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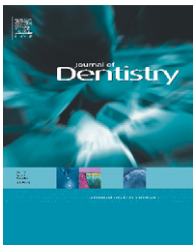

Dentin micro-architecture using harmonic generation microscopy

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ABSTRACT

Objectives: We present a novel way to create high-resolution three-dimensional images of tooth dentin by harmonic generation scanning laser microscopy.
Methods: The images were taken using a pulsed infrared laser. Three-dimensional reconstruction enables the visualization of individual tubules and the collagen fibrils mesh around them with an optical resolution of ~1 μm.
Results: The images show micro-morphological details of the dentinal tubules as well as the collagen fibrils at a depth of up to about 200 μm. The data show that while collagen fibrils are organized in planes perpendicular to the tubules, close to the dentin enamel junction they lie also along the long axis of the tubules.
Conclusions: The unique 3D information opens the opportunity to study the collagen fibril arrangement in relation to the tubule orientation within the dentin matrix, and may be applied to study the micro-morphology of normal versus altered dentin.

1. Introduction

Dentin material comprises the bulk of the tooth mineralized tissues. Its function is to provide elastic support to the overlying hard enamel, enabling the dispersion of mechanical stresses applied during mastication.[1] Dentin is made of tubules of about 0.8–2.5 μm in their inner diameter, filled with the odontoblast cytoplasmatic fluid. The lumen of the tubules is surrounded by a mineralized thin cylinder called peritubular dentin (PTD). The matrix outside those peritubular cylinders, the intertubular dentin (ITD), contains about 30 volume percent mineralized collagen Type I fibrils,[2,3] wrapped around perpendicularly to the tubule long axis.[3] Much smaller amounts of collagen, of the order of 10% by volume, are present in the PTD.[2] Understanding the three-dimensional micro-architecture of dentin will provide a basis for a much improved understanding of the correlation between its structure and function, which can potentially contribute to improving the treatment of the ''cracked tooth syndrome'' and caries disease.

Dentin morphology and composition has been studied for many years. It was first characterized using optical microscopy, and later using electron microscopy.[4] In recent years atomic force microscopy has been utilized to measure both its microstructural and mechanical properties.[5,6] Confocal microscopy using fluorescent labeling was applied to investigate the interface between restorative materials and dentin.[7] To learn about the formation of the interface between the dentin and the filling, the lumen of the tubules was filled with a resin and the tooth tissue was subsequently dissolved. High-resolution electron micrographs show the

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tubules 3D structure.[8] Micro-computerized tomography (μCT) using an X-ray source also produces 3D images of the tooth hard tissues, but the spatial resolution does not allow to detect objects smaller than 10 μm.[9]

Harmonic generation microscopy (HGM) was recently applied to image biological samples.[10,11] This imaging mode is advantageous over traditional fluorescence confocal microscopy in three aspects: First, multiphoton processes are excited only at the focal spot where the photon flux density is high enough for the nonlinear process to occur, eliminating the need for the confocal detector pinhole. Second, the illuminating laser is typically in the infrared rather than in the visible or UV. Using such long excitation wavelengths allows for an improved penetration depth due to lower scattering. Last, the nonlinear susceptibility, which determines the multiphoton processes, is an inherent property of the material. Therefore, there is no need to label the sample with a dye. The image is created by scanning the sample point by point (similar to confocal scanning) and collecting the nonlinearly generated photons from each pixel individually. The penetration depth into the sample is mostly determined by the reduction of the excitation beam power due to scattering in the sample. In samples with low scattering, 3D reconstruction up to several hundreds of microns into the sample is possible, by creating a set of images at various depths into the sample. Commonly, two HGM modes are used: second harmonic generation (SHG[12]) and third harmonic generation (THG[13,14]). Each has its own advantages when applied to a biological tissue. The SHG signal is generated only from non-centrosymmetric materials, like collagen fibrils. The THG signal is generated by every material, however, due to the Guoy phase shift inherent to focused beams, the emitted THG signal generated before the focus of the excitation beam destructively interferes with the signal generated behind the focal plane.[13,15] Therefore, THG will be generated specifically at interfaces between materials of different third order nonlinear susceptibilities.

