RESEARCH

A comparison of the thresholding strategies of micro-CT for periodontal bone loss: a pilot study

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Objectives: Micro-CT provides three-dimensional details and has been widely used for biomedical assessments. This study aimed to determine the most appropriate threshold method for quantitatively assessing the dynamics of periodontal destruction.

Methods: Inflammation was induced by submerging a silk ligature in the sulcus of the maxillary second molars of rats, and the animals were killed prior to ligature placement and after 7 and 21 days. The maxillae were examined for the bone resorptive activities by micro-CT, histology and tartrate-resistant acid phosphatase staining. The imaging threshold was determined by CT phantom, global and local algorithms. A bone fraction measurement from each threshold-determining technique was compared with histomorphometry. The reliability and reproducibility were examined by the intraclass correlation coefficient (ICC) and the coefficient of variation.

Results: Significant reduction of inflammatory infiltration ($p < 0.01$) and active osteoclastic resorption ($p < 0.05$) from Day 7 to Day 21 were noted. High inter- and intraexaminer agreement were demonstrated in both histomorphometric and micro-CT assessments ($ICC > 0.98$). The algorithm-based technique demonstrated stronger correlation to histomorphometry than phantom-based thresholds, and the highest agreement was presented by the local algorithm ($ICC > 0.96$). This, however, was considerably computationally expensive.

Conclusions: The local threshold-determining algorithm is suggested for examining inflammation-induced bone loss. Further investigation will be aimed at enhancing computational efficiency.

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Introduction

Histology had been considered as the “gold standard” to evaluate the structure of bone. However, the invasive and two-dimensional nature and the interference from histological artefacts restricts the accuracy of quantitative measurements.¹ Micro-CT, a non-destructive imaging approach with three-dimensional (3D) mineral

details at the micron level, appears to be a solution for precise assessments of bone. In orthopaedic and dental fields, micro-CT can provide information of bone geometry, mineral density and porosity and profile of the trabecular structure.² The information on tissue-level mineralization can be advantageous for understanding the biomechanical properties, aetiology and diagnosis of bone-associated diseases and the therapeutic effects *ex vivo* and *in vivo*.^{3,4} To correctly analyse micro-CT images, it is essential to reduce radiographic artefacts and look for a proper threshold to segment the image.⁵

Since the reproducible methodology assessing the *in vivo* specimens has been demonstrated by Park et al,⁶ it

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appears that micro-CT can achieve more precise volumetric measurements in periodontal bone destruction than repair groups. Alveolar bone, the supporting structure of tooth, is characterized by high porosity and heterogeneity, and the complexity of surrounding anatomical structures (*i.e.* tooth root, cementum, periodontal ligament) may cause difficulty in radiographic quantification. With the advancement of periodontal disease, alveolar bone destruction takes place by degrading bone matrix to release the calcium minerals, and may significantly alter the mineral density of the bone periphery.⁷ The radiographic intensity of tissue was only based on the local concentration of calcium instead of the configuration of actual bone matrix, and the blurred periphery of alveolar bone in the radiograph caused difficulty for threshold determination and quantification.

At present, several segmentation techniques are used to subtract bone from the surrounding tissue, including visual selection and histogram analysis, corresponding to the radiographic phantoms.⁸ The most widely used segmentation technique is built-in image analysis software: the so-called “global threshold” is based on histogram analysis, in which a Hounsfield unit value is chosen, and voxels above this value are marked as bone.⁸ In combination with the standard edge detection algorithm, a voxel-specific “local segmentation method” was further developed to compensate for the weakness of global segmentation methods in detecting the blurred margin of trabeculae.² As such, a dual segmentation setting was also introduced to determine the threshold of the less dense newly formed bone and very dense cortical bone.⁹

The present study aimed to identify the most appropriate threshold for evaluating the dynamic change of periodontal bone destruction due to inflammation. Several segmentation techniques were used, and the effectiveness was assessed by correlating the volumetric results with histological slides at the relevant level.

