VISUALIZATION AND QUANTIFICATION OF DENTIN STRUCTURE USING CONFOCAL LASER SCANNING MICROSCOPY

Yuichi Kimura,[†] **Petra Wilder-Smith,**[‡] **Tatiana B. Krasieva,**[‡] **Anna Marie A. Arrastia-Jitosho,**[‡] **Lih-Huei L. Liaw,**[‡] **and Koukichi Matsumoto**[†]

[†]Department of Endodontics, Faculty of Dentistry, Showa University, 2-1-1, Kitasenzoku, Ohta-ku, Tokyo 145, Japan; [‡]Beckman Laser Institute and Medical Clinic, University of California, Irvine, 1002 Health Sciences Road East, Irvine, California 92715, U.S.A.

ABSTRACT

Dentin was visualized using a new fluorescence technique and confocal laser scanning microscopy. Thirty extracted human teeth showing no clinical signs of caries were investigated. All teeth were horizontally sectioned to approximately 200 μm thickness and sections were subjected to different pretreatment conditions as follows: vacuum only, ultrasonication only, sodium hypochlorite only, sodium hypochlorite and vacuum, sodium hypochlorite and ultrasonication, and a combination of sodium hypochlorite, vacuum, and ultrasonication. Some samples were left untreated to serve as control. Following pretreatment, rhodamine 123 fluorescent dye was used for staining at concentrations ranging from 10^{-3} to $10^{-7}M$ for 1 to 24 h at pH 6.0, 6.5, or 7.4. Optical staining occurred at pH 7.4 and concentrations $\geq 10^{-5}$ M over 3 h or longer. Surface images obtained using confocal laser scanning microscopy were similar to those observed by scanning electron microscopy without the need for sample-altering conventional scanning electron microscope preparation techniques. Subsurface imaging to a depth of approximately 60 μm was achieved using confocal laser microscope techniques. This fluorescence technique offers a useful new alternative for visualization and quantification of dentin. © 1997 *Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(97)00303-1]

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1 INTRODUCTION

Since the development of a scanning confocal optical system for the microscope in 1957, this device has been extensively modified and applied in 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 for assessment of dental operative procedures and restorations,[3–6] study of the tooth/restoration interface,[7] evaluation of enamel cutting interactions,[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.

Confocal laser scanning microscope (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 the scanning electron microscope (SEM), but subsurface imaging is also possible. Furthermore, confocal microscope images can be reconstructed three dimensionally.[12,13] Conventional dehydration and gold-coating methods utilized for SEM have several disadvantages compared with 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 CLSMs 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 using CLSM to image and study dentin. In addition to identifying appropriate dyes and fluorescence staining techniques, imaging depth and resolution were investigated. The results were

Address all correspondence to Petra Wilder-Smith. Tel: (714) 824-4713; Fax: (714) 824-8413; E-mail: pwsmith@bli.uci.edu

Table 1 Overview of pretreatment conditions.

Sample	Sodium hypochlorite	Vacuum	Ultrasonication	pH	Dye concentration (M)	Dye exposure (h)
1	No	No	No	7.4	10^{-5}	3
2	No	Yes	No	7.4	10^{-5}	3
3	No	No	Yes	7.4	10^{-5}	3
4	Yes	No	No	7.4	10^{-5}	3
5	Yes	Yes	No	7.4	10^{-5}	3
6	Yes	No	Yes	7.4	10^{-5}	3
7	Yes	Yes	Yes	7.4	10^{-5}	3

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.

2 MATERIALS AND METHODS

2.1 SAMPLE PREPARATION

Thirty 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 (approximately 200 μm thickness) using a low-speed saw with coolant (Isomet, Buehler, Illinois).

2.2 PRETREATMENT STUDY

To determine optimal pretreatment conditions and procedures, we undertook the following preliminary investigations. Samples were treated as described in Table 1; sodium hypochlorite (NaOCl) (5.25% by wt, Darrow Co., California) application, vacuum, and/or ultrasonication treatments for 1 h were variable. After pretreatment, samples were subjected to staining procedures. Rhodamine 123 (Eastman Kodak Co., New York) with an absorption peak at 511 nm was used as the fluorescent dye because 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 to 3 times with demineralized water for 5 min. Sections were fixed to glass slides with cyanoacrylate glue and observed through the CLSM. Later, the same samples were prepared for conventional SEM procedures.

