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Parameters

(ヒト変形性膝関節症に伴う軟骨下骨  
変性を捉える超音波指標：  
マイクロCTパラメータと  
の対比による Ex Vivo 研究)

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Ultrasound Parameters for Human Osteoarthritic Subchondral Bone Ex Vivo: Comparison with  
Micro-Computed Tomography Parameters

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## Abstract

This study aimed to identify ultrasound parameters reflecting subchondral porosity ( $P_o$ ), subchondral plate thickness ( $T_{pl}$ ), and bone volume fraction at the trabecular bone region ( $BV/TV_{Tb}$ ). Sixteen osteoarthritic human lateral femoral condyles were evaluated ex vivo using a 15-MHz pulsed-echo ultrasound three-dimensional scanning system. The cartilage–subchondral bone (C-B) surface region (layer 1) and inner subchondral bone region (layer 2) were analyzed; we newly introduced entropy ( $ENT$ ) and correlation ( $COR$ ) of ultrasound texture parameters of the parallel (x) or perpendicular (z) direction to C-B interface for this analysis.  $P_o$ ,  $T_{pl}$ , and  $BV/TV_{Tb}$  were evaluated as reference measurements using micro-computed tomography.  $ENT_{L1x}$  ( $ENT$  of layer 1, x-direction) and  $ENT_{L1z}$  were significantly correlated with  $P_o$  (both  $r = 0.58$ ),  $COR_{L2x}$  with  $T_{pl}$  ( $r = -0.73$ ), and  $COR_{L2z}$  with  $BV/TV_{Tb}$  ( $r = -0.66$ ). These are efficient indicators of the characteristics of osteoarthritis-related subchondral bone; the other texture parameters were not significant.

**Keywords:** osteoarthritis, subchondral bone, micro-CT, ultrasound, texture parameter, porosity, thickness, bone volume fraction, cartilage

## Introduction

Osteoarthritis (OA) of the knee, one of the most common locomotive diseases (Felson, et al. 1987, Hart and Spector 1993, Yoshimura 2009), considerably decreases the quality of life (Norman-Taylor, et al. 1996). The early stages of OA have been characterized by cartilage degeneration, such as damage to the collagen meshwork in the superficial zone of cartilage (Han, et al. 2002, Poole, et al. 2002) and fibrillation of the cartilage surface (Minns, et al. 1977). However, changes in subchondral bone have received increasing attention in recent years despite an early OA stage. Elevated bone remodeling and subchondral bone losses have been seen in the early stages of OA in humans (Bettica, et al. 2002). Thinning and increased porosity of the subchondral bone plate were reported by a canine early OA model (Intema, et al. 2010). After temporal thinning, subsequent thickening of the subchondral bone plate was revealed with OA progression in an animal OA model (Batiste, et al. 2004, Intema, et al. 2010). When including normal to severely degenerated samples, subchondral plate thickness and bone volume fraction ( $BV/TV$ ) in trabecular bone were strongly correlated with OA histopathological grade (Finnilä, et al. 2017). For such OA-related osteochondral changes, ultrasound parameters have been investigated. Amplitude-based ultrasound parameters, such as reflection coefficient ( $R$ ) and integrated reflection coefficient ( $IRC$ ) from the cartilage surface, were sensitive to surface roughness (Chérin, et al. 1998, Kaleva, et al. 2009, Kiyan, et al. 2017, Saarakkala, et al. 2006, Saarakkala, et al. 2004), collagen orientation in the superficial layer of cartilage (Kiyan, et al. 2017), and enzymatic digestion of collagen (Saarakkala, et al. 2004). The wavefront is disturbed by the fibrillated cartilage surface, and the acoustic impedance approaches the surrounding medium by disrupting the collagen meshwork. These are assumed to lower the echo amplitude from the cartilage surface.

For the subchondral bone, comparison of the histological evaluation and quantitative

ultrasound findings showed that *IRC* at the cartilage–subchondral bone (C-B) interface significantly increased in the degenerated osteochondral samples (Saarakkala, et al. 2006). The apparent integral backscatter (*AIB*) from the bone was negatively correlated with bone mineral density (*BMD*) of the subchondral plate (Aula, et al. 2010). The *AIB* from the subchondral bone was also correlated with the surface/volume ratio and trabecular thickness (Liukkonen, et al. 2013). Thus, several studies have shown the sensitivity of *IRC* or *AIB* for determining the *BMD* of the subchondral bone plate or histological score. However, no report has investigated the relationships between ultrasound parameters and porosity at the surface of the subchondral bone plate, subchondral plate thickness, or *BV/TV* in the trabecular region as micro-structural characteristics. Ultrasound with lower frequency (less than 5 MHz) has been used to detect osteoporosis from a macroscopic point of view such as (Hoffmeister, et al. 2006, Karjalainen, et al. 2009). However, focused ultrasound with high and broadband frequency, leading to high resolutions, might be efficient when the aim is limited to the detection of pores on the subchondral plate surface, subchondral plate thickness (Finnilä, et al. 2017, Li, et al. 1999, Milz, et al. 1995), or *BV/TV* just beneath the subchondral plate with sub-millimeter measurements. Furthermore, pores at subchondral plate surface are expected to lower the echo intensity from the C-B interface locally. Internal microstructural characteristics of subchondral bone are also expected to disturb the scattered intensity from the subchondral plate–bone marrow interface because the subchondral plate is not a simple plane plate but complex with areas of the junction to trabecular bone. Therefore, parameters that reflect the spatial distribution of echo intensity might efficiently detect such microstructural changes in the subchondral bone. However, the parameters of the distribution of the echo intensity for microstructural changes in the subchondral bone have not yet been investigated. In addition, *AIB* or *IRC* from bone is affected by attenuation of ultrasound in cartilage (Joiner, et al. 2001). Parameters that are not affected by attenuation in cartilage or thickness of

cartilage are needed to determine the microstructural characteristics of subchondral bone.

In this study, we sought to determine the ultrasound parameters that reflect the microstructural changes in subchondral bone porosity ( $P_o$ ), subchondral plate thickness ( $T_{pl}$ ), and  $BV/TV$  at the trabecular area ( $BV/TV_{Tb}$ ). We focused on detecting subchondral bone changes, not cartilage degeneration, especially in the early to mild stages of OA. For the ultrasound parameters, the  $IRC$  at the C-B interface and the  $AIB$  from the subchondral bone were evaluated.  $IRC$  at the C-B interface and  $AIB$  from the subchondral bone are expected to be influenced by the effect of attenuation through the cartilage. The degree of attenuation in the cartilage layer was considered equal for both  $IRC$  and  $AIB$  because they were attenuated from the same sound pathway in the overlying cartilage. Therefore, we also evaluated the difference between  $AIB$  and  $IRC$ , expecting tolerability of attenuation of ultrasound in cartilage. As indicators reflecting spatial distribution, we introduced texture parameters (Haralick, et al. 1973) for detecting microstructural characteristics in subchondral bone. As reference measurements,  $P_o$ ,  $T_{pl}$ , and  $BV/TV_{Tb}$  were evaluated using micro-CT.

## Materials and Methods

### Samples

The protocol of the present study was approved by the National Hospital Organization, Kyoto Medical Center Review Board (approval number: 09-31). Sixteen human knee osteochondral samples were obtained from 16 OA patients (age  $76 \pm 5$  years: 12 females and 4 males) who underwent total knee arthroplasty. Samples were kept in the freezer at  $-80^{\circ}\text{C}$ . Before the ultrasound measurements were taken, samples from the load-bearing region of lateral femoral condyles were cut to approximately  $14 \times 7 \text{ mm}^2$  (Fig. 1a) sections, left out to thaw, and prepared for ultrasound measurements. Here, we focused on detecting the characteristics in normal-to-mild OA subchondral bone samples, excluding severely degenerated samples. Sixteen samples were visually judged as

International Cartilage Repair Society (ICRS) grade 0–2 (Brittberg and Peterson 1998) by the author YN, who has been an orthopedic surgeon for more than 30 years. Five samples were graded as 0, nine as 1, and two as 2.

#### *Ultrasound measurement system*

The three-dimensional ultrasound scanning system consisted of a computer (NI PXIe-8133; National Instruments, Austin, TX, USA), digitizer with 14-bit resolution and a sampling frequency of 100 MHz (NI PXIe-5122; National Instruments), X-Y stage, stage controller (SHOT-202AM; Sigma Koki, Japan), pulser-receiver (DPR300; JSR Ultrasonics, NY, USA), and point-focused ultrasound probe (V313; Olympus, Japan) with an element diameter of 6.35 mm, f-number of 2, and depth of focus of 2.4 mm (Fig. 1a). In this study, the x- and y-directions were defined as orthogonal to the sound axis, z. The scanning pitch was 0.02 mm in the x-direction and 0.1 mm in the y-direction. The pulser-receiver excited the ultrasound probe. A bandwidth at half-maximum in spectrum amplitude from the plane steel target at the focus was 3.6–22.4 MHz. The beam diameter (–6 dB, at the focus 13.8 mm) was 0.17 mm. The radiofrequency (RF) analog signals were digitized and stored on the computer to analyze the ultrasound parameters. Osteochondral samples were fixed in saline at 20–25°C. Under this condition, the average speed of sound in the saline was 1524 ms<sup>-1</sup>. Before the data acquisition process, the distance between the ultrasound probe and the sample was adjusted with the Z-stage to ensure that the C-B interface was in the focal zone. In addition, the sample was pre-scanned, and cartilage-bone interface was semi-automatically detected. The central region of the sample in X-Y plane was approximated by a plane. On the basis of the coefficients of the fitted plane, the inclination of the sample was adjusted using biaxial goniometer stages so that the ultrasound wave was transmitted almost perpendicularly to the global surface of the bone.

