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Confocal Laser Microscopic Images of *In Vitro* Cariogenic Biofilm and Resulted Demineralized Dentin

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INTRODUCTION

Dental caries or tooth decay is a bacterium induced disease process that initiates with the adhesion, proliferation of oral bacteria, eventual biofilm formation and acid secretion causing dental hard tissue demineralization [1-3]. *Streptococcus mutans* (*S. mutans*) is known as the leading bacteria involved in developing dental caries and even secondary caries as per more than five thousand clinical study and in vitro research reports [4-6]. A good number of researchers all around the world use laboratory strains of *S. mutans* to form biofilms in vitro to find answers to the global issue of tooth decay [6-8]. An oral biofilm reactor (OBR) in our facilities is being used for in vitro formation of cariogenic biofilms aiming to contribute in preventing caries or secondary carie [9,10]. Several methods of analysis have been employed to investigate the *S. mutans* biofilms after formation on enamel or dentin surfaces in those studies - including imaging with light microscope, fluorescence microscope and scanning electron microscope [8-10]. However, images from a confocal laser scanning microscope (CLSM) capable of reproducing surface topography of unstained biofilms with color differentiated depth profile has not been reported.

MATERIALS AND METHODS

Rectangular shaped dentin specimens were prepared from the bovine incisor roots. The specimens' surfaces were ground flat and then polished by diamond slurries with particle sizes up to 0.25 µm. Specimens were washed in an ultrasonic bath with deionized water, ethanol and again with deionized water each for 15 min to remove smear plugs and micro-biomes. Scanning was carried out using a non-contact profilometer (NCP) with a red laser light source (VK-X150/X160, Keyence Co., Ltd., Osaka, Japan) and images were saved.

S. mutans biofilms were formed on the dentin specimens using *S. mutans* MT8148; a laboratory strain, as described elsewhere [8,9]. In short, sucrose supplemented (1% sucrose in Heart Infusion medium) biofilms were grown on the dentin specimen surfaces inside two identical water jacket-encircled chambers of an oral biofilm reactor (OBR) for 24 h. All through the biofilm formation period the pH was continuously recorded. After biofilm formation inside the OBR chambers, each specimen containing the biofilms was gently removed from the Teflon holder.

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One set of specimens with the undisturbed biofilms were immediately scanned by the CLSM and 3D images were saved (1D biofilm). Then the biofilms were cleaned using 0.25 M NaOH and the specimens were washed with distilled water to remove the biofilms. CLSM images were taken again to record the demineralized dentin surfaces (1D biofilm attacked specimen). Another set of specimens were transferred to a 24-well culture plate and were incubated at 37°C in sucrose-HI medium (changed in every alternate day) to demineralize further for 7 days. Thereafter, scanned by CLSM with the undisturbed biofilms on their surface and 3D images were saved same as mentioned above (7D biofilm). Biofilms were then removed using the same method and CLSM images of the 7D demineralized dentin were taken (7D biofilm attacked specimen). All CLSM scanning were carried out using a non-contact profilometer (NCP) with a red laser light source (VK-X150/X160, Keyence Co., Ltd., Osaka, Japan) and Multifile analyser (VK-150-H1XM) software was used for 3-D image construction.

RESULTS AND DISCUSSION

The biofilms that were formed on the dentin specimens in 24 h appeared creamy-white slimy substances on necked eyes and top surface was not smooth characteristically. CLSM 3D images confirmed that showing mountains, valleys and river-like channels running in between (**Figure 1**, left). In this particular image, the height of the biofilm (or vertical thickness) appeared to be more than 60 µm as color gradation displayed unevenness between the biofilm clusters. Most interestingly, the top-most surface of individual clusters was not essentially smooth; porous appearances were remarkable in between already smoothened parts. CLSM 3D images of 7D biofilm have shown further maturation; in vertical direction in exceeded 240 µm (**Figure 1**, right). Color gradation clearly indicated that mountains, valleys continued growing - though river-like channels were disappearing and most of the top surface became significantly smooth. Also, porousness could not be detected - instead they appeared as low-pitched rough surfaces at this stage. Deep ditch-like whole could be detected.

Root dentin surface images are shown in **Figure 2**. The surface was remarkably smooth with open dentinal tubules - open tubule size was very small as obvious in the dentin before biofilm attack (left). After 1D biofilm attack the surface became rougher and tubular orifice became wider which are easily detectable on the image (middle). As biofilm attack continued for 7 days the surface roughened further and tubular orifice widened remarkably as demineralization continued (right). Some notable information with 3D color gradation of the biofilm and dentin demineralization was acquired with this VK-X series of CLSM. The VK-X achieves high-resolution sensing by using a 16-bit photomultiplier as its laser receiving element. Also, it is capable of nanoscale measurements, even from a-

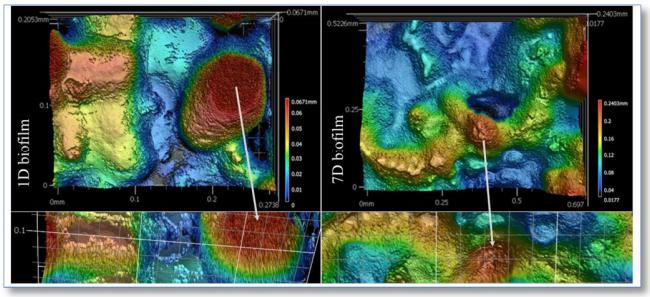

Figure 1. Confocal laser scanning microscopic (CLSM) images of *S. mutans* biofilms formed on dentin surfaces. Left-image is '1 day biofilm' and right-image is '7 day biofilm'. The reverse-sides of respective images are shown below (partially); arrows indicate porous part on 1D biofilms, are not seen on 7D biofilm.

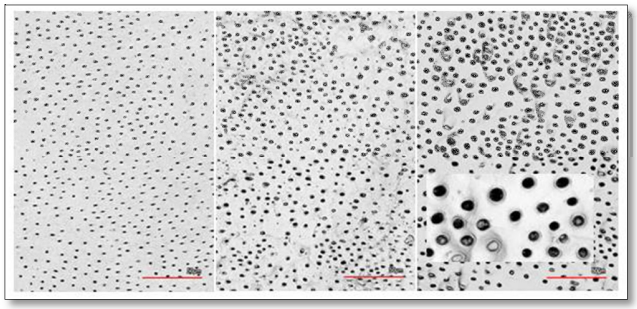

Figure 2. Confocal laser scanning microscopic (CLSM) images of demineralized dentin surface (bars are 50 µm). From left to right images are 'Before biofilm attack', 'After 1 day biofilm attack' and 'After 7 day biofilm attack' respectively. Inset is a high magnification image. In all images many clear open dentinal tubules are seen, some partially or completely blocked dentinal tubules are also seen.

-distance. The non-contact profilometeric image of the unstained biofilm could be taken in less than a minute unlike other biofilm imaging reported so far [11]. Therefore, biofilm maturation can be monitored at any stage without disturbing their growth. Also, the same biofilm specimen can be analyzed using any other method as the partially air dried biofilm remains unharmed. Identification of porous areas was possible due to the penetration and return of CLSM laser through premature or loosely condensed matrix of the biofilms and that would help in determining the time of application of antibacterial agents during in vitro studies. Clear images of normal dentin and demineralized dentin are also helpful for many in vitro experiments; e.g. testing degree of demineralization and remineralization of dental hard tissues [12].

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