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Brillouin microscopy for nondestructive mechanical imaging of dentin

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ABSTRACT

The physical structure of teeth can be altered by diet, age or diseases such as caries or sclerosis. It is of utmost importance to characterize the mechanical properties to predict and understand tooth decay, to design restorative dental procedure, and to investigate the tribological behavior of teeth. Yet, existing imaging techniques are unable to reveal the micromechanics of the tooth, in particular at tissue interfaces. Here we developed a microscope based on Brillouin light scattering (BLS) to probe mechanical changes in tooth tissues. BLS is an inelastic process that uses the scattering of light by acoustic waves in the GHz range. Our microscope thus reveals the mechanical properties at a sub-micrometer scale without contact to the sample. BLS signals show significant differences between healthy tissues and pathological lesions, and allow delineating precisely destructed dentin. We also show maps of the sagittal and transversal planes of healthy tubular dentin that reveal its anisotropic microstructure with a 1 μm resolution, several orders of magnitude below previous reports. Our observations indicate that the collagen-based matrix of dentine is the main load bearing structure, which can be thought of as a fiber-reinforced composite. In the vicinity of polymeric tooth-filling materials, we observed fingering of the adhesive complex into the opened tubules of healthy dentine. The ability to probe the quality of this interfacial layer could lead to innovative designs of biomaterials used for dental restorations in contemporary adhesive dentistry, and would have direct repercussions on the decision-making during clinical work.

INTRODUCTION

Human dentin is an organized, hard, mineralized tissue of the tooth, composed of 70 wt% calcified tissue (hydroxyapatite), 20 wt% organic phase (mostly composed of collagen type I as well as other fibrils), and 10 wt% water [1]. It is perfused with microtubules that allow sensory communication to the underlying pulp and nerves.[2] This fiber-composite-like structure yields an anisotropic distribution of mechanical properties that support masticatory stress concentrated in the surrounding enamel.[3] Tooth mechanical strength can be altered by diet, age or diseases such as caries or sclerosis.[44] It is of utmost importance to characterize mechanical properties during tooth life in order to predict and understand tooth decay, to design restorative dental procedure, and to investigate the tribological behavior of teeth.

44 Most of the characterization means are inherited from material science, and involve destructive testing
45 such as indentation or fatigue tests. [3, 17] Due to the variety of techniques and the complexity of the
46 dentin microstructure, there is a lack of quantified consensus on the mechanical properties of
47 dentin.[38] Moreover, such approaches are clearly difficult to put into practice *in vivo*. In the clinic,
48 dentinal pathological changes can be detected by visuo-tactile and radiographic methods to guide
49 clinicians in establishing a relevant diagnostic, and in proposing an adequate therapy.[4] However,
50 such approaches cannot give precise information on the microstructure, cannot delineate precisely
51 caries margins, [5] and are highly depend on the clinician [6].

52 Acoustic techniques have long been used to probe the mechanics of dentin, initially analyzing the
53 reflection or transmission of ultrasonic pulses emitted from a piezoelectric transducer in the MHz
54 range. [39] Later implementations in tooth tissues in various healthy and pathological states used
55 resonant ultrasonic spectroscopy, [38] acoustic microscopy,[7, 8, 9] surface waves [10] or laser
56 ultrasonics. [11], [12] However, these techniques are limited to the MHz range, i.e. a $\sim 100 \mu\text{m}$
57 resolution, unable to reveal the micromechanics of the tooth, in particular at tissue interfaces.

58 State-of-the-art microscopies, such as multiphotonic or non-linear technologies, [41] can offer optical
59 resolution but cannot provide any information on the mechanics of the sample. Laser spectroscopies
60 have recently rose to prominence, offering quantitative imaging of the chemical structure of tooth
61 tissues and restorative materials. [13, 14, 15, 16] In this paper we propose a new approach to probe
62 acoustic waves in the GHz range by laser spectroscopy, thus offering quantitative microscopy with a
63 contrast based on the mechanical properties of tooth tissues.

64 We developed a microscope based on Brillouin light scattering (BLS) to probe mechanical changes in
65 tooth tissues. BLS is an inelastic process similar to Raman scattering. Raman-based techniques rely
66 on the scattering of light by optical phonons, and operate at THz frequencies. They provide information
67 on the molecular structure of the sample. By contrast, Brillouin scattering uses the scattering of light
68 by acoustic phonons.[18] Since acoustic phonons have lower energies than optical phonons, Brillouin
69 scattering produces frequency shifts in the GHz range that reveal the mechanical properties of the
70 sample. Developed in the 60's for solid-state physics, BLS has only recently emerged as a key
71 technology in life sciences due to its ability to produce non-contact, label-free microscopic images of
72 the mechanical properties of cells and tissues. [19] It has notably been applied for the analysis of
73 biological fibers, connective tissues and muscles [17, 20, 21], eye tissues [22, 23], bone [24, 26] or
74 tumors.[35]

75 We prepared thick slices of teeth extracted in accordance with the ethical requirements for ex-vivo
76 studies. We obtained maps of Brillouin frequency shift and linewidth that can be interpreted as maps
77 of sound velocity and viscosity. Our results show significant changes between healthy tissues and
78 pathological lesions. Such results allow delineating precisely destructed dentin, paving the way to
79 minimally invasive strategies. We also show maps of the sagittal and transversal planes of healthy
80 tubular dentin that reveal its anisotropic microstructure with a $1 \mu\text{m}$ resolution, several orders of
81 magnitude below previous reports.[38] Our observations indicate that the collagen-based matrix of
82 dentine is the main load bearing structure, which can thought of as fiber-reinforced composite. In the
83 vicinity of polymeric tooth-filling materials, we observed fingering of the adhesive complex into the
84 opened tubules of healthy dentine. We confirmed our observations with two-photon excitation
85 fluorescence (2PEF) and second harmonic generation (SHG) microscopies that reveal teeth

86 microstructure due to two-photon excited autofluorescence and collagen-specific second order
87 nonlinear optical susceptibility [27]. The ability to probe the quality of this interfacial layer could lead
88 to innovative designs of biomaterials used for dental restorations in contemporary adhesive dentistry,
89 and would have direct repercussions on the decision-making during clinical work.

