

Bone Mineral Content and Density

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ABSTRACT

The availability of high-throughput biochemical and imaging techniques that can be used on live mice has increased the possibility of undertaking longitudinal studies to characterize skeletal changes such as bone mineral content and density. Further characterization of bone morphology, bone quality, and bone strength can also be achieved by analyzing dissected bones using techniques that provide higher resolution. Thus, the combined use of high-throughput [e.g., biochemical analysis of plasma, radiography and dual-energy X-ray absorptiometry (DEXA)] and secondary phenotyping techniques (e.g., histology, histomorphometry, Faxitron digital X-ray point projection microradiography, biomechanical testing, and micro-computed tomography) can be utilized for comprehensive characterization of bone structure and quality and to elucidate the underlying molecular mechanisms giving rise to musculoskeletal disorders. *Curr. Protoc. Mouse Biol.* 2:365-400 © 2012 by John Wiley & Sons, Inc.

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INTRODUCTION

Disorders of bone and mineral homeostasis are very common and present a major burden on the health service. For example, bone and cartilage disorders, such as osteoporosis and osteoarthritis, affect over 50% of adults over the age of 50 years. Furthermore, osteoporosis is the leading cause of hospital admission for women over the age of 50 years, while osteoarthritis is the second most common cause of absence from work costing an estimated 1% of gross national product. Despite this, current treatments for osteoporosis reduce fracture risk by only 50%, there are few drugs to stimulate bone and cartilage repair, and no therapies are available that can prevent or retard osteoarthritis. Thus, there are urgent clinical and economic needs to enhance our understanding of the pathogenesis of skeletal disorders and develop targeted therapeutic strategies. In this regard, mouse models for bone and cartilage disorders have been very useful in advancing our knowledge of skeletal biology and in providing preclinical models for translational studies. These studies have utilized primary methods that include: plasma or serum biochemistry (Hough et al., 2002, 2004) for markers of bone metabolism; plain radiography (Esapa et al., 2012) for skeletal abnormalities; and dual-energy-X-ray absorptiometry (DEXA; Karunaratne et al., 2012) for alterations in bone and body composition. If indicated by the results of these primary screening methods, which can be used in high-throughput projects involving live mice, secondary phenotyping assessments can also be undertaken

on dissected bones using histology, Faxitron analysis, biomechanical testing, and micro-computed tomography (Esapa et al., 2012) to determine bone morphological parameters, bone mineral content, and bone strength, all of which are critical determinants of a healthy bone. Thus, the combined use of such primary and secondary phenotyping methods provides comprehensive functional characterization to elucidate the underlying molecular mechanisms giving rise to musculoskeletal disorders. This unit provides details of these methods and protocols. Thus, Basic Protocol 1 describes the method for biochemical analysis, Basic Protocol 2 for radiography, Basic Protocol 3 for DEXA analysis, and Basic Protocol 4 for skeletal sample preparation. In addition, Basic Protocol 5 describes the method for histology and histomorphometry, Basic Protocol 6 for quantitative Faxitron digital X-ray microradiography, Basic Protocol 7 for biomechanical testing, and Basic Protocol 8 for quantitative micro-computed tomography (micro-CT). The primary screening procedures described (Basic Protocols 1 to 3) are, in the UK, covered by the Animal (Scientific Procedures) Act 1986; therefore, they must be carried out by persons holding a personal license and under the authority of an approved project license.

BASIC PROTOCOL 1

BIOCHEMICAL ANALYSIS

Bone is a composite material whose extracellular matrix consists mainly of mineral, which is mostly calcium and phosphate in the form of calcium hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], as well as collagen, water, noncollagenous proteins, and lipids. The control of body calcium involves a balance, which is chiefly under the control of parathyroid hormone (PTH), between the amounts of calcium absorbed from the gut, deposited into bone and into cells, and excreted from the kidney. Under normal physiological circumstances, PTH secretion from the parathyroid glands is increased by hypocalcemia and decreased by hypercalcemia. The control of body phosphate similarly involves a balance between the processes in these organs that is regulated by hormones, which include PTH and fibroblast growth factor 23 (FGF23). Thus, biochemical screening of plasma or serum is important in the diagnosis of musculoskeletal diseases that are due primarily to metabolic bone abnormalities and those that produce biochemical changes as a secondary consequence of the disease. Indeed, alterations in tightly regulated biochemical parameters such as calcium, phosphate and alkaline phosphatase activity are helpful in identifying abnormalities in bone metabolism. Moreover, changes in plasma metabolites such as glucose, urea, and creatinine may indicate dysfunction in other organ systems that may subsequently have a secondary impact on bone turnover. The protocol described below is simple and rapid, and involves collection of blood samples from mouse tail vein after application of topical anesthesia, separation of plasma or serum, and utilization of an AU680 automated clinical chemistry analyzer (Beckman Coulter) to screen for a range of metabolites that are routinely investigated in biochemical studies in man.

Materials

- Mice (control and experimental mice of the same strain, age, and sex)
- Eutectic Mixture of Lidocaine and Prilocaine (EMLA) topical cream (Astra Zeneca), or other suitable topical anesthetic cream
- Silver nitrate pencil to stem blood flow from tail vein
- Personal protective equipment (PPE) including gloves and face mask
- Vertical laminar air flow cabinet
- Microvette tubes (Sarstedt) for collecting blood samples, e.g., lithium/heparin or EDTA-coated tubes for plasma, and uncoated/clot activator tubes for serum
- Permanent marker
- Weighing scale
- Heating box set at 37°C
- Mouse restrainer
- Scalpel blades

Ice box to chill blood samples prior to centrifugation
Microcentrifuge, chilled at 4°C
1.5-ml microcentrifuge tubes to hold plasma or serum samples

Fast the mice and collect the blood samples

1. Fast the mice overnight or for 4 to 6 hr (as deemed appropriate for the mouse model under investigation).
2. Label microvette tubes with mouse identification name or number, using a permanent marker.

Unless stated otherwise, the following procedures should be carried out in a laminar flow cabinet using proper personal protection equipment (PPE).

3. Weigh the mice and calculate the maximum volume of blood (<15% of total blood volume) in microliters that can be collected using the formula: body weight (g) × 9.

Since the microvette tubes are not calibrated, determination of the approximate blood volume to be collected can be facilitated by pipetting the sample volume of water as the blood volume that you wish to collect into a reference microvette tube, and making a note of the position on the tube with a marker pen.

4. Anesthetize the tail of the mouse by applying a topical anesthetic cream, such as EMLA cream (or other suitable anesthetic cream) to the area where an incision will be made for blood collection. Leave for 15 min to allow the local anesthetic to take effect.

The mice can be placed in a warmed box at 37°C during this time to facilitate vasodilation, which will speed up sample collection.

5. Slide the door on the front of the restrainer (Fig. 1A) upwards, place the mouse inside the tube, and adjust the nose cone if necessary, leaving the tail exposed.
6. Remove the sleeve from the microvette tube (Fig. 1B), uncap the tube, and place on the work surface.
7. Using a new scalpel blade for each mouse, make a small incision in the lateral tail vein where the anesthetic cream was applied (Fig. 1C).
8. Firmly stroke the opposite side of the tail to encourage blood flow. Collect blood droplets as they form by capillary action, using the microvette tube. When the required blood volume has been collected, replace the caps on the microvette tube, ensuring that the bottom cap is placed on first before the top cap (Fig. 1D). Replace the outer sleeve and place the whole tube on ice.
9. Apply silver nitrate to the incision, if necessary, to ensure that the tail is no longer bleeding, before returning the mouse to its home cage.

Steps 5 to 9 should take no more than 3 min.

Analyze the blood samples

10. For plasma, centrifuge the blood samples within 1 hr of collection in a chilled (4°C) centrifuge located outside the laminar flow cabinet for 10 min at 5000 × g. For serum, leave the samples to clot at room temperature for 30 to 60 min before centrifuging for 10 min at 5000 × g.
11. Transfer the upper layer containing plasma or serum into labeled microcentrifuge tubes and use for clinical chemistry analysis or store up to 1 year at –20°C for later use.

The samples can be sent to a clinical pathology service laboratory that specializes in the analysis of small volume samples obtained from mice (for example, see <http://www.har.mrc.ac.uk/services/clinical-pathology>). Recent analytical methods require only between 2 to 10 µl of sample for each parameter. This allows measurement of a routine

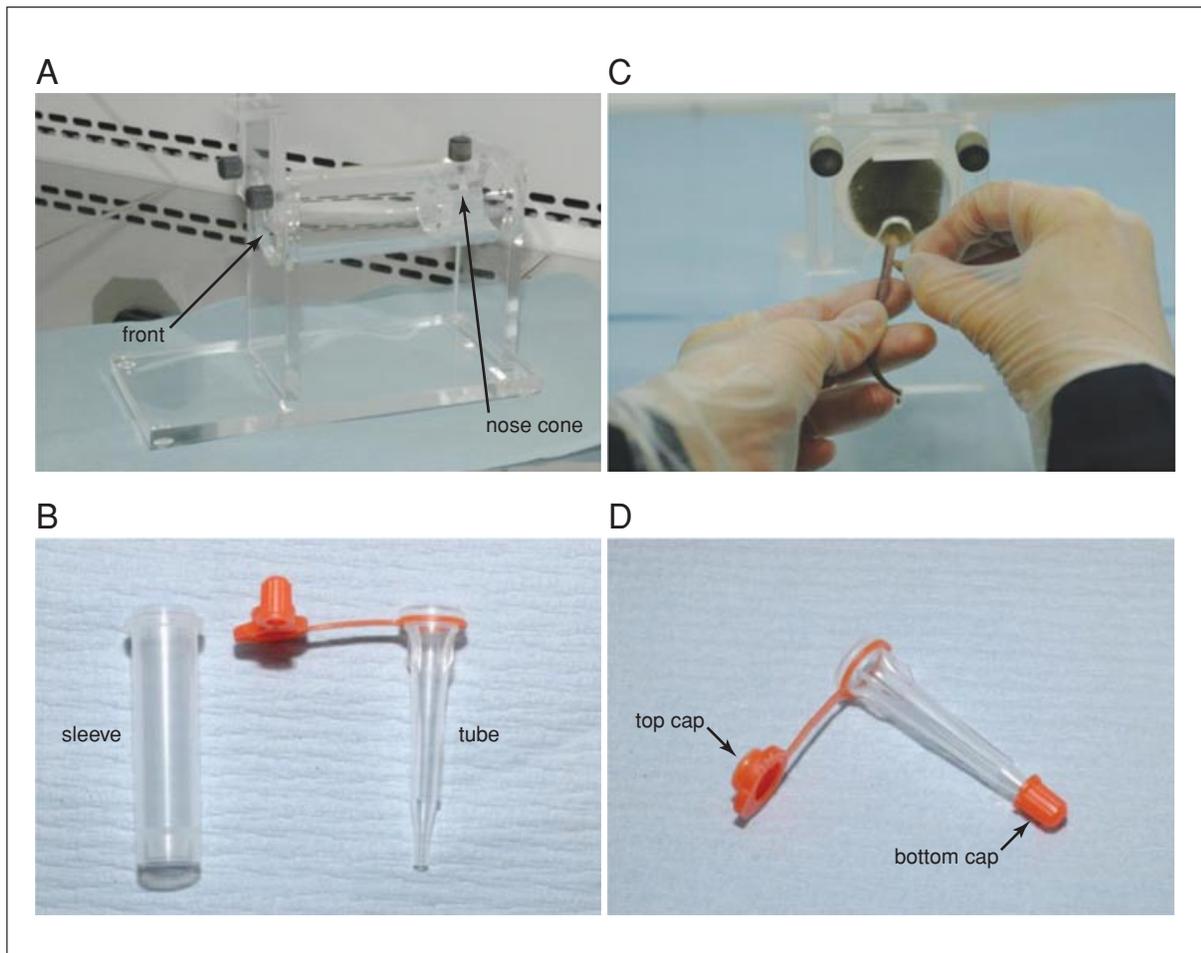


Figure 1 Equipment for blood collection from mouse tail vein. **(A)** Mouse restrainer. **(B)** Microvette tube and sleeve assembly. **(C)** Introduction of incision in the tail of a mouse held in the restrainer. **(D)** Microvette tube used to collect blood by capillary action following incision of mouse tail vein.

biochemical profile of >25 tests (including tests for bone, kidney and liver function) using as little as 200 μ l of total sample volume. The sample volumes to measure specific proteins by using other methods e.g., ELISA or multiplex assays typically vary between 5 to 100 μ l per well.

**BASIC
PROTOCOL 2**

RADIOGRAPHY

Whole-body radiography is a rapid, inexpensive, and non-invasive method for evaluating mice in two dimensions for skeletal abnormalities. Anesthetized mice are placed in the path of an X-ray beam in a shielded cabinet containing safety-interlocked doors, and exposed to X-ray irradiation using appropriate exposure time and power settings. Images are captured using a built-in digital camera and can be stored and analyzed subsequently using different application programs such as Photoshop or DicomWorks (<http://dicomworks.com>). Standardization of mouse handling and positioning of anaesthetized mice for imaging is essential for the production of high-quality images and to ease interpretation of acquired radiographs.

Materials

- Mice
- Anesthetic agent (ketamine at 100 mg/kg body weight)
- Eye lubricant
- Anesthetic reversal agent (xylazine at 10 mg/kg body weight)

Personal protective equipment (PPE) including gloves and face mask
 Faxitron MX-20 digital X-ray system (Faxitron X-ray Corporation)
 Vertical laminar air flow cabinet
 Weighing scale
 Selotape
 DicomWorks software (<http://dicomworks.com>)
 Heating box at 37°C

Calibrate the instrument

1. Switch on the Faxitron machine at least 20 min before use.

Investigator should be wearing proper personal protection equipment (PPE) throughout the entire procedure.

