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Validation of Cortical Bone Mineral Density Distribution using micro-Computed Tomography

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Abstract

Changes in the bone mineral density distribution (BMDD), due to disease or drugs, can alter whole bone mechanical properties such as strength, stiffness and toughness. The methods currently available for assessing BMDD are destructive and two-dimensional. Micro-computed tomography (μ CT) has been used extensively to quantify the three-dimensional geometry of bone and to measure the mean degree of mineralization, commonly called the tissue mineral density (TMD). The TMD measurement has been validated to ash density; however parameters describing the frequency distribution of TMD have not yet been validated. In the current study we tested the ability of μ CT to estimate six BMDD parameters: mean, heterogeneity (assessed by the full-width-at-half-maximum (FWHM) and the coefficient of variation (CoV)), the upper and lower 5% cutoffs of the frequency distribution, and peak mineralization) in rat sized femoral cortical bone samples. We used backscatter scanning electron microscopy (bSEM) as the standard. Aluminum and hydroxyapatite phantoms were used to identify optimal scanner settings (70 kVp, and 57 μ A, with a 1500 ms integration time). When using hydroxyapatite samples that spanned a broad range of mineralization levels, high correlations were found between μ CT and bSEM for all BMDD parameters ($R^2 = 0.92$, $p < 0.010$). When using cortical bone samples from rats and various species machined to mimic rat cortical bone geometry, significant correlations between μ CT and bSEM were found for mean mineralization ($R^2 = 0.65$, $p < 0.001$), peak mineralization ($R^2 = 0.61$, $p < 0.001$) the lower 5 % cutoff ($R^2 = 0.62$, $p < 0.001$) and the upper 5% cutoff ($R^2 = 0.33$, $p = 0.021$), but not for heterogeneity, measured by FWHM ($R^2 = 0.05$, $p = 0.412$) and CoV ($R^2 = 0.04$, $p = 0.469$). Thus, while mean mineralization and most parameters used to characterize the BMDD can be assessed with μ CT in rat sized cortical bone samples, caution should be used when reporting the heterogeneity.

Keywords

Bone mineral density distribution; Mineralization; Micro-computed tomography (μ CT); backscattered scanning electron microscopy

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1. Introduction

Bones undergo constant remodeling, a process that involves removal, deposition and maturation of the matrix, driven by various biological and mechanical factors[1, 2]. The transient nature of the matrix maturation process creates a distribution of mineral content. The average degree of mineralization and its heterogeneity are typically assessed by deriving a bone mineral density distribution (BMDD) using the frequency distribution of grey scale values within calibrated backscatter scanning electron microscopy (bSEM) images[3]. Importantly, the BMDD is known to vary as a function of disease state and with the use of pharmacological agents[4–11] and these changes can affect elasticity, hardness, stiffness and toughness of bone as a material[1, 12–15].

In addition to bSEM, other methods of assessing bone mineral density include ash density, synchrotron micro-computed tomography, quantitative contact microradiography, Fourier transform infrared microscopy, and Raman microscopy[2, 16–19]. Although these techniques provide high accuracy and precision, each requires destructive sample preparation and, with the exception of synchrotron μ CT, none provide information on the distribution of mineral density in 3-D. An imaging modality that has the potential to overcome these limitations is laboratory-based μ CT, which has been used extensively to quantify bone macro- and microstructure in 3-D. The mean mineralization derived from μ CT is commonly reported as “tissue mineral density” (TMD) and is based on the mean x-ray attenuation of the volume of interest. While TMD has been validated against ash density[20, 21], the μ CT-derived mineralization density distribution, has not yet been validated. In the current study, we sought to validate μ CT for the measurement of cortical bone mineralization by comparing to bSEM-derived BMDD parameters using rat-sized cortical bone samples. We chose to focus on the rat because it is a frequently used animal model in preclinical musculoskeletal studies. We extended the range of mineralization found in rat tissue by including cortical bone samples from several other species, which were machined to mimic rat cortical bone geometry. We used a custom calibration set, phantoms and bone samples to establish optimal μ CT voltage and current (two key x-ray tube settings) and integration time (a key detector setting). Then, we assessed the validity of μ CT-derived measures of mean mineralization, peak mineralization, mineralization heterogeneity, and low and high mineral distributions through correlation with bSEM values.

