Title: Local distribution of collagen fibers determines crack initiation site and its propagation direction during aortic rupture

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Abstract

Although elucidation of the mechanism of aortic aneurysm rupture is important, the characteristics of crack initiation and propagation sites remain unknown. To determine the microscopic properties of these sites, the characteristics of local strains and constituents at crack initiation and propagation sites were investigated during biaxial stretching of porcine thoracic aortas (PTAs). PTAs were sliced into approximately 50-μm-thick sections, and the center of the sections was made especially thin using our previously developed technique. Alpha-elastin and cell nuclei were fluorescently labeled as indices of local elastin density and as a strain marker, respectively. Birefringence and second harmonic generation (SHG) light images were used to determine local collagen distributions. The specimens were then stretched biaxially with a laboratory-made tensile tester under a fluorescent microscope equipped with a birefringence imaging system. Local strains were calculated from the local displacement of the cell nuclei. The degree of alignment and density of local collagen fibers were measured from retardance and SHG images. The strain distributions, specifically the first and second principal, and maximum shear strains, fluorescent intensity of α-elastin, and degree of alignment of collagen fibers, showed insignificant differences between the crack initiation sites and other sites. The retardance and intensity of SHG light at the crack initiation sites were significantly lower than those at other sites for all ($n = 6$) specimens. Cracks tended to propagate along the local direction of the collagen fibers. These results indicate that the local density and direction of collagen fibers play an important role in aorta rupture.

1. Introduction

Large aneurysms tend to be at a high risk of rupture due to high wall stress according to Laplace's law. In clinical practices, a critical diameter of approximately 5.5 cm is used as the criterion for surgical repair for patients with thoracic aortic aneurysms (TAAs). However, TAAs smaller than this size can also rupture (Cambria et al. 1995; Coady et al. 1997). Although the thickness of aneurysms should be negatively correlated with the rupture risk because thin walls lead to high local wall stress (Raghavan et al. 2006), no significant difference in thickness has been reported between ruptured and unruptured aneurysmal specimens (Raghavan et al. 2011). With the progression of the disease, the strength of aneurysmal tissue may continue to decrease, and the vessel may finally succumb to the physiological wall stress, resulting in rupture (Raghavan et al. 1996). The tensile strength of TAAs was reported to be significantly lower than that of normal vessels (He and Roach 1994). However, there have also been studies that found no significant differences between healthy and aneurysmal specimens (Garcia-Herrera et al. 2012; Iliopoulos et al. 2009; Raghavan et al. 2011; Sokolis et al. 2012). Thus, the factors that influence aneurysm rupture remain unknown.

Aortic tissue is a composite material mainly consisting of cells, collagen, and elastin. Those constituents are important factors that affect the strength of the aortas. In aneurysms, a decrease in the elastin is commonly reported (He and Roach 1994; Iliopoulos et al. 2009), and this might cause an increase in wall stiffness (He and Roach 1994). The volume fraction of collagen and ground substance was reported to be higher in aneurysms in one study (He and Roach 1994), and collagen content in aneurysmal specimens was not significantly different from that in normal tissue in another study (Iliopoulos et al. 2009). Since the tensile strength of collagen is the highest among the constituents of the aortas (Fung 1981), increased or unchanged collagen content in aneurysmal walls seems paradoxical.

One possible explanation for this paradox is that these studies averaged the parameters in tissues and did not focus on local values in the aortas. Rupture can occur from even a small region with weakened strength in the aortas. We previously developed a pressure-imposed test (bulge test) system (Ohashi et al. 2003) and found that strain distribution in TAA

83   specimens was more heterogeneous than that in healthy porcine thoracic aortas (PTAs)

84   (Sugita et al. 2011). Moreover, all PTA specimens eventually ruptured, whereas some TAA

85   specimens ruptured at much lower stress than average and other TAA specimens did not

86   rupture even under more than 4000 mmHg of pressure (Sugita et al. 2011). These results

87   indicate that locally weak regions in aneurysmal walls are points of failure. Thus, it is

88   important to investigate the relationship between the local properties of the aorta and the

89   rupture site. Although the effects of the histological and mechanical properties on a ruptured

90   site were previously investigated at the millimeter scale (Raghavan et al. 2011), more

91   microscopic analyses should be performed to evaluate tissue heterogeneity.

92   To determine the microscopic properties at the crack initiation site, the bulge test is not

93   suitable for it is difficult to keep specimen focused under a microscope, and thus we

94   developed a biaxial stretch tester placed on an inverted microscope (Sugita and Matsumoto

95   2013b). Using this device, aortic tissues were successfully stretched biaxially until failure in

96   a single focal plane, and their crack initiation points were successfully observed under a

97   microscope (Sugita and Matsumoto 2013b). In the present study, we measured the

98   distributions of elastin and collagen and the strain distributions in the biaxially stretched

99   healthy PTA specimens to investigate the factors that affect crack initiation.

100

101 **2. Materials and methods**

102 **2.1.** *Specimen preparation*

103   Healthy PTA specimens were used. To induce rupture at the center of the aortic

104 specimen under biaxial stretch, aortic slices thinned at the center were prepared according to

105 the procedure described in our previous study (Sugita and Matsumoto 2013b). Briefly, a

106 PTA block with dimensions of 15 mm in the longitudinal direction and 20 mm in the

107 circumferential direction was sandwiched between two metal plates with a hole at the center

108 and compressed by 40% in the radial direction. During this process, the center of the

109 specimen was less compressed than the periphery because of the holes in the metal plates.

110 The block was frozen at −80°C for 10 min to fix the shape of the specimen, embedded in

111 Tissu Mount (Chiba Medical, Saitama, Japan), and sectioned with a cryotome (CM3050SIV,

112   Leica Microsystems, Wetzlar, Germany) into 50-µm slices perpendicular to its radial

113   direction. The specimens were kept at −80°C until tested and only specimens obtained from

114   the middle of one-third of the aorta in the radial direction were used. When the specimens

115   were thawed, the compression in the specimen periphery loosened more than the

116   compression in the center, causing the center of the specimen to appear thinner.

117

118   **2.2. *Staining***

119   The specimens were immersed in 2% bovine serum albumin (BSA) for 1 h for blocking.

120   For elastin staining, specimens were incubated with the antimouse antibody of elastin

121   (Sc-58756, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1/50 in PBS(−)

122   including 0.2% BSA for 1.5 h, followed by incubation with secondary antibody (anti IgG

123   Alexa fluor 488, A11059, Invitrogen, Carlsbad, CA, USA) diluted 1/50 in PBS(−) including

124   0.2% BSA for 1 h. To measure strain distribution in the specimens, the specimens were

125   incubated with 10 µg/ml Hoechst 33342 solution (Invitrogen) for 30 min to visualize the

126   cell nuclei. All processes were performed at room temperature.

127

128   **2.3. *Equi-biaxial tensile test***

129   Specimens were stretched equi-biaxially in both the circumferential and longitudinal

130   directions using a laboratory-made equi-biaxial stretching device (Sugita and Matsumoto

131   2013b). Briefly, a specimen was glued onto polyethylene terephthalate (PET) film sheet and

132   part of PET film with a hole at the center and the sheet was glued onto a metal frame with a

133   hole. The metal frame was then moved stepwise by 0.5 mm at each step toward a metal

134   hollow cylinder to stretch the specimen biaxially. This setup was placed under a fluorescent

135   microscope (IX71, Olympus, Tokyo, Japan). At each step, phase contrast, fluorescent, and

136   retardance images of the specimen were captured on a charge-coupled device camera

137   equipped with a birefringence imaging system Pol-Scope (Abrio-LS, CRi, Woburn, MA,

138   USA) (Oldenbourg 1996) through a 2× objective lens (PLAPON 2×, Olympus). This system

139   enabled us to analyze the retardance as an index of collagen density and slow axis azimuth

140   as the collagen fiber angle of the samples at each pixel on the charge-coupled device. When

141 light passes through a birefringent material, the light wave is separated into two
142 orthogonally polarized waves that travel at different speed, causing a phase shift or
143 retardance and retardance correlated significantly with collagen volume as confirmed in our
144 previous study (Sugita and Matsumoto 2013c). Images were repeatedly captured, and the
145 metal frame was moved continuously until specimen failure. High-speed (300 frames/s)
146 images were recorded simultaneously with a digital camera (Exilim EX-F1, Casio
147 Computer, Tokyo, Japan) to determine the crack initiation site. Analysis was performed six
148 out of ten specimens because cracks initiated from edge of thin sample region in other four
149 specimens perhaps because of the stress concentration in these area (Sugita and Matsumoto
150 2013b).

151

152 **2.4. *Second harmonic generation light observation***

153 Specimen glued onto a PET film was set under the a two-photon-excited microscope
154 (FV1200MPE, Olympus), and the second harmonic generation (SHG) light image of the
155 specimen was captured as previously reported (Sugita and Matsumoto 2017). Briefly, the
156 power of a mode-locked Ti:sapphire laser (wavelength = 800 nm, pulse width = 100 fs,
157 repetition rate = 80 MHz, laser power = 3 mW) was reduced to 4.5%, and the laser was
158 focused on specimens through an $10\times$ objective lens (UMPLan FLN 10XW, NA = 0.30,
159 Olympus). The generated SHG signal was collected in the backward direction through a
160 dichroic mirror (485 nm) and a band pass filter (400 ± 5 nm). Before and after the biaxial
161 stretch test, 4×4 images in both the longitudinal and circumferential directions were
162 captured across the wall with a Z-interval of 10 μm.