2. Set the kV to 26 and time to 300 sec using the panel at the front of the machine (Fig. 2A). Ensuring that the machine is empty, select “Start” to warm up the X-ray tube.

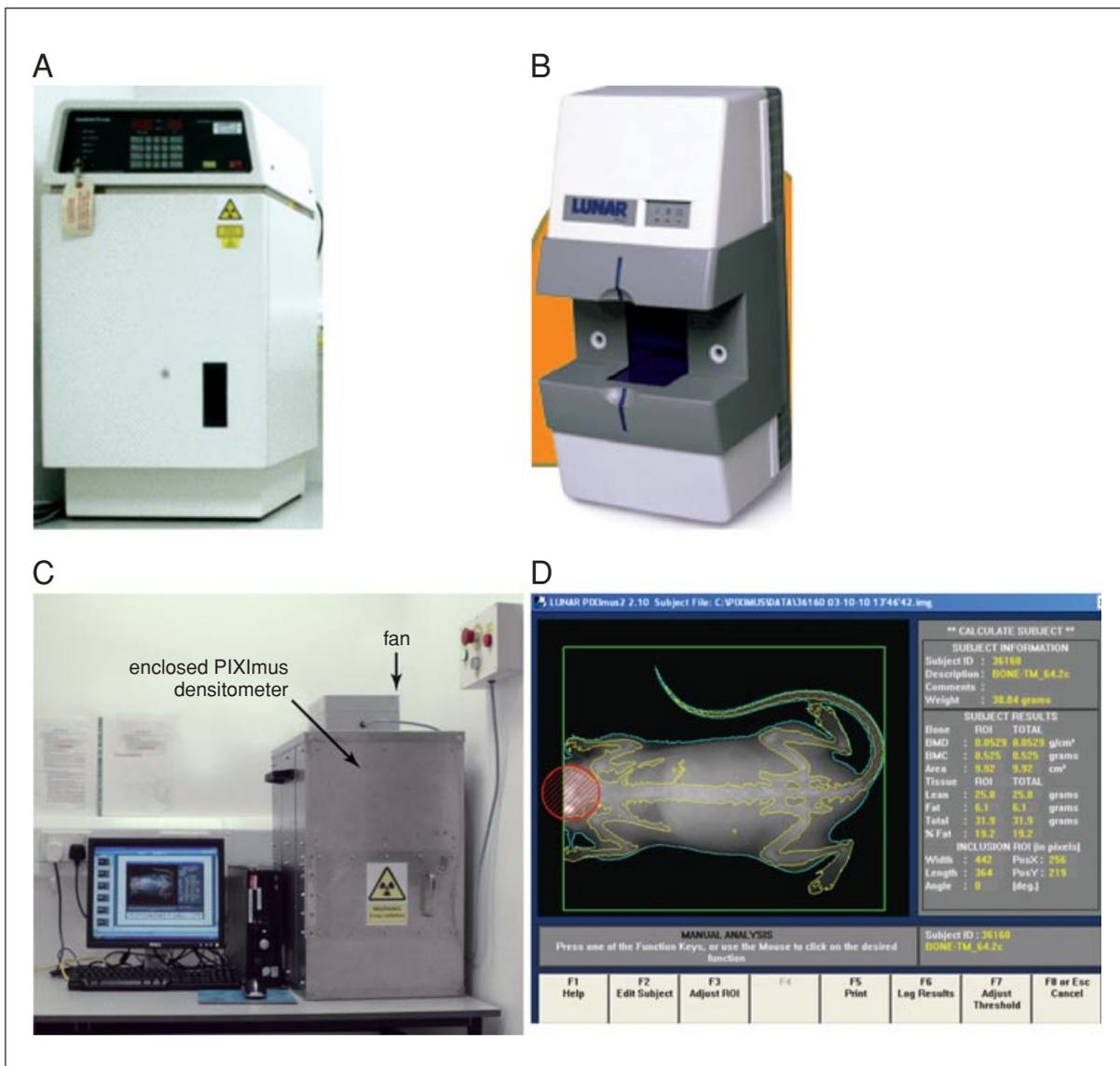


Figure 2 Equipment for radiography and DEXA analysis of mice. (A) Faxitron MX-20 digital X-ray system with appropriate shielding. (B) Lunar PIXImus DEXA scanner without shielding and (C) with shielding and connected to a computer. (D) Sample data readout from the DEXA scanner.

3. Change the time setting on the Faxitron machine to 10 sec.
4. Switch on the attached computer and open the Specimen DR application program.
5. Select the “Service” option. When the “ready for X-ray” message appears at the bottom of the screen, press “Start” on the Faxitron machine and follow the prompts on the computer screen to repeat the process three times.
6. Select “Specimen procedure” on the computer and fill in the mouse details (compulsory fields are marked in asterisks).

Anesthetize the mice and acquire images

7. Weigh the mice in the laminar flow cabinet to determine the amount of anesthetic agent to be administered. Anesthetize the mouse by administering an intraperitoneal injection of an appropriate drug (e.g., ketamine at 100 mg/kg body weight) and place it in a clean cage until sedated (about 2 min).
8. Apply eye lubricant to the cornea for protection, place the mouse dorso-ventrally on a Pyrex plate (supplied with the machine), curl the tail to the left around its body and secure with a piece of selotape, and place the plate on the bottom shelf of the Faxitron machine.
9. Press “Start” on the Faxitron to capture the image. Position the mouse on its side and capture a lateral image; repeat for any specific regions of interest for a maximum of three to five exposures, depending on the single dose of radiation exposure specified in the project license.
10. Save the captured images either as bitmap, or as Dicom files for viewing and processing using the DicomWorks software (<http://dicomworks.com>).
11. Take the mouse out of the Faxitron into the laminar flow cabinet, administer the anesthetic reversal agent subcutaneously (e.g., xylazine at 10 mg/kg body weight), and place the mouse in a heating box set at 37°C for recovery.
12. When all the mice have recovered (15 to 30 min), return them to their cages and to the holding room.

**BASIC
PROTOCOL 3**

DUAL-ENERGY X-RAY ABSORPTIOMETRY (DEXA)

DEXA analysis is a semi-automated, quantitative imaging technique that utilizes relatively low doses of radiation optimized for the separation of mineralized and soft tissue components. Thus, DEXA provides estimates of bone composition including bone size, bone mineral content (BMC), and bone mineral density (BMD) in two dimensions (i.e., areal BMD), as well as soft tissue components including lean mass and fat mass. The protocol below describes the use of a PIXImus densitometer designed for high-throughput whole-body DEXA scanning of mice, and data captured from a whole mouse can be used to extrapolate readouts from specific sites of interest, e.g., the vertebrae or femur.

Materials

Mice

Anesthetic agent (ketamine at 100 mg/kg body weight)

Eye lubricant

Anesthetic reversal agent (xylazine at 10 mg/kg body weight)

Personal protective equipment (PPE) including gloves and face mask

Lunar PIXImus DEXA scanner (GE Lunar Piximus II X-ray bone densitometer)

DEXA phantom mouse

Vertical laminar air flow cabinet

Weighing scale

Animal cages
Ruler
Mouse specimen trays
Selotape
Heating box set at 37°C

Calibrate the instrument

1. Switch on the PIXImus scanner (Fig. 2B and 2C) from the main supply 30 min before use. Switch on the attached computer and open the software that operates the PIXImus.

Investigator should be wearing proper personal protection equipment (PPE) throughout the entire procedure.

2. After 30 min, place the DEXA phantom mouse on the specimen tray and place securely on the tray holder in the machine. Perform quality control (QC) test by pressing F6 on the keyboard, followed by F3.

Mice should not be anesthetized until the PIXImus QC test has passed. If the QC test fails, repeat by pressing F3 on the keyboard. The PIXImus software will not allow mice to be scanned without a successful QC test.

Anesthetize and scan the mice

3. Weigh the mice using the weighing scale in the laminar flow cabinet to determine the volume of anesthetic agent to be administered. Anesthetize the mouse by administering an intraperitoneal injection of an appropriate drug (e.g., ketamine at 100 mg/kg body weight) and place in a clean cage until sedated. If required, measure the length of the mouse (head to anus or tail alone) using a ruler.

Mice can be scanned sequentially using radiography and DEXA under a single anesthetic dose.

4. Apply eye lubricant to the cornea for protection against dryness, place the mouse on the specimen tray, curl the tail to the left around its body and secure with a piece of selotape, and place specimen tray securely in the PIXImus machine.
5. To scan the mouse and take measurements, press F3 on the keyboard, enter mouse details (subject ID, date of birth, sex, weight and length), and select OK.
6. Anesthetize the next mouse, so that it will be sedated when the previous mouse comes out of the PIXImus.

Each mouse is exposed to four rounds of X-rays: 35 kV and 0.5 mA for 15 sec followed by 80 kV and 0.5 mA for 3 sec for each round. The entire scan takes ~3 to 4 min, after which the bone and soft tissue data are displayed on the computer monitor.

7. When the scan is complete, take the mouse out of the PIXImus into the laminar flow cabinet, administer anesthetic reversal agent subcutaneously (e.g., xylazine at 10 mg/kg body weight), and place the mouse in a warmed box set at 37°C for recovery.

Analyze the data

8. To make adjustments to the scanned image displayed on the computer monitor (Fig. 2D), press F3 twice on the keyboard, alter the shape and size of circle/oval around the head using the “Ctrl” button together with the up/down, or left/right keys. Press return on the keyboard to exclude the head from the data analysis.

The adjusted results will be displayed on the computer monitor, next to the scanned image (Fig. 2D).

9. Press F6 on the keyboard to save data, F5 to print, and press F8 twice to exit and return to the main menu, ready to scan the next mouse.

10. When all the mice have recovered, return them to their cages and to the holding room.

Data captured from a whole mouse can be manually adjusted to obtain readouts from a specific region of interest (ROI) such as the spine or femur by altering the size and position of the green rectangle, which is usually placed around the whole of the scanned image by default (Fig. 2D).

11. To adjust the ROI, select F3 and press the “Tab” key so that the rectangle is highlighted in green, becoming the ROI.
12. Use “Ctrl” and the arrow keys to resize the ROI; arrow keys or “Shift” with arrow keys to move the ROI; “Page Up” to rotate clockwise or “Page Down” to rotate counter clockwise by 1°; “Shift” with “Page Up” or “Page Down” for 5° increments.
13. Press “Enter” to recalculate the results after adjusting the ROI, and the ROI data will be displayed on the computer monitor together with the total whole body data.

BASIC PROTOCOL 4

SKELETAL SAMPLE PREPARATION AND FIXATION

Skeletal phenotype analysis can be performed in the mouse at any stage of development or in adulthood. In the pre-natal and peri-natal periods, skeletal “whole-mount” staining of the intact skeleton is often performed together with histological analysis of the proximal tibia growth plate (Fig. 3). In the postnatal period, however, skeletal elements are studied individually and the upper limbs, lower limbs, and vertebrae are frequently analyzed (Fig. 3). Samples may be fixed in 10% neutral buffered formalin to preserve tissue morphology or fixed in 70% ethanol to maintain the biomechanical properties and facilitate subsequent removal of organic material. This protocol describes the preparation and fixation of skeletal samples from postnatal mice for comprehensive skeletal phenotyping.

Materials

Control and experimental mice of the same strain, age, and sex
70% (v/v) ethanol
10% Neutral buffered formalin (NBF; Sigma Aldrich)

Dissecting instruments including:

Scissors, fine
Scalpel
Tweezer
20-ml polystyrene tubes
4°C incubator
Pencil

Additional reagents and equipment for euthanasia (Donovan and Brown, 2006)

Dissect and fix specimen

1. Sacrifice the animal using an approved method (see Donovan and Brown, 2006).
2. Carefully remove the skin from the carcass (skin can be left on the paws) and then remove the abdominal organs with tweezers.
3. To separate the upper limbs, cut under the scapula with fine pair of scissors to detach the upper limb from the thoracic wall and then separate the proximal end of the clavicle from the sternum.
4. To separate the lower limbs while avoiding damage to the femoral head, cut vertically medial to the pelvis and immediately lateral to the sacral vertebrae using a scalpel.