2. Materials and Methods

2.1 Bone Mineral Density Distribution

In the current study we validated the use of μ CT to measure six commonly reported BMDD parameters (the mean, full width at half maximum [FWHM], coefficient of variation [CoV], peak mineralization, and the lower and upper 5% cutoffs of the frequency distribution, Fig. 1)[3, 22]. The FWHM and CoV represent two methods to measure the within-sample heterogeneity: FWHM measured the distribution width at half of the BMDD peak. CoV was calculated as the standard deviation of the distribution divided by the mean of the

distribution: $\frac{\sigma_{\text{BMDD}}}{\mu_{\text{BMDD}}}$. The lower and upper 5% cutoff were quantified by finding the mineral

density value at the 5th and 95th percentile of the frequency distribution, respectively. BMDDs derived from both μ CT and bSEM were characterized with a custom MATLAB script (R2015b, MathWorks, MA).

2.2 Materials

μ CT calibration was performed with either the conventional hydroxyapatite (HA) phantom (5 HA phantoms between 0 and 800 mg/cm³; Scanco Medical, Brüttisellen, Switzerland) or a custom wide-range HA phantom set (7 rectangular HA phantoms between 0 and 1860 mg/cm³; dimensions of 2.4 mm by 5 mm by 10 mm, Roeder lab, University of Notre Dame). The HA phantoms were made with HA mineral suspended in minimally attenuating material (polymethylmethacrylate [PMMA] for the conventional phantoms and polyether ether ketone for the custom phantoms).

μ CT scan optimization was performed using two separate phantom materials. The first was a custom pure aluminum (Al) hollow cylinder phantom with 1 mm thick walls and a 4 mm outer diameter, which mimics rat cortical bone geometry. Aluminum was used because its x-ray attenuation is similar to that of cortical bone and is relatively homogeneous. The second phantom, the HA-test set, was made from the same wide-range HA phantom material used for μ CT calibration. To correlate with bSEM, 2.4 mm by 3.5 mm by 7 mm pieces were machined from the HA-test set and embedded in PMMA after dehydration in a series of ethanol solutions followed by xylene [23]. These plastic-embedded HA-test set pieces were sectioned to obtain 1.5 mm thick slabs (Isomet 5000, Buehler, IL, USA), which were mounted on plastic slides. The slab faces were ground and serially polished with 9 μ m and 3 μ m diamond polishing compounds (Metadi, Buehler, IL, USA) for a mirror finish (Phoenix 4000, Buehler, IL, USA). To prevent charging during bSEM imaging, slabs were then carbon coated for 9 s at 5 V (Carbon Coater 108Carbon/A, Cressington Scientific Instruments, England, UK).

To test the utility of μ CT for BMDD characterization, tibiae from young rats ($n = 3$) and adult rats that received a sham ovariectomy ($n = 3$) or an ovariectomy ($n = 3$) were obtained from previous projects[24]. Bones from several other species were used to increase the range of observed mineralization values[25]. Specifically, frozen femurs from horse, cat, dog, deer, turkey, pig and goat ($n = 1/\text{animal}$) were obtained from a veterinary clinic. All bone samples were fixed in 10% formalin for 2 days followed by storage in 70% ethanol. Samples obtained from species other than rat were machined to create geometries similar to the diaphyseal region of rat long bones (cylinders with an outside diameter of 4 mm, a 1.5 mm inner diameter and a wall thickness 1.25 mm). To match sample regions, all bones were PMMA embedded, sectioned and polished prior to μ CT scanning. A single slab from each animal was used for both μ CT and bSEM imaging. After μ CT scanning, slabs were carbon coated to prevent charging during bSEM imaging. All bone samples were lumped into a single set for all analyses.

2.3. Microcomputed tomography (μ CT) scanning

All scanning was performed on a laboratory-based scanner (μ CT50, Scanco Medical, Brüttisellen, Switzerland). Scan settings were determined by optimization to two sets of μ CT

phantoms (the aluminum phantom and the HA-test set, described in detail above) and validated with the animal samples. All samples were scanned in a 9 mm diameter specimen tube filled with storage media (70% ethanol) and held securely in place with gauze. Standard Scanco beam hardening correction (1200 mg/cc) and a 0.5 mm aluminum filter was utilized for all scans, which effectively reduces beam hardening (Supplemental Fig. 1). A voxel size of 2 μm was selected for all scans based on previous studies which suggested that this voxel size was sufficient to provide the needed spatial resolution while still limiting amounts of noise and required scan times[26]. A 1 mm height of all phantoms and bone samples was scanned and analyzed. MATLAB was utilized to perform the BMDD parameter quantification.

