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Authors
Kimura, Yuichi
Wilder-Smith, Petra BB
Krasieva, Tatiana B
et al.

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Visualization and quantification of healthy and carious dentin structure using confocal laser scanning microscopy

Yuichi Kimura, Petra Wilder-Smith, Tatiana B. Krasieva, Anna Marie A. Arrastia-Jitosho, Lih-Huei L. Liaw, Koukichi Matsumoto*, and Michael W. Berns

Beckman Laser Institute and Medical Clinic, University of California, Irvine, 1002 Health Sciences Road East, Irvine, CA 92715; *Department of Endodontics, Faculty of Dentistry, Showa University, 2-1-1, Kitasenzoku, Ohta-ku, Tokyo 145, Japan

ABSTRACT

In this study, a new fluorescence technique was developed for visualization of dentin using confocal laser scanning microscopy (CLSM). Eighteen extracted human teeth were used: 13 showing no clinical signs of caries and 5 with visually apparent decay. Preliminary study: All teeth were horizontally sectioned to approx. 200 μm thickness and pre-treated as follows: no pretreatment; vacuum only; ultrasonication only; sodium hypochlorite (NaOCl) only; vacuum and NaOCl; ultrasonication and NaOCl; or vacuum, ultrasonication and NaOCl. Samples were stained with Rhodamine 123 fluorescent dye at a concentration of 10^{-5} M in phosphate buffer saline for 1 to 24 hours. Caries study: Dentin surfaces, some with pre-existing caries, were visualized using CLSM. Most dentin tubules in sound dentin appeared open using CLSM, but most dentin tubules in carious dentin appeared closed or narrowed. Surface images obtained using CLSM were similar to those seen by SEM, but additional subsurface imaging was possible using CLSM at depth intervals of 1 μm to a depth of 30-50 μm. This technique shows good potential for non-invasive surface and subsurface imaging of dentin structures.

KEY WORDS: biological images, dentin tubules, dental, imaging, fluorescent dye, three dimension, tooth

2. INTRODUCTION

Extensive modifications in its design since the development of scanning confocal optical system for the microscope in 1957 have allowed the application of this technology to many fields, including medicine.[1] Scanning confocal microscopes offer improved rejection of out-of-focus light and greater resolution than conventional imaging.[2] Today this form of microscopy represents a well recognized technique in the fields of biological and materials science. In the dental field, scanning confocal microscopy has been mainly used

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for assessment of dental operative procedures and restorations,[3-6] study of the tooth/restoration interface,[7] evaluation of enamel cutting effects,[8,9] visualization of enamel surface changes,[10] and clinical dental research.[11] However, this technique has not been used for visualization and quantification of dentin, of the caries process, and more specifically of carious dentin.

CLSM systems can scan a laser beam over stationary samples.[12] Surface images obtained using the confocal microscope are similar in character to those provided by scanning electron microscope (SEM), but subsurface imaging is also possible. Furthermore, confocal microscope images can be three dimensionally reconstructed.[12,13] Conventional dehydration and gold-coating methods utilized for SEM have several disadvantages when compared to sample preparation for CLSM: dehydration and heating can damage specimens and cause a variety of artifacts such as cracking or "bubbling". Tooth samples can be visualized using the confocal laser scanning microscope either by lightly metal-coating the surface, or by using fluorescence techniques.

Two basic types of confocal microscopy exist: real-time direct view tandem scanning microscopy (TSM) and CLSM.[12] The high-frame speed of the TSM allows real-time examination of teeth *in vivo*.[14,15] In contrast, all CLSM's rely on powerful image processing devices and frame stocks for information displays. The acquisition of perfectly registered, serial optical sections by this technique provides an excellent means for the three dimensional reconstruction and quantification of objects.

The purpose of this study was to develop a technique for use of CLSM for imaging and study of dentin. In addition to identifying appropriate dyes and fluorescence staining techniques, imaging depth and resolution were investigated. Results were compared with images from conventional SEM techniques to contrast the levels of information obtained and to assess any structural effects of the staining techniques developed in this study. Development and application of appropriate techniques for CLSM imaging and study of healthy and carious dentin was also priority. Moreover, the sensitivity of the system for imaging natural and induced caries conditions was investigated.

