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Use of a High-throughput In Vitro Microfluidic System to Develop Oral Multi-Species Biofilms

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Corresponding Author:	Alexander Rickard University of Michigan Ann Arbor, Michigan UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	alexhr@umich.edu
Corresponding Author's Institution:	University of Michigan
Corresponding Author's Secondary Institution:	
First Author:	Derek Samarian
First Author Secondary Information:	
Other Authors:	Derek Samarian
	Nicholas Jakubovics
	Ting Luo
Order of Authors Secondary Information:	
Abstract:	There are few high-throughput in vitro systems which facilitate the development of multi-species biofilms that contain numerous species commonly detected within in vivo oral biofilms. Furthermore, a system that uses natural human saliva as the nutrient source, instead of artificial media, is particularly desirable in order to support the expression of cellular and biofilm-specific properties that mimic the in vivo communities. We describe a method for the development of multi-species oral biofilms that are comparable, with respect to species composition, to supragingival dental plaque, under conditions similar to the human oral cavity. Specifically, this methods article will describe how a commercially available microfluidic system can be adapted to facilitate the growth of multi-species oral biofilms derived from a pooled saliva inoculum and grown in pooled cell free human saliva. Furthermore, a description of how the system can be used in conjunction with a confocal laser scanning microscope to generate 3-D biofilm reconstructions for architectural and viability analyses will be presented. Given the broad diversity of microorganisms that grow in the environmentally germane microfluidic-based oral biofilms (including Streptococcus, Neisseria, Veillonella, Gemella, and Porphyromonas), a protocol will also be presented describing how to harvest microfluidic-developed biofilm cells for further subculture- or DNA extraction and analysis. The limits of both the microfluidic biofilm system and the current state-of-the-art data analyses will be addressed. Ultimately, it is envisioned that this article will provide a baseline technique that will improve the study of oral biofilms and aid in the development of additional technologies that can be integrated with the microfluidic platform.
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Title:
Use of a High-throughput In Vitro Microfluidic System to Develop Oral Multi-Species Biofilms

AUTHORS:
Derek Samarian[1], Ting Luo[1], Nicholas S. Jakubovics[2], & Alexander H. Rickard[1]

AFFILIATIONS:
Samarian Derek [1]
[1] Department of Epidemiology, School of Public Health,
The University of Michigan,
Ann Arbor, MI, USA.
dsamari@umich.edu

Luo Ting [1]
[1] Department of Epidemiology, School of Public Health,
The University of Michigan,
Ann Arbor, MI, USA.
tingluo@umich.edu

Jakubovics Nicholas S. [2]
[2] Centre for Oral Health Research,
School of Dental Sciences,
Newcastle University,
Newcastle upon Tyne, UK
nick.jakubovics@ncl.ac.uk

Rickard Alexander H. [1]
[1] Department of Epidemiology, School of Public Health,
The University of Michigan,
Ann Arbor, MI, USA.
alexhr@umich.edu

CORRESPONDING AUTHOR:
A.H. Rickard,
alexhr@umich.edu,
Tel: 734-615-8491;
Fax: 734-764-3192

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SHORT ABSTRACT:

The goal of this methods paper is to describe the use of a microfluidic system for the development of multi-species biofilms that contain species typically identified in human supragingival dental plaque. Methods to describe biofilm architecture, biofilm viability, and approach to harvest biofilm for culture- or culture-independent analyses are highlighted.

LONG ABSTRACT:

There are few high-throughput *in vitro* systems which facilitate the development of multi-species biofilms that contain numerous species commonly detected within *in vivo* oral biofilms. Furthermore, a system that uses natural human saliva as the nutrient source, instead of artificial media, is particularly desirable in order to support the expression of cellular and biofilm-specific properties that mimic the *in vivo* communities. We describe a method for the development of multi-species oral biofilms that are comparable, with respect to species composition, to supragingival dental plaque, under conditions similar to the human oral cavity. Specifically, this methods article will describe how a commercially available microfluidic system can be adapted to facilitate the growth of multi-species oral biofilms derived from a pooled saliva inoculum and grown in pooled cell free human saliva. Furthermore, a description of how the system can be used in conjunction with a confocal laser scanning microscope to generate 3-D biofilm reconstructions for architectural and viability analyses will be presented. Given the broad diversity of microorganisms that grow in the environmentally germane microfluidic-based oral biofilms (including *Streptococcus*, *Neisseria*, *Veillonella*, *Gemella*, and *Porphyromonas*), a protocol will also be presented describing how to harvest microfluidic-developed biofilm cells for further subculture- or DNA extraction and analysis. The limits of both the microfluidic biofilm system and the current state-of-the-art data analyses will be addressed. Ultimately, it is envisioned that this article will provide a baseline technique that will improve the study of oral biofilms and aid in the development of additional technologies that can be integrated with the microfluidic platform.

