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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

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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.

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Keywords

Osteocyte; aging; confocal laser scanning microscopy

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, have been reported to 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 or older, compared with individuals less than 55 years of age (25). However, these age-related differences were not significant. Importantly, two-dimensional (2D) imaging may contribute 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 (Figure 1). It is possible that 2D methods are sufficient for detecting large 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). Submicron-resolution conventional 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 study 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.

2. Materials and Methods

In §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 §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

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 (Figure 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'.

- 1) 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.'
- 2) 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.
- 3) 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.
- 4) Volume filter: segmented objects smaller than $100 \mu\text{m}^3$ or larger than $2000 \mu\text{m}^3$ are removed, as osteocyte lacunae observed in this study generally have volumes between $200\text{--}600 \mu\text{m}^3$.
- 5) 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.
- 6) Analysis: 3D osteocyte lacunae geometry and orientation metrics are extracted from the final segmented image according to the parameters outlined in the following section.

Osteocyte lacunae network parameters—Osteocyte network parameters in 3D and 2D are summarized in Figure 3. The parameters presented here are predominantly focused on describing osteocyte lacunar geometries as well as the relative alignment of osteocyte lacunar networks. These parameters provide a succinct description of 3D osteocyte lacunae geometries and orientation that are well-paired to the capabilities of CLSM. For a thorough description of osteocyte geometries from the local to network scale, the reader is referred to the framework presented by Mader and coworkers (32).

Our work employs “lacunar voxel-fill” and “ellipsoidal fit” definitions of ellipsoidal objects for calculation of volume and surface area. We calculate *lacunar voxel-fill volume* from the number of voxels that comprise the 3D osteocyte lacunar object volume. *Lacunar surface*

area is calculated by performing a Delauney triangulation of the voxels forming the object surface, and summing the area of the triangles. *Ellipsoidal volume* and *ellipsoidal surface area* are calculated consistent with previous work (24, 30, 32). Briefly, an inertia matrix is calculated for each lacuna with reference to its center of mass. The eigenvalues and eigenvectors of this inertia matrix are calculated to yield the principal moments of inertia (I_a , I_b , I_c in increasing order) and principal axes directions, respectively. The principal moments of inertia are related to the ellipsoidal radii (a , b , c) and lacuna voxel-fill volume (m) as follows (30):

$$I_a = m \left(\frac{b^2 + c^2}{5} \right); I_b = m \left(\frac{a^2 + c^2}{5} \right); I_c = m \left(\frac{a^2 + b^2}{5} \right) \quad \text{Equation 1}$$

The solution to this system of equations delivers the following relations for ellipsoidal radii:

$$a^2 = \frac{5}{2m} (I_b + I_c - I_a); b^2 = \frac{5}{2m} (I_a + I_c - I_b); c^2 = \frac{5}{2m} (I_a + I_b - I_c) \quad \text{Equation 2}$$

The radii of this best-fit ellipsoid are then utilized to calculate the volume and surface area of the lacuna.

Sphericity is the ratio of the smallest to largest radii and is effectively a measure of lacunar stretch. For the ellipsoidal approach, radii are calculated from the principle moments of inertia. For the lacunar-fill approach, radii are calculated directly from the center of mass along the principle directions to the object edge. A value of 1 indicates a perfect sphere, with decreasing values indicating a more elongated geometry.

Oblateness is a measure of the tendency of the lacuna to be prolate (rod-shaped) or oblate (plate-shaped). Oblateness is defined by Equation 1, where -1 and +1 indicate perfectly prolate and perfectly oblate geometries, respectively (32). Radii a , b , c are calculated from the best-fit ellipsoid and are in increasing order.

$$\text{Oblateness} = 2 \left(\frac{b - a}{c - a} \right) - 1 \quad \text{Equation 3}$$

Closest center of mass is defined as the shortest distance in 3D space between osteocyte lacunae centers of mass. This value is calculated for each osteocyte lacunae within the sample and provides a measure of how far apart the lacunae are spaced.

Span theta is defined as the absolute value of angle difference (in degrees) between the average of all lacunar principle axes and the principal axes of individual osteocyte lacunae. This parameter estimates the degree of alignment of a group of osteocyte lacunae in 3D space. The name of this variable was chosen as it only exists on the plane that spans the vectors of the individual principal axes and the average principle axis.

