Method for ex-vivo micro-CT analysis of rat bone (proximal tibia, distal femur)

Introduction

The scans of rat bones can be performed on the Skyscan 1072, 1172, 1076 micro-CT scanners. This report sets out a methodology for morphometric analysis of trabecular and cortical murine bone. Results are presented for four rat femur samples, for both trabecular and cortical bone morphometry.

Method

Scan and reconstruction parameters

The rat bones were removed from alcohol storage and dried superficially on paper tissue, before being wrapped in plastic “cling-film” or in parafilm, to prevent drying during scanning (and associated movement artefacts). Each plastic-wrapped bone was placed in a plastic / polystyrene foam tube which was mounted vertically in the Skyscan 1172 / 1072, or horizontally in the 1076 scanner sample chamber, for micro-CT imaging.

The suggested parameters of the scan in the 1172 (10 Mpix model) are as follows:

- X-ray voltage: 70 kV
- X-ray current: 143 µA.
- Filter: 1 mm aluminium
- Image pixel size: 6-8 µm
- Camera resolution setting: Medium or low (2000 or 1000 pixel field width)
- Tomographic rotation: 180°
- Rotation step: 0.4-0.6°
- Frame averaging: 1-2 (depending on required scan time)
- Scan duration: Medium res. 20-30 min.; low res. 8-12 min.
The suggested parameters of the scan in the 1172 (1.3 Mpix model) are as follows:

- X-ray voltage: 70 kV
- X-ray current: 143 µA.
- Filter: 1 mm aluminium
- Image pixel size: 6-8 µm
- Camera resolution setting: High (1280 pixel field width)
- Tomographic rotation: 180°
- Rotation step: 0.4-0.6°
- Frame averaging: 1-2
- Scan duration: 30-40 minutes

The suggested parameters of the scan in the 1072 are as follows:

- X-ray voltage: 70 kV
- X-ray current: 143 µA
- Filter: 1 mm aluminium
- Image pixel size: 6-8 µm
- Camera resolution setting: n/a (1024 pixel field width)
- Tomographic rotation: 180°
- Rotation step: 0.45 or 0.675°
- Frame averaging: 1-2
- Scan duration: 45-70 minutes

The suggested parameters of the scan in the 1076 are as follows:

**In vivo**

- X-ray voltage: 70 kV
- X-ray current: 143 µA.
- Filter: 1 mm aluminium
- Image pixel size: 18 µm
- Camera resolution setting: Medium (2000 pixel field width)
- Tomographic rotation: 180°
- Rotation step: 0.5°
- Frame averaging: 1
- Scan duration: **8 minutes**
**Ex vivo**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray voltage</td>
<td>70 kV</td>
</tr>
<tr>
<td>X-ray current</td>
<td>143 µA.</td>
</tr>
<tr>
<td>Filter</td>
<td>1 mm aluminium</td>
</tr>
<tr>
<td>Image pixel size</td>
<td>8.9 µm</td>
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<tr>
<td>Camera resolution setting</td>
<td>High (4000 pixel field width)</td>
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<tr>
<td>Tomographic rotation</td>
<td>180°</td>
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<tr>
<td>Rotation step</td>
<td>0.4-0.6°</td>
</tr>
<tr>
<td>Frame averaging</td>
<td>1</td>
</tr>
<tr>
<td>Scan duration</td>
<td>20-30 minutes</td>
</tr>
</tbody>
</table>

*Reconstruction parameters suggested (same for all scanners):*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoothing</td>
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<td>Beam hardening</td>
<td>30%</td>
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<tr>
<td>Ring reduction</td>
<td>7</td>
</tr>
<tr>
<td>Post-alignment</td>
<td>use value calculated by Nrecon</td>
</tr>
<tr>
<td>Histogram limits</td>
<td>min 0; max - at right end of high density “tail”; refer to image to get good visible contrast</td>
</tr>
</tbody>
</table>

Reconstruction was carried out employing a modified Feldkamp\(^1\) algorithm using the Skyscan Nrecon\(^2\) software which facilitates network distributed reconstruction carried out on four pcs running simultaneously. Time for reconstruction of on scan dataset is usually much less than the scan duration.