163 These stack images were converted into projection images of 2D sum slices in the
164 circumferential-longitudinal plane using image analysis software (ImageJ 1.47v, National
165 Institutes of Health, Bethesda, MD, USA). The 4×4 images in the longitudinal and
166 circumferential directions were then merged using a "photomerge" function in image
167 analysis software (Photoshop CS6, Adobe Systems, San Jose, CA, USA).

168 Total of eleven unstained specimens were used in this experiment. Six specimens were
169 analyzed and five specimens were excluded because crack initiated in the edge of thin

170   sample region. After imaging, the biaxial test was performed on the specimens as described

171   in the Section 2.3. The specimens were then peeled off from the metal plate carefully not to

172   distort them and their SHG images after crack propagation were captured.

173

174   **2.5. *Strain analysis***

175   Strain distribution was obtained from images of fluorescently labeled cell nuclei during

176   biaxial stretching as we previously reported (Sugita and Matsumoto 2013a). Briefly, local

177   displacement was measured every 10 pixels (approximately 50 μm) with digital image

178   correlation software (Flow-vec v. 4.9, Library, Tokyo, Japan). In a pair of sequential images,

179   an analysis region of 31×31 pixels (approximately 150×150 μm^2) was selected in the first

180   image, and a region with the same size and the highest correlation with the interrogation

181   region was searched in the second image with a search size of 101×101 pixels

182   (approximately 500×500 μm^2). From the displacement data, the components of the 2D

183   strain tensor in the circumferential and longitudinal directions (ε_{CC}, ε_{LL}, $\varepsilon_{CL} + \varepsilon_{LC}$) were

184   locally calculated as follows:

185
$$\varepsilon_{CC} = \frac{X'_{(I+1,J)} - X'_{(I,J)}}{X_{(I+1,J)} - X_{(I,J)}} \tag{1}$$

186
$$\varepsilon_{LL} = \frac{Y'_{(I,J+1)} - Y'_{(I,J)}}{Y_{(I,J+1)} - Y_{(I,J)}} \tag{2}$$

187
$$\varepsilon_{CL} + \varepsilon_{LC} = \frac{X'_{(I,J+1)} - X'_{(I,J)}}{Y_{(I,J+1)} - Y_{(I,J)}} + \frac{Y'_{(I+1,J)} - Y'_{(I,J)}}{X_{(I+1,J)} - X_{(I,J)}}, \tag{3},$$

188   where <I, J> represents the Ith and Jth center points of the analysis window in the

189   circumferential and longitudinal directions, respectively; X and Y are coordinates in the

190   circumferential and longitudinal directions; and the apostrophe (′) indicates the coordinates

191   after displacement. These strains were calculated at each displacement step, and the

192   cumulative strains from the zero load state were calculated. Finally, the first ε_1 and second

193   ε_2 principal strains, the maximum shear strain γ_{max}, and the areal strains ε_{area} were calculated

194   from the following equations:

$$\varepsilon_1 = \frac{\varepsilon_{CC} + \varepsilon_{LL}}{2} + \sqrt{\left(\frac{\varepsilon_{CC} - \varepsilon_{LL}}{2}\right)^2 + \left(\frac{\varepsilon_{CL} + \varepsilon_{LC}}{4}\right)^2} \tag{4}$$

$$\varepsilon_2 = \frac{\varepsilon_{CC} + \varepsilon_{LL}}{2} - \sqrt{\left(\frac{\varepsilon_{CC} - \varepsilon_{LL}}{2}\right)^2 + \left(\frac{\varepsilon_{CL} + \varepsilon_{LC}}{4}\right)^2} \tag{5}$$

$$\gamma = \sqrt{(\varepsilon_{CC} - \varepsilon_{LL})^2 - (\varepsilon_{CL})^2} \tag{6}$$

$$\varepsilon_{Area} = (1 + \varepsilon_{CC})(1 + \varepsilon_{LL}) - \varepsilon_{CL}\varepsilon_{LC} \tag{7}$$

Strain analysis was first performed in all areas of the specimen inside the metal cylinder (6 mm in diameter), and only data obtained from thin center region (~2 mm in diameter at no load state) were used for the analysis to exclude boundary effects.

2.6. Elastin and collagen density analysis

Fluorescent intensities in the elastin image were also averaged for each $50 \times 50 \ \mu m^2$ area, which were the same areas used in the strain analysis, as indices of local elastin density. To investigate the effect of the distribution of collagen fibers on the fracture, retardance was averaged for each $50 \times 50 \ \mu m^2$ area. Since retardance is affected by a combination of density and the alignment agreement of collagen fibers (see Online Resource S1), SHG images were also analyzed. We confirmed that intensity can be used as an index of the density of collagen fibers because the SHG intensity linearly correlated with the density of the collagen fibers, and the degree of alignment for the collagen fibers did not affect the intensity of the SHG image (see Online Resource S2).

2.7. Determination of crack initiation and propagation sites

The crack initiation point was determined using high-speed camera images. The crack initiation site was identified as a local 10×10 pixel ($50 \times 50 \ \mu m^2$) area including the crack initiation point and the surrounding 5×5 area ($250 \times 250 \ \mu m^2$). Other areas were termed "other sites."

Crack positions in SHG images captured before the biaxial tensile test were determined from cracks and collagen fiber patterns in SHG images captured after rupture. For analysis of the alignment and orientation of collagen fibers, $250 \times 250 \ \mu m^2$ areas along the crack in

222 the SHG image were selected as the crack propagation sites. Areas in which the crack

223 propagated in a zigzag fashion were excluded.

224

225 **2.8. *Alignment and orientation of collagen fibers***

226 As described in a previous study (Sugita and Matsumoto 2017), the alignment

227 consistency of collagen fibers was analyzed for the crack initiation and propagation sites

228 determined in Section 2.7. Briefly, local images at the crack initiation and propagation sites

229 were processed using two-dimensional fast Fourier transform to obtain power spectrum

230 images (PSIs) of the fibers. Since high intensities were observed in directions perpendicular

231 to the fiber directions for the obtained PSI (Petroll et al. 1993), average intensity was

232 calculated for every 10° area around the origin in the PSI and was normalized by the

233 summation of the average intensity at each angle to give the probability distribution of

234 collagen fiber orientations. The probability distribution was then fitted with a Gaussian

235 function:

236 $$P = P_{base} + (P_{peak} - P_{base})\exp\left\{\frac{-\left(\alpha - \alpha_{average}\right)^2}{2\sigma^2}\right\},\qquad(8)$$

237 where P is the probability of the fiber angle α, and P_{base}, P_{peak}, $\alpha_{average}$, and σ are fitting

238 constants that indicate the base level, maximum value, average angle, and standard

239 deviation (SD) of the Gaussian distribution, respectively. Since the parameter σ should be

240 high when the alignment consistency of the collagen fibers in the original image is low, σ

241 was used to evaluate the alignment inconsistency of the collagen fibers. When the

242 probability $P(\alpha)$ at angle α was 1.5 times higher than the average of P, α was determined to

243 be the direction in which the collagen fibers were aligned with high probability. The number

244 of directions was 3.7 ± 1.0 (mean $\pm$ SD) in 18 directions of α.

245

246 **2.9. *Crack direction***

247 Crack directions were analyzed both microscopically using SHG images captured under

248 the microscope and macroscopically using images captured with a digital camera.

249 In the microscopic analysis, the crack directions at the crack initiation and propagation

250 sites determined in Section 2.7 were measured. A 100-µm-long line segment was fitted to

251 the shape of each crack visually at the crack initiation and propagation sites in the SHG

252 images captured after rupture, and their angles were measured using ImageJ.

253 In the macroscopic analysis, after whole sample images were captured with a digital

254 camera (CX3, Ricoh Imaging, Tokyo, Japan), the crack direction at the crack initiation

255 position was measured by fitting a 300-µm-long line segment. Similarly, the direction of

256 each crack was determined by fitting a 300-µm-long line segment at both ends of the crack,

257 and these directions were compared with the directions of the cracks at the crack initiation

258 sites identified using microscopic analysis.

259

260 **2.10.** *Statistics*

261 For each specimen, mean $\pm$ SD was calculated both at the crack initiation sites and at

262 other sites, and the results were compared using Student's unpaired t-test within each

263 specimen. The average values at both sites in each specimen were then calculated for all six

264 specimens and compared using Wilcoxon signed-ranks test as non-parametric and paired

265 test. Since the number of the crack end sites were double of the number of the crack

266 initiation sites, comparison of the difference in the crack direction was performed using the

267 Mann-Whitney U-test. The significance level was set at 0.05.

268

269 **3. Results**

270 **3.1.** *Local strains*

271 Figure 1 shows the characteristics of local strains at the crack initiation sites. All strain

272 distributions such as the first principal strain (Fig. 1a), the second principal strain (Fig. 1b),

273 the maximum shear strain (Fig. 1c), and the areal strain (Fig. 1d) during biaxial stretching

274 were heterogeneous as reported in a previous study (Sugita and Matsumoto 2013a). For

275 comparison of strains between the crack initiation sites and the other sites in all six

276 specimens (Fig. 1e–h), only areal strain at the crack initiation sites was significantly higher

277 than that at the other sites (Fig. 1h). However, when strains were compared in each

278 specimen, significantly higher strains were not always observed at the crack initiation sites

279   compared with the other sites (3 of 6 specimens for the first principal strain [Fig. 1e], 0 of 6

280   specimens for the second principal strain [Fig. 1f], 1 of 6 specimen for the shear strain [Fig.

