

1 **VIABILITY GRADIENTS IN BIOFILM: A SLIPPERY SLOPE?**

2 **(Viability Gradients)**

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6

7 **Abstract**

8 *Are the fluorescence profiles observed in biofilm an artefact of confocal*
9 *microscopy and sample topography? A mathematical model has been*
10 *constructed that replicates these profiles in a homogenous 'biofilm'. However;*
11 *direct measurement of metabolic activity in biofilm shows that viability profiles*
12 *do exist and are therefore structural motifs worthy of study.*

13

14 **Keywords**

15 Cell viability, fluorescence, biofilm, confocal laser scanning microscopy

16

17 Biofilms of bacteria are not homogeneous structures and contain many
18 nonviable cells. These phenomena are due in part to the development of
19 physicochemical gradients within the biofilm, which in turn are manifested as
20 gradients of cell viability. Confocal laser scanning microscopy (CLSM) used in
21 conjunction with fluorescent indicators of cell membrane integrity is a powerful
22 technique for measuring viability gradients within biofilm. However; is it
23 possible that the observations are themselves merely an artefact of confocal
24 microscopy?

25

26 The confocal scanning microscope was invented in 1955 as a step towards
27 'the perfect microscope', one that would be able to examine each point in a
28 specimen and measure the amount of light scattered or absorbed by that
29 individual point (Minsky 1988). It was effectively two microscopes bolted
30 together, one to illuminate a point in the sample with an intense spot of light
31 and another to observe this. The foci of these two microscopes were the
32 same; hence they were termed 'confocal'. This equipment was developed in
33 the days before lasers, so intense arc illumination sources were used.
34 Although carbon arcs were the brightest available they were unreliable so
35 zirconium arcs, the second brightest, were used instead. Whilst the optical
36 principles of this device worked well, it was extremely difficult to analyse or
37 visualise the data generated. This changed in the 1980's upon the advent of
38 affordable, reliable computer systems capable of undertaking image analysis
39 and data storage. Modern CLSM uses a focussed spot (or multiple spots or a
40 slit) of laser light to scan across the sample whilst a pinhole aperture blocks
41 aberrant light from areas outside of the focal plane of interest. CLSM has
42 been used for almost two decades to undertake the optical sectioning of
43 microbial biofilms to produce three-dimensional data sets (Lawrence *et al.*
44 1991).

45

46 Fluorescent dyes (fluorophores) are now a fundamental component of CLSM.
47 Fluorescence is the molecular adsorption of a photon which in turn triggers
48 the emission of another photon of a longer wavelength (due to the Stokes
49 shift); the remaining energy is lost as molecular vibration or shed as heat. Put
50 simply, a fluorescent material adsorbs light of one colour and emits another.

51 This phenomenon allows one to illuminate a specific fluorophore with light of
52 one wavelength and selectively collect the resulting emissions using filters to
53 obstruct light of unwanted wavelengths, whilst the confocal optics block light
54 from unwanted focal planes.

55

56 CLSM analysis of biological samples typically uses fluorophores that are
57 associated with specific matrix / biofilm components. In the case of microbial
58 biofilm these fluorophores / targets could included:

- 59 1. Calcofluor white: Binds to β -linked polysaccharides (i.e. to label
60 extracellular polysaccharide).
- 61 2. Fluorescent *in situ* hybridisation (FISH): Detect the presence (or absence)
62 of specific 16s rRNA sequence in multispecies biofilm (i.e. for the
63 identification of microbial species).
- 64 3. Fluorescent indicators of membrane integrity (i.e. to reveal cell viability).
- 65 4. Green Fluorescent Protein integrated into the genome (i.e. used as a
66 reporter of gene expression / metabolic activity / biosensor).

67

68 The molecular Probes™ LIVE/DEAD stain system detects nonviable bacteria
69 by a red fluorescent dye (DEAD - propidium iodide) which is membrane
70 impermeant and as such is excluded from entering intact, healthy cells.

71 Viable bacteria are detected by a complimentary, green fluorescent dye (LIVE
72 – SYTO9™) which is membrane permeable and stains all cells. When these
73 dyes intercalate DNA their fluorescence increases significantly, therefore;
74 unbound dyes in the *milieu extérieur* do not interfere with the detection of the
75 stains within bacterial cells. If both dyes are present in a cell (i.e. a bacterium

76 with a damaged membrane), the DEAD stain displaces the LIVE stain from
77 the nucleic acid due to its much higher affinity to intercalate DNA – such a cell
78 will fluoresce red (DEAD).