2.3.1 μCT Calibration—Previous work by Deuerling et al[27] found that the use of conventional μCT phantoms (0–800 mgHA/cm³) underestimates mineralization by 25–37% for samples denser than 800 mgHA/cm³. As rat cortical bone mineralization exceeds 800 mgHA/cm³, we used a custom, wide-range HA phantom set (0–1869 mgHA/cm³). The most accurate conversion of linear attenuation coefficient values to mineral density values with this phantom set required a nonlinear standard curve (Supplemental Fig. 2), consistent with the finding of Deuerling et al[27]. We limited the effects of tube fluctuation by scanning all samples within 2 weeks of wide-range HA phantom calibration. MATLAB was utilized to perform the mineral density calibration.

2.4 Backscattered scanning electron microscopy (bSEM) imaging

A scanning electron microscope (Ziess SIGMA VP, Oberkochen, Germany) was used to obtain bSEM images using a 4 quadrant solid state backscatter detector. Scanning was performed at 25 kV, 19 mm WD, 150 $\times$ and an aperture of 60 μm in high current mode, resulting in a spatial resolution of 0.74 μm by 0.74 μm . Current was monitored with a Faraday cup and averaged 1.60 nA during sample imaging. At the start of each imaging session, contrast and brightness were set using a line scan across carbon and aluminum standards. Aluminum was set to a grayscale value of ~220 and carbon was set to a grayscale value of ~30. The effective atomic number (Z_{eff}) was used to measure mineral distribution after converting gray scale values using calibration standards of known atomic number[28, 29], including pure aluminum (Al), carbon (C, graphite) and aluminum oxide (Al₂O₃, diameters: ~1.5 mm; ESPI Metals, OR, USA). Images of the standards were taken before and after sample imaging; if the grayscale values from the before and after scans differed by 5% or more, the image set was discarded and the standards and samples were reimaged.

ImageJ (Image J 1.42q, NIH, MD, USA) was used to manually contour the HA-test phantoms and animal samples to exclude surrounding PMMA and to generate gray scale frequency distributions of the calibration standards, phantoms and bone sample images. Frequency distribution grayscale values were converted to effective Z_{eff} by obtaining a linear calibration curve from the above-mentioned standards. The grayscale value for each standard was taken as the average between the images taken before and after each sample imaging session. Z_{eff} for Al was set to 13, Al₂O₃ to 10.5 and C to 6. MATLAB was utilized to perform the grayscale calibration and BMDD parameter quantification.

2.5 μ CT scan optimization: methods – assessment criterion

To optimize the μ CT scanner settings, we concentrated on three scan parameters known to impact mineralization measurements: voltage, current and integration time[21, 30]. Each scan parameter was varied individually while holding other parameters constant. A summary of the specific scan settings evaluated and the order in which they were tested is shown in Figure 2. Scan parameters were assessed using four distinct criteria. The first was the signal-to-noise ratio (SNR) and the contrast-to-noise ratio (CNR) measured using the Al phantom. The SNR was determined by dividing the mean of the phantom frequency distribution by the mean of the distribution associated with the surrounding background peak. CNR was determined by subtracting the peak attenuation of the Al phantom from the background peak normalized to the background.

The second parameter selection criterion was minimizing the FWHM measurement of the Al phantom. The Al phantom is a homogeneous material and, therefore, should have a narrow mineralization distribution. Widening of the FWHM measured would indicate an increase in measurement error either due to partial volume or beam hardening effects.

The third criterion was the accuracy of the μ CT-derived mineralization measurement, which was assessed using the HA-test phantoms. We calculated the percent error between the known mineralization values and μ CT-derived measurement as an absolute difference:

$$\left| \frac{HA_{\text{measured}} - HA_{\text{actual}}}{HA_{\text{actual}}} \right| \times 100$$

The fourth criterion assessed was the correlation coefficient of μ CT-derived and bSEM-derived mineralization measurements made on the HA-test set phantoms. The ideal scan settings were defined as those that maximized both the SNR and CNR, minimized the FWHM of the Al phantom, minimized the % error between μ CT-derived mineralization measurements and the known HA concentration and maximized the correlation coefficient between μ CT and bSEM (Fig. 2).

