Talbot-defocus multiscan tomography using the synchrotron X-ray microscope to study the lacuno-canalicular network in mouse bone

Nobuhito Nango,1 Shogo Kubota,1 Akihisa Takeuchi,2 Yoshio Suzuki,2 Wataru Yashiro,3 Atsushi Momose,3 and Koichi Matsuo4,*

1Ratoc System Engineering Co., Ltd, 1-24-8 Sekiguchi, Bunkyo-ku, Tokyo 112-0014, Japan
2Japan Synchrotron Radiation Research Institute (JASRI/SPring-8), 1-1-1 Kouto, Sayo, Hyogo 679-5198, Japan
3Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan
4Laboratory of Cell and Tissue Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

*matsumo@z7.keio.jp

Abstract: The three-dimensional network of lacunae and canaliculi that regulates metabolism in bone contains osteocytes and their dendritic processes. We constructed a synchrotron radiation X-ray microscope for sequential tomography of mouse tibia first by using a Talbot interferometer to detect the degree of bone mineralization and then by using absorption contrast under a slightly defocused setting to enhance outline contrast thereby visualizing structures of the osteocyte lacuno-canalicular network. The resultant pair of tomograms was precisely aligned with each other, allowing evaluation of mineral density in the vicinity of each osteocyte lacuna and canaliculus over the entire thickness of the cortical bone. Thus, multiscan microscopic X-ray tomography is a powerful tool for analyzing bone mineralization in relation to the lacuno-canalicular network at the submicron resolution level.

©2013 Optical Society of America

OCIS codes: (070.6760) Talbot and self-imaging effects; (110.6960) Tomography; (180.7460) X-ray microscopy; (340.6720) Synchrotron radiation.

References and links

1. K. Matsuo and N. Irie, “Osteoclast-osteoblast communication,” Arch. Biochem. Biophys. 473(2), 201–209 (2008).
2. L. F. Bonewald, “The amazing osteocyte,” J. Bone Miner. Res. 26(2), 229–238 (2011).
3. L. D. You, S. Weinbaum, S. C. Cowin, and M. B. Schaffler, “Ultrastructure of the osteocyte process and its pericellular matrix,” Anat. Rec. A Discov. Mol. Cell. Evol. Biol. 278A(2), 505–513 (2004).
4. L. F. Bélanger, “Osteocytic osteolysis,” Calcif. Tissue Res. 4(1), 1–12 (1969).
5. A. Teti and A. Zallone, “Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited,” Bone 44(1), 11–16 (2009).
6. H. Qing, L. Ardeshirpour, P. Dervieti Pajevic, V. Dusevich, K. Jähn, S. Kato, J. Wyssolomserski, and L. F. Bonewald, “Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation,” J. Bone Miner. Res. 27(5), 1018–1029 (2012).
7. J. J. Wyssolomserski, “Osteocytes remove and replace perilacunar mineral during reproductive cycles,” Bone 54(2), 230–236 (2013).
8. C. Ciani, S. B. Doty, and S. P. Fritton, “An effective histological staining process to visualize bone interstitial fluid space using confocal microscopy,” Bone 44(5), 1015–1017 (2009).
9. H. Kamioka, Y. Kameo, Y. Imai, A. D. Bakker, R. G. Bacabac, N. Yamada, A. Takaoka, T. Yamashiro, T. Adachi, and J. Klein-Nulend, “Microscale fluid flow analysis in a human osteocyte canaliculus using a realistic high-resolution image-based three-dimensional model,” Integr. Biol. 4(10), 1198–1206 (2012).
10. P. Schneider, M. Meier, R. Wepf, and R. Müller, “Towards quantitative 3D imaging of the osteocyte lacuno-canalicular network,” Bone 47(5), 848–858 (2010).
1. Introduction

Bones provide structural support for the body and also store biominerals such as calcium and phosphate in the form of hydroxyapatite crystals, which are integrated into organic bone matrix consisting of collagen fibers and other bone matrix proteins. Adult bones undergo constant dynamic remodeling, a cellular process involving bone resorption by osteoclasts followed by bone formation by osteoblasts [1]. A subset of osteoblasts embeds itself within newly formed bone matrix and differentiates into osteocytes in osteocytic lacunae. Osteocytes are the most abundant cells in mammalian bone [2].

