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Method Article

Targeted 2D histology and ultrastructural bone analysis based on 3D microCT anatomical locations



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A B S T R A C T

Histological processing of mineralised tissue (e.g. bone) allows examining the anatomy of cells and tissues as well as the material properties of the tissue. However, resin-embedding offers limited control over the specimen position for cutting. Moreover, specific anatomical planes (coronal, sagittal) or defined landmarks are often missed with standard microtome sectioning. Here we describe a method to precisely locate a specific anatomical 2D plane or any anatomical feature of interest (e.g. bone lesions, newly formed bone, etc.) using 3D micro computed tomography (microCT), and to expose it using controlled-angle microtome cutting. The resulting sections and corresponding specimen's block surface offer correlative information of the same anatomical location, which can then be analysed using multiscale imaging. Moreover, this method can be combined with immunohistochemistry (IHC) to further identify any component of the bone microenvironment (cells, extracellular matrix, proteins, etc.) and guide subsequent in-depth analysis. Overall, this method allows to:

- Cut your specimens in a consistent position and precise manner using microCT-based controlled-angle microtome sectioning.
- Locate and expose a specific anatomical plane (coronal, sagittal plane) or any other anatomical landmarks of interest based on microCT.
- Identify any cell or tissue markers based on IHC to guide further in-depth examination of those regions of interest.

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Background

Histological processing of mineralised tissues (e.g. bone) allows to identify extracellular matrix (ECM) and cellular components in thin tissue sections following histological staining. Two of the most common embedding methods for mineralised tissue histological processing are paraffin-wax embedding and resin (polymethylmethacrylate; PMMA) embedding. Paraffin embedding is quicker, easier to manipulate and cost-effective. However, the specimen must be decalcified/demineralised before processing and embedding. This disadvantage makes paraffin embedding incompatible with any mineral-based analysis, therefore significantly limiting the possibilities in terms of analysis. PMMA embedding does not require a decalcification step and, thus, is the preferred embedding method for material science examination. However, PMMA processing and embedding take longer, involve the use of more hazardous and expensive chemicals and offer very restricted control over the specimen's position for cutting.

Due to the solid-liquid form of the wax depending on temperature, paraffin embedding allows positioning of any given specimen in a precise manner. Arranging the specimen in a particular position (e.g. femoral condyles facing downwards) allows (i) sectioning the specimen so that the target anatomical plane of interest is exposed and (ii) cutting all specimens in a consistent manner. This controlled-positioning is not possible with resin (PMMA) as the initial liquid consistency of the embedding solution hampers any stable orientation of the specimens in the moulds while the resin is cured. As a result, the embedded specimens often do not have a consistent orientation and the sectioning plane varies between samples, which in turn affects the following measurements and read-outs.

Another limitation, which cannot be addressed by correct sample position during embedding, is the inability to target a specific anatomical location or landmark while sectioning. For instance, metastatic bone lesions might appear in different locations (and sizes) in the bone, which might not be evident when examining the gross morphology of the bone. Another example is the formation of new bone following scaffold implantation; it is difficult to predict where the new bone would grow more extensively and/or which areas of the newly formed bone would be more interesting for examination. Other conditions related to angiogenesis (e.g. abnormal blood vessels) or chondrogenesis (e.g. abnormal growth plate or cartilage tissue) could be investigated more effectively by determining beforehand the location of those areas of interest.

To circumvent these issues, here we propose to define the anatomical location of interest in a PMMA-embedded specimen based on micro computed tomography (microCT), and to use controlled-angle sectioning to expose its surface for further material science characterisation analyses.

Method details

A given mineralised sample (e.g. bone) is embedded in PMMA and, once the PMMA hardens, the entire specimen block (SB) is scanned with microCT. The microCT scan is used to define a specific

anatomical plane (coronal, sagittal) or other landmarks of interest. The main steps of the method involve the rotation of the 3D microCT dataset to identify the anatomical location of interest in a 2D plane and to determine the correcting angles to expose its corresponding surface. To do so, a custom-made correcting block (CB) is designed and attached to the SB to allow for controlled-angle microtome sectioning. Serial histological sections are then collected and stained with histology and immunohistochemistry (IHC). These stainings are used to guide subsequent correlative, multiscale imaging on the corresponding SB's surface, such as confocal laser scanning microscopy (CLSM), second harmonic generation (SHG) imaging and backscattered electron (BSE) microscopy.

Materials and reagents

- PMMA processing and embedding
 - Paraformaldehyde (PFA).
 - Polymethylmethacrylate (PMMA, Technovit 9100, Kulzer, Germany).
 - Moulds.
- Imaging
 - MicroCT scanner.
- Targeted cutting
 - Low speed diamond saw.
 - Superglue.
 - Semi-automated microtome with a D-knife for histological use.
- Method validation
 - Rhodamine 6G (Acros Organics, Belgium).
 - Alcian blue 8GX (A5268-10G, Sigma).
 - Picosirius red F3B (365548-5G, Sigma).
 - Anti-mCherry antibody (NBP2-25157, Novusbio, Bio-Techne GmbH, Germany).

Method

Mineralised tissue specimen

To illustrate the protocol and potential of controlled angle sectioning, here we use a scaffold-implanted murine femur. In short, an electrospun scaffold was implanted together with bone morphogenetic protein 2 (BMP 2) at the lateral side of a murine femur to form new bone at an orthotopic location [1]. After sacrifice, the scaffold-implanted femur and its surrounding soft tissue were dissected and fixed in paraformaldehyde (PFA). As described in the method's background, maintaining the specimen in a controlled position in the process of PMMA embedding is challenging, particularly with this sample, due to the bulkiness of the scaffold and the surrounding soft tissue. Thus, this scaffold-implanted murine femur required informed, controlled-angle cutting based on microCT to expose the area of interest. Moreover, this method can be combined with immunohistochemistry (IHC) to further detect any tissue component of interest (cells, extracellular matrix, proteins, etc.) and guide subsequent higher magnification analysis.