90 MATERIALS AND METHODS.

91

92 *Sample preparation and staining.*

93

94 Teeth were collected at Faculty of Medicine, School of Dentistry, at the Department of Oral Surgery
95 of Dental Clinic of Vojvodina, Serbia. They were extracted for medical reasons and, after the patient's
96 consent, approved to be used for research purposes according to the protocol of Ethical board of Dental
97 Clinic of Vojvodina, Serbia. All types of permanent teeth were analyzed, including incisors, canines,
98 premolars and molars. The teeth were classified in the following groups: healthy teeth with intact hard
99 tissues (two subgroups – teeth cut perpendicularly to their longitudinal axis, teeth cut longitudinally),
100 teeth with caries lesions in dentin [score 5 on the International Caries Detection and Assessment
101 System (ICDAS) scale; distinct cavity with visible dentin], restored teeth with composite resin.

102 Teeth restored with composite used for the imaging of the dentin-adhesive interface were prepared
103 according to the standard dental adhesive placement procedure, after the etching procedure by using
104 37% orto-phosphoric acid for 15 seconds, rinsing it, and placing a single bond universal adhesive (3M
105 Deutschland GmbH, LOT 663414), before restoration with the Filtek Ultimate Body composite. For
106 2PEF imaging of dentin-adhesive interface, a water solution containing 0.5% eosin-Y was added in
107 small quantities into the adhesive in order to distinguish the adhesive from the surrounding dentinal
108 tissue.

109 Teeth were sectioned into ~0.5 mm thick slices along the horizontal and longitudinal axes, by a water-
110 cooled, low speed diamond saw using a hard tissues microtome. Samples were wet-polished with
111 abrasive sandpaper sheets of silicone-carbide of increasing grit numbers (400-2000). This was
112 followed by polishing with a diamond paste on a soft fabric, in order to eliminate the surface
113 corrugations. The smear layer produced by polishing was removed by 10% polyacrylic acid used for
114 5 seconds, after which the samples were rinsed, slightly dry-aired and disinfected in 0,5% chloramine
115 solution and preserved in plastic bags until examined.

116 *Nonlinear laser scanning microscopy*

117 Teeth specimens were observed by a nonlinear optical microscopy setup at the Institute of Physics in
118 Belgrade. The homemade nonlinear laser-scanning microscope used in this study has been described
119 in detail elsewhere.[42, 43] Briefly, a Ti:Sapphire femtosecond laser (Coherent, Mira 900-F) tunable
120 in 700-1000nm range, and a Yb:KGW (Time-Bandwidth Products AG, Yb GLX) femtosecond laser
121 at 1040nm were used as a laser light sources. The samples were imaged using Carl Zeiss, EC Plan-
122 NEOFLUAR, 40×1.3 oil immersion objective or Plan-APOCHROMAT 20x/0.8 air objective for laser
123 focusing and collection of the signal. A visible interference filter (415 nm–685 nm) positioned in front
124 of the detector is used to remove scattered laser light in fluorescence images. Thus, the whole visible
125 range has been detected in two-photon fluorescence images. The tooth auto-fluorescence was excited
126 by the Ti:Sapphire laser at 730nm. The Eosin-Y fluorescence was excited by the Ti:Sapphire laser at

127 820nm. Narrowband interference filters (10 nm FWHM) were used for second harmonic generation
128 imaging. The SHG filter central wavelength was 520nm (FB520-10, Thorlabs) for the Yb:KGW laser.
129 The SHG filter central wavelength was 420nm (FB420-10, Thorlabs) for Ti:Sapphire laser when tuned
130 at 840nm. The SHG excitation wavelength and filter were selected so that fluorescence leakage to
131 SHG channel is minimal.

132 *Brillouin spectroscopy*

133 BLS uses the scattering of light by phonons. Thermal phonons (incoherent vibrations) produce a broad
134 peak at low frequencies due to Rayleigh scattering, while acoustic phonons (coherent vibration) with
135 wavenumbers that fulfill the Bragg condition produce well-defined peaks (**Figure 1**). Using a
136 monochromatic light beam of wavelength λ in a normal incidence backscattering geometry, forward
137 propagating phonons produce a positive shift at a frequency $f = 2nV/\lambda$ (anti-Stokes peak), where
138 V is the sound velocity and n is the refractive index, typically in the GHz range (backward propagating
139 phonons creates a negative Stokes peak at $-f$).

140 Assuming that the sample is homogeneous over the scattering volume, the BLS spectra can be
141 interpreted as the response of a damped harmonic oscillator with frequency position f and full width
142 at half maximum Γ . In this approximation, the storage modulus is $' = \rho(f\lambda/2n)^2$, where ρ is the mass
143 density. The linewidth Γ is ascribed to the loss modulus $M'' = \rho\Gamma f(\lambda/2n)^2$. In other words, the
144 frequency shift and linewidth can be interpreted as the sound velocity (or stiffness) and viscosity of
145 the sample. Note that at the high magnifications we use, Γ can artificially increase due to scattering.