Osteocytes extend numerous dendrites into canaliculi within bone matrix and maintain osteocyte-osteocyte interactions through gap junctions at the tip of dendrites. In mice, the average diameter of osteocytic canaliculi is 260 nm (80 to 710 nm) [3]. Proposed functions of the lacuno-canalicular network include sensing of mechanical load, detection of microfractures, transport of nutrients and waste to and from osteocytes, and, most remarkably, delivery of osteocyte-derived bioactive proteins, such as sclerostin, an inhibitor of osteoblast function, and receptor activator of NF-κB ligand (RANKL), a critical activator of osteoclast differentiation, to the bone surface. Osteoclasts may not be the only cells that demineralize bone, as there is accumulating evidence that osteocytes resorb bone through osteocytic osteolysis [4,5]. Recent reports demonstrate that osteocytes remove and replace mineral around osteocytic lacunae in maternal bone during lactation [6,7]. However, uncertainties remain regarding the direct roles of osteocytes, in particular, osteocytic canaliculi, in bone mineral metabolism.

For three-dimensional analysis of osteocytes and dendrites or the lacuno-canalicular network, investigators have employed light microscopy, confocal laser microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and ultra-high voltage electron microscopy (UHVEM) [3,8–10]. In most analyses, bone specimens are decalcified in acids or chelators such as ethylenediamine tetraacetic acid (EDTA) and sectioned [3,9]. Synchrotron X-ray tomography, using undecalcified specimens, is a promising technology recently applied to image both osteocytic canaliculi and lacunae, the latter being prolate (rugby ball-shaped) structures 10-20 μm in length along the long axis [11–13]. However, quantitative information about the degree of mineralization is limited. Moreover, the canalicular network appears to be beyond the resolution of conventional X-ray microscopes. Recently, X-ray phase tomography has been applied to analysis of the lacuno-canalicular network in human bone [14].

In this study, we overcame these difficulties by developing a sequential Talbot-defocus multiscan technique based on an X-ray microscope with synchrotron radiation. Tomography employing a Talbot interferometer, which enables highly sensitive observation based on X-ray differential phase contrast, can reveal the refractive index decrement (δ) [15], a value that corresponds approximately to electron density and (for materials consisting of light elements)
mass density [16]. Therefore, since bone mineralization positively correlates with the amount of hydroxyapatite, we can estimate the degree of bone matrix mineralization. Tomography using defocus absorption-contrast allowed visualization of the canalicular network (about 0.3 \( \mu \text{m} \) average diameter) along the entire cortical thickness from the endosteum to the periosteum (about 100 to 200 \( \mu \text{m} \)).

2. Materials and methods

2.1 Multiscan method

A multiscan X-ray microscope was constructed at the undulator beamline 20XU (Experimental Hutch II) in the synchrotron radiation facility Super Photon ring 8 GeV (SPRING-8) in Hyogo, Japan. Imaging was carried out using monochromatic 9-keV X-rays through a double-crystal monochromator. An X-ray Fresnel zone plate (ATN/FZP-SiC86/416, NTT Advanced Technology Corporation [NTT-AT], Tokyo, Japan) was used as an objective lens with a focal length of 261 mm and an outer-most zone width of 86 nm. A sample was set 273 mm upstream of the FZP in the on-focus condition. X-ray images were recorded using a charge coupled device (CCD) camera in combination with an optical lens and a phosphor screen, with a pixel size of 4.34 \( \mu \text{m} \). The image detector was placed at the image plane 5.71 meters downstream of the zone plate. The X-ray microscope magnification was thus 20.9, and therefore the effective pixel size was 0.208 \( \mu \text{m} \). Talbot-defocus multiscan analysis was performed sequentially for each sample, as shown in Fig. 1.

The configuration for differential phase contrast combined with the X-ray microscope is shown in Fig. 1(a), where an X-ray Talbot interferometer [17] is located in front of the image detector [18]. A rotating paper diffuser was used to reduce speckle noise from optical elements and windows at the beamline. The X-ray Talbot interferometer consisted of a \( \pi/2 \) phase grating and an amplitude grating, which had gold stripes of pitches of 6.0 \( \mu \text{m} \) and 6.1 \( \mu \text{m} \), respectively on 70-\( \mu \text{m} \) glass plates (Howa Sangyo Co. Ltd., Tokyo, Japan). The distance between the gratings was set to 377 mm, and the phase grating was slightly inclined to reduce the pitch effectively and to remove parallel moiré fringes caused by pitch mismatch between the amplitude grating and the self-image of the phase grating projected onto it. Although it is preferable to place the amplitude grating on the image plane, experimentally it was more convenient to maintain a space of 68 mm between the amplitude grating and the image detector. The resultant spatial resolution was about 1 \( \mu \text{m} \), which was evaluated from the resultant phase tomograms presented below.

