "load for 3D viewing" icon.

24. Use the three views to straighten the sample. Orient all samples in a project the same way.

25. Under the "View" tab, choose "Single Volume of Interest (VOI)," and fit the sample within each of the boxes to crop the dataset and define a volume of interest (VOI).

26. Save the volume of interest (*see* Note 26).

3.4 Bone Mineral Density Calibration

1. Scan, reconstruct, and orient the bone mineral density standards following the steps under "Imaging by Microcomputed Tomography 3.3."

2. Open the CTan software and load one of the density standards by choosing the "open image or dataset" icon.

3. Define the upper bound of the density standard by scrolling to the top and choosing a slice that includes the entire density standard. Right click the slice in

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[20.]In the "Start" tab, select an area in the sample and click the "Fine Tuning" button and select "Post-alignment." Set number of trials to 5 and set parameter steps to 0.5 or 1. Click "Start" and refer to the "Output" tab. Toggle through the choices using the arrow buttons and choose an option that minimizes changes in pores, edges, and dense particles. If the fine tuning does not work in the range selected, manually change the misalignment compensation in the "Settings" tab. Repeat fine tuning for each part of the sample (there should be 3 or more segments for a mouse femur).

[21.]In the "Settings" tab, check "Ring Artifacts Reduction," and set to 10. In the "Start" tab, select an area in the sample, click the "Fine Tuning" button, and select "Ring Artifacts Reduction." Set number of trials to 5 and set parameter steps to 5 Click "Start" and refer to the "Output" tab. Toggle through the choices using the arrow buttons and choose an option that best reduces ring artifacts. This will only be done once per sample.

[22.]This application should only be used if there is noise in the background of the scan, which can be viewed in the "Output" tab. To turn on smoothing, click the "Settings" tab and check the "Smoothing" box. Adjust the levels until the noise in the background is canceled. To see the effect of smoothing, choose the "Start" tab, select an area in the sample and click "Preview." Use the same smoothing settings for the rest of the samples in a project.

[23.]In the "Start" tab, select an area in the sample to view and click "Preview." In the "Output" tab, check "Use ROI," and choose a shape. Move and adjust the size of the ROI in the preview window so that the sample is inside the ROI. Expand the ROI in different segments of the femur to confirm the whole sample is within the ROI (from proximal to distal epiphysis).

[24.]Choose the "Start" tab to see an overview of the scan. Move the red lines at the top and bottom of the scan to include the area of interest.

[25.]There are two ways to reconstruct and save the images. Option 1: Choose the "Start" tab and click the "Start" button. Option 2: Choose the "Start" tab and click the "Add to batch button." Continue to load more datasets to the batch by choosing the "Add to batch button" after reconstructing each sample. When all datasets are complete, click "Start Batch" in batch manager. Folders with reconstructed files will appear in the scan folders.

[26.]Under the "Actions" tab, choose "Save" and "Select a VOI to Save." Establish settings in the "Options for saving a VOI" window under "Saving options." In the Resize X/Y option, choose 1. In the View to save option, choose Transaxial (X-Y). Create a new VOI folder within the reconstruction folder. Click "OK" to save the VOI.

the dataset window and choose "Set the Top of Selection." Repeat for the lower bound but choose "Set the Bottom of the Selection."

4. Define the region of interest (ROI) to include only the density standard (*see* Note 27).

5. Save the ROI (*see* Note 28).

6. Open the new ROI file for the density standard by selecting the "open image or dataset icon" and choosing one of the images in the ROI folder.

7. Apply the ROI to the open file (*see* Note 29).

8. Identify and record the attenuation coefficient (*see* Note 30).

9. Repeat **steps 2–8** for the other density standard.

10. Under "File," choose "Preferences," and under the "Histogram" tab, choose "Calibrate."

11. Enter the known min and max BMD density values of the density standards (Ex: 0.25 and 0.75) followed by the corresponding attenuation coefficients recorded in **step 8**.

12. Click "OK" to calibrate the density range.

3.5 Trabecular Analysis of Microcomputed Tomography Images

1. In the CTan software, select the "open image or dataset" icon. Choose a dataset within a newly created VOI folder.

2. Identify the reference point for the ROI, use a 0.25 mm offset, and create an ROI 2.5 mm in height (*see* Note 31).

3. Create a region of interest to include trabecular bone only, by manually drawing an ROI (*see* Note 32) or use an automated program and save the regions. If manually drawing ROIs, continue to **step 6** after this step. To make an automated program to create an ROI, continue to **step 4**.