146 *Brillouin microscope*

147 The Brillouin spectrometer is based on a tandem Fabry-Pérot interferometer (JRS Scientific
148 Instruments). [35] In order to reduce phototoxicity, we used a $\lambda = 647.1$ nm continuous-wave laser as
149 a light source (instead of classical designs based on a frequency-doubled YAG laser at 532~nm) which
150 gives a $\frac{\lambda}{2n} \approx 200$ nm acoustic resolution, where $n = 1.6$ is the typical refractive index of dentin. The
151 spectrometer offers a 40 GHz free spectral range sampled with a 30~MHz sampling rate. Appropriate
152 signal analysis allows reaching a 6 MHz accuracy at a -10 dB noise level. It is equipped with two sets
153 of mirrors with a 95% reflectivity, and an avalanche photodiode (Count[®] Blue, Laser Components)
154 with a dark count lower than 10 counts/sec (typically 2counts/sec) and a quantum efficiency of 65%
155 at this wavelength. The spectrometer is coupled with an inverted life science microscope (Eclipse Ti-
156 U, Nikon) equipped with a micro-positioning stage (Mad City Labs). The collimated linearly-polarized
157 laser light is focused onto the sample using a 100 × objective lens (NA 0.9), offering a ~ 1 μ m
158 resolution. The typical power used at the focus was 5~mW, resulting in a power density over the
159 scattering volume a hundred times smaller than that previously reported in tissues and cells, again to
160 ensure low phototoxicity. The backscattered light is collected by the same objective lens and is
161 collected outside the microscope using a telescope. A particular attention has been paid to respect the
162 f-number ($f/18$) of the spectrometer in order to maximize photon collection on the photodiode. Spectra
163 were acquired by averaging typically over ~ 100 acquisitions.

164 *Data analysis*

165 Raw spectra sampled at 30 MHz are recorded by the proprietary software of the spectrometer (Ghost
166 software, Table Stable Ltd., Switzerland). When only one peak is observed on each side of the Rayleigh
167 line (see **Fig. 1b** for instance), we fit separately the anti-Stokes and Stokes peaks to a Lorentzian
168 function using Matlab and extract amplitude A , frequency shift f and linewidth Γ . Values for each
169 peak are then averaged. When two closely-spaced peaks are observed, we window sequentially around
170 each peak and fit them individually with a single Lorentzian function. This procedure might
171 underestimate slightly the true spacing between the two peaks, but it reducing the error of the fit and
172 hence allows automating the fitting procedure to analyze large images.

173 The data collected on the different groups of samples were statistically analyzed using Matlab
174 software. The level of significance (p -value) was evaluated according to an unpaired two-tailed t -test.

175

176 RESULTS.

177 *Mechanical signature of caries lesions*

178 Brillouin spectra were recorded for healthy tissues ($n = 34$ in 5 different teeth) and caries lesions ($n =$
179 39 in 5 different teeth). Care was taken to focus the laser beam in the regions that didn't show tubules.
180 For illustration, we plot in **Fig. 1** a typical spectrum. **Figure 1a** shows that the raw spectrum is
181 dominated by Rayleigh scattering (grey box). **Figure 1b** shows a zoom-in on the Stokes (negative
182 frequencies) and anti-Stokes (positive frequencies) peaks for the healthy tissue and caries lesion. We
183 fit these peaks with Lorentzian functions (dashed lines) to extract the frequency shift f and linewidth
184 Γ (**Methods**). The distributions of frequency and linewidth values are plotted in **Figs. 2a** and **2b**,
185 respectively. Note that the spreading of the data is larger in the case of caries because BLS features
186 depend on the position within the highly heterogeneous lesion, as discussed below. Frequency shift is
187 clearly different between healthy and diseased conditions. We don't however observe any significant
188 variation in the linewidth. This observation suggest that BLS features could be used to delineate
189 lesions.

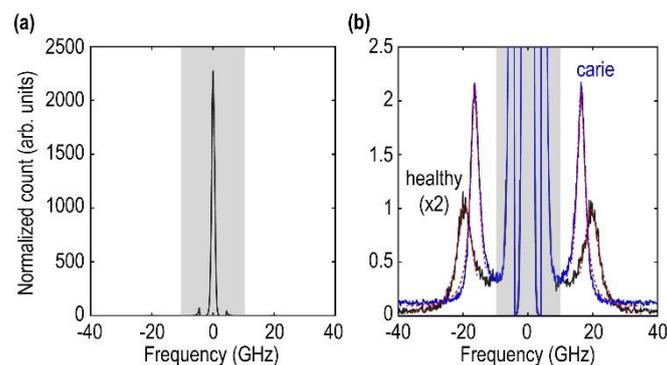


Figure 1: typical spectrum. (a) Raw spectrum where the contribution of Rayleigh scattering is indicated by the grey box. (b) Zoom-in on the Stokes (negative frequencies) and anti-Stokes (positive frequencies) peaks for a healthy tissue (black, intensity multiplied by 2 for clarity) and a caries lesion (blue line). Fits by Lorentzian functions are superimposed (red dashed lines).

190

191 To investigate this, we scanned the laser along a line across the frontier between healthy tissue and a
 192 caries lesion. **Figure 3** shows the frequency shift f and linewidth Γ measured from the healthy region
 193 (left) to the core of the lesion (right). We see that, as already observed in **Fig. 2b**, Γ does not vary. On
 194 the other hand, f decreases slowly, making the precise delineation difficult. As a reference, we indicate
 195 the median (red dashed line) and the 25th and 75th percentiles (grey box) measured in **Fig. 2a**. The
 196 upward arrow indicates where f falls below the 25th percentile. This criterion might be used to define
 197 the limit of the healthy tissue. In this example, f decrease almost linearly by ~ 0.01 GHz/ μm within
 198 the lesion. Given the spreading of the data for healthy dentin measured in **Fig. 2a**, ~ 0.2 GHz between
 199 the mean and the 25th percentile, such decrease would lead to a 20 μm precision in the identification
 200 of the lesion margins.

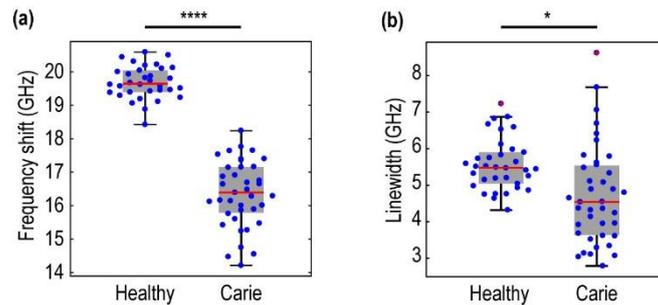


Figure 2: Comparison between healthy tissues ($n=34$) and caries lesions ($n=39$). Distributions of
 (a) frequency shift and (b) linewidth (**** $p<0.0001$, * $p<0.05$, unpaired two-tailed t-test). The
 bottom and top edges of each grey box indicate the 25th and 75th percentiles, respectively. The red
 central line indicates the median. The whiskers extend to the most extreme data points not
 considering outliers ('+' symbol).