A slice from each z-stack (5th from the imaging surface) was analyzed for 2D analogs of the 3D metrics. *Area* measures the area of an ellipse fitted to the segmented 2D osteocyte lacunae. *Perimeter* is the distance around the boundary of the segmented lacuna. *Circularity* is the ratio of minor to major axes of the fitted ellipse, where a value of 1 indicates a perfect circle. *Closest center of mass* is the shortest distance between centers of mass in 2D space. The 2D equivalent of *span theta* is the standard deviation of angles between the major axis and an arbitrary x axis for all lacunae.

Validation—We first verified that the program appropriately quantifies 3D osteocyte lacunar geometries through analyzing phantom objects. These 3D ellipsoids were created with known dimensions and orientations and are available as open-source files along with the 3D Osteocyte Lacunae Analysis program (Figure 4).

We next estimated the percentage of osteocytes present in bone that are segmented by the 3D Osteocyte Lacunae Analysis program and included in the 3D reconstruction for analysis of lacunar network geometries. Some loss of lacunae in an automatic segmentation protocol is expected, as segmentation is affected by inhomogeneity in stain penetration and the presence of bright objects such as bone marrow within the image. In order to estimate an expected number of lacunae to compare with results from automatic segmentation, manual segmentation was performed for three cortical mouse bone z-stacks (*i.e.*, one randomly chosen sample from each of the three age groups described in 2.2) using ScanIP (version 7.0, Simpleware, Synopsys Inc). The manually segmented lacunae were subjected to a volume filtration step analogous to volume filtering within the automatic 3D Osteocyte Lacunae Analysis program (objects with volumes less than 100 μm^3 or more than 2000 μm^3 were excluded). Manually segmented lacunae were not further modified (*i.e.*, no dilation / erosion or smoothing) before analysis of lacunar geometries. The number of osteocyte lacunae segmented by the 3D Lacunar Analysis program was compared with the number obtained through manual segmentation. Lacunar geometries obtained from automatic and manual segmentation for the same samples were then compared.

2.2 Demonstration of technique

We applied the protocol presented in §2.1 for visualization and quantification of 2D and 3D osteocyte lacunar geometries to evaluate osteocyte lacunae in cortical bone from skeletally mature young adult, middle-age, and old mice.

Male C57Bl/6J mice were obtained from Jackson Laboratories at the ages of 3, 15, and 21 months. Mice were housed in a AAALAC-accredited facility individually in polycarbonate cages with standard bedding and ventilation, on light/dark cycles with light from 6 am to 8 pm. Mice were fed Harlan Teklad 2920X chow and allowed free access to water. Mice underwent sham (*i.e.*, placebo surgery) abdominal procedures as a control for another study. Mice were anesthetized with isoflurane (3% inductance, 1.5% maintenance) during procedures and were administered post-operative analgesia (buprenorphine, 0.05 – 0.2 mg/kg²). Mice were euthanized three months after sham procedures *via* CO₂ and cervical dislocation. At the study endpoint, mice were 6 months (young skeletally mature adult, n = 6), 18 months (middle-age, n = 7), and 24 months (old, n = 6) of age (1). All animal

procedures were approved by the Institutional Animal Use and Care Committee at the University of Colorado Denver.

Cortical bone sample preparation and imaging—The left tibia was cleaned of non-osseous tissue and simultaneously histologically dehydrated and stained with 1% basic fuchsin (33). Tibias were then cleared with acetone and embedded in PMMA. Embedded samples were sectioned 1 mm proximal to the tibia-fibula junction with a low-speed saw, with care taken to obtain a consistent cut transverse to the long axis for each sample (Buehler Isomet, Buehler, Lake Bluff, IL). A ground section was prepared from the distal section with target thickness of 200 μm (Exakt 400 CS, Exakt Technologies Inc, Oklahoma City, OK). Final sections had thicknesses of $195 \mu\text{m} \pm 3 \mu\text{m}$. Additional samples were prepared with a variety of thicknesses in order to assess the dependence of measures on specimen thickness and number of visualized osteocyte lacunae.