4. Begin the automated trabecular ROI process by, saving the ROI defined in **step 2** and determining the thresholding scale (*see* Note 33).

27.Select the "regions of interest preview" icon. Select the "interpolated region of interest" drop-down menu. Select "Round." Adjust the circle so that it is slightly smaller than the density standard and place it in the center of the density standard.

28.Under "File," choose "Save from ROI." Create a new ROI folder and un-check "Create folder named VOI." Check "Save ROI-file" and click "OK" to save the ROI file.

29.Select the "regions of interest preview" icon followed by the "load regions of interest" icon. Open the ".roi" file.

30.Select the "binary selection preview" icon. Select the "From dataset" tab in the "Histogram" window. Select the "Attenuation coefficient" tab. Scroll to the bottom and record the mean (total).

31.Scroll through the images to locate where the growth plate separates. In the dataset window right click the slice chosen and click "Selection Reference." Double-click the black bar in the dataset window to open the "Selection" window. Under the "Analytic" tab set offset 0.25 mm and height to 2.5 mm.

32.To manually draw an ROI, choose the "regions of interest preview" icon. Scroll to the lower boundary of the ROI and draw a region that includes trabecular bone but excludes the growth plate and cortical bone. Scroll toward the upper boundary of the ROI and continue drawing skipping several images in between. The ROIs drawn will interpolate automatically. To save, choose "Save from ROI" under the "File" tab. Create a new folder for the trabecular ROI and un-check "Create Folder named VOI" and check "Save ROI-file." Click "OK" to save the ROI.

5. Open the ROI saved in **step 4** by choosing the "open an existing image or dataset" icon and choosing an image within the ROI folder for the sample. Build a task list to create, analyze, and save an automated trabecular ROI (*see* Note 34). After building the task list, skip to **step 9**.

6. Open a sample's manual ROI by choosing the "open an existing image or dataset" icon and choosing an image within the trabecular ROI folder for the sample.

7. Apply the manual ROI to the open file and determine the thresholding scale (*see* Note 35).

8. Choose the "custom processing preview" icon and select the "Internal" tab to build a task list to analyze the trabecular bone (*see* Note 36).

9. Run the task list on multiple samples using the batch manager (*see* Note 37).

33.Save the ROI by choosing "Save from ROI" under the "File" tab. Create a new folder for the trabecular ROI. Uncheck "Create Folder named VOI" and check "Save ROI-file." Click "OK" to save the ROI. To determine the thresholding scale, select the "binary selection preview" icon. Select the "From dataset" tab in the histogram window. Choose the "automatic thresholding" icon in the histogram window. Record the upper and lower threshold values. For a project, the thresholds for all samples should be recorded and then averaged.