201

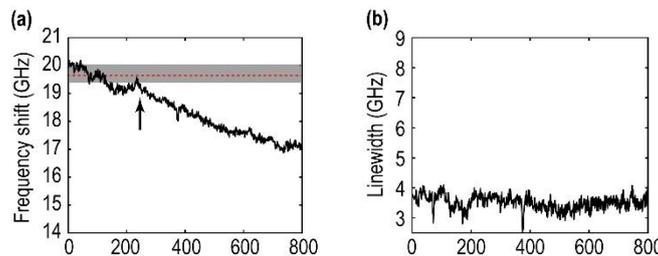


Figure 3: scan across a caries lesion. (a) frequency shift and (b) linewidth. The grey box and red
 dashed line indicate the 25th and 75th percentiles and median identified in Fig. 2a. Upward arrow
 indicates the position where the frequency shift falls below the 25th percentile.

202

203 **Mechanical anisotropy of the dentin**

204 Healthy dentin is composed of a mineralized matrix perfused by microtubes called tubules. This
 205 structure leads to an anisotropic distribution of mechanical properties, largely documented in the
 206 literature on a macroscale.[38] To investigate the anisotropy of the healthy tubular dentin on a
 207 microscopic scale, healthy teeth were cut longitudinally or transversally (**Methods**). The
 208 microstructure of the healthy dentin in the sagittal and transversal planes is well revealed by the
 209 combination of 2PEF image obtained with the Ti:Sapphire tuned at 730 nm and SHG image obtained
 210 with the Ti:Sapphire tuned at 840 nm (**Figures 4 and 5**). The typical tubular structure of dentin appears
 211 clearly on the 2PEF image. Collagen type I, as a triple-helical molecule, the organic component on

212 which the dentin is mostly built, is aligned in a non-centrosymmetric manner, and is responsible for
 213 the contrast in the SHG images. The SHG images of dentin thus reveal the intertubular dentin, which
 214 is less mineralized than the peritubular dentin.[28]

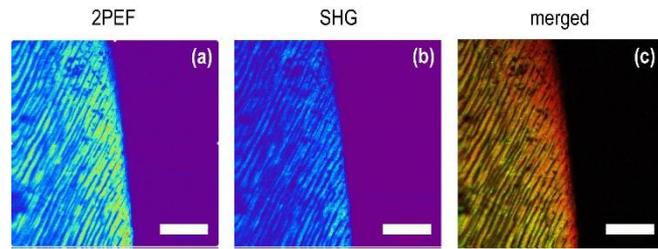


Figure 4: 2PEF and SHG images of healthy dentin in the sagittal plane. (a) 2PEF image reveal the tubular structure of dentin, (b) SHG reveals the presence of collagen type I, (c) merged. Scale bars: 20 μm .

215

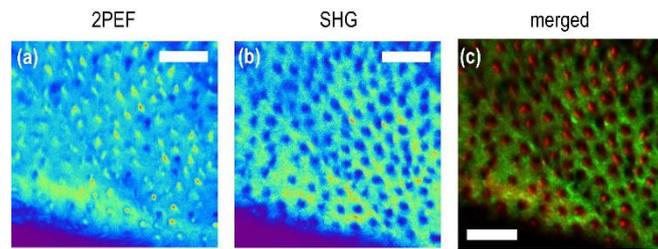


Figure 5: 2PEF and SHG images of healthy dentin in the transversal plane. (a) 2PEF image reveal the tubular structure of dentin, (b) SHG reveals the presence of collagen type I, (c) merged. Scale bars: 20 μm .

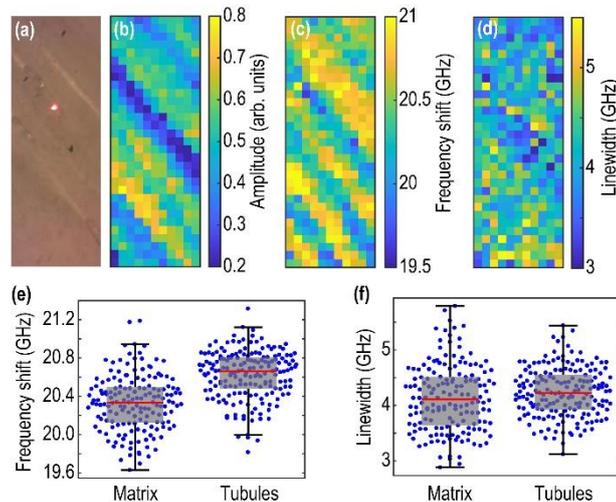


Figure 6: images of healthy dentin in the sagittal plane. (a) optical image, (b) amplitude of the Brillouin peaks, (c) frequency shift and (d) linewidth. Panels (e) and (f) show the distribution of f and Γ -values measured in panels (c) and (d), respectively. The tubules are identified in panel (b) by amplitude values lower than 50% of the maximum amplitude, and the matrix correspond to remaining pixels.

216 The exposed surfaces in the sagittal and transversal planes were mapped with BLS. The laser was
 217 scanned in a raster pattern with a 1 μm resolution and spectra recorded at each pixel. Resulting images
 218 obtained in the sagittal (along the tubules) and transversal planes (perpendicular to the tubules) of the

219 tooth are shown in **Figs. 6** and **7**, respectively. In the sagittal plane, the tubules are clearly visible on
 220 the optical image (**Fig. 6a**), and they are characterized by a lower amplitude (**Fig. 6b**) and a lower f
 221 (**Fig. 6c**). We define an amplitude threshold $A_{th} = 0.5A_m$, where A_m is the maximum amplitude, that
 222 separates the tubules from the surrounding matrix. Pixels in the f and Γ images that correspond to an
 223 amplitude $A < A_{th}$ are ascribed to the tubules, and the remaining pixels to the matrix. The distribution
 224 of pixels for f and Γ are shown in **Figs. 6e** and **6f**. While the linewidth doesn't allow distinguishing
 225 the two components (as observed on **Fig. 6d**), f -values are slightly higher for the tubules.