Step 1: Sample preparation

- (1) PMMA processing and embedding of non-demineralised murine femur: After fixation in fresh 4% PFA, the mineralised specimen is dehydrated in increasing ethanol concentrations for 2 days each step (70, 80, 96, 100, 100%).

Optional: Rhodamine staining can be included after the first 100% ethanol step by adding two additional steps with rhodamine in 100% ethanol (0.417 g Rhodamine 6G per 100 ml ethanol) [1].

Continue with tissue clearing in xylol for 3 h (x2) and immerse the samples in pre-infiltration solution for 3 days. Change to infiltration solution for 3,4 days (x2) and embed the specimen following manufacturer's instructions. Polymerization takes place over 3,4 days at - 20 °C.

Table 1

Summary of tissue processing steps for cold PMMA embedding.

Step	Description	Temperature and duration
1	Fix in 4% PFA	1 day at 4 °C
2	Wash with PBS	1 day at 4 °C
3	70% ethanol	2 days at 4 °C
4	80% ethanol	2 days at 4 °C
5	96% ethanol	2 days at 4 °C
6	100% ethanol	3 days at 4 °C
7	Rhodamine/ethanol 1	1.5 days at 4 °C
8	Rhodamine/ethanol 2	1.5 days at 4 °C
9	Xylene	2 × 3 h at RT
10	Pre-infiltration	3 days at 4 °C
11	Infiltration 1	3 days at 4 °C
12	Infiltration 2	4 days at 4 °C
13	Embedding	RT
14	Polimerisation	3,4 days -20 °C

Tip: To facilitate tissue infiltration with the new solution, directly after each solution exchange (Table 1, beginning of steps 3–12), the samples are subjected to 10 min vacuum in a desiccator.

- (2) Once the PMMA resin is cured, remove the specimen's block (SB) from the mould and trim the excess of PMMA around the sample using a low speed diamond saw. Leave around 2 mm of PMMA around the specimen as this facilitates sectioning.

Optional: Using an engraving pen, a small perforation is made in the SB to serve as landmark, as far as possible from the specimen to avoid any damage (Suppl. Fig. 1). If no engraving pen is available, any particular mark, shape or feature of the SB can be used instead. This characteristic landmark in the SB will help to correlate/orientate the actual SB with the 3D microCT scan dataset.

Step 2: MicroCT scan

The microCT scan should capture the entire SB and the resolution should match the size of the anatomical features of interest. The scanning settings also depend on the tissue and need to be determined individually. In this study, the scanning parameters were: 100 kV, 300 μ A, 30 μ m voxel size, frame rate of 3, average frame of 5 using an EasyTom micro/nano tomograph (RX solutions, France). Reconstruction of the 1120 projections/scan were performed using RX Solutions X-Act software and visualised with DataViewer (Bruker, Kontich, Belgium).

Step 3: Definition of target anatomical location based on microCT

In this step, it is recommended to explore the microCT datasets to get familiar with their general morphology and identify any particular feature(s) of interest. Taking the scaffold-implanted murine femur as an example, we aim to expose the newly formed bone around the midshaft of the femur as well as the native bone itself (Fig. 1 c,d). A longitudinal section of the coronal plane of the femur allows exposing both newly formed bone around the scaffold and native murine bone adjacent and distant from the scaffold (proximal and distal femur). In addition, the coronal plane of the femur allows maintaining a standard sectioning plane for all samples consistently. Therefore, the coronal plane of the femur is defined as the target anatomical location of the SB's microCT scan.

Step 4: Location of target anatomical position using microCT

In the present method we use DataViewer software as it is simple, user-friendly and can be downloaded free of charge from the following website <https://www.bruker.com/products/microtomography/academy/2016/exploring-dataviewer.html>