Materials and methods

Animal model and study design

A total of 15 male Sprague–Dawley rats were used in this study. All animal procedures were performed under protocol 032/10 approved by Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore. Five animals were killed prior to ligature placement and were regarded as the control group. Under general anaesthesia using a mixture of ketamine and xylazine, a 4-0 silk ligature was secured at the gingival sulcus level of the second molar (M2) of all animals as previously described.¹⁰ Animals were killed on Day 7 (early stage, $n = 5$) and on Day 21 (late stage, $n = 5$) and the maxillae were harvested and fixed in 10% formaldehyde at 4 °C for 3 days and transferred to 70% ethanol for subsequent examinations.

Micro-CT settings

Specimens with CT phantoms (Gammex Inc., Middleton, WI) of electron density 1.09 (IB), 1.47 (CB-2) and 1.69 (SB-3) were scanned by an X-ray generator (Siemens InveonTM CT; Siemens Healthcare, Erlangen, Germany) using an 80 W, tungsten anode, 30–80 kVp variable focus X-ray source and a 165 mm detector. Scans were acquired at 2×2 binning, exposure time of 950 ms per projection, high magnification and 56 kVp anode voltage, achieving an ideal dynamic range and an effective pixel size of 19.54 μm . Images were reconstructed with no down-sampling, using the Shepp–Logan algorithm and beam-hardening correction as recommended by the manufacturer.

Histological staining and quantitative measurements

After micro-CT scanning, specimens were decalcified with 12.5% ethylenediaminetetraacetic acid (pH 7.4) for 3 weeks and then embedded in paraffin. A transverse section in the middle of the mesiopalatal root of a second molar (Figure 1a) was selected and 5 μm thick sections were cut. Haematoxylin and eosin stains were performed for histomorphometry and tartrate-resistant acid phosphatase (TRAP) using a leucocyte acid phosphatase kit (Sigma-Aldrich Co. LLC., St Louis, MO) for osteoclastic activity. All the histological images were acquired using a Leica DMD108 system (Leica Microsystems GmbH, Wetzlar, Germany).

Quantifications for inflammatory and bone resorptive (TRAP-positive) cells were performed in five randomly selected areas within the gingival connective tissue and the surface of alveolar bone crest, respectively, at 400 $\times$ magnification, and results are presented as the percentage of inflammatory/bone resorptive cells to the total amount of cells.

Image overlay and region of interest selection

The micro-CT image was re-oriented using Siemens Inveon CT software until the slice corresponding to the histological section was found. The criteria of slice selections were based on the similarity of the morphology of tooth root, root canals and alveolar bone crest. A rectangular-shaped region of interest (ROI) was selected (Figure 1b) using the following parameters: (1) a straight line connecting the cervicoenamel junction (CEJ) and root apex of the mesiopalatal (MP) root of M2 (buccal limit); (2) a perpendicular line connecting the CEJ of MP roots of M2 (cervical limit); (3) a perpendicular line connecting the root apex of MP root of M2 (apical limit); (4) 0.5 mm palatal to the buccal limit (palatal limit).

Based on the above criteria, ROIs were manually selected in histological sections using ImageJ software (National Institute of Health, Bethesda, MD) by two examiners (M-CC and L-YC), and semi-automatically selected in micro-CT images using a customized MATLAB algorithm (MathWorks[®], Natick, MA) by another two examiners (JCL and KL).

Figure 1 Illustration for micro-CT and histomorphometric assessments, 40 $\times$. (a) The transverse section in the middle of the mesiopalatal root of the maxillary second molar (dashed line) was chosen for the plane of assessment. (b,c) A rectangular-shaped region of interest close to the root surface was selected for histomorphometric (b) and micro-CT (c) measurements. (d,e) The image was segmented by the global threshold-determining algorithm (d) and local threshold-determining algorithm (e)

Threshold determination

Three algorithms to determine the threshold were applied to the volumetric data, and the CPU time was recorded. The first was the global iterative process (GT₁), which divided the image intensity histogram of the entire dataset into two portions based on an initial guess.² It calculates the mean intensities in the two regions, and takes the average of these two values for use in the next iteration. Assuming that the distribution is bimodal, the algorithm converges to the midpoint between the two peaks, which represent the background and bone.