3. MATERIALS AND METHODS

3.1. Pretreatment study

Sample preparation

Seven extracted human teeth showing no clinical signs of caries, stored in demineralized water with 0.01 % (w/v) thymol, were horizontally sectioned into thin slices (approx. 200 μm thickness) using a low speed saw with coolant (Isomet, Buehler, IL, U.S.A.).

Pretreatment study

To determine optimal pretreatment conditions and procedures we undertook the following preliminary

investigations. Samples were treated with NaOCl (5.25 % by Wt, Darrow Comp., CA, U.S.A.) solution, vacuum and/or ultrasonication for 1 h. After pretreatment, samples were subjected to staining procedures. Rhodamine 123 (Eastman Kodak CO., NY, U.S.A.) with an absorption peak at 511 nm was used as the fluorescent dye, as this absorption peak corresponds most closely to the excitation wavelength of 488 nm used in our confocal laser microscope. Staining was performed at the concentration of 10^{-5} M for 3 h. After staining, samples were rinsed 2-3 times with demineralized water for 5 minutes. Sections were fixed to slide glasses with cyanoacrylate glue, and observed through the CLSM. Later, the same samples were prepared for conventional SEM procedures.

Staining study

To identify optimal fluorescence staining dyes and procedures we undertook the following preliminary investigations. In untreated samples, Rhodamine 123 concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M were tested at pHs 6.0, 6.5, or 7.4 and staining durations of 1, 2, 3, 4, or 24 h. The buffers used included 50 mM 2(N-morpholino) ethanesulfonic acid (MES) at pH 6.0, 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) at pH 6.5, and 10 mM phosphate buffer saline (PBS) at pH 7.4. After staining, samples were rinsed 2-3 times with demineralized water for 5 minutes. Sections were fixed on slide glasses with cyanoacylate glue, and surface staining intensity was determined by conventional fluorescence microscopy (Carl Zeiss, Germany). The effects on staining of ultrasonication and vacuum application for 1 h were also evaluated.

SEM

SEM was performed to identify the structural effects of the different variables in the fluorescence staining techniques. The samples were dehydrated in a graded series of aqueous ethanol (30, 50, 70, 90, 100 % ethanol) for 10 minutes at each concentration, mounted on stubs using colloidal silver liquid (Ted Pella, CA, U.S.A.) and gold coated on a PAC-1 Pelco advanced coater 9500 (Ted Pella, CA, U.S.A.). Micrographs of the dentin surface were taken on a Philips 515 (Mohawk, NJ, U.S.A.) SEM. Some samples were manually split at 90° to the surface, then gold-coated to allow visualization of sub-surface structures.

Confocal laser microscope device

Stained samples were examined using an LSM 410 inverted Zeiss laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Stacks of thin optical sections were obtained for each sample. The objective lens used was the Plan-Neofluar 100x bright field, n.a.1.3, oil immersion (Carl Zeiss, Oberkochen, Germany). The laser wavelength of 488 nm was used for fluorescence excitation; emission was isolated with a long pass 520 nm filter. The distance between optical sections was 1 μm or 2 μm on the Z-axis. Overall depth of acquisition ranged from approx. 20 μm to 60 μm depending on the depth of penetration of Rhodamine 123 into the sample. The information obtained was stored on 1 GByte optical disc (Panasonic) and 3-dimensional images were generated from stacks of stored images using original LSM 410 software.

3.2. Caries study

Sample preparation

Six extracted human teeth showing no clinical signs of caries and decay were selected. Three of these samples constituted group A (sound teeth). In the remaining 3 samples, caries-like lesions were artificially induced as described below (group B). Five additional teeth with pre-existing naturally occurring dentin caries made up group C of this study. Following factors were set as inclusionary criteria for the "natural caries" group: dentin was used from the tooth crown just below the amelodentinal junction and sections were prepared parallel to the amelodentinal junction; no gross structural cavitation or destruction as determined by the naked eye was apparent in the samples used; only darkly discolored carious dentin was used; and natural caries extended through the entire thickness of the sections under investigation. These criteria were established in an attempt to set a minimal standard of comparability between carious specimens. All teeth were stored in demineralized water with 0.01 % (w/v) thymol, and were horizontally sectioned into thin slices (approx. 200 μm thickness) using a low speed saw machine with coolant (Isomet, Buehler, IL, U.S.A.).