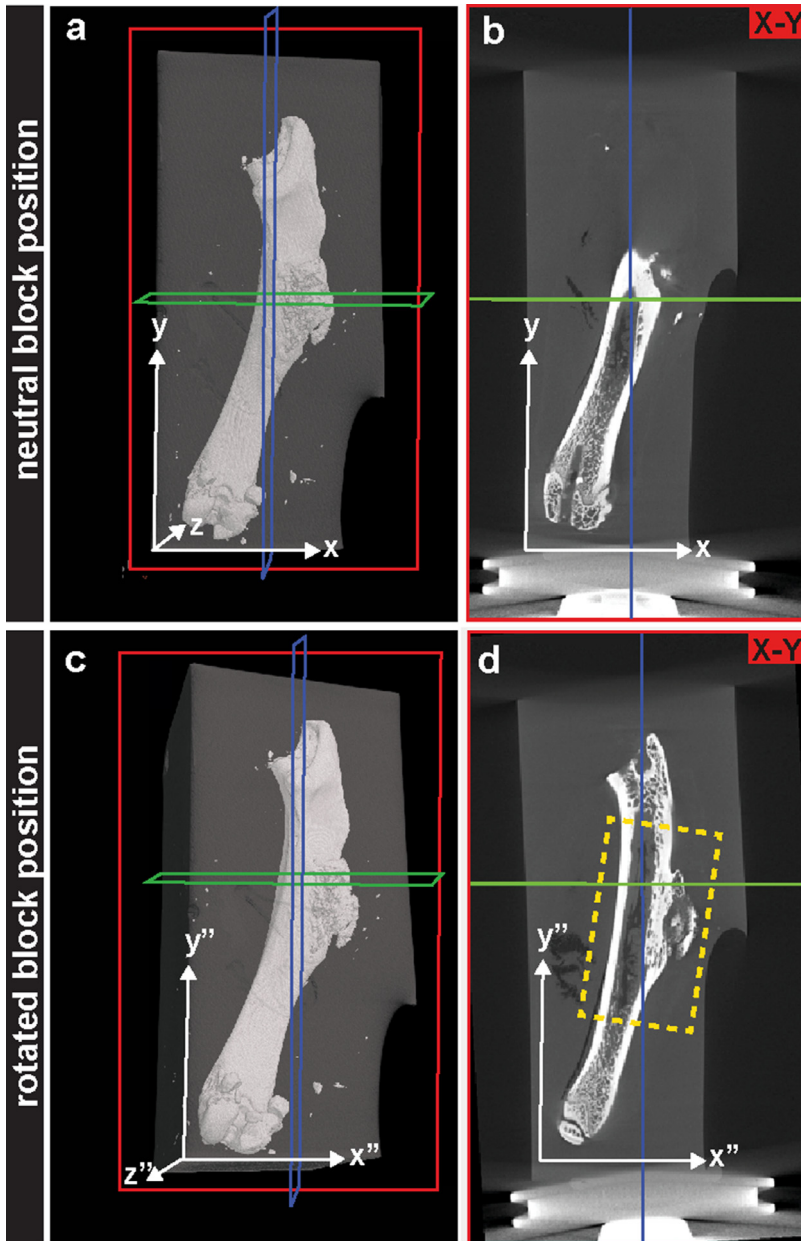


Fig. 1. Resin-embedded bone specimen in neutral and corrected (rotated) position exposing the desired anatomical location in the XY plane. MicroCT scan showing the (a, c) 3D resin block and bone specimen within and (b, d) XY plane of the embedded specimen. Corresponding coordinate system of the scanned block (a,b) in a neutral position (x, y, z) and (c,d) in corrected (rotated) position (x'', y'', z'') to target the desired anatomical location, in this case the newly formed bone on the coronal plane in the midshaft of the femur. Notice the origin of the coordinate system located in the lower left front corner of the SB (a, c), with the axes facing towards the backside of the SB. Yellow dashed line indicates the target region of interest (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

(1) Loading the microCT scan in Data Viewer and define the neutral position.

(a) Introduction to DataViewer.

DataViewer allows to (i) visualise the three orthogonal planes (XZ, XY, ZY) of the microCT scan simultaneously using the 3D panel viewer and (ii) to rotate any of the planes in clock wise (CW) or counter clock wise (CCW) direction. For a tutorial on how to operate DataViewer software see Suppl. Video 1.

Load the SB's microCT dataset in a software that allows to visualise and rotate the scan in 3D. If using DataViewer, load the dataset in 3D and activate the preferences at viewing to show the full cross-hair and the respective labels (XZ, XY, ZY)

The three orthogonal planes (XZ, XY, ZY) appear simultaneously in green, red and blue, respectively. By clicking on any point in one plane, the exact same point is shown in the other two planes. Taking the SB's dataset as an example, the crossing point of the blue and red line in the XZ plane (Fig. 2 a) is the same as the crossing point of the blue and green line in the XY plane (Fig. 2 c) and the red and green line in the ZY plane (Fig. 2 d). Therefore the first and second row in Fig. 2 (Fig. 2 a–e and f–j, respectively) are showing two different crossing points in the SB, without changing the SB position: one in the bottom left front corner of the block (Fig. 1 a–b, Fig. 2e), and another one roughly in the middle of the block (Fig. 2 j).

To rotate any of the planes, press CTRL key on the keyboard, while dragging the left mouse button in either right or left direction. Once you release the CTRL key and the left mouse button, the rotated plane is displayed as well as the corresponding two other orthogonal views, rotated as well. This operation can be repeated sequentially, in any of the planes.

Explore the SB's dataset orthogonal planes and locate the anatomical plane of interest. In the case of the present example, the coronal plane of the femur is the desired location to expose both native and newly formed bone (Fig. 2 r).

Tip: The landmark described in step 1 serves to identify the X, Y and Z planes of the dataset in the actual SB.

(b) Definition of the neutral position and the origin of the coordinate system.

In order to design the correcting block (CB), the required rotation(s) are calculated in terms of plane, angle and direction (CW, CCW). To do so, the SB dataset is placed in a neutral position which will be used to define the origin of the coordinate system. To choose this reference point, namely the origin of the coordinate system, it is recommended to (i) position the SB with all sides roughly parallel to an imaginary straight line, (ii) identify the X, Y and Z planes and (iii) pick one of the SB vertices (*i.e.* corner) as origin. In the present example, the X, Y and Z axes of the coordinate system in the SB are shown in Fig. 1 a and c, with the origin in the front left lower corner of the SB. In this way, the rotation of the planes in CW or CCW direction is defined from the perspective of the origin of the coordinate system in the SB.

Note that the neutral position (and thus the origin of coordinate systems) can be redefined to find the neutral position that requires the simplest rotations (*i.e.* less number of rotations and less angles for each rotation) to expose the target 2D plane. In general, more than 35 ° or 40 ° per rotation might not be feasible with the microtome layout.

Once the neutral position and origin of coordinate systems are defined, it is important to identify the side of the block which will be glued to the CB (*i.e.* opposite side to microtome blade for cutting) and to do so with a thin layer of glue, without creating additional angles.

Tip: Stick post-it® notes to the sides of the SB, each of them indicating the XZ, XY and ZY planes (or X, Y and Z axes) as well as the origin of the coordinate system. Use the post-its to correlate the coordinate axes of the SB's microCT dataset with respect to the faces/sides of the actual SB.

(2) First rotation.

With the SB in a neutral position and the coordinate system origin defined (Fig. 2 a–e), the coronal plane of the femur corresponds to the XY plane (Fig. 2 c, h, m, r). If the SB would be sectioned along the XY plane without any corrections (Fig. 2 f–j), only the lower half of the femur would be exposed, also excluding the newly formed bone on the coronal plane in the midshaft of the femur (Fig. 2 h). To correct this, the ZY plane is rotated around the X axis 10 ° (Fig. 2 n). To define the direction of

