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1 **Micro-cantilever Bending for Elastic Modulus Measurements**

2 **of a Single Trabecula in Cancellous Bone**

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28 **Abstract**

29 Mechanical tests performed on small bone specimens such a single trabecula remain
30 challenging because their isolation, fixation, and precise loading are complicated. Hence,
31 we describe a novel experimental method to measure the elastic properties of a single
32 trabecula using micro-cantilever bending (MCB) testing. The method does not require
33 specimens to be completely separated from the cancellous bone, and the specimen can be
34 easily fixed during the test. In total, 10 trabecular specimens taken from the proximal
35 epiphysis of an adult bovine femur were used in the present study. Measurements were
36 conducted using a small testing device comprising a 1-axial stage, load cell, optical
37 microscope, and small plate with a taper bore for applying load at the edge of the
38 specimen. Each specimen was positioned at the edge of the bore and was deformed by
39 displacing the stage. The deflection of the specimen was observed by optical microscopy.
40 The elastic modulus of the specimen was calculated on the basis of the force–deflection
41 relationship, assuming that the shape of the specimen was a vertical circular cylinder. As
42 a result, an average elastic modulus of 9.1 ± 5.4 GPa was obtained for a single trabecula,
43 including the values in literature. Thus, the MCB test is a novel simple method for

44 biomechanical analysis of a single trabecula. (214 words)

45 **1. Introduction**

46 The cancellous bone is organized into a three-dimensional network of single
47 trabeculae, and the apparent elastic modulus depends on this network. The mechanical
48 properties and nanostructure of each trabecula are important factors in determining the
49 mechanical properties of the cancellous bone. Accordingly, small bone specimens such as
50 a single trabecula must be investigated to understand the impact of aging, osteoporosis,
51 and/or medicines on the risk of cancellous bone fractures. However, few studies have
52 performed mechanical tests on such specimens (Carretta et al., 2013a; Lucchinetti et al.,
53 2000) because such studies remain technically challenging.

54 Tensile tests were performed on a single trabecula in previous studies (Yamada
55 et al., 2014; Carretta et al., 2013b, 2013c; McNamara et al., 2006; Hernandez et al., 2005;
56 Bini et al., 2002; Rho et al., 1993). Although tensile tests present some advantages, it is
57 difficult to completely isolate, fix, and examine small bone specimens. We recently
58 examined trabecular specimens (at least 3 mm in length) by tensile testing; however, the
59 specimens were larger than the standard size and existed in the edges of the cancellous
60 bone (Yamada et al., 2014). Three-point bending tests (Carretta et al., 2013b, 2013c;

61 Hambli and Thurner, 2013; Szabó et al., 2011; Jungmann et al., 2011; Busse et al., 2009)
62 were also performed on a single trabecula. However, such tests generally require
63 complete isolation of the specimen, deflection measurements at high resolution, and very
64 precise loading, which are usually difficult to obtain. Therefore, a simpler and more
65 reliable experimental method is required for biomechanical analysis of a single trabecula.

66 Here we demonstrate a novel experimental method to investigate the elastic
67 properties of a single trabecula on the basis of cantilever bending, as shown in Fig. 1. The
68 micro-cantilever bending (MCB) test does not require the specimen to be isolated
69 completely from the cancellous bone, and the specimen can be fixed easily during the
70 test.

71

72 **2. Materials and Methods**

73 *Specimen preparation*

74 A total of 10 trabeculae were dissected from the proximal epiphysis of an adult
75 bovine femur (two years old) as shown in Fig. 2. First, the proximal epiphysis was sliced
76 vertically to the longitudinal axis of the femur (bone axis; Fig. 2a), and the bone marrow

77 was removed by brief water jetting (Fig. 2b). Second, plate-like cancellous bone samples
78 were cut out from the slices using a low-speed diamond wheel saw (model 650, South
79 Bay Technology Inc., USA). Third, a specific single trabecula of about 1 mm in length
80 aligned in the plane of each plate-like sample was randomly selected and isolated from
81 the cancellous bone, while keeping one extremity connected to the other trabeculae (Fig.
82 2c). Fourth, the remaining attached cancellous bone portion was shaped into a small
83 rectangle such that its height corresponded to the depth of a specimen holder (Fig. 2d).
84 The specimen was placed into the holder almost vertically and fixed by embedding the
85 cancellous bone portion in epoxy resin (Fig. 2e).