2.6 μ CT validation

As with the phantoms, the bone samples were placed in a 9 mm diameter μ CT specimen tube filled with storage media (70% EtOH), held securely in place with gauze and scanned using the optimized parameters identified above (70 kVp, 57 μ A and 1500 ms integration time). The same filter, beam hardening correction and voxel size were utilized as described above (section 2.3). Bones were scanned from the polished surface to a depth of 1 mm. After, μ CT imaging, the samples were mounted on plastic slides and carbon coated and imaged with bSEM.

Reconstructed μ CT images were manually contoured using the manufacturer's software (Scanco Medical V5.15, Brüttisellen, Switzerland) to include only the cortical area. Intracortical porosity was not excluded, but the medullary space and the space external to the periosteal surface were excluded. Frequency distributions in linear attenuation values were generated using an in-house script (OpenVMS IPL). A threshold of 350 mgHA/cm³ was applied to BMDDs when the frequency distribution displayed a bimodal distribution due to high porosity. For a unimodal distribution, the long left-hand tail end was truncated

(Supplemental Fig. 3). The frequency distributions of the animal specimens were converted to mineral density using the nonlinear calibration curve obtained from the custom wide-range HA phantom set (Supplemental Fig. 2). The BMDDs were characterized using the same MATLAB script described above.

After μ CT scanning, embedded and polished sample slabs were carbon coated and imaged with bSEM as described above. ImageJ (Image J 1.42q, NIH, MD, USA) was used to obtain frequency distributions of the calibration standards and sample images. Grayscale values were converted to Z-value using the known values for the standard materials (as described in Section 2.2). Cross-sectional bone images were manually contoured analogous to how the contouring was done with the μ CT images. Specimen frequency distributions were thresholded at a $Z_{\text{eff}} = 7.5$ to remove pore-associated pixels[31]. Distributions were analyzed in the same manner as the phantom images to quantify the BMDD parameters.

To determine whether μ CT-derived BMDDs have confounding pore-associated partial volume effects, pore characteristics were assessed from the bSEM images. The mean pore diameter, number of pores and total pore area were calculated with ImageJ Particle Analysis for all detectable pores and correlated with the μ CT-derived BMDD parameters.

2.7 Statistical analysis

Linear regression analysis was conducted on bSEM and μ CT mineralization measurements made on HA-test set scans and bone tissue scans. Fisher's Z-test was used to assess the coefficient of determination (R^2)[32]. Regression analysis was performed to determine if there was a dependence of μ CT BMDD parameters on sample porosity. All regression analyses were performed with SPSS (IBM SPSS Statistics 19, NY). P-values less than 0.050 were considered significant.

3. Results

3.1 μ CT scan parameter optimization

3.1.1 Voltage—In this experiment, voltage was varied between 45, 55, and 70 kVp while current (200 μ A) and integration time (500 ms) were held constant. At 45 kVp, the frequency distribution of the Al phantom demonstrated signal saturation (Fig. 3a) and, therefore, was excluded from further analysis. Neither of the remaining two voltage settings were found to optimize all four selection criteria. The highest SNR and CNR were obtained with 55 kVp (Fig. 3b). The FWHM of the Al phantom, however, was minimized using a voltage of 70 kVp (Fig. 3c).

Additional optimization analysis was performed using HA-test phantoms of known HA densities. The percent error between the μ CT mineralization and the known phantom mineral density was consistently greater with 55 kVp than 70 kVp (Fig. 4).

bSEM and μ CT-derived BMDD characteristics (mean, peak, FWHM, CoV, upper and lower 5% cutoffs) were correlated for both voltages. Although not significantly different, the correlation coefficients for 70 kVp were higher compared to 55 kVp for the majority of the BMDD parameters (Table 1).

Even though the highest CNR and SNR were obtained with 55 kVp, 70 kVp was chosen as the optimal μ CT voltage setting due to (1) reduction in FWHM of the Al phantom, (2) lower errors in quantifying mineral density and (3) higher correlations for the majority of the BMDD parameters.