Confocal imaging of ground sections was performed in transmission with a Zeiss LSM 710 confocal laser scanning microscope, with 555 nm excitation, 568-1000 nm bandpass filter, and 40x oil-immersion objective. Stacks of 2D images with dimensions of $5.24 \times 10^4 \mu\text{m}^2$ (512×512 pixels, scale $0.447 \mu\text{m} / \text{pixel}$) were obtained through the visible range of osteocyte lacunae (~ 70 - $115 \mu\text{m}$ of imaging depth) with $0.484 \mu\text{m}$ between slices. The region of interest was the same for all bones and is shown in Figure 2. All imaging was performed blinded to specimen age.

Comparison of 2D and 3D osteocyte lacunar geometries—Three-dimensional osteocyte lacunar geometries were analyzed as described in §2.1. In addition, 2D osteocyte lacunar geometries were evaluated from a single slice of the same z-stack for each mouse. The fifth image from the top surface was chosen to take advantage of high contrast lacunae while minimizing effects of surface roughness. The 2D images were saved in .tif format and analyzed with custom Matlab code. The Gaussian blur was the same as for the 3D analysis. An area filter excluded objects less than $5.61 \mu\text{m}^2$ and greater than $191.9 \mu\text{m}^2$, the 2D equivalents of spheres with volume $10 \mu\text{m}^3$ and $2000 \mu\text{m}^3$, respectively. The erosion-dilation scheme was modified such that the structuring object was a disk instead of a sphere. Lastly, objects that were not osteocytes (*e.g.*, bone marrow) were excluded by user specification. From each image, the following 2D geometric parameters were obtained: *area*, *perimeter*, *circularity* (ratio of smallest to largest radii), *closest center of mass*, *lacunar number density* (number of lacunae / image area), and *span theta*. In 2D, the interpretation of span theta is the difference in angle between the direction of the major axis for each osteocyte and the average direction of all lacunae major axes.

2.3 Data analysis

The effect of age on 3D and 2D osteocyte lacunar parameters was evaluated using one-way ANOVA (Minitab v.17). Covariates of imaging depth and number of lacunae were included in additional ANCOVA analyses. Residuals were checked for normality and heteroscedasticity; the dependent variable was transformed when necessary to satisfy ANOVA model assumptions. The Tukey post-hoc procedure was utilized to compare means between ages. For all analyses, alpha was set *a priori* to $p = 0.05$. In addition, correlations for

image depth vs number of lacunae, ellipsoidal vs lacunar metrics, and 3D vs 2D metrics were performed with OriginPro 2016.

3. Results

Validation

The program succeeded in evaluating expected geometries for phantom ellipsoids using the approach outlined in §2.1. A comparison of manual segmentation (ScanIP) and automatic segmentation (3D Osteocyte Lacunae program) demonstrated that 77.1 – 97.8% of the lacunae that were manually segmented were captured with the automatic approach described herein. Lacunar geometries differed for automatic and manual segmentation (Table 1). For example, ellipsoidal volume was substantially larger for automatic segmentation. However, ellipsoidal volume measured by automatic segmentation strongly correlates with volume measured by manual segmentation ($r^2 = 95.6\%$), indicating a systemic bias. Similarly, surface area from automatic segmentation was larger than from manual segmentation, but the two measures show an ideal correlation of $r^2 = 100\%$.

Comparison of ellipsoidal and lacunar-fill metrics

Ellipsoidal and lacunar-fill metrics demonstrated excellent agreement (Figure 8), with the correlation coefficient r^2 approaching 1 for both volume and surface area. Ellipsoidal and lacunar-fill metrics are not independent, but their excellent agreement indicates that lacunae are well-described as ellipsoids. We proceeded with ellipsoidal definitions of all 3D measures for the study of aging on osteocyte lacunar geometries, but lacunar-fill geometries yield similar results (see Supplemental Data). A complete description of the effect of aging on osteocyte geometries measured by the lacunar-fill technique is provided in the Supplementary Data.