2.3 STAINING STUDY

To determine 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 pH 6.0, 6.5, or 7.4 and staining durations of 1, 2, 3, 4, or 24 h. The buffers used included 50 mM (2N-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 to 3 times with demineralized water for 5 min. Sections were fixed on glass slides with cyanoacrylate glue, and surface staining intensity was determined by conventional fluorescence microscopy (Carl Zeiss, Oberkochen, Germany). The effects on staining of ultrasonication and vacuum application for 1 h were also evaluated.

3 SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy was used 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, and 100% ethanol) for 10 min at each concentration, mounted on stubs using colloidal silver liquid (Ted Pella, California) and gold coated on a PAC-1 Pelco advanced coater 9500 (Ted Pella). Micrographs of the dentin surface were taken on a Philips 515 (Mohawk, New Jersey) SEM. Some samples were manually split at 90 deg to the surface, then gold coated to allow visualization of subsurface structures.

3.1 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 $\times$100 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 2 μm on
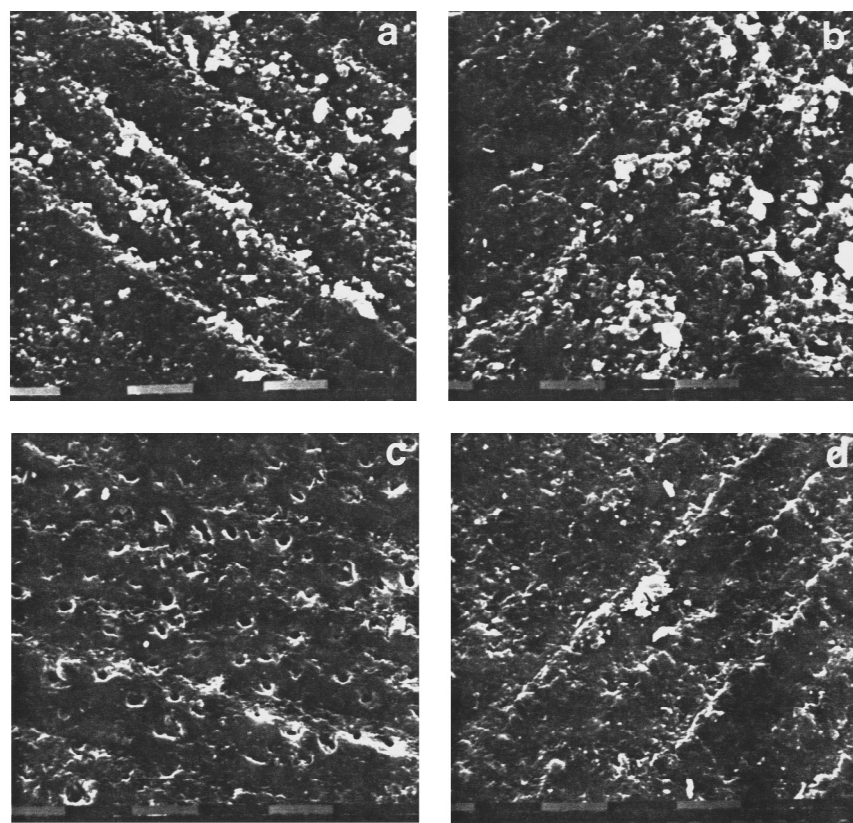
Fig. 1 SEM photographs: (a) no treatment, (b) vacuum treatment (for 1 h), (c) ultrasonication treatment (for 1 h), (d) sodium hypochlorite treatment (for 1 h), (e) sodium hypochlorite and vacuum treatment (for 1 h), (f) sodium hypochlorite and ultrasonication treatment (for 1 h), and (g) sodium hypochlorite, vacuum, and ultrasonication treatment (for 1 h). ×1010. The scale shows 10 μm.

the z axis. Overall depth of acquisition ranged from approximately 20 to 60 μm, depending on depth of penetration of rhodamine 123 into the sample. The information obtained was stored on a 1-GByte optical disk (Panasonic) and three-dimensional images were generated from stacks of stored images using original LSM 410 software.