#### *Pre-processing of ultrasound parameters*

The time positions of the saline-cartilage interface and C-B interface (Fig. 2a) were detected using a threshold-based method. The absolute value of RF signal was spline-fitted. Local asperity was removed, and the profile of the global surface of subchondral bone was defined. The echo signal is affected by the experimental system. The echo signal was normalized in the frequency domain by the reference spectrum as described previously to eliminate the transfer function of the experimental system (Chérin, et al. 1998). The RF signal was gated by a Hanning window with a length of  $0.2 \mu\text{s}$  around the center of the signal of interest (SOI),  $t_{\text{SOI}}$ . The spectrum of the windowed signal ( $S(x, y, f)$ ) was calculated by fast Fourier transform as 512 points with zero padding (Appendix 1: representative spectral amplitude) and normalized by the spectrum of the reference waveform of the perfect reflector at the same depth as the SOI ( $S_{\text{ref}}$ ). The depth ( $z$ ) was calculated by assuming a speed of sound (SOS) of  $1524 \text{ ms}^{-1}$  in saline ( $c_s$ ) and  $1620 \text{ ms}^{-1}$  (Agemura, et al. 1990) in cartilage ( $c_c$ ). The normalized spectrum was averaged around  $x$  with a length of  $\Delta x$ , transformed to a dB value, and averaged within the full width of  $\Delta f$  at the half-maximum  $S_{\text{ref}}(f)$ . The distribution of  $IRC$  or  $AIB$ ,  $B(x, y, t)$ , was calculated by shifting the gate with a pitch of  $0.01 \mu\text{s}$  for the entire area:

$$B(x, y, t) = \frac{1}{\Delta f} \int_{\Delta f} 10 \log_{10} \left( \frac{1}{\Delta x} \int_{x-\frac{\Delta x}{2}}^{x+\frac{\Delta x}{2}} \left| \frac{S(x, y, f)|_t}{S_{\text{ref}}(f)|_{t'}} \right|^2 dx \right) df, \quad (1)$$

$$t = t_{\text{c surf}} + \Delta t_{\text{in}},$$

$$t' = t_{\text{c surf}} + \Delta t_{\text{in}} \times \frac{\tan \theta_r}{\tan \theta_i},$$

$$\theta_i = \arctan(r_{\text{element}}/z_{\text{focus}}),$$

$$\theta_r = \arcsin\left(\frac{c_c}{c_s} \sin \theta_i\right)$$

where  $t_{\text{c surf}}$  was the TOF of the echo from the saline-cartilage interface and  $\Delta t_{\text{in}}$  was the time distance between the saline-cartilage interface and the center of the SOI. The variable  $r_{\text{element}}$  was the radius of

the element of the probe (3.18 mm), and  $z_{focus}$  was the focal length (13.8 mm).  $B(x,y,t)$  was rearranged so that the detected C-B interface was in line (Fig. 2b). Here, time axis  $t$  (Fig. 2b) was redefined so that the rising point of the echo from C-B was the origin. The rising point was set to the -10 dB point from the averaged intensity in the x-direction at each  $y$  position (Fig. 2c).  $B(x,y,t)$  was separated into two layers parallel to the C-B interface as layers 1 and 2 with widths set to 0.2  $\mu s$  and 0.3  $\mu s$ , respectively (Fig. 2b).

#### *Spatial averaged ultrasound parameters*

The reflection components from the C-B interface are considered the main components of layer 1. The averaged intensity of layer 1 was calculated as follows:

$$IRC_{L1} = \frac{1}{L_x L_y} \iint \max(B(x, y, t) \mid 0 \leq t < \Delta T) dx dy, \quad (2)$$

where  $L_x$  and  $L_y$  were the lengths of the side for the rectangle region of analysis in the  $x$  and  $y$  directions, respectively,  $\max(* \mid \text{section})$  denotes the function that outputs the maximum value within the section, and  $\Delta T$  is 0.2  $\mu s$ . The analyzed region was selected from the central region without artifacts of the sample edge, and  $L_x$  and  $L_y$  were approximately 5 mm and 3 mm, respectively.

For layer 2, the averaged backscatter intensity,  $AIB_{L2}$ , was defined as follows:

$$AIB_{L2} = \frac{1}{L_x L_y \Delta T} \iiint_{0.2 \mu s}^{0.5 \mu s} B(x, y, t) dt dx dy, \quad (3)$$

$IRC_{L1}$  and  $AIB_{L2}$  are expected to be influenced by the effect of attenuation through the cartilage. Therefore, in this study, we focused on extracting the characteristics of subchondral bone without influence from the overlying tissues. The degree of attenuation in the cartilage layer was considered equal for both  $IRC_{L1}$  and  $AIB_{L2}$  because they were attenuated from the same sound pathway in the overlying cartilage. We introduced the difference (in dB value) of  $AIB_{L2}$  to  $IRC_{L1}$  expecting a reduced attenuation effect through the cartilage:

$$D_{L21} = AIB_{L2} - IRC_{L1}, \quad (4)$$

In addition, the spatial averaged pulse width of  $B$  was evaluated.  $B(x, y, t)$  was normalized by the maximum level of  $B$  at each position  $(x, y)$ . The summation of the section that exceeded the threshold value  $V_{th}$  for normalized intensity  $nB(x, y, t)$  was defined as pulse width  $PW_b$ :

$$PW_b(x, y) = \iiint_{t=0}^{t=0.75\mu s} [nB(x, y, t) \geq V_{th}] dt dx dy, \quad (5)$$

where operation  $[*]$  returned 1 if the condition  $*$  was true and otherwise  $[*]$  returned zero.  $V_{th}$  was set at -6 dB.

#### *Texture parameters*

Texture parameters by Gray Level Co-occurrence Matrix (GLCM) (Conners, et al. 1984, Haralick, et al. 1973) were applied to extract the characteristics of subchondral bone.  $B$ , eq (1), was normalized by the maximum value of each x-z 2-D cross-section and the maximum value of each layer. The normalized  $B$  of each layer was converted to the gray level,  $B_g$ , with the dynamic range of 30 dB. The number of gradations,  $N_g$ , was set to 256. After gradation, GLCM ( $P$ ) was calculated as follows:

$$P(i, j | \Delta x, \Delta z) = \frac{1}{(N_x - \Delta x)(N_z - \Delta z)} Q(i, j | \Delta x, \Delta z), \quad (6)$$

$$Q(i, j | \Delta x, \Delta z) = \left( \sum_{n=1}^{N_z - \Delta z} \sum_{m=1}^{N_x - \Delta x} A \right),$$

$$\text{where } A = \begin{cases} 1 & \text{if } B_g(m, n) = i \text{ and } B_g(m + \Delta x, n + \Delta z) = j \\ 0 & \text{elsewhere} \end{cases}.$$

The cases in which combinations of the two gray levels at relative positions specified by distance  $(\Delta x, \Delta z)$  was  $(i, j)$  were counted and accumulated throughout the whole image with the size of  $N_x \times N_z$  to obtain the co-occurrence frequency matrix with a size of  $N_g \times N_g$  ( $Q$ ).  $P$  was obtained by normalizing the total number of combinations. The texture parameters of entropy ( $ENT$ ) and correlation ( $COR$ ) (Albregtsen 2008, Conners, et al. 1984, Haralick, et al. 1973) were calculated from

$P$  as follows:

$$ENT(\Delta x, \Delta z)_{Li} = - \sum_{i=0}^{Ng-1} \sum_{j=0}^{Ng-1} P(i, j | \Delta x, \Delta z) \times \log_2 P(i, j | \Delta x, \Delta z). \quad (7)$$

$$COR(\Delta x, \Delta z)_{Li} = \sum_{i=0}^{Ng-1} \sum_{j=0}^{Ng-1} \frac{ijP(i, j | \Delta x, \Delta z) - \mu_x \mu_y}{\sigma_x \sigma_y}, \quad (8)$$

where

$$\mu_x = \sum_{i=0}^{Ng-1} i \sum_{j=0}^{Ng-1} P(i, j | \Delta x, \Delta z),$$

$$\mu_y = \sum_{i=0}^{Ng-1} \sum_{j=0}^{Ng-1} j P(i, j | \Delta x, \Delta z),$$

$$\sigma_x^2 = \sum_{i=0}^{Ng-1} (i - \mu_x)^2 \sum_{j=0}^{Ng-1} P(i, j | \Delta x, \Delta z),$$

and

$$\sigma_y^2 = \sum_{j=0}^{Ng-1} (j - \mu_y)^2 \sum_{i=0}^{Ng-1} P(i, j | \Delta x, \Delta z).$$

Each texture parameter obtained from the x-z cross-sectional image was averaged in the y-direction to be compared to each micro-CT parameter.

$ENT$  expresses the disorder of the image and amount of information. When an image is uniform, only specific combinations of two gray levels occur at a high probability, and the image decreases the  $ENT$ . On the other hand, when an image is complex, and there are many combinations of two gray levels, the image increases the  $ENT$ . In principle,  $ENT$  can range from 0 to 16 in an image with 256 gradations.  $ENT$  was introduced to detect microstructural differences in subchondral bone.

$COR$  expresses the correlation between the combinations of two gray levels and can range

from -1 to 1. When an image has a banded or linear pattern, the combinations of two levels tend to be close and the image increases *COR*. When the ultrasound scans the subchondral bone, an echo band parallel to the subchondral surface is observed. We expected the porosity or subchondral microstructural changes to destroy the band or continuously influence the *COR*.

These texture parameters were analyzed with a focus on two directions: parallel (toward the x-direction) and perpendicular (toward the z-direction) to the C-B interface. The distance for the x-direction ( $\Delta x$ ) was set 0.6 mm from the viewpoint of the subchondral bone microstructure and acoustic field in this experimental system. Preliminarily, we analyzed the diameters of surface pore for a few samples by micro-CT and confirmed that it has a maximum diameter of 0.6 mm. The distance of two positions should be set to the same or greater order to detect such structures. If the two relative positions are too close, such as 0.1 mm (one pixel), the texture parameters might have a lower ability to detect structural changes because the gray levels between two positions are too highly correlated to distinguish structural differences. In cases wherein the distance (in the x-direction) between two relative positions was set to less than the beam diameter, which was 0.17 mm in this experimental system, two gray levels,  $B_g(m, n)$  and  $B_g(m + \Delta x, n + \Delta z)$ , were too close, and the ranges of texture parameter tend to be small. In the z-direction, the distance was set to 0.10  $\mu s$ , which corresponded to the half width at -15 dB maximum of  $B$  for a perfect reflector (in Fig. 1c). Thus, texture parameters were calculated in the x-direction (parallel to the C-B interface,  $(\Delta x, \Delta z) = (0.6 \text{ mm}, 0 \mu s)$ ) and the z-direction (perpendicular to the C-B interface,  $(\Delta x, \Delta z) = (0 \text{ mm}, 0.10 \mu s)$ ). In this study, subscript x or z in the variable names of texture parameters shows the direction, whereas subscript  $L_1$  or  $L_2$  shows the layer number.

### *Micro-CT parameters*

The osteochondral samples were scanned by a micro-focus X-ray CT system

(SMX-100CT-SV3 Type II; Shimadzu, Japan) with a voxel size of  $31 \times 31 \times 31 \mu\text{m}^3$ , cone beam mode, tube voltage of 45 kV, and the tube current of 42  $\mu\text{A}$ . Reconstructed images were output by the manufacturer provided software. The X-Y plane was defined as the plane approximately parallel to the cartilage surface, and the Z-axis was defined as an axis of depth direction, the same as in Fig. 1a. First, cross-sections including the artificial cut edges of samples were removed, and cuboid areas of CT images were prepared. Three interfaces of air-cartilage, C-B, and backside of the subchondral plate (subchondral plate-bone marrow) were detected as follows.