Stability of osteocyte lacunar geometries with respect to imaging depth and number of imaged lacunae

3D osteocyte lacunar geometries were observed to depend on imaging depth and number of osteocyte lacunae for thin sections and small numbers of segmented lacunae (Figure 5-6). *Surface area* and *volume* only weakly depend on imaging depth (Figure 5a-b) and number of osteocyte lacunae (Figure 6a-b). *Closest center of mass* appears to be biased towards larger values when measuring thin sections and small numbers of lacunae (Figures 5d and 6d). It is possible that *closest center of mass* is artificially high for thin sections because a relatively high number of lacunae touch an edge and are excluded from analysis, thus increasing the distance between lacunae. This bias is mitigated when measuring sufficiently thick sections that supply larger numbers of lacunae (*i.e.*, greater than about 60 micrometers of imaging depth and 50 lacunae per 3D image). *Sphericity* (Figures 5c - 6c), *Span theta* (Figures 5e - 6e), and *Oblateness* (Figures 5f - 6f) did not depend on imaging depth or number of lacunae. We chose to analyze samples with 50 or more lacunae to ensure adequate sampling of lacunae size, shape, and orientation. The necessary imaging depth (the depth of the z-stack from CLSM imaging) to obtain 50 or more segmented lacunae was approximately 60 μm (Figure 7), which matched the depths at which we observed the onset of parameter stability from Figure 6.

Comparison of 2D and 3D osteocyte lacunar geometries

3D geometric measures were regressed against 2D analogues generated from an in-focus slice of the same z-stack (Figure 9). In general, 2D measurements insufficiently predicted 3D outcomes from the same (*i.e.*, matched) lacunae. Of the 2D measures, *Lacunar number density* was the best predictor of a 3D measure with $r^2 = 0.426$. *Closest center of mass* in 2D modestly predicted the same measure in 3D ($r^2 = 0.362$). 2D *Area* was also modestly predictive of 3D *volume* ($r^2 = 0.313$), whereas 2D *Perimeter* was poorly predictive of 3D *surface area* ($r^2 = 0.0902$). 2D *span theta* was poorly predictive of the same measure in 3D ($r^2 = 0.129$). Finally, *circularity* was not predictive of *sphericity*, with $r^2 = 0.000$.

Effect of age on osteocyte lacunar geometries

Age was seen to influence 3D *volume*, *surface area*, *sphericity*, *oblateness*, and *span theta* with $p < 0.05$ for the main effect of age (Table 2, Figures 10 - 11). The following post-hoc comparisons between ages were adjusted by the Tukey procedure to set family-wise error at $p = 0.05$. *Volume* and *surface area* were reduced by -39.8% ($p = 0.027$) and -30.6% ($p = 0.019$), respectively, in old mice compared with young mice. *Sphericity* was +10.3% higher ($p = 0.036$) for middle-age than young mice and +16.3% higher ($p = 0.004$) for old mice than young mice, while *oblateness* was +72.3% greater ($p = 0.003$) for old versus young mice. *Span theta* was +54.1% higher ($p = 0.026$) for old mice compared with young mice. The lacunar number density, the number of osteocytes per volume of segmented bone, was reduced by -37.7% ($p = 0.010$) for old mice compared with young mice. Properties from middle-age mice were generally intermediate between those of old and young mice. The only measure not affected by age was 3D *center of mass*. To confirm that these age-related changes to 3D osteocyte lacunar geometries were not trivially explained by differences in imaging depth or number of lacunae, these analyses were again evaluated with imaging depth and number of lacunae as covariates. All parameters for which there was a significant main effect of age (*i.e.*, *volume*, *surface area*, *sphericity*, *oblateness*, *span theta*, *lacunar number density*) continued to show a significant effect of age despite these covariates. Other parameters (*i.e.*, *closest center of mass*) did not depend on age regardless of whether covariates were included in the analysis.

Meanwhile, age did not significantly affect 2D *area*, *perimeter*, or *circularity* (Table 2). In two-dimensions, *closest center of mass*, *span theta*, and *lacunar number density* changed with age ($p < 0.05$). Lacunae from old mice had +34.0% larger ($p = 0.005$) *closest center of mass*, -52.2% smaller *span theta* ($p = 0.049$), and -52.1% smaller *lacunar number density* ($p = 0.002$).

