The osteocyte lacuno-canalicular network in bone investigated by synchrotron radiation-based tomography techniques

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ABSTRACT

Bone has a complex hierarchical structure, which is essential for its performance. Bone is typically replete with cells called osteocytes that are embedded in the mineralized bone matrix in osteocyte lacunae, which are interconnected by canaliculi only a few hundred nanometer wide to form a vast cellular network. Our understanding of the osteocyte lacuno-canalicular network has been limited because of difficulties to image the cellular network within the opaque bone matrix in 3D. Synchrotron X-ray computed tomography is ideally suited to study the lacuno-canalicular network in bone because it combines the high penetration power of X-rays with sub-micron resolution while retaining a fast acquisition time and thus high throughput. We discuss how synchrotron radiation-based tomography techniques have given insights into the osteocyte network in bone both in the form of regular tomography and, for higher resolution studies, in the form of nanotomography such as holotomography. These studies have provided quantitative measures of osteocyte lacunar properties and their relation to location within bones and bone challenges such as immobilization or lactation. Nanotomography revealed new features of the canalicular network that we term canalicular junctions, which are likely to play an important but hitherto hidden role in fluid flow dynamics within the bone cellular network. The examples illustrate how tomography provides information on complex biological materials like bone and we foresee that these capabilities will continue to improve with future/upgraded synchrotron X-ray sources.

Keywords: Tomography, synchrotron, holotomography, bone, X-ray tomography, nanotomography, osteocyte lacunae

1. INTRODUCTION

Bone has a fascinating but complex hierarchical structure [1]. In mammals, bone is replete with cells called osteocytes that play several important roles in bone biology [2-7]. The cells are located in lacunae on the order of \(4\times10^7\) µm\(^3\) in size, which are interconnected by canaliculi only a few hundred nanometers wide that allow intercellular communication; see Figure 1. The bone matrix is predominantly formed by a hierarchical composite of nanoparticulate apatite and collagen supplemented by a number of additional biomolecules and water. Our understanding of the bone matrix has undergone several important changes recently, e.g. in terms of microstructural motifs and the nature of the bone mineral nanocrystals [8-10]. Indeed, much remains to be learned about bone hierarchical structure and function. To this end, we have applied synchrotron X-ray tomography over several length scales to study various aspects of the osteocyte lacuno-canalicular network as schematically illustrated in Figure 1. Herein, we review a selection of these results.

The osteocyte canaliculi have complex structures and the characterization of this network demands 3D spatially resolved information. Due to the opaque nature of bone, X-ray tomography is an ideally suited technique even if other methods are also valuable [11]. Lab-source \(\mu\)- and nano-CT instruments have proven successful in studying the size and shape of osteocyte lacunae [12-14]. While lab-based CT systems have in recent years achieved spatial resolution down to a few hundred nanometers, this comes at a cost of very long scan time per probed volume compared to synchrotron tomography [15]. To map the small lacuno-canalicular network, one would ideally wish for ~100 nm resolution to resolve canaliculi, a probed volume of \(\sim1\) mm\(^3\), and the ability to investigate tens to hundreds of specimens to afford the statistical basis needed to answer biological questions. This combination of requirements cannot be fulfilled by a single experiment. Instead, we have benefitted from the high-throughput nature of ‘standard’ attenuation-based synchrotron \(\mu\)CT with voxel sizes of the order 0.3-0.6 µm depending on experimental details. We have conducted a number of studies of rodent animal models [16-21] and human bone [22] to understand the nature of the osteocyte lacuno-
Figure 1. Investigation of bone structure requires access to 3D imaging on several length scales ranging from lab-scale µCT (left) over synchrotron µCT (center), affording information on for example the lacunae housing the osteocyte bone cells, to nano-CT that provides information on the full cellular network in bone, the lacuno-canalicular network. A single osteocyte lacuna has dimensions on the order of $4 \times 10 \times 18 \ \mu m^3$.

canalicular network. In these experiments, many specimens can be investigated in a realistic amount of synchrotron beamtime [18, 19, 22, 23].

For higher resolution, both ptychography [24] and holotomography [25] was used. Both these techniques depend on the partial coherence of synchrotron X-ray sources to improve resolution through phase information. A number of studies have been conducted on bone using holotomography to afford ~100 nm resolution, e.g. references [16, 26-28]. As we will discuss in section 3, the improved resolution afforded by these techniques yields surprising insights into the bone lacuno-canalicular network [16].

2. OSTEOCYTE LACUNAE IN RODENTS BY HIGH THROUGHPUT SR-µCT

Long bones form through a complicated process called endochondral ossification where cartilage provides initial growth, is calcified, and thereafter replaced by bone. Additional bone can be added by apposition or removed by resorption from the bone surface as the bone grows. In humans and some other animals, the bones are additionally remodeled by Haversian remodeling, where bone-degrading cells, osteoclasts, drill tunnels through the bone, which are then refilled by bone building cells called osteoblasts. This results in the formation of osteons [1, 29].