281   1g], and 2 of 6 specimens for the areal strain [Fig. 1h]). These results indicate that the crack

282   initiation sites were not determined by high local strain at this scale (~50 µm).

283

284   **3.2. *Local elastin distribution***

285   Figure 2a shows a typical image of fluorescently labeled elastin. The intensity of

286   fluorescently labeled elastin was heterogeneous and significantly higher at the crack

287   initiation sites (white box area in Fig. 2a) for 2 of 6 specimens and lower for 2 of 6

288   specimens (Fig. 2b). These results indicate that the crack initiation sites were not

289   determined by elastin distribution either.

290   **3.3. *Retardance distribution***

291   Figure 3 shows the characteristics of birefringence distribution just before rupture. In the

292   retardance image, which shows an index of collagen density distribution (Fig. 3a),

293   retardance was low at the crack initiation sites. Statistical analysis showed that retardance at

294   the crack initiation sites was significantly lower than that at other sites for both the average

295   value of all the specimens and comparison within a single specimen (6 of 6 specimens) (Fig.

296   3c).

297   A typical image of azimuth distribution in a specimen is shown in Fig. 3b. Collagen

298   fibers aligned in the circumferential direction and the azimuth changed slightly in several

299   local areas. The slow axis azimuth at the crack initiation sites was significantly higher in 2

300   of 6 specimens than that at the other sites but was lower in 2 of 6 specimens (Fig. 3d).

301   We had expected that the retardance might change at failure because collagen fibers

302   might be broken locally at the crack initiation sites. However, changes in the retardance

303   between the period just before rupture and the previous loading step at the crack initiation

304   sites showed no clear trend: the changes in the retardance was significantly higher in some

305   specimens (3 of 6 specimens) but lower in others (2 of 6 specimens; Fig. S3). Since

306   collagen density during a stretch should not change at each site, changes in retardance may

307   indicate changes in the alignment consistency of collagen fibers. Thus, these results indicate

308 that changes in the alignment consistency of collagen fibers do not correlate with the crack

309 initiation.

310

3.4. *SHG light distribution at crack initiation site*

312 The retardance is affected by both the collagen density and alignment consistency of

313 collagen fibers. We thus analyzed the SHG signal, which is generated from collagen fibers

314 in the aorta. Figure 4 shows a typical image of SHG light in the specimen before (Fig. 4a)

315 and after (Fig. 4b) the biaxial test. The average intensity at the crack initiation sites for all

316 specimens was significantly lower than that at the other sites (Fig. 4c). This significant

317 difference was also found in each specimen (6 of 6 specimens).

318 Fitting parameter σ, which shows the alignment inconsistency of collagen fibers, shows

319 insignificant differences between the crack initiation sites and the other sites (Fig. 4d). This

320 indicates that low retardance at the crack initiation sites is due to low collagen density rather

321 than high alignment inconsistency in the collagen fibers.

322 These results indicate that cracks propagate from low collagen density sites.

323

3.5. *Crack propagation in the aorta*

325 An SHG image obtained after rupture (Fig. 4b) showed that the cracks observed under

326 the microscope propagated in zigzags, and the cracks partially ran along the longitudinal

327 direction in the macroscopic view. In 64% (25 of 39) of the crack propagation sites, crack

328 directions corresponded to the directions of the collagen fibers with high probability for

329 collagen fibers aligned in the circumferential direction (Fig. 5a) or approximately 45° from

330 the circumferential and longitudinal (Fig. 5b) directions, indicating that the cracks tended to

331 propagate along the directions of the collagen fibers. However, some cracks ran in other

332 directions (Fig. 5c), indicating that other factors may be involved in crack direction. Crack

333 angle distribution at crack propagation areas is shown in Fig. 5d. The local angle of the

334 crack direction, which was determined using a 100-µm-long line segment fitted locally to

335 the crack in the microscopic image, seems to run in several preferred direction such as ±30°

336 and ±0° from the circumferential direction.

337   Macroscopically, the cracks seem to run in the circumferential direction rather than in the

338   longitudinal direction (Fig. S4). Figure 5e shows the absolute value of the local crack angle

339   from the circumferential direction, which was determined to be the 300-µm-long line

340   segment in images captured with a digital camera. The angle at crack initiation sites (41° ±

341   20°) tended to be higher than that at the ends of the crack sites (20° ± 19°) though it was

342   insignificant ($p = 0.06$), indicating that the cracks run in the relatively longitudinal direction

343   at the crack initiation sites, then propagate in the relatively circumferential direction on the

344   macro scale.

345

346   **4. Discussion**

347   In this study, the effects of local mechanical and morphological properties on crack

348   initiation and propagation were investigated using healthy PTA specimens. We found that

349   crack initiation and propagation depended on the local structure of collagen fibers; cracks

350   were initiated at regions with a low density of collagen fibers and propagated along the

351   direction of the fibers. These results are reasonable because the elastic modulus and tensile

352   strength of collagen fibers are higher than those of other constituents in the aortas (Fung

353   1981), and collagen fibers are considered principal load-bearing elements in high strain and

354   stress regions. In addition to that, recent studies have stated mechanics of arterial wall

355   focusing on collagen fiber distributions which affects material characterization of artery

356   (Gasser et al. 2006) and ascending thoracic aortic aneurysms (Sassani et al. 2015). However,

357   this is the first study, to our knowledge, to confirm that the local density of collagen fibers

358   plays an important role in the wall strength of biaxially stretched aortas on the microscopic

359   scale. Strain distribution in TAA specimens was more heterogeneous than that in healthy

360   PTAs (Sugita et al. 2011), and decreased elastin has been reported in aneurysms (He and

361   Roach 1994; Iliopoulos et al. 2009). Such heterogeneity and changes in constituents may

362   produce local weak points with a low density of collagen fibers in aneurysmal tissues. Stress

363   concentration occurs at these points, and the crack may initiate from these points. This

364   might be the reason for the rupture of smaller aneurysms (Cambria et al. 1995; Coady et al.

365   1997).

366   Although the retardance and intensity in SHG images were significantly lower at the
367   crack initiation sites than at other sites in this study, the values at these sites were not always
368   the lowest. Furthermore, holes were sometimes observed in specimens, and cracks were
369   initiated in other regions. Although we had anticipated that cracks would initiate from sites
370   with the lowest retardance, not all cracks initiated from that regions. These results indicate
371   that other factors must be involved in rupture, and such factors should be elucidated to
372   precisely determine the rupture risk. Since the aortic media comprise both types I and III
373   collagen and a decrease in type I and an increase in type III collagen correlate significantly
374   with a decrease in the elastic modulus of tendons (Wan et al. 2014), the density
375   heterogeneity of collagen subtypes may affect the local strength of the aorta. The
376   connecting force between fibers is another possible candidate that may affect the local
377   strength.

378   One might think that other possible reason influencing on the position of the crack
379   initiation sites can be a local thickness. The local thickness of the specimen was evaluated
380   from intensity of a bright field image of the specimen after confirming in a preliminary
381   study that the intensity correlated significantly with local thickness of specimen measured
382   directly with a laser microscope. The cracks initiated from the thinner area in only 2 out of 6
383   specimens, indicating that local thickness was not a determinant factor of the crack
384   initiation sites in this study.

385   Basically, cracks run in the same direction as collagen fibers. In the thoracic aortas, two
386   distinct collagen fiber families were observed, and their azimuthal mean angles in the
387   circumferential-axial plane were reported to be $-27.19°$ and $27.75°$ from the circumferential
388   direction (Schriefl et al. 2011). This may be the reason why the probability of a large local
389   crack angle was high at approximately $\pm30°$ from the circumferential direction in this study
390   (Fig. 5d). After crack initiation, most cracks propagated and changed direction to the
391   circumferential direction (Fig. 5e), resulting in crack shapes that were aligned in the
392   circumferential direction (Fig. S4a). This result was in good agreement with a previous
393   study in which all cracks ran in the circumferential direction in pressure-imposed tests for
394   PTA specimens (Sugita et al. 2011).

We previously reported that the first principal strain was inversely correlated with retardance (Sugita and Matsumoto 2013a). This is likely because cracks run from high strain regions because the cracks were initiated at low retardance regions in this study. However, this study showed that the first principal strain was not always higher at the crack initiation sites than at the other sites (Fig. 1e) although areal strains tended to be high (Fig. 1h). Since the correlation coefficient was relatively low (−0.077) in a previous study (Sugita and Matsumoto 2013a), collagen distribution may play a more important role than strains in vessel wall strength. In bulge test of ascending aortic thoracic aneurysms, rupture occurred at one of strain concentration area (Davis et al. 2016). Since thickness of the specimen in the Davis et al. might not be uniform, thin specimen area tends to become high strain area, which may result in rupture. In this study, since specimen with relatively uniform thickness was prepared by cutting specimen, collagen distribution might play a more dominant role than the strain on the wall strength. Heterogeneous distribution in mechanical properties of collagen fibers might cause crack initiation in the area where strain was not so high.

To evaluate the size of the crack initiation sites, analysis using other sizes for crack initiation sites was also performed. When the size of the crack initiation site was changed from 250×250 μm^2 to 350×350 and 150×150 μm^2, retardance at the crack initiation sites was still significantly lower than that at other sites (Fig. S5a and b). Thus, the size of the crack initiation sites did not have much impact on the result in this study.