INTRODUCTION:

Biofilms are architecturally complex communities of bacteria that are aggregated on surfaces [1]. These communities typically contain numerous species that interact with one another, within the biofilm, and with the environment [2]. Oral biofilms, the most visually conspicuous being dental plaque, are a persistent problem in humans and their uncontrolled development leads to taxonomically diverse multi-species communities [3]. The component bacteria of these diverse communities can be up to 1000-times more resistant to antimicrobials than their free-floating (planktonic) counterparts [4-6]. Failure to treat these oral biofilm communities, which can cause dental caries and periodontal disease, has resulted in a significant public health burden: over 500 million visits to the dentist office per annum in the US, and an approximately $108 billion to treat or prevent periodontal disease and dental caries [7].

"*While many microbiologists advocate studying microbial behavior under natural conditions, few of them do so. This is because their morale for overcoming the difficulties is constantly sapped by the attractive ease of working with laboratory cultures*." Smith [8].

At present, oral biofilm research is conducted using a variety of *in vivo* and *in vitro* approaches, each with their own advantages and disadvantages [9,10]. *In vitro* approaches often use model biofilm systems that are relatively easy to set up but may lack clinical/real-world relevance [10,11]. *In vivo* approaches typically rely upon animal model systems that may reproduce certain aspects of the human oral environment, but again suffer from limitations due to differences in anatomy, physiology, microbiology and immunology between animals and humans [12,13]. It should be noted that oral biofilms can also be developed on enamel surfaces held in a stent within the mouths of human volunteers, but this approach is currently relatively costly and labor-intensive [14,15]. Ultimately, novel agents or technologies to improve oral healthcare are tested in humans under controlled clinical trial conditions [11]. At present, an often-used *modus operandi* for identifying and evaluating new oral healthcare agents is to perform laboratory studies first, to discern potential efficacy, and then perform animal studies and "field trials" that employ clinicians to evaluate the success of the technology [9,16,17]. Unfortunately, lab studies tend to rely on model systems that occupy a large footprint, are technologically challenging to use, and often contain simplified communities of one or at most a few species to derive potential real-world meaning [10,18]. Given that dental plaque biofilms, and indeed nearly all biofilms in the broader environment, contain multiple species and develop in a complex milieu under flow, developing biofilms that contain one or a few species in artificial media is likely to provide outcomes that are not representative of the real-world scenario [10,19]. To address the time, cost, training requirements, and the poor representative nature of laboratory model biofilm systems to the real-world environment, we recently developed a high throughput and environmentally germane biofilm system [20] (Fig. 1). The system benefits from the use of cell-free pooled human saliva (CFS) as medium and untreated pooled human (bacterial) cell-containing saliva (CCS) as an inoculum. Uniquely, the system also combines microfluidic technology, a confocal laser scanning microscopy platform, and culture-independent bacterial diversity analysis technology. Taken together, the model system is environmentally germane (using saliva as an inoculum to grow multi-species biofilms at 37^{o}C in flowing saliva) and the oral biofilms contain species (including *Streptococcus*, *Neisseria*, *Veillonella*, and *Porphyromonas* species) in abundances representative of those found in early supragingival plaque [20].

When considering this work that describes the use of the newly developed model system, particular attention to the amalgamation of CLSM, microfluidics, and the capability to perform culture-independent diversity analyses must be given. The union of these technologies by our research group was intentional and not only adds a high-throughput capability to the newly developed model system but also allows questions to be asked that could not be easily addressed before with other systems. Firstly, CLSM has distinct advantages over traditional microscopy as it allows for the three-dimensional analysis of biofilms. Often unappreciated, this is extremely important as