226 In the transverse plane, the tubules appear as disks on the optical image (**Fig. 7a**). They are also clearly
 227 visible on the BLS images, characterized here too by a low amplitude (**Fig. 7b**). In this case however,
 228 the low amplitude of the Brillouin peaks does not allow to identify f or Γ -values in the tubular regions,
 229 and only the matrix appears in the f and Γ images, **Figs. 7d** and **7f**. This is because the tubules cause
 230 a depression in the transverse plane that defocuses the laser beam. Considering pixels where $A < A_{th}$
 231 thus only reveals the distributions of values for the matrix, plotted in **Figs. 7c** and **7e**, respectively. The
 232 f and Γ values differ slightly in the sagittal and transverse planes, suggesting that the matrix itself is
 233 anisotropic.

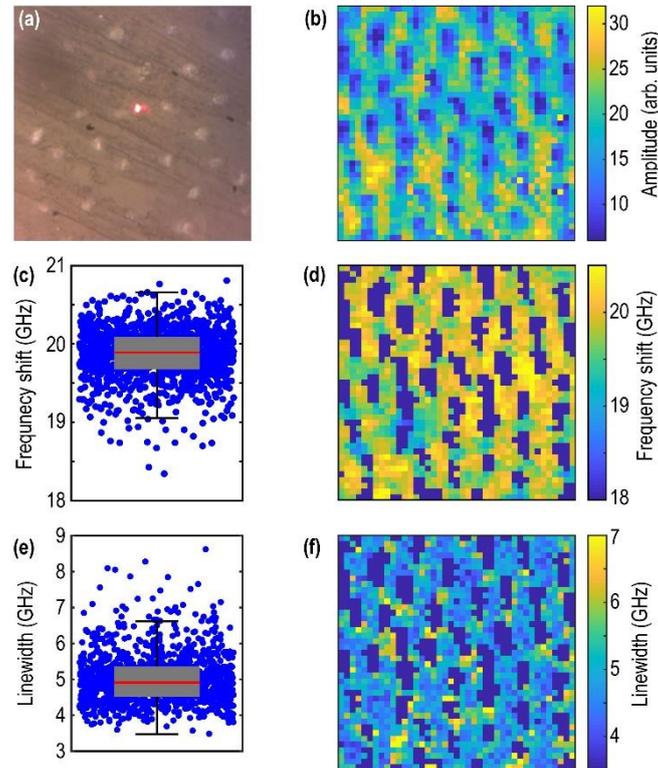


Figure 7: images of healthy dentin in the transversal plane. (a) optical image, (b) amplitude of the Brillouin peaks, (d) frequency shift and (f) linewidth. Panels (c) and (e) show the distribution of f and Γ -values measured in panels (d) and (f), respectively.

234

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236

237

238 **Characterization of the dentin-resin interface**

239 In this section we examine the adhesive interface between healthy dentin and tooth-filling resin. **Figure**
240 **8a** shows a typical sample with adhesive interface indicated by the arrow. Merged 2PEF image
241 obtained with the Ti:Sapphire tuned at 820 nm and SHG image obtained with the Yb:KGW (**Figure**
242 **8b**) reveals the typical structure of the interface between dentin (SHG, blue pseudocolor) and adhesive
243 (labelled by eosin in 2PEF, red pseudocolor). This image reveals that the low-viscous monomer
244 adhesive fingers in-between the exposed dentinal collagen fibers of intertubular dentin, or in the
245 tubules themselves, forming the so-called hybrid layer. Extensions of monomer entering a few
246 individual dentinal tubules, i.e. resin tags, are also observable (a typical one is pointed at by a white
247 arrow).

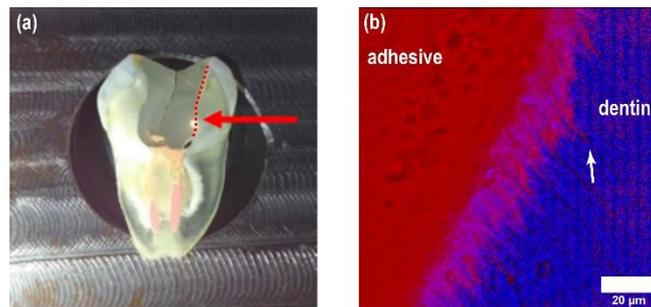


Figure 8: structure of a typical dentin adhesive interface. (a) photograph of dentin-resin adhesive interface shown by dashed line and the arrow. (b) Merged 2PEF and SHG images of a dentin-adhesive interface. 2PEF micrograph reveals the adhesive colored by eosin Y (red pseudocolor) and SHG micrograph reveals dentin (blue pseudocolor). Arrow points to a typical resin tag. Scale bar: 20 μm .

248

249 We probed the interface between healthy dentin and resin with BLS. **Figure 9a** shows a typical
250 spectrum where we recognize the signature of healthy dentin at around 20 GHz (blue area). In addition,
251 we also observe a second peak at around 13 GHz (pink area), revealing the presence of the adhesive.
252 This result demonstrates that BLS is able to detect the presence of the two components mixed in the
253 hybrid layer. To investigate this, we recorded BLS images in a typical hybrid layer. The frequency
254 shift (**Fig. 9b**) and linewidth (**Fig. 9c**) both allow to observe the adhesive, hybrid layer and dentin. This
255 clearly highlights the ability of BLS to quantify adhesive interfaces, and to reveal the fingering of the
256 adhesive in the hybrid layer. Such results should pave the way for novel strategies for tooth repair.