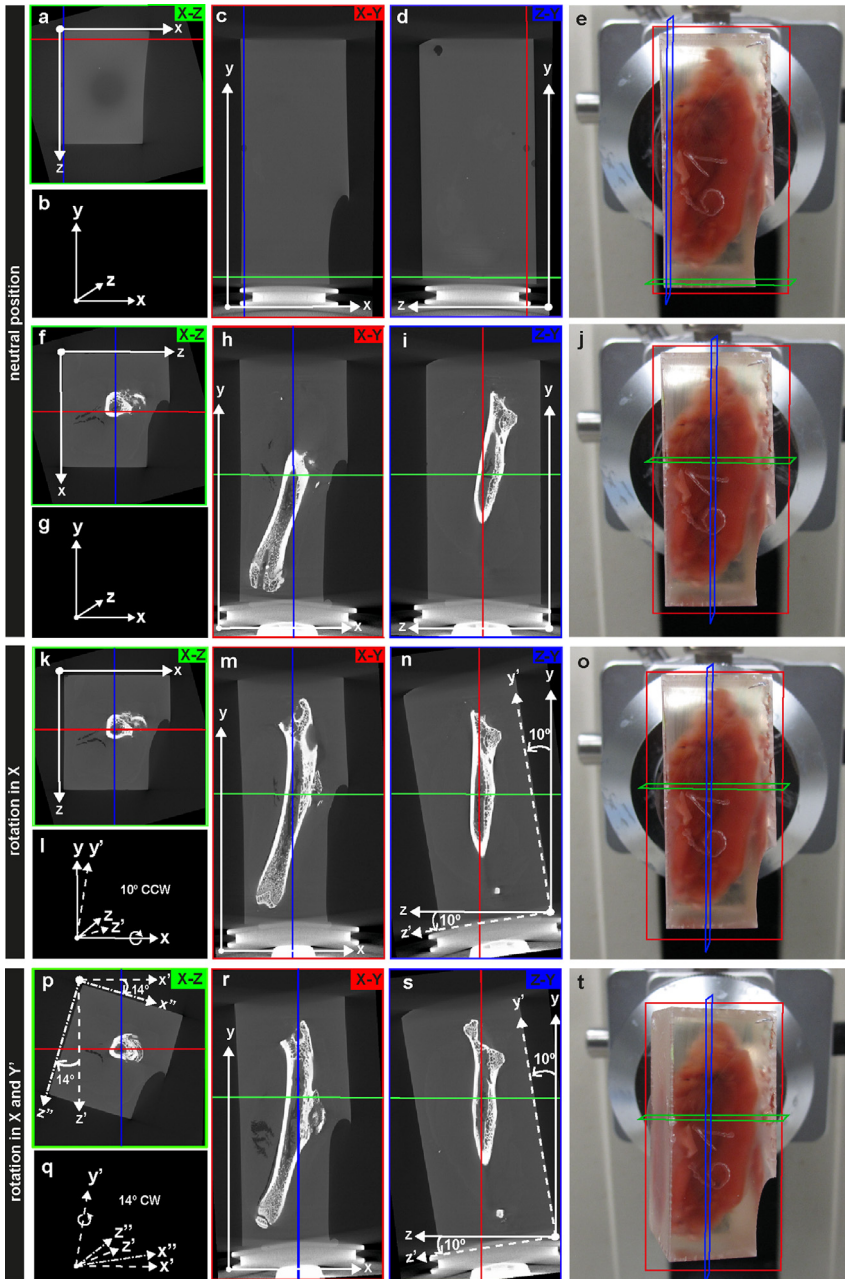


Fig. 2. Step-by-step rotations of the SB dataset to expose the anatomical location and plane of interest. MicroCT scan of the SB (resin-embedded scaffold-implanted murine femur with surrounding soft tissue, stained with rhodamine) showing the 2D planes: (a, f, k, p) XZ in green, (c, h, m, r) XY in red and (d, i, n, s) ZY in blue, and (b, g, l, q) the corresponding coordinate system. Axes of the coordinate system are indicated with a solid line corresponding to the block's neutral position. Regular dashed lines correspond to the block following rotation around the X axis, and irregular dashed lines correspond to the block following rotation around X and Y' axis. Image of the block in the microtome positioned in (e, j) neutral position, (o) following CCW rotation around X axis, and (t) following CCW rotation around X axis and CW rotation around Y' axis (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

rotation, the same 10° ZY rotation is performed from the perspective of the coordinate system origin (Fig. 2 l), which indicates the CCW direction around the X axis:

First rotation	
Rotating plane	ZY
Rotating axis	X
Rotation angle (degrees)	10°
Rotation direction (CW or CCW)	CCW

Note that the axes post-rotation are labelled as Y' and Z' and sketched as regular discontinued lines (Fig. 2 l, n). After this rotation (Fig. 2 k–o), the coronal plane exposes now the whole femur, however the newly formed bone is only partially exposed (Fig. 2 m).

(3) Second rotation.

To expose both femur coronal plane and newly formed bone (Fig. 2 r), the XZ plane is then rotated around the Y' axis 14° (Fig. 2 p). To define the direction of rotation, the same 14° XZ' rotation is performed from the perspective of the coordinate system origin (Fig. 2 q), which indicates the CW direction around the Y' axis:

Second rotation	
Rotating plane	XZ'
Rotating axis	Y'
Rotation angle (degrees)	14°
Rotation direction (CW or CCW)	CW

Note that after the second rotation the axes are labelled as X'' and Z'' and sketched as irregular discontinued lines (Fig. 2 p, q).

Thus, the sequential rotations around the X (CCW 10°) and around Y' (CW 14°) axes allow to expose the desired target anatomical plane. To measure the exact angles of any rotated plane using ImageJ [2] please see Suppl. Video 2.

Note: Most commonly, the SB will only require a single rotation step, simplifying the process.

Before continuing to Step 5, it is recommended to go over the previously calculated rotations with the actual SB:

- Identify the origin of the coordinate system and the corresponding planes (XZ, XY and ZY) in the actual SB to reproduce the positions and movements in the first and second rotation.
- Identify the side of the SB immediately facing the microtome blade as the side facing towards you.
- Identify the side of the SB having to be glued to the CB as the side facing away from you.

Tip: To visualise the expected histological sections when cutting through the block, slide through the dataset's slices in the direction of cutting. See as an example the yellow arrow in Fig. 4 a,b, where the pointy end of the arrow indicates the sectioning direction of the microtome blade or knife.

