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Superimposition of sequential scans to measure erosion on unpolished and curved human enamel

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Abstract

**Objectives:** To determine if superimposition of sequential scans can discriminate between different fluorides at step heights less than 5 μm on natural human enamel surfaces.

**Materials and methods:** Natural, unpolished, human enamel specimens (n = 60) were randomly assigned to one of three pre-treatment toothpaste slurries with a calcium silicate/fluoride, fluoride-only and a control. Baseline and post treatment scans, from a non-contacting profilometer with a 0.01 μm z-axis and <1 μm lateral scanning resolution were imported into superimposition software to define change in mean 3D step height and surface roughness following erosion in 0.3 % citric acid for 15 min. Statistical analysis conducted with two-way repeated measures ANOVA and post-hoc Tukey’s multiple comparisons.

**Results:** Confidence and resolution of superimposition and subtraction of repeated profiles from unpolished enamel revealed accuracy within 1-2 μm. The technique was able to discriminate between the fluorides demonstrating statistical differences in mean (SD) 3D step height (μm) of 1.96 (0.40) and 2.75 (0.49) (p=0.0024). There was a statistically significant increase in surface roughness for all groups after 15 min erosion compared to baseline. But no statistically significant difference between the interventions after 15 min erosion but there was compared to no fluoride (p=0.006).

**Conclusion:** Superimposition and subtraction of profiles could discriminate between fluoride interventions, which showed statistical differences in enamel loss differences less than 3 μm.
**Clinical Significance:** This erosion model and data analysis workflow was able to distinguish differences between scans of 3 μm on unpolished enamel following the interaction with fluoride.
Introduction:

Polished enamel surfaces are commonly used in in-vitro research investigating dental erosion [1,2], abrasion/wear [3], and demineralisation/cariology [4]. These surfaces are easier to measure with microhardness, profilometry or other techniques as they are flat and provide consistent results. But polished enamel is more susceptible to acid-dissolution compared to the natural enamel surfaces as the outer surface layer contains the highest concentrations of fluoride and calcium ions and is acid-resistant and is removed by polishing [1,5,6]. The resistance to acids and the interaction with modifying products may differ between polished and the natural enamel surface. Preserving the outer layer of enamel also more closely matches the clinical situation.

Scanning and profiling curved natural enamel surfaces with a profilometer cannot use standard techniques for step height calculation, because the natural surface is not flat. The step height is calculated using a centralised wear scar or trough surrounded either side by reference area [7]. On polished surfaces the reference areas are flat, and software is used to ensure they are horizontal so that the difference in z-height between the base of the trough and the reference areas can be recorded [1]. But natural surfaces are more complex with a generalised curve and non-uniform local areas formed during mineralisation on the enamel. To overcome the general curve and localised irregularities, superimposition of successive digital scans, taken from before and after acid immersion is needed. Our team developed workflows that allow accurate measurement of curved surfaces and so allow preservation of the mineral rich layer [7]. These scans use circular reference zones on the enamel surface and following acid immersion a zone of erosion surrounded by a reference area is amiable to subtraction calculation of digital maps to produce mean step heights and volume
calculation[7]. But we do not know if these techniques can discriminate at the level of 1-3 μm. The aims of this study were to use an erosion model to assess if superimposition of sequential profilometric scans of natural enamel specimens can detect the impact of different fluoride interventions. The null hypothesis was that superimposition of sequential scans cannot discriminate between different fluorides at step heights less than 5 μm on natural human enamel surfaces.