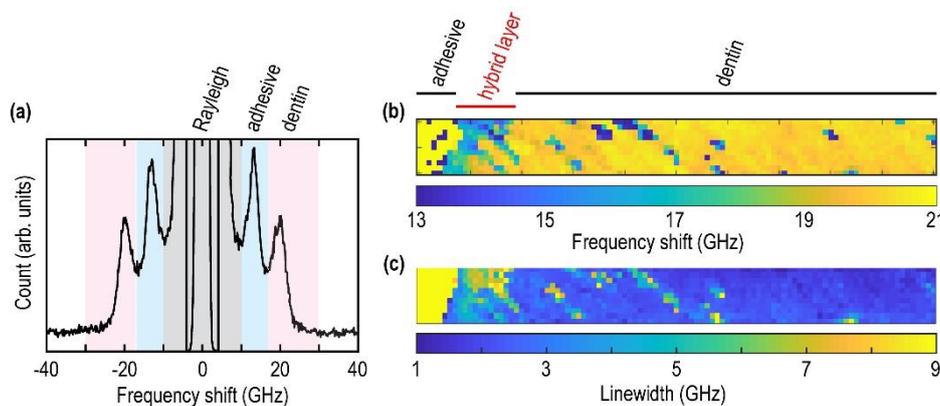


Figure 9: imaging the hybrid layer. (a) typical spectrum at the dentin-adhesive interface. The peaks arising from dentin and adhesive are shown in blue and pink, respectively. (a) Frequency shift and (b) linewidth maps of the hybrid layer.

257

258 DISCUSSION.

259 We have demonstrated the potential of BLS to examine non-destructively and with high resolution the
260 mechanical properties of different healthy, diseased or restored dental tissues. The BLS maps in the
261 sagittal and transverse planes clearly reveal the tubular structure of dentin. Our results showed $f_s \sim$
262 20.4 GHz in the sagittal plane, and $f_t \sim 19.8$ GHz in the transverse plane for the matrix alone. These
263 values correspond to sound velocities $V_s = \frac{f\lambda}{2n_s} \approx 4100$ m/s and $V_t = \frac{f\lambda}{2n_t} \approx 4300$ m/s, assuming
264 refractive indices $n_s = 1.6$ and $n_t = 1.5$ in the sagittal and transverse plane, respectively.[37] From
265 these values we can estimate the storage moduli $M'_s = \rho V_s^2 \sim 36$ GPa and $M'_t = \rho V_t^2 \sim 38$ GPa, where
266 $\rho \sim 2100$ kg/m³ is the mass density (**Methods**).[36] This matches very well with previous results,
267 $\sim 37\text{--}39$ GPa, obtained by ultrasonic interferometry.[40]

268 Our data shows an anisotropy of 6% and stiffer values in the transverse plane. Since the tubules are
269 aligned in the softer sagittal plane, this observation demonstrates that the tubules do not contribute to
270 mechanical strength of dentine. Instead, the mineralized collagen fibrils, aligned in the stiffer
271 transverse plane, act as a load bearing structure, as suggested previously.[38] This means that the
272 anisotropy of dentine originates from the anisotropy of the matrix itself, and that its structure could be
273 thought as a fiber-reinforced composite material in the transverse plane.

274 The difference between frequencies of healthy dentin and those of caries lesions is very clear ($p <$
275 0.0001). The transition from healthy dentin to caries allows delineation with a 20 μm precision for the
276 samples we probed. The linewidth $\Gamma = 2\pi\eta(2n/\lambda)^2$ is constant across this region, meaning that both
277 n and kinematic viscosity η are constant. The change in f can thus be ascribed to a reduction in the
278 stiffness of the tooth in the lesion. This observation demonstrates the high specificity of BLS, and the
279 ability to produce quantitative indicators of tooth decay. In contemporary clinical practice, the visuo-
280 tactile method is the dominant approach to caries delineation, and is obviously less resolved than BLS
281 and operator-dependent. In order to avoid inter-examiner variations, visual examination can be aided
282 by dyes, light-fluorescence based methods, or fiber-optic transillumination. In this context, BLS has a
283 unique potential to provide quantitative information at a microscale, in a label-free, non-invasive, non-
284 ionizing manner. Further comparison with histological sections of lesions with different scores should
285 reveal the sensitivity of BLS, and grant new minimally invasive strategies in dentistry. As has been
286 done for cancer therapy [35], ophthalmology [30] or bone,[25] BLS could be used as a platform for
287 early diagnosis of tooth decay, micrometric precise caries detection during clinical work, but also for
288 the monitoring of efficacy of the therapeutic techniques. [31, 34]

289 BLS images of the hybrid layer bring new information on the spatial distribution of mechanical
290 properties in the adhesive junction. In future, BLS could be used to test the adhesion strength of
291 different types of adhesives under preclinical investigation, or the weakening of the hybrid layer by
292 enzyme-caused degradation of collagen or adhesive, mineral-depleted dentin[31, 32] or caries lesion
293 [33]. Moreover, the hybrid layer is subjected to multiaxial stresses during functional use, and it has

294 been predicted that stress concentration in the underlying hard dental tissues depends on the stiffness
295 of this interface [29]. In future, BLS could also help studying the impact of wear on this interface at a
296 microscale. Beyond dentistry, BLS could also provide new tools to investigate the link between
297 microwear, stiffness and diet, offering important information in paleobiology.

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307 REFERENCES.