34.To build a task list to create a trabecular ROI and run analysis, select the "custom processing preview" icon and select the "Internal" tab to build a task list. Click + to add a function to a task list. Do not run the task list yet. Add the functions in the following order with the described settings: thresholding (Global—enter the values determined in **step 4**), despeckle (type: sweep, 3D space, remove: all except largest object, and apply to: image), ROI shrink-wrap (mode: shrink-wrap, 2D space, check "stretch over holes," diameter: 32 pixels—adjust as needed), bitwise operations (image = image XOR region of interest), morphological operations (type: opening, 3D space, kernel: round, radius: 2, and apply to: image), morphological operations (type: closing, 3D space, kernel: round, radius: 2, and apply to: image), morphological operations (type: opening, 3D space, kernel: round, radius: 4, and apply to: image), morphological operations (type: closing, 3D space, kernel: round, radius: 4, and apply to: image), morphological operations (type: opening, 3D space, kernel: round, radius: 6, and apply to: image), morphological operations (type: closing, 3D space, kernel: round, radius: 6, and apply to: image), morphological operations (type: opening, 3D space, kernel: round, radius: 8, and apply to: image), morphological operations (type: closing, 3D space, kernel: round, radius: 8, and apply to: image), morphological operations (type: opening, 3D space, kernel: round, radius: 9, and apply to: image), morphological operations (type: closing, 3D space, kernel: round, radius: 9, and apply to: image), morphological operations (type: opening, 3D space, kernel: round, radius: 10, and apply to: image), morphological operations (type: closing, 3D space, kernel: round, radius: 10, and apply to: image), morphological operations (type: erosion, 2D space, kernel: round, radius: 3, apply to: image), bitwise operations (region of interest = COPY Image), reload (apply to: image), despeckle (type: sweep, 2D space, remove: all except largest object, apply to: region of interest), despeckle (type: remove pores, 2D space, detected: by image borders, apply to: region of interest), save bitmaps (apply to ROI, file format: BMP, check "custom sub-folder" and name the folder, check "convert to monochrome" 1 bit), histogram (unit: bone mineral density, choose 3D space, check "Inside VOI," choose a file name and location), thresholding (global thresholding, use the values previously recorded for thresholding), despeckle (remove white speckles, choose 3D space, volume: less than 6 voxels, apply to image), despeckle (remove black speckles, choose 3D space, volume: less than 6 voxels, apply to image), 3D analysis (check "basic values," "additional values," "trabecular thickness" and "trabecular separation," under the "save results as" region, select a format to save the data output, and name the output of the dataset), and 3D model (apply to: image inside ROI, type of file: *.p3g, algorithm: Double-Time Cubes, check "Custom filename" and name the file).

35.To apply the ROI to the open file, select the "regions of interest preview" icon. Select the "load regions of interest" icon and open the ".roi" file. To determine the thresholding scale, select the "binary selection preview" icon. Choose the "From dataset" tab in the histogram window and select the "automatic thresholding" icon in the histogram window. Record the upper and lower threshold values. For a project, the thresholds for all samples should be recorded and then averaged.

36.To add functions to a task list, click + on a highlighted function. Add the following functions to the task list in the following order with the described settings: histogram (unit: bone mineral density, choose 3D space, check "Inside VOI," choose a file name and location), thresholding (global thresholding, use the values previously recorded for thresholding), despeckle (remove white speckles, choose 3D space, volume: less than 6 voxels, apply to image), despeckle (remove black speckles, choose 3D space, volume: less than 6 voxels, apply to image), 3D analysis (check "basic values," "additional values," "trabecular thickness" and "trabecular separation," under the "save results as" region, select a format to save the data output, and name the output of the dataset), and 3D model (apply to: image inside ROI, type of file: *.p3g, algorithm: Double-Time Cubes, check "Custom filename" and name the file).

37.Select the "BATMAN" icon. Choose the "Add" button and load a trabecular ROI dataset (from **step 3** if manual method was used and **step 4** if automated method was used). Under the "Add" arrow button, choose "Load ROI" and select the ".roi" file. Add all samples within a project and click "Start" to run the task list on all samples.

3.6 Cortical Analysis of Microcomputed Tomography Images

1. In the CTan software, select the "open image or dataset" icon.

2. Identify the reference point for the ROI and set the height to 0.6 mm (*see* Note 38).

3. Create a region of interest to include the entire area of the bone (*see* Note 39).

4. Save the cortical ROI (*see* Note 40).

5. Open a sample ROI by choosing the "open an existing image or dataset" icon and choosing an image within the cortical ROI folder for the sample.

6. Apply the ROI to the open file and determine the thresholding scale (*see* Note 41).

7. Build a 2D analysis task list using a combination of functions until the cortical bone that remains in the "binary selection preview" closely matches the raw images (*see* Note 42).

8. Build a histogram Task List by constructing a task list the same as the "2D analysis Task List" except for the 2D analysis and 3D model functions. Add ROI shrink-wrap, reload, histogram functions to the end of the list (*see* Note 43).

9. Run the task list on multiple samples using the batch manager. Repeat for each task list (*see* Note 44).

[38.] To define a reference point, first select the "regions of interest preview" icon. Define the length of the bone by using the Z-position in the "Dataset" window and calculate 45% of the femur's length. Move the distance calculated from the distal end of the femur. Select the slice in the "Dataset" window. To make the position the selection reference, right-click and choose "Selection Reference." Double-click the black bar in the dataset window to open the "Selection" window. Under the "Analytic" tab set offset 0 mm and height to 0.6 mm.

