An analysis of the effects of chlorhexidine on oral biofilm vitality and structure based on viability profiling and an indicator of membrane integrity

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Abstract
Multispecies biofilms modelling interproximal plaque were grown on a hydroxyapatite substratum in a constant-depth film fermenter and then immersed in a viewing solution containing fluorescent indicators of membrane integrity. Confocal laser scanning microscopy (CLSM) revealed the structure and spatial distribution of cell vitality through the biofilms. Chlorhexidine gluconate (CHX) was added to the viewing solution to achieve concentrations of 0.05% and 0.2% w/v before further CLSM time-lapse series were captured. Image analysis showed that exposure to 0.2% CHX caused the biofilm to contract at a rate of 1.176 μm min$^{-1}$ along the z-axis and also affected changes in total fluorescence and viability profiles through the biofilms after a delay of between 3 and 5 minutes. At a concentration of 0.05% CHX, total fluorescence measurements of the biofilm exhibited barely detectable changes after 5 minutes. Fluorescence profiles (fluorescence vs time vs depth) however clearly demonstrated that a time-dependent effect was present, but the clearest indicator of the effects of dilute CHX over time was viability profiling. These findings suggest the possibility of using fluorescent indicators of membrane integrity in conjunction with viability profiling to evaluate the penetration of the bactericidal effects of membrane-active antimicrobial compounds into biofilm.

Keywords
Biofilm, chlorhexidine, confocal laser scanning microscopy (CLSM), antimicrobial, penetration, viability
**Introduction**

Bacteria which are members of a biofilm community are generally less susceptible (10 – 1000 times) to the effects of antimicrobial compounds than their planktonic counterparts (19). There are two aspects to this phenomenon; that which is conferred by (direct) and that which is coincidental to (indirect) the biofilm modality. Direct resistance adaptations are acquired by, or activated in response to, cell density such as slower growth rates, the production of persister cells and the activation of a suite of mechanisms collectively termed quorum sensing (20). Slow growing bacteria are intrinsically less susceptible to the effects of antimicrobial compounds (5) and whilst persister cells may only form a small fraction of the biofilm community, they are “essentially invulnerable to killing” (30). Quorum sensing bacteria release autoinducer molecules into the local microenvironment, the concentration of which increases as a function of population density. When a critical threshold concentration of these molecules is detected by a bacterium, changes in gene expression are induced; these include the activation of virulence systems, competence and sporulation (2). Indirect resistance to the antimicrobial agent is conferred by the physical properties of the biofilm, including the co-operative adherence to surfaces (18) and the impedance of the penetration of macromolecules to the inner regions of the biofilm by the exopolysaccharide matrix (33). The latter may also function as an ion-exchange matrix and so hinder the penetration of positively-charged low-molecular mass antimicrobial agents (32).

Dental plaque is a biofilm which forms on the non-shedding surfaces of the oral cavity (34). If left untreated, the succession of dental plaque development can lead to serious complications such as caries, gingivitis and periodontitis. A typical plaque removal regime might involve brushing with either a manual or electric
toothbrush, followed by the use of a proprietary antimicrobial mouthrinse for 30 seconds. These products are commonly based upon a dilute alcohol solution containing an antimicrobial compound such as chlorhexidine (CHX), although many formulations are available (29). It has been shown, in an in vitro model, that CHX exposure times of the order of 30 seconds have very little effect upon the number of viable bacteria which can be recovered from oral biofilms. In particular, 0.2% CHX has been shown to be ineffective against dental plaque in an in vitro model after 5 minutes exposure, requiring 60 minutes exposure to achieve a 2-log$_{10}$ to 5-log$_{10}$ kill (24).

Chlorhexidine gluconate ($\text{C}_{22}\text{H}_{30}\text{Cl}_2\text{N}_{10}\cdot2\text{C}_6\text{H}_{12}\text{O}_7$) is a cationic bisbiguanide with a molecular mass of 897.77 Da. It possesses broad antibacterial activities which are combined with low mammalian toxicity and the ability to bind to skin and mucous membranes. The CHX molecule reacts with negatively-charged groups on the cell surface causing an irreversible loss of cytoplasmic constituents, membrane damage and enzyme inhibition. At high concentrations, CHX results in extensive cell damage, coagulation of cytoplasm and precipitation of proteins and nucleic acids. CHX is the active ingredient in many commercially available disinfectants, antiseptics and antimicrobial mouthrinses. A CHX concentration of 0.2% CHX is deemed to be the most effective as a mouthrinse and as such is considered to be the “gold standard” (15).

