Influence of the Sonicare toothbrush on the structure and thickness of laboratory grown *Streptococcus mutans* biofilms

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ABSTRACT: ***Purpose:*** To evaluate the effect of powered brushing with the Sonicare electronic toothbrush on the structure and thickness reduction of *S. mutans* biofilms using digital time-lapse microscopy (DTLM) and confocal microscopy (CM) techniques. ***Materials and Methods:*** *S. mutans* biofilms grown on glass microscope slides on BHI and 2% sucrose were exposed to Sonicare for 15 seconds with the bristle tips just contacting the slide, and at distances of 0.5, 1.0, and 1.5 mm above the slide. ***Results:*** With direct bristle contact, the reduction in biofilm thickness was greater than 99%. DTLM showed the break up and detachment of biofilm caused by the shear forces generated by the rapid bristle motion in real time. The Sonicare was shown to significantly reduce biofilm thickness even when the bristles were 1 mm above the biofilm. The percent biofilm thickness reduction was inversely proportional to the bristle distance. This study demonstrates the Sonicare toothbrush effectively removed biofilm from hard flat surfaces both by direct bristle contact and by fluid dynamic shear forces alone. (*Am J Dent* 2003;16;79-83).

CLINICAL SIGNIFICANCE: The effectiveness of the Sonicare to remove biofilm, even when the bristles were not in direct contact, suggests that the generated shear forces may help remove dental plaque in hard-to-reach areas such as interproximal spaces and periodontal pockets.

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Introduction

Dental plaque is a complex, multispecies biofilm that grows on the enamel and tissues of the oral cavity.[1-4] Routine removal of dental plaque is critical in maintaining high-quality oral health, since the succession of microorganisms that form plaque[1] are the causative agents for caries,[5,6] gingivitis and eventually periodontal disease.[7]

The most common way to remove dental plaque and disrupt this progression is through routine oral hygiene such as tooth brushing. Mechanical removal of dental plaque is achieved through direct contact and movement of toothbrush bristles across tooth and gum surfaces. Toothbrush movement can be performed manually or by the use of mechanically powered toothbrushes in which the bristle motion is generated by the device. Other plaque removing techniques, such as the use of dental floss or proxibrushes, also rely on direct contact with the plaque on the tooth surfaces. An alternative mechanism to remove dental plaque is an oral irrigator, which projects a high velocity fluid jet to shear away the dental plaque in hard-to-reach areas (*i.e.* interproximal surfaces). However, it is not certain that oral irrigators are effective plaque removers.[8,9]

Recently, a variety of powered toothbrushes have been developed to improve the efficiency of plaque removal in hard-to-reach areas using increased bristle velocity, brush stroke frequency, and new bristle patterns and motions. These characteristics utilize the fluid activity generated in the mouth by the electronic toothbrush. The short term use of mechanically powered toothbrushes has been shown to be equivalent to brushing with a manual toothbrush and superior for long term use.[10-12] The Sonicare[a] toothbrush uses a combination of direct mechanical brushing and fluid activity *via* a high velocity bristle motion at 260Hz.

Previous *in vitro*[13,14] and *in vivo*[15] studies involving the Sonicare have shown that similar fluid dynamic effects are capable of altering cell surface structures or removing adherent oral bacteria (*Streptococcus mutans, Actinomyces viscosus*, and *Porphyromonas gingivalis*).

Clinical studies[16,17] have shown that routine use of the Sonicare toothbrush performs better than routine use of a manual toothbrush in removing supragingival plaque, especially in hard-to-reach areas such as the interproximal regions and the posterior teeth. Studies[13,15,18] have also shown the Sonicare to be effective at removing attached bacteria from model dental surfaces or removing naturally formed dental plaque from human tooth enamel without direct bristle contact on the hard surface. Previous *in vitro* studies have relied on physical removal of biofilm remaining on the model surface after exposure and assessing removal efficacy using viable enumeration of organisms.[13-15] These studies were able to show nearly complete bacterial removal when bristles were touching the surface on which the bacteria were grown and significant bacterial removal when bristles were 3-4 mm away from the surface. The removal effect when bristles were not touching the surface was attributed to the fluid motion generated by the Sonicare toothbrush.[13,15,18]