Harmonic generation microscopy is well suited to study the special architecture of dentin: the tubules are ideal to be observed using the THG signal and the collagen can be imaged using the SHG signal. Dentin thin sections are partially transparent to the infrared light, enabling 3D reconstructions of the data. By embedding the sample in a medium of matched refractive index the light penetration is much improved. The lateral (x–y plane) optical resolution of the images is of the order of 1 μm and the longitudinal (z direction) resolution, is of the order of 3 μm. The aim of this study was to image in three-dimensions the dentin microstructure using harmonic generation microscopy (HGM), in particular the architecture of the dentin tubules, and the surrounding collagen distribution. We present here 3D reconstructions of a healthy cow incisor dentin using SHG and THG microscopy.

2. Materials and methods

2.1. Sample preparation

Two cow incisors from a freshly slaughtered mature animal were extracted and kept in Hank's balanced salt solution (HBSS) and 1% penicillin solution of 10,000 U/∝L for 1 month, and then transferred to HBSS and 0.1% ammonium azide solution for another month. After separating the crown from the root, the teeth were embedded in epoxy resin (Epofix®, Struers, Copenhagen, Denmark). The root and the crown were sectioned using a water cooled diamond blade (Minitom® Struers, Copenhagen, Denmark) through the pulp cavity in the mesial–distal direction. Thereafter, the pulpal sides of each slice were metallographically ground through a series of SiC abrasive papers (800 mesh) and polished using diamond suspensions of 9.0 and 1.0 μm particle size, on a soft polishing cloths (LaboForce-3® & LaboPol-2®, Struers, Copenhagen, Denmark). The samples were rinsed copiously under water and cleaned ultrasonically after each polishing step.

One tooth was imaged using an environmental scanning electron microscope (ESEM). The second tooth was prepared for HGM. Thin sections of about 200 μm were cut from the pulpal aspect of the root (sections A and B) and from the pulpal aspect of the crown (section C), using the diamond blade. Another thin section was produced at the crown by first grinding and polishing the dentin enamel junction (DEJ) aspect as described, and then grinding the pulp aspect down to about 200 μm on a SiC abrasive paper (800 mesh) (section D). Description of the samples is available in Table 1.

The root section A was sonicated for 45 min in HBSS aqueous solution before HGM measurement. The other sections were sonicated for 45 min in methanol and then transferred to Murray index matching solution (2:1 mixture of benzyl benzoate and benzyl alcohol) and again sonicated for 45 min before the HGM measurement. Murray solution has a refractive index close to that of dentin, and when it penetrates into the lumen of the tubules, the dentin becomes nearly transparent. Using HBSS solution, only the dental part which is constructed from tubules perpendicular to the section surface is transparent.

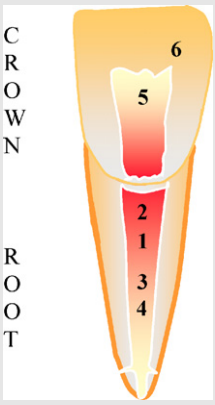
2.2. Electron scanning microscopy

Polished surface of the dentin were scanned in the back-scattered electron mode of the Philips XL-30 environmental scanning electron microscope (ESEM). Acceleration voltage was 10 kV, beam spot size number 4, working distance was 6.9 mm, and water pressure was 0.8 Torr. Images were taken at magnification of 2000.