There were some limitations in this study. First, tissues were frozen to shape the specimens. This may have caused changes in the mechanical properties of the specimens. However, as discussed in our previous paper (Sugita and Matsumoto 2013b), freezing has been reported to affect the mechanical behavior of a small strain region either significantly (Venkatasubramanian et al. 2006) or insignificantly (Masson et al. 2009; Stemper et al. 2007), and the effect of freezing on the collagen fibers appears to be low (Goh et al. 2010). Thus, we believe that the influence of freezing on the results of this study was negligible. Second, specimens were equi-biaxially stretched because we plan to apply this analysis method to aneurysmal tissue, which is thought to be stretched biaxially in vivo, in a future study. However, healthy PTA, which is not biaxially stretched in vivo, was used in this

424   study because the aortas have a hollow cylindrical shape. Third, for modification of the
425   shape of the specimens, which enabled us to observe the crack initiation sites during biaxial
426   stretching and to observe SHG light from the specimens, the specimens were sliced into
427   50-μm sections, which may have affected the collagen structure around the surface area.
428   However, thick specimens, which were sliced into 150-μm sections, showed a similar result
429   in that retardance was significantly lower at the crack initiation sites than at the other sites
430   (Fig. S6), indicating that the effect of slicing the specimen would have been very small.
431

432   **5. Conclusions**

433   Constituent and mechanical characteristics of crack initiation sites were studied in
434   healthy PTAs subjected to equi-biaxial stretching. Although there were insignificant
435   differences between the sites with regard to local strains and elastin distribution, the
436   retardance and intensity of SHG images were significantly lower at the crack initiation sites,
437   indicating that low local collagen density increases the risk of rupture. Furthermore, cracks
438   tended to propagate along the direction of the collagen fibers. These results indicate that the
439   local density and direction of collagen fibers play an important role in aorta rupture.
440

441   **Conflicts of interest statement**

442   There are no conflicts of interest.

443

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530   medial collateral ligaments PLoS ONE 9:e103363

531   doi:10.1371/journal.pone.0103363

532

533 **Figures Captions**

534

535 **Fig. 1** Characteristics of local strains at crack initiation sites just before rupture. (a–d)

536 Typical distributions of (a) the first principal, (b) the second principal, (c) the maximum

537 shear, (d) and areal strains in specimen #1. Red boxes in images show the crack initiation

538 sites. Bar in (d) = 1 mm applies to all images. (e–h) Comparison of (e) the first principal, (f)

539 the second principal, (g) the maximum shear, (h) and areal strains between the crack

540 initiation site and other sites for each specimen. Linear and broken lines show data for $p <$

541 0.05 and NS, respectively, for comparison between the crack initiation site and other sites

542 within a single specimen. Each mark represents the average within a single specimen

543

544 **Fig. 2** Characteristics of local amount of elastin at crack initiation sites just before rupture.

545 (a) Typical fluorescent image of elastin in specimen #2. A white box shows the crack

546 initiation sites. Bar = 1 mm. (b) Comparison of mean intensities of elastin I_{Ela} between the

547 crack initiation site and other sites in each specimen. Linear and broken lines show data for

548 $p < 0.05$ and NS, respectively, for comparison between the crack initiation site and other

549 sites within a single specimen. Each mark represents the average within a single specimen

550

551 **Fig. 3** Characteristics of local retardance *Ret* and slow axis azimuth *Azi* at crack initiation

552 sites just before rupture. (a and b) Typical (a) retardance and (b) slow axis azimuth

553 distribution in specimen #1. Boxes in images show the crack initiation sites. Bar in (a) = 1

554 mm applies to both images. (c and d) Comparison of (c) mean retardance and (d) slow axis

555 azimuth from the circumferential direction between the crack initiation site and other sites

556 in each specimen. Linear and broken lines show data for $p < 0.05$ and NS, respectively, for

557 comparison between the crack initiation sites and the other sites within a single specimen.

558 Each mark represents the average within a single specimen

559

560 **Fig. 4** Characteristics of collagen fibers in second harmonic generation (SHG) images

561 obtained from crack initiation sites. (a and b) Typical SHG images captured (a) before and

562 (b) after biaxial stretch testing in no load state for specimen #12. Bar in (a) corresponds to

563 250 μm and applies to both images. White arrowheads in (b) show cracks. The center of the

564 red boxes shows the crack initiation point. Color arrows show the same position. (c)

565 Comparison of intensity of SHG images (I_{SHG}) between the crack initiation site and other

566 sites in each specimen. (d) Comparison of standard deviation of the probability distribution

567 of collagen fibers σ between the crack initiation site and other sites in each specimen. Linear

568 and broken lines show data for $p < 0.05$ and NS, respectively, for comparison between the

569 crack initiation site and other sites within a single specimen. Each mark represents the

570 average within a single specimen

571

572 **Fig. 5** Crack direction at crack propagation sites. (a–c) Typical SHG images at crack

573 propagation sites and the probability distributions of collagen fiber orientation along with

574 crack direction. Most cracks propagated in (a) the circumferential direction (0° and ±180°)

575 and (b) approximately 45° from the circumferential and longitudinal directions and the

576 direction of the cracks corresponded to those of the collagen fibers with high probability

577 although (c) some cracks deviated from this trend. The bar in (c) = 50 μm applies to all

578 images. (d) Histogram of crack angle from the circumferential direction obtained at the

579 crack propagation sites. (e) Angle of a crack ~300 μm in length from the circumferential

580 direction at the crack initiation and end sites.

581

582   **Supplementary Figures Captions**

583

584   **Fig. S1** Change in retardance at the intersection of two cellophane tapes placed on a glass

585   slide at various angles. (a) Illustration of two strips of cellophane tape placed on a glass

586   slide at angle α. (b) Retardance images of one and two strips of cellophane tape placed in

587   parallel ($\alpha = 0°$). (c) Retardance images of two strips of cellophane tape placed at various

588   angles. (d) The relationship between angle α and retardance

589

590   **Fig. S2** Effect of the alignment consistency of collagen fibers on the intensity of SHG

591   images. (a) A typical image of collagen fibers. (b) Enlarged view of boxed area indicated

592   with an arrow in (a). (c) Relationship between intensity at the intersection point of two

593   collagen fibers $I_{intersection}$ and the sum of the intensities of the two fibers $I_1 + I_2$. The triangle

594   plot shows the average intensity of a single fiber, fibers 1 I_1 and 2 I_2. The broken line

595   indicates the data when the value in the horizontal axis is the same as that in the vertical

596   axis. (d) Effect of angle β between two collagen fibers on the intensity ratio *Ir* at the

597   intersection point to the sum of the two fibers

598

599   **Fig. S3** Effect of changes in retardance *ΔRet* between the period just before rupture and the

600   previous loading step on the crack initiation sites. (a) A typical image of changes in

601   retardance *ΔRet*. A black box in the image shows the crack initiation sites. Bar = 1 mm. (b)

602   Changes in retardance *ΔRet* at the crack initiation site and at other sites in each specimen
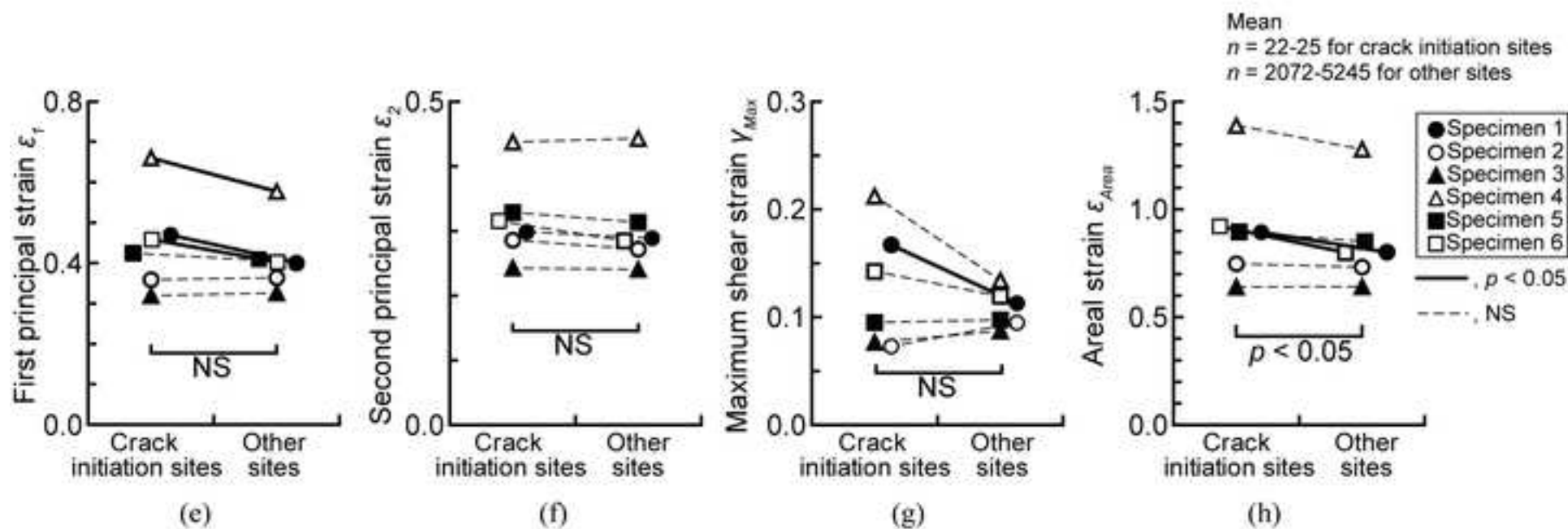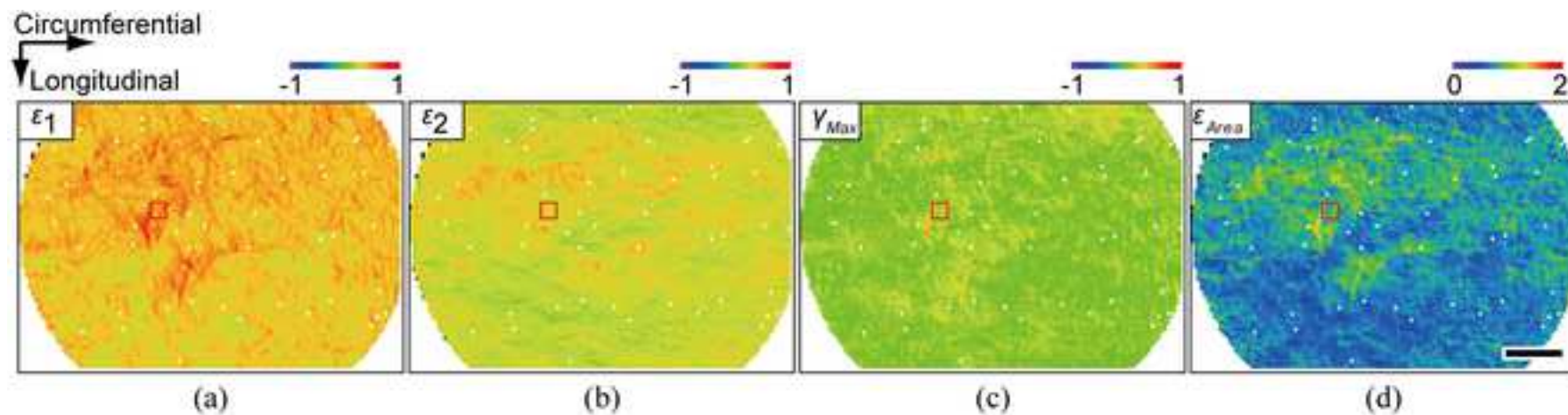
603