The CT images were binarized using a global threshold set to 12 % of maximum value (8 bit, 255) and median filter with the kernel size of (3,3) for X-Z cross-section was adopted to remove the isolation points. The air-cartilage interface was easily detected as a first positive edge (position of the first 1 of binarized image) toward depth direction (Fig. 3a green line).

For the detection of C-B interface, two steps were set. In the first step, as a rough detection of C-B interface, the global threshold of 35 % was set, and the first edge was detected toward depth direction. The outliers of detected positions were removed and linear-interpolated (Fig. 3a red line). In the second step, original CT images were extracted so that the roughly detected C-B interface (Fig. 3a red line) was in line. Rearranged CT images were pre-thresholded to 24 %, and remaining pixels were binarized with adaptive thresholding using the mean of minimum and maximum values within a window with the size of (15, 35) for the X-Z cross-section (Bernse 1986) (Fig. 3b). Positions of the first 1 toward to depth direction were detected, and the outliers at pore were removed and linear-interpolated to obtain the C-B interface.

In the detection of backside of subchondral plate, 3-D median filter with the kernel size of (3, 3, 3) was adopted for the rearranged and binarized images. After median filtering, the opening processing of morphological operations with the kernel size of (7,7) for the X-Y cross-section was

adopted to separate the junction area of the subchondral plate and trabecular bone (Fig. 3c). The backside of the subchondral plate was detected as the first negative edge (first 0) from the C-B interface. The outliers were removed and linear-interpolated as the same as the processing of detection of C-B interface. If there was no negative edge because of pore area, backside position was defined as 0.09 mm depth (3 in pixel) from the interpolated C-B interface for the later calculation of porosity in the subchondral plate.

For trabecular bone analysis, the volume of interest (VOI) was defined as 0.5–1.5 mm depth from the C-B interface. The trabecular area of the original CT images was binarized with an adaptive threshold using local mean and standard deviation by (Sauvola and Pietikäinen 2000). The window size was set to (31, 31) for X-Z plane so that both the trabecular bone and bone marrow region were included. A parameter of a bias setting (k) was set to 0.5, and the dynamic range of standard deviation (R) was set to 128 (i.e. 50 %) (Fig. 3d).

After segmentation, the volume and the area of each segment were calculated by volumetric marching cubes method (Lorensen and Cline 1987, Muller and Ruegsegger 1995) (Fig. 3e). The thickness of cartilage ( $T_c$ ) was obtained by dividing the volume of cartilage by the interpolated subchondral plate area ( $S_{plate}$ ). The subchondral plate thickness ( $T_{pl}$ ) was obtained by dividing the volume of the subchondral plate by  $S_{plate}$ . For the calculation of the porosity in superficial zone of the subchondral plate, VOI was selected as the depth of 0 to the smaller depth of 0.09 mm or backside from C-B interface.  $BV/TV$  in the VOI of the subchondral plate ( $BV/TV_{plate}$ ) was calculated and porosity in subchondral bone ( $P_o$ ) was calculated as  $1 - BV/TV_{plate}$ .  $BV/TV$  in the trabecular bone region of 0.5–1.5 mm depth from the C-B interface ( $BV/TV_{Tb}$ ) were calculated as the ratio of bone volume in the trabecular bone region to total volume, which was equal to  $S_{plate} \text{ mm}^2 \times 1.0 \text{ mm}$ .

*Statistical analyses*

Normality was tested by Shapiro-Wilk test for all the ultrasound and micro-CT parameters. The correlations between ultrasound parameters and micro-CT parameters were evaluated by Pearson's correlation coefficients or Spearman's rank correlation coefficients to investigate the efficiency of detecting each microstructural characteristic of the subchondral bone. In addition, the partial correlations between the ultrasound parameters and the micro-CT parameters when the control variable was set to  $T_c$  were evaluated to investigate whether each parameter reflected the microstructural characteristics of subchondral bone without being affected by attenuation through the cartilage. Similarly, for parameters without normality, partial rank correlation coefficients were evaluated.

Samples were grouped into two, intact (ICRS grade = 0) and degenerated (ICRS grade = 1 or 2) and the differences in micro-CT parameters between two groups were tested to investigate the relationship between degeneration in cartilage surface and micro-CT parameters. Before the test, the normality and equality of variances were confirmed for each group and each parameter by Shapiro-Wilk's test and Levene's test, respectively. On the basis of the results of normality and the equality of variances, Student's t-test, Welch's t-test, or Mann-Whitney U test was selected.

All results were considered significant at a two-tailed p-value < 0.05.

## Results

### *Representative images*

Figs. 4 show representative images of two samples (#1 and #2) obtained by micro-CT and ultrasound measurements. Sample #1 showed higher porosity and lower thickness of the subchondral plate ( $P_o = 13.0\%$  and  $T_{pl} = 0.12\text{ mm}$ , Fig. 4-a1) than those of sample #2 ( $P_o = 3.1\%$  and  $T_{pl} = 0.29\text{ mm}$ , Fig. 4-a2).  $BV/TV_{Tb}$  was almost the same value both in sample #1 (32.0 %, Fig. 4-2a) and sample #2 (32.9%, Fig. 4-2b). Corresponding to these microstructural characteristics of subchondral bone, the

cross-sectional echo intensity  $B$  showed qualitative differences (Figs. 4-c1, 4-c2). In sample #2, there was much scattering in layer 2 (Fig. 4-c2), and the pulse width was larger in sample #2 (Fig. 4-d2) than in sample #1 (Fig. 4-d1). We expected the qualitative agreement with porosity distribution (Figs. 4-a1, 4-a2) and ultrasound pulse width distribution (Figs. 4-d1, 4-d2) but did not observe obvious agreement.

#### *Correlation coefficients between ultrasound parameters and micro-CT parameters*

Shapiro-Wilk test showed the normality for the all parameters except  $PW_b$  and  $COR_{L1x}$ . Descriptive statistics for all the parameters are shown in Table 1.

For porosity in the subchondral plate,  $ENT_{L1x}$ ,  $ENT_{L1z}$ , and  $ENT_{L2z}$  of the texture parameters significantly correlated with  $P_o$  ( $r = 0.58$ ,  $0.58$ , and  $0.52$ , respectively; Table 2). Partial correlations between these parameters and  $P_o$  also showed significance, especially in  $ENT_{L1x}$  and  $ENT_{L2z}$  ( $r = 0.58$  and  $0.57$ ; Table 3).

For subchondral plate thickness,  $D_{L2l}$  significantly correlated with  $T_{pl}$  ( $r = 0.64$ ; Table 2).  $D_{L2l}$  was also significantly correlated with  $T_c$  ( $r = 0.63$ ; Table 2). However, the partial correlation coefficient between  $D_{L2l}$  and  $T_{pl}$  showed a significant correlation ( $r = 0.54$ ; Table 3). All the texture parameters of layer 1 did not show significant correlation with  $T_{pl}$ . Of texture parameters, only  $COR_{L2x}$  in layer 2 was significantly correlated with  $T_{pl}$  ( $r = -0.73$ ; Table 2).  $COR_{L2x}$  also showed significance in partial correlation with  $T_{pl}$  ( $r = -0.69$ ; Table 3).  $PW_b$  had a tendency to increase with the increase in  $T_{pl}$  ( $r = 0.47$ ,  $p = 0.07$ ; Table 2). The rank correlation coefficient between  $PW_b$  and  $T_{pl}$  was not significant ( $r = 0.33$ ,  $p = 0.23$ ; Table 3).

For  $BV/TV_{Tb}$ , all the spatial averaged parameters did not show significant correlations.  $COR_{L1z}$  and  $COR_{L2z}$  showed a significant correlation with  $BV/TV_{Tb}$  ( $r = -0.58$  and  $-0.66$ , respectively; Table 2). In partial correlations,  $COR_{L2z}$  showed significance ( $r = -0.60$ ; Table 3) while  $COR_{L1z}$  did not show the significance ( $r = -0.48$ ,  $p = 0.07$ ; Table 3).

With  $T_c$ , the parameters of  $IRC_{LI}$ ,  $D_{L2I}$ ,  $PW_b$ , and  $COR_{LIz}$  were significantly correlated ( $r = -0.56, 0.63, 0.66$  and  $0.72$ , respectively; Table 2).

The relationship between surface degeneration and micro-CT parameters are shown in Fig. 5. Only  $T_{pl}$  showed a significant difference between the intact and the degenerated groups ( $p = 0.005$ ; Fig. 5c).

## Discussion

The present study revealed for the first time to our knowledge the efficiency of the ultrasound parameters that reflected  $P_o$ ,  $T_{pl}$ , and  $BV/TV_{Tb}$  for normal to mild OA samples. Focusing on detecting these characteristics in the superficial region of subchondral bone, we used focused and broadband high-frequency (3.6–22.4 MHz) ultrasound.  $IRC$  or  $AIB$  itself was considered to be affected by attenuation in the cartilage layer. Therefore, we newly introduced the  $D_{L2I}$ ,  $PW_b$ , and texture parameters, which were relatively calculated from the intensity. We expected that they would be parameters that were independent of the overlying cartilage.

$ENT_{Llx}$  and  $ENT_{LIz}$  showed significant positive correlations with  $P_o$ , although the correlation coefficients were not strong ( $r = 0.58$  and  $0.58$ , respectively; Table 2). Significant partial correlation coefficients between  $ENT_{Llx}$  or  $ENT_{LIz}$  and  $P_o$  also showed less influence of  $T_c$ . Therefore, we conclude that  $ENT_{Llx}$  and  $ENT_{LIz}$  were efficient parameters reflecting the porosity of the subchondral plate. Pores at the subchondral surface locally lower the echo intensity at the C-B interface and increase the amount of information in the image, resulting in an increase in the  $ENT$ . This is assumed to be the reason for the positive correlations between  $ENT_{Llx}$  or  $ENT_{LIz}$  and  $P_o$ . The reason that the correlation coefficients were not strong ( $r = 0.58$  and  $0.58$ ) might be due to the asperity at the subchondral surface. Asperity would also affect  $ENT_{Llx}$  and  $ENT_{LIz}$ . In addition, it is noticeable that the beam diameter at the C-B interface is considered to be larger than that in saline due to the downshift of central frequency. As

shown in Fig. A of appendix 1, the central frequency at the C-B interface was downshifted to the 7.5 MHz compared to the central frequency of 13 MHz without attenuation by cartilage layer. Although the 13 MHz component still existed within -6 dB level at the C-B interface (Fig. A), deterioration of lateral beam resolution is obvious. Further investigation for the effect of this on the texture parameters is needed.