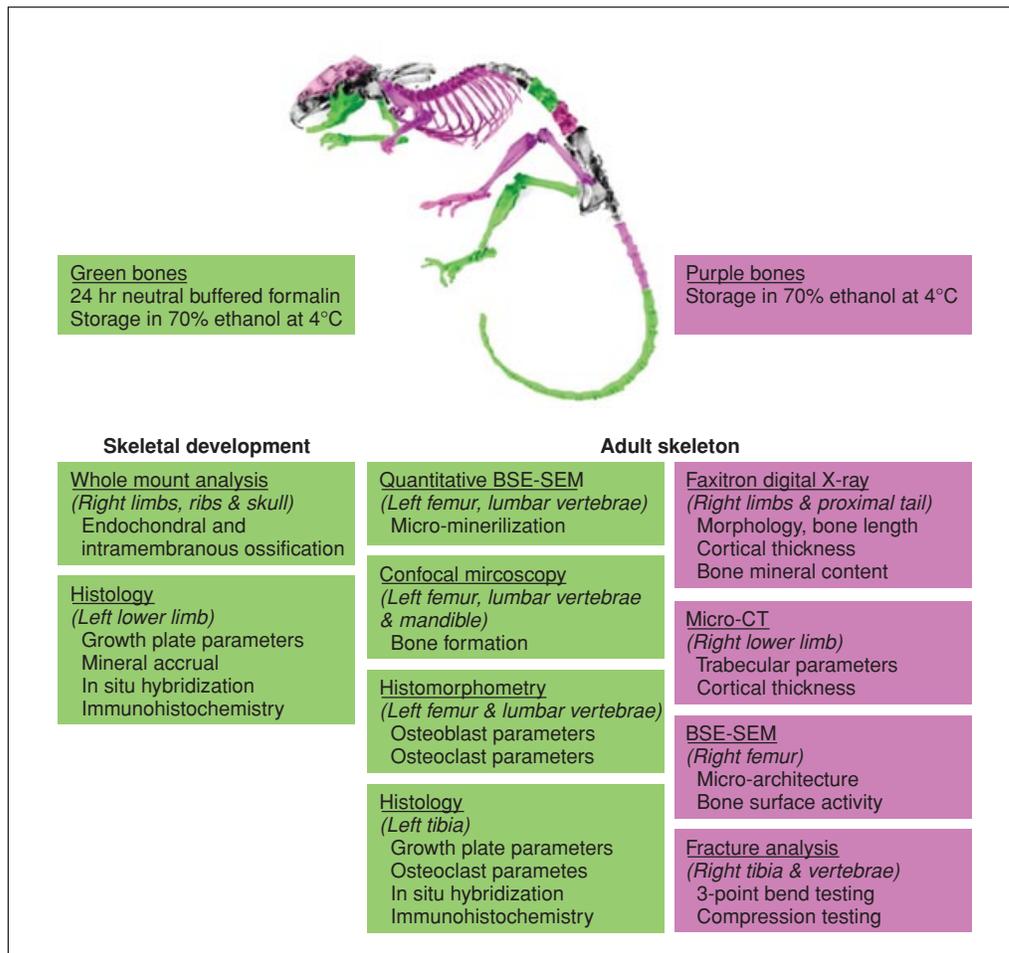


Figure 3 Sample collection, fixation, and analysis in mouse skeletal phenotyping.

- To separate the caudal vertebrae (Ca), remove the skin from tail, proximal to distal, and then separate the tail from the body at its base. Count down inter-vertebral spaces and detach the six most proximal vertebrae using a scalpel.
- To separate four lumbar vertebrae, cut the vertebral column at an intervertebral space just below the ribs with a scalpel. Count down inter-vertebral spaces and detach 4 lumbar vertebrae using a scalpel.
- Separate the skull at its base using a scalpel ensuring all skin has been removed.
- Place the intact right upper and lower limbs, the proximal six tail caudal vertebrae, and two proximal lumbar vertebrae into 70% ethanol in a 20-ml polystyrene tube and fix for at least 48 hr at 4°C (Fig. 3). Use a pencil to label the tube clearly indicating the specimen number and fixation method.
- Place the intact left upper and lower limbs, the distal caudal vertebrae, and two distal lumbar vertebrae and the skull into 10% NBF in a 20-ml polystyrene tube and fix for 12 hr at 4°C. Use a pencil to label the tube clearly indicating the specimen number and fixation method. After 12 hr, replace the 10% NBF with 70% ethanol and store at 4°C for at least a further 48 hr (Fig. 3).
- After fixation, dissect the muscle and soft tissues carefully away from the bones and joints of the upper and lower limbs using small curved dissecting scissors. Detach the humerus from the scapula and the paw from the radius and ulna using small curved dissecting scissors. Detach the femur from the acetabulum being especially

careful to avoid damage to the femoral head and then separate the paw from the tibia and fibula using small straight scissors.

11. Return the dissected bones to their original 20-ml tubes replacing with fresh 70% ethanol and store at 4°C.

Skeletal samples can be stored for many months and may, depending on the fixation method, be used for histology, histomorphometry, micro-CT analysis, back-scattered electron scanning electron microscopy, confocal microscopy, or biomechanical testing (Fig. 3).

BASIC PROTOCOL 5

HISTOLOGY AND HISTOMORPHOMETRY

Histology analysis can be used to assess the organization of articular cartilage and growth plates in long bones, and to assess the proliferation and differentiation of chondrocytes by determining their height in the resting, proliferating, and hypertrophic growth plate zones (Fig. 4A and 4B). Histology can also be used to visualize bone structure and the cellular components of bone, including osteoclasts, osteoblasts and osteocytes, in both growing and adult mouse bones. This is typically coupled with histomorphometric techniques, which can be either “static,” which is used to quantitate cell number, or “dynamic,” which is used to measure mineralization and bone formation rates. Dynamic histomorphometric analysis requires injecting mice with calcein, which labels newly formed bone and is performed on undecalcified bone samples (Fig. 4C). Mice are injected intraperitoneally with calcein (10 mg/kg body weight) at intervals, e.g., 6 days and 2 days, respectively, before the mice are sacrificed and bones dissected for processing. Static histomorphometry can be performed on decalcified or undecalcified samples.

Hematoxylin and eosin (H&E) staining may be used for embryonic, neonatal, and adult mouse limbs. However, in our experience alcian blue and van Gieson staining, which stains cartilage blue and osteoid red, is superior to H&E and can be used to stain specimens from E17.5 through development and adulthood. In some cases, specific staining procedures are required to identify individual cell types. For example, the presence of tartrate resistant acid phosphatase activity is often used to identify osteoclasts.

Materials

- Dissected bones (see Basic Protocol 4)
- 10% EDTA, pH 7.4
- Graded ethanol series
- Paraffin wax (Leica Microsystems)
- Pathcentre (Thermo Shandon)
- Xylene (VWR) *or* Sub-X (Leica Microsystems)
- Scott's tap water (17.5 g sodium bicarbonate, 100 g magnesium sulfate, 25 ml formaldehyde, made up to 5 liters with distilled water)
- Gill 3 Hematoxylin (Fisher Scientific)
- Acid alcohol (1% hydrochloric acid in 70% Ethanol)
- 20% lithium carbonate
- Eosin (Fisher)
- Isopropyl alcohol
- Clearium mounting medium (Leica Microsystems)
- 2% Alcian Blue 8GX (Fisher): prepared in 3% acetic acid, pH 2.5
- Weigerts Hematoxylin (see recipe)
- van Gieson (Fisher)
- Acetate buffer, pH 5.2 (see recipe)
- Acetate-tartrate buffer (see recipe)
- Naphthol AS-BI phosphate solution (see recipe)
- Pararsosaniline (see recipe)

4% sodium nitrite (see recipe)
DPX (VWR)
LRWhite medium resin (Taab Laboratories)
Automated tissue processing system, optional
Paraffin embedding system (Leica), optional
Microtome (Leica Microsystems)
SuperFrost Plus slides (VWR)
37°C incubator
Coplin jar, 37°C
Measuring cylinder, 37°C
Glass bottles
Leica DMRB microscope
Osteomeasure bone histomorphometry system (OsteoMetrics)
UV lamp

I. Histology: Decalcified samples

1. Use dissected bones fixed in 10% neutral buffered formalin (see Basic Protocol 4).
2. Decalcify the fixed bones in 10% EDTA (pH 7.4) for 14 days, which may be insufficient for older (adult) animals for which we use 3 weeks.

Use ~100 ml of 10% EDTA per bone in a histological specimen pot at room temperature. Completion of decalcification can be assessed by Faxitron imaging.

3. Dehydrate the decalcified bones through graded ethanol (70% for 1 hr; 95% for 1 hr; and 3 changes in 100% ethanol for 2 hr, 1 hr, and 1 hr, respectively) and process into paraffin wax using an automated system such as Pathcentre.

This processing step can be achieved using an automated tissue processing system.

Processed samples can be embedded in paraffin using a heated paraffin embedding system (Leica).

4. Using a microtome (Leica RM2255), cut sections (3- to 4- μ m thick), float on warm distilled water, and lift onto SuperFrost Plus slides. Dry the samples overnight at 37°C.
5. Deparaffinize the tissue using xylene or Sub-X for 15 min, and repeat twice.
6. Rehydrate the tissue by placing for 1 min in descending grades of alcohol (100%, 90%, 80% and 70%).

The tissue sections can then be stained as follows.

Hematoxylin and eosin staining

7. Wash the sections for 1 min under running tap water, stain with hematoxylin for 5 min, and wash again with water for 1 min.
8. Treat the sections with acid alcohol (1% hydrochloric acid in 70% ethanol) for 5 sec to reduce background and wash in tap water.
9. Treat the sections with 20% lithium carbonate for 30 sec and wash in running tap water for 1 min.
10. Counterstain the sections using 1% eosin for 3 min and wash in running tap water for 30 sec.
11. Dehydrate the sections by placing in isopropyl alcohol for 2 min.
12. Mount the sections in a fume hood using Clearium mounting medium and leave the slides to dry for 5 min at room temperature.

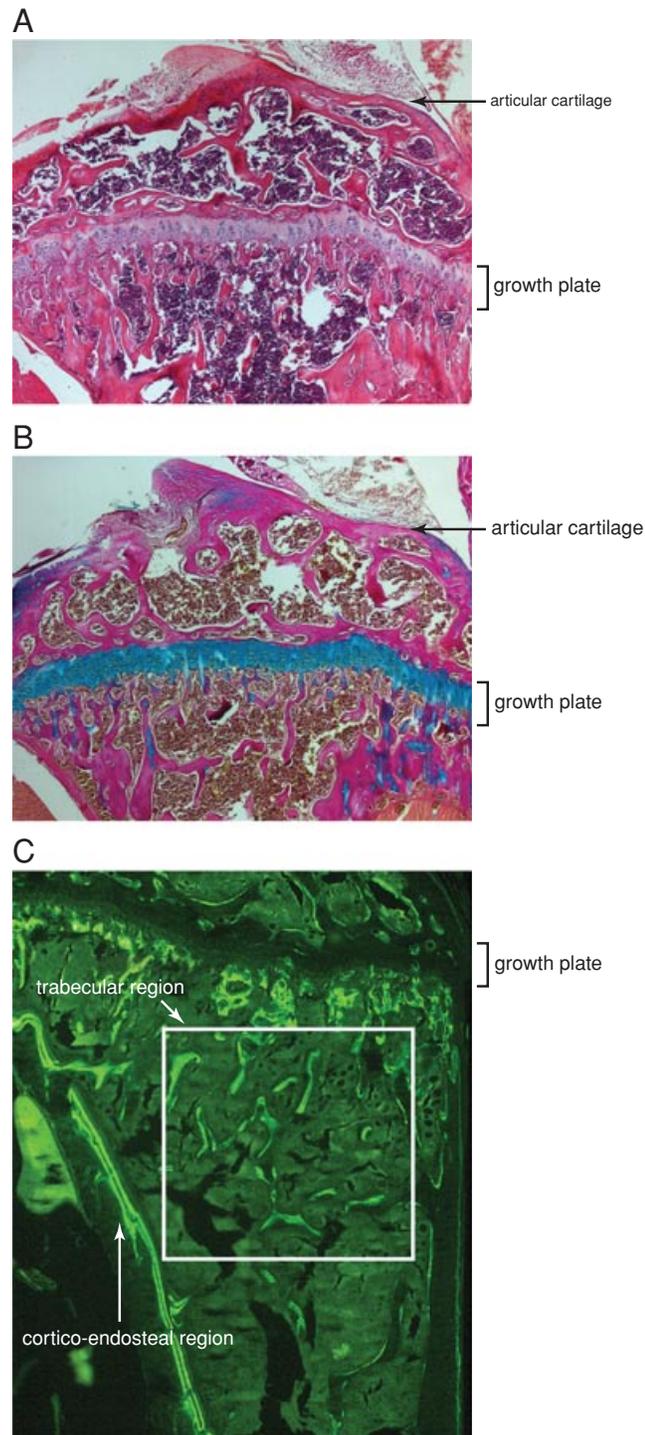


Figure 4 Histology and histomorphometric analysis. Proximal tibia from a 22-week-old mouse stained with (A) Hematoxylin and eosin and (B) Alcian blue (cartilage) and van Gieson (osteoid) revealing the articular cartilage and growth plate. (C) Histomorphometry, using calcein to label new bone. For the color version of this figure, go to <http://www.currentprotocols.com/protocol/mo120124>.

Alcian Blue and van Gieson staining

Steps 13 to 21 should be performed at room temperature.

13. Deparaffinize the sections in xylene (twice for 15 min each).
14. Rehydrate the sections in descending grades of ethanol (100%, 80%, 60%, 30%, and 0%) for 5 min in each.
15. Stain with Alcian blue 8GX for 30 min and wash in distilled water for 3 min.
16. Stain with Weigerts hematoxylin for 5 min and wash in distilled water for 3 min.
17. Differentiate the sections by placing in 1% acid alcohol for 20 sec.
18. Neutralize in Scott's tap water for 30 sec.
19. Stain with van Gieson for 5 min, and wash briefly in distilled water.
20. Dry the slides for 15 min at 37°C.
21. Mount the sections in a fume hood using Clearium mounting medium.

Tartrate-resistant acid phosphatase (TRAP)

TRAP is an isoenzyme of acid phosphatase present in osteoclasts. TRAP activity can be revealed using an azo dye coupling method. The following part of the protocol provides sufficient reagents to stain ten slides in a Coplin jar. Warm a Coplin jar and measuring cylinder to 37°C before starting the procedure.

22. Dewax the sections through ethanol into distilled water.
23. Incubate the sections in acetate buffer warmed to 37°C in a Coplin jar.
24. Prepare solution A by adding 50 ml acetate-tartrate buffer to 1 ml naphthol AS-BI phosphate solution in a glass bottle.
25. Incubate the sections in solution A for 30 min at 37°C.
26. Prepare solution B in a fume hood as follows. Hexazotise the pararosaniline by mixing 2 ml pararosaniline with 2 ml of 4% sodium nitrite and allow to stand for 4 min. Immediately prior to use add 2.5 ml of solution B to 50 ml acetate-tartrate buffer.
27. Incubate sections in solution B for 15 min at 37°C.
28. Rinse the sections in gently running tap water.
29. Counterstain in Gill's hematoxylin and restore blue coloration by washing in running tap water.
30. Dehydrate in ethanol (~ 5 sec in 70%, 95%, and 100%, respectively), clear in xylene, and mount with DPX.