[39.] At this location in the diaphysis, mostly cortical bone remains. To create an ROI that contains the cortical bone, start by choosing the "regions of interest preview" icon. Scroll to the lower boundary of the ROI. Click the "interpolated region of interest" dropdown and choose "Elliptic." Include the entire area of the bone within the ellipse. Scroll toward the upper boundary of the ROI and continue moving the ellipse to cover the entire tissue, skipping several images in between. The ellipses will interpolate automatically.

[40.] To save the cortical region of interest (ROI) choose "Save from ROI" under "File." Create a new cortical ROI folder. Uncheck "Create folder named VOI" and check "Save ROI-file." Click "OK" to save the ROI.

[41.] To apply an ROI, select the "regions of interest preview" icon. Select the "load regions of interest" icon and open the ".roi" file. To determine a thresholding scale, start by selecting the "binary selection preview" icon. Select the "From dataset" tab in the histogram window. Choose the "automatic thresholding" icon in the histogram window. Record the upper and lower threshold values. For a project, the thresholds for all samples should be recorded and then averaged.

[42.] Choose the "custom processing preview" icon and select the "Internal" tab to build a task list. Add the following functions to the task list in the order with the described settings: thresholding (global threshold, use values recorded from **step 6**), despeckle (remove white speckles, choose 3D space, volume: less than 6 voxels, apply to image), despeckle (remove black speckles, choose 3D space, volume: less than 6 voxels, apply to image), ROI shrink-wrap (mode: shrink-wrap, 2D space, check "stretch over holes," choose appropriate number of pixels for phenotype for diameter, morphological operations and bitwise operations (use a combination as needed to rid the sample of any trabecular bone so that only cortical bone remains), despeckle (use in between morphological operations as necessary to rid the region of trabecular bone, type: sweep, 2D or 3D space depending on sample, remove: all except largest object, apply to: image), 2D analysis (under the "add to report" region choose "all results," check "Append Summary results to file," and choose a file name and location), and 3D model (apply to: image inside ROI, type of file: *.p3g, algorithm: double-time cubes, check "custom filename" and name the file).

[43.] Add the following functions to the end of the task list in order with the described settings: ROI shrink-wrap (mode: shrink-wrap, 3D space to ensure that only the bone will be measured when calculating BMD, check "stretch over holes," diameter: choose appropriate number of voxels for phenotype), reload (apply to: image), and histogram (unit: bone mineral density, 3D space, check "Inside VOI" and "Append Summary results to file," and choose a file name and location).

[44.] To use Batch Manager, select the "BATMAN" icon. Choose the "Add" button and load a cortical ROI dataset. Under the "Add" arrow button, choose "Load ROI" and select the ".roi" file. Load all samples within a project and click "Start" to run the task list on all samples.

3.7 Modeling Skeletal Phenotypes

Several modifications can be made when generating visual representations of scanned samples. The guidelines below are for a basic, gray-scale model.

1. Using CTvol, open a 3D file by selecting the "open 3D object" icon and choosing the p3g file within the ROI folder of the desired sample.

2. Use the wheel on the computer mouse to zoom in and out on the sample.

3. Select "Options" and choose "Objects" to open up the "Objects" window. Adjust color, emission, diffusion, reflection, and opacity as desired within this window.

4. Select the "move camera" or "move objects" icon and use your mouse to adjust the sample to the desired position and angle.

5. Save the Image by selecting "Save Image" from the "File" dropdown menu.

3.8 Mouse Fluorochrome Injections

Labeling schedules will vary depending on anticipated phenotype and project design. The following schedule is for mice 6 months of age with fluorochrome labeled surfaces at four different time points.

1. At 4 weeks of age, perform intraperitoneal (IP) injections on mice with 0.10% working calcein solution. Inject 0.2 mL per 20 g of body weight for a 10 mg/kg dose (*see* Note 45).

2. At 10 weeks of age, perform IP injections on mice with 0.20% working alizarin complexone solution. Inject 0.2 mL per 20 g of body weight for a 20 mg/kg dose (*see* Note 45).

3. At 24 weeks of age, perform IP injections on mice with 0.75% working demeclocycline hydrochloride solution. Inject 0.250 mL per 20 g mouse 150 IP/100 subcutaneous for a 75 mg/kg dose (*see* Note 45).