**Materials and methods:**

Natural human enamel specimens were prepared by sectioning the crown from extracted sound human permanent molar teeth of unknown origin and collected under ethical approval (REC: 12/LO/1836) with patients written consent and using previously published protocols [1]. Each tooth produced a single section and teeth were randomly assigned to one of three slurry pre-treatment groups (n = 20/group): calcium silicate and sodium phosphate salts with 1,450 ppm as sodium monofluorophosphate, a regular toothpaste (SMFP, 1,450 ppm), and non-fluoride toothpaste (NF) of the same formulation, but without fluoride or calcium products; all were manufactured in unmarked tubes (Unilever Oral Care, Liverpool, UK). Toothpaste slurries were mixed in a 1:2 ratio (toothpaste:deionised water, w:v), rapidly homogenised at 25,000 RPM (Ultra-Turrax; Janke & Kunkel, Germany) and immediately used. Each enamel sample was immersed in 10 ml of a toothpaste/slurry for 3 min, followed by a rinse in deionised water for 1 min, dried for 5 sec with oil-free airflow and then placed in artificial saliva for 30 min. Artificial saliva was produced according to method previously described by Eisenburger et al [8] and without proteins. Samples were eroded for three, 5 min cycles (cumulative times: 5, 10, and 15 min), with one erosion cycle consisting of immersion in 10 ml 0.3 % citric acid (titratable acidity 18.0 ml) for 5 min under constant
agitation at 62.5 RPM (Stuart mini-Orbital Shaker, Bibby Scientific, Stone, England), each followed by 2 min rinse with deionised water. A reference area of tape, consisting of attaching a 1.5 mm diameter round hole cut from PVC tape using 1.5 mm surgical punch biopsy (Kai Medical; Seki, Japan), was placed over the maximum bulbosity of each specimen. An overview of the experimental protocol can be seen in Figure 1.

Baseline scans, and after each 5 min cycles, were recorded with z non-contacting laser profilometry (NCLP; Taicaan, XYRIS 4000, Southampton UK), consisting of a red light displacement laser (655 nm) with a 2 μm laser spot size, 0.01 μm z-axis resolution and lateral scanning resolution of <1 μm and surface form detection threshold of 0.26 μm [7,9]. Surface profiles before and after erosion consisted of a 3.5 × 3.5 mm rectilinear grid with 351 points measured at 10 μm step over distance to produce a digital data cloud consisting of 123,201 scanned points per sample [1]. A bespoke measurement jig was used to ensure exact repositioning of samples for scanning before/after erosion. Three-dimensional step height was calculated for each sample for each erosion cycle.

A 25 μm Gaussian filter removed roughness data for all before/after erosion scans, to leave only the surface form, which was then analysed using automated surface metrology software (Digital Surf; Besançon, France) [10]. Pre-and post-erosion scans of the unpolished surface were superimposed digitally using natural fiducial markers on the enamel surface. Following superimposition the scans were subtracted to produce a residual data set [7]. Using this residual data set, the mean 3D step heights, were calculated for each erosion timepoint and treatment group using ISO 5436-1, which determined the z-height difference between the eroded trough and reference regions.
Surface roughness scans, were randomly selected over the surface, consisting of a cluster of five scans, each with dimensions $0.2 \times 0.2$ mm ($51 \times 51$ points and a $4 \mu m$ step-over), to give 2601 point cloud [1,11]. Surface roughness analysis was conducted after applying a $25 \mu m$ Gaussian filter removing the form data to produce surface roughness ($S_a$) for each scan according to ISO 25178-2 [12].

Qualitative two-dimensional images were obtained for representative samples from each pre-treatment group using TSM (Tandom Scanning Microscope, Noran Instruments; Middleton, WI, USA) with a $20 \times$ objective lens ($20 \times /0.35$ NA objective) and filtered light projection (green, $550$ nm). Images were processed using image processing software (ImageJ, Abramoff et al [13]), co-localised using natural fiducial markers and assessed qualitatively to determine visual differences between samples for each pre-treatment group, according to previously published protocol [1].