86

87 *Trabecular axis and morphology*

88 The longitudinal orientation of each trabecula within the femoral epiphysis was
89 visualized by scanning the plate-like samples (Fig. 2c) using a microfocus X-ray CT
90 instrument (inspeXio SMX-90CT, Shimadzu Corporation, Japan) at a tube voltage of 90
91 kV, tube current of 110 μ A, and voxel size of 0.092 mm/voxel. The trabecular orientation,
92 α , was defined as the angle between the longitudinal direction of the trabecula and bone

93 axis (Fig. 3).

94 The shape of the trabecula was determined by high-resolution scanning of the
95 specimens fixed to the jigs (Fig. 2e) using a voxel size of 0.009 mm/voxel. The area (A),
96 circularity, and aspect ratio of the cross-sections were analyzed using ImageJ software.

97

98 *MCB*

99 The MCB tests were conducted using a small testing device (Fig. 4),
100 comprising an acrylic plate to apply the displacement and load on the specimen, a 1-axial
101 stage (ALS-4011-G1M, Chuo Precision Industrial Co., Ltd., Japan), and a load cell
102 (LVS-1KA, Kyowa Electronic Instruments Co., Ltd., Japan). The tests were conducted
103 under optical microscopic observation at a 3- μ m resolution (VH-5000, Keyence
104 Corporation, Japan). Each specimen was positioned at the edge of a taper bore drilled into
105 the acrylic plate, with the contact position in close proximity with the free end. The plate
106 was displaced horizontally in a stepwise manner using the stage, and microscopic images
107 were captured vertically (Fig. 4a). The deflection, d , was defined as the horizontal
108 displacement of the contact position in the direction of plate displacement and the load, F ,

109 in the same direction was measured by the load cell connected to the plate. The maximum
110 d and F values were $178 \pm 16 \mu\text{m}$ and $0.68 \pm 0.75 \text{ N}$, respectively. A drop of water-based
111 ink was applied to the edge of the bore, and the contact position at the surface of the
112 specimen was observed by optical microscopy after the test. Then, the distance, l ,
113 between the fixed end of the specimen and contact position was measured for the
114 calculation of the elastic modulus. Specimens were loaded and then unloaded three times
115 under air-dried conditions.

116 The shape of the specimen was assumed to be a vertical circular cylinder of
117 orthotropic material and the shear stress was considered to be negligible during loading.
118 Then, the elastic modulus, E , in the longitudinal direction of the single trabecula was
119 calculated as in Eq. (1) from the plane curve of the bending axis (e.g., Lekhnitskii, 1963).

120

121
$$E = \frac{l^3}{3I} \frac{F}{d} \quad (1)$$

122

123 , where I is a second moment of area and is expressed by Eq. (2) (e.g., Timoshenko and
124 Young, 1968).

125

126
$$I = \frac{\pi D^4}{64} \quad (2)$$

127

128 , where D is the diameter of cylinder. D value was calculated from the average

129 cross-sectional area of each specimen as $2\sqrt{A/\pi}$.

130

131 **3. Results**

132 The morphological characteristics and orientation of the trabecular specimens

133 are shown in Table 1. The specimens appeared to be rod-like in shape according to

134 microscopic and X-ray CT observations. The longitudinal orientation of the trabecular

135 specimens varied widely from 14° to 87° with respect to the bone axis.