With  $T_{pl}$ ,  $COR_{L2x}$  showed significant negative correlations ( $r = -0.73$ ; Table 2) as well as negative partial correlation coefficients between  $COR_{L2x}$  and  $T_{pl}$  ( $r = -0.69$ ; Table 3). One of the main components from layer 2 might be the scattered echo from the backside of the subchondral plate. In this study, the range of  $T_{pl}$  was 0.12–0.29 mm. Assuming that the speed of sound in the cortical plate is 3635 m/s (Collins 1999), the scattered wave originated from the back side of the subchondral plate, the interface between the subchondral plate, and the bone marrow, and arrives 0.07–0.16  $\mu$ s after the reflected wave arrives at the C-B interface. When the plate is thin, such as 0.1 mm, two waves are superimposed almost inside layer 1. When  $T_{pl}$  become thick, the echo from the backside shifts backwards (Appendix 2: a simple reflection model of a thin plane plate). However, in fact, the backside was a complex shape with junctions to the trabecular bone, as shown in Figs. 3a-b. Moving backward of the backside echo and the backside echo with the time of flight variation could explain the negative correlation between  $COR_{L2x}$  and  $T_{pl}$ . In addition, the trabecular thickness at the junction is assumed to increase as  $T_{pl}$  increases (Finnilä, et al. 2017). This would lower  $COR_{L2x}$ .

$D_{L2I}$  also showed significant correlations with  $T_{pl}$ . Partial correlations between  $D_{L2I}$  and  $T_{pl}$  still showed significance although  $D_{L2I}$  showed significant correlations with  $T_c$ . Therefore,  $D_{L2I}$  are also considered efficient parameters reflecting subchondral plate thickness.  $D_{L2I}$  is the difference between  $AIB_{L2}$  and  $IRC_{LI}$ . Both  $AIB_{L2}$  and  $IRC_{LI}$  were assumed to be affected by the same amount of attenuation in the cartilage. The attenuation effect was considered canceled by taking the difference (in

dB value). On the other hand,  $PW_b$  and  $T_{pl}$  did not show the significance although  $PW_b$  had a tendency to increase with the increase in  $T_{pl}$ . In this study, we could not show the efficiency. The small sample size and inability to ensure the normality might be the reason for the inefficiency. Further consideration is needed.

With  $BV/TV_{Tb}$ ,  $COR_{L2z}$  showed a significant negative correlation ( $r = -0.66$ ,  $p = 0.01$ ; Table 2) and partial correlation ( $r = -0.60$ ,  $p = 0.02$ ; Table 3). As OA progresses, a fine mesh structure with a thin trabecular layer reportedly turned into a coarse mesh structure with a thicker trabecular layer (Bergman, et al. 1994, Li, et al. 2013). These structural changes in the trabecular area might increase the responsiveness of the ultrasound wave in this study

Concerning  $AIB_{L2}$ , there was no significant correlation between  $AIB_{L2}$  and  $T_{pl}$ . However, to combine the previous reports of the negative correlation between  $AIB$  and  $BMD$  at the subchondral plate (Aula, et al. 2010) and the positive relationship between  $BMD$  and  $T_{pl}$  (Li, et al. 2013),  $AIB_{L2}$  and  $T_{pl}$  might have shown a negative correlation. On the other hand, the positive correlation between subchondral plate thickness and trabecular thickness (Goldring 2012) and the positive correlation between  $AIB$  bone and trabecular (Liukkonen, et al. 2013) thickness suggests the positive correlation between  $AIB$  and  $T_{pl}$ . The behavior of  $AIB_{L2}$  for  $T_{pl}$  was considered to differ from positive (including speculation), negative (including speculation), or no correlation (in this study). We speculate that the main factor of  $AIB_{L2}$  may be the echo from the backside of the subchondral plate (Appendix 2). When the  $T_{pl}$  is thick enough compared to the wavelength, the backside echo would not be detected due to high attenuation in the subchondral plate, which might lower the  $AIB$ . On the other hand, when the  $T_{pl}$  is in the range of less than several wavelengths, the backside echo is separated from the front side echo with increasing  $T_{pl}$ , which might increase  $AIB$ . Limited to normal to mildly degenerated samples,  $AIB_{L2}$  might increase with OA progression but might decrease when thicker or more severely degenerated

samples are included.  $AIB_{L2}$  is also affected by attenuation in the overlying cartilage. In this study,  $AIB_{L2}$  was not correlated with  $T_{pl}$ . This might be due to the cancellation effect by the increase in  $AIB_{L2}$  as  $T_{pl}$  increased and  $AIB_{L2}$  decreased with an increasing  $T_c$  (Appendix 3, Table B correlation between micro-CT parameters).

Regarding the applicable range of the ultrasound parameters in the present study, a highly-degenerated sample with thick  $T_{pl}$ , such as 1 mm thickness with sclerosis, would be principally out of the range for the detection of  $T_{pl}$  because high attenuation in cortical plate would interfere with detection of the backside echo. In this situation,  $D_{L2I}$  might be higher because a highly sclerotic subchondral bone with a thicker plate is expected to work like as a metal plate.  $D_{L2I}$  might be limited for normal to moderate OA. Clarification of the applicable range is a future issue.

With respect to the samples in this study, a simple judgment of ICRS grade was used to confirm the correlation between cartilage surface degeneration and subchondral degeneration by micro-CT. The decreasing tendency in  $P_o$  with large variance in the degenerated group (Fig. 5b) was consistent with that of the previous study that reported temporally increased porosity in early OA in animal models (Botter, et al. 2011). In  $BV/TV_{Tb}$ , no marked difference was observed between the intact and degenerated group (Fig. 5d). On the other hand,  $BV/TV$  at the trabecular bone was reported to strongly ( $r = 0.78$ ) correlate with Osteoarthritis Research Society International (OARSI) grade (Finnilä, et al. 2017). One reason for the difference could be that the samples were selected within normal to mainly early degenerated samples in this study for our purpose while (Finnilä, et al. 2017) including severe OA samples. Besides, the resolution of the cartilage evaluation might be another reason. ICRS grade, a visual judgment of the cartilage surface, includes less information than the histological judgment of OARSI grade. In  $T_{pl}$ , the degenerated group showed a significant increase (Fig. 5c) compared to the intact group. In addition, the observed wide variance of  $T_{pl}$  in the degenerated group is

suggested to be related to the previous reports that showed temporal thinning and then thickening of the subchondral plate in the early stages of OA (Batiste, et al. 2004, Intema, et al. 2010, Li, et al. 2013).

Thus, subchondral characteristics might not necessarily change monotonically as OA progresses, especially in the early stages. Therefore, a technique that can diagnose the subchondral characteristics longitudinally is needed. For this demand, ultrasound could have advantages of being non-invasive, low-cost, and convenient compared to magnetic resonance imaging or X-ray CT. In addition, ultrasound parameters for subchondral bone could be used to judge drug efficacy for OA treatment targeting subchondral bone, although, in practical situations, these parameters should overcome soft-tissue disturbance. In the future, the abilities of these echo parameters must be investigated in vivo. In practical situations, to ensure the uniformity of beam property in the region of interest is critical and the settings for calculation of the texture parameters, such as dynamic range,  $N_g$ , and relative position ( $\Delta x$ ,  $\Delta z$ ), should be optimized. In addition, the allowable range of deterioration in beam characteristics due to the overlying tissues should be clarified.

In this study, we carefully set the sample, including the process of pre-scan for adjusting the sample inclination. The depth of the sample was also adjusted so that the C-B interface was in the focal zone. However, we measured each sample only once. Although it was under well-controlled ex-vivo environment, the result includes the measurement error. The reproducibility should be clarified before clinical application in the future. Ideally, all manual processes should be removed. Optimal beam forming to ensure the perpendicularity to the C-B interface would be useful.

In conclusion, here we identified the ultrasound parameters for subchondral porosity and thickness of subchondral, and  $BV/TV_{Tb}$ .  $ENT_{L1x}$  and  $ENT_{L2z}$  were efficient for detecting porosity.  $DL_{21}$  and  $COR_{L2x}$ , in particular,  $COR_{L2x}$ , efficiently detected  $T_{pl}$ . For  $BV/TV_{Tb}$ ,  $COR_{L2z}$  showed efficiency. These parameters are considered less dependent on cartilage thickness and reflect microstructural

changes in the subchondral bone. This technique is expected to be applied to in vivo evaluations in the future.

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## Appendix 1

Representative spectral amplitude of the echo from subchondral bone was shown in Fig. A. For the evaluation of noise floor, the signal of interest (SOI) with no scatter area was extracted and short-time Fourier transformed with a Hanning window with a length of  $0.2\ \mu\text{s}$  (Fig. A-i). The representative echo signal ( $0.2\ \mu\text{s}$  before cartilage-subchondral (C-B) interface to  $0.8\ \mu\text{s}$  after C-B interface) was extracted and short-time Fourier transformed, as the same manner (Fig. A-ii). Within a bandwidth (3.6 - 22.4 MHz), the spectral amplitude of the signal exceeded the noise floor (Fig. A-iii, spectra at 0.1, 0.35, 0.6, and  $0.8\ \mu\text{s}$ ).

## Appendix 2

Ultrasound pulsed echoes from the subchondral (cortical) plate were calculated using a simple reflection model (Fig. B-i) to confirm the interference between the front side echo (that from the cartilage–bone interface) and back side echo (that from the subchondral plate–bone marrow interface). The input waveform was set as the Gaussian pulse with a central frequency of 13 MHz and a relative bandwidth of 80% (Fig. B-ii). The acoustic parameters of the medium were assumed as shown in Table A. The front and back side echo was calculated for two cases with a thickness cartilage ( $T_c$ ) of 0 mm (Fig. B-iii) and 2 mm (Fig. B-iv). Thickness of the subchondral plate was set at 0.1–0.5 mm with 0.1-mm steps. The echo from the back side ( $V_{back}$ ) is superimposed onto the echo from the front side ( $V_{front}$ ) when  $T_{pl}$  is 0.1 mm. When  $T_{pl}$  is larger than 0.3 mm, the  $V_{front}$  and  $V_{back}$  waves are completely separated. Although attenuation in the cartilage layer results in the downshift of central frequency, the envelope keeps its shape. In addition to attenuation in the cartilage, attenuation in the cortical bone lowers the central frequency ( $f_c$ ) of  $V_{back}$ .

### Appendix 3

The relationship between micro-CT parameters was shown in Table B.  $T_{pl}$  significantly correlated with  $P_o$  ( $r = -0.61$ ).  $T_{pl}$  had weak increasing tendency with increase in  $T_c$  ( $r = 0.41$ , n.s.).  $P_o$  had weak decreasing tendency with increase in  $T_c$  ( $r = -0.41$ , n.s.).  $BV/TV_{Tb}$  had weak increasing tendency with increase in  $T_{pl}$  ( $r = 0.43$ , n.s.).