- 308 [1] M. Goldberg, A. B. Kulkarni, M. Young, and A. Boskey, “Dentin: structure, composition and
309 mineralization,” *Front. Biosci. (Elite Ed)*, vol. 3, p. 711, 2011.
- 310 [2] S. R. Stock, A. C. Deymier-Black, A. Veis, A. Telser, E. Lux, and Z. Cai, “Bovine and equine
311 peritubular and intertubular dentin,” *Acta Biomater*, vol. 10, p. 3969, 2014.
- 312 [3] J. H. Kinney, M. Balooch, S. J. Marshall, G. W. Marshall JR, and T. P. Weihs, “Hardness and
313 Young’s modulus of human peritubular and intertubular dentine,” *Arch. Oral Biol.*, vol. 41,
314 no. I, pp. 9–13, 1996.
- 315 [4] S. K. Makhijaa, M. E. Robinson, J. D. Bader, D. A. Shugars, M. S. Litaker, H. R. Im, D. B.
316 Rindal, D. J. Pihlstrom, C. Meyerowitz, V. V Gordan, M. H. Buckberg, and G. H. Gilbert,
317 “Dentists’ decision strategies for suspicious occlusal caries lesions in a National Dental PBRN
318 study,” *J. Dent.*, vol. 69, p. 83, 2018.
- 319 [5] F. Schwendicke, S. Paris, and Y.-K. Tu, “Effects of using different criteria for caries removal:
320 A systematic review and network meta-analysis,” *J. Dent.*, vol. 43, no. 1, pp. 1–15, 2015.
- 321 [6] G. J. Joves, G. Inoue, A. Sadr, T. Nikaido, and J. Tagami, “Nanoindentation hardness of
322 intertubular dentin in sound , demineralized and natural caries-affected dentin,” *J. Mech.
323 Behav. Biomed. Mater.*, vol. 32, pp. 39–45, 2014.
- 324 [7] R. Maev, L. Denisova, E. Maeva, and D. AA, “New data on histology and physico-mechanical
325 properties of human tooth tissue obtained with acoustic microscopy.,” *Ultrasound Med Biol*,
326 vol. 28, pp. 131–136, 2002.
- 327 [8] K. Raum, K. Kempf, H. J. Hein, J. Schubert, and P. Maurerb, “Preservation of microelastic
328 properties of dentin and tooth enamel in vitro—A scanning acoustic microscopy study,” *Dent.
329 Mater.*, vol. 23, pp. 1221–1228, 2007.
- 330 [9] A. Shelke, M. Blume, M. Mularczyk, C. Landes, R. Sader, and J. Bereiter-Hahn,
331 “Visualisation of localized elastic properties in human tooth and jawbone as revealed by
332 scanning acoustic microscopy,” *Ultrasound Med. Biol*, vol. 39, no. 5, pp. 853–859, 2013.

- 333 [10] S. Toda, T. Fujita, H. Arakawa, and K. Toda, "Nondestructive testing in human teeth using a
334 leaky Lamb wave device," *Ultrasonics*, vol. 44, pp. e1151–e1155, 2006.
- 335 [11] K. Sun, L. Yuan, Z. Shen, Q. Zhu, J. Lu, and X. Ni, "Experimental and numerical studies for
336 nondestructive evaluation of human enamel using laser ultrasonic technique," *Appl. Opt.*, vol.
337 52, no. 28, pp. 6896–6905, 2013.
- 338 [12] H.-C. Wang, S. Fleming, Y.-C. Lee, M. Swain, S. Law, and J. Xue, "Laser ultrasonic
339 evaluation of human dental enamel during remineralization treatment," *Biomed. Opt. Express*,
340 vol. 2, no. 2, pp. 345–355, 2011.
- 341 [13] H. Salehi, E. Terrer, I. Panayotov, B. Levallois, B. Jacquot, H. Tassery and F. Cuisinier,
342 "Functional mapping of human sound and carious enamel and dentin with Raman
343 spectroscopy", *J. Biophotonics* 6, 765-774 (2013).
- 344 [14] R. Ramakrishnaiah, G. ur Rehman, S. Basavarajappa, A. A. Al Khuraif, B. H. Durgesh, A. S.
345 Khan, and I. ur Rehman, "Applications of Raman Spectroscopy in Dentistry: Analysis of
346 Tooth Structure," *Appl Spectrosc Rev*, vol. 50, no. 4, pp. 332–350, 2015.
- 347 [15] C. de C. A. Lopes, P. H. J. O. Limirio, V. R. Novais, and P. Dechichi, "Fourier transform
348 infrared spectroscopy (FTIR) application chemical characterization of enamel, dentin and
349 bone," *Appl. Spectrosc. Rev.*, vol. 53, no. 9, pp. 747–769, 2018.
- 350 [16] Z. A. Steelman, D. S. Ho, K. K. Chu and A. Wax, "Light-scattering methods for tissue
351 diagnosis", *Optica* 6, 479-489 (2019).
- 352 [17] R. Harley, D. James, A. Miller, and J. W. White, "Phonons and the elastic moduli of collagen
353 and muscle," *Nature*, vol. 267, pp. 285–287, 1977.
- 354 [18] L. Brillouin, "Diffusion de la lumière et des rayonnés X par un corps transparent homogène;
355 influence de l'agitation thermique," *Ann. Phys.*, vol. 17, pp. 88–122, 1922.
- 356 [19] F. Palombo and D. Fioretto, "Brillouin Light Scattering: Applications in Biomedical
357 Sciences", *Chem. Rev.* 119, 7833-7847 (2019).
- 358 [20] J. Randall, J. M. Vaughan, and S. Cusak, "Brillouin scattering in systems of biological
359 significance," *Philos. Trans. R. Soc. A*, vol. 293, pp. 341–348, 1979.
- 360 [21] F. Palombo, C. P. Winlove, R. S. Edginton, E. Green, N. Stone, S. Caponi, M. Madami, and
361 D. Fioretto, "Biomechanics of fibrous proteins of the extracellular matrix studied by Brillouin
362 scattering," *J. R. Soc. Interface*, vol. 11, p. 20140739, 2014.
- 363 [22] J. M. Vaughan and J. T. Randall, "Brillouin scattering, density and elastic properties of the
364 lens and cornea of the eye," *Nature*, vol. 284, no. 5755, pp. 489–491, 1980.
- 365 [23] G. Scarcelli and S. H. Yun, "Confocal Brillouin microscopy for three-dimensional mechanical
366 imaging," *Nat. Photonics*, vol. 2, no. 1, p. 39, 2008.
- 367 [24] S. Lees, N. J. Tao, and S. M. Lindsay, "Studies of compact hard tissues and collagen by means
368 of brillouin light scattering," *Connect. Tissue Res.*, vol. 24, no. 3–4, pp. 187–205, 1990.
- 369 [25] R. Tsubota, K. Fukui, and M. Matsukawa, "Local ultrasonic wave velocities in trabeculae
370 measured by micro-Brillouin scattering," *J. Acoust. Soc. Am.*, vol. 2, pp. EL109-EL114,
371 135AD.
- 372 [26] V. Mathieu, K. Fukui, M. Matsukawa, M. Kawabe, R. Vayron, E. Soffer, F. Anagnostou, and
373 G. Haiat, "Micro-Brillouin Scattering Measurements in Mature and Newly Formed Bone