136 The specimens deflected in the direction of the plate displacement and torsional

137 deformation was not observed. Figure 5 demonstrates the high repeatability of the $F-d$

138 relationships obtained for a specimen. The linear region of the relationships was used to

139 calculate the average F/d (N/mm) value of the three measurements, which was then used

140 to determine the elastic modulus. According to the Wilcoxon signed-rank test, the ratios

of force–deflection trend (F/d) in the second and third measurements compared to the first measurements had no significant difference with the first measurements ($P > 0.05$). In the cancellous bone of a bovine femur, the average elastic modulus of the 10 trabecular specimens was 9.1 ± 5.4 GPa.

In the present study, the elastic modulus had no significant correlation with the orientation, size, or shape of the trabeculae. Furthermore, the trabecular orientation had no significant correlation with the size and shape.

4. Discussion

The present study found that the elastic modulus of trabecular specimens from a bovine femur was 9.1 ± 5.4 GPa. These values fall within the range reported for a single trabecula under dry conditions (2–16 GPa; Yamada et al., 2014; Carretta et al., 2013b, 2013c; Busse et al., 2009; Bini et al., 2002; Rho et al., 1993), including different species, ages, locations, size, and types of mechanical test. There was also no significant difference with the elastic modulus values obtained in our previous study using the same age bovine femurs (Yamada et al., 2014). These findings indicate that the new MCB

157 protocol proposed herein can be used to determine the elastic modulus of a single
158 trabecula.

159 Tensile and three-point bending tests have been performed on a single trabecula.

160 Tensile tests are among the simplest mechanical tests, and are suited for the observation
161 of the deformation behavior of mineral crystals within a single trabecula by X-ray
162 diffraction (Yamada et al., 2014). However, it is rather difficult to completely isolate, fix,
163 and examine small bone specimens, such as those used in the present study. On the other
164 hand, three-point bending tests do not require fixation of the specimen to a jig with a resin
165 or glue, while complete isolation of the specimens from the cancellous bone is generally
166 required. Furthermore, the rod-shape specimen may move and roll on the support during
167 loading; precise loading therefore needs to be taken into account. High-resolution
168 measurements for the displacement are also required; in fact, the deflection generated by
169 the same force is 16 times smaller by three-point bending than by cantilever bending of a
170 uniform beam. These tests may not be suited for a single trabecula due to technical
171 difficulty. Lorenzetti et al. (2011) conducted fixed-fixed beam tests on a single trabecula
172 in a cancellous bone sample by applying force with a nylon wire. Although this method is

173 quite convenient, it may generate displacement artifacts at the trabecular ends that must
174 be removed from the calculations, and controlling the loading position and direction may
175 be technically challenging. In contrast, the MCB test allows the trabecular specimens to
176 remain attached to the cancellous bone at one extremity and allows for better control of
177 the position during the test, which is a simple and useful experimental method for
178 biomechanical analysis of a single trabecula.

179 In Wolff's law the trabecular orientation plays an important role in the
180 mechanical strength of the bone; however, the elastic modulus of single trabeculae had no
181 significant correlation with the orientation in the present study. Our previous study also
182 did not show this correlation, although the effects of the hydroxyapatite crystal strain
183 ratio on the elastic modulus of single trabeculae were observed (Yamada et al., 2014). It
184 suggests that the variation of the elastic modulus of single trabeculae may depend on its
185 hierarchical structure. Furthermore, the variation may be relatively small locally since the
186 single trabeculae were extracted from the same region, and the trabecular networks are
187 important for the apparent strength of the cancellous bone in the region.

188 The present study has some limitations. First, we examined the 1-mm single

189 trabeculae taken from a relatively close area to the diaphysis, which were easy to extract.