Confocal laser scanning microscopy (CLSM) uses a tightly focussed beam of laser light and pinhole aperture to capture a series of thin optical sections, termed an image stack, through a (biological) sample. Analysis of CLSM images has been used in conjunction with vital staining techniques to determine the architecture and spatial distribution of vitality for organisms within oral biofilm (1,21,36). These techniques
have been refined by treating the viable and nonviable bacteria as distinct populations and then comparing the spatial distribution trends between these two cohorts to produce viability profiles (9). These processes employ a function of image fluorescence against the depth of the optical section into the confocal image stack. Viability profiles typically show the progression from viable fluorescence to nonviable fluorescence with increasing depth into the biofilm. This phenomenon is also apparent in the horizontal plane, showing the outer layers of the biofilm surface features to exhibit more viable fluorescence than the inner regions, which contain nonviable fluorescence (12,22).

*Bac*Light LIVE/DEAD stain (Molecular Probes, OR, USA) is a two component system using SYTO9™ (proprietary nomenclature) and propidium iodide to differentiate between viable and nonviable bacterial cells respectively. Bacteria with damaged membranes are stained as nonviable, whereas bacteria with intact membranes are deemed viable. Whilst this may not hold true for every bacterium in every circumstance (27), the fidelity of the system is sufficient to elucidate depth-related trends in cell vitality within multi-species populations of bacteria comprising a biofilm. Since CHX damages bacterial membranes, its bactericidal action can be measured in “real-time” with LIVE/DEAD stain.

The aim of this study was to monitor the effects of CHX upon oral biofilms via changes in their viability profiles, in real-time.
Materials and Methods

Biofilm Growth and Sampling

The methodology of growing microcosm dental plaques in a constant-depth film fermenter (CDFF) has been described in detail previously (10,11,17,35). In this instance hydroxyapatite discs (Clarkson Chromatography Products, South Williamsport, PA) recessed to a depth of 200µm were used to support the plaque biofilms, modelling the thickness of interproximal plaque. The contents of the CDFF (University of Wales, Cardiff, UK.) were exposed to the atmosphere via a 0.2µm filter (Whatman, Poole, Dorset, UK.). An aliquot of a saliva pool was used to inoculate 1 litre of a complex mucin-containing artificial saliva (11) which was pumped into the CDFF at a rate of 0.72 ml min$^{-1}$ until exhaustion of the volume after 24 hours. At the same time, sterile artificial saliva was also pumped into the CDFF at 0.72 ml min$^{-1}$ until cessation of the experiment after approximately 30 days.

Biofilms were removed from the CDFF at various times, ranging from 17 to 27 days after inoculation. A single HA disc containing a biofilm was immersed in 1ml of saline to remove excess medium and unattached cells.

Confocal Laser Scanning Microscopy and the Application of Chlorhexidine

Two solutions were prepared; a 10% chlorhexidine digluconate solution from a 20% stock (Sigma-Aldrich Co. Ltd., Gillingham, UK) and a viewing solution comprising of 8ml distilled water (dH$_2$O) containing 2µl each of components A and B of BacLight™ LIVE/DEAD stain (Molecular Probes, Eugene, OR).

A biofilm laden disc was placed, biofilm upwards, into a small cell culture dish (Bibby Sterilin Ltd, Stone, UK) and the viewing solution carefully poured in. This volume of liquid submerged the entire disc and biofilm, which was then allowed
to develop in the dark for 10 minutes. The biofilms were examined by a fixed stage microscope (DMLFS, Leica Microsystems, Milton Keynes, U.K.) incorporating a TCS SP2 (Leica) laser scan head, mounted on a vibration-free platform. The objective lenses used were 20x and 40x water immersion dipping lenses (Leica).

A preliminary scan of a random area of the biofilm was taken to determine the optimum photomultiplier (PMT) and z-axis settings (upper and lower positions of the image stack). It was later found that the PMT gain and offset values were best set so as to give a slightly wider range than was dictated by the preliminary scan. While this strategy reduced image quality, in terms of the range of pixel brightness information, it allowed the quantification of fluorescence values above and below the levels of the original image. Shifting the PMT settings in this manner helped to minimise saturation.

