Preliminary Study of *In Vivo* Formed Dental Plaque Using Confocal Microscopy and Scanning Electron Microscopy

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**Abstract:**

**Objective:** Confocal laser scanning microscopy (CLSM) is relatively a new light microscopical imaging technique with a wide range of applications in biological sciences. The primary value of CLSM for the biologist is its ability to provide optical sections from a three-dimensional specimen. The present study was designed to assess the thickness and content of *in vivo* accumulated dental plaque using CLSM and scanning electron microscopy (SEM).

**Materials and Methods:** Acroflat lower arch splints (acrylic appliance) were worn by five participants for three days without any disturbance. The formed plaques were assessed using CLSM combined with vital fluorescence technique and SEM.

**Results:** In this study accumulated dental plaque revealed varied plaque microflora vitality and thickness according to participant’s oral hygiene. The thickness of plaque smears ranged from 40.32 to 140.72 µm and 65.00 to 128.88 µm for live (vital) and dead accumulated microorganisms, respectively. Meanwhile, the thickness of plaque on the appliance ranged from 101 µm to 653 µm. CLSM revealed both dead and vital bacteria on the surface of the dental plaque. In addition, SEM revealed layers of various bacterial aggregations in all dental plaques.

**Conclusion:** This study offers a potent non-invasive tool to evaluate and assess the dental plaque biofilm, which is a very important factor in the development of dental caries.

**Key Words:** Microscopy, Confocal; Microscopy, Electron; Dental Plaque; Fluorescent Antibody Technique

**INTRODUCTION**

An important factor in the development of oral diseases is accumulation of dental biofilm on tooth surfaces. The general term for the diverse microbial community accumulated on the teeth surfaces is dental plaque. Dental plaque is defined as bacterial aggregation on the teeth or other solid oral structures [1]. Similar to other biofilms, dental plaque exhibits an open architecture. The open architecture consisting of channels and voids facilitates the flow of nutrients, waste products, metabolites, enzymes, and oxygen through the biofilm [2]. Because of this structure, a variety of microbial organisms including both aerobic and anaerobic bacteria can make up biofilms. The microbial composition of dental biofilms includes over 700 species of bacteria and archaea, which all exist in a relatively stable environment called microbial homeostasis [3].
Dental plaque biofilms are responsible for many of the common diseases of the oral cavity including dental caries, periodontitis, gingivitis, and the less common peri-implantitis. There is increasing evidence that the amount and composition of plaque is directly related to the degree of periodontal health [4]. Biofilms are present on healthy teeth as well. In fact, biofilm accumulate on all surfaces in the mouth, regardless of whether these are natural, artificial or dental materials’ surfaces.

In vivo investigation of oral biofilms is extremely difficult, as they are very thin and usually inaccessible. Moreover, the use of chemical and radioactive markers in the oral cavity is unacceptable. Several methods have been used to view the microstructure of these biofilms including light microscopy [5], transmission electron microscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Electron microscopy usually requires specimen preparation involving dehydration, which may cause disruptive shrinkage and loss of biofilm matrix [6,7].

CLSM provides penetrative views for specimens. This method was initially applied almost exclusively in cell biology and, more recently, CLSM has found potential applications in dental caries and dental plaque investigations [8]. CLSM is based on the principle of eliminating stray light from out-of-focus planes by means of confocal apertures. Images are acquired by scanning the sample with a spot-sized light source (~1 µm diameter) and by recording the light reflected from the in-focus plane. Topography is made possible by recording a series of consecutive images in both the x-y and x-z planes. Depth movement of the sample is made possible by a fine-focusing object table, which is moved in the z-direction. CLSM allows for the study of unsectioned, naturally moist teeth. When used to visualize the outermost surface/subsurface areas, CLSM requires no sample preparation [8]. Previous studies of dental plaque have shown that using CLSM it is possible to examine the structure of oral biofilms grown under conditions similar to those which would exist in vivo [1,9].

The present study was designed to assess the thickness and content of in vivo accumulated dental plaque using CLSM and SEM. This study was designed to investigate the microstructure of intact dental plaque (oral biofilms), grown in acrylic appliance (acroflat lower arch splint) worn by highly oral hygienic dental student volunteers without disturbing the biofilm structure using CLSM and SEM.