The second global algorithm (GT₂) is the Otsu method,¹¹ which non-iteratively maximizes the between-class variance (BCV) of the bimodal distribution. For each intensity bin in the image histogram, the BCV was calculated and the intensity that corresponded to the

maximal BCV was selected as the threshold. This threshold generally corresponded to the bottom of the valley between the two peaks in the distribution, to minimize the overlap between each cluster.

The third was a novel local algorithm (LT) that involved dividing the 3D dataset into separated 3 $\times$ 3 $\times$ 3 (in voxels) regions, and applying the above-mentioned iterative algorithm within each region to find a local threshold (Appendix A). This assumed a bimodal distribution in each region. The 3D dataset was first filtered for low-strength edges to remove noise and artefacts from the image dataset. This was done by calculating the Sobel gradient and setting a pre-set cut-off for low-intensity gradient voxels. The remaining voxels were then divided into regions and the local thresholds were determined automatically. For calculated local thresholds that were below 50% or above

Figure 2 Dynamic change in ligature-induced periodontal bone loss. (a) Intact connective tissue attachment and alveolar bone level in control (100 $\times$). (b) Inflammatory cell infiltration, disruption of connective tissue and erosive alveolar bone crest after 7 days of ligature placement, 100 $\times$. (c) Reduction of inflammation, recovery of connective tissue attachment with established bone loss at day 21 (100 $\times$). (d) Well-aligned collagen fibre and osteoclast lining with occasional Howship's lacunae on the bone crest in control (400 $\times$). (e) Extensive resorptive lacunae on the bone crest on day 7 (400 $\times$). (f) Newly formed extracellular matrix within gingival connective tissue with less prominent resorptive lacunae on the bone surface at day 2 (400 $\times$)

200% of the global threshold (*i.e.* entire background or bone regions that were essentially unimodal and gave skewed values with the algorithm), the global threshold was instead applied in these regions. The resultant thresholds were then smoothened with a 3D Gaussian filter to achieve better continuity between regions.

CT phantoms were used to determine thresholds. The phantoms were scanned under the same setting as tested specimens, and the threshold was determined by the Otsu technique.¹¹

Customized software was used to load the image volumes and apply the threshold-determining algorithms. Calculation of a bone fraction (bone area/total area) of the selected ROI was based on the threshold-determining results of a single image slice (Figure 1c–e).

In histological sections, the bone areas within the ROI were manually selected by two examiners (M-CC and L-YC) based on the morphology of the bony structure, and the bone fraction of ROI was calculated and compared with micro-CT imaging.

Examiner reliability and statistical analysis

Intra- and interexaminer validation measures were conducted for the bone fraction in histological and micro-CT slices. Examiners independently selected the ROI and bone area in all the specimens, and each

specimen was evaluated twice by each examiner with a minimum interval of 24 h. The reliability and reproducibility were determined by the intraclass correlation coefficient (ICC) and the coefficient of variation (CV) [$CV = (\text{standard deviation}/\text{mean}) \times 100\%$]. Values for the ICC range from 0 to 1. ICC values greater than 0.75 show good reliability,¹² and the CV demonstrates the precision error as an indicator for reproducibility.¹³ Quantitative measurements of inflammatory cells, bone resorptive cells, bone fraction and the value of threshold within each group were presented as mean and standard deviation and analysed by unpaired *t*-tests, with a *p*-value of less than 0.05 being considered statistically significant. The correlation between micro-CT imaging and histology was also evaluated by the ICC and CV stated above.

Results

All the animals survived until the time of killing without any significant gross pathology observed. Intraorally, progressive and persistent bleeding upon probing accompanied by hypermobility of M1 and M2 were noted in most of the ligature-placed animals after 3 days.