II. Histology: Undecalcified samples

Use undecalcified dissected bones fixed in 10% neutral buffered formalin for 12 hr (Basic Protocol 4).

31. Process the bones through 70% (2 changes, 48 hr each), 80% (2 changes, 48 hr each), 90% (2 changes, 48 hr each), and 100% (3 changes, 48 hr each) ethanol and embed in LRWhite medium resin.
32. Using a Leica RM2265 microtome with a tungsten-carbide blade, cut 10- μ m sections onto SuperFrost Plus slides.

33. Stain the sections as described above (see steps 7 to 12 or 22 to 30) or leave unstained for visualization of calcein labels and assessment of bone formation rates.
34. Mount the sections in DPX.

III. Histomorphometry

Bone histomorphometry is used to obtain quantitative information about bone structure and the cellular components of bone. A number of dedicated bone histomorphometry systems are available including the Osteomeasure system (OsteoMetrics) and the Bioquant Osteo system (Bioquant Image Analysis Corporation). These systems use the American Society for Bone and Mineral Research nomenclature for reporting bone histomorphometry (Parfitt et al., 1987).

Static histomorphometry

35. Switch on the Leica DMRB microscope and position a TRAP-stained section on the microscope stage.
36. Switch on the OsteoMeasure bone histomorphometry system.
37. For trabecular bone, define the region of interest.

For the tibia, this typically starts 250- μ m below the growth plate and will comprise a number of fields of view that traverse the section and a number of fields that move away from the growth plate. For C57BlK^{al}Wrij mice, this is usually 3-fields by 3-fields each measuring 250 μ m \times 250 μ m.

This will differ for each bone, strain of mouse, and the age of the mouse.

38. Using the OsteoMeasure system, trace the bone perimeter.
39. Select “osteoblast” from the measurement list and click on each individual osteoblast, then activate “osteoblast surface” and trace the bone surface lined by osteoblasts.
40. Select “osteoclast” from the measurement list and click on each individual osteoclast, then activate “osteoclast surface” and trace the bone surface lined by osteoclasts.
41. Having completed measurements for one field of view, move to the next field and repeat the above for all fields.
42. Having completed the measurement, activate the “summary data” tab and the calculated parameters will be listed. This data can be stored and used for subsequent analysis.

Dynamic histomorphometry

43. Switch on the UV lamp and allow to warm up for at least 5 min.
44. Position an unstained section on the DMRB microscope stage.
45. Switch on the OsteoMeasure bone histomorphometry system.
46. For trabecular bone, define the region of interest. As with static measurements for the tibia this typically starts 250- μ m below the growth plate and will comprise a number of field of view that traverse the section and a number of fields that move away from the growth plate.

For C57BlK^{al}Wrij mice, this is usually 3-fields by 3-fields each measuring 250 μ m \times 250 μ m.
47. Using the OsteoMeasure system, trace the bone perimeter.
48. Select “Single Label” from the measurement list and trace the bone surface occupied by a single calcein label.

49. Select “Double Label” from the measurement list and trace the bone surface occupied by a double calcein label.

The mean inter-label width will be calculated automatically.

50. Having completed measurements for one field of view, move to the next field and repeat the above for all fields in a section.
51. Having completed the measurements, activate the “summary data” tab and all primary and calculated parameters for the section will be listed.

These include the percentage mineralizing surfaces (MS, %) using $[(dL+0.5sL)/BPm.] \times 100$, where dL = double label perimeter, sL = single label perimeter and $BPm.$ = total bone perimeter; the mineral apposition rate (MAR, $\mu\text{m}/\text{day}$) derived from the interlabel width ($Il.W$)/number of days between injections; and the bone formation rate (BFR, $\text{mm}^2 \times 10^{-3}/\text{mm}/\text{day}$) calculated using $(MAR \times (dL+0.5sL))/BPm.$ $BFR = MAR \times MS$. This data can be stored for subsequent analysis.

QUANTITATIVE FAXITRON DIGITAL X-RAY MICRORADIOGRAPHY

Faxitron digital point projection X-ray microradiography is a rapid, cost-effective, and sensitive method to quantify relative bone mineral content and determine morphometric parameters, such as bone length and cortical thickness in post-mortem skeletal specimens. The Faxitron MX20 can generate 16-bit grayscale images of mouse skeletal elements at a 10- μm pixel resolution. This protocol describes image acquisition and calibration and montage generation and analysis (Bassett et al., 2010).

Materials

- Bones in 70% ethanol (see Basic Protocol 4)
- Faxitron MX20 variable kV point projection X-ray source and digital image system (Qados, Cross Technologies)
- ImageJ 1.45 software (download at <http://rsb.info.nih.gov/ij/>)
- Calibration standards including:
 - 1-mm diameter steel wire
 - 1-mm diameter aluminum wire (Hollinbrow Precision Products))
 - 1-mm diameter polyester fiber
- Mitutoyo CD-6 CP Digital calipers (Mitutoyo)
- Paper towels
- Microsoft Excel
- Adobe Photoshop CS5 (Adobe Systems)

Acquire the image

In mice, Faxitron imaging can be used at any time after 4 weeks of age. For skeletal phenotype screening, image an upper limb, a lower limb and the proximal 6 tail vertebrae. The Faxitron X-ray source is shielded by lead lining. Inadvertent radiation exposure is prevented by a trip switch locking mechanism that is activated when the X-ray source is switched on. It is essential that these safety features are tested at installation and checks are included as part of a maintenance contract.

1. Insert the key and switch on the Faxitron machine at least 15 min prior to use in order to ensure stability of the X-ray source.
2. The Faxitron should be calibrated each time it is used and it is necessary to remove the polycarbonate sample stage prior to calibration. A 15-sec exposure at 26 kV is suitable for most adult mouse bones. Using the software provided, calibrate the Faxitron by running four flat dark = field images and save the calibration settings.

3. Ensure that the steel, aluminum, and polyester calibration standards are attached to the under surface of the polycarbonate sample tray using thin tape. Arrange the standards at the periphery of the field (Fig. 5A). Place the sample tray on top of the thick plastic support and insert into the uppermost level in the Faxitron cabinet (5× magnification) and close the door.
4. Ensure that the Auto Level, Auto Exposure Control, Contrast Assist and Sharpen Assist toggles are all deselected. Then select the “start exposure” button to commence imaging. The image will display on the computer screen. Ensure that all three standards are within the field of view.
5. Set the digital calipers to 15 mm and place it flat on the sample tray within the imaging area. Select the “start exposure” and save the image for future linear image calibration.
6. Only remove the bones (from Basic Protocol 4) from the 70% ethanol solution when you are ready to start imaging. Remove the samples from the 70% ethanol for as short a period of time as possible to prevent drying out. Blot the samples briefly on a paper towel to remove the excess ethanol and then arrange them on the polycarbonate sample tray within the imaging area bound by the three standards (Fig. 5A). Replace the sample tray in the uppermost slot in the Faxitron.
7. Select “start exposure” and acquire the image. Check that the orientation of bones and standards is correct, taking care to make sure that all elements are within the field of view. Should any adjustment be necessary, remove the sample stage, reposition the samples, and repeat the imaging.
8. Save the image as a 16-bit DICOM file.
9. Once all the samples have been successfully imaged, switch the Faxitron off and transfer the data for image calibration and analysis.

Image calibration

10. Open the 2368 × 2340 DICOM image in ImageJ and press “h” to reveal a histogram showing the distribution of grayscale pixels within the image (Fig. 5B).
11. Record the minimum and maximum gray level values in an Excel spreadsheet. Use the ImageJ “Rectangular Selections Tool” to select as large an area as possible of the polyester standard without including background. Press “h” and record its modal gray level. Similarly, select the steel standard and record its modal gray level. This procedure is not applied to the aluminum standard, which acts as an internal reference for the grayscale stretch procedure described below.
12. Using ImageJ, stretch each of the 16-bit DICOM images (16,383 gray levels) to the modal gray level values of the plastic and steel standards noted above. This stretched image is inverted using ImageJ and then converted to an 8-bit TIFF file (256 gray levels). In the resultant image the plastic and steel standards are thus always assigned gray levels of 0 and 255, respectively (Fig. 5C).
13. From the ImageJ “Image” drop-down menu select “Lookup Tables” and “16_colors.” This procedure applies a pseudo-color scheme in which gray levels are divided into 16 equal intervals each represented by a different color (Fig. 5C), thus greatly aiding visual presentation of digital images. Name and save the image as a Tiff file.
14. Repeat this procedure for all the images.

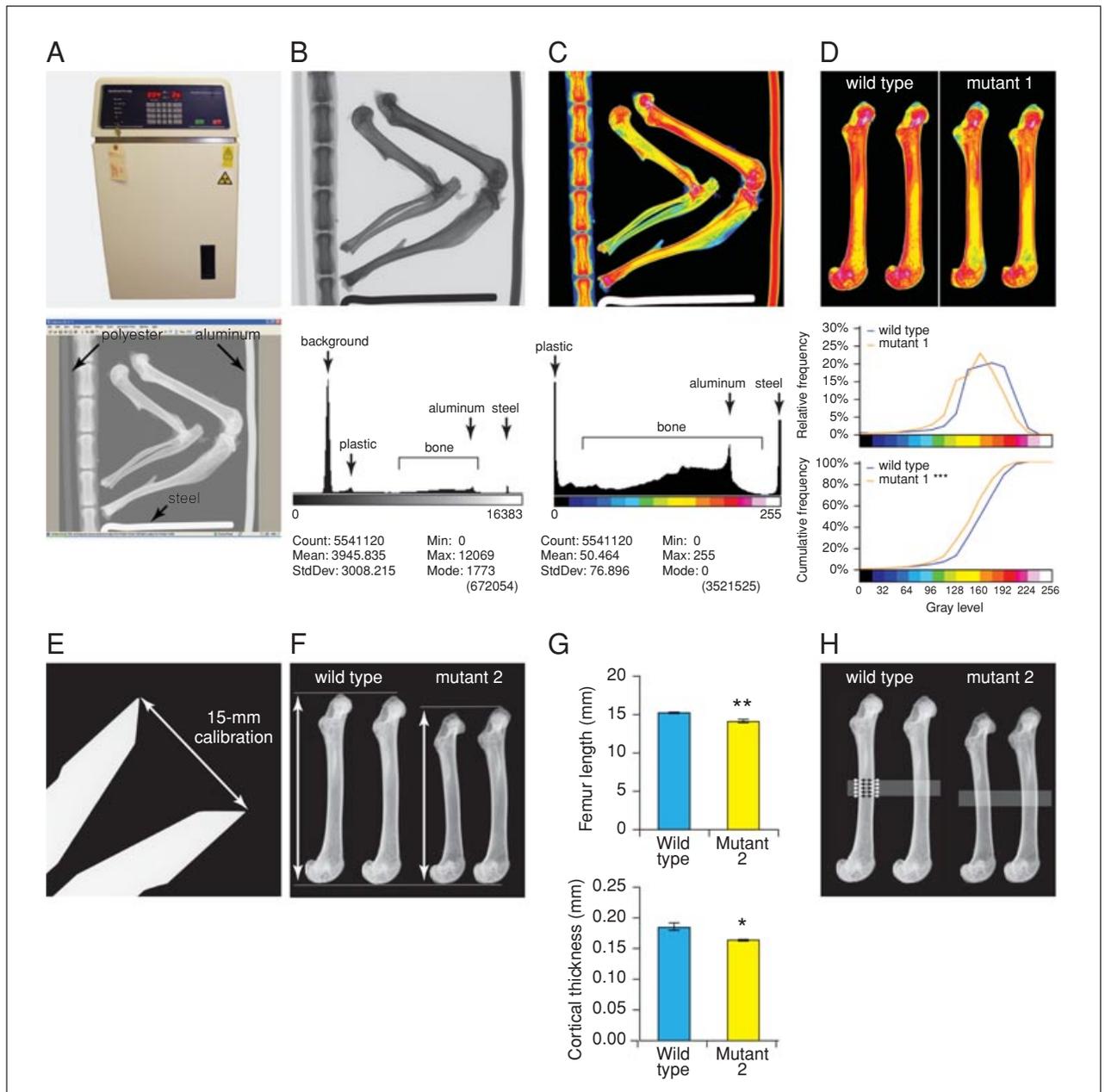


Figure 5 Quantitative Faxitron digital X-ray microradiography. **(A)** Use of a Faxitron MX-20 showing PC imaging software below to illustrate recommended organization of limbs and vertebrae alongside polyester, aluminum, and steel calibration standards. **(B)** Original 16-bit DICOM image. The histogram below shows the grayscale pixel distribution with location of the three standards relative to skeletal samples. The large peak on the left represents the background. **(C)** Pseudo-colored 8-bit TIFF image following stretch processing. The histogram below shows the stretched grayscale distribution in relation to the 16 color bins. **(D)** Pseudo-colored 8-bit TIFF image of two representative, cleaned femurs from wild-type and mutant montages. Relative and cumulative frequency histograms show reduced bone mineral content in mutant mice ($n = 4$). Kolmogorov-Smirnov test, mutant 1 versus wild-type, *** $p < 0.001$. **(E)** Faxitron image of digital micrometer set at 15 mm for ImageJ calibration. **(F)** Grayscale images of two representative, cleaned femurs from wild-type and mutant montages showing determination of femur length. **(G)** Graphs illustrating reduced bone length and cortical thickness in mutant mice ($n = 4$). Student's t test (mutant 2 versus wild-type), * $p < 0.05$, ** $p < 0.01$. **(H)** Images showing determination cortical bone thickness by measurement of the external and internal diameter at five separate mid diaphyseal locations.