4. Discussion

We sought to characterize changes to osteocyte lacunae occurring with aging. In order to analyze osteocyte lacunar geometries in 3D, we developed a facile and open-source segmentation and analysis method based on CLSM. In this method, depth-stacks of 2D images acquired with CLSM are first segmented into 3D osteocyte lacunae and then analyzed for lacunar geometries using the open-source, Matlab-based '3D Osteocyte Lacunae Analysis' program described here. This method pairs common CLSM imaging with

time-efficient segmentation and analysis procedure, while avoiding the bias and user error intrinsic to manual segmentation. Our approach avoids pitfalls inherently associated with 2D imaging including difficulty distinguishing osteocytes from non-osteocyte objects and variance in geometries due to sectioning planes that lie away from the major ellipsoid axes.

Two aspects of our method were validated. First, we analyzed geometries of ellipsoidal “phantoms” and achieved agreement between measured and expected dimensions. Next, we compared the number of segmented lacunae for automatic and manual segmentation for one randomly-selected sample from each age group. The time-intensive nature of manual segmentation (approximately 10 hours per sample) prohibited a comparison of manual and automatic segmentation for every specimen. For our small comparison ($n = 3$), we found that 77-98% of manually segmented lacunae were automatically segmented with the 3D Osteocyte Lacunae Analysis program. It was expected that inhomogeneity in stain penetration and contrast from bone marrow would preclude 100% automatic segmentation of lacunae. While ideal to segment and analyze all lacunae, the limitations of automatic segmentation are expected to similarly affect all samples in the present study. Most importantly, the automatic segmentation strategy measured a sufficient number of lacunae to yield stable group comparisons of lacunar geometries (Figure 6).

Automatic and manual segmentation yielded somewhat different osteocyte lacunar geometries, yet the automatic method still holds predictive value. Lacunae were substantially larger and more spherical from automatic than from manual segmentation (Table 1), but the two sets of measurements are strongly correlated. Thus systemic bias exists between automatic and manual measurement of lacunar geometries. There are several possible reasons for this bias. Automatically segmented lacunar geometries depend on choice of threshold. And, importantly, manual segmentation should not be considered as a gold standard for measuring lacunar geometries. Manual segmentation is prone to user error and bias, and in our experience may be particularly prone to exclusion of low-contrast pixels on the edge of lacunar cross-sections. Further, the manually segmented lacunar objects were also not subjected to the erosion and 18 dilation procedures that are present in the automatic procedure. Erosion and dilation would be expected to result in somewhat smoother and more spherical lacunar objects. While automatic and manual segmentation may each err in estimating absolute lacunar geometries, the excellent correlation from linear regression between these two measurements suggests that they access qualitatively similar information, and that automatic segmentation could therefore be utilized for facile comparison of group differences.

We applied the automatic segmentation and analysis method introduced herein to study the effects of age on osteocyte lacunar geometries from cortical mouse bone. Lacunae were seen to become smaller, more spherical, more oblate, and more spatially disorganized with age (Figures 10 - 11). These effects were large; from 6-24 months osteocyte lacunae decreased in volume by -39.8%, became +16.3% more spherical, +72.3% more oblate, and +54.1% more spatially disorganized. Consistent with reports from other studies, aging also reduced the number density of lacunae (22-25). In our study, old mice had 37.7% fewer lacunae per volume than young mice. Importantly, CLSM is known to introduce distortion, particularly with respect to image depth, and thus the values reported here for osteocyte lacunae are not

intended to be interpreted as exact measurements (34). However, the effect of age on lacunar shape and size was still significant when image depth was included as a covariate, indicating that our group differences are stable with respect to depth-related distortion.

The effects of aging were largely not discernable from 2D analyses of the same images. In 2D, the mean *area* and *perimeter* decreased from young to old mice, but these changes were not significant. *Circularity*, the 2D analog of *sphericity*, also did not reproduce the age dependence seen in 3D. The only measures demonstrating a significant effect of age for both 2D and 3D were *span theta* and *lacunar number density*. Yet while 2D measures show *span theta* to decrease with age, indicating greater lacunar alignment, this effect was reversed in 3D. *Lacunar number density* was thus the only measure to be affected by age in both 2D and 3D and also show agreement in the direction of the effect. *Lacunar number density* is not sensitive to the size, shape, or alignment of lacunae. It is therefore not surprising that our study and others, spanning 2D and 3D metrics and several imaging modalities, have found agreement of decreasing number density of lacunae with aging.