Figure 3 Osteoclastic activities by tartrate-resistant acid phosphatase staining. (a) Intense osteoclastic activities after 7 days of the silk ligature placement, 400 $\times$. (b) Reduced osteoclastic bone resorption at Day 21, 400 $\times$

Dynamic destruction from histological observations

In the control specimens, collagen fibres were uniformly oriented and perpendicular to the root surface within the gingiva. The surface of the alveolar bone crest was lined with multinucleated osteoclastic cells, and Howship's lacunae were occasionally detected on the surface of the alveolar bone crest (Figure 2a,d). Compared with the control animals, all specimens in experimental groups demonstrated a significant increase in distance from the alveolar bone surface to the CEJ (Figure 2a–c). Diffuse inflammatory cells infiltrating adjacent to the gingival sulcus with liquefaction degeneration of connective tissue and evident epithelium enlargement with disruption of connective tissue attachment were noted on Day 7 (Figure 2b). Inflammatory cell accumulation was also noted in the connective tissue next to the alveolar bone crest, and the surface of the bone crest was irregular owing to extensive osteoclastic activities (Figure 2e). At Day 21, the gingival connective tissue was occupied by newly formed extracellular matrix. Inflammation had subsided, and connective tissue reattachment to the root

surface could be occasionally found. The density of osteoclastic cells was decreased, and a reversal line was present (Figure 2c,f). The bone resorptive activities were also assessed by TRAP staining, demonstrating intense osteoclastic activities adjacent to the alveolar bone crest at Day 7 but dramatically subsided to scanty expression at Day 21 (Figure 3). Significant reduction of inflammation ($p < 0.01$) and alveolar bone resorption ($p < 0.05$) from Day 7 to Day 21 in further confirmed by the quantitative measurements (Figure 4).

Reliability and reproducibility of assessments

The reliability and reproducibility were evaluated by inter- and intraexaminer correlations. In the histological sections, the bone fraction was compared within the ROI between two examiners. Table 1 reveals high inter- and intraexaminer agreement, while a high ICC and low CV demonstrated that the procedure was standardized with precise errors between measurements. By further dividing the specimens into control, early stage (Day 7) and late stage (Day 21), our results demonstrated

Figure 4 Statistical analysis of inflammation and bone resorption at Days 7 and 21. The data are presented as the percentage of inflammatory/bone resorptive cells to the total amount of cells in the gingival connective tissue and the surface of alveolar bone crest (* $p < 0.05$; ** $p < 0.01$)

Table 1 Reliability and reproducibility of the bone fraction measurements

Groups	Histomorphometry		Micro-CT (IB threshold)	
	ICC	CV (%)	ICC	CV (%)
Overall	0.998	2.74	0.996	2.66
Control	0.998	1.62	0.989	1.22
Ligature (D7)	0.998	3.25	0.974	2.94
Ligature (D21)	0.992	3.75	0.986	3.27

CV, coefficient of variation; IB, CT phantom of electron density 1.09; ICC, intraclass correlation coefficient.

that the agreement was slightly higher in the control (ICC > 0.99, CV < 2%) than in the destructive stages (ICC > 0.99, CV < 4%).

In the micro-CT slice, the bone fraction within the ROI was compared under the fixed threshold determined by CT phantoms (Table 2). The overall reliability and reproducibility were high (CV < 5%, data not shown), and the highest agreement was noted with the IB threshold (ICC > 0.98, CV < 3%). In Table 1, under the IB-determined threshold, the reliability and reproducibility was still slightly higher in the control (ICC > 0.98, CV < 2%) than in the bone loss specimens (ICC > 0.98, CV < 4%). The results under the SB-3 threshold were excluded as hardly any bone appeared in this threshold.

The effect of threshold: correlations between micro-CT and histomorphometry

Among all the threshold-determining algorithms, the CPU time was longest for the LT method, whereas the global method, using GT₂, took longer than the GT₁. The range of the threshold determined and the correlation between micro-CT and histomorphometry are

shown in Table 2. The LT threshold was not available because of local variance leading to variable thresholds. No significant difference in the threshold value was demonstrated between GT₁ and GT₂. However, compared with the control, the threshold in ligature-placed specimens appeared to be higher with global threshold-determining algorithms, especially at Day 7.