4 RESULTS

4.1 PRETREATMENT STUDY

Figures 1(a) through 1(g) show SEM photographs of samples after various pretreatments. Figures 2(a) through 2(g) show CLSM images of the same samples as in Figure 1. In Figures 1(a) and 2(a) (untreated samples), SEM showed surface debris and ridges from sample preparation with the saw. With CLSM, the surface image obtained was difficult to capture in focus, due to surface unevenness and focusing limitations. However, saw marks and surface debris are again visible. Subsurface images at 2 μm and deeper, showing dentin tubules, are much clearer than surface images and appear in focus.

In Figures 1(b) and 2(b) (vacuum treatment samples), SEM showed surface debris and ridges from sample preparation with the saw. With CLSM, the surface image was again difficult to capture in

focus, due to surface unevenness and focusing limitations. However, saw marks and surface debris are again visible, and subsurface images at 2 μm and deeper, showing dentin tubules, are much clearer and appear in focus.

In Figures 1(c) and 2(c) (ultrasonication treatment samples), surface images obtained by SEM and CLSM are comparable. No surface debris was evident. Subsurface imaging was successful to a depth of 18 μm.

Surface images obtained by SEM and CLSM are very similar for Figures 1(d) and 2(d) (sodium hypochlorite treatment samples). 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.

In Figures 1(e) and 2(e) (sodium hypochlorite and vacuum treatment samples), surface images obtained by SEM and CLSM are 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.

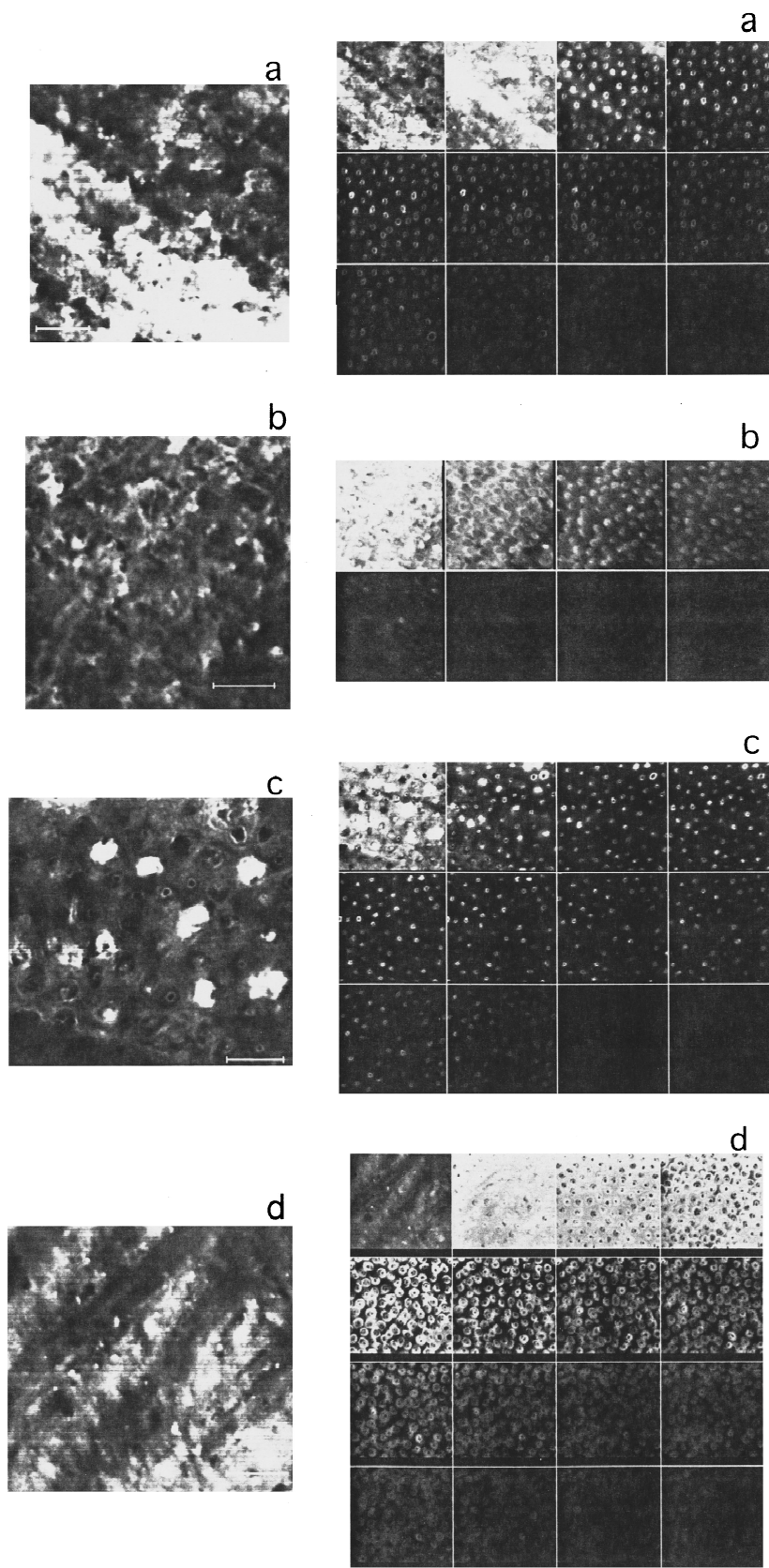In Figures 1(f) and 2(f) (sodium hypochlorite and ultrasonication treatment samples), surface images