This study investigated the effect of the Sonicare toothbrush on thickness and structure of a laboratory biofilm grown from a hard surface and quantified the effect at different bristle distances using direct visualization techniques of digital time-lapse microscopy (DTLM) and confocal microscopy (CSLM). The test organism was *Streptococcus mutans*, an early colonizer of tooth surfaces which is in part responsible for acid production and the progression of caries.[1,2,6]

Materials and Methods

Inoculum culture preparation - Thawed frozen stock culture (100 µl) of *Streptococcus mutans* UA159 (ATCC # 700610, www.atcc.org) was added to 100 ml Brain Heart Infusion

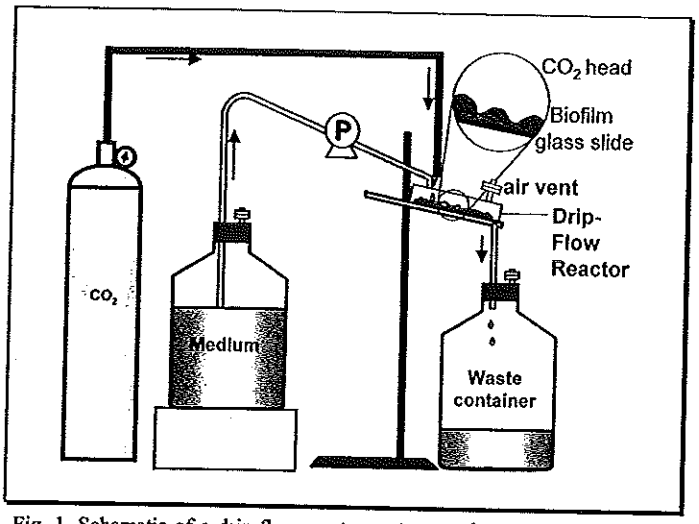

Fig. 1. Schematic of a drip-flow reactor system used to grow the *S. mutans* biofilms. Drip-flow reactor is shown in the continuous-flow mode of operation. An inset of the reactor shows the biofilm growing on a glass microscope slide, and the headspace being supplemented with CO_2.

(BHI) supplemented with 2% sucrose. Cultures were incubated at 35°C overnight in an anaerobic chamber with CO_2 gas generating cartridges. The viable cell density of the inoculum was determined by serially diluting and plating onto solid BHI agar + 2% sucrose. To disperse aggregates the culture was vigorously vortexed for 30 seconds initially and then for 5 seconds between each transfer and again for 5 seconds before plating. The plates were incubated overnight in an anaerobic chamber with CO_2 gas generating cartridges. The inoculum concentration was $1.9 \pm 1.7 \times 10^8$ CFU/ml (mean ± 1 S.D., n=5).

Drip-flow reactor preparation and sterilization - Biofilms were grown on glass microscope slides in four channels of a drip-flow reactor (Fig. 1).[19,20] The microscope slides were cleaned with soap and water, rinsed with 95% ethanol, then rinsed thoroughly with reagent grade water. The glass slides were placed in each chamber of the drip-flow reactor and secured with autoclave tape. Tubing was attached to the drainage system of the drip-flow reactor and the entire system was wrapped in foil and autoclaved for 20 minutes. Silicon tubing that feeds the continuous flow nutrients into the drip-flow reactor was autoclaved at 121°C for 20 minutes.

Biofilm growth - Approximately 15 ml of BHI + 2% sucrose was added to each of four chambers of a sterile drip-flow reactor and 3 ml of an overnight culture of *Streptococcus mutans UA159* were added to each chamber of the drip-flow reactor. The channels were sealed using lids tightened down with Teflon screws. CO_2 flowed through the headspace of each chamber. The drip-flow reactor was incubated in batch at 37°C in a level position for 24 hours to allow the *S. mutans* cells to attach to the glass slide. After the batch phase, the drip-flow reactor was placed onto a block inclined at 10°, the effluent opened, and 1/10 BHI + 2% sucrose was pumped into each chamber at approximately 0.5 ml/minute for an additional 48 hours (Fig. 1). The CO_2 continued to flow through the headspace of each chamber during the continuous flow period.