Osteocyte lacunae across cortical bone: central vs lamellar bone.

Since rodents do not undergo Haversian remodeling the structure of rodent long bones differs from that of, for example, humans. Rodent long bones contain a central bone band remnant from the endochondral ossification process, which we showed contained islands of highly mineralized calcified cartilage remaining from the formation process [21]. The increased mineralization is easily detectable by synchrotron µCT as shown in Figure 2C as high contrast region [20, 21]. These data were collected using monochromatic X-rays at 17 to 18 keV and isotropic voxel sizes of 325 to 370 nm at beamline TOMCAT at the Swiss Light Source, Paul Scherrer Institute, Switzerland, depending on the study in question. At this resolution, osteocyte lacunae can easily be isolated by segmentation [18-21]. By separating osteocyte lacunae into those originating from central bone and those from lamellar bone, we showed that the properties of osteocyte lacunae are tightly connected to the organization of the mineralized collagen fibrils. In lamellar bone, the osteocyte lacunae were more asymmetric in shape than in central bone. Their orientation was determined by investigating the orientation of the eigenvectors of best fit ellipsoids to the lacunae. We found that the shortest axis was very strongly aligned perpendicular to the bone long axis while it was not oriented in central bone. This shows a tight interconnection between the 3D organization of the bone matrix and the osteocyte lacunae [20].
Figure 2. Rodent long bones contain a central band of bone remnant from the endochondral formation process surrounded by interior and exterior regions of appositional bone termed lamellar bone. (A) Sketch of a cross-section through a rat long bone showing the central, endochondrally derived, bone part surrounded by lamellar bone grown by apposition. (B) Osteocyte lacunae and blood vessels (bv) abound in bone as seen by void spaces detected by 0.3 to 0.4 µm voxel size synchrotron tomography. The osteocyte density is larger in central bone but the osteocyte lacunae are more rounded in shape and less co-orientated than in lamellar bone. (C) Section through the gray scale images of the stack shown in B at the location of the vertical arrow. Gray levels have been digitally adjusted. Calcified cartilage islands are visible as bright scalloped regions.

We then proceeded to study the impact of animal age on osteocyte lacunae in rat cortical bone [18]. Using high throughput synchrotron tomography at 18 keV with a 325 nm isotropic voxel size, we investigated 42 bone rods in six age groups (i.e. seven animals in each age group). Three consecutive 702 µm high scans were collected across a rectangular bone rod cut to a maximum base area of 0.58×0.58 µm² to match the field of view for highest image quality. In total, 7.4·10⁵ osteocyte lacunae were quantified allowing us to make strong statistical statements on the properties of osteocyte lacunae and their dependence on age. A selection of results is shown in Figure 3. The animals grew as expected (Figure 3B), but the geometric properties of the osteocyte lacunae such as the lacunar volumes (Figure 3C) did not depend on animal age. The lacunar density, i.e. the number of lacunae per bone volume, showed slight variations with age in a nonsystematic fashion (Figure 3D), perhaps influenced by the relative fractions of central to lamellar bone sampled. The degree of mineralization of the bones could be determined from the synchrotron tomography gray levels, since the synchrotron tomography was done at a small sample to detector distance to minimize phase effects. It grew with age, see Figure 3E. These results, together with mechanics and lab-µCT data, allowed us to conclude that the geometrical properties of osteocyte lacunae are almost independent on age, whereas higher order structural features as well as the degree of mineralization varied significantly with age resulting in age-related changes in whole bone mechanical properties [18].
On the proposed ability of osteocytes to break down bone, osteocytic osteolysis.

Osteocytic osteolysis has been proposed to play several physiological roles from sensing damage to signaling to other bone cells and even other organs [2-7]. It has long been debated whether osteocytes are also able to break down the bone matrix in their immediate surroundings [30-32]. Consequently, a number of studies have addressed this topic [19, 33-49]. The general view has swung to be in favor of osteocytic osteolysis as a common effect resulting from e.g., lactation [37, 40-42, 44, 46]. One way to establish osteocytic osteolysis has been to study lacunar cross-sectional areas by various microscopies. Since osteocyte lacunae are highly anisotropic in shape (Figure 1), cross-sectional areas are unlikely to give an unbiased estimate of the lacunar size. Instead, tomography affords information on the volume of the lacunae.

Immobilization results in a lack of muscle-forces on bones, which leads to bone demineralization e.g. in astronauts or during long bed-rest. Immobilization induced osteocytic osteolysis remains a subject of debate [19, 34, 35, 37, 38, 47, 48]. We used synchrotron tomography to study lacunar volumes of 1.85 million lacunae in rats with one hind leg immobilized by injection with botulinum toxin (‘Botox’) [19]. We found that immobilization resulted in lower bone volume and load to fracture in mechanical tests. However, synchrotron tomography showed no changes in osteocyte lacunar volumes, density, shape or orientation upon immobilization or during subsequent recovery. Additionally, no changes in bone mineral density were detected. Thus osteocytes did not participate in bone demineralization upon this mode of immobilization [19].