Prepare the montage

15. In order to compare samples from different groups of mice (e.g., wild-type and mutant) it is necessary to generate a single montage for each group. Most frequently, femurs and caudal vertebrae are selected for comparison.

Montages can be produced using imaging software such as Adobe Photoshop.

16. Open the TIFF files (8-bit indexed-color) of the pseudo-color images you wish to combine in Adobe Photoshop.
17. To generate a femur montage, select the first image and use the “Crop Tool” to select the femur, ensuring that the entire bone is included. Using the “Image” and “Image rotation” drop-down menu, rotate the image so the femur is oriented vertically. Increase the montage canvas size, using a black background, so that it is large enough to accommodate all the individual femurs from the group.
18. Crop and rotate the femur from each image and then paste into the montage canvas positioning appropriately (Fig. 5D). Name and save the montage as a TIFF file without data compression.
19. Prior to analysis of remaining soft tissue, tibia and standards must be removed from the montage image. Select the “Pencil Tool” and black for the pencil color then right-click and adjust the pencil size to ~33-pixel diameter.
20. Use the “Zoom Tool” to increase the magnification to at least 200% and then use the pencil to black out any nonfemur tissue, being careful not to black out any of the bone itself.
21. Select the “Magic Wand Tool” with a 5% tolerance and click adjacent to the first femur. A line should now appear around the bone and if the edges are not clearly highlighted, brush over to black out any remaining soft tissue. Repeat this process for each femur in the montage. Name and save the montage as a new TIFF file (Fig. 5D).
22. Repeat steps 1 to 6 to give a separate montage of each group of mice.
23. To generate montages of caudal vertebra a slightly different method is required to remove soft tissue from the vertebral images. Crop two equivalent vertebrae from each image and paste them into the group montage. Name and save the montage. Select a black “Pencil Tool” with a diameter of 13 pixels and zoom the image to at least 300%. Carefully draw a continuous line around the outside of each individual vertebra. Select the “Paint Bucket Tool” and fill in the background outside the vertebrae with black. Name and save the montage as a new TIFF file.

Analysis of bone mineral content

24. To determine the relative mineral content of each group, open the cleaned montage file in ImageJ.
25. Under “Plugins,” select “Macro,” Install, and Open “CustomHistogram.” If the CustomHistogram macro is not present in the Macro folder, it can be downloaded from the ImageJ Web site (<http://rsb.info.nih.gov/ij/>).
26. While holding down the Shift key, select all bones in the montage using the “Wand (tracing) tool.”
27. Under “Plugins,” select “Macro” followed by “CustomHistogram.” Select the following values in the dialog box: X Min: 0, X Max: 255, Y Max: auto, Bins: 16 and press OK. Select the “List” button in the displayed histogram and copy the “bin start” and “count” columns and paste into an Excel spreadsheet. This gives the number of pixels in each of 16 equal gray level divisions (bins). The frequency distributions

thus comprise summed grayscale values from each bone included in montages and therefore facilitate comparison of relative gray level distributions between groups (Fig. 5D).

28. Perform statistical analysis of the difference between the cumulative frequency distribution of the gray levels of different montages (Fig. 5C) using the Kolmogorov-Smirnov test in an Excel spreadsheet. This statistical method is described by Demidenko (<http://www.springerlink.com/content/wpc6c4aw8mpup66g/>).

Analysis of bone length and cortical thickness

29. Prior to determining morphometric parameters, it is necessary to remove the pseudo-color scheme from the montage images. Open the cleaned montage file in ImageJ, select “Lookup Tables” from the “Image” drop-down menu, and select “Grays.” Rename and save the grayscale montage file.
30. To quantify morphometric parameters, it is essential to calibrate ImageJ first. Open the DICOM image of the digital caliper acquired in step 5. Select the “Straight” tool and right click to select the “Straight Line” option. Draw a straight line between and perpendicular to the two surfaces of the digital caliper (Fig. 5E). Select “Set Scale” from the “Analyze” drop-down menu, type “15” in the “Known distance” box and “mm” in the “Unit of length” box. Finally, tick the “Global” box and click OK. Select “Set Measurements” from the “Analyze” drop-down menu, and deselect all the boxes except “Display label.”
31. To determine bone length, open the grayscale montage file in ImageJ, uncheck the “Disable Global Calibration” box, and check the “Disable these Messages” box in the calibration dialog box and then click OK.
32. Selecting the first bone in the montage, use the “Straight” tool to draw a line from one end of the bone to the other and then type “m” (Fig. 5F). A results window will appear showing the montage name and bone length. Repeat this step for each bone in the montage.
33. Use the “Open” drop-down menu in ImageJ to open the next grayscale montage file and repeat step 32. Finally, copy the results window into an excel spreadsheet. Calculate the mean bone length and standard deviation for each montage (Fig. 5H).
34. Cortical thickness is normally determined at the mid-femoral-diaphysis using a medio-lateral projection. Open the grayscale femur montage with Photoshop and use the “Rectangle Tool” to place a 100-pixel high white rectangle over the mid-diaphyseal region of the femurs, setting opacity at 15%. Rename and save the cortical thickness montage.
35. Use the “Open” drop-down menu in ImageJ to open the cortical thickness montage file. This ensures that the images remain calibrated.
36. Select the first bone in the montage and use the “Straight” tool to draw the external cortical diameter and then type “m” (Fig. 5G). Use the highlighted rectangle to guide a total of five equally spaced measurements of the external cortical diameter. Similarly use the rectangle to guide 5 equally spaced measurements of the internal cortical diameter. Repeat this step for each bone in the montage.
37. Use the “Open” drop-down menu in ImageJ to open the next cortical thickness montage and repeat step 8. Finally, copy the results window into an Excel spreadsheet.
38. Calculate the mean cortical thickness for each femur in a montage by subtracting each internal cortical thickness measurement from the corresponding external cortical thickness. Calculate the mean cortical thickness for each femur from these five

independent measurements of cortical thickness. For each montage, determine the mean femur cortical thickness and standard deviation (Fig. 5G).

BIOMECHANICAL TESTING

The biomechanical properties of the mouse skeleton change rapidly with skeletal development and are dependent upon multiple variables including the bone matrix, its mineralization, and skeletal geometry. Destructive 3-point bend and compression testing (Fig. 6A-D) can be used to determine the functional characteristics of mouse bone. Key biomechanical parameters of bone strength, rigidity, and toughness can be calculated from load displacement curves and skeletal dimensions (Fig. 6E-L). This protocol describes 3-point bend testing of the femur and compression testing of caudal vertebrae and calculation of key biomechanical parameters (Bassett et al., 2010).

Biomechanical testing of skeletal elements can be performed at any age in mice but is technically more challenging before cessation of linear growth, which occurs at ~8 weeks of age. The femur is chosen frequently for destructive 3-point bend testing, as the geometry of the mid-femoral-diaphysis can be approximated to a uniform elliptical tube. Thus, the moment of inertia/second moment of area (I) for each femur can be obtained directly from micro-CT analysis (Basic Protocol 8) or calculated from cortical dimensions derived from anterior–posterior and medio–lateral Faxitron images (Fig. 6E-H). Similarly, compression testing is performed commonly using proximal caudal vertebrae, as the geometry can be approximated to a cylinder (Fig. 6I-L). The essential caudal vertebra dimensions can be obtained from Faxitron (Basic Protocol 6) or micro-CT (Basic Protocol 8) analysis.

Materials

Cleaned long bones or vertebrae from experimental mice of the same strain, age and sex, fixed and stored in 70% ethanol at 4°C (Basic Protocol 4)

70% ethanol

Cyano-acrylate glue (Loctite Precision)

Instron 5543 load frame using 100 N or 500 N load cells and Bluehills2 software (Instron Limited)

Custom mounts for destructive 3-point bend and compression testing of mouse bones (Quality Test Solutions)

Mitutoyo CD-6 CP Digital calipers (Mitutoyo)

Aluminum foil

Safety glasses

Paper towels

Dissecting instruments including a scalpel with size 22 disposable blades (Swann-Morton)

6-cm polystyrene petri dish

Fine forceps

Femur 3-point bend test

1. Attach the 100 N load cell to the Instron 5543 load frame (Fig. 6A).
2. Attach the 3-point bend test custom mounts to the base plate of the Instron 5543 load frame and to the 100 N load cell.
3. Using the digital calipers, set the separation (span L) of the support pins carefully and ensuring the loading pin is located centrally (Fig. 6B).

The span should be constant for all groups of femurs analyzed and should be approximately half the total femur length determined by Faxitron analysis (Basic Protocol 6, step 33).

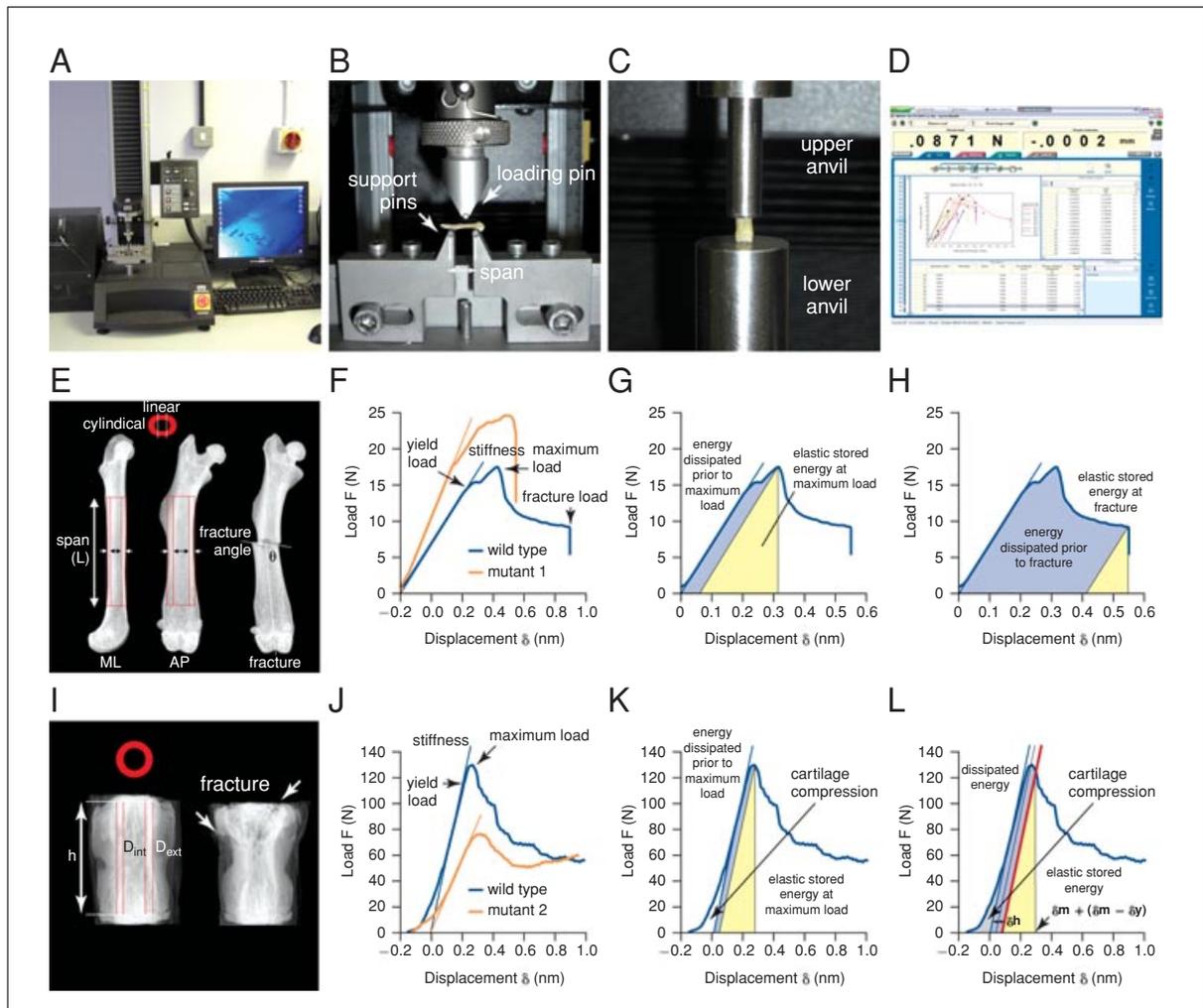


Figure 6 Biomechanical analysis. (A) Instron 5543 load frame. (B) Custom mount for destructive 3-point bend testing of mouse bones incorporating rounded support and loading pins to minimize cutting and shear forces. The location of a femur during testing is shown. (C) Custom mounts for mouse vertebral compression testing showing upper and lower anvils. The location of proximal caudal vertebrae during testing is shown. (D) Instron Bluehills 2 software illustrating load displacement values and curves. (E) Grayscale Faxitron images showing medio-lateral (ML) and anterior-posterior (AP) views of a femur prior to fracture and an AP view post fracture. Small black and white arrows show the ML and AP cortical thickness and the red lines indicate how the femur cross-section, shown above, was determined. The larger white arrows indicate the site of fracture. (F) Femur load displacement curves from wild-type and mutant mice showing a strong but brittle phenotype in mutant 1. (G-H) Femur load displacement curves showing the stored (orange) and dissipated (purple) energy at maximum load and at fracture. (I) Grayscale images showing a Ca5 before and after fracture. White arrow shows the cylindrical height (B), which excludes the vertebral end plates. Small black arrows indicate internal diameter (D_{int}) and small white arrows the external diameter (D_{ext}). The red lines indicate how the cross-section, shown above, was determined. Larger white arrows indicate the site of the crush fracture (J). Caudal vertebrae load displacement curves from wild-type and mutant mice showing a weak and flexible phenotype in mutant 2 and the lack of a clear point of fracture. (K) Caudal vertebra load-displacement curve showing elastic stored energy at maximum load [(ESE_m) in orange] and dissipated energy at maximum load (DE_m) in purple. Initial cartilage compression is shown in dark gray. (L) Determination of “Toughness” by calculating the dissipated energy per unit strain around maximum load. Dissipated energy (DE) is shown in purple and elastic stored energy (ESE) in orange. The compressive extension (δh) is indicated by the double-headed black arrow. For the color version of this figure, go to <http://www.currentprotocols.com/protocol/mo120124>.