Sampling for biofilm viable cell density - After the continuous flow period of 48 hours, one of the slides was aseptically removed from the reactor and placed into a 100 ml sterile glass beaker containing 50 ml sterile dilution buffer. The biofilm was scraped from the surface of the slide using the flat end of a flame-sterilized chemical spatula. The resuspended biofilm was homogenized at 20,500 rpm for 1 minute using a Tekmar tissue homogenizer, serially diluted, and drop plated onto BHI agar + 2% sucrose.

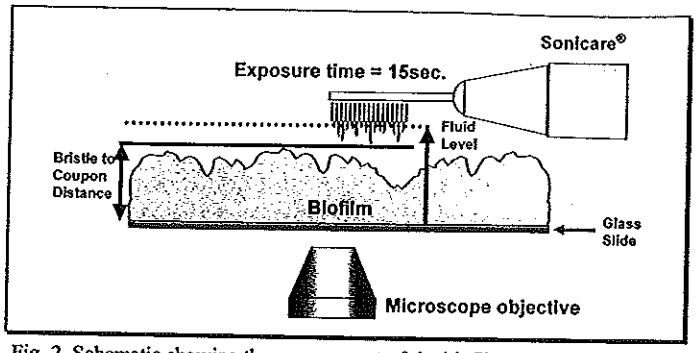

Fig. 2. Schematic showing the arrangement of the biofilm colonized slide, the toothbrush, and the microscope objective during exposure.

Exposure of intact biofilm to the Sonicare - Remaining slides were aseptically removed and placed onto a tissue to blot the underside of the slide while the surface with the biofilm remained hydrated. The coupon was glued to the inside of a lid of a sterile Petri plate using super-glue to prevent the slide from moving during powered brushing. The Petri plate was placed onto the stage of an inverted microscope, and approximately 20 ml of nanopure water was gently pipetted over the biofilm. The toothbrush was positioned so that the longest bristles of the brush head were at a known distance from the coupon surface (0.0, 0.5, 1.0, or 1.5 mm). The brush head of the Sonicare toothbrush was kept in a stationary horizontal position and activated for 15 seconds (Fig. 2). The Sonicare exposure parameters in this study were chosen to establish a comparison between this biofilm and dental plaque models described previously.[15] The brushing motion was digitally monitored by CCD camera (COHU 4612-5000[b]) and a Scion VG-5 PCI[c] framestore board using Scion Image software (available at http://www.scioncorp.com/). Each biofilm coupon was exposed to powered brushing in two separate locations, once on each end of the coupon, avoiding the edge of the slide. After exposure to the Sonicare, the biofilm was stained with the BacLight[d] live/dead kit using 1.5 µl SYTO9 + 1.5 µl propidium iodide per ml of nanopure water and incubated for 1 hour before visualization with confocal microscopy. The live/dead kit is sensitive to membrane integrity and when excited at 488 nm "live" cells appear green and "dead" cells appear red. These stains were used to aid in visualization of the biofilm and to assess the influence of brushing on the viability of cells within the biofilm that had not been mechanically removed.

Biofilm thickness measurements and biofilm inspection using confocal scanning laser microscopy (CSLM) - The influence of powered brushing on the biofilm thickness and structure was also assessed by confocal microscopy using a Leica TCS-NT.[e] Biofilm thickness was measured in five randomly chosen locations in an area on the slide that was either exposed or unexposed to brushing. In the "exposed" location thickness measurements were taken in the center region, at