4. At 25 weeks of age, perform IP injections on mice with 0.10% working calcein solution. Inject 0.2 mL per 20 g of body weight for a 10 mg/kg dose.

5. Sacrifice animals at 26 weeks of age.

3.9 Infiltration

1. Fix the femurs in 10% NBF for 48 h, followed by storage in 70% ethanol.

2. Remove excess tissue from femurs and move to individual histocassette tissue cassettes.

3. Using the Dremel hand tool, notch each bone ¼ to ½ of the way through in the midshaft region. Return each bone to its cassette.

4. Dehydrate and clear the samples for 2–4 h in 70% ethanol, 95% ethanol, 100% ethanol, and xylene (*see* Note 46).

[45.]To minimize complications with IP injections, dosage can be split between IP and subcutaneous injections.

5. Transfer the specimens to xylene until infiltration.

6. Within the RIP unit infiltrate the samples under 15 psi using 100% MMA for 24 h (infiltration 1), the infiltration solution for 24 h (infiltration 2), followed by the infiltration solution for at least 2 days (infiltration 3).

7. Transfer bone tissue to individual, prefabricated embedding molds for coronal sectioning or glass culture tubes for cross-sectioning.

3.10 Femoral Embedding and Cross-Sectioning—When processing tissues that are light sensitive due to fluorochrome labeling, keep in the dark in between processing steps.

1. Add a few drops of glass culture tube embedding solution into a labeled glass culture tube and insert the femur, ensuring the head of the femur is closest to the open end of the tube (*see* Note 47).

2. Cap the glass tube with a rubber stopper and transfer the beaker into the bead bath at 25 °C for optimal polymerization.

3. Check the glass tubes once daily until the polymerization process is complete (*see* Note 48).

4. Remove the polymerized rod containing the femur from the glass culture tube by gently shattering the surrounding glass.

5. Load the resulting plastic rod into the specimen holder of the Extech Labcut 150. Secure the rod into the specimen holder by tightening down the adjustment bolts with an Allen wrench.

6. Line up the plastic rod with the diamond wafering blade to the midshaft of the femur and move the specimen arm so it sits at the back of the Labcut 150.

7. Place either the unattached reed switch magnet or the canopy in place so that the reed switch is covered.

8. Turn the instrument on, set the speed to 166.2 rpm, select the "rev" button to start the blade.

9. Turn on the lubricant pump and slowly pull the specimen arm forward.

10. Sit the rod atop the saw blade and begin to section the bone at the midshaft or slightly more distal (*see* Note 49).

11. Save the distal end for MMA embedding and coronal cross- sectioning or discard it.

46.In a tissue processor, in order, clear the samples for 2–4 h each, in the following solutions: 70% ethanol, 95% ethanol 2×, 100% ethanol 3×, and xylene 2×.

47.Add a paper label containing the specimen ID to the end of the tube. Fill the remainder of the tube with embedding solution until the tube is approximately ¾ full.

48.Monitor the volume of embedding solution for evaporation and add additional embedding solution to ensure the samples remain submerged, when necessary. Polymerization is complete when the embedding solution has turned to a hardened plastic, the tube is smooth, and no longer tacky.

49.When the saw blade cuts completely through the rod, the saw blade will automatically stop as it sits on the specimen arm.

12. Adjust the micrometer on the Extech Labcut 150 with the diamond wafering blade to the desired thickness for the first wafer. Ensure the rod is advancing over the blade (*see* Note 50).

13. Section desired number of wafers.

14. Remove any plastic dust particles from the wafers by dipping each in a sonicator filled with deionized water for 3–5 s. Dab off any excess water.

15. Place a nickel size pool of Eukitt mounting media onto the back of a labeled glass slide and mount the wafers in the pool.

16. Cover the glass slide with 2–3 layers of 1–2 ply paper towel cut into slide size strips.

17. Place a blank glass slide on top of the paper towel strips and fasten both slides together with 2–3 binder clips and allow the slide to air dry overnight.

18. Remove the binder clips, blank slide, and paper towel strips from the surface of the slide with mounted wafers to be sanded.

19. Using a table micrometer, zero out the slide thickness by placing a portion of the glass slide that does not contain a wafer between the clamps. Tighten the clamp on the slide and clear the counter.