The CLSM control software was set to take a series of time-lapse scans (xyzt) at intervals of either 30 or 60 seconds, depending upon the thickness of the image stack. Scans were taken in 8-bits at a resolution of 512x512 pixels. This relatively low image quality was used in order to minimise the scan time, an estimate of which was displayed by the controller software. The z-axis step size was the major determinant of the scan time and this was the value which was manipulated in order to balance image quality with time-point resolution. The length of the time-lapse series was typically 10 or 15 minutes.

Immediately after the first scan of the time-series was completed, 160μl of the 10% CHX solution was added to the cell culture dish to give a final concentration of CHX in the 8ml of viewing solution of 0.2% w/v. The CHX solution was injected via pipette (Eppendorf AG, Hamburg, Germany) at an angle perpendicular to the disc; with the aim of maximising mixing whilst avoiding the direct disruption of the
biofilm by fluid shear. Similar experiments were conducted whereby 40µl of the 10% CHX solution was added to the viewing solution to achieve a final concentration of 0.05% w/v CHX. The resulting collections of confocal sections were archived onto optical discs as time-depth-PMT channel coded image files (tagged image file format - *.tiff) by Leica TCS NT software.

A series of complementary time-lapse control experiments were also undertaken where CHX was not added to the viewing medium.

**Image Analysis**

Image stacks were analysed by a Java™ based image analysis program, ImageJ (8,25). The individual time-depth-PMT channel coded tiff files were first reassembled into usable tiff stacks using a specially written ImageJ plug-in.

Projection images were assembled for each time-point and fluorescence channel using ImageJ’s “Z-project” function, which produces a single image based upon the sum of pixel brightness through the image stack. The projections were converted from 32-bits, back to 8-bits using a scaled method and the resulting time-coded images formed into a separate stack. This time-lapse stack was then analysed by ImageJ’s “plot z-axis profile” function to divulge time-related trends in total image fluorescence for both the viable and nonviable channels. Sagittal (elevation view) time-lapse projections were also constructed using similar methods.

Fluorescence intensity profiles through the image stacks over time were determined for both the viable and nonviable fluorescence channels by employing the “plot z-axis profile” function. These raw data were then exported to a spreadsheet program (Microsoft Excel™) and the process repeated for each point in the time-lapse series. Once the z-axis profiles were in spreadsheet format, the data were normalised,
i.e. the maximum fluorescence value for a particular channel at a particular time point was allocated a value of 1 unit. Viability profiles were then constructed by subtracting the normalised nonviable z-axis profile values from their corresponding viable values and plotting against depth of the optical section into the biofilm (9).

The rates of biofilm contraction, as revealed by the sagittal projections, were measured by object tracking. This involved noting the z-axis position of distinguishing features within the image stack, such as the top of the biofilm or a particularly bright microcolony, and tracking the movement of these features over time. The corresponding increase in image brightness due to increasing fluorescence per unit volume was calculated and factored into the sum of projected fluorescence where appropriate.
Results

Three examples of CHX-exposed biofilms are presented in this study; biofilm #1 was used to measure total fluorescence and biofilm contraction at 0.2% CHX, whilst biofilms #2 and #3 were used to produce depth related profiles at 0.2% and 0.05% CHX respectively.

Preliminary experiments showed that CHX exposure caused optical section fluorescence within the biofilm to increase, often resulting in saturation of the captured images, i.e. too many pixels at the maximum brightness value (data not shown). This phenomenon was attributed to the observed contraction of the biofilm causing a corresponding increase in the fluorescence per unit volume (i.e. within the thickness of an optical section).

On exposure to 0.2% CHX, time-lapse projection images showed evidence of changes in oral biofilm #1 vitality and structure (figure 1). Sagittal (side-on) projections (figure 2) showed that 0.2% CHX caused the biofilm (#1) to contract at a rate of 1.176µm min\(^{-1}\) which was measured by object tracking over a period of 15 minutes (figure 3). The influence of sample contraction on increasing image fluorescence per optical section is given in equation 1.

Quantitative analysis of total image fluorescence (Z-projections of biofilm #1, figure 1) revealed that raw nonviable fluorescence fluctuated immediately after the exposure to 0.2% CHX before increasing after 4 minutes (figure 4a) whereas raw viable fluorescence remained relatively constant. The initial fluctuations in fluorescence values were likely due to the unavoidable turbulence caused by the addition of the CHX solution which disturbed the biofilm. When the effect of the rate of biofilm contraction upon optical section fluorescence was taken into account
viable fluorescence was shown to have actually decreased whilst nonviable fluorescence increased (figure 4b), as one might have predicted.