4. Turn on the Instron 5543 load frame. Start the Bluehills2 software when the transducer indicator shows “2.”
5. In Bluehills2 select a “flex_load_test” with a constant rate of displacement of 0.03 mm/second and a sample rate of 20 Hz. Raw data logging should include time, extension, and load.

The “End Test” criteria should include a >40% change in load, a >5 mm displacement or a load >95 N.

6. Ensure the vertical position of the loading pin is appropriate for the start of the test and then
 - a. Calibrate the load cell.
 - b. Balance the load.
 - c. Reset the gauge length.
7. Fashion a screen of aluminum foil around the 3-point bending mount with sufficient opening to allow placement of a femur on the supporting pins. This will ensure the bone fragments are retained even following a brittle fracture.
8. Ensure that safety glasses are worn.
9. Select the sample for testing. Remove excess 70% ethanol from the cleaned lower limb (Basic Protocol 4) using a paper towel. Check the sample has not been fixed in neutral buffered formalin. Separate the femur and tibia carefully at the knee joint using a scalpel. Ensure that all soft tissue has been removed and place the femur in a petri dish containing 70% ethanol.
10. Enter the sample information into Bluehills2.
11. Place the femur carefully onto the supporting pins with the anterior surface upward. Ensure that it is stably located.
12. Start the test (samples should be removed from the 70% ethanol for as short a period of time as possible to prevent drying out). If the specimen slips or rotates at initial loading stop the test, place the specimen back into 70% ethanol and repeat step 11.
13. Following fracture, return the loading pin to its start position, save the data and replace the bone fragments in the sample tube.

The fractured sample can be imaged by Faxitron if required (Fig. 6E).

14. Return to step 9 and repeat the 3-point bending test for all the femur samples.
15. On completion of the last sample, end the test and save and export the raw data.

An Excel file of raw data will be generated for each sample.

Femur 3-point bend test data analysis

16. Combine all the raw data files into one Excel file. For each sample, plot a load-displacement (extension) curve. Use the “LINEST” function (least squares method) to determine the trend line for the linear elastic phase of deformation. Correct the displacement so that the trend line passes through the origin (zero displacement at zero load) and re-plot the graph (Fig. 6F).
17. The 3-point bend test load-displacement curve can be subdivided into:
 - a. A phase of linear elastic deformation ending at the yield point.
 - b. A phase of plastic deformation including the maximum load.
 - c. The point of failure or fracture.
18. Use the load-displacement curve and the trend line to determine the load (F) and displacement (δ) values at yield (F_y , δ_y), maximum load (F_m , δ_m), and fracture (F_f , δ_f) (Fig. 6F).

To calculate stiffness (k), use the formula:

$$k = \frac{F_y}{\delta_y} \text{ (the slope of the trend line).}$$

19. If the moment of inertia/second moment of area (I) is not available directly from micro-CT analysis (Basic Protocol 8) it can be calculated from the femur anterior-posterior mid-diaphyseal cortical thickness (T_{ap}) and diameter (D_{ap}), and the medio-lateral cortical thickness (T_{ml}) and diameter (D_{ml}) obtained from Faxitron imaging (Fig. 6E).
20. The moment of inertia (I) is the sum of its cylindrical and upper and lower linear components (Fig. 6E).

$$I_{\text{total}} = I_{\text{tube}} + I_{\text{linear}}$$

$$I_{\text{tube}} = T_{\text{ml}} \times r^3 \times \pi$$

$$r = \left(\frac{D_{\text{ap}}}{2} \right) - \left(\frac{T_{\text{ml}}}{2} \right)$$

where

$$I_{\text{linear}} = 2(D_{\text{ml}} - D_{\text{ap}}) \times T_{\text{ap}} \left[\frac{(D_{\text{ap}} - T_{\text{ap}})^2}{4} \right]$$

21. To calculate Young's modulus (E) use the formula:

$$E = F_y \times \left(\frac{[L]^3}{\delta_y} \right) \times 48 \times I_{\text{total}}$$

where L = the span.

22. To calculate the Yield Stress (σ_y) use the formula:

$$\sigma_y = F_y \times L \times \frac{D_{\text{ap}}}{2} \times I_{\text{total}} \frac{1}{4}$$

23. The total work energy (TWE) is the area under the load-displacement curve.
24. The elastic stored energy (ESE) is the area of a right-angled triangle with the vertex on the load displacement curve and a hypotenuse with a slope equal to that the linear elastic phase of deformation (Fig. 6G and H)
25. The dissipated energy or plastic work (DE) is calculated by subtracting the elastic stored energy from the total work energy (Fig. 6G and H).

At yield: $DE_y = 0$ (all work is elastic before yield).

At maximum load: $DE_m = TWE_m - ESE_m$.

At fracture: $DE_f = TWE_f - ESE_f$.

26. To calculate the fraction of energy dissipated at maximum load and at fracture (plastic work as a fraction of total work) (Fig. 6G-H) use the formula:

$$\text{At maximum load} = \frac{DE_m}{DE_m + ESE_m}$$

$$\text{At fracture} = \frac{DE_f}{DE_f + ESE_f}$$

If the fracture surface areas are similar between samples then the fraction of energy dissipated at fracture is proportional to the material's "Fracture toughness."

27. "Toughness" is the ability of a material to dissipate energy and deform plastically without fracturing, thus requiring a balance of strength and ductility. In bone, plastic deformation, the period after yield and before fracture, is characterized by nonpropagating microfractures that absorb energy but do not result in failure until considerable internal microdamage has occurred. "Fracture toughness" can be calculated by dividing the energy dissipated at fracture by the area of the fracture surface. An estimate of the fracture surface can be made by correcting the femur cortical cross sectional area for the angle of the fracture θ (Fig. 6E).

Caudal vertebra compression test

28. Attach the 500 N load cell to the Instron 5543 load frame.
29. Attach the custom mounts for compression testing to the Instron 5543 load frame and to the 100 N load cell.
30. Turn on the Instron 5543 load frame. Start the Bluehills2 software once the transducer indicator shows "2."
31. In Bluehills2 select a "compression_test" with a constant rate of displacement of 0.03 mm/second and a sample rate of 20 Hz. Raw data logging should include time, compressive extension and compressive load. The "End Test" criteria should include a >40% change in load, a >2 mm displacement or a load >400 N.
32. Ensure the vertical position of the upper anvil is appropriate for the start of the test and then:
- Calibrate the load cell.
 - Balance the load.
 - Reset the gauge length.
33. Ensure that safety glasses are worn.
34. Select ethanol-fixed samples for testing (Basic Protocol 4). Remove excess 70% ethanol from the sample using a paper towel. Straighten the proximal tail vertebrae carefully and place on a flat surface. Identify the location of the intervertebral discs between Ca4, Ca5, and Ca6. Using a scalpel with a sharp size 22 blade cut between Ca4 and Ca5, ensuring the scalpel blade is vertical and perpendicular to the long axis of the tail. Similarly, cut between Ca5 and Ca6 and then Ca6 and Ca7. Ensure that all cuts are perpendicular through the intervertebral discs and parallel to each other. Place Ca5 and Ca6 in a petri dish containing 70% ethanol and return the remainder of the tail to the specimen container.
35. Enter the sample information in to Bluehills2.
36. Put a small drop of cyano-acrylate glue at the center of the lower anvil (Fig. 6C). With fine forceps pick up Ca5, remove excess ethanol, and carefully place it on the

lower anvil ensuring it is aligned vertically (Fig. 6C). Allow 20 sec for the glue to bond.

37. Start the test. Samples should be removed from the 70% ethanol for as short a period of time as possible to prevent drying out.
38. At the end of the test, usually following 1 mm of compressive extension, return the upper anvil (Fig. 6C) to its start position, save the data and place the vertebrae in the sample tube.

The fractured sample can be imaged by Faxitron if required.

39. Repeat the compression test with Ca6 to test reproducibility.
40. Return to step 34 and repeat the compression test for all the tail samples.
41. On completion of the last sample, end the test and save and export the raw data. An Excel file of raw data will be generated for each sample.

Caudal vertebra compression test data analysis

42. Combine all the raw data files into one Excel file. For each sample, plot a load-displacement (extension) curve. Use the “LINEST” function (least squares method) to determine the trend line for the linear elastic phase of deformation. Correct the displacement so that the trend line passes through the origin (zero displacement at zero load) and re-plot the graph (Fig. 6J).
43. Use the load-displacement curve and the trend line to determine the load (F) and displacement (δ) values at yield (F_y, δ_y) and maximum load (F_m, δ_m) (Fig. 6J).
44. To calculate stiffness (k) use the formula:

$$k = \frac{F_y}{\delta_y} \text{ (the slope of the trend line).}$$

45. If caudal vertebra geometry is approximated to a cylinder, it is necessary to determine vertebra height (excluding the endplates) (h), and the internal and external diameter D_{int} and D_{ext} (Fig. 6I). These parameters may be obtained from micro-CT analysis (Basic Protocol 8) or by Faxitron imaging.

$$\text{Wall thickness: } t = \frac{D_{\text{ext}} - D_{\text{int}}}{2}$$

$$\text{Wall cross-sectional area: } A = \pi \times t \times (D_{\text{int}} + t)$$

Yield “stress” and “strain” can then be calculated

$$\text{Yield stress: } \sigma_y = \frac{F_y}{A}$$

$$\text{Yield strain: } \epsilon_y = \frac{\delta_y}{h}$$

Young’s modulus (E) is defined as stress per unit strain thus:

$$E = \frac{F_y \times h}{A \times \delta_y} \text{ (stress/strain).}$$

46. The total work energy (*TWE*) is the area under the load-displacement curve (*AUC*). In vertebral compression studies it is important that the area representing cartilage compression (*ACC*) is subtracted from the *AUC* (Fig. 6K and L). Thus:

$$TWE = AUC - ACC$$

47. The elastic stored energy (*ESE*) is the area of a right-angled triangle with the vertex on the load-displacement curve and a hypotenuse with a slope equal to that of the linear elastic phase of deformation (Fig. 6K and L)
48. The dissipated energy (*DE*) is calculated by subtracting the elastic stored energy from the total work energy (Fig. 6K).

At yield: $DE_y = 0$ (all work is elastic before yield).

At maximum load: $DE_m = TWE_m - ESE_m$.

49. To calculate the fraction of energy dissipated at F_m (Fig. 6K) use the formula:

$$\text{Fraction of energy dissipated} = \frac{DE_m}{DE_m + ESE_m}$$

50. "Toughness" is defined as "plastic work or dissipated energy per unit strain." Toughness can be calculated by determining the dissipated energy (*DE*) during a similar magnitude of compression before and after maximum load. Figure 6L illustrates this for the period between the yield point and maximum load and for a similar period after maximum load.

$$DE \text{ (at extension } \delta_m + (\delta_m - \delta_y)) = TWE - ESE$$

$$\delta_h \text{ (compressive extension)} = 2 \times (\delta_m - \delta_y)$$

$$\text{Strain} = \frac{\delta_h}{h}$$

$$\text{Toughness} = \frac{TWE - ESE}{\frac{\delta_h}{h}}$$

**BASIC
PROTOCOL 8**

QUANTITATIVE MICRO-COMPUTED TOMOGRAPHY (MICRO-CT)

Quantitative micro-computed tomography is a sensitive technique used to measure trabecular and cortical bone mineral density, bone volume, and structure. The structural parameters that can be measured include trabecular bone volume, thickness, number and spacing and indices of connectivity, cortical bone volume, and thickness. The protocol described is based upon the analysis of bones excised from mice and uses the SkyScan 1172, although the protocol could be adapted for other micro-CT scanners. The micro-CT technique can also be performed on live mice to facilitate the longitudinal study of changes in bone structure. The protocol describes the 3 steps of data acquisition, image reconstruction, and data analysis.

Micro-CT imaging can be performed on mice once mineral is present. Typically, studies are performed on mice of 12 to 16 weeks of age and undertaken on tibia, femora or vertebra.