20. Open the micrometer clamp and move the slide so one of the mounted wafers is measurable. Close the clamp down onto the wafer and obtain a starting measurement of the wafer. Repeat this step for all wafers.

21. Using 600 grit sandpaper, gently sand the surface of the wafers until each measures 40–50 μm. Remeasure each wafer using the micrometer throughout the sanding process in order to determine when the desired thickness has been obtained.

22. Once all wafers have been sanded to the desired thickness, wipe the sanding remnants from the surface of the slide with a damp paper towel.

23. Add a thin layer of Eukitt mounting media to the surface of the wafers and add a glass coverslip.

24. Add 2–3 small weights to the top of the coverslip to ensure any air bubbles are removed and allow the slides to air dry overnight.

3.11 MMA Embedding and Coronal Sectioning of Femurs—When processing tissues that are light sensitive due to fluorochrome labeling, keep in the dark in between processing steps.

1. Add a thin layer of prefabricated mold embedding solution to each prefabricated mold and allow the reagent to become sticky/tacky.

50.Example: a 15 mm setting on the micrometer will yield a 35 μm wafer.

2. Orient the tissue for coronal sectioning and fill remainder of the mold with prefabricated mold embedding solution.

3. Add a paper label containing the specimen ID, and a paper clip bent to a 45°–90° angle to each mold.

4. Cap the top of the mold with a silicone stopper and transfer each mold to the bead bath at 25 °C, ensuring the containers are covered.

5. Remove molds from the bead bath daily and check until polymerization is complete (*see* Note 51).

6. Remove fully polymerized molds to grind and polish using the MetaServ 250 Single Grinder/Polisher (*see* Note 52).

7. Apply a thin layer of the coating solution to a glass slide.

8. Place the embedded tissue mold in the microtome chuck and face into the mold until the desired tissue is exposed.

9. Brush the exposed face of the block with a fine tip paint brush, soaked in the cutting solution, and initiate the automatic microtome to begin slicing a section from the block at 5 μm.

10. Cut a small rectangle from a Kimwipe, and soak in 70% ethanol.

11. Apply the Kimwipe to the surface of the mold and initiate cutting on the microtome at a very slow speed.

12. As the Kimwipe and plastic section are slowly cut, grasp them together with a pair of forceps. Continue to carefully pull the section off the block as it is cut away by the microtome.

13. Transfer the section and wipe into a petri dish of 75% ethanol. Carefully stretch the section if wrinkles are visible using two pairs of forceps.

14. Once flat, place a piece of plastic film, just larger than the section, on top of the section to keep it free of wrinkles.

15. Transfer the section and plastic film combination to the coated slide.

16. Cover the slide with a thin sheet of plastic and lightly squeeze out any excess ethanol using a gentle wiping motion with a Kimwipe.

17. Cover the section with paper towel and roll flat with rubber roller.

18. Each day sectioning is completed, stack the slides together with a blank slide on the top and bottom of the stack and place in a clamp. Transfer each stack to an oven set at 60 °C, overnight.

19. Remove slides from the oven and inspect for damage.

[51.]Polymerization is complete when the embedding solution has turned to a hardened plastic, the mold is smooth, and no longer tacky.
[52.]Expose the surface of the polymerized block using the grinder by holding the back of the block so that the face is in contact with the abrasive paper. While the block is still wet, apply pressure as needed and rotate the paper to polish away the surface of the mold.

20. Begin deplasticizing slides by soaking them in cellusolve for 1 h.

21. Place slides in acetone for 2–5 min.

22. Rinse slides in DI water for 2–5 min.

23. Continue to "Goldner's Trichrome Staining 3.12" **step 1** for staining or **step 10** to only coverslip for fluorochrome imaging.