3.1.2 Integration Time—Integration times of 500, 1000 and 1500 ms were tested while holding voltage constant at 70 kVp and current at 200 μ A. There were no differences between 500 and 1000 ms integration times for SNR and CNR, however 4% increases in SNR and CNR were observed at 1500 ms compared to the 500 and 1000 ms integration times (Fig. 5a). Noise measured by the FWHM of the Al phantom was decreased by 31% at 1000 compared to 500 ms, with a further reduction of 19% when integration time was increased from 1000 to 1500 ms (Fig. 5b). The percent error between the known mineral density of the HA-test set and the μ CT-derived mineralization measurement ranged from 0.3 – 4.2% (Fig. 5c). The lowest percent error over all phantoms, except the 930 mgHA/cm³ phantom, was observed with the longest integration time (1500 ms).

High correlations were found between bSEM and μ CT-derived BMDD parameters at all three integration times for the HA-test set (Table 2). The 1500 ms integration time was chosen because (1) it had a slightly higher SNR and CNR, (2) reduced noise as measured by the FWHM of the Al phantom, (3) the lowest overall percent error associated with quantifying the mineral density of the phantoms (excluding the 930 mgHA/cm³ phantom), and (4) the majority of the correlation coefficients, from μ CT and bSEM regression analysis, were higher at 1500 ms compared to the other integration times.

3.1.3 Current—Voltage and integration time were held constant at 70 kVp and 1500 ms while two current settings were tested, 57 and 200 μ A. A 32% and 33% increase in SNR and CNR, respectively, was observed at 57 μ A when compared to 200 μ A (Fig. 6a), but a 60% increase in the FWHM measurement of the Al phantom was found when using 57 μ A (Fig. 6b).

Percent error in quantifying the known mineral density of the HA-test set was generally lower with 57 μ A compared to 200 μ A (Fig. 6c). The higher current, however, had lower percent errors for two mid-range phantoms, 930 and 1550 mgHA/cm³. High correlations were found between bSEM and μ CT derived BMDD parameters for both currents, with 57 μ A tending to be higher than 200 μ A, although the differences were not statistically significant (Table 2).

Although the FWHM of the Al phantom was higher at a current of 57 μ A as compared to 200 μ A, the lower current (57 μ A) was chosen for the following reasons (1) higher SNR and CNR, (2) lower overall error in quantifying actual mineral density and (3) BMDD parameter correlations were consistently higher with 57 μ A compared to 200 μ A.

3.2 Validation with Animal Samples

From the HA and Al phantom studies it was determined that the optimal μ CT scanner settings are 70 kVp, 57 μ A and 1500 ms integration time at a voxel size of 2 μ m. Animal samples were μ CT scanned using the optimized scanner settings followed by imaging with

bSEM. High correlations between bSEM and μ CT were observed for most of the BMDD parameters (Fig. 7), including mean ($R^2 = 0.65$, $p < 0.001$), peak mineralization ($R^2 = 0.61$, $p < 0.001$), and the lower 5% cutoff ($R^2 = 0.62$, $p < 0.001$). A significant correlation was observed for the upper 5% cutoff ($R^2 = 0.33$, $p = 0.021$), but the correlations for heterogeneity were not significant: FWHM ($R^2 = 0.05$, $p = 0.412$) and CoV (Figure 7c, $R^2 = 0.04$, $p = 0.469$).

To determine if excess background noise was potentially affecting the heterogeneity measurements (FWHM and CoV), a subset of samples was chosen (maximizing the variation in the FWHM measurement) and rescanned with three frame averages per projection. Increasing frame averaging did not improve the correlation between bSEM and μ CT for the FWHM measurement compared to a single frame averaging ($p = 0.495$).

3.3 Pore-associated partial volume effects

Pore characteristics (max pore diameter, mean pore diameter, total pore area and total number of pores) were measured with bSEM and tested for correlation with the μ CT-derived BMDD parameters to assess the impact of porosity on μ CT-derived BMDD. Significant correlations were found for total pore area with mean mineralization ($p = 0.015$), peak mineralization ($p = 0.013$) and the lower 5% cutoff ($p = 0.021$), but not for FWHM ($p = 0.313$), CoV ($p = 0.074$) or the upper 5% cutoff ($p = 0.101$). No significant correlations were found between maximum pore diameter or mean pore diameter with any μ CT-derived BMDD parameter. Significant correlations were found between total number of pores and mean mineralization ($p = 0.019$), peak mineralization ($p = 0.021$) and the lower 5% cutoff ($p = 0.027$), but not for the upper 5% cutoff ($p = 0.150$), FWHM ($p = 0.323$) or CoV ($p = 0.074$).

