INTRODUCTION: Osteocytes, the most abundant cells in mature bone, are strategically placed to regulate the homeostasis and mechanical adaptation of bone [1]. Interstitial fluid flow and solute transport have been hypothesized to be involved in osteocyte metabolism and mechanosensitivities [2]. Recent experimental and theoretical studies have shown that the anatomical features of the lacunar-canalicular system (LCS), the fluid pathway in bone, are determining factors of the magnitude of fluid flow and cellular stimulation [3, 4]. Using a newly developed fluorescence recovery after photobleaching (FRAP) approach, we have measured fluid flow and solute transport in the cortex of long bone [4, 5, 6]. However, for trabecular bone, there are few studies on load-induced fluid flow or solute transport. In theory, single trabeculae are of the same dimension (200 µm) as the osteons in cortical bone, thus convective transport is also critical for the most inner cells of trabecular bone. To quantify transport characteristics of trabecular bone, the anatomy of the osteocyte LCS has to be determined as previously done for cortical bone [7]. The objective of this study was to characterize osteocyte LCS anatomical features relevant to fluid transport using confocal imaging.

METHODS:
Sample Preparations. Skeletally mature C57BL/6J male mice (n = 3, 16 weeks old; Jackson Laboratory) were sacrificed under IACUC approval, and the right femurs dissected, fixed, and bulk stained in basic fuchsin before embedding in methyl methacrylate [7]. The distal 1/3 of the femurs were cut into 0.2 mm thick slices along the sagittal plane using an Isomet saw (Buehler), polished using sandpaper and polishing liquids, and mounted on glass slides with Eukitt’s mounting medium and a cover slip.

Confocal Imaging. Both cortical bone (femoral cortex) and trabecular bone (1.25 mm below the epiphyseal growth plate) were imaged using an inverted confocal laser-scanning microscope (Zeiss LSM 510). For measurements of lacunar size and canalicular number density, lacunae were randomly selected from both regions and subjected to 3D high-resolution imaging. Regularly spaced grids were overlaid on a preview image and only the lacunae that fell on the grid intersections were chosen for 3D imaging. Z-stacks of 2048×2048-pixel images were captured of the chosen lacunae with a z step of 200-nm using a 40× (1.2NA) water immersion objective (Zeiss C-Apochromat Korr) under 561/650 nm excitation/emission and a pin hole of 1 Airy unit. A total of 60 lacunae in the cortical bone and 45 lacunae in the trabecular bone were imaged from three murine femurs. For measurements of lacunar density, 40 images (2048×2048-pixel) of each bone type were captured using a 10× (0.30NA) objective (Zeiss EC Plan-NeoFluar).

Quantitative Measurements. 3D renderings of individual lacunae with their associated canaliculi were reconstructed from the z-stack images (Fig. 1A) using VOLUENCY software (PerkinElmer). The number of canaliculi emanating from the lacunae surface were counted directly from the 3D renderings. Segmentation of lacunae and their canaliculi was achieved using the software AMIRA (Visage Imaging, Inc. (Fig. 1B). Osteocyte lacunae were isolated from the surrounding canaliculi and their volume and surface area were measured.

RESULTS: In both cortical and trabecular bone, the lacunae resembled scalene ellipsoids in which the length of the three axes differed from one another (one-way ANOVA test P < 0.05). However, in trabecular bone, the length of the major axis was shorter than that in cortical bone, while one minor axis length was longer, which indicated a shape difference between lacunae in the two types of bone. Lacunar volume, surface area, and canaliculi number did not show significant difference between cortical bone and trabecular bone. Osteocyte lacunae number density per unit area in cortical bone was 1200.7 ± 149 cells/mm², higher than that in trabecular bone (1078.2 ± 213.9 cells/mm², P < 0.05).

DISCUSSION: In this study we found that osteocyte lacunae in cortical bone do not differ from those in trabecular bone in volume, surface area and canaliculi number. The lacunae volume values were similar to previous reported values (350 µm³) found in rabbit long bone at the mid-diaphysis [8]. Canaliculi number values differed from those in a previous study that found the canaliculi numbers to be 41 for human trabecular bone, 54 for chick bone and 115 for equine bone [9]. However, the previous studies were based on measurements performed using 2D light microscopy and scanning electron microscopy. The lacunae number density in this study was smaller in murine trabecular bone, although similar to previously reported values in adult rat trabecular bone (810–1060 cells/mm³) [10]. This study demonstrated that lacunae are more like triaxial ellipsoids since the lengths of the three axes are significantly different. Our lacunae values were also similar to previous measures in adult rat tibia that found three axes lengths to be 17.6 ± 0.3 µm, 6.1 ± 0.3 µm, 4.0 ± 0.2 µm [11].

A potential limitation of the current study is the absence of z-direction optical correction, used to adjust for the distortion of images in confocal microscopy. We are currently attempting to quantify the correction factor in our system, but do not expect this factor to exceed ~0.9 based on previous studies [12]. In summary, our confocal images revealed that osteocyte lacunae and their associated canaliculi could be clearly visualized, allowing us to better characterize the lacunar-canalicular microstructure in both trabecular and cortical bone. These measures provide anatomical data for experimental studies on solute diffusivity and fluid flow velocity in trabecular bone under mechanical loading [4 – 7].

SIGNIFICANCE: The trabecular osteocyte LCS characteristics obtained in this study are crucial for better understanding the role of fluid flow in trabecular bone's mechanotransduction, which is critically involved in normal bone adaptation and pathological bone loss.

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