Bone 110 (2018) 115–127

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

A new open-source tool for measuring 3D osteocyte lacunar geometries from confocal laser scanning microscopy reveals age-related changes to lacunar size and shape in cortical mouse bone

Chelsea M. Heveran [a], Adam Rauff [b], Karen B. King [c], R. Dana Carpenter [d,1], Virginia L. Ferguson [a,*,1]

[a] Department of Mechanical Engineering, University of Colorado Boulder, United States
[b] Department of Bioengineering, University of Colorado Denver, United States
[c] Department of Orthopaedics, University of Colorado School of Medicine, United States
[d] Department of Mechanical Engineering, University of Colorado Denver, United States

ARTICLE INFO

Article history:
Received 11 September 2017
Revised 15 December 2017
Accepted 14 January 2018
Available online 31 January 2018

Keywords:
Osteocyte
Aging
Confocal laser scanning microscopy

ABSTRACT

Osteocytes can participate in systemic mineral homeostasis through perilacunar maintenance and remodeling, where changes to osteocyte lacunar morphology may affect bone structural integrity, tissue strains, and osteocyte mechanosensitivity. Though aging is associated with both decreased bone quality and altered mineral metabolism, it is not known if osteocyte lacunae undergo age-related changes in geometry. In order to survey lacunar changes with age, we developed an open-source program whereby 3D osteocyte lacunae are automatically segmented and then subsequently reconstructed from confocal laser scanning microscopy (CLSM) depth stacks for quantitative analysis of geometry and orientation. This approach takes advantage of the availability and speed of CLSM while avoiding time-consuming and bias-prone manual segmentation. Unlike conventional approaches used to quantify osteocyte lacunar morphology, CLSM enables facile analysis in three-dimensions with clear identification of osteocyte lacunae. We report that 3D osteocyte lacunae measured by CLSM become smaller, more spherical, more oblate, more spatially disorganized, and more sparsely populated with increased age in C57Bl/6 mouse cortical bone in groups spanning 6–24 months old. Critically, these age-related changes are in large part not observed in 2D analyses from the same samples. These results (1) demonstrate proof-of-concept of an efficient method to quantitatively assess osteocyte lacunae in 3D for application to a wide range of studies and (2) motivate further inquiry into how changes to osteocyte lacunar geometries and perilacunar material contribute to diminished bone quality in aging.

1. Introduction

Bone quality and fracture resistance are decreased with aging [1–4]. Osteocytes, which account for approximately 95% of bone cells, participate in several ways to maintain fracture resistance-determining bone quality [5,6]. In addition to critical roles in mechanotransduction and in modulating bone turnover, the osteocyte has been more recently appreciated for its ability to both resorb and replace perilacunar bone in a process referred to as perilacunar remodeling [5–14]. These changes to perilacunar remodeling, which can result in altered osteocyte lacunar geometries, occur in a variety of physiologic conditions and diseases that alter mineral metabolism such as lactation [7–9], microgravity [15,16], glucocorticoid therapy [10,14,17], and vitamin D deficiency [18]. Aging may also change perilacunar remodeling and osteocyte lacunar geometries. Aging changes mineral metabolism, increases the prevalence of senescent osteocytes, and may change physical activity and loading patterns, all of which could influence the behavior of the osteocyte and its interaction with the perilacunar environment [19–21]. Several groups have noted a decreased number density of osteocytes with increased age, but it is unknown if and how osteocytes alter the lacunar geometry and orientation with aging [22–25]. The lacunar-canalicular network is voluminous, accounting for at least 1% of cortical bone volume [6]. Thus, changes to the sizes and shapes of osteocyte lacunae in aging could affect bone structural integrity, tissue strains, and osteocyte mechanosensitivity.