604   **Fig. S4** Typical images of a crack captured at (a) the macroscopic scale and (b) the

605   microscopic scale. The circle in (a), which shows the circle at the metal frame of the biaxial

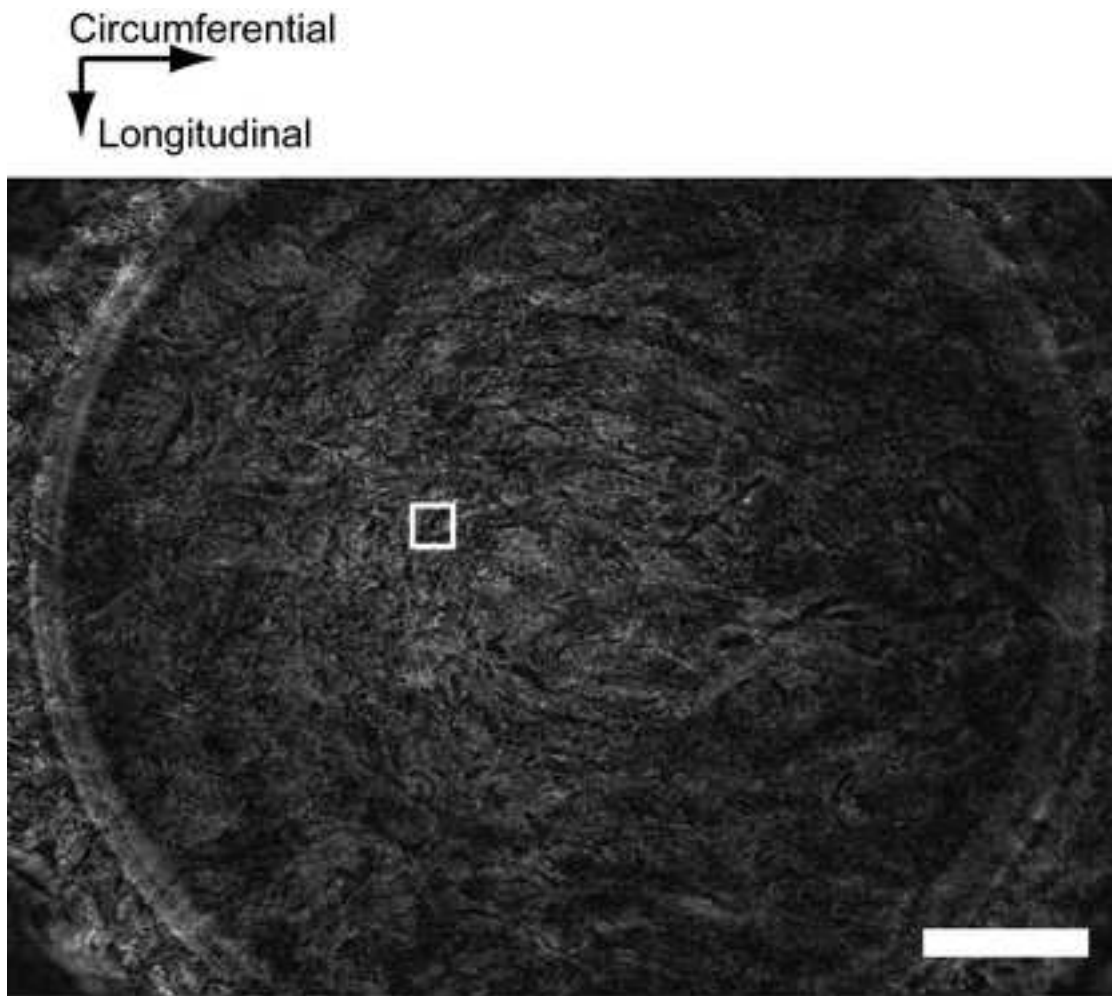606   stretch tester, corresponds to 10 mm and the bar in (b) = 350 μm

607

608   **Fig. S5** Effect of the analysis size used for the crack initiation sites on retardance between

609   the crack initiation sites and other sites. The crack initiation sites were changed to (a) 7×7

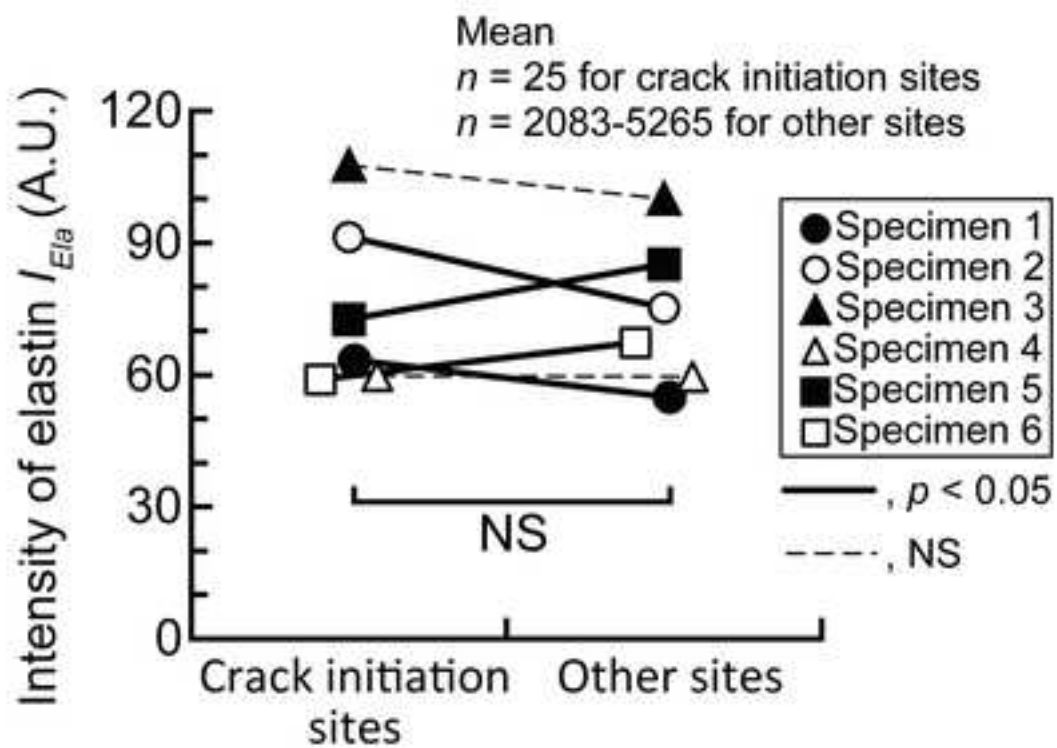610   (350×350 μm^2) and (b) 3×3 (150×150 μm^2) areas