79

80 CLSM data can be studied by a variety of image analysis techniques to yield
81 numerical data regarding biofilm architecture (Wood *et al.* 2000), metabolic
82 activity (Macfarlane and Macfarlane 2006), composition (Daims *et al.* 2006)
83 and commensal interactions (Egland *et al.* 2004). Our previously published
84 work regarding the spatial distribution of cell vitality in biofilm is based upon
85 depth related trends observed using fluorescent indicators of membrane
86 integrity. These data were gathered by CLSM and derived from plotting the
87 total image fluorescence values against the depth of the optical section into
88 the biofilm (Hope *et al.* 2002). To facilitate a degree of reproducibility between
89 experiments, a biofilm tower (i.e. a high point) was centred in the confocal
90 image stack (figure 1). The results typically produced a bell shaped curve of
91 depth-related fluorescence distribution (figure 3). These data were then
92 normalised (i.e. maximum image fluorescence = 1) and used to compare the
93 distribution of viable and nonviable bacteria in biofilm (Hope and Wilson
94 2003a; Hope *et al.* 2002). Variations of this technique have also been applied
95 by other groups and their findings have been similar to ours (Table 1) (Auschill
96 *et al.* 2001; Arweiler *et al.* 2004; Netuschil *et al.* 1998; Pratten *et al.* 1998;
97 Zaura-Arite *et al.* 2001; Hope *et al.* 2002; Hope and Wilson 2003b; Hope and
98 Wilson 2006; Watson and Robinson 2005; Dalwai *et al.* 2006).

99

100 We were initially concerned that the viability profiles revealed by analysing
101 image fluorescence could be an artefact caused by the inter-relationship of
102 biofilm topology and the loss of image contrast which occurs with increasing
103 depth in CLSM imaging. Since the biomass contained within an optical
104 section (generally) increases with depth into the biofilm, this will correspond
105 with an increase in fluorescence due to the presence of more fluorescent
106 material. This effect will be apparent up to a depth of approximately 40 μm ,
107 being the point where the absorption of photons emitted by the fluorophore
108 causes image fluorescence to decrease towards zero (Vroom *et al.* 1999).

109

110 A mathematical model was constructed to demonstrate this perceived
111 phenomenon based upon an idealised hemi-spherical biofilm of radius 80 μm
112 (figure 2). In this model, the amount of fluorescent material within the 'optical
113 section' increases with depth into the hemi-sphere. Fluorescence is
114 distributed homogenously within this hemi-spherical model. Quenching of
115 emitted photons is modelled at a linear rate beyond 40 μm depth until total
116 absorbance at the base of the biofilm where no fluorescence is detectable.
117 The resulting 'fluorescence profile' through this *in silico* model (figure 3) is
118 similar to those which have been previously reported *in actu* (figure 1) and the
119 conformity between these two facets would no doubt be even closer if a more
120 complicated shape and a non-linear co-efficient of adsorption were
121 incorporated into the mathematical model.

122

123 The result of this mathematical model was initially thought to be the reason
124 why the 'typical' viability profiles we had previously observed in supragingival

125 plaque biofilms were again evident in subgingival plaque – even though
126 individual optical sections presented a nonviable outer layer of bacteria
127 surrounding a viable interior (Hope and Wilson 2006). This contradiction in
128 subgingival plaque biofilm was discussed and it was suggested that if the
129 extent of nonviable bacteria in the outer layers was minimal, then although it
130 might present itself to the observer as nonviable outer layer, it would not affect
131 image analysis of depth related viability / fluorescence profiles.

132

133 It now seems as though our initial concerns have been allayed after the
134 results published in a recent study (Beyenal *et al.* 2004). In these
135 experiments, an optical microsensor was used to probe biofilms of
136 *Staphylococcus aureus* which were engineered to express Yellow Fluorescent
137 Protein. The microsensor measured fluorescence at different points within a
138 biofilm microcolony (figure 4) and reported depth related profiles. They
139 suggested that metabolic activity (vitality) increases with depth in the outer
140 layers of the biofilm before decreasing in the deeper regions. The relative
141 fluorescence profiles produced by the direct microsensor technique, which
142 physically penetrated into the biofilm, were similar to those produced by
143 CLSM. This suggests that viability profiles produced by CLSM are not an
144 artefact of the process by which the images are captured (Table 1). It would
145 be interesting to see if the fluorescence profiles through the *S. aureus*
146 biofilms, as captured by CLSM, matched those produced by the microsensor.
147
148 Biofilm topography is without doubt an important consideration when using
149 CLSM to capture transverse optical sections and subsequently measure the

150 spatial distribution of cell vitality in relation to depth. The reproducibility of this
151 and similar techniques will be improved by taking steps to standardise which
152 structural motifs of biofilm are analysed, along with more advanced 3-
153 dimensional analysis (Hope and Wilson 2003b).