To summarise, in order to expose the coronal plane of the SB, it is necessary to cut the SB repositioned as follows: rotation of 10° (CCW) around the X axis and rotation of 14° (CW) around the Y' axis.

Step 5: Design of the custom-made correcting block (CB)

Once the one or two correction angles are defined, a CB needs to be designed. This CB will be glued to the SB and positioned in the microtome holder (back face). By doing so, the microtome blade will not cut parallel to the front face of the SB as in standard sectioning, but with a corrected controlled angle (Fig. 3).

These CBs are made from a 25 mm PMMA cylinder with parallel top and bottom sides (Fig. 3 a), which then are cut with a low-speed circular saw with the desired angles (Suppl. Fig. 2).

To break down the steps and illustrate the process, we created CBs with 0° , 10° , 14° and both 10° and 14° and positioned the CB + SB together in the microtome holder (Fig. 3).

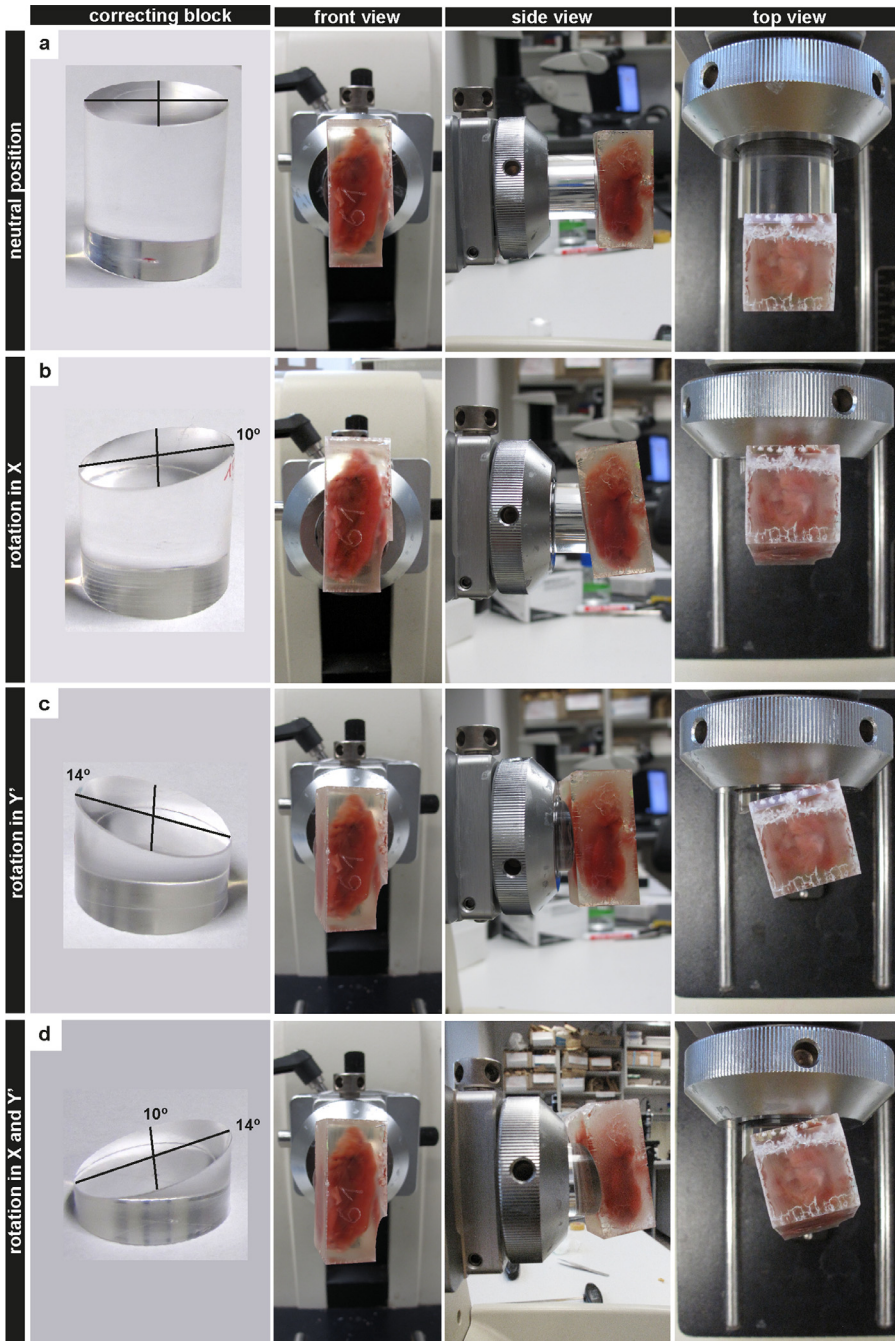


Fig. 3. Different perspective's views of the specimen block together with the correcting block positioned in the microtome holder. (a–d) Images of the CBs and SB+CB cut with (a) no angle (flat), (b) 10 °CCW around the X axis, (c) 14 °CW around the Y axis, (d) 10 °CCW around the X axis and 14 °CW around the Y axis. Second, third and fourth column show the SB + CB positioned in the microtome viewed from the front, side and top, respectively. Note that the rotation around X and Y are for illustration purposes and in practice both correction angles are applied in one same CB (d).

Fig. 3 shows each of the CBs alone (Fig. 3, first column) and positioned in the microtome holder together with the SB (CB+SB) in each of the rotation steps: neutral position (Fig. 3 a), rotation around X (Fig. 3 b), rotation around Y (Fig. 3 c) and rotation around X and Y (Fig. 3 d). For each step, three views of the CB + SB positioned in the microtome are shown: from the front (Fig. 3, second column), from the side (Fig. 3, third column) and from the top (Fig. 3, fourth column).