611

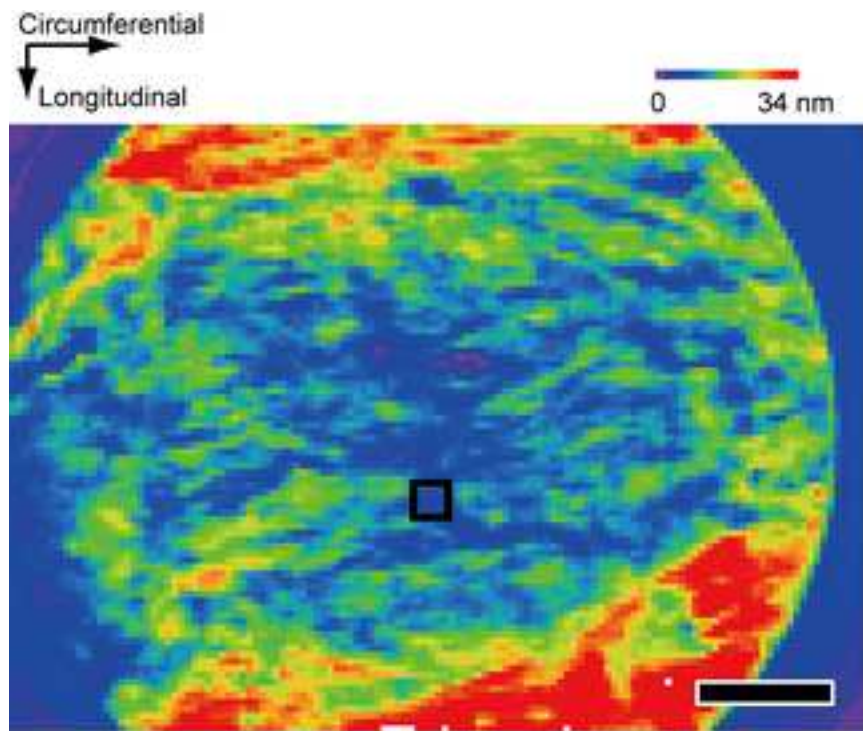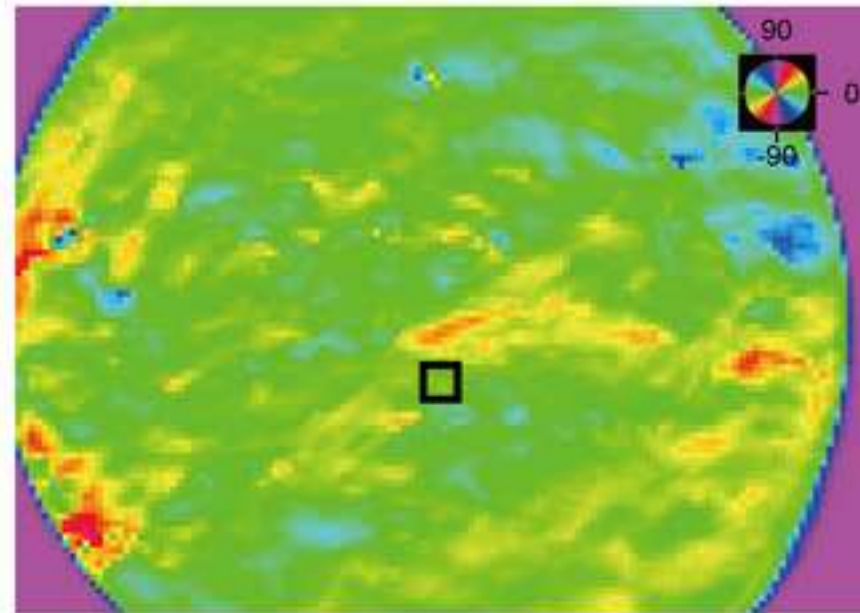612   **Fig. S6** Retardance at the crack initiation site and other sites for thick (150 μm) specimens

Figure 1

Click here to download Figure Fig1_v8.tif
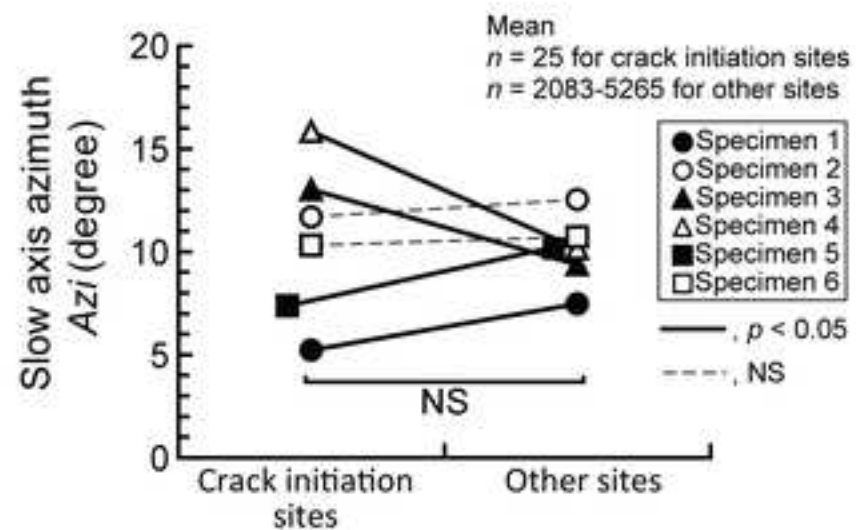

(a) (b) (c) (d)

(e) (f) (g) (h)

Figure 2

Circumferential

Longitudinal

(a)

Mean
$n = 25$ for crack initiation sites
$n = 2083\text{-}5265$ for other sites

Intensity of elastin I_{Ela} (A.U.)

- ● Specimen 1
- ○ Specimen 2
- ▲ Specimen 3
- △ Specimen 4
- ■ Specimen 5
- □ Specimen 6
- —— , $p < 0.05$
- ---- , NS

NS

Crack initiation sites   Other sites

(b)

Figure 3

Circumferential
Longitudinal
0   34 nm
90
0
-90

(a)   (b)

Retardance *Ret* (nm)

p < 0.05

Crack initiation sites   Other sites

(c)

Slow axis azimuth *Azi* (degree)

Mean
n = 25 for crack initiation sites
n = 2083-5265 for other sites

● Specimen 1
○ Specimen 2
▲ Specimen 3
△ Specimen 4
■ Specimen 5
□ Specimen 6

——— , *p* < 0.05
------ , NS

NS

Crack initiation sites   Other sites

(d)

Figure 4

Click here to download Figure Fig4_v9.tif ±

(a)

(b)

(c)

(d)

Figure 5   Click here to download Figure Fig5_v6.tif ⬇

Figure 5

1   **Supplementary Materials (Online Resource)**

2

3   **Title: Local distribution of collagen fibers determines crack initiation site and its**

4   **propagation direction during aortic rupture**

5

6   **Biomechanics and Modeling in Mechanobiology**

7

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1 **S1. Effect of alignment consistency of birefringent material on retardance**

2   Retardance is used as an index of the local density of collagen fibers in the aorta (Sugita and

3 Matsumoto 2013). However, it is well known that retardance is also affected by the alignment

4 consistency of birefringent materials, suggesting that retardance decreases when birefringent

5 collagen fibers are not aligned in the same direction. Since we already showed that retardance

6 is proportional to the volume of collagen fibers (Sugita and Matsumoto 2013), we attempted

7 to confirm the effect of the alignment consistency of birefringent materials on retardance using

8 cellophane tape, which is known to be a birefringent material because it is stretched in one

9 direction during the manufacturing process.

10   The alignment consistency of birefringent material was evaluated based on the retardance

11 at the intersection point of two strips of cellophane tape. The two strips of tape were placed on

12 a glass slide with various angles α (Fig. S1a), and their resultant retardance was measured with

13 a birefringent imaging system (Abrio-LS, CRi, Woburn, MA, USA).

14   When the two strips of tape were placed in parallel, retardance in the area covered by the

15 two strips was twice that in the area covered by one strip (Fig. S1b). As the angle between the

16 two strips of tape increased, the retardance in the area covered by the two strips decreased (Fig.

17 S1c and d). When the angle $\alpha = 90°$, the resultant retardance was almost 0 (Fig. S1d). This

18 indicates that retardance is affected by the alignment consistency of birefringent materials.