154

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159

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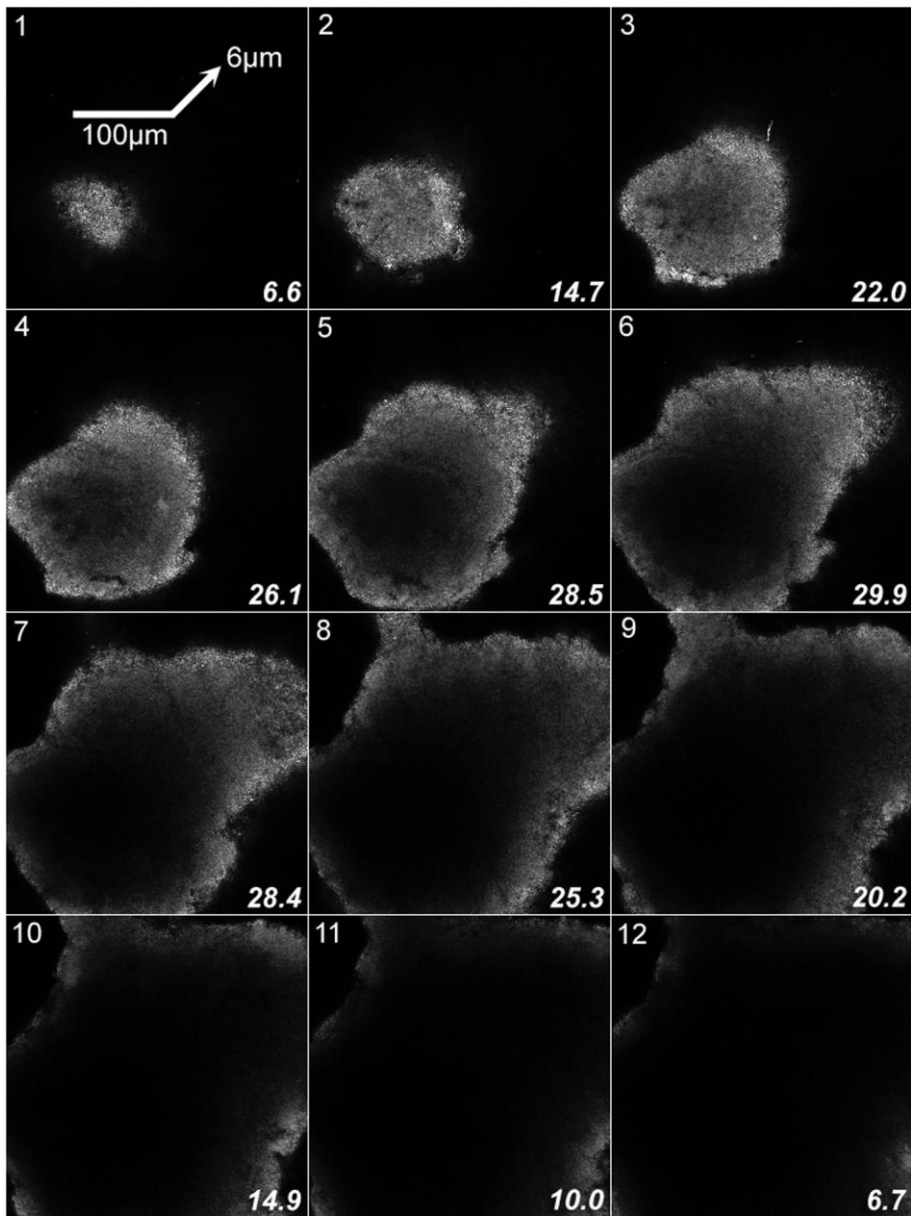
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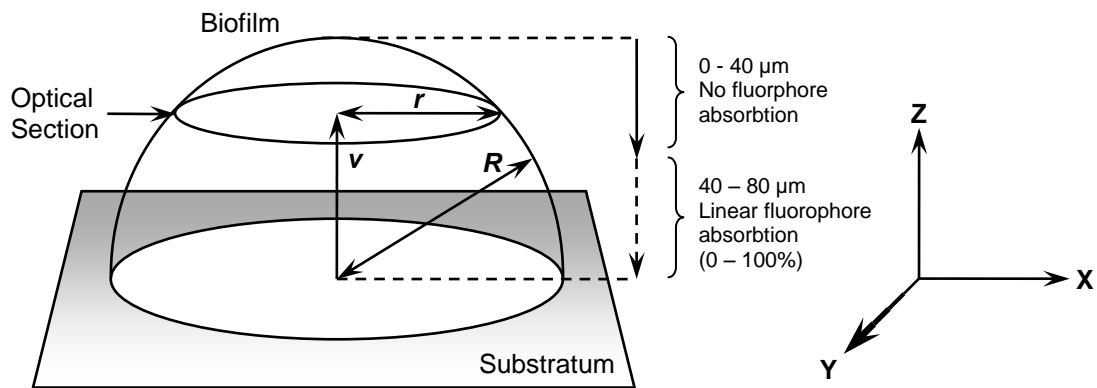
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226