Data were collected, tabulated, and statistically analysed (GraphPad Prism 7 Software; California USA). A power calculation (GPower 3.0.1 Universität Düsseldorf; Düsseldorf, Germany), based on ANOVA comparing between treatment groups was conducted indicated a sample size of 8 per treatment group (54 total sample size) would be required for an effect size $0.45$ yielding $80\%$ power. Data were assessed for normal distribution using Shapiro-Wilks and Komogorov-Smirnov tests and visually assessed with boxplots and histograms. Data were normally distributed, therefore mean and standard deviations reported. Inter-group analysis was conducted with two-way repeated measures ANOVA with post-hoc Tukey’s test for multiple intra group comparisons.
Results

Superimposition of the scans and then measurement of step height produced data in the range of 1-3 μm with standard deviation less than 1 μm and so was able to discriminate between the interventions. Mean (SD) 3D step height formation, from the curved natural surfaces, after 5, 10, and 15 min for each treatment group and their statistical associations are summarised in Figure 2. The lowest mean (SD) 3D step height formation after 15 min erosion was demonstrated by calcium silicate and fluoride with 1.96 (0.4) μm. This was statistically significantly lower (p = 0.0024) than fluoride only, 2.75 (0.49) μm and control 2.75 (0.79) μm. There was no statistical difference between the interventions after 5 min of erosion (p = 0.41).

Mean (SD) surface roughness for each treatment group can be seen in Figure 3. There was a statistically significant increase in surface roughness for all groups after 15 min erosion compared to baseline. But no statistically significant difference between the interventions after 15 min erosion but there was compared to no fluoride (p = 0.006).

Qualitative analysis of TSM images group showed progressive erosive destruction of the surface enamel with increase of erosion time. After 15 min erosion, no visual differences could be noted between the pattern of erosive destruction between the pre-treatment groups.
Discussion

This study shows superimposition and subtraction of scans, taken from natural unpolished enamel in an erosion model, was able to discriminate between two fluoride interventions with 3D mean step heights at less than 3 μm. Using unpolished human enamel preserved the outer mineral rich layer and means that 1,450 ppm fluoride and calcium silicate were absorbed onto enamel and contributed to protection from the acids. Polished enamel surfaces have uniform reference zones and an erosion trough and involve subtraction of the reference zones is relatively straightforward. But the complex topography on unpolished teeth means finding a flat reference zone is not possible. Therefore, superimposition and subtraction of digital scans, using fixed reference zones, was needed to show z height differences. This study evaluated the reaction to calcium silicate/phosphate and fluoride toothpaste on natural human enamel molar surfaces. Previous studies investigating dental erosion, abrasion/wear, and demineralisation/cariology in vitro have been conducted on polished enamel surfaces using either of bovine [3,14,15] or human [4,16,17] origin. The method was sensitive enough to discriminate less than 3 μm and so our null hypothesis was rejected.

There are significant morphological and histological differences between natural and polished human enamel. Natural human enamel has higher acid resistance [5] and superior mechanical properties [6,18] compared to polished human enamel, and the effects of acid-erosion may therefore be potentiated on polished enamel surfaces. These properties of natural human enamel are due to the amorphous surface layer of natural enamel that comprises the highest concentration of fluoride, phosphate, and proportion of fluorapatite [19] when compared to
other enamel layers, such as, the dentino-enamel junction (DEJ). This amorphous surface layer is largely lost during the polishing process to produce flat enamel samples.

Previous erosion studies on polished enamel samples have used reference regions either side of a erosion trough to measure z heights using ISO 5436-1 [20]. However, this form-removal technique cannot be used on natural enamel surfaces. Profile subtraction of successive scans is required because of the curvature and complexity of the outer surface enamel layer. The utilisation of profile subtraction techniques has been previously demonstrated for determining enamel loss in natural enamel samples [21–23]. This requires that the samples are scanned before and after each erosion phase and to supplement this a relocation jig with fiducial markers was needed to identify reference regions to ensure consistency and reliability of scanning [7]. This process is analogous, but more complex, to the simple form removal techniques used in studies on polished enamel. It overcomes the issues of mathematically trying to use form-removal which would not work on eroded natural enamel samples, and allows the analysis of 3D step height using ISO 5436-1 [1,10,24].

Our data indicated that whilst the 3D step height was lowest in order of calcium silicate/fluoride, fluoride and then controls, which were statistically significant, the surface roughness data did not show significant difference between the fluorides. This suggests that using the $Sa$ parameter alone, was unable to discriminate between the differences in surface texture detail of the eroded natural enamel surface. But it was able to discriminate between the fluorides and control at a level of 0.07-0.08 $\mu m$. 