biofilms are heterogeneous with respect to species composition and spatial position as well as the physiological conditions being imposed at different spatial locations within the biofilm [6,21]. In concert with three-dimensional rendering software and image analysis software, the biofilm architecture, spatial relationships between composition species, and extent of antimicrobial killing can be analyzed [22-24]. Such abilities are not possible using standard transmitted light or epifluorescence microscopy. Next, microfluidics has garnered particular attention in the field of microbiology as it enables the study of biofilms under carefully controlled conditions (flow, temperature, pH, etc) and only requires small volumes of liquid [25-27]. As a point of comparison, growing an oral biofilm in human saliva within a flow cell model system (a system that is arguably considered the mainstay model for many oral biofilm studies) for 22 hours at a similar flow-rate and shear as that achieved in a microfluidic system requires at least 200 mL, as opposed to 800 µL in the microfluidic device [28-31]. Thus, a microfluidic model biofilm system enables the study of quantity-limited material under defined conditions. Finally, pyrosequencing technology has been optimized in the last decade to require only small amounts of material to perform a community analysis and is sufficiently versatile to control depth of sequencing to obtain the identity of even rare biofilm species. The use of this technology, such as bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), has allowed for pertinent questions concerning the ecology of biofilms to be addressed [32,33]. Such questions imbued difficulties in the past when pyrosequencing was not available because of the time and costs required to create plasmid libraries and the complex technological and analytical steps required to derive data [33,34]. Of course, a great advantage with culture-independent approaches, such as pyrosequencing, is that the bacterial species that cannot be grown in isolation conventional laboratory media (i.e. viable but non-cultivable species) can be identified and their relative abundance in the community quantified [35 36]. To add perspective, as early as 1963, the late Sigmund Socransky estimated that approximately 50% of the bacteria in material isolated from the human oral gingival crevicular crevice could not be cultured using laboratory growth conditions [37].

The objective of this methods paper is to describe the approach to develop oral multi-species biofilms in a commercially available microfluidic (Bioflux) system under conditions representative of the human oral cavity and with a species composition and abundance that is comparable to supragingival plaque. Furthermore, using both freeware and commercial software, we highlight how basic biofilm architecture measures can be derived from CLSM data, with a focus on approaches to quantify biofilm biomass, roughness, and viability (based upon Live/Dead staining). Finally, the steps required to harvest biofilm material for diversity analysis by bTEFAP are described.

PROTOCOLS:

With regard to institutional reviews for human subject work of this type, prior arrangements and permissions should be garnered from the host institution. As an aid to preparing an application, a useful NIH algorithm/chart can be found here:http://grants1.nih.gov/grants/policy/hs/PrivateInfoOrBioSpecimensDecisionChart.pdf

1) PREPARATION OF POOLED SALIVA FOR USE AS AN ENVIRONMENTALLY GERMANE GROWTH MEDIUM

1.1. Recruit ≥5 individuals for saliva donation. Do not take any identifying information, ensure no individuals will donate saliva if they are ill, or have taken oral antibiotics in the past 3 months, or have consumed food or liquid, with the exception of water, in the previous 2 hours before donation.

1.2. Collect saliva in 50 mL plastic tubes. Pool the collected saliva in a plastic beaker, keeping it on ice. Do not use glass as polymers in the saliva will adhere to the internal glass surfaces

1.3. Add Dithiothreitol (DTT) to a final concentration of 2.5 mM from a 100X stock. (Stock is frozen in single-use aliquots at –20^{o}C). Stir for 10 minutes in a plastic beaker on ice.

1.4. In order to remove particulate matter, centrifuge the pooled saliva in a Sorval SS-34 centrifuge for 30 minutes at 17,500 x g.

1.5. Dilute the saliva with 3 volumes of dH_2O to give one-fourth concentrated saliva.

1.6. Filter-sterilize saliva using 0.22 µm polyethersulfone (PES), low protein binding filter. Use filter with large surface area, or use several small area filters. Keep saliva in a plastic container on ice while filtering.

1.7. Freeze the pooled saliva at –80^{o}C in 50 ml plastic tubes until needed to grow bacteria. Each plastic tube is for one use only and should contain no more than 35 mL as each microfluidic well holds a maximum of 1.2 mL (1.2 mL x 24 wells = 28.8 mL) and space is needed in each tube as frozen saliva expands during freezing.

1.8. For use, thaw the pooled saliva at room temperature. Once thawed, filter-sterilize once more (0.22 µm polyethersulfone, low protein binding filter to remove any precipitates.

2) PREPARATION OF POOLED SALIVA FOR USE AS AN INOCULUM

2.1. Recruit ≥5 individuals for saliva donation. Do not take any identifying information, ensure no individuals will donate saliva if they are ill, have taken oral antibiotics in the past 3 months, or have consumed food or liquid, with the exception of water, in the previous 2 hours before donation. Samples should be collected over 30 min.