MATERIALS AND METHODS
This study was approved by the Ethical and Research Committee of University Science,
Fig 2. Profile paragraph for the plaque accumulated. (left) Shows the distribution of living microorganisms, (right) Shows the distribution of dead microorganisms.

Malaysia. Participants already signed written consent form prepared by the research group (School of Dental Sciences, University Sains Malaysia). Five dental students with a high level of oral hygiene were selected for this study (School of Dental Sciences, University Sains Malaysia). The participants wore acroflat lower arch splint (Acrylic appliance) for three days without any disturbance. The appliance was removed off at the end of period. The appliance specimens were carefully transferred to physiological saline for five minutes and cut into two parts.

In order to study the morphology of plaque microorganism's contents, smears from plaque adhering to the first part of the appliance were spread on glass slides and immediately stained with fluorescein diacetate and ethidium bromide according to established procedures [9,10]. These samples were examined using Laser scanning confocal microscopy (Leica TCS SP II, Germany) and examined with an argon (514/488 nm) and HeNe (543 nm) laser, using × 20 objective lens. For further processing, the data were stored. Series sections were done for each image and the Z-step was 5.0 μm. By xyz mode scanning of sample at various focal planes along the Z-axis, a three-dimensional data set was acquired for samples using three-dimensional reconstruction software. Confocal microscopy combines fluorescence microscopy, laser light, and computer image processing. The ability of the laser beam to be focused with high precision enables breakout of samples parts that are out of focus, resulting in visualization of a single focal plane of a bulk sample over a given time period. The image was analyzed using a profile program for thickness of the plaque area.

CF2D (visualize the frequency distribution of intensities in a two-dimensional cytofluorogram), CF3D (visualize the frequency distribution of intensities in a three-dimensional cytofluorogram) and densitometric analysis were performed for each plaque to measure plaque intensity.

The following control and test series were run with CLSM: 1. Estimation of blank Acrylic to record their virgin structure (background control); 2. Recording of vital-stained plaque smears on Acrylic ("staining control"); 3. Evaluation of undisturbed human dental plaque and analysis of undisturbed plaque biofilms accumulated on acroflat lower arch splint. One plaque-covered spot located in the center of each specimen was analyzed in the confocal microscope.

The second part of the specimens were kept in a 1% cacodylate buffer solution containing 1% gluteraldehyde and 4% formaldehyde at +4°C for 24 hours. As for the post-fixation procedure; after the fixative agents washed away with cacodylate buffer, samples were again kept for two hours in another solution (1% osmium tetroxide [OsO₄] buffered with cacodylate buffer) at a low room temperature (2-10°C). Following the repeated cleansing procedure with cacodylate buffer, samples were dehydrated in increasing concentrations (25%, 50%, 75%, and 100%) of ethanol. The samples then were immersed 3 times in hexamethyldisilazane (HMDS) (each time for 10 min) and
air-dried at room temperature. Samples were mounted on stainless stubs with double sticky tabs. The samples were coated with gold and examined with SEM (Leica Cambridge S360 at 10 KV). Blank Acrylic was used as control.

RESULTS
Confocal microscopy observations a subsurface aspect of blank Acrylic appliance showed no self-fluorescence (auto-fluorescence). It appeared as black surface as visualized by CLSM.
All participants revealed pleomorphic microflora, variable from coccoid and cocco-bacilli to bacilli in morphology. Living plaque flora appeared as green coccoid to cocco-bacilli mixed with dead bacterial material in addition to green desquamated cell remnants. In the topography of living and dead bacterial colonies in plaque smear, the layers of both live bacteria and dead bacteria appeared as multiple accumulations varied in heights due to the differences in microorganism accumulation.
Densitometric analysis revealed that the mean plaque intensity ranged from 40.32 to 140.72 µm for live (vital) microorganisms, and from 65.00 to 128.88 µm for dead microorganisms. Plaques were found in all participant specimens. Differences in the thickness and distribution of plaques among all the participants were found.
Plaques from all participants revealed green vital microflora and red dead bacteria except participant number 3, who showed only dead bacteria with a profile graph showing the absence of the live microorganisms. Plaques layer thicknesses were 207 µm, 653 µm, 148 µm, 101 µm, and 273 µm in participants 1, 2, 3, 4, and 5, respectively. The topography of all plaques showed layers of live and dead microorganisms (Figs 1, 2).
Moreover, the profile paragraphs revealed the distribution of the living and the dead microorganisms in each plaque. Densitometric