- (1) When the CB is flat (0°), the SB keeps the neutral position (Fig. 3 a), which corresponds to the same neutral position in Fig. 2 a–j.
- (2) With the rotation of 10° CCW correction around X axis (Fig. 2 k–o), the bottom part of the SB points towards the front, where the microtome blade would be (Fig. 3 b).
- (3) With the rotation of 14° CW correction around Y axis, the left side of the SB points towards the front (Fig. 2 i–l), where the microtome blade would be (Fig. 3 c).
- (4) With the rotations of (i) 10° CCW around X axis and the (ii) 14° CW correction around Y axis, both the bottom part and the left side of the SB points towards the front, where the microtome blade would be (Fig. 3 d).

The custom designed CB is then glued in the appropriate position, onto the correct side of the SB, opposite to the microtome blade:

- (a) Using sand paper, the two facing surfaces of the CB and the SB are ground to generate a rough surface to better glue the parts.
- (b) Superglue is applied to one of the surfaces and both rough surfaces are pressed together for 5 seconds.
- (c) Allow 10 min to dry and the CB+SB is ready for microtome sectioning.

Optional: At this point, the CB+SB can be scanned again to check the correct positioning of the CB+SB before sectioning.

Step 6: Controlled-angle cutting of the corrected SB in the microtome and validation with microCT scans

While cutting, the histological sections should match the predicted slices of the XY (coronal) plane of the rotated dataset. Small discrepancies can be corrected by manual tilting of the microtome holder. Before collecting tissue sections, the SB is trimmed to remove the PMMA before reaching the actual bone (see shaded yellow area in Fig. 4 a,b). Once the surface of the SB starts to expose bone tissue, histological sections are collected at different depths throughout the SB (Fig. 4 d, f, h, j, l, n), before reaching the target region (Fig. 4 p). Note the resemblance between the predicted XY planes of the rotated dataset (Fig. 4 c, e, g, i, k, m, o) and the corresponding histological sections (Fig. 4 d, f, h, j, l, n, p).

Start collecting serial sections of the SB about $100\ \mu\text{m}$ before reaching the target anatomical plane. The optimal thickness of the histological sections will vary between tissues and specimens, although $5\text{--}10\ \mu\text{m}$ is generally recommended.

Step 7: Multiscale, correlative imaging of the target anatomical location defined by microCT

Once the SB has been sectioned, the last serial sections collected from the block can be correlated with the corresponding surface of the SB. To illustrate this, Fig. 5 shows another example of scaffold-implanted murine femur with a microCT scan of the coronal plane (Fig. 5 a).

- (1) Consecutive sections are stained with histological stainings such as Alcian Blue (proteoglycans in blue) and Picrosirius Red (collagen in red) (Fig. 5 b), and Goldner's Trichrome (Fig. 5 c), where green is collagen, bright red is cell cytosol and connective tissue is pink/red.
- (2) The corresponding surface of the SB can then be imaged with any microscopy technique compatible with resin embedding. Here we list a few examples:
 - a. CLSM to visualise the lacunar-canalicular network (LCN) stained by rhodamine (Fig. 5 d). Z-stacks can be acquired with CLSM and quantitative data can be obtained from the cellular network (Suppl. Videos 3–6).

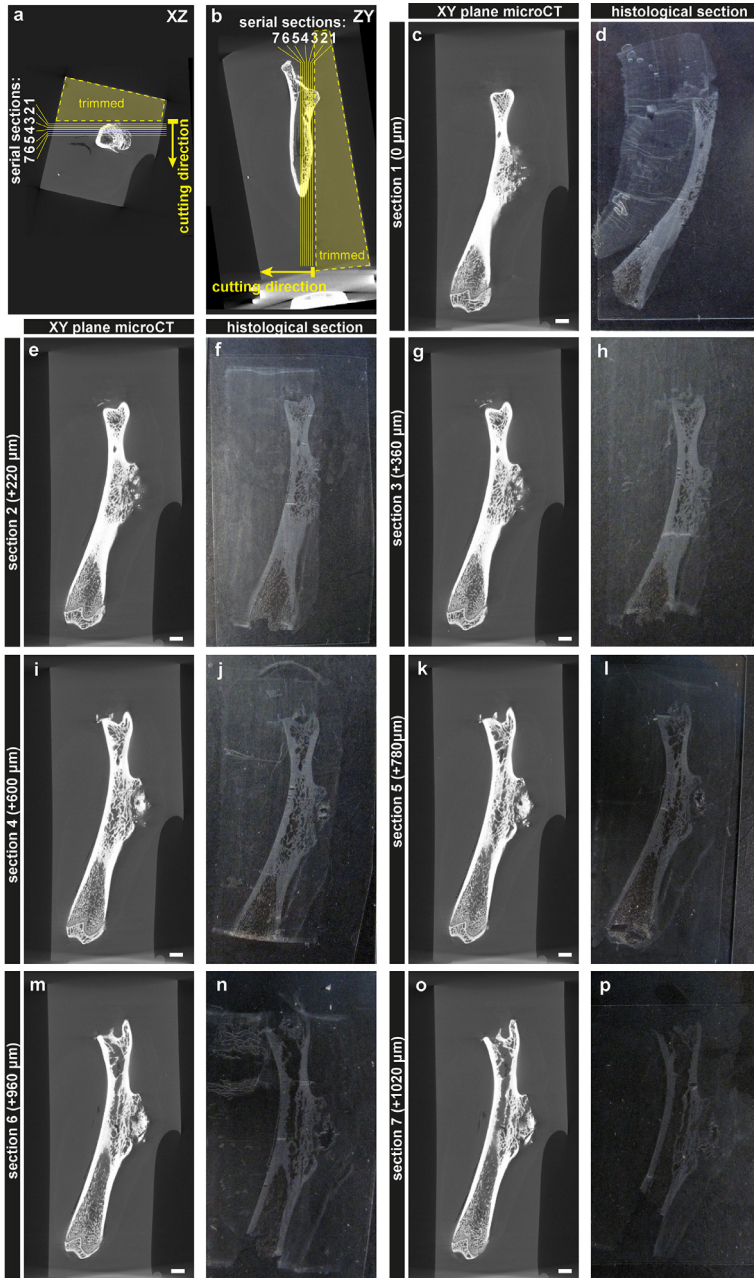


Fig. 4. Predicted microCT XY planes correspond to the histological sections collected while microtome sectioning. (a) microCT XZ and (b) microCT ZY planes of the SB containing scaffold-implanted femur following correcting angle rotations. Solid arrow indicates the cutting direction. Dashed yellow lines indicate the position of seven representative sections collected while cutting. (d, f, h, j, l, n, p) Representative histological sections collected while sectioning and corresponding to (c, e, g, i, k, m, o) the predicted XY planes based on microCT, (c,d) at 0 µm, (e,f) 220 µm, (g,h) 360 µm, (i,j) 600 µm, (k,l) 780 µm, (m,n) 960 µm and (o,p) 1020 µm (target anatomical location). Histological sections were photographed with a black background to enhance contrast between the bone tissue and the resin in the section. Scale bars equal 1 mm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