Overall, measurement from the algorithm-based threshold was more highly correlated with histomorphometry than the phantom-based threshold (Table 2). The bone fraction under the LT-determined threshold demonstrated the strongest correlation among all determined thresholds (CV < 7%), whereas the closest correlation among the phantom-based threshold was by CB-2 (CV = 13.45%). The bone fraction from global threshold-determining algorithms revealed higher deviation from histomorphometry than the CB-2 threshold in controls and on Day 7, and the deviation appears to be lower in the global method at Day 21. By contrast, measurements by the LT-determined threshold consistently revealed high agreement with histomorphometry in all specimens and the closest correlation in the specimens with bone destruction, especially at Day 7 (CV < 5%). The bone fraction within the ROI also demonstrated a significant difference among the algorithm-based threshold-determining techniques in the Day 7 group, whereas no significant difference was noted in the control and the Day 21 group.

Discussions

In this study, we submerged a multithread silk stitch to simulate plaque-induced periodontal destruction. Plaque

Table 2 Correlation of threshold-determining techniques to histomorphometry: measurement from the bone fraction

Threshold-determining technique	Histomorphometry	GT ₁	GT ₂	LT	IB	CB-2	SB-3
<i>Overall (n = 15)</i>							
Value of threshold	N/A	2296.52 (430.39)	2296.49 (430.79)	N/A	994.3	1754.8	4096.5
CPU time (s)	N/A	0.15 (0.04) ^b	0.31 (0.07) ^b	6.53 (2.61)	N/A	N/A	N/A
Bone fraction	0.31 (0.11)	0.29 (0.16)	0.29 (0.16)	0.32 (0.12)	0.60 (0.14) ^{a,b}	0.39 (0.14)	0.00 (0.01) ^{a,b}
Correlation	N/A	0.955 (17.62)	0.955 (17.61)	0.993 (6.20)	0.946 (39.81)	0.949 (13.45)	N/A (137.18)
<i>Control (n = 5)</i>							
Value of threshold	N/A	1952.92 (114.83)	1952.59 (114.41)	N/A	994.3	1754.8	4096.5
Bone fraction	0.43 (0.08)	0.46 (0.09)	0.46 (0.09)	0.46 (0.05)	0.76 (0.05) ^{a,b}	0.52 (0.09)	0.00 (0.00) ^{a,b}
Correlation	N/A	0.900 (8.29)	0.900 (8.29)	0.989 (6.31)	0.979 (27.90)	0.990 (4.90)	N/A (141.40)
<i>Ligature D7 (n = 5)</i>							
Value of threshold	N/A	2811.07 (49.30)	2811.57 (49.19)	N/A	994.3	1754.8	4096.5
Bone fraction	0.24 (0.05)	0.17 (0.04) ^b	0.17 (0.04) ^b	0.24 (0.04)	0.54 (0.08) ^{a,b}	0.35 (0.09) ^{a,b}	0.01 (0.01) ^{a,b}
Correlation	N/A	0.960 (29.57)	0.960 (29.57)	0.960 (4.19)	0.925 (51.54)	0.851 (20.89)	N/A (136.99)
<i>Ligature D21 (n = 5)</i>							
Value of threshold	N/A	2020.76 (87.91)	2020.26 (88.11)	N/A	994.3	1754.8	4096.5
Bone fraction	0.31 (0.10)	0.31 (0.06)	0.31 (0.04)	0.33 (0.05)	0.50 (0.08) ^{a,b}	0.34 (0.06)	0.00 (0.00) ^{a,b}
Correlation	N/A	0.986 (9.61)	0.986 (9.62)	0.999 (6.99)	0.103 (38.07)	0.964 (12.89)	N/A (141.42)

CB-2, electron density 1.47; GT₁, global iterative process; GT₂, second global algorithm (Otsu method); IB, electron density 1.09; LT, local algorithm; N/A, not applicable; SB-3, electron density 1.69.

The values of threshold and bone fraction are presented as mean (standard deviation), and correlation is presented as the intraclass correlation coefficient (coefficient of variation).

^aSignificant difference to histomorphometry, $p < 0.05$.