Table 1. Showing the plaques thickness and the intensity of living and dead microorganisms

<table>
<thead>
<tr>
<th>Specimens No.</th>
<th>Plaque Thickness (µm)</th>
<th>Densitometric analysis (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vital microorganisms</td>
</tr>
<tr>
<td>1</td>
<td>207</td>
<td>1.92</td>
</tr>
<tr>
<td>2</td>
<td>653</td>
<td>2.95</td>
</tr>
<tr>
<td>3</td>
<td>148</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>101</td>
<td>1.54</td>
</tr>
<tr>
<td>5</td>
<td>273</td>
<td>6.09</td>
</tr>
</tbody>
</table>
analysis revealed the mean plaque intensity (Table 1) of vital (green) and dead (red) microorganisms separately (Fig 3). SEM observations Control acrylic appliance sample revealed smooth feature without appearance of any granularity compared to the test specimens.

Test specimens revealed deposit of bacterial accumulation on the surface with differences in appearance and thickness among participants. Typical micrographs from all participants were obtained with varied magnification.
for plaque accumulation at 72 hours (Fig 4). The morphology of the plaques microorganisms was very clear in all specimens. In addition to epithelial cells, two types of organisms were found on the specimens No. 1, and 4: the short bacilli (predominant) and the coccoid. The specimen No. 2 showed heavy distributions of different types of microorganisms, including coccoid, coco-bacilli, short bacilli and long bacilli, which were accumulated together as heavily patches. Microorganisms were of various types including coccoid, coco-bacilli, short bacilli and long bacilli, which were accumulated together as heavily patches. This sample revealed the morphology of plaque accumulation sites which was quite classical with a clearly distinguishable thread-like interplaque matrix. In this matrix, many coccal and rod shaped microorganisms had been dispersed. In the specimen No. 3, only one kind of microorganisms was found which appeared as clearly typical long bacilli accumulated heavily on the surface of the specimen. The higher SEM magnification revealed that these microorganism colonies accumulated as multiple layers covering most specimen surface. Heavily classical plaque accumulation with clear typical microorganisms was appeared in specimen No. 5. Thin, filament-like structures had radiated from individual coccal and bacilli bacteria and intertwined with filaments radiating from other bacteria while the interbacterial matrix was very dense. All specimens revealed differences in amount of bacterial colonization. The coccal bacteria were found to be clustered independently on the surface, whereas some other like bacilli had formed agglomerates.