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## Figure Captions

Fig. 1. Experimental system for ultrasound parameters. (a) Schematic representation of the three-dimensional ultrasound scanning system. (b) Block diagram for data acquisition. (c) Echo intensity for perfect reflector along the depth direction obtained by equation (1). A steel plate with sufficient thickness compared with the wavelength was set as the focus.

Fig. 2. Pre-processing for ultrasound parameters. (a) Radiofrequency image of an osteochondral sample. Cartilage-subchondral bone (C-B) and saline-cartilage interfaces were detected. (b) Intensity distribution,  $B$ , after rearrangement in line with the C-B interface. Time axis was redefined as  $t'$  for the rearranged data. (c) Averaged envelope in the x-direction at a certain y position. The origin was forwardly defined at 10 dB down from the first peak. Layers 1 and 2 were defined as the 0–0.2  $\mu\text{s}$  and 0.2–0.5  $\mu\text{s}$  sections, respectively. The second peak, marked with the red circle, was often observed as a marked feature (compared to the perfect reflector of Fig. 1c), suggesting interference with the echo that originated from the back side of the subchondral plate (Appendix 2).

Fig. 3. Calculation of micro-computed tomography (CT) parameters. (a) A cross-sectional image of an osteochondral sample. Cartilage surface and cartilage-subchondral bone (C-B) interfaces were detected. For the pore area, interpolation was adopted and the global C-B interface was defined (red line). (b) A binarized image of subchondral bone. The image was rearranged so that the C-B interface was in line. Some trabecular bones were not separate with subchondral plate. (c) A binarized image of subchondral plate without trabecular bone. Median filter and opening processing removed the trabecular bone. (d) Binarized image of trabecular bone within volume of interest. The depth region of 0.5–1.5 mm from C-B interface was selected. (e) Volumetric image of the sample by marching cubes surface rendering.

From volume and area for each segmented tissue, thickness of cartilage, thickness of subchondral plate, porosity in the surface area of subchondral plate,  $BV/TV$  at trabecular area were calculated.

Fig. 4 Micro-computed tomography (CT) (a and b) and ultrasound (c and d) images of subchondral bone. Two representative samples with thin subchondral plate thickness ( $T_{pl}$ ) (sample #1) and thick  $T_{pl}$  (sample #2) are displayed. Subscript 1 is for sample #1 and 2 is for #2. (a) Micro-CT images of subchondral bone plate. (b) The volume of interest in trabecular region. (c) Distribution of ultrasound echo intensity,  $B$ . Both images are displayed with the dynamic range of 30 dB. An image of the 0.2–0.5  $\mu$ s region (layer 2) showed relatively higher scattering level in sample #2. (d) Pulse width distribution in the x-y plane. A larger pulse width is seen in sample #2.

Fig. 5. The differences of micro-computed tomography (CT) parameters between intact ( $N = 5$ ) and degenerated ( $N = 11$ ) group. (a) Thickness of the cartilage ( $T_c$ ). (b) Porosity of the subchondral surface ( $P_o$ ). (c) Thickness of the subchondral plate ( $T_{pl}$ ). A significant difference was recognized. In the degenerated group, large variance, including a lower value than that of the intact group, was observed. (d) Bone volume fraction at the trabecular area ( $BV/TV_{Tb}$ ).

The p-values (two-tailed) were evaluated by Student's t-test in a and d, Welch's t-test in c, and Mann-Whitney U test in b. Horizontal lines denote mean  $\pm$  standard deviation except that the line in degenerated group in (b) denotes median.

Fig. A Representative spectrum of the echo from subchondral bone. (i) Short-time Fourier transform (STFT) of the echo without scatter for evaluation of noise floor. (ii) STFT of the echo from the subchondral bone. (iii) Spectral amplitude at each time position.

Fig. B. Simple calculation for interference of the echoes from cartilage-subchondral bone interface and backside of the subchondral plate.

## Tables

Table 1. Descriptive statistics values of all parameters. Normality was confirmed by Shapiro-Wilk test

| Parameter        | Mean or median† | SD or interquartile range‡ | Range |
|------------------|-----------------|----------------------------|-------|
| $T_c$ [mm]       | 1.78            | 0.43                       | 1.44  |
| $P_o$ [%]        | 5.4             | 4.5                        | 13.0  |
| $T_{pl}$ [mm]    | 0.18            | 0.05                       | 0.17  |
| $BV/TV_{Tb}$ [%] | 34.4            | 2.6                        | 9.3   |
| $IRC_{L1}$ [dB]  | -32.7           | 5.6                        | 22.5  |
| $AIB_{L2}$ [dB]  | -43.8           | 2.4                        | 9.2   |
| $D_{L21}$ [dB]   | -11.1           | 4.5                        | 15.7  |
| $PW_b$ [μs]      | 0.24†           | 0.08‡                      | 0.29  |
| $ENT_{L1x}$      | 9.9             | 0.33                       | 1.3   |
| $ENT_{L1z}$      | 9.1             | 0.29                       | 1.2   |
| $COR_{L1x}$      | 0.38†           | 0.1‡                       | 0.46  |
| $COR_{L1z}$      | 0.31            | 0.2                        | 0.60  |
| $ENT_{L2x}$      | 10.4            | 0.3                        | 1.3   |
| $ENT_{L2z}$      | 10.0            | 0.3                        | 1.2   |
| $COR_{L2x}$      | 0.51            | 0.17                       | 0.62  |
| $COR_{L2z}$      | 0.53            | 0.14                       | 0.45  |

(All parameters except  $PW_b$  and  $COR_{L1x}$  showed normality)

629 Table 2. Correlation coefficients between ultrasound and micro-CT parameters

| Ultrasound<br>parameter | Micro-CT parameters                   |                |                                    |               |                                       |                |                                       |               |
|-------------------------|---------------------------------------|----------------|------------------------------------|---------------|---------------------------------------|----------------|---------------------------------------|---------------|
|                         | $T_c$ [mm]                            |                | $P_o$ [%]                          |               | $T_{pl}$ [mm]                         |                | $BV/TV_{Tb}$ [%]                      |               |
|                         | r (p-value)                           |                | r (p-value)                        |               | r (p-value)                           |                | r (p-value)                           |               |
|                         | [95 % CI]                             |                | [95 % CI]                          |               | [95 % CI]                             |                | [95 % CI]                             |               |
| Spatial averaged        |                                       |                |                                    |               |                                       |                |                                       |               |
| $IRC_{LI}$ [dB]         | <b>-0.56</b><br><b>[-0.83, -0.09]</b> | <b>(0.02)</b>  | 0.24<br>[-0.29, 0.66]              | (0.38)        | -0.45<br>[-0.77, 0.06]                | (0.08)         | 0.08<br>[-0.43, 0.55]                 | (0.76)        |
| $AIB_{L2}$ [dB]         | -0.13<br>[-0.58, 0.39]                | (0.64)         | -0.02<br>[-0.51, 0.48]             | (0.96)        | 0.16<br>[-0.36, 0.61]                 | (0.54)         | 0.27<br>[-0.26, 0.67]                 | (0.31)        |
| $D_{L2I}$ [dB]          | <b>0.63</b><br><b>[0.19, 0.86]</b>    | <b>(0.01)</b>  | -0.30<br>[-0.69, 0.23]             | (0.26)        | <b>0.64</b><br><b>[0.21, 0.86]</b>    | <b>(0.01)</b>  | 0.04<br>[-0.46, 0.53]                 | (0.87)        |
| $PW_b$ †[μs]            | <b>0.66</b>                           | <b>(0.01)</b>  | -0.33<br>(0.22)                    |               | 0.47<br>(0.07)                        |                | -0.01<br>(0.96)                       |               |
| Layer 1 texture         |                                       |                |                                    |               |                                       |                |                                       |               |
| $ENT_{L1x}$             | -0.14<br>[-0.60, 0.38]                | (0.59)         | <b>0.58</b><br><b>[0.12, 0.84]</b> | <b>(0.02)</b> | -0.45<br>[-0.77, 0.06]                | (0.08)         | -0.15<br>[-0.60, 0.37]                | (0.58)        |
| $ENT_{L1z}$             | -0.15<br>[-0.60, 0.38]                | (0.59)         | <b>0.58</b><br><b>[0.12, 0.83]</b> | <b>(0.02)</b> | -0.45<br>[-0.77, 0.06]                | (0.08)         | -0.14<br>[-0.59, 0.39]                | (0.62)        |
| $COR_{L1x}$ †           | -0.02<br>(0.93)                       |                | -0.09<br>(0.75)                    |               | 0.08<br>(0.76)                        |                | 0.27<br>(0.31)                        |               |
| $COR_{L1z}$             | <b>0.72</b><br><b>[0.35, 0.90]</b>    | <b>(0.002)</b> | 0.02<br>[-0.48, 0.51]              | (0.95)        | -0.11<br>[-0.57, 0.41]                | (0.68)         | <b>-0.58</b><br><b>[-0.83, -0.11]</b> | <b>(0.02)</b> |
| Layer 2 texture         |                                       |                |                                    |               |                                       |                |                                       |               |
| $ENT_{L2x}$             | -0.01<br>[-0.50, 0.49]                | (0.98)         | 0.48<br>[-0.02, 0.79]              | (0.06)        | -0.30<br>[-0.69, 0.23]                | (0.26)         | -0.19<br>[-0.63, 0.33]                | (0.47)        |
| $ENT_{L2z}$             | -0.06<br>[-0.54, 0.45]                | (0.83)         | <b>0.52</b><br><b>[0.03, 0.81]</b> | <b>(0.04)</b> | -0.36<br>[-0.73, 0.17]                | (0.17)         | -0.17<br>[-0.61, 0.36]                | (0.53)        |
| $COR_{L2x}$             | -0.35<br>[-0.72, 0.18]                | (0.19)         | 0.26<br>[-0.27, 0.67]              | (0.33)        | <b>-0.73</b><br><b>[-0.90, -0.37]</b> | <b>(0.001)</b> | -0.31<br>[-0.70, 0.22]                | (0.24)        |
| $COR_{L2z}$             | 0.39<br>[-0.13, 0.74]                 | (0.13)         | 0.17<br>[-0.35, 0.62]              | (0.52)        | -0.36<br>[-0.73, 0.17]                | (0.17)         | <b>-0.66</b><br><b>[-0.87, -0.24]</b> | <b>(0.01)</b> |

630  $T_c$ , thickness of the cartilage;  $P_o$ , porosity at the subchondral surface;  $T_{pl}$ , thickness of the subchondral  
631 plate;  $BV/TV_{Tb}$ , bone volume fraction at the trabecular region. CI: confidence interval.  $PW_b$  and  $COR_{L1x}$   
632 did not show the normality. The correlation coefficients between  $PW_b$  or  $COR_{L1x}$  and micro-CT  
633 parameters were evaluated by Spearman's rank correlations † while the others were by Pearson's  
634 correlations.