^bSignificant difference to measurement by LT, $p < 0.05$.

can rapidly accumulate on the stitches and induce a pronounced inflammatory reaction in 3 days.^{14,15} Inflammatory infiltrate extended to the deep connective tissue, and a significant number of active osteoclasts lined the front of the alveolar bone at Day 7 (Figure 3a).^{15,16} The osteoclastic activities subsided at Day 21 (Figure 3b). Kuhr *et al*¹⁷ confirmed correspondence to the speed of bone loss, demonstrating that bone loss progressively increased in the first 2 weeks but consistently slowed down later. Thus, the specimens at Day 7 were likely to present with progressive bone resorption, whereas specimens at Day 21 predominantly displayed tissue adaptation and bone remodelling.

Good correlation between histomorphometry and micro-CT had been seen in previous investigations,^{1,18–21} and micro-CT appeared to be more suitable for measurement of the trabecular bone according to its variable spatial structure.²² However, the ROI usually required manual selection, in which some operational errors cannot be eliminated. The difference in resolution may potentially affect the outcome of measurements. Since the average width of bone trabeculae is 70–100 μm in rats,²³ the resolution of our specimens (19.54 μm) is considered suitable to estimate the structure indices of trabecular bone. Any higher resolution would lead to generation of a huge number of data without contributing significantly to the morphometric results.²⁴

This is the first study comparing several imaging segmentation methods to find a threshold specifically for inflammatory bone destruction. Although the study was limited by the relatively low number of specimens, significant differences among threshold-determining methods were revealed. Bone destruction takes place by degrading bone matrix to release calcium minerals,⁷ leading to a significant disruption of the collagen matrix and alteration of local mineral density. This phenomenon appears to cause difficulty identifying the bone border, and a greater interexaminer variance was noted in histomorphometry (Table 1). The blurred margin of bone under CT imaging may also influence determination of the threshold,⁵ and our results demonstrated a significant difference in bone fraction between local and global threshold-determining algorithms (Table 2). Later, at Day 21, while bone remodelling was taking place, the bone crest was replaced by a more mature and uniformly mineralized tissue. In accordance with the well-defined edges, our results indicated high reliability and reproducibility using histomorphometry as well as the high agreement of bone fraction between the local and the global threshold at this stage.

Fixed thresholds have been used in volumetric measurement in previous studies.^{4,9} Our study revealed that image segmentation by an appropriate fixed threshold was able to provide similar bone volume measurements to histomorphometry while no progressive bone loss was taking place (Table 2). The lower threshold determined by IB demonstrated higher deviation from histomorphometry than CB-2 owing to the unavoidable beam-hardening effects which led to overestimation of

the foreground.² Utilizing the global threshold-determining algorithm, researchers reported an ICC of 0.87–0.92 and CV of 8.3–27.2% of the bone fraction measurement,²⁵ whereas our study demonstrated equivalent results in controls; the correlation appeared higher while bone resorption was taking place. However, a significant increase in variation between histomorphometric and micro-CT measurements on Day 7 indicated that the blurred edge of mineralized tissue may affect threshold determination by global algorithms. As such, a local threshold based on the standard edge detection algorithms appears to be a solution to compensate for the effects of image degradation.^{26,27} Our study confirmed that LT threshold determining was able to provide a more precise measurement of trabecular structure than global threshold-determining algorithms in micro-CT imaging, and the correlation with histomorphometry was stronger and the variation was significantly less than with global methods. On the basis of the gradual loss of mineral content causing blurring of the bone edge during active osteoclastic resorption, LT threshold determining was suggested for image segmentation (Table 2).

The global threshold-determining method was computationally efficient for very large uncropped datasets, where convergence was reached in 1 s or less (Table 2). However, there was usually significant overlap between the statistical distributions of the voxels representing bone and background data in the histogram because of variable lighting and shading effects, meaning that a single selected threshold would inevitably misrepresent relatively brightly shaded background voxels, and newly synthesized bone voxels that were less dense and consequently less bright than most other bone. Images at this threshold tended to be washed out in areas of bone with fine morphological detail, and background artefacts were sometimes erroneously determined to be bone. By contrast, although the local threshold-determining method was far more computationally expensive, by taking local thresholds in small neighbourhoods even relatively brightly shaded background voxels could be successfully differentiated from bone. As such, the image resolution and selectivity of images with thresholds that were locally determined tended to be superior to those that were globally determined, and in certain cases the local method was hypersensitive to noise or artefact.