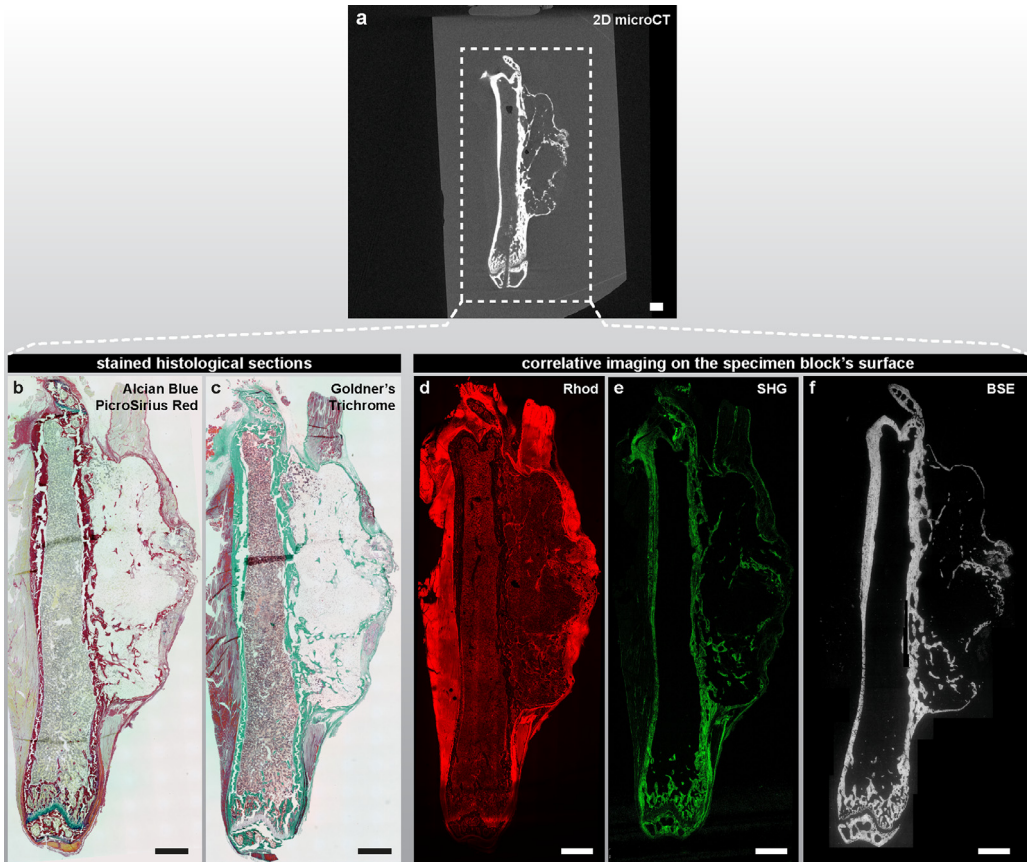


Fig. 5. Multiscale, correlative imaging of the target anatomical location defined by microCT. (a) Visualisation of the coronal plane of the scaffold-implanted at the murine femur, which was defined as the anatomical region of interest using microCT. (b,c) Serial histological sections stained with (b) Alcian Blue and Picrosirius Red and (c) Goldner's Trichrome. Correlative imaging on the corresponding block surface with (d) confocal laser scanning microscopy CLSM of rhodamine (Rhod) staining, (e) second harmonic generation (SHG) imaging of fibrillar collagen, and backscattered electron (BSE) microscopy of the mineral content. The intense red signal surrounding the bone tissue (d) corresponds to soft tissue, which was intensely stained with rhodamine. Scale bars equal 1 mm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

- b. SHG imaging to visualise the collagen organisation of the bone (Fig. 5 e)
- c. BSE microscopy of the mineral content (Fig. 5 f).

Notice the similarity between the target anatomical location on the SB microCT dataset (Fig. 5 a) and the stained histological sections (Fig. 5 a,b) as well as the corresponding SB surface (Fig. d-f), validating the accuracy of the technique.

Note: After sectioning, avoid additional polishing of the block to maintain the correlation between the last histological sections and the surface of the specimen block (SB).

Step 8: Immunohistochemistry-based identification of regions of interest for higher magnification analysis

Next, IHC on the resin sections is used to look for specific markers in the tissue and identify areas of interest for further correlative, higher magnification imaging and analysis. We have used as an example another scaffold-implanted murine femur containing mCherry-labelled osteoblast cells. In order