190 Second, a stepwise deformation was applied to all specimens. Although the measured

191 force did not show significant time dependence, a small decreasing force was observed

192 immediately after the applied deflections. Accordingly, deflection was measured from

193 optical images captured during the period of stable force. Third, the linear region of the

194 force–deflection relationship was used to calculate the elastic modulus. In general, a

195 non-linear relationship is observed with curved specimens, inhomogeneous shapes, large

196 deformations, and non-linear elastic properties as well as after yielding. In the present

197 study, the region used for the calculations had high linearity ($R^2 = 0.96 \pm 0.02$), and the

198 specimens appeared as straight rods with linear elastic properties. Fourth, the specimen

199 was assumed to be a vertical circular cylinder for the calculation of the elastic modulus to

200 simplify the method. In general, asymmetrical shape and anisotropic material properties

201 generate oblique deflection and rotation, which complicate the calculation; however, such

202 phenomenon was not detected in the study. Furthermore, although the sample may

203 slightly tilt with respect to the vertical axis, it has less impact on the results because the

204 effects of the cosine of the tilt angle are negligibly small. Fifth, the measured elastic

205 moduli are considerably affected by the elastic properties around the bottom region of the
206 trabeculae in the bending test, and we assumed that the trabeculae had homogenous
207 mechanical properties. Last, the specimens were assumed to be rigidly fixed to the
208 holders. Although the fixation may represent a source of error, the embedded cancellous
209 bone portion was larger than the single trabecula and the stiffness of the portion was
210 considered to be sufficiently high to resist the applied load.

211

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214

215 **Conflict of interest statement**

216 There is no actual or potential conflict of interest associated with this project.

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 278

1 **Legends of the figures and table**

2 **Figure 1** Schematic of micro-cantilever bending for a single trabecula.

3 **Figure 2** Specimen preparation: the proximal epiphysis of a bovine femur (a) was sliced
4 vertically to the longitudinal axis of the femur (bone axis) and the bone marrow was
5 removed using a water jet (b). Plate-like cancellous bone samples were cut out from the
6 slices, and a specific single trabecula of about 1 mm in length aligned in the plane of each
7 plate-like sample was randomly selected. The cancellous bone surrounding the target
8 single trabecula was removed (c). The remaining cancellous bone portion was shaped
9 into a small rectangle (d). The specimen was placed into a holder almost vertically and
10 fixed by embedding the cancellous bone portion into epoxy resin (e). The trabecular
11 specimen was observed by optical microscopy (f). White arrows indicate the bone axis.

12 **Figure 3** Definition of the trabecular orientation α .

13 **Figure 4** Experimental setup (a) and small testing device designed to conduct
14 micro-cantilever bending tests on a single trabecula (b). The device comprised an acrylic
15 plate (2.3-mm thickness), a 1-axial stage (ALS-4011-G1M, Chuo Precision Industrial Co.,
16 Ltd., Japan) equipped with a high-resolution (2 μm) and high-repeatability (0.3 μm)

17 actuator, and a load cell (LVS-1KA, Kyowa Electronic Instruments Co., Ltd., Japan) with
18 high repeatability ($\leq 0.5\%$) and small hysteresis ($< 0.5\%$). The diameters of the taper bore
19 drilled into the acrylic plate at the upper and bottom side were 6.2 and 4.3 mm,
20 respectively, and the edge of the bore was sharp (approximately 20- μm radius).

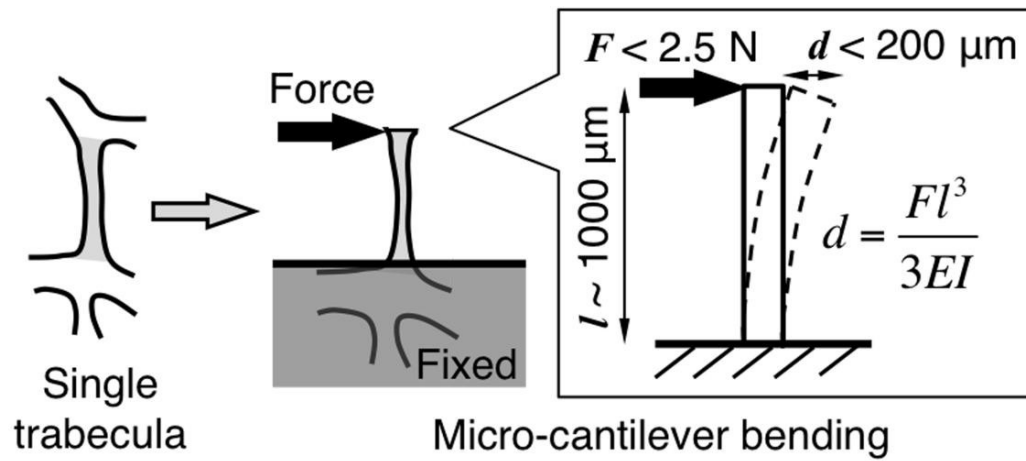
21 **Figure 5** Force–deflection relationships in a single trabecula. Tests of each specimen
22 were conducted three times, with the same color dots indicating values from the same
23 test.