Although micro-CT imaging provides a more objective and precise quantification of the bony structure, this state-of-the-art technique still cannot provide information regarding bone apposition labelled by fluorescence markers,¹ and cellular evaluation is still not possible. Micro-CT imaging also cannot evaluate the alignment of collagen fibril and osteoid deposition.¹ It is also difficult to distinguish the host bone and calcium-containing grafting materials in micro-CT imaging.¹⁸ In these regards, accompanying micro-CT imaging with conventional histological sections is still required to evaluate bone dynamics.²⁰

In conclusion, micro-CT imaging is capable of offering detailed 3D information of periodontal structures. For

progression of periodontal destruction, the edge-detected local algorithm is suggested for determining a suitable threshold, whereas the results from a fixed-value threshold or global algorithms, commonly built in imaging software, are considered questionable and may be only appropriate to investigate steady-state mineralized structures. However,

the computationally expensive nature may restrict the application of the local algorithm to extensive image analysis. Thus, further investigation will aim at improving the computational efficiency of the local algorithm, even validating this methodology on specimens while repair or regeneration is in progress.

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Appendix A: The source code of local threshold calculation (under the MATLAB interface)

```

function thresholded = local_threshold(nimg3)
% This local threshold algorithm is to detect the area above the threshold
% of radiographic images.
%
% Originated by Mr. Kaicheng Liang (kliang933@gmail.com)
% Modified by Dr. Po-Chun Chang (dencpc@nus.edu.sg)
%
% Definition of variables
% nimg3 -- the array of a slice of radiographic image (containing the
% value of hounsfield units of each voxel)
% thresholded -- the array recording the voxel with the value of hounsfield
% unit above the threshold (the value of the voxel below the
% threshold is set as '0' )

NO_threshold = 0;
h = waitbar(0, 'Please wait...');
nimg2 = nimg3;
nimg3 = int16(nimg2);
nimg3 = sort(nimg3(:));
Tk = 0; Tkprev = 1000; iter = 1;

% the threshold from global algorithm (GT1) is generated firstly
% Global -- the threshold from global algorithm

for k = 1:100
    while ((abs(Tkprev-Tk)/Tkprev)*100)>.1
        background = nimg3(find(nimg3<= Tk));
        bone = nimg3(find(nimg3>Tk));
        Tkprev = Tk;
    end
    Tk = .5*(sum(bone)/length(bone)+sum(background)/length(background));
    iter = iter+1;
end

Global = Tk

% thresholded -- the array recording the voxel with the value of hounsfield
% unit above the threshold (the value of the voxel below the
% threshold is set as '0' )

thresholded = (nimg2>Tk);

load sobel

nimg2 = smooth3(nimg2, 'gaussian', 5, .001);

gradz = imfilter(nimg2, sobelz, 'same', 'conv');
grady = imfilter(nimg2, sobely, 'same', 'conv');
gradx = imfilter(nimg2, sobelx, 'same', 'conv');
gradmag = sqrt(gradx.^2+grady.^2+gradz.^2);

kernelsize = 3;
gradthresh = str2num(get(handles.edit4, 'String'));