A differential phase image, which maps the variation of X-ray refraction at the sample, was generated using a fringe-scanning (FS) method [17] as follows. The amplitude grating was displaced by a step of 1/5 of its pitch using an FS motor. Five fringe patterns were thus acquired by the image detector, and a differential phase image was calculated from the patterns using a formula for the FS method. Because the differential image imposes built-in contrast resulting from imperfection in the gratings and their alignment, a background differential phase image was measured without a sample in the field of view prior to the tomographic scan. The differential phase image was corrected for by the background differential phase image prior to tomographic image reconstruction. The exposure time for acquiring a fringe pattern was 4 s. This procedure was then repeated at 500 angular positions of stepwise sample rotation over 180 degrees. The total scan time was 6.5 h/sample, including data transfer and storage.

The defocus-contrast X-ray microscope configuration is shown in Fig. 1(b). The Talbot interferometer was removed from the optical axis. The Fresnel zone plate on the defocus stage was displaced 6 mm downstream along the optical axis, while keeping the positions of the
Fig. 1. Setup of multiscan X-ray microscope. (a) Differential phase-contrast mode in combination with a Talbot interferometer consisting of a phase grating and an amplitude grating. (b) Defocus absorption-contrast mode achieved by moving the Fresnel zone plate (thick arrow). (c) Block diagram of control system. FS, fringe scanning; GR, grating remove; DA, defocus arrangement; CR, CT scan rotation.

Sample and image detector fixed, in order to enhance edge contrast of the object by Fresnel diffraction effects [19,20]. The exposure time for acquiring a defocus image was 2 s. The images measured were processed with a normal filtered-backprojection CT algorithm, and the reconstructed tomogram exhibited a linear absorption coefficient with enhanced edge contrast, which outlined the boundaries of fine structures. In this study, 1800 projection images were acquired by a tomographic scan over 180-degree sample rotation using a CT scan rotation (CR) motor on the rotation stage (Fig. 1(c)). The total scan time was 2.5 h/sample, including data transfer and storage.

For each sample, Talbot and defocus tomographic scans were performed sequentially with minimal time between scans in order to avoid sample deformation or drift. Multiscan 3D images were superimposed using 3D image analysis software TRI/3D-BON (Ratoc System Engineering Co., Ltd., Tokyo, Japan). Briefly, osteocytic lacunae and canaliculi of the two 3D images were superimposed and the non-overlapping volume was calculated. By rotating and moving the second 3D image, a position was identified at which the non-overlapping volume became the minimum.
2.2 Preparation of bone samples

Tibias were isolated from euthanized 12 to 16 week-old female C57BL/6J mice (Clea, Tokyo, Japan). After removing muscles and soft tissues, tibias were fixed in 70% ethanol, infiltrated for three days in LR white resin (Electron Microscopy Sciences, Fort Washington, PA, USA) and embedded in the same resin. After waiting a day for specimens to solidify, the shaft of the long bone (diaphysis) was cut as a piece approximately 3 mm long and the posterior cortical bone was vertically sliced twice to make a 300 μm or less wide column with intact periosteum and endosteum surfaces (Fig. 2(a)). After immersing samples in acetone for 10 min, extra resin was removed using forceps. The cortical bone sample in the form of pseudo-square rod was glued onto the tip of a sample holder (a 1 mm-diameter brass column with a notch protruding from a 3 mm-diameter column) using double-sided adhesive tape (Fig. 2(b)). Samples glued to the sample holder were equilibrated to room temperature before use. All animal experiments were performed according to the guidelines for animal experiments of Keio University and were approved by Keio University Institutional Animal Care and Use Committee.

![Figure 2](image)

Fig. 2. Preparation of mouse bone sample. (a) Schematic presentation of tibia. The cortical bone sample used for imaging is represented in green. (b) The sample (green arrow) attached to the holder using double-sided adhesive tape (arrowhead). Scale bar, 1 mm.