Previous studies have investigated the protective and remineralising effects of different calcium-silicate/sodium phosphate formulations with or without fluoride. The efficacy of 2 min application of calcium silicate (1 mg/ml) slurry in phosphate buffer (pH 7) in remineralising eroded bovine enamel has been previously demonstrated [14]. The same group additionally demonstrated the efficacy of calcium silicate slurry after immersion in fluoride solution (2 min, 1000 ppm sodium fluoride) in protecting bovine enamel against subsequent acid erosion (nitric acid; pH 3). Their study utilised scanning electron cell microscopy (SCCM) and atomic force microscopy (AFM) to demonstrate that both volume of the erosion pits and the rate of calcium loss were significantly reduced in those samples treated with calcium silicate/sodium phosphate [14]. Additionally, the erosive-protective benefits of calcium silicate/sodium phosphate and fluoride toothpaste against citric-acid mediated erosion compared with a sodium fluoride control toothpaste has been previously demonstrated [15,25]. This was shown by a reduction in microhardness loss in those samples treated with CSSP versus fluoride (NaF and SMFP) and non-fluoride control toothpastes. However, these studies used polished enamel derived from either bovine [14] or human teeth [15,25], which have different erosion kinetics and characteristics compared with natural (unpolished) human enamel [1]. The workflow used in this study has potential for further work to understand what happens to teeth in the mouth and this relevance to interventions used to prevent erosion.

The challenge with natural surfaces is a more complex surface topography, but this necessitates a different approach to the analysis. This needed more complex stages, with the potential for errors, but despite this the overall accuracy remained around 3 μm. Polished surfaces create a more uniform structure to allow controlled measurement, but the process
removes the outer mineral rich layer. Whether this matches the clinical situation is not known but taken with other work emphasises the action of fluoride. The authors used citric acid as the erosive agent and whilst commercial products might be more clinically relevant, most have complex formulae with more than one acid, and so we chose a simpler more controlled method. The same concept was related to the formulation of toothpastes. We could have chosen different products, but our selection varied slightly in formulation and so tested the workflow. Finally, the choice of a remineralising artificial saliva, without proteins, simplified the method and so it would be interesting to investigate how natural saliva interacts with the toothpastes and whether the workflow can distinguish any difference.

**Conclusion** The methods used in this in-vitro study, on unpolished human enamel and using of superimposition and subtraction scans was able to discriminate between different fluorides at step height less than 3 μm. Our null hypothesis was rejected and we were able to distinguish between the action of calcium silicate and sodium phosphate salts with 1,450 ppm as sodium monofluorophosphate formulation and controls
Figure 1 - Experimental protocol outlined in three steps: Sample Preparation, Sample Pre-treatment, Sample Erosion Protocol
Step 1: Sample Preparation
- Sample preparation and cleaning
- Dry 24 hrs
- Pre-erosion measurement with NCLP
- Tape samples

Step 2: Sample Pre-treatment
- Toothpaste pre-treatment
  - Immersion + agitation 62.5 RPM
  - 10 ml toothpaste slurry (per sample)
  - **3 min**
  - Fluoride or non-fluoride toothpaste slurry
- Wash and Dry
  - Deionised water
  - **1 min**
  - Dry with oil-free air 5 sec
- Wait period and Dry
  - Immersion (stagnant)
  - 10 ml artificial saliva (per sample)
  - **30 min**
  - Dry with oil-free air 5 sec

Step 3: Sample Erosion Protocol
- Erosion
  - Immersion
  - 10 ml 0.3% citric acid pH 3.2 (per sample)
  - **5 min**
- Post-erosion measurement with NCLP
- Repeat 3 times
Figure 2 – Mean (SD) 3D step height for each erosion time point and treatment group
Figure 3 – Mean (SD) surface roughness for each treatment group
Figure 4 – TSM qualitative images for each treatment group demonstrating the impact of citric acid erosion after each erosion cycle
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