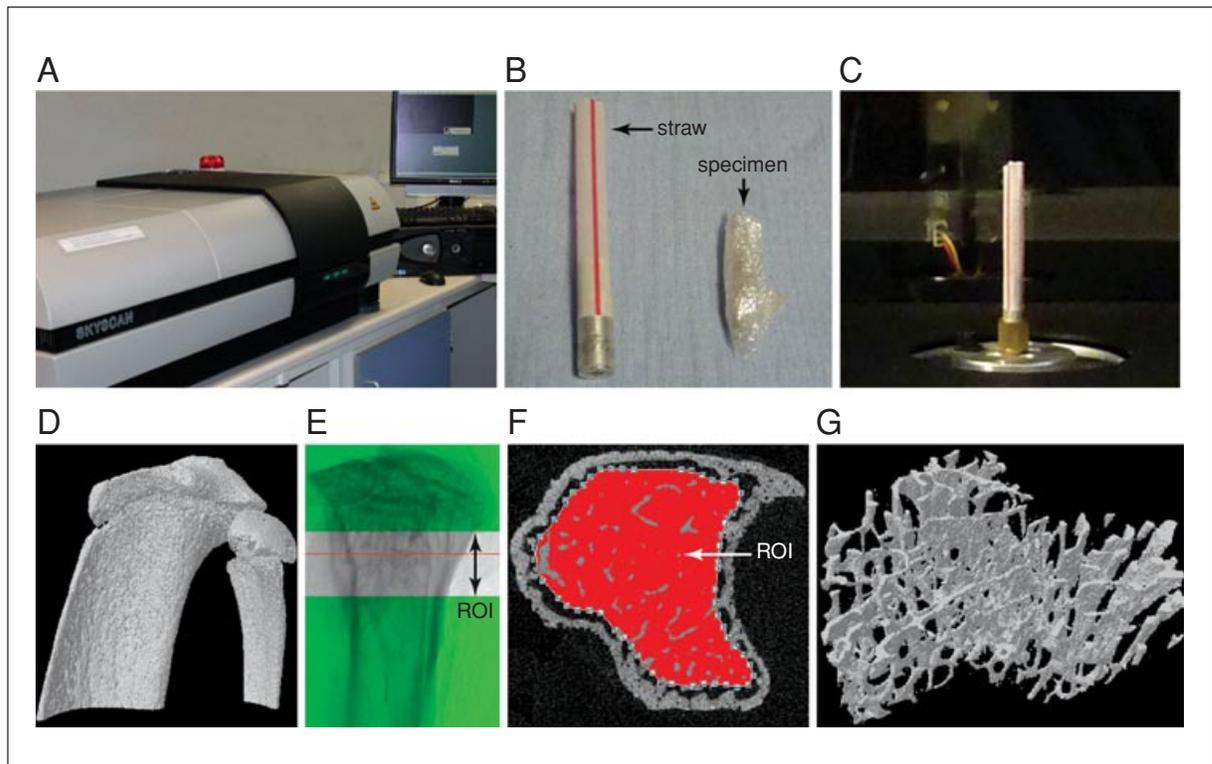


Figure 7 Micro-computed tomography. **(A)** SkyScan 1172 micro-CT scanner. **(B)** Plastic straw used to hold specimens in the sample holder and mouse tibia (specimen) wrapped in cling-film to prevent drying. **(C)** Tibia mounted within the straw inside the sample chamber. **(D)** 3-dimensional reconstruction of the tibia. **(E)** X-ray projection of the tibia through the longitudinal axis. Gray bar shows the region of interest (ROI) with arrow indicating extremes. The red line is the position of the transverse slice shown in F. **(F)** Single transverse slice showing the ROI in red. **(G)** 3-dimensional reconstruction of the trabecular bone ROI upon which assessment of structural characteristics is undertaken. For the color version of this figure, go to <http://www.currentprotocols.com/protocol/mo120124>.

Materials

Bone samples
 SkyScan 1172 high-resolution micro-CT system (e2v technologies)
 Computer for image reconstruction
 Small BMD phantom set (SP-4002; e2v technologies)
 Cling-film
 Plastic straws or pipet tips or polystyrene tubes

Acquire the data

The first step is to acquire the data required in order to reconstruct the chosen bone. This often referred to as “scanning.” The micro-CT is an X-ray-based technique with a shielded X-ray source housed within the scanner. Safety features are incorporated into the device to prevent exposure. These are tested when the equipment is installed and are checked as part of a routine maintenance.

1. Switch on the computers. Insert the key, switch on the SkyScan 1172, and start the SkyScan program (Fig. 7).
2. Switch on the X-ray source. Allow 15 min to ensure stability of the X-ray source.
3. Complete flat-field and alignment checks immediately after the installation of the scanner, and every 4 and 8 weeks afterwards, respectively.

This ensures the scanner is correctly calibrated with respect to background readings and X-ray detection.

4. Scan both phantoms at the resolution to be used for subsequent bone scans immediately after installation, and every 4 weeks afterwards.

Once each phantom has been reconstructed, an appropriate ROI can be set and the corresponding area analyzed to give a grayscale value. This value can be used to monitor any fluctuations that occur. Additionally, the known densities of the standards can be plotted against their corresponding grayscale values to produce a linear graph. The equation of this line can be used to acquire a real bone mineral density (gm/cm^3), as opposed to a relative bone density, for any subsequent bones.

5. Prepare the bone sample by removing bone from fixation or storage medium and wrapping it in cling-film to prevent drying. Mount the samples in an appropriate holder (for tibia and femora this can be a plastic straw, for vertebra this can be a pipet tip, and for skulls this can be a polystyrene tube). Ensure bones fit tightly using additional cling-film, if necessary (Fig. 7).
6. Use the “Open door” command on the task bar to open the sample chamber. Mount the sample in the specimen holder and close the sample chamber door using the icon.
7. Activate the TV icon to visualize the specimen in the sample chamber.
8. Select the camera resolution—low (1024×500), medium (2000×1024) or high (4000×2000)—using the icon on the toolbar at the bottom of the screen. For small bone samples, such as mouse tibia, medium resolution is typically used.
9. Select the relevant filter—no filter, aluminum filter or copper filter—so that maximum absorption of the sample is between 60% to 80%. For small bone samples, the aluminum filter is used. If correct absorption cannot be achieved through the filter alone, adjust the voltage of the scanner.
10. Adjust the height of the sample in the chamber to ensure it is with the ROI.
11. Adjust the pixel size— $4.3 \mu\text{m}$ is appropriate for small bone samples.
12. Check the rotation of the sample. If the sample “wobbles” when rotating, open the chamber and adjust the sample.
13. Activate the circular arrow on the main task bar to display chosen settings.

Type in filename.

Use browse facility to give the folder a new name: one folder is used for each bone.

Set rotation step: 0.7° is the default.

Set averaging to 2 if doing an 180° scan.

Set rotation to 180° , which is appropriate for long bones. Use 360° for skulls or asymmetrical bones.

Click OK to accept settings.

14. If using the camera setting on medium, with 0.7° rotation, 2 times averaging through 180° the scan will take approximately 11 min. Confirm timings and, if necessary, check settings.
15. Start the scan.
16. Once the scan is completed, remove the sample from the sample chamber and return to fixation/storage medium. Insert the next sample for scanning.
17. Close the SkyScan program using the “EXIT” function on the actions menu.

Image reconstruction

The second step is to take the data acquired through the scanning step and reconstruct it to obtain a 3-dimensional image of the bone.

18. Switch on the SkyScan computer and the second reconstruction PC.
19. Launch the NRecon program by opening the program from either the desktop or the taskbar.
20. Click on the “Open” icon and locate the scan to be reconstructed. Open the file (TIFF file).
21. Click the “Preview” button on the “Start” tab. After ~30 sec the “Start” tab will change to the “Output” tab and a cross-section of the bone will appear.
22. Ensure the image is sharp and clean; lack of sharpness may indicate movement during scanning and the bone should be re-scanned.
23. Adjust the grayscale range for maximum contrast between the sample and background by altering the number range on the tab.

For small bones, including tibia, femora and vertebrae the value is typically 0-0.16.

24. Select the destination for the reconstruction data by clicking the “Browse” tab and creating a new folder.

Each image reconstruction is stored in a unique folder.

25. Activate the “Start” tab and click the “Add to Batch” tab; the sample should be added to the batch list. Once all samples are added, click the “Start Batch” button.

The files within the batch list will each be reconstructed and stored in the destination folder. Reconstruction time will vary depending upon the number and the processor power of the PCs.

Micro-CT analysis

Having scanned and reconstructed a bone, the third step is the data analysis. The nature of this is dependent upon the question being addressed. The example described below is based upon the analysis of trabecular bone in a mouse tibia or femur. However, the protocol can be adapted for cortical bone and other bone structures.

26. Switch on the computers and start the CT_{AN} analysis program.
27. Locate the folder containing the files of interest and load the reconstructed dataset.
28. *Defining the ROI:* For measuring trabecular bone density, bone volume and structural parameters, an area of trabecular bone must be selected. To standardize the ROI a standard reference point is identified, for example the most distal point of the growth plate.
29. Double-click the dark blue vertical bar on the right of the data table to open the selection window. Click the “Analytic” tab. The identified reference line is shown. Define the distance from the reference line from which measurements will be made. A typical ROI for a tibia is 1-mm high, set 0.2 mm from the most distal point of the growth plate. However, these values will depend upon experiment specific considerations, including the strain of mice, their age and sex, and the bone to be analyzed.
30. Click the ROI icon on the tool bar and the image will change to red. Select the lowest file level in the ROI, which is shown by yellow markers against the file number.

31. Draw the ROI for the trabecular bone and re-draw at different levels through the length of the bone. After each re-draw allow the software to interpolate the ROI. Save the ROI as a new dataset in a new folder (Fig. 7).
32. Go to the “Open folder” icon on the tool bar and open the ROI dataset (denoted xxxxx.voi.roi).
33. Select the “Binary image” icon and then click the “From selection” tab, which will average across all slices rather than a single slice.
34. Set the grayscale threshold value to define bone. The upper value is 255 but the lower value can be adjusted until a clear image is obtained i.e., such that individual pixels are not defined (value too low) and bone is not artificially thick (value too high). Lower values are typically 65-90.
35. Analyze data sets individually or as part of a “batch.” See the “Batch Manager” at the end of this protocol for details.
36. Select the “Custom processing” icon in the tool bar, which displays a list of available tasks and select the tasks to run (see “Batch Manager” for details).
37. Select the “Play” button to start the analysis.

**SUPPORT
PROTOCOL**

USE OF THE BATCH MANAGER

The Batch Manager (BatMan) protocol allows large numbers of reconstructed images to be analyzed in the absence of the operator. For materials, see Basic Protocol 8.

1. Start Batch Manager and the window displayed will show three tabs. The “task list” tab shows a list of tasks to be performed. The “Internal task” shows all the available tasks. To add a task, select it and click the Add button.
2. For the analysis of trabecular bone three tasks are required, Thresholding, Despeckle, and 3D Analysis, which should be undertaken in that order. Each requires selecting and individual settings checking.

Thresholding should have the “default” option checked—this will then use the value inputted at the “Binary image” stage.

Despeckle should be set to “remove white speckles” within “3D space” with an area “less than” “10” voxels in the “region of interest.”

3D analysis should have all parameters checked and a destination for the results text file chosen.

3. Once the task list has been created, add all data sets to be analyzed and select the “Start batch” button to begin the batch analysis.

The data is output in the form of a text file. This should then be converted into an appropriate spreadsheet format by opening the text file and resaving as a .csv file format. This file can then be modified and saved in the latest Excel format prior to data analysis.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Acetate buffer, pH 5.2

Dissolve 5.44 g sodium acetate trihydrate in 200 ml distilled water
Adjust pH to 5.2 with 50 to 60 ml of 1.2% acetic acid
Store up to 2 weeks at 4°C

Acetate-tartrate buffer

Dissolve 4.6 g sodium tartrate (dehydrate) in 200 ml acetate buffer (see recipe) and warm to 37°C for 2 to 4 hr
Prepare fresh

Naphthol AS-BI phosphate solution

Dissolve 20 mg naphthol AS-BI phosphate into distilled water in a glass bottle
Add 1 ml dimethylformamide in a fume hood
Prepare fresh

Pararosaniline

Add 1 g pararosaniline to 20 ml distilled water in a conical flask
Carefully add 5 ml hydrochloric acid, mix gently and cover loosely with foil
Heat on a hotplate (set to 160°C) for 30 to 45 min, but do not allow to boil.
Bring to a simmer for 5 min, cool to room temperature, filter and store in bottle protected from light
Store up to 2 months at room temperature protected from light

Sodium nitrite, 4%

Dissolve 80 mg sodium nitrite in 2 ml distilled water
Prepare fresh

Weigerts Hematoxylin

Solution A:
1 g hematoxylin in 100 ml absolute alcohol

Solution B:
4 ml of 30% aqueous ferric chloride, 1 ml hydrochloric acid, and 95 ml distilled water

Make Weigerts Hematoxylin by combining equal volumes of solutions A and B
Prepare fresh

COMMENTARY

Background Information

The phenotype-driven approaches described here provide rapid, inexpensive, and non-invasive methods for investigating the presence of musculoskeletal abnormalities in mice. These strategies can be applied to longitudinal studies aimed at establishing disease onset and progression. Moreover, the use of multiple screening techniques on the same group of mice maximizes the amount of phenotypic data that can be obtained from a limited group of mice, and mice with phenotypic abnormalities can be utilized for in depth phenotypic characterization using techniques such as micro computed tomography, peripheral quantitative computed tomography, and fracture and histomorphometric studies, which require the use of expensive equipment, often time consuming, and require dissected bone specimens.

Critical Parameters and Troubleshooting

Biochemical analysis

Collection of blood samples from mouse tail vein requires great skill that can be acquired through training. This is critical not only for the welfare of the mice, but also for the quality of the blood samples, as it is important to minimize the occurrence of hemolysis, which may introduce artifacts resulting in data inconsistency. Moreover, individuals should be experienced in animal handling to reduce stress and anxiety to the mice, be skilled in experimental procedures, and follow standard operating procedures. Mice should be fasted for the same duration (usually 4 to 6 hr) and blood samples collected at approximately the same time of day to minimize the fluctuations in biochemical and physiological parameters

that normally change throughout the day. Mice can also be fasted overnight before blood sampling. However, fasting duration should be selected based on the intended study and an understanding of the mouse strain under investigation.

Radiography

Application of a lubricant on the eyes is important in improving the welfare of the mice, as it prevents drying of the cornea during the scanning and recovery period. Similarly, placing the mice in a heated box at 37°C during the recovery period significantly reduces the risk of hypothermia that may ensue due to a drop in metabolic rate while under general anesthesia. Mice should be monitored during recovery from anesthesia and if there is evidence of poor recovery, appropriate action should be taken according to standard operating procedures and project license guidelines. If an acquired image is of poor quality (“ghost”-like), it is likely due to the fact that the mouse was not fully sedated and had moved during the scanning process. Unless permitted by the project license, the anesthetic dose should not be topped-up in order for the scan to be repeated straightaway. Such a mouse can be scanned at a later date as determined by the project license restrictions. Individuals performing radiography scans should be familiar with the anatomy of a mouse, and be able to position the mouse appropriately before scanning, to ensure that the quality of the acquire images permit a detailed assessment of all the bones, especially those of the limbs and digits, and also to avoid positional artifacts.