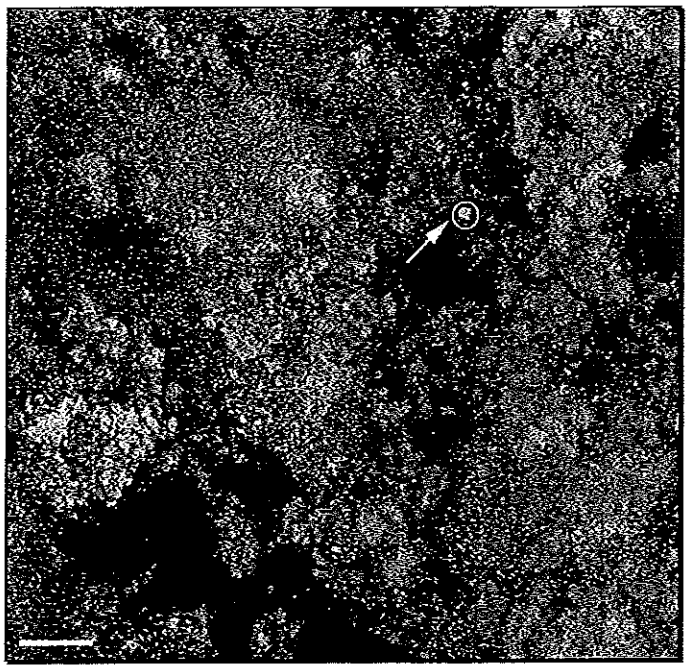

Fig. 3. 72-hour *S. mutans* biofilm prior to exposure to Sonicare. Individual cells aggregated into spherical structures (indicated by arrow) which further aggregated to form larger secondary structures of cell clusters separated by interstitial voids and channels (dark areas). Scale bar = 100μm.

least 2 mm inside the perimeter of where the overlying brush head had been positioned, to avoid the thickness gradient at the exposed edge. Thickness measurements in the "unexposed" region were taken at locations between 2 mm and 1 cm beyond the area overlaid by the brush head. The percent biofilm thickness reduction was calculated from:

$$\frac{\text{(Unexposed biofilm thickness – Exposed biofilm thickness)}}{\text{Unexposed biofilm thickness}} \cdot 100\%$$

Statistical analysis - Linear regression and one-way ANOVA were conducted using Quattro Pro 9.[f] Differences between means were considered significant for $P < 0.01$.

Results

After 72 hours, *S. mutans* had formed extensive biofilms on the glass slides. The biofilms were heterogeneous, ranging in thickness from approximately 100 μm to 500 μm. The *S. mutans* cells in the biofilm were aggregated in spherical clusters varying in size from several cells to 250 μm in diameter (Fig. 3). The average concentration of viable cells in the unexposed biofilm was $8.6 \pm 4.1 \times 10^7$ CFU/cm^2 (n = 30).

When the toothbrush was turned on, the rapid vibration of the bristles caused regions of localized turbulence in which entrapped air bubbles and fluid were seen swirling over the surface of the glass slide. The swirling motion caused biofilm to break up and detach. After the brushing was complete, pieces of detached biofilm were observed floating above the glass slide (time-lapse and 3D confocal movies showing the bristle motion and biofilm removal can be viewed at http://www.erc.montana.edu/Res-Lib99-SW/Movies/2002/02-M010_M013.htm). When the bristles contacted the slide surface (bristle distance = 0 mm), almost all of the biofilm was removed apart from a few small patches of cells (Fig. 4). When the bristles were positioned above the biofilm, an