4. Discussion

Bone remodeling and mineralization are dynamic processes that affect the distribution of mineralization levels within the tissue at any given time. Further, both the mean level and the distribution of mineralization contribute to material and structural strength. μ CT is often used to measure tissue mineral density, a measurement of the mean degree of mineralization in the volume of interest. A more complete characterization of bone mineralization can be obtained from the bone mineral density distribution (BMDD), which is currently measured using destructive imaging methods, such as backscattered scanning electron microscopy (bSEM). A method to measure the BMDD non-destructively would not only save researchers effort in sample preparation, but would also allow for mechanical characterization on the same samples. In the current study, we have demonstrated that with proper calibration and optimized scanner settings, the mean mineralization, peak mineralization, upper 5% cutoff and lower 5% cutoff of the mineralization distribution can be accurately quantified in rat size cortical bone samples with μ CT. Our validation study did not show a significant correlation for the two heterogeneity measurements (FWHM and CoV) in the animal bone samples. When using HA test phantoms, we found strong correlations between bSEM and μ CT for all BMDD parameters.

μ CT is commonly used to measure bone structure and architecture in rodents and the μ CT-derived measure of global mineralization, tissue mineral density, has been correlated with ash density [19, 20, 33]. The distribution of mineralization is also thought to be important to the mechanical properties of bone and is known to change as a function of disease and pharmaceutical treatments [3, 9, 34–37]. Although various studies have demonstrated that μ CT settings impact the linear attenuation coefficient and therefore the mineralization measurement, these studies have primarily measured the change in attenuation values [21, 30, 38] and have not evaluated whether μ CT can accurately predict BMDD parameters.

Laboratory μ CT instruments have several limitations that could prevent the accurate measurement of BMDD. These limitations include polychromatic x-ray spectra that could lead to beam-hardening artifacts and limited spatial resolution that could lead to partial volume effects, especially in relatively porous samples such as bone. In the current study, we limited the effects of beam-hardening by calibrating the μ CT system with a wide range of mineral density values. As shown in previous publications, the use of these wide range phantoms more accurately represents the mineralization level of bone tissue and reduces the errors associated with extrapolating beyond conventional calibration materials (Supplemental Fig. 4) [27, 30]. In this way, we were able to correct for the non-linear relationship between mineral density and x-ray attenuation induced by beam-hardening artifacts [38]. Further, the x-ray tube spectrum can fluctuate over time and updating the calibration curve frequently will minimize experimental error over the course of a study. In the current study, we limited the effects of tube fluctuation by scanning all samples within 2 weeks of phantom calibration.

The limited spatial resolution achievable with μ CT can induce partial volume effects, which can also reduce the accuracy of mineralization measurements. Voxel size should be chosen according to the desired measureable outcome because of potential induced error in architectural measurements and porosity [26, 39–41]. Therefore, in the current study we chose to use a voxel size of 2 μ m, which should limit the contribution of partial volume artifacts by accounting for the majority of the microporosity present in bone tissue, such as Haversian canals, which are between 4 and 11 μ m in rats [42]. We assessed the contribution of porosity to the μ CT-derived BMDD measurements and demonstrated that despite the high resolution utilized, porosity did impact the mean and peak mineralization levels and the 5% cutoffs. Despite these potential confounding effects, the correlation between μ CT and bSEM-derived mean and peak mineralization, and 5% cutoff remained significant.

Several μ CT scan parameters are thought to impact the mineral density measurement, including voltage, current, orientation, media, voxel size, the beam filter and integration time [21, 26, 27, 30, 41]. In this study, we focused on optimizing the voltage, current and integration time due to the importance of these parameters in controlling the x-ray spectrum in polychromatic sources, such as μ CT, and to limit the variable matrix. All other parameters were kept constant. Voltage controls the photon energy of the incident x-rays, current controls the number of photons interacting with the sample, and integration time controls the amount of time that the detector is collecting photon information. We chose to first optimize the voltage parameter as the x-ray attenuation coefficient and subsequently the mineralization measurement, is strongly dependent on voltage. We then tested the

integration time followed by the current settings, to identify the optimum scan parameters, which were determined to be 70 kVp, 57 μ A, and 1500 ms for rat-sized cortical bone.