Following exposure to 0.05% CHX, the total viable fluorescence in biofilm #3 remained unchanged over 15 minutes whilst nonviable fluorescence increased marginally after a delay of 5 minutes (figure 5a). It was not appropriate to incorporate the brightness adjustment due to the contraction of the biofilm in this instance since the amount of biofilm shrinkage was small and non-uniform; an annular contraction around the base of the biofilm tower was observed causing the biofilm around the edges of the image stack to depress a little whilst the height of the central portion fluctuated slightly (data not shown).

Fluorescence profiles through biofilm #2 revealed that the peak values for both viable and nonviable fluorescence moved deeper into the image stack over time after exposure to 0.2% CHX (figures 6a and 6b). The viable fluorescence values in this instance increased after 5 minutes; this was due to contraction of the biofilm (figure 6a). Nonviable fluorescence also increased after 3 minutes (figure 6b). The fluorescence values of these data emphasise the disparity between viable (0 - 10) and nonviable (0 - 45) image fluorescence pixel brightness values; it cannot be inferred from these data that nonviable bacteria are predominant in the image stack since the laser power and PMT settings are essentially user-definable.

Fluorescence profiles through biofilm #3, which was subjected to CHX at 0.05%, showed smaller changes (figures 7a and 7b) over time than the biofilms exposed to CHX at 0.2%. The changes over time in the fluorescence profiles where more apparent than those observed in the total fluorescence images (figure 5a).

Figure 8 shows viability profiles (normalised LIVE minus DEAD fluorescence against depth) through the biofilm on exposure to 0.2% CHX against time.
zero, before exposure to 0.2% CHX, viability profiles through the image stack show the ‘typical’ motif of the biofilm possessing viable upper layers and moving down through to nonviable fluorescence (9). Between 0 and 5 minutes the viability profile changed little, although it shifted deeper into the image stack as the biofilm contracted. After 5 minutes exposure, the viability profile moved deeper still into the image stack and became less distinct until after 7 minutes, the ‘typical’ viability profile was no longer evident.

At 0.05% CHX changes in the viability profiles over time were apparent (figure 9), although to a lesser degree than those occurring at 0.2% CHX (figure 8). The changes in cell vitality at this relatively low concentration of CHX were revealed much more effectively by viability profiling than by total fluorescence (figure 5a).

Control experiments showed that no changes occurred in membrane integrity in oral biofilms immersed in viewing solution when CHX was not present (figure 5b).
Discussion

Fluorescent indicators of membrane integrity have been used in conjunction with flow cytometric techniques to discriminate between viable and nonviable planktonic bacteria (14). This technique was subsequently used to evaluate the biocidal activity of three common oral antiseptics; CHX, cetylpyridinium chloride and triclosan (23). It is likely, therefore, that the bactericidal action of other antimicrobial compounds which affect the cell membrane, will also be detectable by fluorescent indicators of membrane integrity. Antimicrobial compounds which do not directly affect the cell membrane, such as those which interfere with protein or nucleic acid synthesis, are normally considered to be bacteriostatic and will not be suitable agents for study by these methods. The results presented in this study demonstrate that the penetration of the bactericidal effect of membrane-active antimicrobial compounds into biofilms can also be tracked using indicators of membrane integrity.

Examination of the biofilms while they were immersed in the viewing solution allowed the diffusion of aqueous solutes into the biofilms to be studied whilst in their ‘natural’ hydrated state. This is important as the major component of any biofilm is water. Dehydration and/or fixing in resin disrupts the structural motifs and spatial distribution of bacteria within the biofilm. Phosphate buffered saline (PBS), which would normally be used to simulate physiological conditions, could not be incorporated into the viewing solution because CHX reacts with phosphate and chloride radicals resulting in double decomposition and the slow crystallisation of insoluble salts. Control experiments showed that exposing the biofilm to the dH₂O-based viewing solution had no adverse osmotic effects upon the biofilm with regard to structure or viability (figure 5b).
An exposure time of 30 seconds is most often quoted to describe typical mouthrinse use, although poor user-compliance may reduce the exposure time still further. A recent study suggested that there was no significant difference in plaque index between patients using a 30 second rinse with a 0.12% CHX solution compared to those employing a 60 second rinse with a 0.2% CHX solution (16). CHX has been shown to persist in the mouth by binding to mucosal surfaces and also to the pellicle and saliva. Two hours after rinsing with 0.2% CHX, saliva retains antibacterial properties (26) and suppresses counts of bacteria in saliva for over 12 hours (28). This phenomenon has been attributed to the gradual desorption of CHX back into the mouth, creating a bacteriostatic milieu (13), however it is more likely that the antimicrobial effect is due to tooth-bound CHX (15).

