

Chapter 29

Quantitative X-ray Imaging of Rodent Bone by Faxitron

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Abstract

This chapter describes the use of digital micro-radiography with the Faxitron machine as a means of imaging and quantitating bone mineral content in mice and rats.

Key words: Bone mineral content, Digital X-ray analysis, Faxitron, ImageJ, Mouse, Skeletal phenotype analysis

1. Introduction

Over the past 10 years there has been an explosion of interest in skeletal phenotyping of different mouse strains, driven in part by initiatives such as ENU Mutagenesis programmes and the systematic generation of genetically modified mice with targeted inactivation of all genes in the genome. Each of these strains needs to be screened for phenotype abnormalities across a range of physiological systems, including the musculoskeletal system. Detailed characterization of skeletal phenotypes in a high-throughput manner using techniques such as MicroCT and quantitative CT represents a specific challenge. Developmental abnormalities may occur early in utero and during neonatal life (skeletal dysplasias) or in the post-natal period (linear growth and endocrine disorders), and such abnormalities impact on bone mass and mineralization in adulthood. By contrast, phenotypes such as osteoporosis do not manifest until adulthood and may display sexual dimorphism. Thus, analyses need to be performed separately in both sexes and at several ages if abnormalities are to be captured efficiently. To address this issue, we have developed and refined a cost-effective, rapid, and highly sensitive method for the quantitative assessment of bone mineral content from fixed post-mortem specimen using the

Faxitron point projection digital micro-radiography machine. In addition to quantitating bone mineral content, the Faxitron can also be used to obtain morphological information and direct measurements of anthropomorphic parameters from digital X-ray images.

2. Materials

2.1. General Reagents/ Materials

1. Dissecting instruments.
2. 20-ml Polystyrene tubes.
3. 70% Ethanol solution.

2.2. Equipment and Computer Software

1. Faxitron MX20 variable kV point projection X-ray source and digital image system (Qados, Cross Technologies plc, Berkshire, UK) (see Note 1).
2. Calibration standards – 1-mm steel wire; 1-mm diameter spectrographically pure aluminium wire [Hollinbrow Precision Products (UK) Ltd., Shropshire, UK]; 1-mm diameter polyester fibre.
3. ImageJ 1.43n software (available free of charge at <http://rsb.info.nih.gov/ij/>).
4. Adobe Photoshop 7.0.1 or later software (Adobe Systems Inc., San Jose, CA, USA).

3. Methods

3.1. Dissection and Preparation of Skeletal Elements

Bones can be imaged from mice at any age from 4 weeks onwards. For initial screening we usually analyse an upper limb, a lower limb, and the proximal six tail vertebrae following careful dissection post-mortem as described below:

1. Sacrifice the animal using an approved method.
2. Carefully remove the skin from the carcass.
3. To remove the upper limb, cut under the scapula with a scalpel blade to detach from the thorax and cut through the clavicle as close to the sternum as possible.
4. To remove the lower limb, cut vertically medial to the pelvis immediately lateral to the vertebrae thereby avoiding the femoral head.
5. To remove the proximal six tail vertebrae, peel off the skin, cut the tail at the base. Count down inter-vertebral spaces and detach the six most proximal vertebrae.

6. Place the intact limbs and tail vertebrae into 70% ethanol in a 20-ml polystyrene tube and fix at 4°C for at least 48 h.
7. After fixation, carefully dissect the muscle and soft tissues away from the bones of the upper and lower limbs (see Note 2). Carefully detach the humerus from the scapula and the femur from the acetabulum to leave the intact limb bone exposed.
8. Place the dissected bones in a polystyrene tube containing fresh 70% ethanol and store at 4°C until further use (see Note 3).