2.2. Collect saliva in 50 ml plastic tubes at room temperature and pool the collected saliva in a plastic beaker at room temperature.

2.3. Dilute pooled saliva with autoclaved general reagent grade glycerol to yield a stock with a final ratio of 25% glycerol, 75% cell-containing saliva [CCS].

2.4. Freeze saliva at –80^{o}C in 3 mL single-use aliquots until needed.

2.5. When required to inoculate the microfluidic system, aliquots are thawed at room temperature and agitated gently on a vortexer for 5 seconds before being pipetted into the microfluidic system as described in step 3 of the protocol, below.

3) GROWTH OF ORAL MULTI-SPECIES BIOFILMS

3.1. *CFS pre-treatment* – First coat the channels with CFS. Add 100 µL of CFS to each outlet well, do this by using an automatic experimental controls software, such as Bioflux by selecting “manual” and set flow from channels “B1-B24” to be used.

3.2. Next, set shear to 1.0 dyne/cm² and flow for 2 minutes at room temperature to ensure homogenous distribution of the CFS throughout the channel. Ensure there is fluid in each inlet channel to verify that CFS flowed through all channels evenly.

3.3. Incubate plate at room temperature for 20 minutes.

3.4. Aspirate out CFS/pretreat solution that remains in the outlet wells and transfer to the inlet wells. This total ~100 µL will serve to balance against the pressure being applied to the inoculum in the outlet well.

3.5. *Inoculation* – To each outlet well, add 100 µL of CCS inoculum. Place the microfluidic plate on the heat plate set at 37°C, select manual within the control software and set flow from outlet wells to the inlet wells (ie reverse) at 1.0 dyne/cm² for exactly 6 seconds.

3.6. Incubate plate on heating plate at 37°C for 40 min to allow for initial adherence and growth of the inoculum.

3.7. *Overnight Growth* - Aspirate all of the inoculum from each of the outlet wells for channels being used. Add up to 1 mL total volume of CFS into each of the inlet wells (can be done on top of existing CFS).

3.8. Incubate the plate on the heating plate at 37°C, select manual and set the program to run at 0.2 dyne/cm² for 22 hours.

3.9. *Stain prewash* - Aspirate all fluid from the inlet and outlet wells and add 100 µL of PBS (pH 7.4) to each of the inlet wells. Flow for 20 minutes at 0.2 dyne/cm².

3.10. *Stain mixture addition* – For cell viability staining make 100 µL of stain mixture for each channel to be stained. Specifically, Add 3 µL of SYTO 9 and 3 µL of propidium iodide per 1mL of PBS using commercial cell viability staining kit such as LIVE/DEAD.

This generates a staining mixture containing 10.02 µM of SYTO 9 and 60 µM of propidium iodide.

3.11. Aspirate the remaining PBS from the inlet wells and then add 100 µL of the cell viability stain mixture to each inlet well. Set to flow at 0.2 dyne/cm^2 and run the solution from inlet to outlet for 45 minutes at room temperature.

3.12. . *Post-staining wash* - Aspirate the remaining stain in each of the inlet wells and add 100 µL of PBS to each inlet well. Set to flow at 0.2 dyne/cm^2 and run the PBS solution from inlet to outlet for 30 minutes at room temperature to remove any excess stain.

4) IMAGE COLLECTION, 3D RENDERING, AND IMAGE ANALYSIS

4.1 Use an inverted confocal laser scanning microscope (CLSM) to highlight key steps and considerations when collecting biofilm data from the microfluidic system.

NOTE: Given the dimensions of the microfluidic channels, the need for sensitivity to discern biofilm structure, and the requirements to detect fluorescence signal of varying intensity, the fit the CLSM with a 40X 1.25NA objective lens or one with similar optical quality, magnification, and numerical aperture.

4.2. Standardize the emission capture gates with gain and offset measurements being kept constant. Ensure that the laser power does not exceed 25% as this will result in photo-bleaching.

4.3Convert CLSM files from the Leica Image File type (LIF) to OME (Open Microscopy Environment) file type. This allows for greater ease of access and compatibility between software programs. Use commercially available software such as, IMARIS to convert files to this type.

3D Rendering for Images/Figures

4.4. Once converted to OME format, use IMARIS to render the images in 3D. Perform this using a combination of "*Easy3D"* and "*Surpass"* options.