24 **Table 1** Mean values and standard deviations of each parameter measured on the
25 trabecular specimens ($n = 10$): the distance between the fixed end of the specimen and
26 contact position (l) observed by microscopy, which was almost the same as trabecular
27 length; trabecular orientation (α), average cross-sectional area (A), circularity (Cir), and
28 aspect ratio (AR) measured by micro CT scans; diameter of the specimen (D) measured
29 by the approximation of a circular cylinder; elastic modulus (E) and R^2 value of
30 force–deflection relationship from the micro-cantilever bending (MCB) test.

1 Figure and table

2

Figure 1

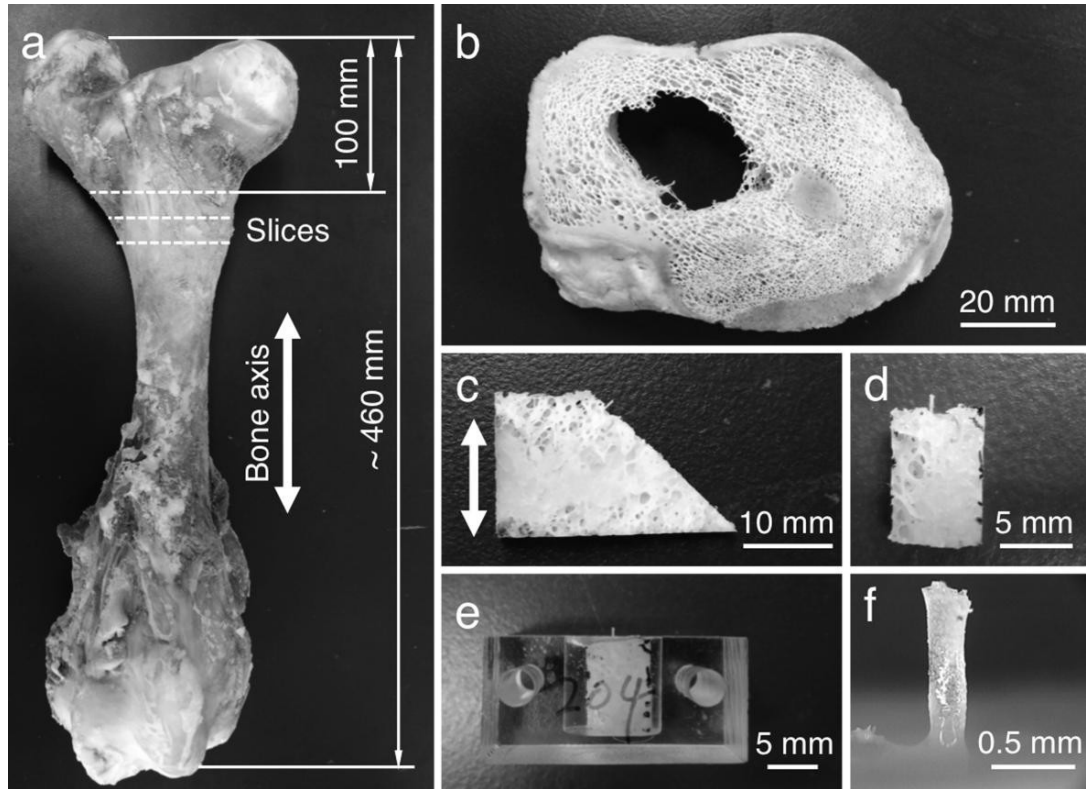


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Figure 2