2.3. Harmonic generation microscopy

As an imaging platform a Zeiss Axiovert-135 microscope was used, which was modified into a scanning microscope. The laser source was a Spectra Physics OPAL that provides linearly polarized 100 fs pulses at a wavelength of 1.5 μm at a repetition rate of 80 MHz. The laser beam was coupled through one of the microscope ports and was focused into the sample by a X60-0.85 NA microscope objective. The focal point was scanned in the x–y plane using two optical scanners, and along the z-axis using the motorized objective turret of the microscope. The SHG signal, at a wavelength of 0.75 μm, and the THG signal, at a wavelength of 0.5 μm, were collected by a condenser and measured by a photomultiplier tube (Hamamatsu R4220) and a radio frequency lock-in amplifier (Stanford Research Instruments, model SR844) which synchronizes with the 80 MHz pulse train. A typical

Table 1 – A lower anterior bovine incisor

Slice (immersion solution)	Approximate observation points		Density (tubules/mm^2)	Angle (°)
A (HBSS)	Deep dentin (1)		22,440	1
B (Murray)	Deep dentin (1)		20,550	21
	Deep dentin (2)		26,110	7
	Deep dentin (3)		24,440	10
	Deep dentin (4)		28,330	4
C (Murray)	Deep dentin (5)		40,550	1
	Shallow dentin (6)		n/a[a]	~90[a]
D (Murray)	Shallow dentin (6)		15,000	11

Various locations of the 3D HGM observation points are indicated on the illustration. The results of the 3D reconstruction present tubule density and orientation (angle). Slices A, B and C were facing the pulpal wall (deep dentin in the center, shallow dentin at the periphery) at various root locations. Tubules cut crosswise at different angles. Slice D was cut from the proximal side near the DEJ.

[a] Tubule orientation was almost parallel to the surface, thus no 3D reconstruction was possible; the angle was estimated.

integration time was 300 μs per pixel. The output signal from the lock-in amplifier was fed into a computer, which synchronized the scanning process and the data collection. Sets of images of a 30 μm × 30 μm region, each containing 100 × 100 pixels, were collected.

2.4. *Image analysis*

HGM and ESEM images were viewed using ImageJ (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). The SHG images were overlaid on the THG images using the colocalization finder plugin of ImageJ. Three-dimensional images were reconstructed using Amira 3.1.1 (TGS Inc.). A set of SHG images overlaid on the THG images (produced by ImageJ) were analyzed using the Orthoslice tool. The THG data was reconstructed by drawing surfaces of equal intensity using the Isosurface tool. The SHG data was imaged using the Voltex tool.

The angles at which the tubules reach the polished surface were measured from the THG reconstructions. First we found the plane at which the long axes of the tubules lie, and then we measured the angle between this plane and a line perpendicular to the physical surface of the sample. The tubule density was calculated twice for each 3D structure at the top and bottom of the scanned box, by dividing the number of tubules reaching the surface by 900 μm.[2] The average density is reported.

3. Results

Images of four polished dentin sections were obtained by measuring third harmonic generation (THG) and second harmonic generation (SHG) signals at the sample surface and focusing beneath the surface to depths of up to about 200 μm. The THG displays interfaces inside the sample, whereas the SHG reveals the collagen fibrils due to their non-centrosymmetric structure. Fig. 1a and b shows the harmonic generation microscopy (HGM) images collected