3.12 Goldner's Trichrome Staining

1. After deplasticizing samples, place slides in Wiegerts Hematoxylin for 20 min.

2. Rinse slides in running Tap Water for 10 min.

3. Place slides in working solution of Ponceau Acid Fuchsin for 10 min.

4. Place slides in 1% acetic acid for 30 s.

5. Place slides in filtered PTA-Orange G for 4 min.

6. Place slides in 1% acetic acid for 30 s.

7. Place slides in Light Green solution for 10 min.

8. Place slides in 1% acetic acid for 1 min.

9. Rinse slides in 2 changes of distilled water, for 1 min each.

10. Coverslip using the Ventana Symphony by using the proprietary "Coverslip Only" program.

3.13 Paraffin Embedding and Sectioning of Femurs

1. Fix the femurs in 10% NBF for 48 h, followed by storage in 70% ethanol.

2. Remove excess tissue from femurs and move to individual histocassette tissue cassettes.

3. Decalcify samples in 10% EDTA pH 7.4, stirring for 10–14 days at 4 °C (*see* Note 53).

4. Dehydrate, clear, and infiltrate the samples in cassettes within a tissue processor using 70% ethanol for 60 min, 80% ethanol for 60 min, 95% ethanol for 60 min 2×, 100% ethanol for 60 min 3×, xylene for 30 min 2×, paraffin for 30 min, and paraffin for 45 min.

5. Remove the cassettes from the tissue processor and move them to the embedding center.

6. Set the heated areas of the embedding center between 60 °C and 70 °C.

7. Remove the tissue from the cassette and place it in an appropriately sized heated mold. Orient all the samples the same way depending on the type of cut desired (sagittal or coronal).

[53.]Replace with fresh EDTA after 7 days. Samples should be flexible before processing.

8. Hold the tissue specimen down with forceps while partially filling the mold with molten paraffin. Secure the tissue by quickly cooling the base of the mold (*see* Note 54).

9. Place the cassette bottom atop the mold and fill with paraffin.

10. Cool the blocks in a cooling area to set the paraffin until it has solidified.

11. Remove the blocks from the mold by pulling upward on the cassette.

12. Rough cut/face each block to expose the desired surface area of the tissue(s). Precool paraffin blocks with the tissue side down in a tray of ice water to facilitate sectioning.

13. Using a steel microtome knife or disposable blade, cut the appropriate sections (*see* Note 55).

14. For histological sections, place the sections on the pre-labeled glass slides with the IDs that correspond to each block being sectioned. Mount sections by floating a paraffin ribbon into a water bath set between 36 °C and 42 °C.

15. Dry paraffin sections in a lab oven set to 60 °C for 10–20 min.

16. Remove the sections from the oven and allow the slides to cool at room temperature.

17. Deparaffinize slides by soaking in fresh xylene for 3 min 3×, hand-dip slides into 100% ethanol 10×, repeat in fresh 100% ethanol, hand-dip slides in 95% ethanol 10×, repeat in fresh 95% ethanol, and rinse in DI water.

18. Store slides in DI water until TRAP staining.

3.14 TRAP Staining

1. Use the Sigma-Aldrich product 387-A: Acid Phosphatase, Leukocyte (TRAP) kit and protocol to stain deparaffinized samples.

2. Coverslip the slides using an aqueous mounting media and glass coverslips.

3.15 Imaging Slides

1. Image the trichrome stained samples at 20× magnification using a brightfield microscope (Leica Aperio AT2—High Volume, Digital Whole Slide Scanning).

2. Image fluorochrome labeled samples at 20× magnification (Zeiss Axio Imager.A2, Lumen Dynamics X-Cite Series 120 Q, QImaging QIClick Digital CCD Camera) using objective filter ET-DAPI/FITC/Texas Red (Chroma product # 69002). Use BIOQUANT OSTEO 19.2.6 software and follow the BIOQUANT OSTEO 19.2.6 "Image Region and Image Menu Introduction."

[54.]To prevent cross-contamination, wipe the forceps with a clean Kimwipe prior to placing them back into the forceps warmers.
[55.]For histological sections, 5 μm is the standard thickness.

3.16   Histomorphometry—The analysis steps are specific to BIOQUANT OSTEO 19.2.6 Manual.

1.   To quantify trabecular bone parameters, open BIOQUANT OSTEO 19.2.6 software and create a new dataset following "The Data Region: Introduction."

2.   Measure trabecular parameters (Goldner's Trichrome stained, Fluorochrome labeled, TRAP stained slides) by following the "Rodent Trabecular Bone Protocol."

3.   To quantify cortical bone parameters, start by creating new dataset following "The Data Region: Introduction."

4.   Measure cortical bone parameters by following the "Basic Cortical Bone Protocol."

Acknowledgements

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