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A quantitative framework for the 3D characterization of the osteocyte lacunar system

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ABSTRACT

Assessing the role of osteocyte lacunae and the ways in which they communicate with one another is important for determining the function and viability of bone tissue. Osteocytes are able to play a significant role in bone development and remodeling because they can receive nourishment from, interact with, and communicate with other cells. In this sense the immediate environment of an osteocyte is crucial for understanding its function. Modern imaging techniques, ranging from synchrotron radiation-based computed tomography (SR CT) to confocal laser scanning microscopy, produce large volumes of high-quality imaging data of bone tissue on the micrometer scale in rapidly shortening times. These images often contain tens of thousands of osteocytes and their lacunae, void spaces which enclose the osteocytes. While theoretically possible, quantitative analysis of the osteocyte lacunar system is too time consuming to be practical without highly automated tools. Moreover, quantitative morphometry of the osteocyte lacunar system necessitates clearly defined, robust, and three-dimensional (3D) measures. Here, we introduce a framework for the quantitative characterization of millions of osteocyte lacunae and their spatial relationships in 3D. The metrics complement and expand previous works looking at shape and number density while providing novel measures for quantifying spatial distribution and alignment. We developed model, *in silico* systems to visualize and validate the metrics and provide a concrete example of the attribute being classified with each metric. We then illustrate the applicability to biological samples in a first study comparing two strains of mice and the effect of growth hormone. We found significant differences in shape and distribution between strains for alignment. The proposed quantitative framework can be used in future studies examining differences and treatment effects in bone microstructure at the cell scale. Furthermore, the proposed strategy for quantitative bone cell morphometry will allow investigating structure–function relationships in bone tissue, for example by linking cellular morphometry to bone remodeling.

Introduction

Over the last years, sound evidence has demonstrated that osteocytes, the most abundant cells in bone, play a role in mechanosensation [29,27], mineral homeostasis [62,25], and bone mass regulation [30,64]. Groups of osteocytes form a cellular network through their slender cell processes called canaliculi, which link the individual osteocytes together and with other bone cells. This cellular network, embedded inside the bone tissue, forms the lacuno-canalicular network (LCN) [52]. The LCN consists of microstructural porosity elements, which enclose the osteocytes and their cellular processes. The traditional approach for studying the osteocyte network and the LCN in three dimensions (3D) uses confocal laser scanning microscopy (CLSM), which, if combined with appropriate fluorescent labels, allows for functional bone imaging. Yet, this advantage comes at a price since the samples require extensive preparation and that only a limited depth can be probed. These factors ultimately limit the number and size of samples to be studied and make studies with more than 10 samples very time consuming. Standard 3D imaging techniques with higher penetration power, such as desktop micro-computed tomography (μCT) or magnetic resonance imaging (MRI) still lack the ability to visualize the LCN with typical dimensions of osteocyte lacunae 10 μm and volumes ranging from 200–600 μm^3 in a stable, time-efficient manner [52]. More recent developments have further increased the resolution of desktop μCT scanners, but issues with stability, and extremely long measurement times (>10 h) make such techniques impractical for larger studies. Higher spatial resolution techniques (<1 μm), such as electron microscopy, capable of visualizing the canaliculi are impractical because their range of depth is typically limited to a few micrometers for bone tissue [22]. In addition, coherent X-ray experiments can be used to visualize canaliculi, but they are usually time and sample size prohibitive [52,25]. Furthermore post-processing techniques [41] in combination with high-resolution phase sensitive

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tomographic imaging [33] have recently been able to extract the canaliculi from small regions, but while they offer a many fold improvement on speed, they still have limited fields of view and require cutting the sample. Given the rapid pace of development, this technique may soon be practical for more detailed studies of the osteocyte lacunar network on a larger scale.

High-resolution 3D imaging technologies, such as synchrotron radiation-based CT (SR CT), have been applied on bone tissue to assess thousands of lacunae in cortical bone microstructure with millimeter-sized field of views and sub-micron voxel-sizes [2,7,10,52,33,26]. Although measurements and analyses have already been conducted within many biological studies, there has been little work to develop a consistent, well-defined, technique and scale independent set of quantitative metrics for describing the osteocyte lacunar network. In order for results to be comparable and more abstract notions like spatial distribution, organization, and alignment to be quantified, a number of new metrics need to be introduced. Since many of these ideas are new and initially abstract, a set of *in-silico* lacunae where shape and position are specified, is required to show how the metrics characterize changes in the lacunae and provide a baseline for comparing the results with random values.