Mineralization heterogeneity, measured by the FWHM and CoV, is the only BMDD characteristic that was not validated in the animal samples. As both FWHM and CoV were highly correlated in the HA test set ($R^2 = 0.96$ and $R^2 = 0.92$, respectively), it is possible that the narrow range of heterogeneity values in the animal samples (μ CT FWHM: 277 – 585 mgHA/cm³ and CoV: 14.19 – 25.34 mgHA/cm³) inhibited our ability to detect significant correlations. It is also possible that significant partial volume effects were induced by porosity within the bone matrix below the 2 μ m voxel size used. However, we did not find a significant correlation between bone porosity and heterogeneity measured by μ CT, suggesting that this potential confounding variable did not account for the lack of correlation for the heterogeneity measurements.

Additional factors that could contribute to the poor heterogeneity correlations in the animal samples include the polychromatic x-ray source employed by laboratory μ CT systems. Although, our calibration and optimization strategies were designed to limit the effects of the polychromatic source on the mineralization measurement, a polychromatic source produces a broad range of x-rays. The attenuation of a material is highly dependent on the x-ray energy and, therefore, a distribution of x-rays within the beam would lead to variable attenuation across the sample [19]. It is possible to overcome the limitations associated with polychromatic sources by using synchrotron computed tomography which is able to obtain a near monochromatic x-ray spectrum[19]. However, these instruments tend to have low x-ray fluxes, requiring that samples be machined before scanning and generally require the sample to be dry, which means this modality also suffers from the limitation of being destructive to the sample.

We used areal bSEM measurements as the comparison to volumetric μ CT measurements, which may have contributed some additional errors in the form of spatial variability within the μ CT dataset not present in the bSEM data set. We could have limited the μ CT analysis to a smaller volume of interest by analyzing, for instance, only one slice. However, this would negate one of inherent advantages of μ CT as a 3D measurement tool. While this difference in sampling may have contributed to some of the variance between the two modalities, significant correlations between μ CT and bSEM were found for the majority of the BMDD parameters.

In conclusion, we validated the quantification of many bone mineral density distribution parameters with high-resolution, laboratory-based μ CT, including mean mineralization, peak mineralization and the high and low cutoffs in rat sized cortical bone samples. Heterogeneity, however, was not found to be correlated between the two modalities in the bone samples, although all parameters were well correlated when using HA test phantoms. It is important for researchers to optimize their scanner settings before quantifying mineral density with μ CT. The optimal settings for quantifying the BMDD of rat sized cortical bone using the Scanco μ CT50 are 70 kVp, 57 μ A, 1500 ms integration time at a voxel size of 2 μ m. The ability to accurately assess rat sized cortical bone BMDD non-destructively may

prove useful when studying bone disease and adaptation to altered mechanical environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- μ CT represents a non-destructive method to quantify the BMDD.
- μ CT BMDD was validated against the current gold standard, bSEM.
- High correlation was found between μ CT and bSEM when using phantom materials.
- Two measures of mineralization heterogeneity were not correlated in cortical bone samples.

Mean, peak, low and high mineralization values were correlated in cortical bone samples.

Figure 1.

Approach to validation. Images from (a) μ CT and (b) bSEM were used to generate the (c) bone mineral density distribution (BMDD). The correlation between the two imaging modalities for each BMDD parameter was assessed, including mean mineralization, peak mineralization, lower 5% cutoff, upper 5% cutoff, full-width-at-half-maximum (FWHM) and the coefficient of variation of the distribution (σ /mean). Each data set was calibrated against standards so that differences in native brightness and contrast between μ CT and bSEM were negated. These two images are from the same specimen, but at slightly different locations. Scale bar = 1 mm.

Experiments			
	Exp 1 (Voltage)	Exp 2 (Integration Time)	Exp 3 (Current)
Voltage (kVp)	45, 55 & 70	70	70
Integration Time (ms)	500	500, 1000 & 1500	1500
Current (μ A)	200	200	57 & 200

Assessment Criteria for each Experiment			
Aluminum Phantom		HA Test Set	
Maximize	Minimize	Minimize	Maximize
↑SNR	↓FWHM	↓% Error from Actual Density	↑ bSEM Correlation
↑CNR			

Figure 2.