A study of the mass transport of fluorescently-labelled dextrans of different sizes (3K-Dex to 240K-Dex) into oral biofilms (33) showed that biofilms did indeed inhibit the diffusion of macromolecules and the extent of the inhibition was more than that which was predicted. This inhibition of diffusion was attributed to the tortuosity of the biofilm matrix, i.e. the extremely convoluted diffusion paths which the molecules must traverse. A model was produced for the diffusion of dextrans above 10K-Dex, however it did not cover the diffusion of 3K-Dex. This was ascribed to the EPS possessing “pore” diameters wider than 2.6nm but narrower than 4.6nm. These “pore” diameters were based upon the predicted diameters of the dextrans and since the molecular mass of chlorhexidine gluconate (897.77 Da) is well below that of the 3K-Dex, it is unlikely that the diffusion of CHX into biofilms is significantly retarded by a molecular sieving effect. It is more probable that ionic interactions are occurring between the positively-charged CHX molecules and the negatively charge extracellular matrix. These ionic interactions are understood to reduce diffusion.
coefficients of fluorescent probes within biofilms of *Lactococcus lactis* and *Stenotrophonas maltophilia* around 50 fold (7). Conversely, the diffusion of aminoglycosides, such as tobramycin, into biofilms of *Pseudomonas aeruginosa* have been studied and it was determined that the interactions between the positively charged molecules and the negatively charged matrix was not a major mechanism of biofilm resistance (3). Mathematical modelling has also suggested that the diffusion of stoichiometrically reacting solutes through biofilms is not significantly retarded (31).

Mathematical modelling has been applied specifically to the diffusion of CHX into dental plaques (32). If the aqueous diffusion coefficient ($D_{aq}$) of CHX in water at 30ºC (an assumption of the temperature of the solution held in the mouth) is $4.2 \times 10^{-6}$ cm$^2$ s$^{-1}$, then for a dental plaque 260 µm thick, the diffusion time through the biofilm to the substratum is 298 s. If one supposes that the centres of the biofilm structures examined in our study were at most 50 µm from the bulk fluid flow (figure 1), then the diffusion time would be of the order of 1 minute. This does not wholly account for the delay of approximately 5 minutes before the CHX was observed to affect cell membrane integrity, suggesting that oral biofilms possess an intrinsic resistance to CHX which goes beyond their ability to impede diffusion (6).

Exposure to CHX does not kill bacteria immediately; a delay of 20 seconds has been reported in *Escherichia coli* and *Pseudomonas aeruginosa* (4) and 30 seconds for oral streptococci (23). In this study using biofilms of oral bacteria, the bactericidal effects of both 0.05% and 0.2% CHX were detectable after between 3 and 5 minutes. This anomaly could indicate that biofilms of oral bacteria are intrinsically less susceptible to the effects of CHX than their planktonic counterparts. It is reasonable to assume that there will be no significant delay between the cell
membrane becoming compromised and the penetration of the DEAD stain into the cell since the fluorophores will already be present in close proximity to the cells.

The mechanisms underlying the contraction of oral biofilm on exposure to CHX are probably related to ionic interactions between the negatively-charged extracellular polysaccharide (EPS) matrix, which comprises the bulk of the volume of biofilm, and the positively-charged CHX molecules. These interactions will change the physico-chemical properties of the EPS; solubility, hydrophobicity and localised charge along the polymer chains. Changes in charge will in turn affect the tertiary structure of the EPS chains and the degree of bonding with adjacent strands. As the positive CHX interacts with the negative EPS the net charge of the matrix will shift towards neutral, reducing the repulsive forces between charged moieties allowing closer associations to occur between polymeric strands, reducing the volume occupied by the biofilm. A more compact matrix may further inhibit the diffusion of solutes, including CHX, into the biofilm due to the tightening of the apparent molecular sieve.

The phenomenon of biofilm contraction appears to be related to the concentration of CHX, since the contraction observed at 0.2% was distinct and quantifiable, whilst at 0.05% the contraction was very slight and non-uniform. Observations made during the exposure of biofilm #3 to 0.05% CHX showed a water channel which appeared to open up. Image analysis estimated that the area (i.e. the number of pixels) occupied by the water channel increased 17% over 15 minutes. It is possible that conformational changes in biofilm structure, such as the opening up of this water channel could actually assist in the diffusion of CHX further into the deeper layers. Biofilm exposed to CHX at 0.5% also exhibited marked shrinkage (data not shown due to image saturation – see methods).
Biofilm contraction is unlikely to be due to any synergistic effect between CHX and the osmotic pressure of distilled water. Control experiments were undertaken whereby PBS was incorporated into the viewing solution. Although the double decomposition of the salts was apparent, as the formation of a milky precipitate, the biofilm was still seen to contract and shift towards nonviable fluorescence (data not shown).