3.2. Faxitron Digital X-ray Imaging of Excised Bones

1. Switch on Faxitron machine for at least 15 min prior to use (see Note 4).
2. Remove the polypropylene sample stage prior to the calibration step.
3. Calibrate the Faxitron by running four flat dark field images using time set at 15 s and voltage at 26 kV and save the calibration settings (see Note 5).
4. When you are ready to image the bones, remove them from the 70% ethanol solution, place them on a paper towel to remove the excess ethanol and arrange them, within the imaging area of the polypropylene sample tray along with the steel, aluminium, and polyester calibration standards (see Note 6).
5. Insert sample tray into the ×5 magnification uppermost slot in the Faxitron machine and close the door.
6. Ensure that the Auto Level, Auto Exposure Control, Contrast Assist and Sharpen Assist toggles are deselected. Then select the “start exposure” button to commence imaging of the samples. The image will display on the computer screen.
7. Check that the orientation of samples and standards is correct taking care to make sure that all elements are included in the image.
8. Should any adjustment be necessary, remove the polypropylene sample stage, reposition the samples as necessary, replace the sample stage, and repeat step 6.
9. When you are happy with the image save it as a DICOM file, which is the default format.
10. Switch the Faxitron off using the key when you have completed the analysis session.

3.3. Image Processing

1. Open DICOM image in ImageJ and press “h” to reveal a histogram showing the distribution of grey scale pixels within the image (Fig. 1a).
2. Record the minimum and maximum grey level values.
3. Using the Rectangular Selections Tool within ImageJ, select as large an area as possible of the polyester standard without

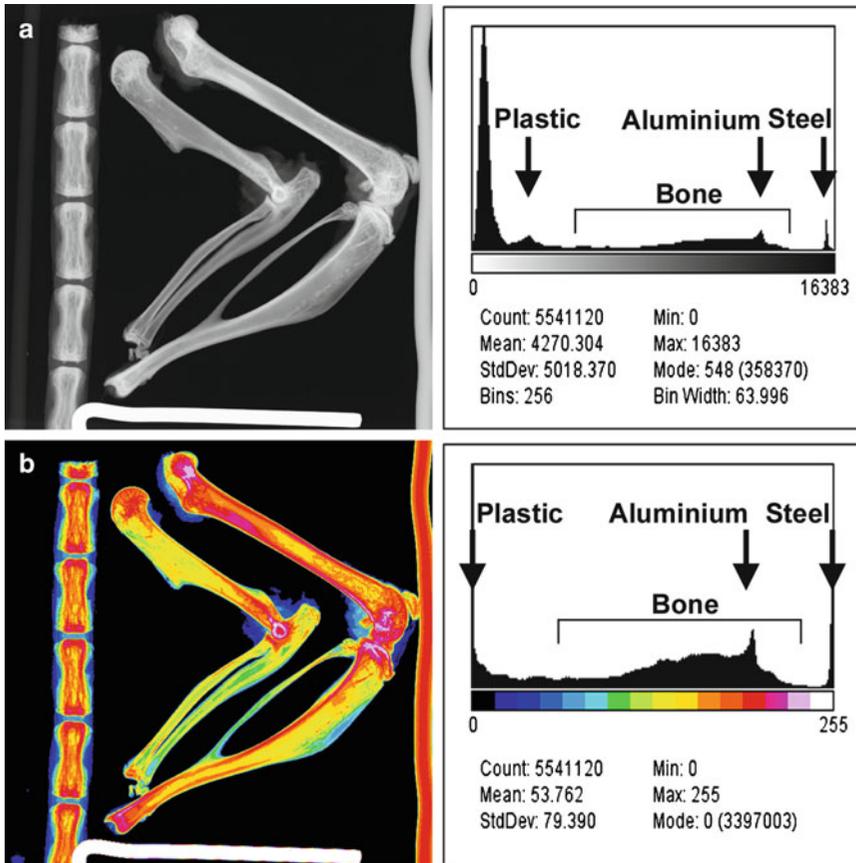


Fig. 1. (a) Original DICOM image showing the recommended organisation of limbs and vertebrae alongside polyester (left), aluminium (right), and steel (bottom) calibration standards. The histogram on the right shows the grey scale distribution and location of the three standards relative to mineralised tissue. The large peak on the left represents the background. (b) Pseudo-colour 8-bit TIFF image following stretch processing. The histogram shows the stretched grey scale distribution in relation to the 16-colour bins.

including background and press “h”. Note the value of the modal grey level. Similarly, select as large an area as possible of the steel standard and press “h”, noting the value of its modal grey level. This procedure is not applied to the aluminium standard, which acts as an internal reference for the grey scale stretch procedure described below.