Table 3. Partial correlation coefficients between ultrasound parameters and micro-CT parameters

| Ultrasound parameter      | $P_o$ [%]   |               | $T_{pl}$ [mm] |                | $BV/TV_{Tb}$ [%] |               |
|---------------------------|-------------|---------------|---------------|----------------|------------------|---------------|
|                           | r           | p-value       | r             | p-value        | r                | p-value       |
| Spatial averaged          |             |               |               |                |                  |               |
| $IRC_{LI}$ [dB]           | 0.01        | (0.97)        | -0.29         | (0.30)         | -0.17            | (0.55)        |
| $AIB_{L2}$ [dB]           | -0.07       | (0.80)        | 0.24          | (0.39)         | 0.24             | (0.39)        |
| $D_{L2I}$ [dB]            | -0.06       | (0.82)        | <b>0.54</b>   | <b>(0.04)</b>  | 0.39             | (0.16)        |
| $PW_b^\dagger$ [ $\mu$ s] | -0.13       | (0.64)        | 0.33          | (0.23)         | 0.31             | (0.25)        |
| Layer 1 texture           |             |               |               |                |                  |               |
| $ENT_{L1x}$               | <b>0.58</b> | <b>(0.02)</b> | -0.43         | (0.11)         | -0.22            | (0.43)        |
| $ENT_{L1z}$               | <b>0.57</b> | <b>(0.03)</b> | -0.43         | (0.11)         | -0.21            | (0.46)        |
| $COR_{L1x}^\dagger$       | -0.10       | (0.72)        | 0.10          | (0.73)         | 0.28             | (0.31)        |
| $COR_{L1z}$               | 0.49        | (0.06)        | <b>-0.64</b>  | <b>(0.01)</b>  | -0.48            | (0.07)        |
| Layer 2 texture           |             |               |               |                |                  |               |
| $ENT_{L2x}$               | <b>0.52</b> | <b>(0.05)</b> | -0.33         | (0.24)         | -0.21            | (0.45)        |
| $ENT_{L2z}$               | <b>0.54</b> | <b>(0.04)</b> | -0.37         | (0.18)         | -0.20            | (0.46)        |
| $COR_{L2x}$               | 0.14        | (0.63)        | <b>-0.69</b>  | <b>(0.005)</b> | <b>-0.51</b>     | <b>(0.05)</b> |
| $COR_{L2z}$               | 0.40        | (0.14)        | <b>-0.62</b>  | <b>(0.01)</b>  | <b>-0.60</b>     | <b>(0.02)</b> |

(thickness of cartilage ( $T_c$ ) was set as a control variable)

$P_o$ , porosity at the subchondral surface;  $T_{pl}$ , thickness of the subchondral plate;  $BV/TV_{Tb}$ , bone volume

fraction at the trabecular region.  $^\dagger$  : Partial rank correlation coefficient.

640 Table A. Acoustic parameters for calculating reflection echo in a thin plate

| Term                                                | Value | Reference                          |
|-----------------------------------------------------|-------|------------------------------------|
| Attenuation in cartilage $\alpha_1$ [dB/mm/MHz]     | 0.185 | (Joiner, et al. 2001) <sup>a</sup> |
| Speed of sound in cartilage [m/s] <sup>+</sup>      | 1620  | (Agemura, et al. 1990)             |
| Density of cartilage [kg/m <sup>3</sup> ]           | 1100  | (Joseph 1999) <sup>b</sup>         |
| Speed of sound in cortical bone [m/s] <sup>+</sup>  | 3635  | (Collins 1999)                     |
| Density of cortical bone [kg/m <sup>3</sup> ]       | 1920  | (Collins 1999)                     |
| Speed of sound in bone marrow [m/s]                 | 1500  | ++                                 |
| Density of cortical bone [kg/m <sup>3</sup> ]       | 1000  | ++                                 |
| Attenuation in cortical bone $\alpha_2$ [dB/mm/MHz] | 1.4   | (Collins 1999) <sup>d</sup>        |

641 <sup>+</sup> Longitudinal speed of sound.

642 <sup>a</sup>The attenuation value was obtained from the figure by reading the value and extrapolation.

643 <sup>b</sup>Bovine articular cartilage.

644 <sup>++</sup>The acoustic impedance of water was simply used, alternative to it of bone marrow.

645 <sup>d</sup>The lowest value listed in the Collins 1999 was used.

646 **Table B.** Pearson’s correlation coefficients between micro-CT parameters

|          | $P_o$         |         | $T_{pl}$       |         | $BV/TV_{Tb}$  |         |
|----------|---------------|---------|----------------|---------|---------------|---------|
|          | r             | p-value | r              | p-value | r             | p-value |
|          | CI (95%)      |         | CI (95%)       |         | CI (95%)      |         |
| $T_c$    | -0.41         | (0.12)  | 0.41           | (0.12)  | -0.37         | (0.16)  |
|          | [-0.75, 0.11] |         | [-0.11, 0.75]  |         | [-0.73, 0.15] |         |
| $P_o$    | 1             | -       | <b>-0.61</b>   | (0.01)  | -0.28         | (0.30)  |
|          | -             | -       | [-0.85, -0.16] |         | [-0.68, 0.25] |         |
| $T_{pl}$ |               |         | 1              | -       | 0.43          | (0.09)  |
|          |               |         | -              | -       | [-0.08, 0.76] |         |

647  $T_c$ , thickness of cartilage;  $P_o$ , porosity at the subchondral surface;  $T_{pl}$ , thickness of the subchondral  
648 plate;  $BV/TV_{Tb}$ , bone volume fraction at the trabecular area