Induction of artificial caries-like lesion

All samples in group B were subjected to a demineralization solution for 3 days. The demineralization solution contained 0.1 M lactic acid, 0.2 mM Methylene Diphosphonic Acid (MHDP) at pH 4.8 and 0.01 % thymol.[16]

Fluorescence staining

After pretreatment with NaOCl (5.25 % by Wt) for one hour under vacuum and ultrasonication, samples were stained using Rhodamine 123 in PBS as the fluorescent dye at a concentration of 10^{-5} M. The dye was applied for one hour under vacuum and ultrasonication. After staining, sections were washed 2-3 times with PBS, blotted and fixed to slide glasses with cyanoacrylate glue.

Confocal laser microscope device

Same as in 3.1. Preliminary section.

4. RESULTS

4.1. Preliminary study

Untreated samples: SEM showed surface debris and ridges from sample preparation with the saw. Using CLSM, the surface image obtained was difficult to capture in focus, due to surface unevenness and focusing limitations. However, saw marks and surface debris were again visible. Subsurface images at 2 μm and deeper, showing dentin tubules, were much clearer than surface images and appeared in focus.

Vacuum treatment samples: SEM showed surface debris and ridges from sample preparation with the saw.

Using CLSM, the surface image was again difficult to obtain in focus, due to surface unevenness and focusing limitations. However, saw marks and surface debris were again visible, and subsurface images at 2 μm and deeper, showing dentin tubules, were much clearer and appeared in focus.

Ultrasonication treatment samples: Surface images obtained by SEM and CLSM were comparable. No surface debris was evident. Subsurface imaging was successful to a depth of 18 μm.

NaOCl treatment samples: Surface images obtained by SEM and CLSM were very similar. Minimal surface debris was observed. Subsurface imaging was successful to a depth of 30 μm. Tubule diameters appeared larger than in previous samples, with tubular walls showing marked fluorescence.

NaOCl and vacuum treatment samples: Surface images obtained by SEM and CLSM were comparable. No surface debris was evident. Subsurface imaging was successful to a depth of 22 μm. Using CLSM, the surface image obtained was difficult to capture in focus, due to surface unevenness and focusing limitations.

NaOCl and ultrasonication treatment samples: Surface images obtained by SEM and CLSM were comparable. Subsurface imaging was successful to a depth of 38 μm, and no surface debris was observed. Tubule diameters appeared larger than in previous samples, with tubular walls showing marked fluorescence.

NaOCl, vacuum and ultrasonication treatment samples: Surface images obtained by SEM and CLSM were similar. No surface debris was observed and subsurface imaging was successful to a depth of 38 μm. Tubule diameters appeared larger than in previous samples, with tubular walls showing marked fluorescence.

Especially in untreated samples, the smear layer caused by sample sectioning prevented a clear view of dentin tubular structure. Pretreatment with a combination of NaOCl, vacuum and ultrasonication permitted visualization at the deepest level approx. 40 μm. The CLSM surface views of the sections were very similar to SEM images. Additionally, subsurface structures could be observed with CLSM due to permeability of the smear layer to the fluorescence dye, Rhodamine 123. Dye penetration, and subsurface visualization, were improved using NaOCl and ultrasonication or a combination of NaOCl, vacuum and ultrasonication. This methodology permitted visualization of samples to a depth of approx. 30 - 50 μm after pretreatment, as compared to control samples, where visualization was possible only to a depth of approx. 20 μm. In generally, dye penetration was good after pretreatment, but inter-sample variability measured by up to a factor of three.

Staining study

Rhodamine 123 concentrations ranging from 10^{-3} M to 10^{-7} M were evaluated at pH 7.4 for 3 h exposure time. A concentration $\geq 10^{-5}$ M resulted in good quality images. Staining effectiveness at a pH of 6.0, 6.5, or 7.4 was investigated. Rhodamine 123 staining was best at a pH of 7.4. Immersion times $\geq$ 3 h resulted in good images. Effects of ultrasonication and vacuum pretreatment were small, but these techniques reduced the required immersion duration.

Comparison between SEM and CLSM

The images obtained by SEM or CLSM were very similar at a magnification of up to 1000x. This is the magnification limit of CLSM, but additional subsurface imaging is also possible.

4.2. Caries study

CLSM results in teeth with naturally-occurring caries fell into one of 3 groups:

The first type; Many dentin tubules in the carious lesion were closed or extensively narrowed, usually at a subsurface level. Thus, on the surface of the carious lesion, tubules are visible, but at deeper levels many could no longer be visualized. Dye penetration into the carious area was evidently very poor compared to the sound zone.