NOTE: Attention to background signal and thresholding should be made. The histogram function in IMARIS can be used to obtain the collection range and this should be kept constant between images being analyzed.

4.5. Save images as different file types using the "*snapshot*" function and make careful consideration to image resolution before saving file types.

4.6 Assemble images into figures using software such as CorelDraw or Adobe Illustrator.

3D Image Analysis for Graphs and Tables

4.7 Use freely available IMAGEJ [23] and COMSTAT/COMSTAT 2 [38] for this 3D image analysis. Download the packages from http://rsb.info.nih.gov/ij/ and http://comstat.dk/, respectively.

NOTE: The software platform JAVA will need to be installed prior to the use of the image analysis software.

4.7.1. Use the IMAGEJ software with COMSTAT2 plugin and the most recent Java update installed. Import the OME files for each biofilm image in "*hyperstack"* mode and view with "*split channels*".

4.7.2. Analyze individually the two channels (red/propidium-iodide/dead being channel 0, green/SYTO-9/LIVE being channel 1) using the "*histogram"* function of IMAGEJ. This function lists the total number of pixels of 8-bit OME files (shown as "count") at each color intensity from 0-255 (shown as "value"), with 0 being pixels with no signal (background) and 255 being pixels with complete signal saturation.

4.7.3. Export the data into Microsoft Excel and standardize all signal values by weighting each pixel by the corresponding signal intensity. This is performed by multiplying the total count at a given signal intensity by the numerical 8-bit value (0-255) of that signal intensity.

4.7.4. Sum all weighted values, 0-255, for both channels for each biofilm image captured. Take the sum of the weighted values for both channels are and find the percent total signal from either channel, do this to determine the relative ratio or percent red and percent green signal (i.e. the percent "DEAD" and percent "LIVE" for each treatment).

4.7.5. Perform statistical analyses, for example using two-tailed Student's T-test modified for unequal variance. Values of $p < 0.05$ are considered significant and those values of $p < 0.01$ are considered highly significant.

5) HARVESTING BIOFILM CELLS FOR CULTURE-INDEPENDENT ANALYSIS

5.1 Remove all spent and unused saliva from inlet and outlet wells. Wash the wells with 1 mL sterile distilled water three times.

5.3. Add 100 µL of sterile distilled water to the inlet wells and, using the software control interface, pass the sterile distilled water forward through the channels at $\geq$8.0 dyne/cm^2 (flow rate $\geq$745 µL/h, shear of 800 s^{-1}) for at least 10 min. Repeat in the reverse direction for at least 10 minutes and then repeat the forward and reverse washing step process.

5.4. Check by light or by epifluorescence microscopy (if cells were stained/labelled) for thorough biofilm removal (Fig. 2). Collect the 100 µL cell-suspension and store at -80^{o}C for culture-independent analysis. A cost-effective analysis of community composition can be through using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) using the approach described in Nance et al. [20]

FIGURE AND TABLE LEGENDS:

Figure. 1. The Bioflux microfluidic CSLM oral biofilm system. (**A**) A diagram showing a vertical cross-section of the Bioflux system while being mounted on a Leica SPE CLSM. (**B**) An annotated (black lines) photograph highlighting two microfluidic channels and the inlet and outlet wells. (**C**) An example of a rendered two-dimensional Live/Dead stained oral biofilm that has been treated with a 0.01% CPC solution resulting in heterogeneous killing, in part due to reaction diffusion limitation. Green color indicates live cells, stained with Syto 9; red colored cells are dead or damaged and stained with propidium iodide. Fig 1A and 1B from Nance et al. [20] with permission. Bar in Fig. 1C represents 30 µm.

Figure. 2. Two dimensional and three-dimensional views of rendered 22 h biofilms developed in pooled cell-free saliva (CFS) that was inoculated from pooled cell-containing saliva (CCS). An untreated biofilm community (left column of images) is compared against a 70% ethanol treated biofilm community and a representative biofilm is shown in different planes of view (XY, XZ, and ZYZ). Histograms showing the differences in red (dead/damaged cells) and green (live cells) are shown for each image stack (logged data shown in black and non-logged data shown in grey). All data is derived from 8 bit images and thus on a scale of 0-255 (256 increments). Scale bars represent 30 µm.