Our observations highlight the limitations of 2D analysis and also reveal instances where 2D metrics could be misleading. Specifically, 2D analysis of osteocyte lacunae is inherently limited by assuming that cross-sectional geometries represent the complex ellipsoidal shapes of lacunae. A 2D section cannot reliably capture the largest dimensions of a network of lacunae, or their true orientations in space. Variance in 2D sample cutting and preparation could potentially yield quite different results for the same samples. In addition, 2D analyses may include cross-sections of non-osteocyte objects, such as vasculature, that are difficult to distinguish from osteocyte lacunae unless visualized in 3D. Further, as demonstrated by the *closest center of mass* and *span theta* measurement in this study, changes to osteocyte spatial arrangement observed in 2D do not necessarily extend into the third dimension and could be misleading. For the above reasons, we recommend that geometries and orientations of osteocyte lacunae are measured in 3D.

It is important to consider whether our findings that osteocyte lacunar geometries change with age could be confounded with age-related differences in bone tissue organization. With aging in mice, the proportion of cortical lamellar bone increases relative to less-organized non-lamellar central or woven bone (1). Following this prior work, we anticipated that 6 month old C57Bl/6 cortical bone would include a mix of lamellar and non-lamellar bone, while cortical bone from 24 month old mice would include predominantly lamellar bone (1). Yet we do not expect that our changes to lacunar geometries with age are simply a result of changing bone composition. In a 2003 review, Currey described more rounded lacunae in woven as opposed to lamellar bone (35). In two SR-CT studies of osteocyte lacunae, lacunar geometries in lamellar bone in rats were significantly more stretched than those from less-organized central bone (29), and osteogenesis imperfecta in mice was associated with lacunae that were rounder and less aligned in bone lacking a definitive lamellar structure (36). The direction of changes in osteocyte lacunae with age in the present study are more in line with non-lamellar than lamellar bone, though lamellar bone increases with advancing age. Thus, while it was outside the scope of this study to separate imaged bone into lamellar and non-lamellar components, we do not expect that bone compositional changes explain the alteration of lacunar morphologies with age.

Another possibility is that age-dependent changes to osteocyte lacunar geometry could be explained by older mice having more mineral-dense bone that decreases penetration of histological stain. In this case, the autofluorescence of cell nuclei, which are smaller and more spherical than lacunae, could dominate the contrast and lead to segmentation of nuclei instead of lacunae. However, this effect is unlikely for the current study. The lower limit of the volume filter during segmentation ($100 \mu\text{m}^3$) would exclude nuclei. Thus, age-related changes to osteocyte lacunae observed in this study are not expected to be trivially explained by differences in bone composition or staining that may occur with age.

Our findings indicate that lacunae become smaller, rounder, less rod-shaped, and more randomly oriented with increasing age. The change from elongated, oriented lacunae in young mice to rounder, more disorganized lacunae in old mice is reminiscent of altered lacunar morphologies that occur with unloading. In a SR-CT study, Britz *et al* reported that osteocytes from the cortical tibia of rats unloaded *via* sciatic neurectomy become less abundant and have smaller volumes (37). Osteocyte lacunae from the cortical femur of male 23-week old C57Bl/6 mice subjected to one month of spaceflight were smaller and more spherical as assessed by SR-CT than lacunae from control mice (38). Spatial organization of lacunae was not measured in either of these studies. However, osteocyte lacunae from the non-load bearing calvaria were rounder as well as more spatially disorganized compared with the load-bearing fibula in young adult C57Bl/6 mice (39). The similar osteocyte geometries between unloaded and aged load-bearing bone might be attributed to either differences in activity with aging or, intriguingly, altered osteocyte mechanosensitivity with age. Both scenarios require further investigation.