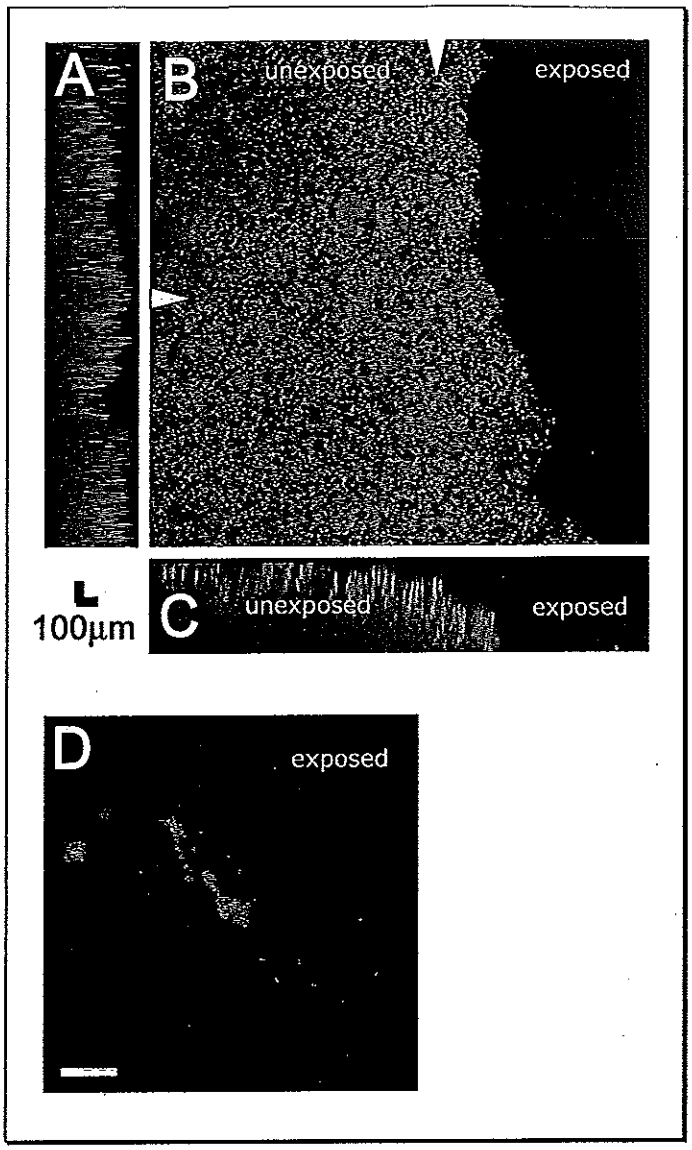

Fig. 4. Confocal image of *S. mutans* biofilm after a 15-second exposure to Sonicare when bristles were contacting the surface of the glass slide. Scale bar equal to 100 μm. This micrograph shows almost complete removal of biofilm in the area exposed to Sonicare (exposed region of panel B). At this magnification the bright dots are the spherical aggregates formed from single cells. Panels A and C are the vertical and horizontal cross sections through the biofilm in the locations indicated by the white arrows. Panel C transects the exposed area. Panel D is a high magnification image showing a small group of individual cells which remained attached in the exposed area. Scale bar = 50 μm.

opaque layer of remaining biofilm could be seen after exposure. CSLM cross sections across the exposed and unexposed areas showed the effect of powered brushing on biofilm thickness reduction (Fig. 5). The average biofilm thickness in the unexposed areas for all experiments was approximately 300 μm. Figure 6 shows the biofilm thickness in exposed and unexposed areas for all bristle heights. The percent reduction in thickness in the exposed areas relative to the unexposed areas decreased in linear proportion with increasing bristle distance from greater than 99% reduction at 0 mm to approximately 20% reduction at 1.5. One way ANOVA showed that these thickness reductions were significant at the 99% confidence level ($P < 0.01$) for bristle distances of 0.0, 0.5, and 1.0 mm. Linear regression showed that the percent thickness reduction was inversely proportion-