The second type; Here, typically, some dentin tubules in the carious area were narrowed, or completely blocked. The margins of the dentin tubules affected by caries were stained strongly compared to tubules in sound dentin. Dye penetration in these samples was reduced compared to healthy samples with visibility becoming problematic already at a depth of 15 μm or less.

The third type; Some dentin tubules were narrowed, only a few were completely blocked, and the margins of dentin tubules were again strongly stained. The dark zones seen in the CLSM images corresponded to patches of extensive carious destruction of the dentin surface.

Artificial caries-like lesions were investigated after 3 days induction. Many dentin tubules in the artificial caries-like lesion were still open, but dye penetration into the carious lesion was reduced as compared to sound dentin. The margins of the dentin tubules were strongly stained compared to sound dentin. The etched surface appearance was attributed to caries induction methods causing dentin surface dissolution.

5. DISCUSSION

A limited range of applications for scanning confocal microscopy has been investigated in the field of dentistry. Usually these involve methods for assessment of dental operative procedures and materials.[3-11] By eliminating the need for problematic and tedious specimen preparation techniques, confocal microscopy is well suited to the observation of dental and material surfaces, and to monitoring effects of various agents and factors on their microstructure. In these previous studies, however, TSM was mainly used, providing poor horizontal resolution.[12] Moreover, this technique has not been used for visualization and quantification of caries processes, and more specifically of carious dentine. Kodaka et al. reported dental applications of CLSM, but using metal-coated samples rather than fluorescent staining technique.[10] In addition to its costly, practically demanding and time-consuming nature, metal coating has the disadvantage of usually requiring sample dehydration, a process which can easily damage, alter or distort sample structure and produce multiple artifacts.

Compared with SEM, magnification provided by confocal microscopy and fluorescence techniques is low (maximum x1000), but within this range, CLSM allows surface and sub-surface visualization in three dimensions. Dimensional quantification is easy and accurate: thus we were able to measure the depth of carious lesions easily using marker systems on the computer screen. Disadvantages of CLSM techniques include its lack of suitability for clinical investigations, as relatively thin sections are needed. However, the high-frame speed of the TSM enables real-time examination of teeth *in vivo*.[14,15]

Our investigations of fluorescence staining techniques for dentin showed that NaOCl application only did not enhance dye uptake, but was very effective together with ultrasonication in achieving good staining. Similar surface images were obtained using SEM and CLSM, but it was possible to perform also non-invasive subsurface imaging in dentin using CLSM. The presence of a smear layer did reduce the effectiveness of fluorescence staining, but the smear layer was partially permeable to the fluorescent dye. Using the fluorescence techniques developed in this investigation, we were able to achieve good surface and subsurface images of dentin samples. Optimum staining conditions for visualization and quantification of dentinal structure were identified in this study. However, further improvements in subsurface visualization would be beneficial.

In the second part of this investigation, decayed dentin samples without obvious gross carious destruction or cavitation were used. Caries-induced zones of dentin destruction, demineralization and tubular occlusion were clearly visible using CLSM in our "natural caries" samples. These findings correspond with histopathological observations described by other authors.[17,18] Demineralization of the dentinal tubular walls and their immediate surroundings was also observed in some carious dentin samples. This observation may be attributed to the caries process. However, we noticed a similar effect in some healthy dentin tissues, after sample preparation using NaOCl and ultrasonication to enhance dye uptake. The same sample preparation technique with NaOCl and ultrasonication was used in these specimens. Thus, at this stage the exact origins of this observation remain to be elucidated, and further studies are necessary to assess optimal sample preparation techniques for CLSM.

The difference in appearance of "natural" vs "induced" caries in the CLSM was striking. Very few occluded or narrowed dentin tubules were observed in the "artificial caries" samples, and the entire sample surface had a strongly demineralised appearance. These results are in agreement with histopathological observations by others,[17,18] and are mainly attributed to the differences between the chemical induction techniques used in artificial caries, and the bacterially-related processes and pulpal reactive events occurring during natural decay.

We used CLSM for visualization and quantification of caries process and more specifically of caries dentin, and obtained useful and novel results. However, this technique requires development to optimize its potential.

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