Figure. 3. Flow chart demonstrating the outcome of extraction/harvesting of 22 h oral multi-species biofilm (developed from a CCS inoculum in flowing CFS) from the Bioflux microfluidic device using the elevated shear/flow technique. Dotted lines have been applied to the images to aid in determining the location of the channel walls. The community composition of the harvested biofilm can be analyzed by 454 pyrosequencing approaches such as bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and expressed at multiple taxonomic levels (phylum through species) depending upon requirements. Shown is an example of the community composition, at the phylum level, of biofilms harvested from three microfluidic channels. Bars reprent scale in micrometers.

Table 1. Quantification of biofilm properties of untreated and 70% ethanol treated 22 h biofilms developed in pooled cell-free saliva (CFS) from an inoculum cell-containing saliva (CCS). Bold values are the averages and non-bolded values in brackets are the standard deviations). Data is from at-least five images from three microfluidic channels. Significant differences to the untreated control are highlighted by an asterisk (*: $P < 0.05\%$; ** $P < 0.01\%$).

REPRESENTATIVE RESULTS:

3D RENDERING OF BIOFILMS

Representative results are shown in Figure 3. A useful capability to IMARIS software is the contained tools to examine each slice of the collected biofilm stack and to render them to create three-dimensional reconstructions. In addition, artificial shadowing effects can be added to help visually interpret three-dimensional structures. Furthermore, the biofilms can be orientated in any direction with almost limitless useful magnification to explore biofilm or micro-colony structure. The images presented in Fig. 3 show the outcome of the treatment of an oral multi-species biofilm. Of particular note is that treatment with 70% ethanol resulted in significant color change for most of the biofilm (from green to red) suggesting significant cell-death or cell-damage. The large micro-colonies appeared to contain more dead cells than the underlying (and now exposed) micro-colony cells. Such an outcome is conceivably due to de-adhesion of biofilm cells because ethanol is a membrane-active antimicrobial. By converting the files to OME format and analyzing the rendered stacks in IMAGEJ, it can be seen by applying histogram functions that the amount of red and green signal is inversely proportional between the untreated biofilms and those treated with ethanol.

QUANTIFICATION OF BIOFILM PROPERTIES

Table 1 shows the average and standard deviations of key structural parameters. A key advantage to using IMARIS, IMAGEJ and COMSTAT, is that if IMARIS is used first, OME files can be generated to allow files to be pipelined through IMAGEJ and COMSTAT. The sample data presented in Table 1 shows that treatment of the oral biofilms with 70% ethanol resulted in a highly significant drop in viability from 80.8% to 28.3%. Ethanol can cause some cell damage and structural changes in biofilms but this is often-times not measurably significantly different. Here, no differences in measures of average biovolume, average thickness and average roughness were detected (Table 1).

DISCUSSION:

This methods paper highlights the basic steps required to setup and run a microfluidic system in a manner to allow for the development of oral multi-species biofilms derived from pooled saliva and grown in filter-sterilized 25% human saliva. Approaches to characterize the biofilm are given but it should be remembered that these described approaches are modifiable and additional technologies such as, for example, stains or labels can be introduced. As a matter of example, one could conceivably use labeled antibodies or introduce a fluorescent strain to visualize and examine the spatial position of certain species within the oral multi-species biofilm. Furthermore, depending upon the model of the microfluidic system being used, it is possible to develop biofilms under anaerobic conditions (in the case of the Bioflux system, a technical note is available at Fluxion.com on this subject).

A key aspect to the microfluidic systems are their compatibility with multiple technologies and this is highlighted here by the ability to perform culture-independent diversity analyses (Fig. 3). While the internal surfaces of the microfluidic system are not easily accessible, vigorous forward and reverse flow does enable substantial biofilm biomass to be removed. While all of the biofilm cannot be easily removed and this could be considered a weakness to the system, it is relevant to note that many model biofilm systems also have a similar issue and the claims from any such collected data have to

be tempered with this knowledge. For example, it is possible that the abundance of streptococci might be higher in the biofilm than actually determined because many *Streptococcus* species have exceptionally good abilities to bind to saliva coated surfaces [39].

As with all model biofilm systems, one has to be mindful of the questions being asked. For example, this system would not be suitable for long-term longitudinal studies. A model system such as a constant depth film reactor, a sorbarod system, or a drip reactor might be more suitable [40-42], although the problem of limited quantities of saliva for a model system becomes an issue as these are not microfluidic-based designs. In a similar light though, the Bioflux system described here could also be adapted for studies of biofilms in other environments where biological fluids are only available in small quantities. For example, this could include urine and wound exudate.