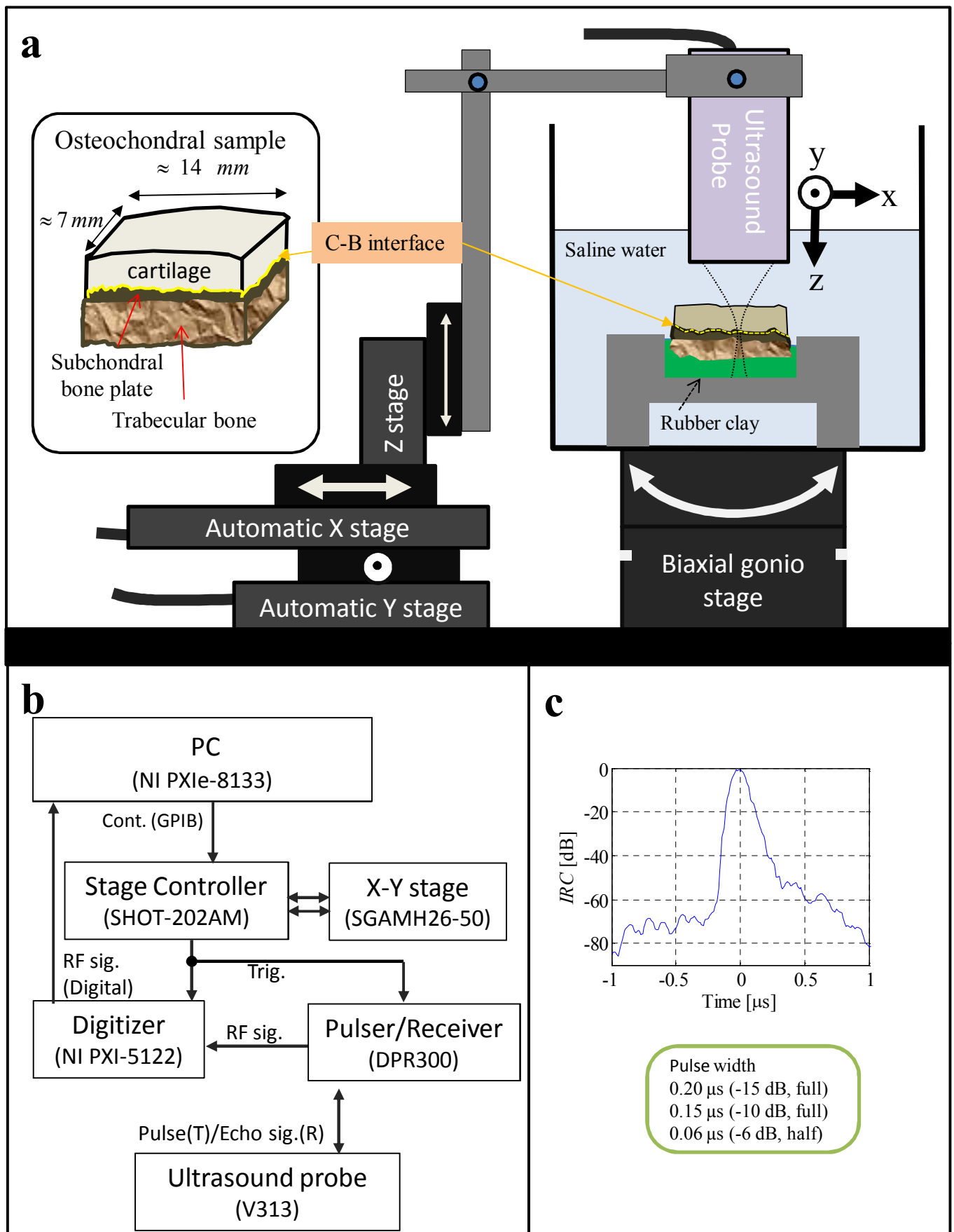


Fig.1

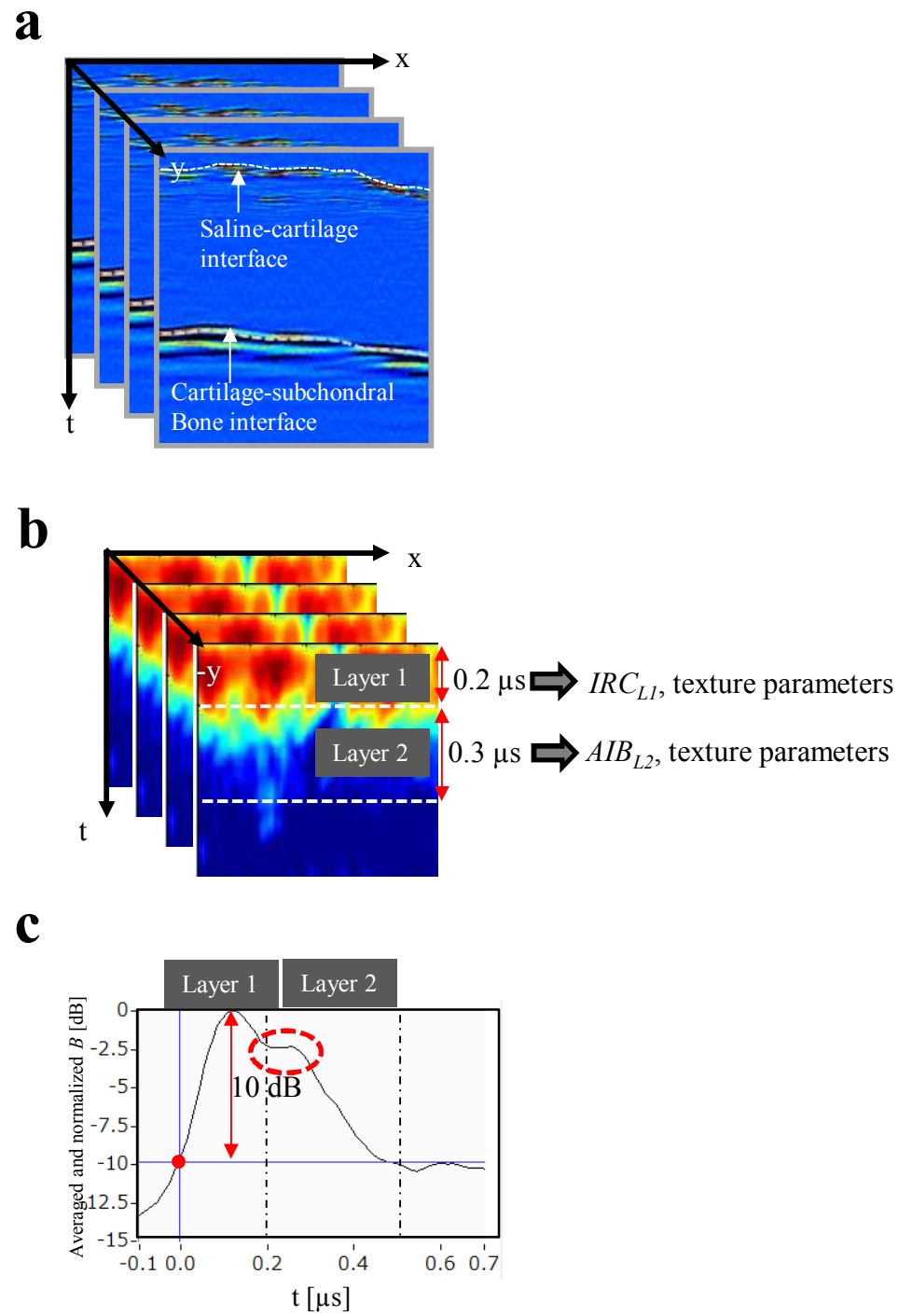
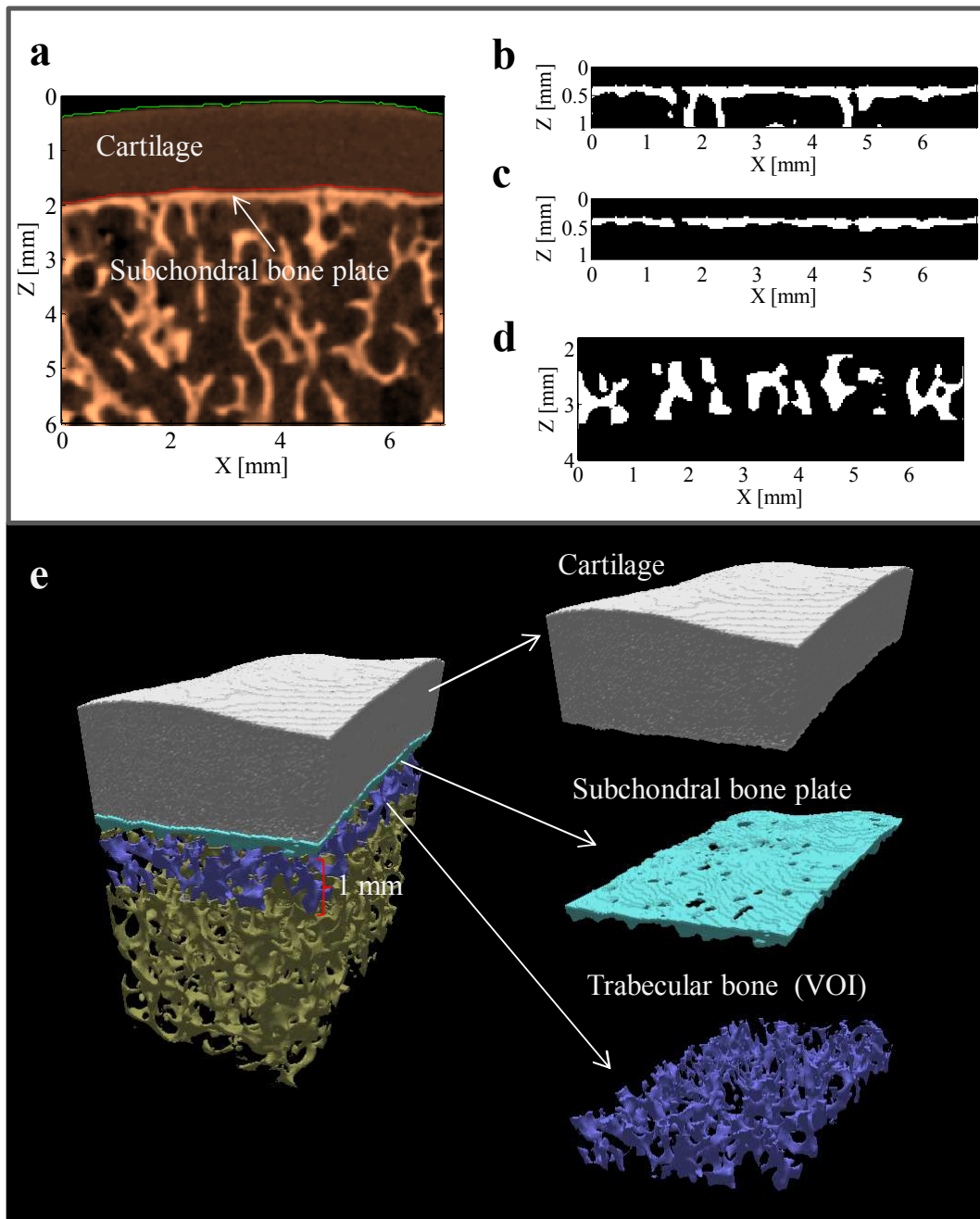
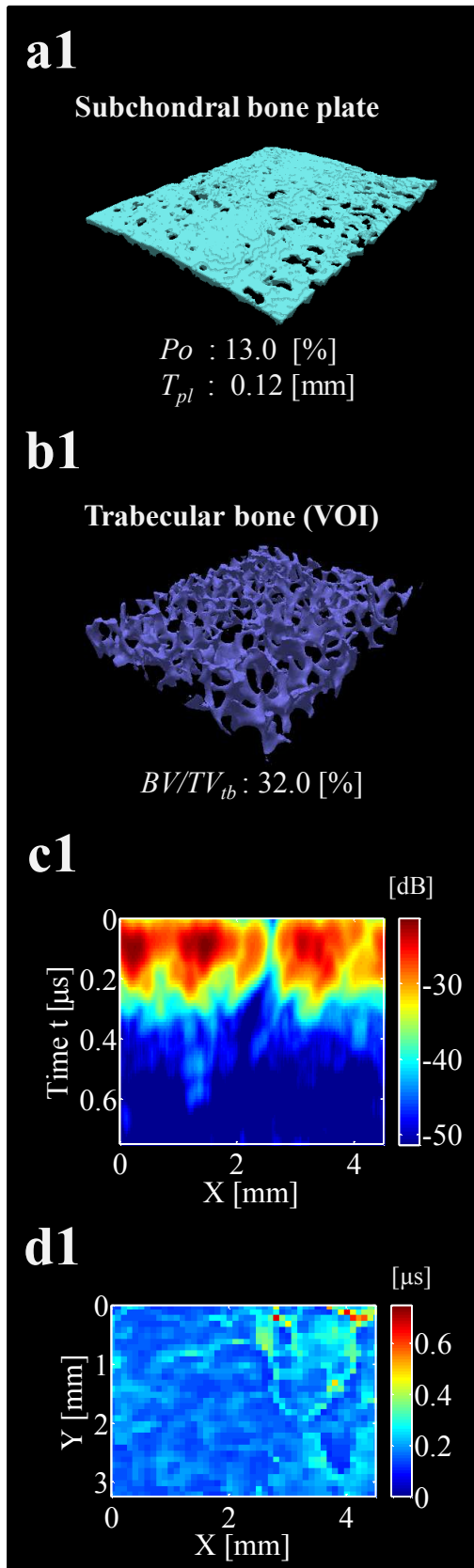