**DISCUSSION**

Dental plaque has been defined as bacterial aggregation on the teeth or other solid oral structure [1]. Most of the information on the composition of dental plaque has resulted from the *in vivo* cultural and morphological studies. For this purpose, various materials have been used to imitate enamel surface [10]. In this study, acrylic appliance was used to substitute the teeth surface in order to study structural aspects of oral microbial colonization. Viewing of plaque smears on glass slides using CLSM showed green vital and red dead microorganisms in addition to green desquamated cell remnants. The topography showed the distribution of both living and dead microorganisms. These findings are compatible with the previous studies [11-13]. While the control acrylic surface revealed black non-autofluorescence surface, the surface of all appliances worn by the participants and vitally stained with fluorescein diacetate and ethidium bromide were fluorescent. These results indicate the accumulation of plaque on the worn appliance. Plaques belonging to different participants were different in shapes, thickness, and vitality status. Three of five participants were heavy or rapid plaque formers, while the others were light or slow plaque formers. The confocal microscopy images from the rapid plaque former participants stained for vitality exhibited relatively complex plaque morphology. In vital staining, the plaques appeared as green vital microorganisms embedded within the red non-vital microorganisms. Although some difficulties existed in the interpretation of the results of the light plaque formers, the features could be easily appreciated in the heavy plaque formers. These results are in agreement with the previous studies [9,11]. Apparently, the biofilms generated in our study within three days are consistently thicker than those generated within four days reported previously by Wood et al [14]. Wood et al placed two devices in the mouths of each of eight healthy volunteers and left them to generate biofilm within four days. Then, immediately upon removal of the devices from the mouth, the intact, undisturbed biofilms were imaged by the non-invasive technique of confocal microscopy in both reflected light and
fluorescence mode. The depth measurements indicated that the plaque formed in the devices was thicker round the edges at the enamel/nylon junction (range: 75-220 µm) than in the center of the devices (range: 35-215 µm). The reflected-light confocal images showed a heterogeneous structure in all of the plaque biofilms examined: channels and voids were clearly visible [14]. The results of our study showed the feasibility of the combination of two different techniques: vital fluorescence staining of the dental plaque flora for assessment of its vitality and non-invasive confocal microscopy of the plaque biofilm to visualize its three-dimensional topography. The recorded data from this study revealed the general architecture of undisturbed early dental plaque formation in vivo.

The application of confocal microscopy in dental medicine has mainly been limited to the examination of hard tissues [15]. Only few studies have been conducted using confocal microscopy to evaluate dental plaque [11,14]. The vital fluorescence technique allows the visualization of single bacterial cells according to their vitality status. The morphological sub-structures are also attainable.

In the present study, the green live or red dead coccoid and bacilli could be appreciated and distinguished as well as the desquamated epithelial cells. Calculation of thickness, distribution, width, geometric and densitometric analysis for each plaque in the present study resulted in findings, which was in agreement with previous data reported by other investigators [11,16]. Application of confocal microscopy software, the CF2D and CF3D, and the statistical multicolor mask analysis for the examined plaque are some of the advantages of CLSM in supporting the analysis of the plaque. To our knowledge, no previous study has used these facilities in analysis of the dental plaque. Thus, this study can be considered as the first study using CF2D and CF3D and its statistical multicolor mask analysis to evaluate dental plaque.

The analysis of data revealed light sparse plaques in two participants. The dental plaques in these two cases consisted of more dead material than living microorganisms, which was proven by 3D and topographical analysis. Indeed, the dead material was found especially when thin plaque specimens were examined. Topographical appearance of others' plaques revealed that the living plaque microorganisms were embedded between and on the top of major dense dead material. This finding is in agreement with the previous studies [4,17,18]. Some investigators assessed dental plaque using histochemical techniques and found that microorganisms in the inner layers of old plaque had a low vitality [17,18]. Other investigators found that dead cellular material was a major component of the plaque mass during the initial stages of plaque development and accumulation [10].

The results of SEM are compatible with and support the result of confocal microscopy. Two participants revealed light plaque formers. These two specimens showed similarities in the morphological features of microorganisms. Bacilli were the major microorganisms in case of participants No. 1, while coccoid was the major in No. 4. Meanwhile both forms, the bacilli and the coccoid, were found in participants’ No. 1 and No. 4 who showed only primarily sparse plaque distribution. The other participants revealed heavy accumulation, aggregation, and co-aggregation of microorganisms.

The combining and comparing the results of SEM and CLSM enabled us to classify the participants of this study into two groups: slow or light plaque formers, and rapid or heavy plaque formers. This is compatible with the previous studies.

In some studies, investigators reported the use of rapid and slow plaque former groups to investigate predominant cultivable organisms in supragingival plaque [16]. They demonstrated
statistically significant differences in the mean relative proportion of the Gram-positive and Gram-negative bacteria between the rapid and slow formers plaque [16].

CONCLUSION
The results of the present study revealed that in all participants the microorganisms colonized in the substitute enamel surface via the surface coating material, which is likely to be salivary pellicle. First, individual organisms attached to the surface and then they formed micro colonies. These micro colonies coalesced to form larger colonies and then spread out as a monolayer. Co-aggregation allowed multilayer formation. This pattern was investigated in CLSM as well as SEM.

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REFERENCES