Lactation has been claimed to result in osteocytic osteolysis [37, 40-42, 44, 46]. Several mice strains have been studied: In CD1 mice, lacunar areas were increased upon lactation [37] accompanied by reduced pH in lacunae [42]. Increased osteocyte lacunar sizes were found in lactating C57BL/6 mice [40, 41]. We investigated lactating NMRI mice. We used both SEM and optical microscopy – the tools of most preceeding studies – to map lacunar areas supplemented by synchrotron tomography to study lacunar volumes [17]. We found no change upon lactation in neither lacunar areas nor volumes showing that osteocytic osteolysis did not contribute to calcium release from the skeleton in this mouse strain. The use of 3D tomographic data strongly increased our confidence in the results, since the volumetric measure can be expected to be more accurate than 2D area-based estimates of lacunar sizes. The degree to which osteocytic osteolysis occurs across species remains an open question for future investigations.
3. HIGH-RESOLUTION TOMOGRAPHY REVEALS CANALICULAR JUNCTIONS

The osteocyte lacunae are interconnected by canaliculi a few hundred nanometer in diameter. To study these therefore requires higher resolution than that achievable by standard tomography techniques. To this end, ptychography [24] and holotomography have been used [16, 26-28]. We undertook studies of the lacuno-canalicular network in mice using holotomography at ID16 at the ESRF using 17 keV photons. In holotomography [25], a focused X-ray beam is used and the sample placed at several positions downstream of the focus to sample various degrees of phase contrast propagation allowing to recover the phase information from the sample. We used local tomography at four distances to generate images at 130 and 50 nm isotropic voxel sizes on rods of mouse femoral bone with a maximum diameter of 400 µm. The data allowed extracting the details of the lacuno-canalicular network as illustrated in Figure 4.

We found small void volumes at places, where several canaliculi crossed (Figure 4B). Their geometrical properties were characterized from 130 nm voxel size data. Fitting best fit ellipsoids resulted in the smallest and largest diameters having medians around 1.3 and 4.4 µm, respectively. This compares to median lacunar volumes of 154 µm³. The median junction to lacunar distance was 14 µm, which corresponded well to simulations of distance distributions assuming a random distribution of junctions compared to lacunae. However, junction to junction distances were 8 µm, which is shorter than those predicted from a random junction distribution indicating that junctions cluster, possibly in regions of higher canalicular density. The density of junctions was of similar order of magnitude as lacunae, 44 ∙ 10⁹ mm⁻³ compared to 76 ∙ 10⁹ mm⁻³. This observation has significant bearings on our understanding of the osteocyte lacuno-canalicular network. First, junctions may provide a means for multicellular communication suggesting that the intercellular communication in bone may be more complex than hitherto assumed. Secondly, osteocytes are suggested to sense mechanical damage to bone through changes in fluid flow through the lacuno-canalicular network. We could show by confocal scanning laser microscopy on dye infused samples that junctions were connected to the fluid flow system of the osteocyte lacuno-canalicular network. Secondly, fluid flow simulations showed that the presence of junctions resulted in drastic changes to the fluid flow dynamics. This suggests that junctions play a significant role in osteocyte biology. They were only discoverable through 3D imaging with ultrahigh resolution as made possible by modern nanotomography techniques. We expect that these techniques will progress to teach us many more lessons about bone biology as techniques continue to improve.

![Figure 4. Osteocyte lacuno-canalicular network from mice based on 50 nm voxel size holotomography data [16]. (A) Selected sub-volume through the void space left by the osteocyte lacuno-canalicular network featuring four osteocyte lacunae (1-4) with lacunae 2-4 only being partially within the field of view. (B) Lacuna 1 from (A). Two canalicular junctions are seen, each at the junction of five canaliculi (white circles).](https://www.spiedigitallibrary.org/conference-proceedings-of-spie)

4. CONCLUSION

Tomography is invaluable in bone research, since bone is intrinsically three dimensional in nature. Synchrotron radiation-based computed tomography techniques are especially useful for studies of the osteocyte lacuno-canalicular network. These studies benefit from the power of synchrotrons in a number of ways, in particular

1. The high throughput made possible by the high flux of synchrotron light sources allows investigating a large number of samples, which is essential for addressing biological questions due to the large biological variation between individual animals.
2. The near monochromatic nature of many synchrotron tomography setups affords access to quantitative determination of the bone mineral content without the negative effects of beam hardening. Combined with the high spatial resolution, this means that measures of the true bone matrix mineral content can be determined accurately and precisely.

3. Nanotomography techniques, such as ptychography and holotomography, afford ultrahigh resolution, which allows assessing the full lacuno-canalicul network in detail and in 3D in large volumes. This information is essential for improving our understanding of this intriguing cellular network.

With the emergence of diffraction limited storage rings with very high coherence, these advantages will only increase. We therefore foresee a bright future for synchrotron tomography in bone science.

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