- Using ImageJ, stretch each of the $2,368 \times 2,340$ DICOM images (16,383 grey levels) to the modal grey 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 grey levels). In the resultant image, the plastic and steel standards are thus assigned grey levels of 0 and 255, respectively.

3.4. Generating Pseudo-coloured Images of Individual Bones

1. Open the stretched 8-bit grey scale TIFF image in ImageJ.
2. Select the “Image” menu, then select Lookup Tables, 16 Colours (see Note 7).
3. Save the image as a TIFF file and repeat the procedure for all images.

3.5. Generating a Montage of Pseudo-coloured Images of All Bones

To compare images from different groups of mice (e.g. wild-type and mutant), it is desirable to display them side by side in a single montage. This can be achieved using Adobe Photoshop as described below.

1. Open the TIFF files of all pseudo-colour images of the bones you wish to analyse in Adobe Photoshop.
2. Select the first image and adjust the canvas size so it is big enough to accommodate all the individual colour images in two rows – wild-type above mutant.
3. Copy and paste each colour image into the enlarged canvas and position appropriately.
4. Save the montage as a TIFF file with selection of the following settings: Image Compression: NONE; Byte Order: Macintosh.

3.6. Processing and Cleaning Montages for Analysis of Mineralisation

1. Open the montage TIFF file in Adobe Photoshop.
2. Select the Brush Tool and adjust brush size to approximately 33 pixel diameter, select black for the brush colour.
3. Zoom in to at least 200% and use the Brush Tool to black out any soft tissue beyond the edge of the bone being careful not to black out any of the bone itself (see Note 8).
4. Select the Magic Wand Tool and click adjacent to the first bone. A yellow line should now appear around the bone and if the edges are not clearly highlighted brush over to black out any soft tissue remaining.
5. Repeat this process for each bone being analysed (see Note 9).
6. Save the processed and cleaned montage as a new TIFF file.
7. Repeat steps 1–6 to give separate TIFF files containing montages of the bones to be analysed.

3.7. Determination of Relative Mineral Content

1. Open the cleaned, montage file in the ImageJ programme (Fig. 2a).
2. Under Plugins, select Macro, Install, and open CustomHistogram.txt (see Note 10).
3. Select all bones to be analysed using the Wand (tracing) tool whilst holding down the Shift key.