```

```
edgeint = nimg2.* (gradmag>gradthresh);

origsize = size(nimg2);
tempsize = ceil(origsize/kernelsize)*kernelsize;
nimg2(tempsize(1),tempsize(2),tempsize(3)) = 0;

edgeint(tempsize(1),tempsize(2),tempsize(3)) = 0;
for i = (origsize(3)+1):tempsize(3)
    edgeint(:, :, i) = edgeint(:, :, origsize(3));
end
localthresh = zeros(size(nimg2));

for i = 1:kernelsize:size(nimg2,1)
    for j = 1:kernelsize:size(nimg2,2)
        for k = 1:kernelsize:size(nimg2,3)
            [ i,j,k] ;

            testcube = edgeint(i:i+kernelsize-
1,j:j+kernelsize-1,k:k+kernelsize-1);
            testcube = testcube(:);
            Tk = mean(testcube); Tkprev = 1000000; iter = 1;

            for l = 1:100
                while ((abs(Tkprev-Tk)/Tkprev)*100)>.1
                    background = testcube(find(testcube<= Tk));
                    bone = testcube(find(testcube>Tk));
                    Tkprev = Tk;

Tk = .5*(sum(bone)/length(bone)+sum(background)/length(background));
                    iter = iter+1;
                end
            end
            if Tk < .5*Global | isnan(Tk) == 1
                Tk = Global;
            elseif Tk > 2*Global
                Tk = Global;
            end
            Tk;
            localthresh(i:i+kernelsize-1,j:j+kernelsize-1,k:k+kernelsize-1) = Tk;
        end
    end
    waitbar(i / size(nimg2,1))
end
localthresh2 = smooth3(localthresh, 'gaussian', 5, 300);
nimg2 = nimg2(1:origsize(1),1:origsize(2),1:origsize(3));
localthresh = localthresh(1:origsize(1),1:origsize(2),1:origsize(3));

localthresh2 = localthresh2(1:origsize(1),1:origsize(2),1:origsize(3));
thresh2 = nimg2>localthresh;
thresh2 = nimg2>localthresh2;

thresholded = thresh2(:, :, 3);
close(h)
end

Global = Tk
```

```

% thresholded -- the array recording the voxel with the value of hounsfield
% unit above the threshold (the value of the voxel below the
% threshold is set as '0' )
thresholded = (nimg2>Tk);

load sobel

nimg2 = smooth3(nimg2, 'gaussian', 5, .001);

gradz = imfilter(nimg2, sobelz, 'same', 'conv');
grady = imfilter(nimg2, sobely, 'same', 'conv');
gradx = imfilter(nimg2, sobelx, 'same', 'conv');
gradmag = sqrt(gradx.^2+grady.^2+gradz.^2);
kernelsize = 3;
gradthresh = str2num(get(handles.edit4, 'String'));
edgeint = nimg2.*(gradmag>gradthresh);
origsize = size(nimg2);

tempsize = ceil(origsize/kernelsize)*kernelsize;
nimg2(tempsize(1), tempsize(2), tempsize(3)) = 0;
edgeint(tempsize(1), tempsize(2), tempsize(3)) = 0;
for i = (origsize(3)+1):tempsize(3)
    edgeint(:, :, i) = edgeint(:, :, origsize(3));
end
localthresh = zeros(size(nimg2));

for i = 1:kernelsize:size(nimg2, 1)
    for j = 1:kernelsize:size(nimg2, 2)
        for k = 1:kernelsize:size(nimg2, 3)
            [ i, j, k ];

            testcube = edgeint(i:i+kernelsize-
1, j:j+kernelsize-1, k:k+kernelsize-1);

            testcube = testcube(:);
            Tk = mean(testcube); Tkprev = 1000000; iter = 1;

for l = 1:100
    while ((abs(Tkprev-Tk)/Tkprev)*100)>.1
        background = testcube(find(testcube<= Tk));
        bone = testcube(find(testcube>Tk));
        Tkprev = Tk;

Tk = .5*(sum(bone)/length(bone)+sum(background)/length(background));
        iter = iter+1;
    end
end

if Tk < .5*Global | isnan(Tk) == 1
    Tk = Global;
elseif Tk > 2*Global
    Tk = Global;
end

Tk;
localthresh(i:i+kernelsize-1, j:j+kernelsize-
1, k:k+kernelsize-1) = Tk;

```

```
        end
    end

    waitbar(i / size(nimg2,1))
end

localthresh2 = smooth3(localthresh, 'gaussian', 5, 300);

nimg2 = nimg2(1:origsize(1), 1:origsize(2), 1:origsize(3));

localthresh = localthresh(1:origsize(1), 1:origsize(2), 1:origsize(3));
localthresh2 = localthresh2(1:origsize(1), 1:origsize(2), 1:origsize(3));

threshed = nimg2 > localthresh;
threshed2 = nimg2 > localthresh2;

thresholded = threshed2(:, :, 3);

close(h)
```