19 Thus, when the alignment consistency of collagen fibers in the aorta is low, the retardance is

20 not adequate as an index of collagen density.
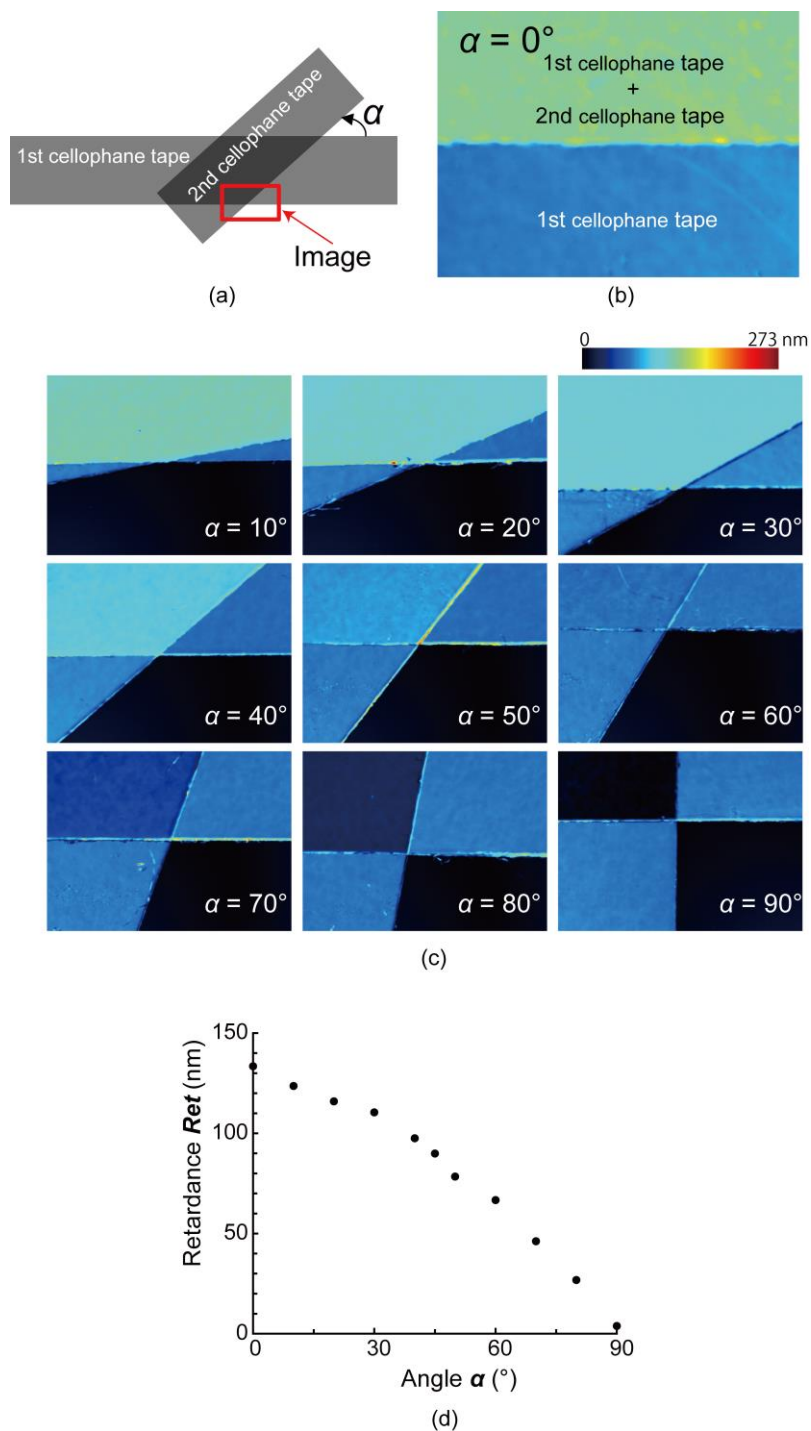
Fig. S1 Change in retardance at the intersection of two cellophane tapes placed on a glass slide at various angles. (a) Illustration of two strips of cellophane tape placed on a glass slide at angle α. (b) Retardance images of one and two strips of cellophane tape placed in parallel ($\alpha = 0°$). (c) Retardance images of two strips of cellophane tape placed at various angles. (d) The relationship between angle α and retardance

S2. Effect of alignment consistency of collagen fibers on SHG intensity

To evaluate collagen density with no effect from the alignment consistency of collagen fibers, second harmonic generation (SHG) signals were employed in this study. To evaluate the effect of the alignment consistency of collagen fibers on the SHG signal, the intensity at the intersection point of two collagen fibers was investigated.

Collagen fibers obtained from mouse tail tendons were mounted on a glass slide and observed under the SHG microscope (FV1200MPE, Olympus) as stated in the *Second harmonic generation (SHG) light observation* subsection in the Materials and methods section. Animal experiments were approved by the institutional review board for animal care at the Nagoya Institute of Technology. Image analysis was performed with image analysis software (ImageJ, National Institutes of Health). In the obtained SHG image (Fig. S2a), areas showing the intersection of two collagen fibers in a confocal plane were selected (Fig. S2b). Intensity at the intersection point of two collagen fibers $I_{intersection}$ and the average intensity along each collagen fiber I_1, I_2 were measured, and the results were compensated by subtracting the background intensity around the fibers. The sum of the average intensities of the two collagen fibers $I_1 + I_2$ was then compared with the intensity at the intersection point $I_{intersection}$, as the intensity ratio Ir, to exclude the influence of the width of the collagen fibers as follows:

$$Ir = \frac{I_1 + I_2}{I_{int\,er\sec tion}}.$$
(S1)

The ratio Ir was then plotted against the angle β to evaluate the effect of the angle β between two fibers on SHG intensity.

Figure S2c shows the relationship between intensity at the intersection point of two collagen fibers and the sum of the intensities of the two fibers (fiber 1 + fiber 2). Intensity at the intersection point was almost the same as the sum of the intensities of two fibers. This indicates that SHG intensities can be used to quantify the density of collagen fibers. Figure S2d shows the relationship between the angle β of two collagen fibers and the intensity ratio Ir at the intersection point to the sum of the two fibers. The correlation was insignificant ($p = 0.30$), and the intensity ratio Ir was not significantly affected by angle β, indicating that the alignment consistency of the collagen fibers does not affect the intensity of the SHG image of collagen

1   fibers.

(a)   (b)

(c)   (d)
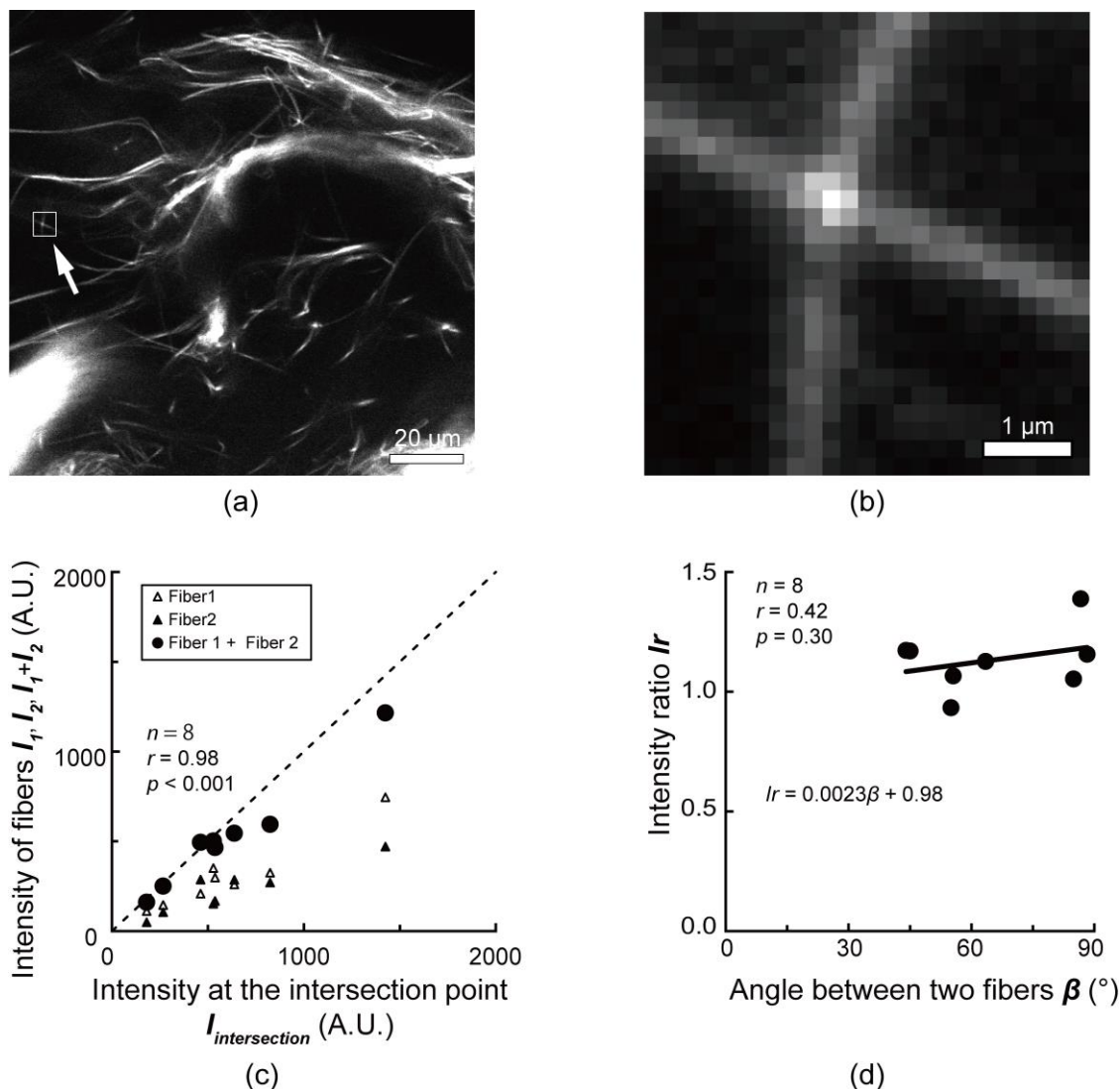
Fig. S2 Effect of the alignment consistency of collagen fibers on the intensity of SHG images.
(a) A typical image of collagen fibers. (b) Enlarged view of boxed area indicated with an arrow
in (a). (c) Relationship between intensity at the intersection point of two collagen fibers
$I_{intersection}$ and the sum of the intensities of the two fibers $I_1 + I_2$. The triangle plot shows the
average intensity of a single fiber, fibers 1 I_1 and 2 I_2. The broken line indicates the data when
the value in the horizontal axis is the same as that in the vertical axis. (d) Effect of angle β
between two collagen fibers on the intensity ratio Ir at the intersection point to the sum of the
two fibers

S3. Characteristics of changes in retardance between the period just before rupture and the previous loading step at the crack initiation sites

   The effect of changes in retardance on the crack initiation sites was studied because the network of collagen fibers at the crack initiation sites might change just before rupture. For this purpose, we calculated the changes in retardance ΔRet by subtracting the retardance just before rupture from the retardance at the previous loading step at each local area.