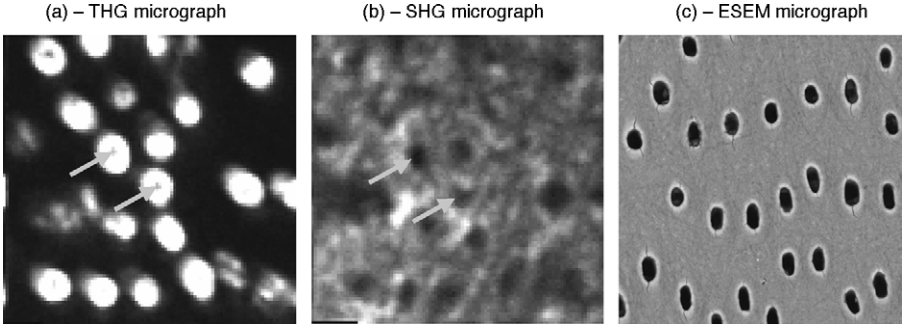


Fig. 1 – (a) A 30 μm × 30 μm THG image of deep root dentin immersed in HBSS (section A) at a depth of about 30 μm under the polished surface. The bright circles are a result of THG signal created at the tubule lumen–PTD interface. The spatial resolution of the THG sometimes enables us to see the uniform lumen within the tubules (arrows). (b) The same field of view was depicted using the SHG mode. The signal originates from the collagen fibrils traveling in a plane perpendicular to the laser propagation direction. The black circular bodies are the tubules, which show no collagen signal. The arrows point to the same tubules marked in panel a. (c) A 30 μm × 30 μm ESEM micrograph of a different sample of deep root dentin, taken in the backscattered electron mode.

from a deep root dentin sample immersed in HBSS (section A) at a depth of about 30 μm under the polished surface. To better understand the features imaged by HGM, an ESEM micrograph of deep root dentin is also presented (Fig. 1c). The ESEM backscattered electron mode enables us to recognize variation in the mineralization level. Thus, the lumen of the tubules appears in black, while their highly mineralized peritubular dentin (PTD) cuffs appear in white, and the partially mineralized intertubular dentin (ITD) in gray. Based on the ESEM data we found that the averaged radius of the lumen is about 0.9 μm. The THG image displays bright circular features with an average radius of 1.54 μm (Fig. 1a). The spatial resolution of the THG image is about 1 μm. This means that a feature of about 0.9 μm in radius, like the lumen, will appear as ~1.5 μm in radius. This convinced us that the THG signal was created only at the lumen–PTD interface and not at the PTD–ITD interface. The spatial resolution of the THG sometimes allowed us to see the uniform lumen within the tubules. In Fig. 1b the SHG signal is depicted. This signal originates from the collagen fibrils. In this representation the tubules and the PTD appear in black because they contain much less collagen than the ITD. Table 1 summarizes the morphological observations from the sections measured. We found that tubule density increases from the dentin enamel junction (DEJ) towards the pulp, in agreement with previously published data.[4]

In Fig. 2 the two HGM signals were combined in a single image: the collagen signal is in green and the lumen–ITD interfaces are in red. The direction of the tubules in relation to the sample surface was found to influence the image quality. As long as the tubules were viewed from the top, their signal was very clear and the collagen signal showed spatial variation (Fig. 2a and b). When the tubules were lying parallel to the sample surface it was more difficult to separate individual tubules (Fig. 2c). This may be because the focus depth of field is larger than the distance between the tubules. Thus, in one image tubules lying below or above the scanned surface were also detected. In this geometry we could still see variation in the collagen signal, though less pronounced than when the tubules are perpendicular to the surface (compare Fig. 2b and c). When the tubules were tilted at an angle of 11° to the surface the variation in the collagen signal was minor (Fig. 2d).

Slices of SHG and THG were reconstructed to show the dentin 3D structure (Fig. 3). In Fig. 3a, the data from section A of root deep dentin was sliced in three directions to show the course of the tubules within the collagen mesh. Tubules could be seen cutting through the surfaces chosen to represent the volume of the dentin. In Fig. 3b the THG data was used, demonstrating our ability to reconstruct the tubule surface of the same sample. The tubules lie parallel to each other, deviating coordinately from a perfect straight course. The tubules appear to narrow towards the bottom. This is an artifact caused by loss of the signal at increased depths. From this data the inclination angle of the tubules was calculated in relation to a line perpendicular to the scanned surface (Table 1). Fig. 3c shows the collagen fibrils signal in 3D as it was reconstructed from the SHG images. (Here too the signal collected at the sample surface is more intense than closer to the bottom.) This set of data represents the ITD collagen. Observing the 3D reconstruction it was discovered that the collagen fibrils form lumps parallel to the tubules. These structures could not be visualized in the 2D data.