Fig. 2 CLSM images on the same samples as in Figure 1: (a) no treatment, (b) vacuum treatment (for 1 h), (c) ultrasonication treatment (for 1 h), (d) sodium hypochlorite treatment (for 1 h), (e) sodium hypochlorite and vacuum treatment (for 1 h), (f) sodium hypochlorite and ultrasonication treatment (for 1 h), and (g) sodium hypochlorite, vacuum, and ultrasonication treatment (for 1 h). Each figure is a pair of surface view (left) and a series of 2-μm sections (right). $\times$1000. The scale shows 10 μm.
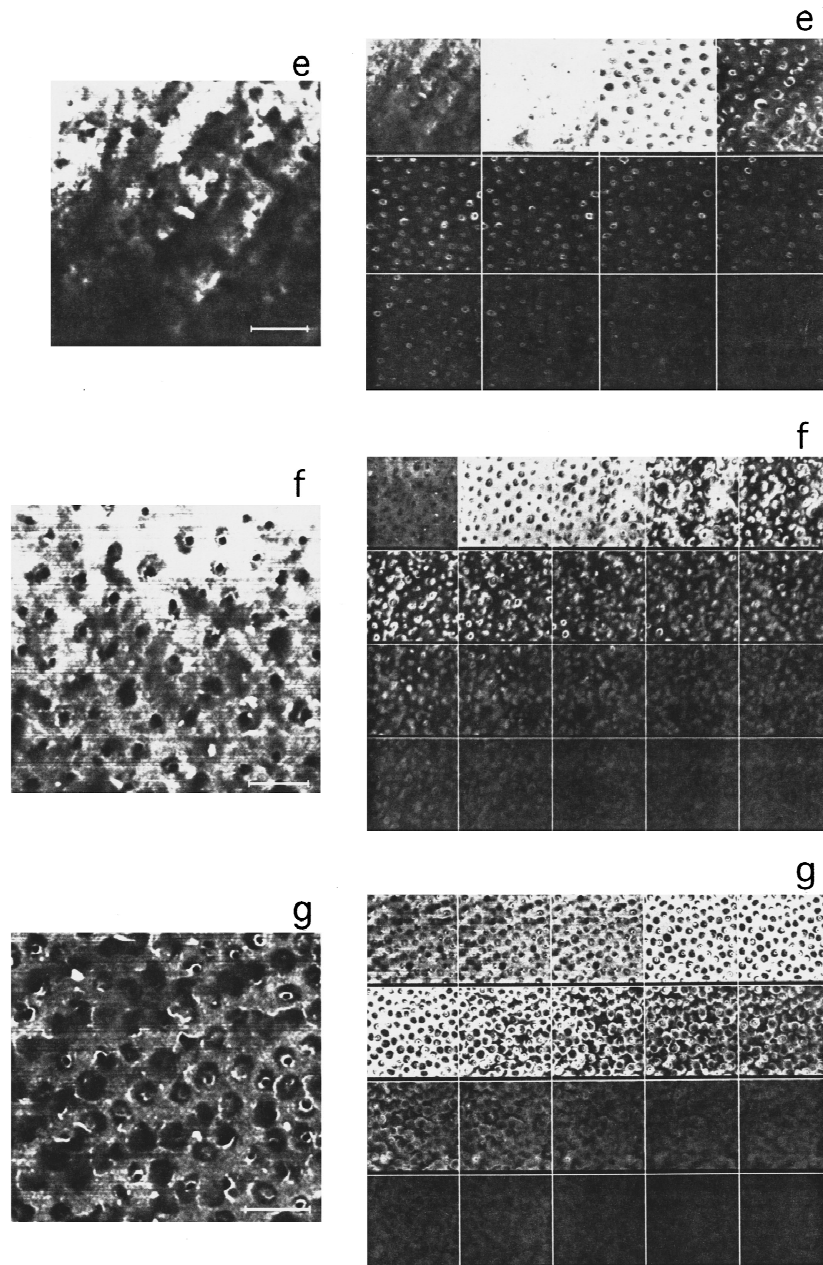
Fig. 2 (*Continued*).