227 **Figure 1** Sequence of twelve optical sections through subgingival oral biofilm
 228 stained with SYTO9 (300 x 300 x 72 µm; 6 µm slice separation). The average
 229 pixel brightness for individual images is given in the bottom right of each slice.

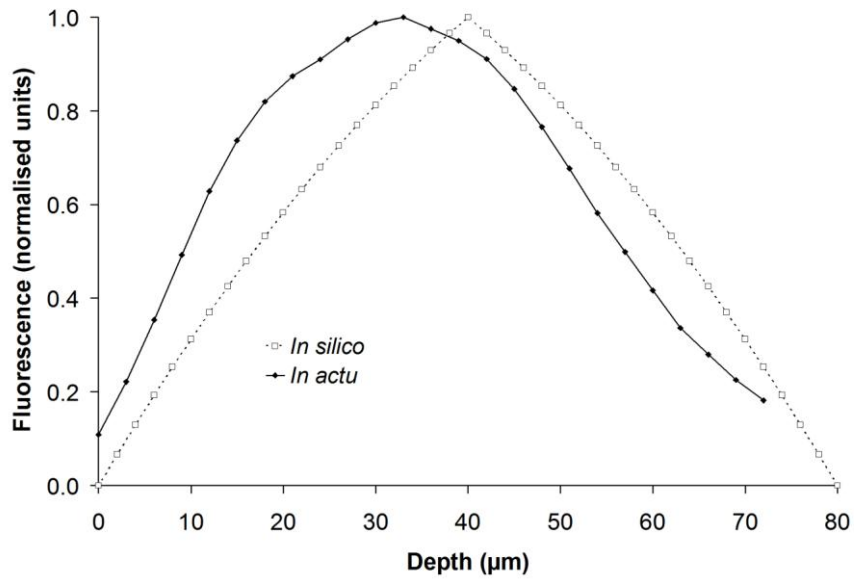
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232 **Figure 2** Confocal image stack model based upon an idealised hemi-
 233 spherical biofilm where; R is the radius of the sphere ($80 \mu\text{m}$), v is the height
 234 of the optical section in the image stack and r is the radius of the circle formed
 235 by the biofilm (at height v). In this model, the area (a) of a particular optical
 236 section can be calculated using the equation, $a = \pi (R^2 - v^2)$ and is equal to
 237 the image fluorescence. The fluorescence within the hemi-sphere is
 238 distributed homogenously. The absorption of fluorophore photons by the
 239 sample is modelled by a linear decrease in fluorescent intensity from $40 \mu\text{m}$ to
 240 $80 \mu\text{m}$ depth (0 to 100% adsorption).