Fig. 2



**Fig. 3**

Sample #1



Sample #2

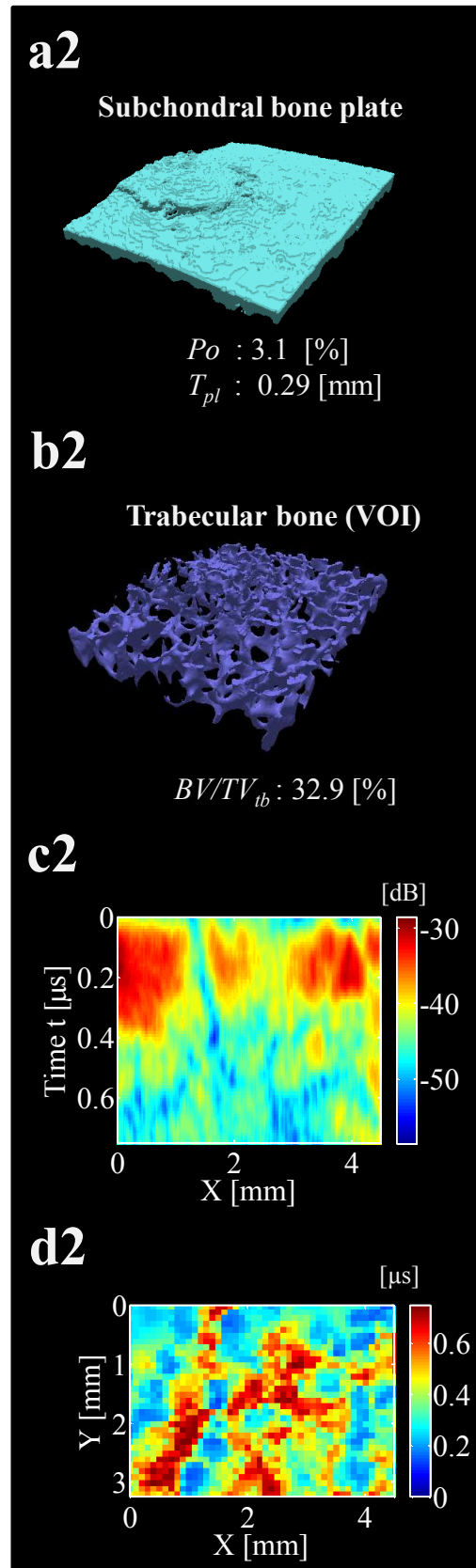


Fig. 4

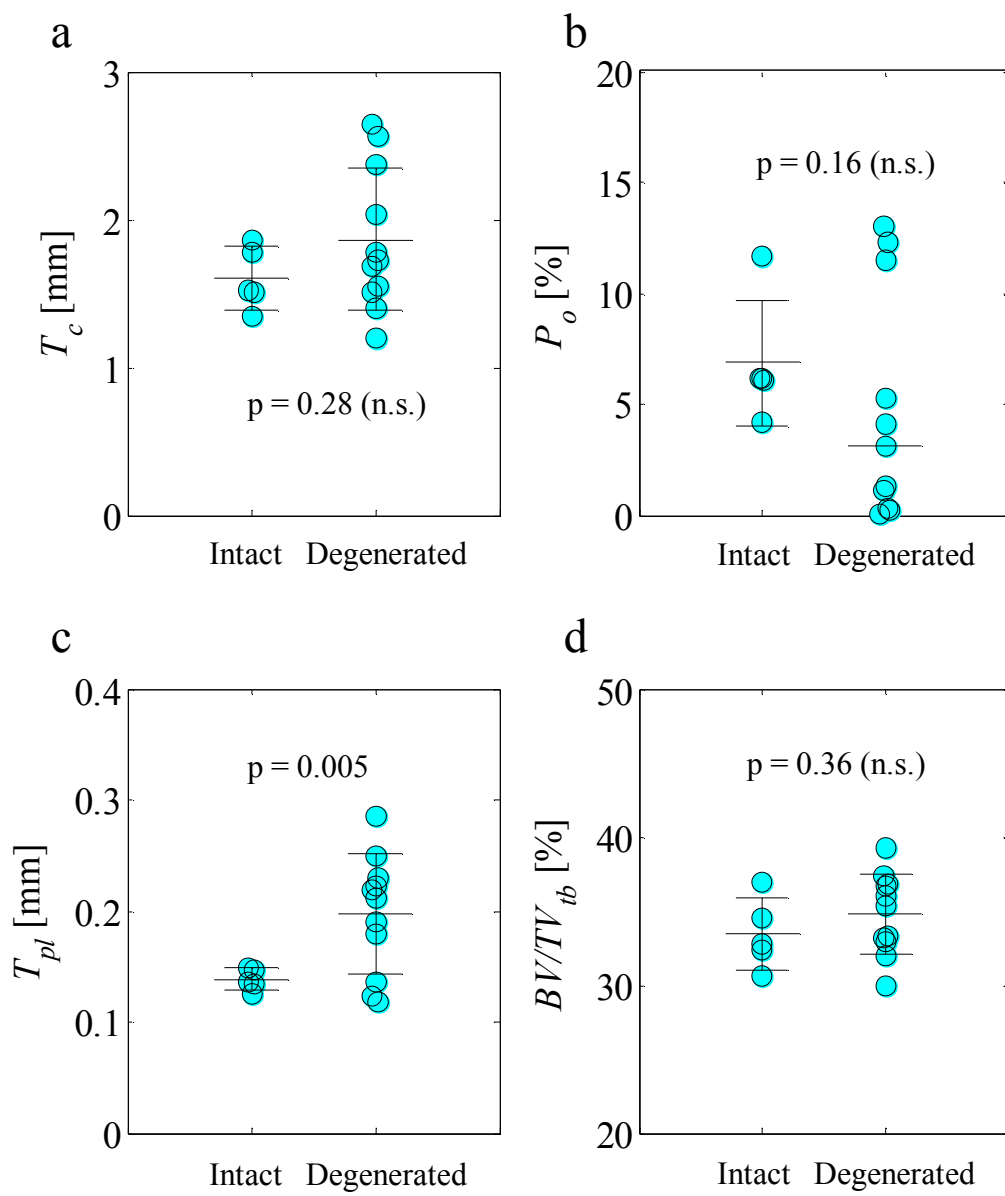
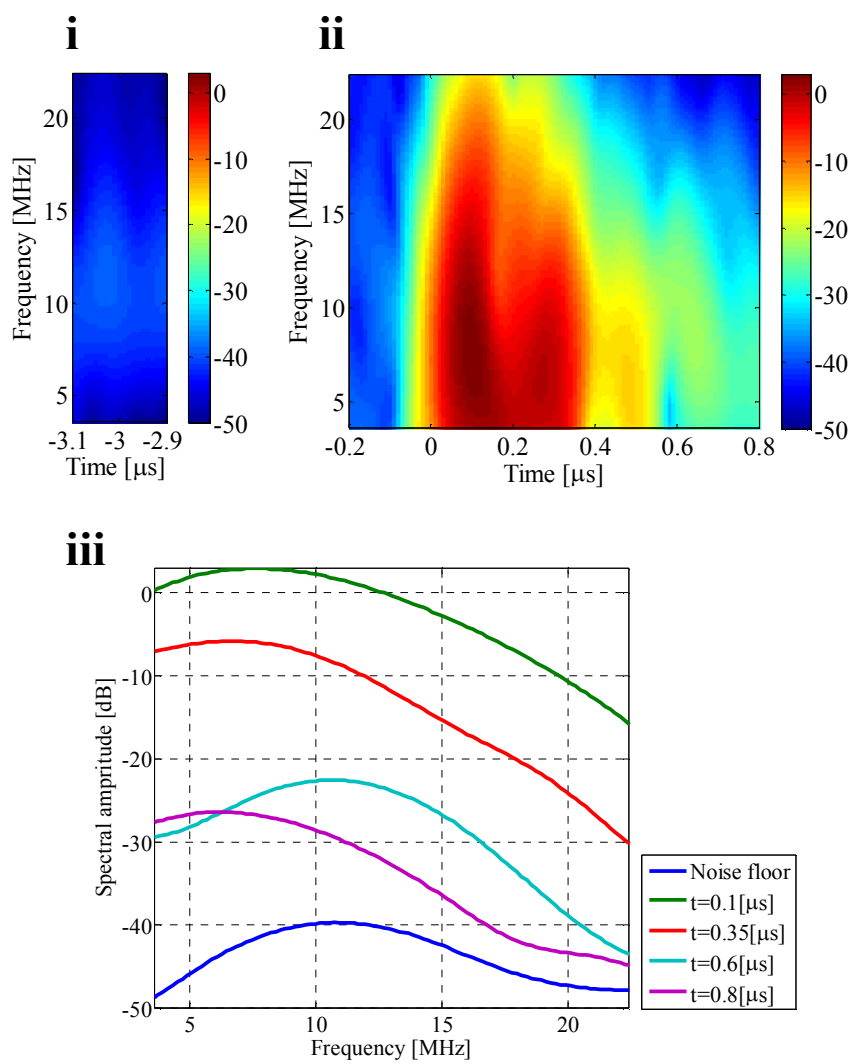
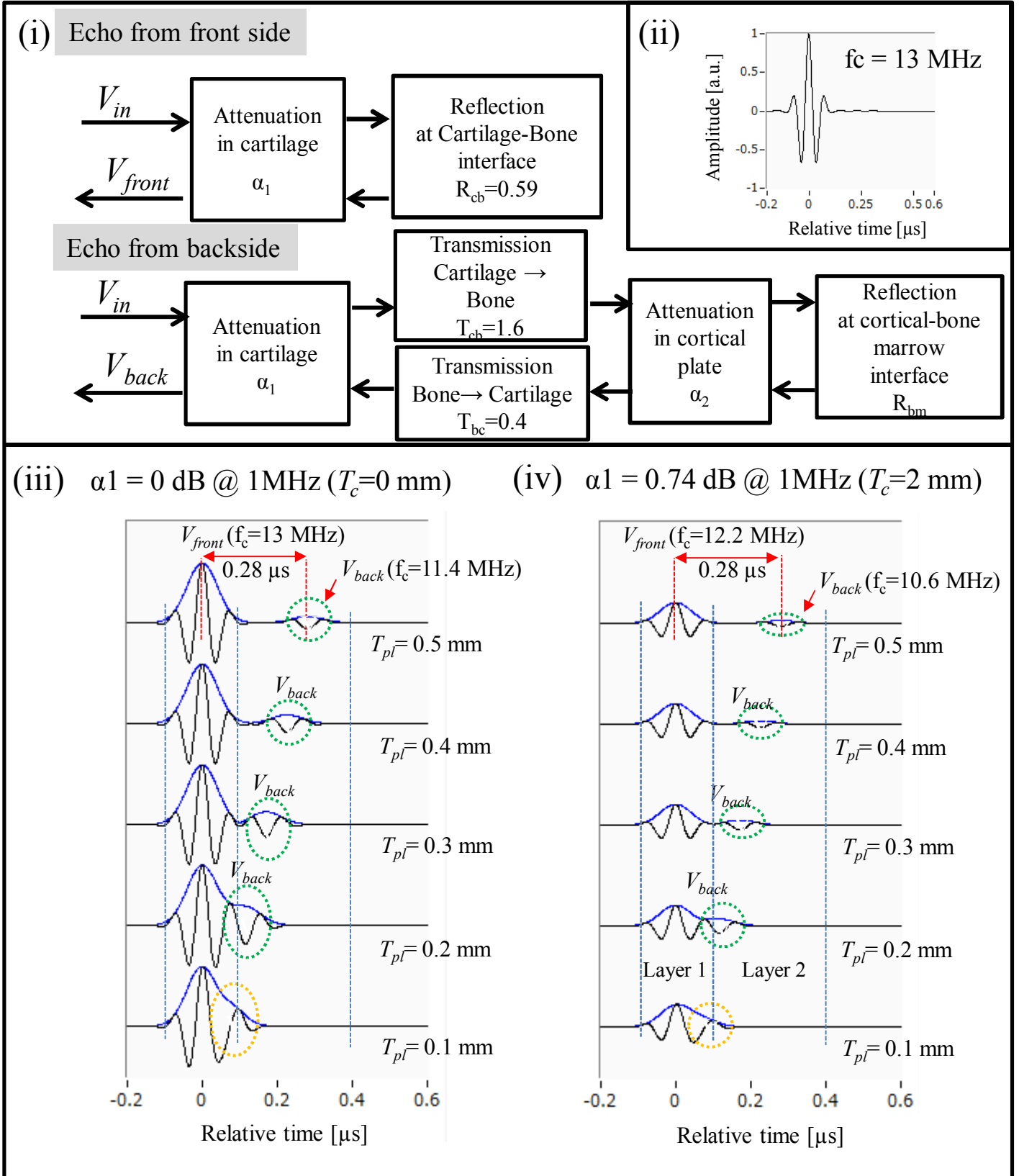


Fig. 5



Appendix 1 , Fig. A



Appendix 2 Fig. B