obtained by SEM and CLSM are 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.

In Figures 1(g) and 2(g) (sodium hypochlorite, vacuum and ultrasonication treatment samples), surface images obtained by SEM and CLSM are 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 sodium hypochlorite, vacuum, and ultrasonication permitted visualization at the deepest level, 38 μm. The CLSM surface views of the sections were very similar to SEM images.

In addition, subsurface structures could be observed with CLSM due to the permeability of the smear layer to the fluorescence dye, rhodamine 123. Dye penetration and subsurface visualization were improved by using sodium hypochlorite and ultrasonication or a combination of sodium hypochlo-
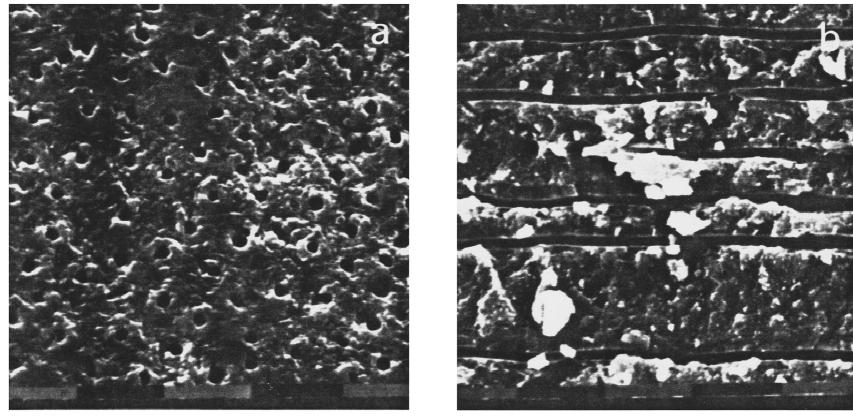
Fig. 3 SEM photographs: (a) surface view and (b) lateral view. Sample was pretreated with a combination of sodium hypochlorite, vacuum, and ultrasonication (for 1 h). ×1010. The scale shows 10 μm.

rite, vacuum, and ultrasonication. This methodology permitted visualization of samples to a depth of approximately 30 to 50 μm after pretreatment, compared with control samples, where visualization was possible only to a depth of approximately 20 μm. In general, dye penetration was good after pretreatment, but intersample variability measured up to a factor of 3.

4.2 STAINING STUDY

Rhodamine 123 concentrations ranging from 10^{-3} to $10^{-7} M$ were evaluated at pH 7.4 for 3 h exposure time. 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 time for immersion.

4.3 COMPARISON BETWEEN SEM AND CLSM

Figure 3 shows SEM photographs. Figure 3(a) shows the surface view and Figure 3(b) shows the lateral view. Figure 4 shows CLSM images in the same sample as in Figure 3. Figure 4(a) shows the surface view; Figure 4(b) shows subsurface scans; Figure 4(c) shows the accumulated 3-D view (0 deg); and Figure 4(d) shows the accumulated 3-D lateral view (90 deg). The images obtained by SEM or CLSM were very similar.