While the images from the discussed techniques are impressive, appropriate and automated quantitative tools for evaluation of the LCN are needed to take full advantage of a number of new avenues in imaging at the cell scale. For osteocytes and osteocyte lacunae, several works have addressed the task of providing descriptive metrics about overall shape, orientation, global number densities, and occupancy ratio [7,10,53,60,65,67]. The work of Ascenzi et al. [2] has gone further, looking locally and quantifying analytically the properties of individual osteocyte lacunae in human secondary osteons. Specifically, the osteocyte lacunar orientation with respect to lamellae was calculated by fitting an ellipse to lacuna images obtained by confocal microscopy. The limitations of this study are that it is a two-dimensional (2D) approach and the need for substantial user interaction, which is problematic for reporting on reproducible results. More recent works such as Ref. [25] have examined a large number of metrics related to connectivity and spatial distribution, but only on small samples. With such large data sets, a clear, flexible, and power set of tools are required to analyze the data well. Following the guidelines put forth in recent "Reproducible Research" initiatives, the quantitative analysis should be performed using open or as open as possible software packages, and statistical analysis should be made publicly available, a task which is currently not possible with many packages [44,57,40]. The availability of the raw or cached data and analyses performed allows detailed inspection of the results obtained and the possibility to easily do further analyses.

BoneJ, a toolbox that was developed by Doube et al. [15] for standard bone measures in the form of an ImageJ plug-in (http://rsb.info.nih.gov/ij/; U.S. National Institutes of Health, Bethesda, Maryland, USA), makes a major step towards these goals by offering an open-source technique for consistently calculating several whole-bone, trabecular, and osteocyte lacunar measures. While the tools are principally designed for lower resolution investigation looking at trabecular bone and entire specimens, a basic 3D shape analysis tool is provided for extracting osteocyte lacunar measures, such as surface area, volume, and orientation for segmented osteocyte lacunae. However, this toolset lacks the flexibility to perform a number of different analyses, necessary for large-scale studies and detailed analysis of distribution, alignment, and other osteocyte lacunar measures. The analysis of osteocyte lacunae in a given bone specimen presents several challenges, particularly in scale, because even a small tissue volume of 1 mm^3 contains between 10,000 and 100,000 individual osteocyte lacunae [52]. Consequently, novel image post-processing methods for quantitative morphometry, measuring shape, orientation, and characterizing distribution of osteocyte lacunae must be fully automated. Yet, until now, there have only been limited efforts to systematically define, verify, and validate the 3D shape, orientation, and distribution measures for the quantification of osteocyte lacunae on the same level as well-established quantitative bone morphometry metrics [6,43]. Therefore, the main goal of this study was to introduce, validate, and apply a novel framework to quantitatively assess 3D shape, orientation, and distribution of osteocyte lacunae in a fully automated, high-throughput manner. Furthermore, we wish to provide a set of easily visualized metrics to illustrate the quantitative differences between groups using a few simple figures. Inspired by existing measures in the field of bone research, material science, and soft matter physics, we adopt some of the pertinent metrics to provide a comprehensive and robust scheme for characterizing osteocyte lacunae in bone. A wider aim of the study is to introduce a consistent instrumentation-independent pipeline for taking microstructural 3D imaging data to meaningful quantitative information on the sample. Due to the abstract nature of the metrics and the oversimplification of synthetic data, we present a study, where we assess the lacunar descriptors for femoral bones from two different mouse strains with and without growth hormone measured using SR CT. The study shows the utility of the given metrics to quantify real systems and insight into which metrics are conserved between strains and how growth hormone might affect microstructure.

Materials and methods

To establish a framework for the quantitative assessment of osteocyte lacunae, we first define mathematically the osteocyte lacunar descriptors (definition). We then verify the descriptors using *in silico* models (verification). We then use the osteocyte lacunar descriptors to compare femoral bones from two different inbred mouse strains as measured using SR CT (application). In the Supplementary materials, we provide extensive details about the generation of the *in silico* models and the comparisons made between model generation parameters and numerically calculated osteocyte lacunar descriptors. We provide as well more details on the examinations of resolution dependencies and comparisons between statistical to physical osteocyte lacunar measures.

Definition of osteocyte lacunar descriptors

The following descriptors for the osteocyte lacunae are defined with a view to quantify their individual morphology, their spatial relationship to each other, and their position within the context of the whole-bone geometry. To this end, the osteocyte lacunar descriptors are grouped into three categories (Fig. 1): shape and orientation, local environment, and global environment. This hierarchical organization allows analysis of the osteocyte lacunar system beginning from the cellular level (shape and orientation) going up to the whole-bone scale (global environment). The shape descriptors characterize the dimensions, orientation, and anisotropy of each lacuna individually. The local environment measures provide ways to collectively characterize the surroundings of a particular lacuna including its neighbors and the amount of surrounding bone. The metrics provide the ability to determine the relationship between lacunae and their immediate neighboring lacunae and the corresponding distances and orientations. Finally, the distribution and alignment metrics provide the ability to assess the entire bone or regions of interests with a single metric. For the summary statistics (e.g. average stretch), the numbers represent first finding the average lacuna and then calculating the metric for that average lacuna, which reduces the effect of measurement error and noise.

We start by establishing a tensor framework for addressing this problem, which provides 3D descriptors that are independent of the imaging frame. The first tensor is the shape tensor (S) describing the individual lacuna, which will be discussed in Shape and orientation section. We then introduce two tensors in Section Local environment for characterizing the collective metrics for a group of lacunae. The distribution tensor (D) describes the spatial distribution and spacing between lacunae and the alignment tensor (T) describes the collective orientation.