3. Results

We applied the Talbot-defocus multiscan method to each cortical bone sample (Fig. 2) by scanning it twice at an identical position using the setup shown in Fig. 1. Figure 3 shows a sagittal section (from anterior to posterior) of the tibial cortical bone sample with the endosteum and periosteum as the anterior and posterior border (left and right border in the photograph), respectively. The top of each panel corresponds to the proximal direction. The Talbot phase tomogram revealed the degree of mineralization in the vicinity of osteocyte lacunae and canaliculi (Fig. 3(a)). The high mineralization zone observed near the periosteum and endosteum corresponds to the lamellar zone. The low mineralization zone located at the center of the cortical bone corresponds to the non-lamellar (turbulent) zone. About ten osteocyte lacunae were visualized as semi-oval black spaces (arrowheads). The cement line, the junction between the original resorbed surface and new bone, showed high bone mineral density (double arrowhead in Fig. 3(a)).
Fig. 3. Reconstructed tomograms. (a) Phase tomogram, in which gray scale indicates the refractive index decrement $\delta$ ranging 0 (corresponding to low (L) mineral density) to $7 \times 10^{-6}$ (corresponding to high (H) mineral density). Arrows, osteocyte canaliculi; Arrowheads, osteocyte lacunae; Double arrowhead, cement line. (b) Tomogram with defocus edge enhancement. The outer edges of the lacunae and canaliculi are enhanced. Note that black and white are reversed. (c) Combined image of (a) and (b). The defocus image is pseudocolored red. Scale bars, 25 $\mu$m. The dotted rectangle in (a-c) is magnified in the left image.

The grayscale of the resultant absorption contrast image acquired under a slightly defocused setting was reversed to demonstrate the lacuno-canicular network (Fig. 3(b)), revealing that the darker region exhibited the lower absorption coefficient. Canaliculi were enhanced as bright lines due to the defocus effect. Near the endosteum and periosteum in the lamellar zone, lacunae were oriented longitudinally and aligned parallel to the bone surface, and canaliculi ran parallel to each other extending toward or away from the bone surface (arrows). In contrast, at the middle of the cortical bone in the turbulent zone, lacunae orientations were irregular, and the parallel arrangement of canaliculi was no longer apparent in the anterior-posterior plane. In this study, the defocused image in Fig. 3(b) was reconstructed without phase retrieval [21]. We relied instead on the fact that most fine canalicular structures were effectively visualized by the defocus contrast method, even if...
canaliculus diameter is equivalent to or even less than the system spatial resolution. Nonetheless, future analysis using phase retrieval is worth attempting.

The Talbot-defocus tomograms shown in Figs. 3(a) and 3(b) almost precisely corresponded with each other when they were overlaid using auto-alignment (see 2.1), as illustrated by the appearance of canaliculi, as indicated by yellow arrows in Fig. 3(c). Thus, we detected a 3D structure of osteocyte canaliculi and the degree of mineralization in the vicinity of the lacuno-canalicular network in the merged image.

4. Conclusions

To detect the structure of a canaliculus, which is about 260 nm in diameter [3], we employed a defocus absorption-contrast method to enhance the edge structure. Although the diameter of the canaliculus cannot be determined using this method, the location of abundant canaliculi was mapped as distinct lines in the defocus tomogram obtained. The Talbot phase tomogram revealed the distribution of mineralization, and combining the two tomograms allowed us to understand changes in mineralization in regions surrounding the lacuno-canalicular network. One potential limitation of these experiments is possible degradation of bone samples. Currently, we do not know whether embedding in resin results in better preservation than would simple air drying after ethanol fixation.

We found that the degree of mineralization varied with individual samples of osteocyte peri-lacunar and peri-canalicular bone tissue. The fact that mineralization changes spatially suggests that osteocytes directly participate in mineral metabolism through the lacuno-canalicular network (manuscript in preparation). Further work is needed using mice in different physiological and pathological states, such as lactation, osteoporosis, or osteopetrosis (lack of osteoclastic bone resorption) to reveal the biological significance of the degree mineralization change near the lacuno-canalicular network.

Acknowledgments

We thank Yoshihiro Takeda and Yasunari Takada for technical assistance, and Elise Lamar for critical reading of the manuscript. This work was supported by JSPS KAKENHI Grant Numbers 19390399, 21390425, 24659685 to KM and 19350027 to AM. The experiments were performed under the approval of the SPring-8 committee (Proposal No. 2005B0976, 2006A1377, 2006B1141, 2007A1137, 2007A1848, 2007B1130, 2007B1787, 2008A1279, 2008B1508, 2009A1195, 2009A1871 and 2010B1438).