   Figure S3a shows a typical image of changes in retardance ΔRet. Although the changes in retardance ΔRet seem to be heterogeneously distributed, statistical analysis shows no clear tendency; changes in retardance ΔRet were significantly higher at the crack initiation site in some specimens (#1, #5, and #6) and lower in other specimens (#3 and #4; Fig. S3b). These results indicate that the changes in retardance ΔRet are not related to the crack initiation.
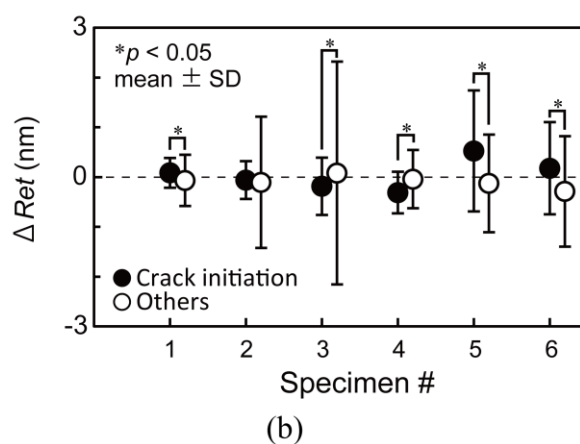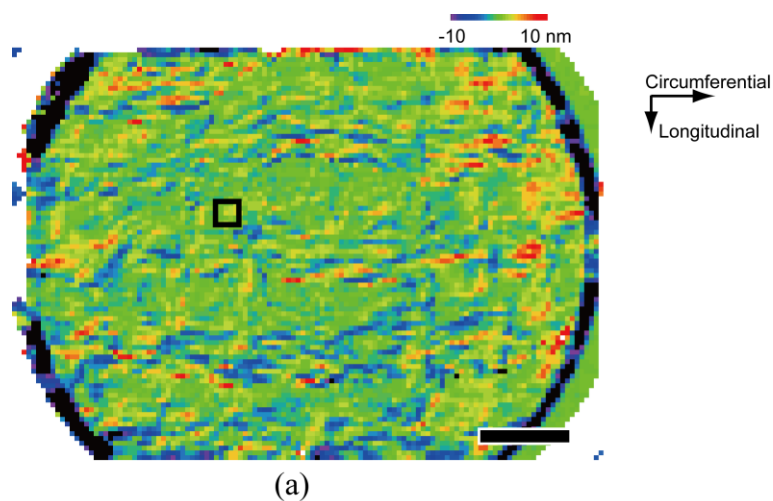

(a)

(b)

Fig. S3 Effect of changes in retardance ΔRet between the period just before rupture and the previous loading step on the crack initiation sites. (a) A typical image of changes in retardance

1   *ΔRet*. A black box in the image shows the crack initiation sites. Bar = 1 mm. (b) Changes in

2   retardance *ΔRet* at the crack initiation site and at other sites in each specimen

1 **S4. Crack direction observed at the microscopic and macroscopic scales**

2   Figure S4a and b show typical specimen images captured with a digital camera and SHG

3 microscope, respectively, after the biaxial tensile test. Although the crack around the crack

4 initiation sites appears to run in the longitudinal direction, cracks far from the crack initiation

5 sites run in the circumferential direction in the macroscopic image. Microscopically, this crack

6 runs in a zigzag direction around the crack initiation site as the crack seems to run along the

7 collagen fibers (Fig. S4b).

8

(a)   (b)
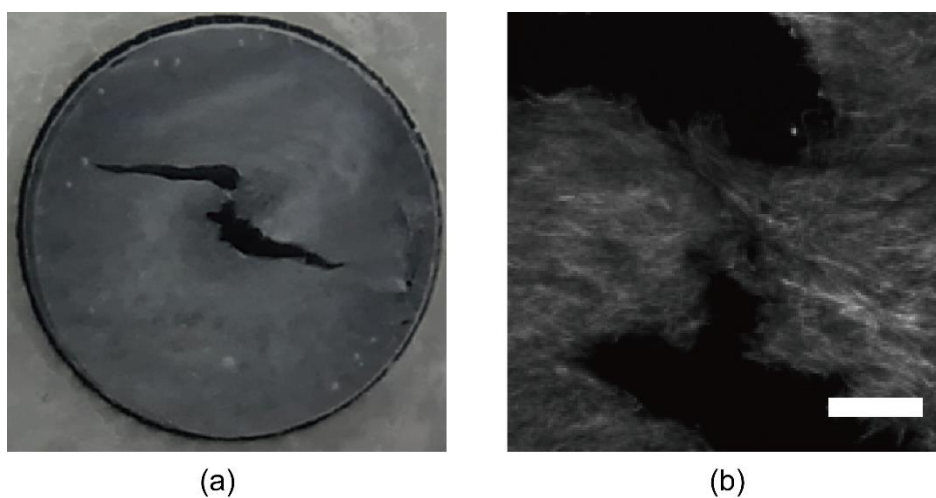
10 **Fig. S4** Typical images of a crack captured at (a) the macroscopic scale and (b) the microscopic

11 scale. The circle in (a), which shows the circle at the metal frame of the biaxial stretch tester,

12 corresponds to 10 mm and the bar in (b) = 350 μm

1 **S5. Evaluation of the analysis size of the crack initiation sites**

2   The crack initiation sites were identified as 5×5 areas of 10×10 pixels (50×50 μm^2), that

3 is, 250×250 μm^2. To evaluate the analysis size used for the crack initiation sites, the size was

4 increased to 7×7 (350×350 μm^2) and decreased to 3×3 (150×150 μm^2).

5   Fig. S5a and b show the comparison between the retardance at 7×7 and 3×3 regions for

6 the crack initiation sites, respectively, and retardance at other sites within a single specimen.

7 Even though the size of the crack initiation sites was changed, the retardance at the crack

8 initiation sites remained significantly lower than the values at other sites for all specimens.

9

10

(a) 7×7 (b) 3×3
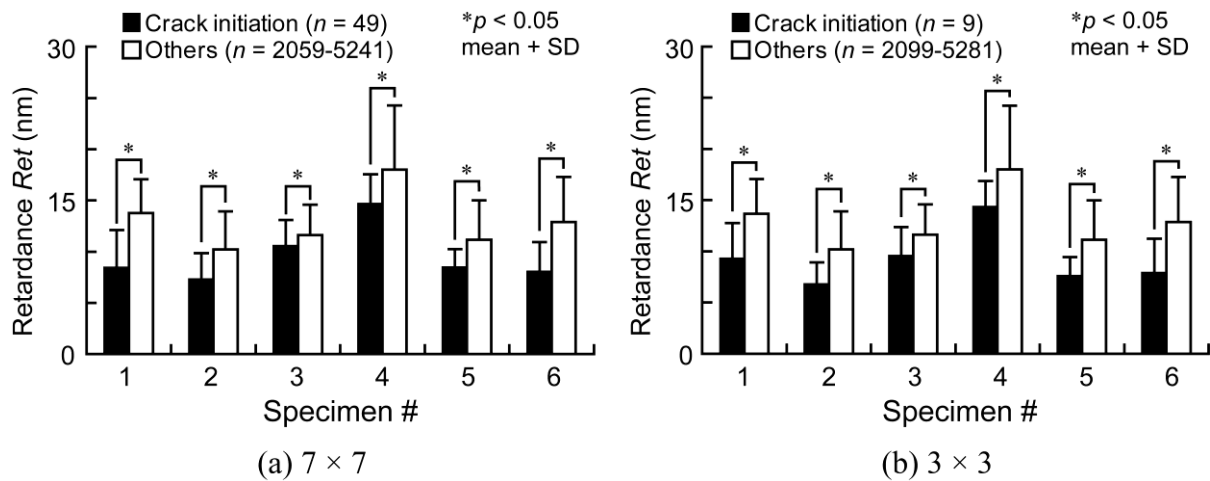
11 **Fig. S5** Effect of the analysis size used for the crack initiation sites on retardance between the

12 crack initiation sites and other sites. The crack initiation sites were changed to (a) 7×7 ($350 \times$

13 350 μm^2) and (b) 3×3 (150×150 μm^2) areas

1   **S6. The effect of specimen slice thickness**

2   To modify the shape of the specimen that enabled us to observe the crack initiation sites

3   during biaxial stretching and to observe SHG light from the specimens, specimens were sliced

4   into 50-µm sections, which might affect collagen structure around the surface area. To evaluate

5   the slice thickness of the specimens, we performed a biaxial stretch test for thick specimens,

6   which were sliced into 150-µm sections, and obtained the retardance distribution ($n = 3$).

7   Figure S6 shows the retardance at both the crack initiation site and other sites in thick

8   specimens. Retardance was significantly lower at the crack initiation sites than at other sites

9   within the same specimen for all three specimens (Fig. S6). This indicates that the effect of

10   slicing the specimen would have been very small.

11
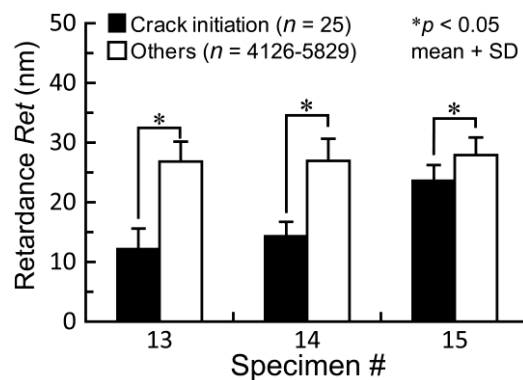
12

13   **Fig. S6** Retardance at the crack initiation site and other sites for thick (150 µm) specimens

1   **Reference**

2   Sugita S, Matsumoto T (2013) Quantitative measurement of the distribution and alignment of

3   collagen fibers in unfixed aortic tissues. J Biomech 46:1403-1407

4   doi:10.1016/j.jbiomech.2013.02.003