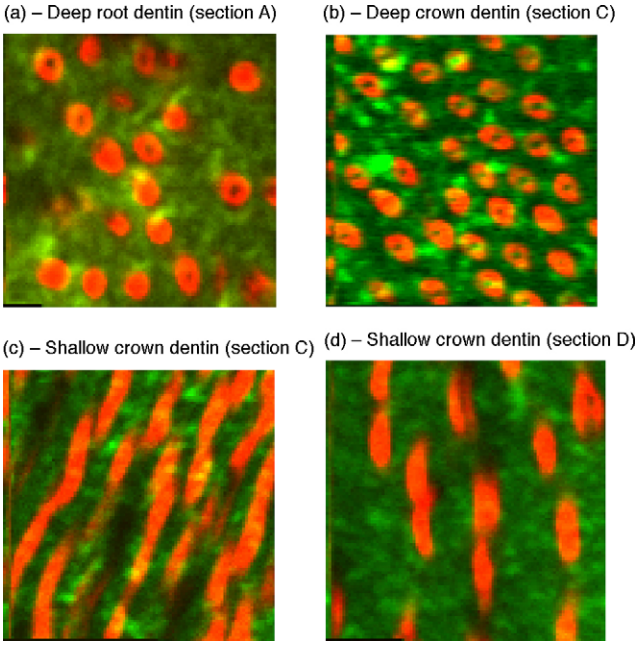


Fig. 2 – Overlays of second harmonic generation (SHG, in green), on third harmonic generation (THG, in red) images of three of the samples described in Table 1: (a) section A, deep root dentin immersed in aqueous buffer (HBSS); (b) section C, deep crown dentin immersed in organic solution (Murray solution); (c) section C again, this time measured at the tooth periphery where the tubules are almost parallel to the scanned surface; (d) section D, shallow crown dentin immersed in organic solution (Murray solution). In Fig 1d the tubules run at an angle of 11.4° to the sample surface. Note that the variation in the collagen signal is stronger when the tubules travel perpendicularly or parallel to the scanned surface. Size of field is 30 μm × 30 μm. The slices are located about 30 μm (a), 60 μm (b), 30 μm (c), and 21 μm (d) under the surface of the sample.

4. Discussion

This work presents the micro-architecture of tooth dentin reconstructed from harmonic generation micrographs. The major advantage over other imaging methods is the ability to locate features within the dentin 3D space. The information includes two features: The tubule surfaces, observed using the third harmonic generation (THG) signal, and collagen, observed through the second harmonic generation (SHG) signal. This allows the reconstruction of the collagen fibril distribution in relation to the tubule path.

The THG signal is created at interfaces between materials of different third order nonlinear susceptibilities. We showed that the THG is generated only at the lumen–PTD interface and