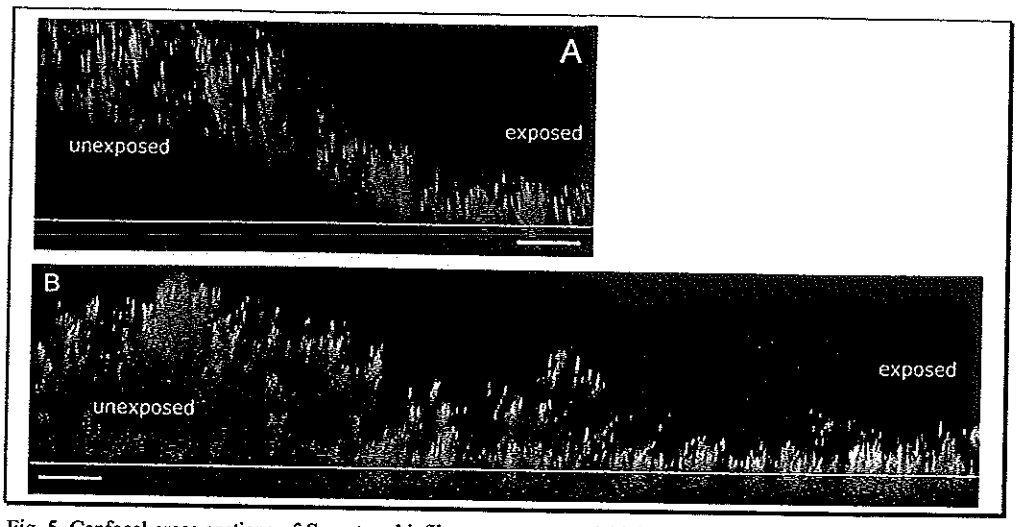

Fig. 5. Confocal cross-sections of *S. mutans* biofilms across exposed (right) and unexposed (left) areas at bristle to surface distances of 0.5 mm (panel A) and 1.0 mm (panel B) showing thickness reduction of biofilm caused by powered brushing. The thin horizontal white line indicates the surface of the glass slide. The "hollow" region in the unexposed area where the biofilm was thicker was an artifact caused by the limits of penetration of the confocal laser. Scale bar = 50 μm.

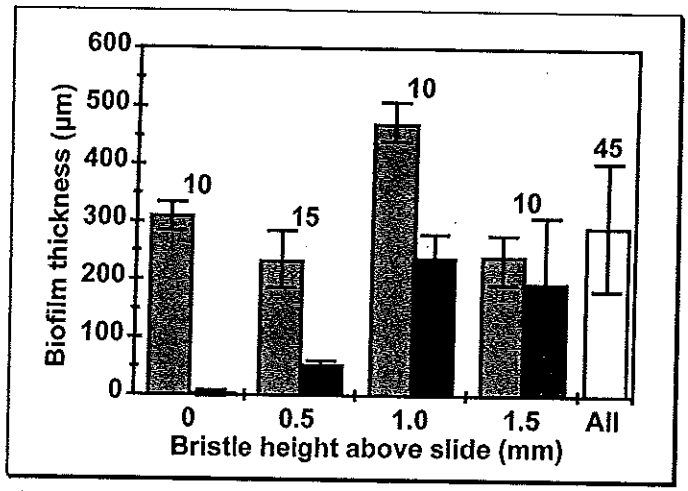

Fig. 6. Biofilm thickness measurements in unexposed areas (gray bars) and exposed areas (black bars) after 15-second exposure to powered brushing. Bars = ± 1.0 S.D. of the sample mean, n= 5 measurements from 2 or 3 slides given above bars. The open bar shows the mean biofilm thickness for all unexposed biofilm measurements.

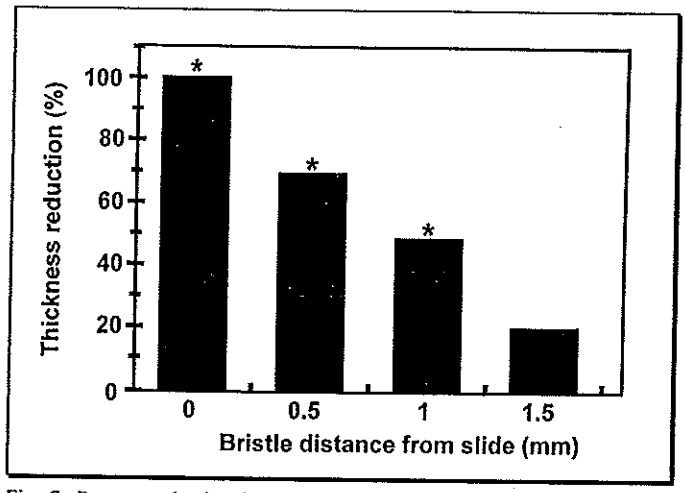

Fig. 7. Percent reduction in biofilm thickness in the exposed areas as a function of bristle height above the glass surface. Asterisks above bars indicate the bristle heights that gave a significant reduction in biofilm thickness ($P < 0.01$). $P = 0.20$ for bristle height of 1.5 mm.