- 374 Tissue Surrounding an Implant,” *J. Biomech. Eng.*, vol. 133, no. February, pp. 021006-1-
375 021006-6, 2011.
- 376 [27] M. Oheim, D. Michael, M. Geisbauer, M. Dorte, and R. Chow, “Principles of two-photon
377 excitation fluorescence microscopy and other nonlinear imaging approaches,” *Adv. Drug*
378 *Deliv. Rev.*, vol. 58, pp. 788–808, 2006.
- 379 [28] R. Elbaum, E. Tal, A. I. Perets, D. Oron, D. Ziskind, Y. Silberberg, and H. D. Wagner,
380 “Dentin micro-architecture using harmonic generation microscopy,” *J. Dent.*, vol. 35, no. 2,
381 pp. 150–155, 2007.
- 382 [29] A. Misra, V. Singh, and R. Parthasarathy, *Material-tissue interfacial phenomena: Challenges*
383 *in mathematical modeling*. Elsevier Ltd, 2016.
- 384 [30] S. H. Yun and D. Chernyak, “Brillouin microscopy: assessing ocular tissue biomechanics,”
385 *Curr. Opin. Ophthalmol.*, vol. 29, no. 4, pp. 299–305, 2018.
- 386 [31] S. Sauro, V. P. Feitosa, T. F. Watson, and D. H. Pashley, “Therapeutic bonding approaches to
387 remineralize dentin-bonded interfaces,” *Dent. Mater.*, vol. 32, no. Supplement 1, pp. e3–e4,
388 2016.
- 389 [32] M. Hashimoto, H. Ohno, H. Sano, M. Kaga, and H. Oguchi, “In vitro degradation of resin–
390 dentin bonds analyzed by microtensile bond test, scanning and transmission electron
391 microscopy,” *Biomaterials*, vol. 24, pp. 3795–3803, 2003.
- 392 [33] M. Oliveir, F. Chasqueira, S. Arantes-Oliveira, and S. Pessanha, “The use of micro-Raman
393 spectroscopy for the analysis of caries-affected dentin adhesive interfaces.” p. In Press.
- 394 [34] S. Sauro, R. Osorio, T. F. Watson, and M. Toledano, “Influence of phosphoproteins’
395 biomimetic analogs on remineralization of mineral-depleted resin – dentin interfaces created
396 with ion-releasing resin-based systems,” *Dent. Mater.*, vol. 31, no. 7, pp. 759–777, 2015.
- 397 [35] J. Margueritat, A. Virgone-Carlotta, S. Monnier, H. Delanoë-Ayari, H. C. Mertani, A.
398 Berthelot, Q. Martinet, X. Dagany, C. Rivière, J.-P. Rieu, and T. Dehoux, “High-frequency
399 mechanical properties of tumors measured by Brillouin light scattering”, *Phys Rev. Lett.* 122,
400 018101 (2019).
- 401 [36] Regine Gradl, Irene Zanette, Maite Ruiz-Yaniz, Martin Dierolf, Alexander Rack, Paul
402 Zaslansky, and Franz Pfeiffer, “Mass Density Measurement of Mineralized Tissue with
403 Grating-Based X-Ray Phase Tomography”, *PLoS ONE* 11, e0167797 (2016).
- 404 [37] I. Hariri, A. Sadr, Y. Shimada, J. Tagami, and Y. Sumi, “Effects of structural orientation of
405 enamel and dentine on light attenuation and local refractive index: An optical coherence
406 tomography study”, *J. Dentistry* 40, 2012
- 407 [38] J. H. Kinney, S. J. Marshall and G. W. Marshall, “The mechanical properties of human dentin:
408 a critical review and re-evaluation of the dental literature”, *Crit. Rev. Oral. Biol. Med.* 14, 13-
409 29 (2003)
- 410 [39] G. Kossoff and C. J. Sharpe, “Examination of the contents of the pulp cavity in teeth”,
411 *Ultrasonics* 4, 77-83 (1966).
- 412 [40] S. Lees and F. R. Rollins, “Anisotropy in hard dental tissues”, *J. Biomech.* 5, 557-566 (1972).
- 413 [41] T. Cloitre, I. V. Panayotov, H. Tassery, C. Gergely, B. Levallois and F. J. Cuisinier,
414 “Multiphoton imaging of the dentine-enamel junction”, *J. Biophotonics* 6, 330-337 (2013).

- 415 [42] M. Rabasović, D. Pantelić, B. Jelenković, S. Čurčić, M. Rabasović, M. Vrbica, et al.,
416 "Nonlinear microscopy of chitin and chitinous structures: a case study of two cave-dwelling
417 insects", J. Biomed. Opt. 20, 016010 (2015).
- 418 [43] K. Bukara, S. Z. Jovanić, I. T. Drvenica, A. Stančić, V. Ilić, M. D. Rabasović, D. V. Pantelić,
419 B. M. Jelenković, B. Bugarski and A. J. Krmpot, "Mapping of hemoglobin in erythrocytes and
420 erythrocyte ghosts using two photon excitation fluorescence microscopy" J. Biomed. Opt. 22,
421 026003 (2017)
- 422 [44] D. Zaytsev, S. Grigoriev and P. Panfilov, "Deformation behavior of root dentin under
423 Sjogren's syndrome", Mat. Lett. 65, 2435-2438 (2011).
- 424