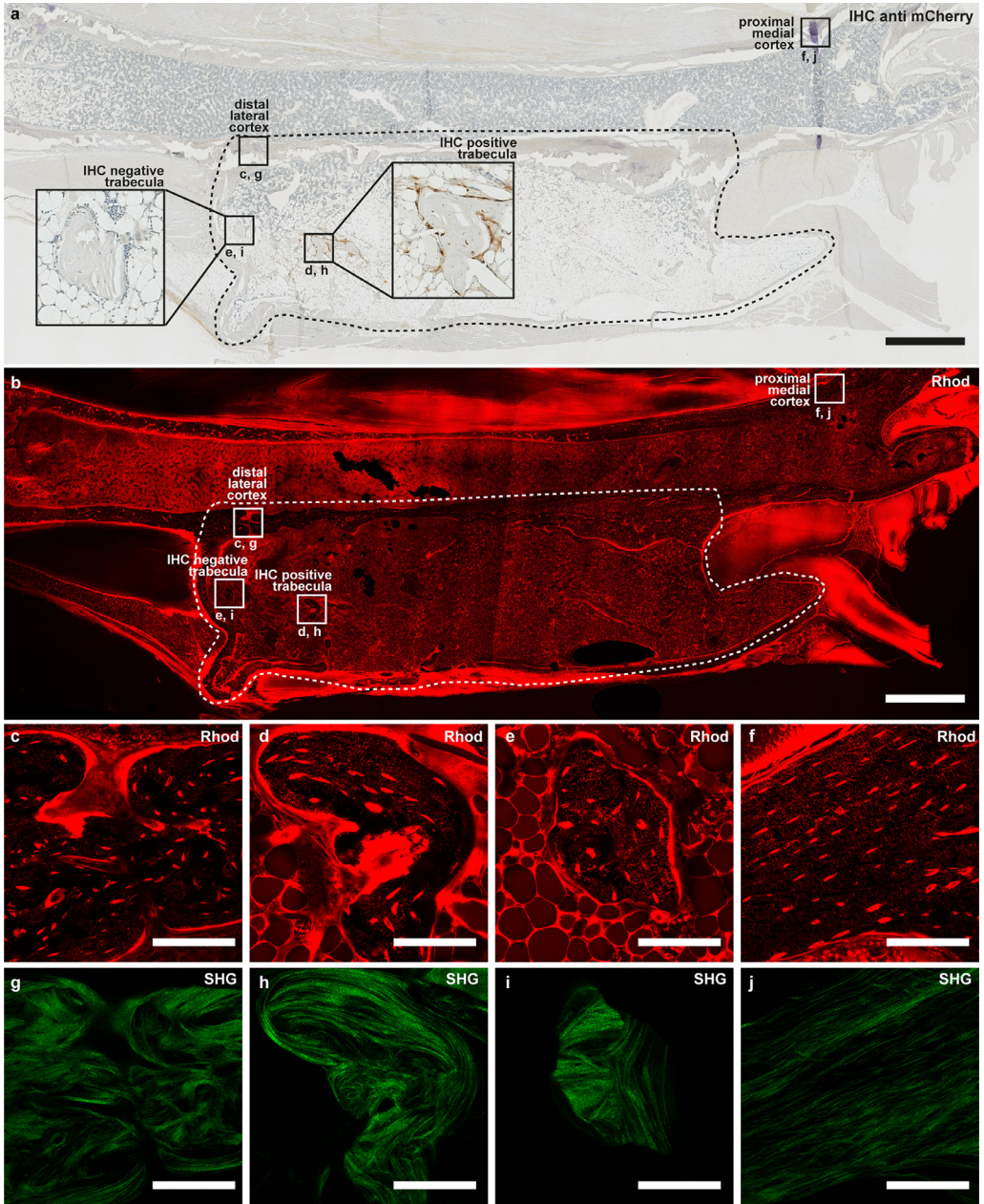


Fig. 6. Correlative ultrastructural characterization at the target anatomical location identified by IHC. mCherry-labelled mouse osteoblast-seeded scaffolds were processed and controlled-angle sectioned to expose the coronal plane of the implanted femur. **(a)** Longitudinal histological sections were probed with anti-mCherry (brown) immunohistochemistry (IHC) and counterstained with haematoxylin (blue). **(b)** Corresponding block surface was imaged with **(b–f)** confocal microscopy to visualise osteocytes and LCN with rhodamine staining and **(g–j)** second harmonic generation to visualise collagen organisation. Regions of **(c, g, e, i, f, j)** negative and **(d, h)** positive mCherry IHC staining located **(c, g, d, h)** within the newly formed bone ossicle (dashed line) or within the native murine bone **(e, i)** in contact or **(f, j)** not in contact with the scaffold were imaged. Scale bars equal **(a, b)** 1000 μm and **(c–j)** 100 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to identify the location of those mCherry-labelled osteoblasts within the newly formed bone in the scaffold area, IHC against mCherry is performed in one of the last sections collected from the SB (Fig. 6 a), where brown indicates positive staining and pale blue indicates negative tissue counterstaining (Fig. 6 a). Based on the location of the positive (brown) and negative (pale blue) staining, four regions of interest (ROIs) are identified:

- Trabecular bone (IHC negative) formed within the newly formed bone ossicle (Fig. 6 c, g).
- Trabecular bone (IHC positive) formed within the newly formed bone ossicle (Fig. 6 d, h).
- Native murine bone (IHC negative) in the distal lateral cortex, in direct contact with the scaffold (Fig. 6 e, i).
- Native murine bone (IHC negative) in the proximal medial cortex, not in contact with the scaffold (Fig. 6 f, j).

These four ROIs are then imaged at higher magnification (40x) to visualise the LCN in 2D (Fig. 6 c-f) and in 3D (Suppl. Video 3–6), as well as the corresponding collagen organisation visualised with SHG (Fig. 6 g–j). Based on the LCN it is possible to assess differences between regions of newly formed bone mCherry positive and negative. Moreover, the effects of the scaffold in the native murine bone can be observed when comparing the highly organised collagen of the medial cortex with the region in the lateral cortex (compare Fig. 6 i, j).

In conclusion, this method offers a protocol to specifically expose any anatomical location of interest in a precise manner based on microCT scans, which then can be imaged with correlative, multiscale imaging techniques. Moreover, this method can be combined with immunohistochemistry to further identify any component of the bone microenvironment (cells, extracellular matrix, proteins, etc.) and guide subsequent in-depth analysis. Although this method might be initially challenging to introduce as routine sample analysis, laboratories that already have histology and microCT facilities will benefit extensively from it.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.mex.2021.101480](https://doi.org/10.1016/j.mex.2021.101480).

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