5 DISCUSSION

A limited range of applications for scanning confocal microscopy has been employed in the field of dentistry. Usually applications involve methods for assessing dental operative procedures and materials.[3–11] By eliminating the need for problematic specimen preparation techniques, confocal microscopy is well suited to the observation of dental and material surfaces and to monitoring the effects of various agents and factors on their microstructure. In these earlier studies, however, TSM was mainly used, although the use of CLSM was also reported.[12]

Other dental applications of CLSM have been reported,[10] but using metal-coated samples, rather than fluorescent staining techniques. In addition to its costly, demanding, and time-consuming nature, metal coating has the added disadvantage of usually requiring sample dehydration, a process that 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 ×1000), but within this range CLSM allows surface and subsurface visualization in three dimensions using a fluorescent dye. Dimensional quantification is easy and accurate: thus we were able to follow and measure the course and dimensions of dentin tubules easily using marker systems on the computer screen. The disadvantages of CLSM techniques include its lack of suitability for clinical investigations because relatively thin sample 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 sodium hypochlorite 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 also perform noninvasive subsurface imaging in dentin using CLSM. The presence of a smear layer reduced 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.
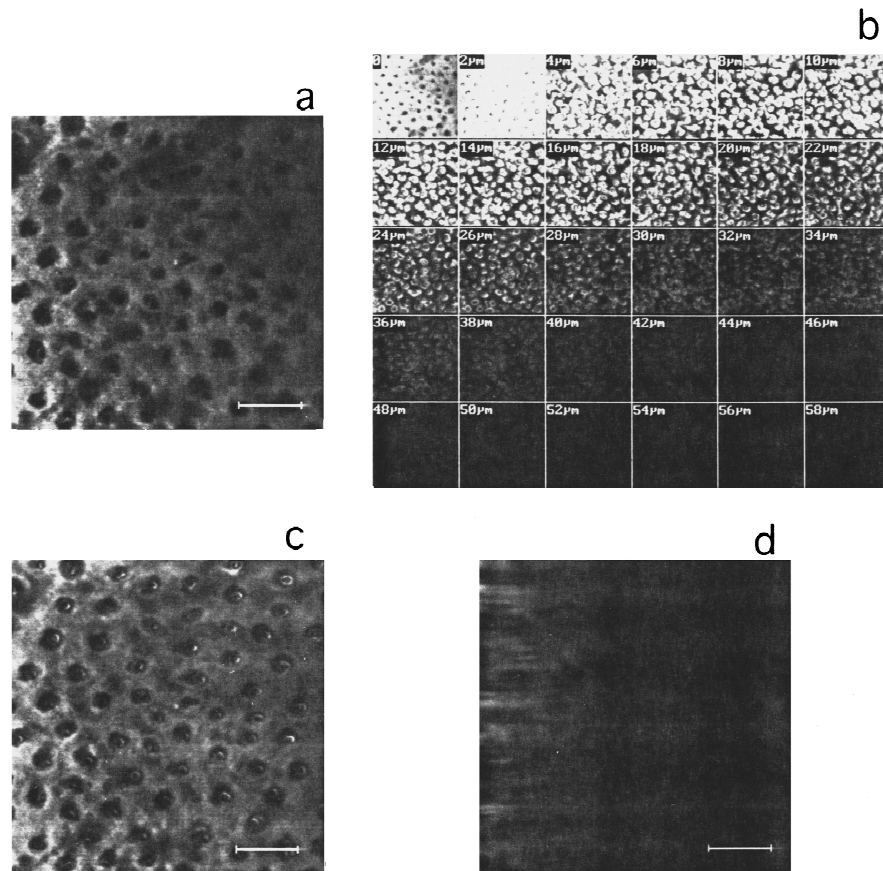
a

b

c

d

Fig. 4 CLSM images of the same sample as in Figure 3: (a) surface view, (b) series of 2-μm sections, (c) accumulated 3-D view (0 deg), and (d) accumulated 3-D lateral view (90 deg). ×1000. The scale shows 10 μm.

However, further improvements in subsurface visualization would be beneficial.

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