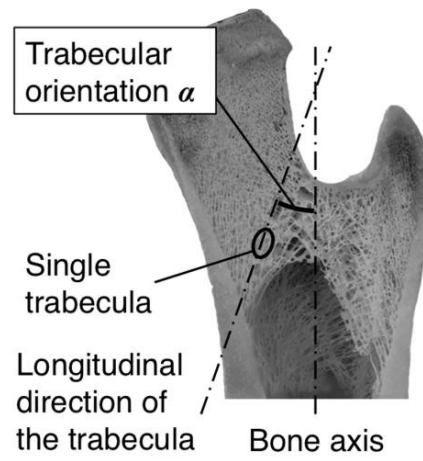


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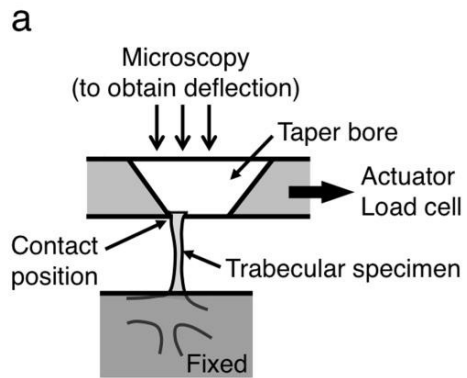
Figure 3



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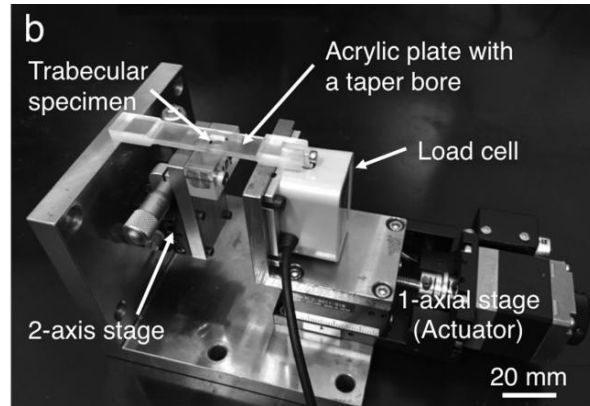
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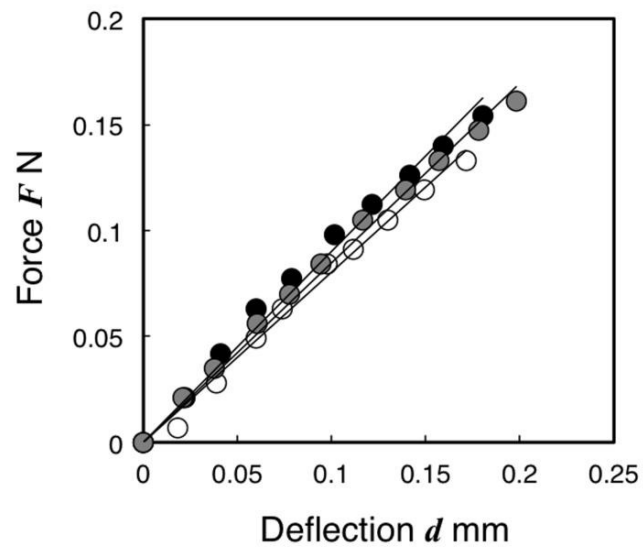
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Figure 4



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Figure 5



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Table 1

Experiments	Microscopy	μ -CT					MCB	
Parameters	l	α	A	Cir	AR	D	E	R^2
Unit	mm	$^{\circ}$	mm^2	-	-	mm	GPa	-
Mean	1.12	49	0.0575	0.67	1.4	0.25	9.1	0.96
S.D.	0.17	24	0.0515	0.10	0.1	0.10	5.4	0.02

18

19