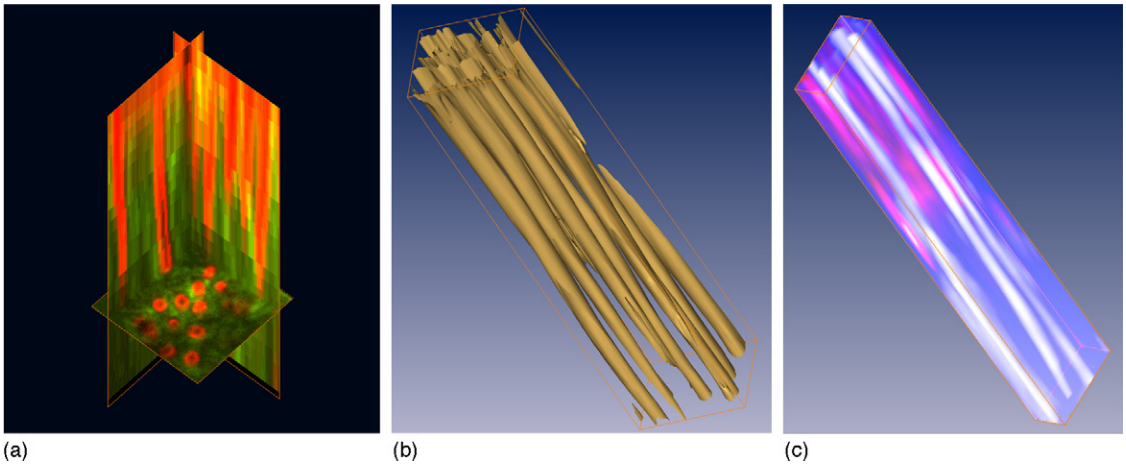


Fig. 3 – Three-dimensional reconstruction of section A, a root deep dentin sample immersed in aqueous buffer. (a) Second harmonic generation signal (SHG, in green) overlaid on third harmonic generation signal (THG, in red) (box dimensions are 30 μm × 30 μm × 110 μm), (b) third harmonic generation (THG) signal represented through plot of surfaces of similar brightness (box dimensions are 20 μm × 15 μm × 90 μm), and (c) collagen signal as a result of second harmonic generation (SHG) represented using a temperature color map (length of the reconstructed box is 110 μm). It is possible to distinguish the tubule lumen in white, and regions of strong collagen signal in pink. Both THG and SHG signals are decreased with the penetration depth.

not at the PTD–ITD interface. The SHG signal is created from collagen fibers that lie in a plane perpendicular to the light direction. Maximal signal is obtained when the laser light polarization is parallel to the collagen fibrils.[16] Observing the 3D reconstruction of the SHG we found that regions of high intensity signal form structures parallel to the tubules long axis (Fig. 3c). We relate these features to locations where collagen fibrils are oriented at the same direction. Such structures may be important with respect to the mechanical properties of the dentin, as it was shown that the collagen arrangement influences the fracture toughness of dentin.[17]

In bulk dentin the collagen fibrils are arranged in planes perpendicular to the tubules long axis.[3] Therefore, collagen signal may be detected when the tubules are arranged at a right angle to the scanned surface. These conditions are fulfilled in Fig. 2a and b. However, in Fig. 2d the tubules are tilted at an angle of 11° to the scanned surface, and therefore the collagen signal (in green) is weaker and more uniform. In Fig. 2c spots of high intensity SHG signals are observed even though the tubules are perpendicular to the laser propagation direction. This could be explained by the presence of collagen fibrils traveling along the tubules, which is at variance with the observation of Kinney et al.[3] Collagen fibrils that lie parallel to the tubules long axis were observed near the dentin enamel junction (DEJ),[18,19] possibly in agreement with our results showing strong collagen SHG signals close to the DEJ. This point will require further investigation.

Three-dimensional reconstruction tools for directly imaging the tooth hard tissues include micro-compound tomography (μCT)[20,9] and nuclear magnetic resonance (NMR).[21] These methods have a resolution reaching tens of micrometers. A different approach is to construct a 3D object from sequential images of sections at a known distance. In this method the 2D resolution may be high but the z-direction is very poorly resolved (for example, work by Arnold et al.[22]). We add to these methods a tool with superior resolution to study micrometer scale features. Questions concerning the direction of the dentinal tubules in relation to dentin bonding,[23] or to dentin mechanical properties[17] may be approached using the HGM reconstructions.

5. Conclusions

Our initial results demonstrate the possibility to apply HGM to tooth dentin to create 3D maps of the tubules, and of the arrangement of the intertubular collagen fibrils. We have shown that some of the collagen fibrils are oriented perpendicularly to the tubules in the bulk dentin, forming lumps of ordered collagen fibrils along the tubules. Collagen fibrils oriented along the tubules long axis were found close to the DEJ. The HGM imaging enabled us to minimize artifacts caused by processes during sample preparation: we showed that we are able to measure HGM signals under aqueous solution and this allowed handling the sample in conditions closer to the *in vivo* state minimizing artifacts caused by drying. Penetration of the laser light into the sample eliminated artifacts caused during the preparation of the surface, and in addition, images were taken without any chemical labeling. This method may be incorporated in many topics related to the research on dentin, such as the hybridization of dentin with artificial materials, primary dentin development versus secondary dentin, dentin bacterial infection, and crack formation.

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