al to the bristle height above the slide ($r^2 = 0.995$, n=4) (Fig. 7). There was no discernable difference in the distribution of red and green cells in the biofilm between the unexposed biofilm and the remaining biofilm in the exposed areas. Cells appearing yellow indicated that cells had taken up both stains and the membrane integrity and viability of these cells was, therefore, ambiguous.

Discussion

After 2 days, *S. mutans* formed an extensive, structurally heterogeneous, biofilm up to 500 μm thick in the drip flow reactor. The biofilm architecture consisted of cell clusters separated by channel and voids, and was similar to oral plaque biofilms grown *in vivo*.[21] The drip flow reactor is a good model system for growing oral biofilms because it provides an environment with similarities to that of the oral cavity. Growth surfaces are bathed in a thin (< 0.5mm) film of media providing the biofilm with constant moisture and nutrients. The CO_2 headspace facilitates gas exchange. Additionally, the drip flow reactor is inexpensive and simple to operate. Growth of biofilms on transparent glass microscope slides allowed direct microscopic visualization of the biofilm before, during, and after exposure to the Sonicare. Although *S. mutans* does not represent the complex microbial community found in dental plaque, this species is an early colonizer that readily forms biofilms and is often used as a study model.[1,2] The thickness and heterogeneity of the *S. mutans* biofilm suggests that localized micro-aerophilic and anaerobic regions would develop in the biofilm[22] demonstrating the potential of this system to cultivate more ecologically complex dental biofilms which could be achieved by using human saliva as an inoculum. Also, for future studies, *in vivo* conditions may be better mimicked by coating the surface of the glass slide with hydroxyapatite.

DTLM was used to visualize the turbulence created by the bristles and the effect of this on the mechanical detachment and structural deformation of biofilm in real time from a hard flat surface in an *in vitro* model. CSLM clearly showed the influence of Sonicare on biofilm structure and thickness at

various bristle heights above the substratum. Although Sonicare was shown to physically remove biofilm, the live/dead stain suggested that there was no influence on the viability of the remaining biofilm bacteria in the exposed areas. The percent of biofilm thickness reduction increased as the bristles were positioned closer to the glass slide, as expected. However, even at a bristle distance of 1.5 mm there was a 20% reduction in biofilm thickness. At a bristle to surface distance of 1.0 mm, the reduction in biofilm thickness was approximately 50% after 15 seconds of mechanical brushing. This trend complements a previous study by Stanford *et al.*[15] who used viable cell counts as a method of assessment of biofilm removal which reported a nearly 80% removal of human dental plaque cells adhering to hydroxyapatite at a bristle distance of 3 mm and over 99% reduction of biofilm/cells when bristles were contacting the surface after 15 seconds of exposure to Sonicare. Specific differences between percent reductions and the present study may be attributed to the different methods used to grow biofilms and the difference between culturing and direct observation methods used to assess biofilm removal.

It is possible that the removal of biofilm through the generation of projected fluid shear away from the bristles, which we directly observed, may explain clinical studies in which the Sonicare was more effective at removing supragingival plaque from hard-to-reach areas, including interproximal regions, than manual brushing. In future studies our microscopic techniques, which allow both the visualization of flow patterns generated by powered brushing and the resulting detachment of biofilm plaque in real time, may be used to test this hypothesis by the use of *in vitro* models more closely representing interproximal spaces and gingival crevices.

a. Philips Oral Healthcare, Inc., Snoqualmie, WA, USA.
b. Cohu, Inc., San Diego, CA, USA.
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