This study had several limitations. The automatic segmentation process identified many but not all lacunae, due to inhomogeneity in lacunar contrast and also bright bone marrow influencing thresholding. Additionally, we did not assess changes to perilacunar remodeling activity with age. We also did not survey changes to bone quality near osteocyte lacunae. These data will be important for understanding how changes to osteocyte lacunar geometries observed with increased age influence bone strength and toughness. Importantly, our animal model involved male mice who were subjected to a sham surgery. To better generalize the effects of age on osteocyte lacunar morphologies, it will be important to include aging male and female mice without surgical interventions. Lastly, we were unable to determine from this study whether perilacunar remodeling rate is increased or decreased, and note that a change in osteocyte activity in either direction could result in the observed changes in osteocyte lacunae geometries.

Our work points to the exciting possibility that osteocyte geometries may change in aging bone. In addition to aging, changes to osteocyte lacunae may occur with lactation (7-9), metabolic diseases (18, 40), osteoporosis (25), drug therapies (*e.g.*, glucocorticoid therapy) (10, 14, 17), exercise (13) and microgravity (15-16), and may affect bone quality, tissue strains, and mechanosensitivity. Thus, measuring 3D osteocyte lacunar geometries is important for understanding the effects of age, disease, and therapeutic interventions. It is anticipated that the efficiency and accessibility of our 3D Osteocyte Lacunae Analysis method will assist with these investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- An open-source tool for automatic segmentation and analysis of 3D osteocyte lacunar morphologies from confocal laser scanning microscopy is introduced
- Osteocyte lacunae from cortical mouse bone become smaller, more spherical, and more spatially disorganized with increasing age.
- Age-related changes to osteocyte lacunar morphologies are apparent in 3D but not 2D analyses of the same lacunae.

Figure 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.

Figure 2.

A) Cortical bone is stained with basic fuchsin, dehydrated, and embedded in PMMA. Ground sections 200 μm thick are prepared for imaging. B) Z-stacks of images are acquired with CLSM. C) The 3D Osteocyte Lacunae Analysis program is used to segment osteocytes from raw images, using thresholding, filtering, and erosion / dilation steps. D) Potential osteocytes are reconstructed in 3D. The user can select non-osteocytes (*e.g.*, vasculature) for exclusion. This final 3D matrix is analyzed for osteocyte lacunar geometries.

Figure 3.
Definitions of osteocyte lacunar geometries

Figure 4.
The program accurately calculates A) orientation and B) surface area for ellipsoidal phantoms.

Figure 5. 3D measures of osteocyte lacunar geometries, especially closest center of mass (D), may be sensitive to imaging depth below approximately 60 micrometers.

Figure 6.

3D measures of osteocyte lacunar geometries, especially closest center of mass (D), may be sensitive to number of lacunae for approximately 50 or fewer osteocytes.

Figure 7. Image depth from CLSM of approximately 60 micrometers or greater is adequate for achieving 50 or greater lacunae.

Figure 8.

Ellipsoidal and lacunar calculations demonstrate excellent agreement for A) volume and B) surface area, with correlation coefficients approaching 1. Best-fit ellipsoids have been employed to describe osteocyte lacunar geometries in previous efforts (24, 30), but yield essentially equivalent information as a voxel-fill lacunar approach.

Figure 9. 3D parameters of osteocyte lacunae are poorly to moderately predicted by 2D analogs measured from a single in-focus slice of the same z-stack.

Figure 10.

3D osteocyte lacunar geometries change with age. Compared with young mice, lacunae from old mice have A) smaller surface area, B) smaller volume, C) greater sphericity, E) greater span theta, F) greater oblateness, and G) smaller lacunar number density. The distance between lacunae, given by D) closest center of mass, does not change with age.

Figure 11. 3D osteocyte lacunar geometries change with age. Compared with young mice (A), old mice (B) have smaller, more spherical, and sparser lacunae.

Table 1

Comparison of osteocyte lacunar geometries between manually and automatically segmented lacunae.