Very few studies have evaluated the effects of aging on osteocyte lacunar geometries. Mullender and coworkers, using 2D histomorphometry, found that osteocyte lacunae from trabecular bone of the iliac crest had smaller mean lacunar cross-sectional area for men and women aged 55 years and older, compared with individuals <55 years of age [25]. However, these age-related differences were not significant. Importantly, two-dimensional (2D) imaging may contribute

* Corresponding author.
E-mail address: virginia.ferguson@colorado.edu (V.L. Ferguson).
[1] VL Ferguson and RD Carpenter contributed equally as last authors.

https://doi.org/10.1016/j.bone.2018.01.018
8756-3282/

to the uncertainty about whether osteocyte lacunae change in aging due to several critical limitations. Osteocyte lacunae can appear indistinguishable from non-lacunar objects (*e.g.*, a vascular cross-section in bone). Furthermore, the ellipsoidal shape that generally characterizes osteocyte lacunae can yield a range of 2D shapes depending on the sectioning plane (Fig. 1). It is possible that 2D methods are sufficient for detecting large magnitude changes to osteocyte lacunae as observed with lactation, but 2D techniques have insufficient sensitivity to capture more subtle changes in lacunar morphologies that may result from with aging, disease, and therapeutic interventions.

Three-dimensional (3D) images can surmount the challenges of 2D quantification of osteocyte lacunar geometries. Previous efforts have utilized synchrotron-radiation microCT (SR-CT) to generate 3D images of osteocyte lacunae and surrounding canaliculi [14,26–29]. Conventional, submicron-resolution microCT (termed 'nanoCT') can also be employed for osteocyte lacunae visualization. However, both of these technologies are prohibitively expensive and unavailable for many researchers. By contrast, confocal laser scanning microscopy (CLSM) provides a relatively common, fast, and inexpensive technique with the ability to capture depth stacks (z-stacks) of osteocyte lacunae at submicron resolution. Some prior work has considered CLSM for the purpose of studying 3D osteocyte network geometries [24,30,31]. However, this technique has not been widely utilized, perhaps because of the necessity of either manually segmenting osteocytes, which is both time intensive and prone to user bias, or constructing algorithms to appropriately segment 2D osteocyte lacunae slices and then reconstruct and analyze 3D osteocyte geometries. An open-source tool for analyzing 3D osteocyte lacunar geometries from CLSM z-stacks would enable facile and time-efficient quantitative analysis of osteocyte lacunar morphologies.

CLSM has only been sparingly employed to characterize 3D age-related changes to osteocyte lacunae. Lai et al. reported that osteocyte lacunae from trabecular and cortical bone of male C57Bl/6 mice had smaller mean lacunar volume and surface area at 32 weeks of age than at 15 weeks, but these differences were not significant [24]. The potential effect of aging on osteocyte geometries reported by these authors is exciting. However, it is important to note that only three mice were studied at each age, and only 10 lacunae were analyzed for each cortical and trabecular compartment per study animal. In order to confirm the effects of aging on osteocyte lacunar morphology, it is necessary to involve larger numbers of aging animals as well as robustly large populations of lacunae from individual bone specimens.

In the present study, we employ CLSM to evaluate how 3D osteocyte lacunar geometries change with aging in large groups of lacunae imaged from mouse cortical bone. We present an open-source automatic segmentation method for convenient and time-efficient analysis of 3D osteocyte lacunae. The validity and limits of our approach are considered by analysis of phantom ellipsoidal objects and comparison with manual segmentation.