In conclusion, as with any *in vitro* model system, one has to be cognizant of the strengths and weaknesses to the model microfluidic system for the growth of oral biofilms. While the microfluidic system is arguably environmentally germane and allows for the development of biofilms that are compositionally *similar* to the *in vivo* situation, it is not the *same* as the *in vivo* situation and will likely never be so. A laboratory model system is only as good as its assumptions and while the microfluidic system has many overlaps with conditions within the human oral cavity, factors such as host-based effects and external biotic and abiotic challenges (e.g. through drinking and eating) cannot be easily replicated. With this in mind, however, it is clear that the high throughput nature, and environmental, and microbiological overlaps with the real-world oral environment make this an alluring system for making predictions before preparing for lengthy, expensive, and logistically challenging clinical studies.

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REFERENCES:

1 Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. Biofilms as complex differentiated communities. *Annual review of microbiology.* **56** 187-209, doi:10.1146/annurev.micro.56.012302.160705, (2002).

2 Wimpenny, J. Microbial metropolis. *Advances in microbial physiology.* **56** 29-84, doi:10.1016/S0065-2911(09)05602-1, (2009).

3 Jakubovics, N. S. & Kolenbrander, P. E. The road to ruin: the formation of disease-associated oral biofilms. *Oral diseases.* **16** (8), 729-739, doi:10.1111/j.1601-0825.2010.01701.x, (2010).

4 Mah, T. F. & O'Toole, G. A. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in microbiology.* **9** (1), 34-39, doi:10.1016/S0966-842X(00)01913-2, (2001).

5 ten Cate, J. M. & Zaura, E. The numerous microbial species in oral biofilms: how could antibacterial therapy be effective? *Advances in dental research.* **24** (2), 108-111, doi:10.1177/0022034512450028, (2012).

6 Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H. & Whyte, F. W. The physiology and collective recalcitrance of microbial biofilm communities. *Advances in microbial physiology.* **46** 202-256, doi:10.1016/S0065-2911(02)46005-5 (2002).

7 Anon. *Centers for Disease Control and Prevention (CDC): Oral health: Preventing cavities, gum disease, tooth loss, and oral cancers.* , < http://www.cdc.gov/chronicdisease/resources/publications/AAG/doh.htm#aag> (2011).

8 Smith, H. in *Microbial behavior, "in vivo" and "in vitro"* eds H. Smith & J. Taylor) 1-29 (Cambridge University Press, 1964).

9 Haffajee, A. D. & Socransky, S. S. Introduction to microbial aspects of periodontal biofilm communities, development and treatment. *Periodontology 2000.* **42** 7-12, doi:10.1111/j.1600-0757.2006.00190.x, (2006).

10 McBain, A. J. Chapter 4: In vitro biofilm models: an overview. *Advances in applied microbiology.* **69** 99-132, doi:10.1016/S0065-2164(09)69004-3, (2009).

11 Baehni, P. C. & Takeuchi, Y. Anti-plaque agents in the prevention of biofilm-associated oral diseases. *Oral diseases.* **9 Suppl 1** 23-29, doi:10.1034/j.1601-0825.9.s1.5.x (2003).

12 Graves, D. T., Kang, J., Andriankaja, O., Wada, K. & Rossa, C., Jr. Animal models to study host-bacteria interactions involved in periodontitis. *Frontiers of oral biology.* **15** 117-132, doi:10.1159/000329675, (2012).

13 Chun, J., Kim, K. Y., Lee, J. H. & Choi, Y. The analysis of oral microbial communities of wild-type and toll-like receptor 2-deficient mice using a 454 GS FLX Titanium pyrosequencer. *BMC microbiology.* **10** 101, doi:10.1186/1471-2180-10-101, (2010).

14 Diaz, P. I. *et al.* Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Applied and environmental microbiology.* **72** (4), 2837-2848, doi:10.1128/AEM.72.4.2837-2848.2006, (2006).

15 Auschill, T. M. *et al.* Effect of two antimicrobial agents on early in situ biofilm formation. *Journal of clinical periodontology.* **32** (2), 147-152, doi:10.1111/j.1600-051X.2005.00650.x, (2005).

16 Coenye, T. & Nelis, H. J. In vitro and in vivo model systems to study microbial biofilm formation. *Journal of microbiological methods.* **83** (2), 89-105, doi:10.1016/j.mimet.2010.08.018, (2010).

17 Donlan, R. M. Role of biofilms in antimicrobial resistance. *ASAIO journal.* **46** (6), S47-52, doi:10.3201/eid0809.020063, (2000).