**Region of interest selection for trabecular and cortical bone**

Both trabecular (metaphyseal) and cortical (metaphyseal-diaphyseal) were selected with reference to the growth plate. A crosssectional slice was selected as a growth plate reference slice, in the following way. Moving slice-by-slice toward the growth plate from the metaphysis/diaphysis, a point is reached where a clear “bridge” of low density cartilage (chondrocyte seam) becomes established from one corner of the crosssection to another. This bridge is established by the disappearance of the last band of fine primary spongiosal bone interrupting the chondrocyte seam (see figure 1). This landmark allows a reference level to be defined for the growth plate: trabecular and cortical volumes of interest are then defined relative to this reference level. (Note that the “bridge” can be at different locations in the growth plate crosssection in difference bone scans, depending on the orientation of the scanned bone.) Such a reference slice can be identified in all micro-CT scans of the proximal tibia and distal femur.
Note that while in the femur there are four circular “islands” delineated by chondrocyte seams in crossections through the upper growth plate, in the tibia there are only two such islands; none-the-less, the criterion for a growth plate reference described here can be reliably applied both for the femur and tibia. The only possible exception is in very old mice or rats where the growth plate is inactive and very little or no chondrocyte seams are present. In such cases, a similar landmark can be found involving connection of the bony structures of the vestigial growth plate.

Figure 1. Moving slice-by-slice toward the growth plate from the metaphysis/diaphysis, a point is reached where a clear “bridge” of low density cartilage (chondrocyte seam) becomes established from one corner of the crossection to another, as shown by the arrow. The establishment of this bridge coincides with the disappearance of the last band of fine primary spongiosal bone interrupting the chondrocyte seam. This landmark allows a reference level to be defined for the growth plate: trabecular and cortical volumes of interest are then defined relative to this reference level.

The regions of interest (ROI) were selected with reference to a growth plate reference slice, selected as described above. Pixel size here is 7.7 micron. Trabecular and cortical regions were defined as positions along the long axis of the femur relative to the growth plate reference. The trabecular region commenced about 0.77 mm (100 image slices) from the growth plate level in the direction of the metaphysis, and extended from this position for a further 3.08 mm (400 image slices). The vertical extent of the trabecular and cortical ROIs is indicated in figure 2. Within the trabecular region, separation of the trabecular from cortical bone was done using a freehand drawing tool for delineating of complex regions of interest (figure 3). The boundaries of the selected trabecular ROI ran parallel and close to the endocortical boundary - but excluding peripheral vestiges of the growth plate and associated
Micro-CT rat bone analysis, Skyscan

primary spongiosa, as shown in fig. 3. In Skyscan CT-analyser the volume of interest (VOI) automatically interpolates or morphs the edited ROI shapes (such as freehand drawn shapes) between edited levels. The frequency of edited levels depends on how rapidly the bone crosssection shape changes along the bone axis (in the z direction) and becomes less as you move from the growth plate in the direction of the diaphysis (bone shaft).

The cortical region commenced about 3.08 mm (500 image slices) from the growth plate level in the direction of the metaphysis, and extended from this position for a further 0.77 mm (100 image slices). This probably represents a diaphyseal site - there were relatively few remaining thin trabecular structures, compared to the much higher relative volume and number density of trabecular structures at the metaphysis close to the growth plate. The trabecular and cortical bone ROIs were delineated as shown in figure 3.

Figure 2. The vertical extent of the trabecular and cortical ROIs, defined with reference to a standard growth plate reference level (see text).

Figure 3. Trabecular and cortical ROIs delineated by the Skyscan CT-analyser software ROI tool, by freehand drawing.
Morphometric analysis

3D and 2D morphometric parameters were calculated for the trabecular and cortical selected ROIs. Single grey threshold values were applied for the trabecular and cortical ROIs respectively (68 and 120). (The lower density threshold for trabecular bone allows segmentation of the finer structures; the thicker cortical bone can have higher threshold to represent thickness and porosity more accurately.) The signal to noise ratios of the reconstructed images were high so that further image processing steps on the binarised images such as despeckle were not likely to significantly improve measurement precision. 3D parameters were based on analysis of a Marching Cubes\textsuperscript{3} type model with a rendered surface. Calculation all of 2D areas and perimeters was based on the Pratt\textsuperscript{4} algorithm. Morphometric parameters measured by CT-analyser have been validated on both virtual objects and aluminium foil and wire phantoms\textsuperscript{5}. Structure thickness in 3D was calculated using the local thickness or “sphere-fitting” method\textsuperscript{6}, and structure model index (an indicator of the relative prevalence of plates and rods) was derived according to the method of Hildebrand and Ruegsegger\textsuperscript{7}. Degree of anisotropy was calculated by the mean intercept method\textsuperscript{8}.

3D Model construction

Rendered 3D models were constructed for 3D viewing of trabecular and cortical analysed regions. Model construction was by the “Double time cubes” method\textsuperscript{9}, a modification of the Marching cubes method\textsuperscript{3}.