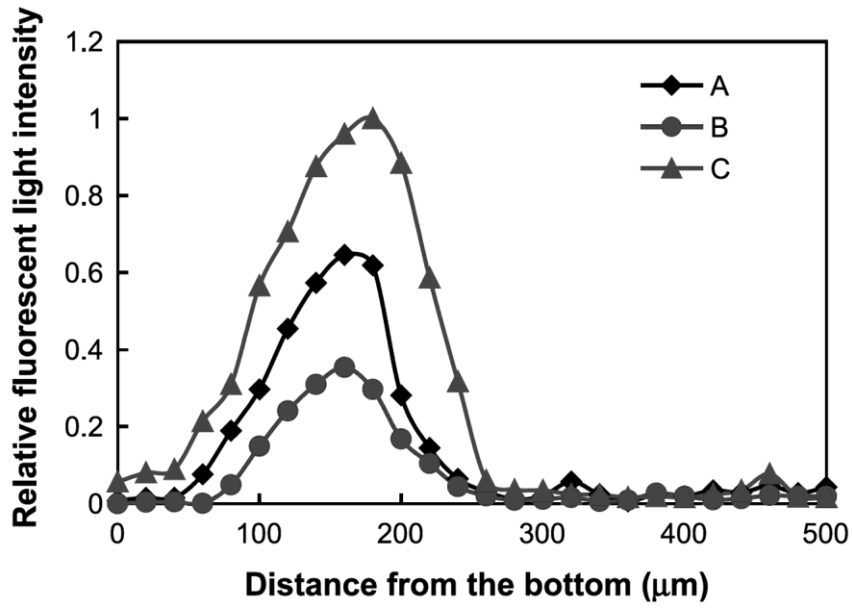
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242

243 **Figure 3** Fluorescence profile through *in actu* subgingival plaque biofilm
 244 grown in a CDFP (corresponding to figure 1) compared to a mathematical
 245 model showing the fluorescence profile through an idealised biofilm *in silico*
 246 (corresponding to figure 2).

247



248

249 **Figure 4** Relative fluorescence intensity profile measured in biofilm of

250 *Staphylococcus aureus*. Plots A, B and C refer to different sites in a

251 microcolony (Beyenal *et al.*, 2004).

252

253 **Table 1** Studies which comment upon on the spatial distribution of cell vitality
 254 in oral biofilm. Asterisks (*) denote the common thread of cell vitality /
 255 biomass distribution shown in figure 3.

Study	Principle	Findings
Arweiler 2004	CLSM. Vital staining; measured as percentage vitality in different optical sections.	1. Dead lower layers, live middle / upper layers, less live in outermost layers.* 2. Live lower layers, dead upper layers. 3. Thin disorganised layers.
Auschill 2001	CLSM. Vital staining; measured as percentage vitality in different optical sections.	Dead lower layers, live middle / upper layers, less live in outermost layers.*
Dalwai 2006	CLSM. Vital staining; viable and nonviable fluorescence measured in different optical sections.	Fluorescence distribution low in deep biofilm, higher in the middle layers, decreasing in the outermost layers.*
Hope 2002	CLSM. Vital staining; viable and nonviable compared by normalising fluorescence values in different optical sections.	Dead lower layers, live middle / upper layers, less live in outermost layers.*
Hope 2003	CLSM. Vital staining; analysis of data in 3-dimensions	Dead inner layers, live outer layers.*
Hope 2006	CLSM. Vital staining; viable and nonviable compared by normalising fluorescence values in different optical sections. Subgingival oral biofilm model.	1. Low fluorescence in lower layers, higher in middle / upper layers, lower in outermost layers.* 2. Horizontal sections suggested dead outer layers.
Netuschil 1998	CLSM. Vital staining; measured as percentage vitality.	Dead lower layers, live middle / upper layers, less live in outermost layers.*
Pratten 1998	CLSM vital staining.	Dead lower layers, live upper layers.*
Watson 2005	CLSM. Vital staining. Biomass recorded as total image fluorescence.	Low biomass in outer layers, increasing in middle layers, decreasing in deeper layers.*
Zaura-Arite 2001	CLSM. Vital staining. Comparison of percentage vitality at different depths.	No definitive pattern of vitality reported.

Other Related Studies

Study	Principle	Findings
Beyenal 2004	Direct measurement of Yellow Fluorescent Protein by an optical microsensors in <i>S. aureus</i> . (not oral biofilm, not CLSM)	Low fluorescence (metabolic activity) at biofilm surface, increasing with depth, decreasing in deeper layers.*
Egland 2004	CLSM. Distribution of specifically labelled bacteria (FISH) in a dual-species oral biofilm. (not vital staining)	Fluorescence distribution (biomass) low in deep biofilm, higher in the middle layers, decreasing in the outermost layers.*
Pratten 1998	Transmission electron microscopy. Comparison of cells from different depths in biofilm. (not CLSM)	Higher proportion of 'ghost' cells (assumed to be nonviable bacteria) in lower layers.*

256