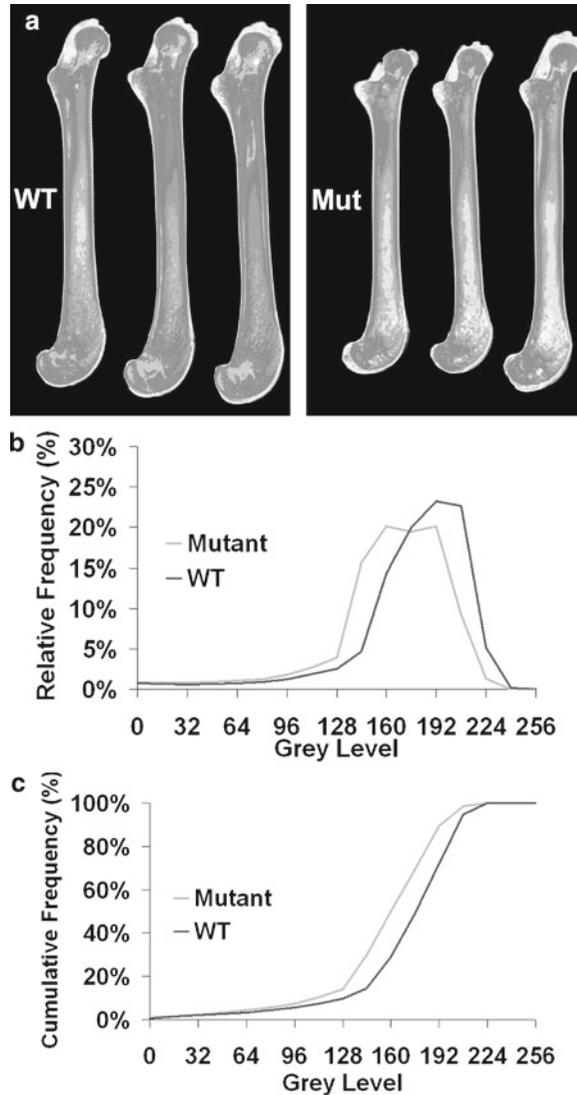


Fig. 2. (a) Black and white representation of pseudo-colour 8-bit TIFF files containing cleaned femur montages. (b) Relative frequency distribution plot showing difference in grey scale distributions between WT and mutant femurs. (c) Cumulative frequency plot showing difference between WT and mutant femurs.

4. 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.
5. Select the “List” button in the displayed histogram and copy the “bin start” and “count” columns and paste into an Excel spreadsheet. This provides the number of pixels in each of 16 equal grey level divisions (bins) to give a frequency distribution

of grey level values for the whole montage. The wild-type and mutant frequency distributions thus comprise summed grey scale values from each bone included in the respective montages and therefore facilitate comparison of their relative grey level distributions (Fig. 2b).

6. Statistical analysis of the difference between the cumulative frequency distribution of the grey levels of wild-type versus mutant montages (Fig. 2c) can be performed using a Kolmogorov–Smirnov test on each of the grey scale images to determine if these differ from one another (1).

4. Notes

1. The radiation source in the Faxitron 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.
2. Muscle and soft tissues should not be removed at the time of dissection. Complete removal is much easier and results in less risk of damage to bone when this is done after fixation in 70% ethanol for at least 48 h.
3. Samples can be stored in 70% ethanol at 4°C for many months and can be re-used for additional analyses including micro-CT imaging, back-scattered electron scanning electron microscopy, and investigation of biomechanical properties by destructive three-point bend and compression testing.
4. The X-ray source needs to warm up in order to maintain stability.
5. The Faxitron requires to be recalibrated each time it is restarted.
6. Samples should be removed from the 70% ethanol solution for as short a period of time as possible to prevent drying out.
7. This procedure applies a pseudo-colour scheme in which grey levels are divided into 16 equal intervals each represented by a different colour (Fig. 1b), thus greatly aiding visual presentation of digital images.
8. If in doubt regarding the identity of bone or soft tissue, it is better to erase a small bit of bone rather than leaving areas of soft tissue because the markedly lower grey levels of soft tissue would introduce artefacts and skew the mineralisation data. By contrast, erasing a bit of bone is readily compensated and has

little if any overall effect. Nevertheless, if a mistake is made using the Brush Tool it can be rectified using CTRL+Z.

9. A slightly different method is required to erase soft tissue from the vertebral images. Using the Pencil Tool in Adobe Photoshop, select a diameter of 13 pixels with 100% hardness. Zoom to 300% and draw a line along the outside of individual vertebrae. For the inexperienced eye, it can be difficult to distinguish soft tissue and ligament from bone and training from an experienced operator is necessary. Select the Paint Bucket Tool and fill in the background outside the vertebrae with black.
10. If the CustomHistogram.txt macro is absent from the Plugin menu, it can be downloaded from the ImageJ website (<http://rsb.info.nih.gov/ij/>).

Reference

1. Demidenko E. Kolmogorov–Smirnov Test for Image Comparison. In: Analysis in Computer Science and its Applications, Lagana A. (Ed.), Springer, Berlin (2004), Volume 3046; 933–39.