Further work is necessary to understand the interplay between the delay of CHX’s action upon cell viability and the apparently immediate contraction of the biofilm in order to determine the effects which these phenomena exert upon the observations described in this study, to determine if contraction of the biofilm assists or impedes CHX penetration. Future experiments are being devised to evaluate the effects of different concentrations of CHX and other antimicrobial compounds. It would also be of great interest to use longer time-frames to determine when the biofilm ceases contraction in response to CHX, however, the massive amounts of data generated are a limiting factor.

Whilst total fluorescence measurements were capable of measuring the antimicrobial effects of CHX at a concentration of 0.2% w/v (figures 4a and 4b), these techniques were not sensitive enough to effectively elucidate the bactericidal effects of 0.05% CHX (figures 5a and 5b). When depth was included as a variable, the time-dependent bactericidal effects of 0.05% CHX were much more easily visualised (figures 7a, 7b) and this effect was magnified further by the construction of viability profiles (figure 9). We have shown that viability profiles through oral biofilm change upon exposure to CHX and suggest that they are more sensitive at detecting the antimicrobial effects of lower concentrations of CHX, and presumably other
membrane-active antimicrobial compounds, than total fluorescence techniques (i.e. non-confocal fluorescence microscopy).

These results suggest that viability profiling can be used to investigate the penetration and antimicrobial effects of existing, and novel, membrane-active biocides on biofilm. By incorporating a depth-related function into the analysis of the image stack, the sensitivity of total fluorescence measurements was improved upon considerably. The effects of the shrinkage phenomenon on the susceptibility of the biofilms to CHX remains unclear. On the one hand, compaction of the EPS matrix would be likely to inhibit diffusion of the CHX molecules into the biofilms whilst the opening up of water channels would facilitate the transport of the antimicrobial to the inner regions of the biofilm.

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Figure Legend

Figure 1. Z-axis projections from a CLSM time-lapse series showing oral biofilm (#1) after exposure to 0.2% CHX in the presence of BacLight LIVE/DEAD stain. (Green = viable channel/LIVE, blue = nonviable channel/DEAD)

Figure 2. Sagittal projections through the biofilm (#1) showing contraction after exposure to 0.2% CHX (viable channel only).
Figure 3. Contraction of the biofilm (#1) as measured by object tracking after exposure to 0.2% CHX. The z-axis positions of three features within the image stack were tracked over time, ♦ representing the uppermost optical section containing biofilm. Total image depth was 79.35µm; the rate of contraction was 1.176µm min⁻¹.

Equation 1. Adjustment of total image fluorescence values to compensate for biofilm contraction in a time-lapse series

\[
\left( \frac{d - (\Delta ct)}{d} \right) \times f = F
\]

- $\Delta c$ = Rate of biofilm contraction (µm min⁻¹)
- $t$ = Time point (mins)
- $d$ = Total image depth (µm)
- $f$ = Total image fluorescence (units)
- $F$ = Total image fluorescence adjusted for contraction of biofilm (units)
Figure 4. Total image stack fluorescence against time showing oral biofilm (#1) after exposure to 0.2% CHX, a) raw data, b) total image fluorescence compensated for biofilm contraction using equation 1. Trendlines are included for reference only.
Figure 5.  a) Total image stack fluorescence against time showing oral biofilm (#3) after exposure to 0.05% CHX. The rate of biofilm shrinkage was not incorporated into these data since the contraction was slight and non-uniform.  b) Control, no CHX.
Figure 6. Viable (a) and nonviable (b) fluorescence profiles through biofilm (#2) after exposure to 0.2% CHX.
Figure 7. Viable (a) and nonviable (b) fluorescence profiles through biofilm (#3) after exposure to 0.05% CHX.
Figure 8. Time-lapse of viability profiles through oral biofilm (#2) after exposure to 0.2% CHX, corresponding to data shown in figure 5.

Figure 9. Time-lapse of viability profiles through oral biofilm (#3) after exposure to 0.05% CHX, corresponding to data shown in figure 7.