DEXA

All operators must understand the potential hazards and be familiar with the safety and emergency procedures relating to the use of X-ray systems.

The PIXImus DEXA scanner is supplied with an open imaging area (Fig. 2B), and is stipulated to have an external radiation field in a rectangle at the entrance of the detector that is 1 m × 0.8 m (3.9 × 3.1 inches). Thus, the manufacturer recommends that operators should stand at a minimum distance of 1 m from the source during use. However, appropriate local radiation, health and safety agencies should be consulted to ensure compliance with their regulations on radiation safety. One way of improving the safety of the PIXImus is to introduce lead shielding with an interlocking safety door (Fig. 2C). This, however, introduces potential problems with over-heating,

which can lead to inactivation of the X-ray source during scanning. However, heat dissipation can be improved by installing a fan as an integral part of the shielding.

DEXA scanning cannot be performed unless the scanner passes a QC test using the DEXA phantom mouse, which has preset parameters for BMD and %fat. Since the %fat can drift slowly over time, it is not unusual to have multiple attempts (>6 times) before the QC is successful. Persistent QC failure can be rectified by performing a Field calibration to re-establish the DEXA phantom mouse settings—this process takes 1 hr, and should be factored into the daily planned usage of the scanner. For instance, it is not recommended to start anesthetizing mice for DEXA scanning unless the PIXImus passes the QC test.

Skeletal sample preparation and fixation

Appropriate care should be taken when using dissection instruments, neutral buffered formalin and neutral buffered formalin-fixed samples. When dissecting samples from juvenile animals particular care should be taken not to break the digits when removing the skin from the upper and lower limbs or to damage the hip joint when separating the femur from the pelvis. It is also important to ensure that all sample tubes clearly indicate the method of fixation (ethanol or formalin then ethanol) and are labeled in pencil. When removing the muscle and soft tissue from juvenile bones particular care should be taken to avoid displacing the distal femur and proximal tibia growth plates.

Histology and histomorphometry

Care needs to be taken with the dehydration and embedding steps in order to ensure embedding medium is fully infiltrated into the tissue. Failure to do this will lead to difficulties in cutting sections, damage to the marrow space, and cells within the marrow “pulling away” from the bone surface. This will make subsequent histomorphometric analysis of cells lining bone surfaces difficult. Care also needs to be taken when using the microtome and preparing histological sections. The quality of sections plays a critical role in the subsequent histological and histomorphometric analyses. For H&E staining, samples are “blued” to differentiate the color within the sections. This can be achieved in Scott’s tap water or in running tap water. The choice will depend upon the pH of the tap water. If the tap water is alkaline, this will be adequate to “blue” the sections; if it is more acidic, Scott’s tap water will be required. Sections will require

“clearing” in xylene and then mounting. This can be done either with xylene followed by mounting in DPX or using Clearium mounting medium, which eliminates the need for xylene.

For histomorphometric analysis, it is critical that new users receive appropriate training from a skilled bone histomorphometrist. It is anticipated that new users will be trained on a “standard” set of slides and both intra and inter-observer variability determined. Prior to commencing analysis, consideration needs to be given to determining the regions of interest to be analyzed. Typically, this will be influenced by the questions being addressed and will differ between mouse strain and maybe dependent upon age and gender.

Quantitative Faxitron digital X-ray microradiography

When calibrating the Faxitron prior to imaging it is essential to remove the polycarbonate stage to which the standards are attached. If this is not done, negative images of the standards will appear in all subsequent images. When acquiring the Faxitron image it is essential that the Auto Level, Auto Exposure Control, Contrast Assist, and Sharpen Assist toggles are all deselected. If these functions remain selected, unwanted stretching of the original DICOM image will occur. When comparing skeletal samples by quantitative Faxitron analysis it is important that skeletal elements are images in a similar orientation. This is best achieved by retaining the integrity of the knee and elbow joints and imaging the upper and lower limbs at ~90% flexion (Fig. 5A). When preparing montages of skeletal elements from one experimental group it is important to maintain the stretched pseudo-colored images as “Indexed color” files rather than RGB or CMYK. This is best achieved as described in Basic Protocol 6, steps 15 to 23. If the final montage is not Indexed Color the “custom histogram” analysis in ImageJ will give incorrect results.

Biomechanical testing

Appropriate care should be taken using dissection instruments, and safety glasses should be worn at all times during biomechanical testing. Biomechanical testing should not be performed on neutral buffered formalin-fixed bones and it is important to ensure that samples do not become dehydrated during preparation or testing. The upper and lower pins of the 3-point bend test supports should have a convex profile to minimize cutting of the specimen.

Prior to commencing a 3-point bend test it is important to determine a suitable separation between the lower support pins. “The span” and approximately half the total length of the shortest femur is appropriate. To obtain reproducible and reliable results from 3-point bend testing it is important that the femur be placed in a consistent and stable orientation on the lower support pins. This is most frequently obtained with the anterior surface of the femur uppermost, but it is advisable to gain some experience with test specimens prior to analyzing critical samples. Similarly, ensuring that cuts between caudal vertebrae are perpendicular, through the intervertebral discs and parallel to each other is essential to obtain consistent results.

Quantitative micro-computed tomography

The maximum sample size possible is ~50 mm in diameter, and samples can be scanned either fixed or unfixed. Samples should be dissected to remove all soft tissue beforehand; otherwise, the soft tissue elements may influence the density of the samples and the resulting values will not reflect data generated from the standards. Samples should always be wrapped in cling-film within the sample holder, as this will both prevent the sample from moving during the scan and stop the sample from drying out, which will be detrimental to any subsequent techniques such as histology. Cling-film that does not contain significant amounts of chlorine is preferred, as this may show up on the scan. If the reconstructed sample image looks smeared or blurred, or has a double-image, then the sample has moved during scanning and should be re-scanned. For any study it is preferred that all samples are scanned on the same machine and that a single observer be responsible for drawing the regions of interest. These considerations reduce potential sources of variability.

Anticipated Results

Biochemical analysis

Biochemical phenotypic abnormalities can be identified in progeny from *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized mice (Acevedo-Arozena et al., 2008) in which there is no prior knowledge of causative genes, or in progeny from transgenic/knock-out mice whereby the genotypes are clearly established. In the former, baseline data has to be assembled from a cohort of normal mice, and results of individual ENU-mutagenized mice compared with the mean value of the baseline

mice. ENU progeny identified with values of any given biochemical parameter that is ≤ -3 or $\geq +3$ standard deviations from the mean baseline value should be considered as an outlier. Such mice should be bled again one month later and their plasma retested to confirm the biochemical abnormality. In the latter, biochemical data can be compared between wild-type, heterozygous, and homozygous littermates, to establish mean and standard deviation values in each group. Significance differences can be revealed by performing statistical analysis for p values < 0.05 .

Radiography

Whole-body radiography is a rapid and inexpensive technique for visualizing a wide range of skeletal phenotypes that may be due to skeletal dysplasia or defects in mineralization and ectopic calcification, and these can be accomplished in small and large cohorts of mice. In studies of ENU-mutagenized progeny (Acevedo-Aroza et al., 2008) in which there is no prior knowledge of a causative gene, inheritance of such phenotypic abnormalities can be confirmed and map locations identified using 12 or more progeny for dominantly inherited phenotypes, and 30 or more for recessively inherited phenotypes. A similar number of mice would be adequate to confirm a phenotype-genotype association in cohorts of transgenic and knock-out mice.

DEXA

Measurement of whole-body areal BMD by DEXA analysis is highly reproducible within a given mouse inbred strain but shows inter-strain differences (Beamer et al., 1996). Thus, for identification of mice with alterations in BMD such as in progeny from ENU mutagenized mice (Acevedo-Aroza et al., 2008), experimental data from each mouse should be compared against baseline data obtained from mice of a similar background strain and age. Values that are ≤ -3 or $\geq +3$ standard deviations from the baseline means should be considered as outliers and therefore followed up for further investigation. In studies whereby genotypic information is available from mice of a similar background strain, differences of 2 standard deviations from the mean of wild-type littermates (or p values < 0.05) should be sufficient to confirm mutant phenotypes. As described in Basic Protocol 3, BMD data can be obtained from specific skeletal sites; however, the results may not be reproducible due to difficulties in consistently identifying the same

region of interest in each mouse. More accurate site-specific BMD data can be obtained by dissecting out the bones of interest, storing them at -70°C , or fixing in 10% formalin and storing in 70% ethanol until DEXA analysis (Franco et al., 2005; Barbaric et al., 2008).

Skeletal sample preparation and Faxitron

Meticulous sample preparation and fixation will facilitate both rapid skeletal phenotype screening and accurate quantitation of bone mineral content. This is a critical stage of the analysis procedure and also enables samples to be used in further detailed studies where appropriate.

Histology and histomorphometry

The use of histology analysis facilitates the visualization of abnormalities in cartilage, bone and chondrocytes, and may reveal any disorganization of the growth plates, enabling the relative sizes of the growth plate zones to be measured and used as a determinant of linear bone growth and development. Measurement of the number of osteoclasts and osteoblasts and/or the proportion of bone surface covered by these cells can be used to determine the cellular mechanisms responsible for any structural change in the skeleton. Measurement of the separation between two calcein labels will give a measure of the mineral apposition rate (MAR). In conjunction with a measure of the bone surface covered by single calcein labels or double calcein labels the bone formation rate can be calculated. This is a reflection of the rate at which new bone is deposited within the region being analyzed.

Given the tight coupling between resorption and formation consideration needs to be given to sample point(s). A single "snap shot" may mask changes in one cell type at the expense of another.

Quantitative Faxitron digital X-ray microradiography

Quantitative Faxitron analysis can rapidly and reproducibly deliver both morphological and bone mineral content parameters. A study of skeletal samples from more than 70 age- and sex-matched wild-type mice from an identical genetic background demonstrated coefficients of variation of 1.7% for bone mineral content, 2.0% for bone length and 5.1% for cortical thickness. To detect a 10% to 15% difference in phenotype we recommend analyzing between five and six age- and sex-matched mice and a similar number of control animals.

Biomechanical testing

Destructive 3-point bend and compression testing can provide important biomechanical parameters of bone strength and toughness. A study of skeletal samples from than 70 age- and sex-matched wild-type mice from an identical genetic background demonstrated coefficients of variation of 9.8% for yield load, 8.5% for maximum load, 26.6% for fracture load, 13.6% for stiffness and 11.0% for toughness. To detect a 10% to 15% difference in phenotype we recommend analyzing between ten and twelve age- and sex-matched mice and a similar number of control animals.

Quantitative micro-computed tomography

Micro-CT analysis can provide a detailed analysis of bone structure in three dimensions. Analysis of the trabecular bone compartment will provide information on bone volume and structural parameters including trabecular number, separation and thickness. Indices of trabecular connectivity can also be determined. In addition, micro-CT analysis can also be used to provide information on cortical bone, including cortical bone volume and cortical thickness. Variability in microCT data is greater than that seen for Quantitative Faxitron Digital X-ray Microradiography. This is likely to reflect the relative small volume of bone analyzed by micro-CT compared to the whole bone analyzed by Faxitron x-ray micro-radiography. When comparing phenotypes or determining the effects of interventions, we recommend using ten age- and sex-matched mice and a similar number of control animals.

Time Considerations

Fasting of mice for biochemical analysis (Basic Protocol 1) can take 4 to 15 hr depending on the study to be carried out. The minimum duration required for local anesthetic to take effect is 15 min, and blood sampling takes ~3 min per mouse. Centrifugation of blood samples takes 10 min and collection of plasma samples, which will depend on the number of mice being investigated, should take no more than 5 min. Overall, it should be possible to collect and process samples from 20 mice per hour.

For radiography (Basic Protocol 2), instrument warm up time is 20 min and calibration takes about 2 min. Induction of anesthesia takes ~2 min, X-ray and data acquisition takes ~2 min, and the duration for mice to recover is ~15 to 30 min. Overall, 20 mice can be analyzed per hour.

For DEXA analysis (Basic Protocol 3), instrument warm up time is 30 min and calibration takes ~5 min. Induction and recovery from anesthesia takes the same duration as in Basic Protocol 2 and scan time per mouse is ~3 to 4 min. Overall, ten mice can be analyzed per hour. If desired, radiography (Basic Protocol 2) and DEXA analysis (Basic Protocol 3) can be performed sequentially using a single anesthetic dose and recovery.

Skeletal sample preparation (Basic Protocol 4) takes ~5 min per animal.

For histology and histomorphometry (Basic Protocol 5), time considerations have been described in the section. In summary, after preparation of bone sections, hematoxylin and eosin staining takes ~20 min, alcian blue and van Gieson staining ~1 hr 45 min, TRAP staining ~1 hr 45 min, static histomorphometry analysis ~10 min per slide, and dynamic histomorphometry analysis ~30 min.

X-ray microradiography imaging (Basic Protocol 6) takes ~1 min per mouse whereas analysis takes ~20 min per sample.

Biomechanical testing (Basic Protocol 7) takes ~5 min per sample whereas analysis takes ~20 min per sample.

MircoCT scanning (Basic Protocol 8) takes ~15 min per bone, including sample preparation time. Analysis takes ~10 min per bone. TRAP staining takes approximately half a day, taking into account time required to warm up the buffer solution and to counterstain and coverslip slides at the end.

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