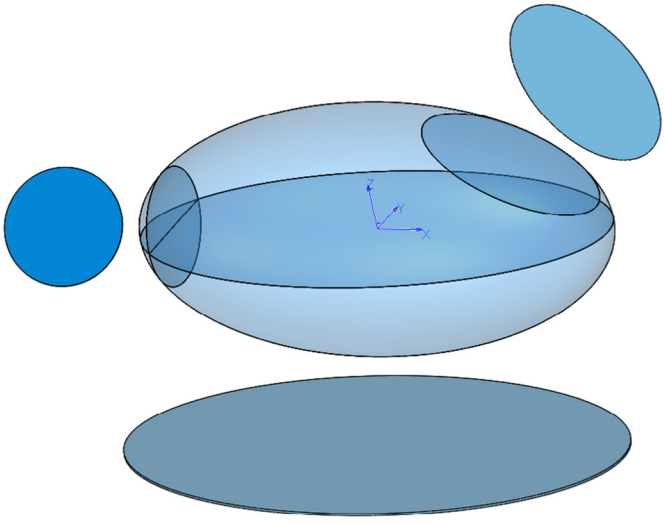

Fig. 1. Different 2D cross-sections of a 3D ellipsoid produce a variety of geometries. Accordingly, 3D methods are most appropriate for comparing osteocyte lacunar geometries between samples.

2. Materials and methods

In Section 2.1, a Matlab-based process to segment osteocyte lacunae from 2D images within z-stacks from CLSM to generated 3D osteocyte lacunae is described. Then, in Section 2.2, we describe an experiment in which cortical bone from aging mice was imaged with CLSM and evaluated to quantify the effects of age on osteocyte lacunar morphology.

2.1. Visualization and quantification of 3D osteocyte lacunar geometries

2.1.1. 3D osteocyte lacunae segmentation and reconstruction

The "3D Osteocyte Lacunae Analysis" program for segmenting and reconstructing osteocyte lacunae from confocal z-stacks involves the following steps (Fig. 2):

1) Thresholding: Images are first stored as individual 2D image slices. Each image within a z-stack first undergoes smoothing *via* Gaussian filter and then thresholding. The image is filtered with a 2D smoothing kernel specified by sigma, (0.65 in our program). The mean of the smoothed image is obtained and used for the thresholding parameter. Thus, each image slice has a different threshold which is proportional to the mean intensity of that slice. The thresholding parameter is the mean of the Gaussian smoothed image multiplied by 1.9. This value, as well as sigma, worked well with our mouse bone image data, and could be easily modified to better fit other data sets. The binarized 2D images within each stack are then stored in a 3D matrix, referred to here as '3D_Segmented_Image'.
2) Filling holes: Holes are filled for objects within the 3D matrix using the Matlab function imfill(3DSegmentedImage, 'holes'). Specifically, black pixels within the white outlines of the 3D lacunar edges are converted to white pixels so that each lacuna becomes a filled volume. The resulting image is called '3D_Segmented_Image_Filled.'
3) Erosion and dilation: These imaging processing operations are commonly used to eliminate small, unwanted objects remaining from thresholding. The functions used in the program operate on 3D volumes. A spherical (radius = 5 pixels) structuring element (SE) is chosen for the erosion and dilation procedure. Objects in the 3D matrix are eroded with reference to the structuring element to remove osteocyte lacunae processes and canaliculi and are then dilated back to their original volumes. The Matlab function imopen (3D_Segmented_Image_Filled, SE) achieves the erosion and dilation procedure, using the same SE for each step.
4) Edge filter: segmented objects are removed if any portion of the object touches an image boundary. This prevents incomplete objects from inclusion in the final analysis.
5) Volume filter: segmented objects smaller than 100 μm^3 or larger than 2000 μm^3 are removed, as osteocyte lacunae observed in this study generally have volumes between 200 and 600 μm^3.
6) User selection and removal of non-osteocyte lacuna objects: following steps 1–5, the segmented and filtered 3D image is visualized in a custom Graphical User Interface (GUI) named Visualize Lacunae GUI. Users can visualize the segmentation results and select objects (*e.g.*, vasculature) for exclusion.
7) Analysis: 3D osteocyte lacunae geometry and orientation metrics are extracted from the final segmented image according to the parameters outlined in the following section.

2.1.2. Osteocyte lacunae network parameters

Osteocyte network parameters in 3D and 2D are summarized in Fig. 3. The parameters presented here are predominantly focused on