Experimental breakdown. Left box: 3 experiments (Exp 1, Exp 2, and Exp 3) were conducted to optimize voltage, integration time and current. Right box: the assessment criteria for each experiment are listed (SNR = signal-to-noise ratio, CNR = contrast-to-noise ratio, FWHM = full-width-at-half-maximum of the frequency distribution, bSEM = backscatter scanning electron microscopy).

Figure 3.

(a) Histogram distributions of background (left peaks) and Al phantom (right peaks), normalized to maximum frequency. Increase in frequency at the high end of attenuation (arrow) with 45 kVp indicates detector saturation. (b) Signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) for three voltage settings and (c) FWHM measurements of the Al phantom. These scans were performed holding the current and integration time constant at 200 μA and 500 ms, respectively.

Figure 4.

The HA-test set was scanned with μ CT at 55 and 70 kVp and the absolute value of the error between measured and actual mineral density was plotted for each phantom (200 μ A and 500 ms integration time). Percent error for the 0 mgHA/cm³ phantom was not quantified because the embedding media only slightly attenuates x-rays and would result in artificially large errors.

Figure 5.

(a) SNR and CNR, (b) FWHM measurements of the Al phantom and (c) percent error in quantifying the actual mineral density of the phantoms at integration times of 500, 1000 and 1500 ms. All scans were performed at 70 kVp and 200 μ A. Percent error for the 0 mgHA/cm³ phantom was not quantified because the embedding media only slightly attenuates x-rays and would result in artificially large errors.

Figure 6.

(a) SNR and CNR and (b) FWHM calculated using the Al phantom and (c) percent error in calculating actual mineral density of the HA-test set at 57 μA and 200 μA . All scans were performed at 70 kVp and 1500 ms integration time. Percent error for the 0 mgHA/cm^3 phantom was not quantified because the embedding media only slightly attenuates x-rays and would result in artificially large errors.

Figure 7. Correlations between bSEM and μ CT with bone samples

Cortical bone samples from various species machined to mimic rat cortical bone geometry were scanned with μ CT using the optimal parameters (70 kVp, 57 μ A and 1500 ms integration time) and bSEM. (a) Mean mineralization, (b) upper and lower 5% cutoffs, (c) peak mineralization, specimen heterogeneity measured by (d) FWHM and (e) CoV. All BMDD parameters were significantly correlated between modalities with the exception of FWHM and CoV. Regression equations: (a) $y = 0.0021x + 8.45$, (b) upper: $0.0015x + 8.90$ and lower: $0.0019x + 9.05$, (c) $y = 0.0019x + 8.66$ (d) $y = -0.0001x + 0.66$ and (e) $y = 0.019x + 0.03$.

Table 1

Correlation coefficients between μ CT and bSEM for the HA-test set (200 μ A and 500 ms integration time) at 2 voltage settings 55 and 70 kVp. The 1240 mgHA/cm³ phantom was excluded as it was lost during sample preparation and bSEM scanning could not be performed on it. Significant correlations were found for mean mineralization, FWHM, lower 5%, upper 5% cutoff, peak mineralization and CoV between μ CT and bSEM ($p < 0.010$ for all correlations).

Coefficient of Determination (R^2)		
BMDD Parameter	55 kVp	70 kVp
Mean	0.995	0.996
Peak	0.962	0.957
FWHM	0.855	0.879
CoV	0.891	0.881
Lower 5%	0.905	0.915
Upper 5%	0.866	0.878

Table 2

The correlation coefficients between bSEM and μ CT using the HA-test set at varying μ CT integration times
All scans were performed at 70 kVp and 200 μ A ($p < 0.020$ for all correlations).

Coefficient of Determination (R^2)			
BMDD Parameter	500 ms	1000 ms	1500 ms
Mean	0.997	0.996	0.996
Peak	0.953	0.956	0.961
FWHM	0.892	0.938	0.977
CoV	0.856	0.911	0.934
Lower 5%	0.899	0.919	0.919
Upper 5%	0.869	0.881	0.890

Table 3

The correlation coefficients between bSEM and μ CT using the HA-test set at two μ CT current settings. All scans were performed at 70 kVp and 1500 ms integration time ($p < 0.010$ for all correlations).

Coefficient of Determination (R^2)		
BMDD Parameter	55 kVp	70 kVp
Mean	0.996	0.996
Peak	0.975	0.959
FWHM	0.956	0.910
CoV	0.925	0.904
Lower 5%	0.958	0.916
Upper 5%	0.932	0.877