Age	6 mo			18 mo			24 mo		
Segmentation procedure	Manual	Automatic		Manual	Automatic		Manual	Automatic	
Volume (μm^3)	321 $\pm$ 104	457 $\pm$ 223		313 $\pm$ 147	462 $\pm$ 348		276 $\pm$ 114	349 $\pm$ 127	
Surface area (μm^2)	323 $\pm$ 72.4	346 $\pm$ 123		313 $\pm$ 94.7	335 $\pm$ 180		265 $\pm$ 72.2	286 $\pm$ 71.9	
Sphericity	0.189 $\pm$ 0.0525	0.286 $\pm$ 0.0709		0.201 $\pm$ 0.0534	0.311 $\pm$ 0.0825		0.252 $\pm$ 0.0499	0.316 $\pm$ 0.0447	
Oblateness	-0.317 $\pm$ 0.381	-0.380 $\pm$ 0.339		-0.0629 $\pm$ 0.405	-0.157 $\pm$ 0.379		0.0466 $\pm$ 0.302	-0.020 $\pm$ 0.351	
Closest Center of Mass (μm)	19.0 $\pm$ 3.89	19.1 $\pm$ 5.39		20.2 $\pm$ 4.19	21.6 $\pm$ 4.68		19.4 $\pm$ 3.92	19.7 $\pm$ 3.71	
Span theta (degrees)	18.0 $\pm$ 15.3	19.8 $\pm$ 17.7		26.32 $\pm$ 23.3	28.9 $\pm$ 23.9		25.30 $\pm$ 21.6	29.7 $\pm$ 21.9	
Number of lacunae	125	107		105	81		90	88	
Lacunar number density ($\mu\text{m}^3 \times 10^{-5}$)	3.11	2.64		2.16	1.66		2.23	2.18	

Osteocyte lacunae geometries were calculated from manual (ScanIP) and automatic (3D Osteocyte Lacunar Analysis) procedures for a randomly-selected bone from each age group. Lacunar geometries were calculated from best-fit ellipsoids. Data represent the mean and standard deviation of each sample's lacunar geometries.

Table 2

Comparison of osteocyte lacunar geometries in 3D and 2D for cortical bone from young through old mice

	3D metrics			2D metrics		
	6 mo	18 mo	24 mo	6 mo	18 mo	24 mo
Volume (μm^3) $p = 0.033$	488 $\pm$ 33.9	390 $\pm$ 68.8	294 $\pm$ 27.8 *	23.2 $\pm$ 1.70	19.6 $\pm$ 1.48	20.3 $\pm$ 1.59
Surface area (μm^2) $p = 0.024$	361 $\pm$ 14.6	300 $\pm$ 35.3	251 $\pm$ 18.2 *	17.5 $\pm$ 0.638	16.5 $\pm$ 0.714	17.2 $\pm$ 1.09
Sphericity $p = 0.004$	0.283 $\pm$ 0.00233	0.313 $\pm$ 0.00853 *	0.329 $\pm$ 0.0115 *	0.564 $\pm$ 0.0201	0.555 $\pm$ 0.0238	0.541 $\pm$ 0.0274
Oblateness $p = 0.005$	-0.316 $\pm$ 0.0344	-0.204 $\pm$ 0.0476	-0.0875 $\pm$ 0.0333 *	N/A		
Closest center of mass (μm) $p > 0.05$	19.9 $\pm$ 0.367	20.0 $\pm$ 0.499	21.2 $\pm$ 0.540	21.0 $\pm$ 1.35	25.2 $\pm$ 1.60	28.0 $\pm$ 0.888 *
Span theta (degrees) $p = 0.030$	17.6 $\pm$ 1.23	23.9 $\pm$ 2.21	27.2 $\pm$ 2.94 *	20.4 $\pm$ 3.60	19.0 $\pm$ 2.54	9.76 $\pm$ 1.35 *
Lacunar number density ($1/\mu\text{m}^3 \times 10^{-5}$) $p = 0.012$	3.06 $\pm$ 0.280	2.30 $\pm$ 0.239	1.91 $\pm$ 0.164 *	7.03 $\pm$ 0.970	4.72 $\pm$ 0.540	3.37 $\pm$ 0.28 *

Data are presented as mean $\pm$ standard error. Osteocyte lacunar geometries were calculated from best-fit ellipsoids. For each geometric parameter, the p-value for the main effect of age is reported.

* Indicates $p < 0.05$ in Tukey post-hoc comparison with young mice.