18 Wimpenny, J. W. The validity of models. *Advances in dental research.* **11** (1), 150-159, doi:10.1177/08959374970110010601 (1997).
19 Umland, T. C. *et al.* In vivo-validated essential genes identified in Acinetobacter baumannii by using human ascites overlap poorly with essential genes detected on laboratory media. *mBio.* **3** (4), doi:10.1128/mBio.00113-12, (2012).
20 Nance, W. C. *et al.* A high-throughput microfluidic dental plaque biofilm system to visualize and quantify the effect of antimicrobials. *The Journal of antimicrobial chemotherapy.* **68** (11), 2550-2560, doi:10.1093/jac/dkt211, (2013).
21 Werner, E. *et al.* Stratified growth in Pseudomonas aeruginosa biofilms. *Applied and environmental microbiology.* **70** (10), 6188-6196, doi:10.1128/AEM.70.10.6188-6196.2004, (2004).
22 Rueden, C. T. & Eliceiri, K. W. Visualization approaches for multidimensional biological image data. *BioTechniques.* **43** (1 Suppl), 31, 33-36, doi:10.2144/000112511, (2007).
23 Collins, T. J. ImageJ for microscopy. *BioTechniques.* **43** (1 Suppl), 25-30, doi:doi:10.2144/000112517, (2007).
24 Rao, D., Arvanitidou, E., Du-Thumm, L. & Rickard, A. H. Efficacy of an alcohol-free CPC-containing mouthwash against oral multispecies biofilms. *The Journal of clinical dentistry.* **22** (6), 187-194 (2011).
25 Rusconi, R., Garren, M. & Stocker, R. Microfluidics expanding the frontiers of microbial ecology. *Annual review of biophysics.* **43** 65-91, doi:10.1146/annurev-biophys-051013-022916, (2014).
26 Kim, J., Park, H. D. & Chung, S. Microfluidic approaches to bacterial biofilm formation. *Molecules.* **17** (8), 9818-9834, doi:10.3390/molecules17089818, (2012).
27 Mosier, A. P. & Cady, N. C. Analysis of bacterial surface interactions using microfluidic systems. *Science progress.* **94** (Pt 4), 431-450 (2011).
28 Foster, J. S. & Kolenbrander, P. E. Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. *Applied and environmental microbiology.* **70** (7), 4340-4348, doi:10.1128/AEM.70.7.4340-4348.2004, (2004).
29 Cuadra-Saenz, G. *et al.* Autoinducer-2 influences interactions amongst pioneer colonizing streptococci in oral biofilms. *Microbiology.* **158** (Pt 7), 1783-1795, doi:10.1099/mic.0.057182-0, (2012).
30 Rickard, A. H. *et al.* Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. *Molecular microbiology.* **60** (6), 1446-1456, doi:10.1111/j.1365-2958.2006.05202.x, (2006).
31 Corbin, A., Pitts, B., Parker, A. & Stewart, P. S. Antimicrobial penetration and efficacy in an in vitro oral biofilm model. *Antimicrobial agents and chemotherapy.* **55** (7), 3338-3344, doi:10.1128/AAC.00206-11, (2011).
32 Diggle, M. A. & Clarke, S. C. Pyrosequencing: sequence typing at the speed of light. *Molecular biotechnology.* **28** (2), 129-137 (2004).
33 Hiyari, S. & Bennett, K. M. Dental diagnostics: molecular analysis of oral biofilms. *Journal of dental hygiene : JDH / American Dental Hygienists' Association.* **85** (4), 256-263 (2011).

34 Filoche, S., Wong, L. & Sissons, C. H. Oral biofilms: emerging concepts in microbial ecology. *Journal of dental research.* **89** (1), 8-18, doi:10.1177/0022034509351812, (2010).
35 Xu, J. Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. *Molecular ecology.* **15** (7), 1713-1731, doi:10.1111/j.1365-294X.2006.02882.x, (2006).
36 Rogers, G. B., Carroll, M. P. & Bruce, K. D. Studying bacterial infections through culture-independent approaches. *Journal of medical microbiology.* **58** (Pt 11), 1401-1418, doi:10.1099/jmm.0.013334-0, (2009).
37 Socransky, S. S. *et al.* The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts and counts of specific organisms. *Archives of oral biology.* **8** 275-280 (1963).
38 Heydorn, A. *et al.* Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology.* **146 (Pt 10)** 2395-2407 (2000).
39 Nobbs, A. H., Lamont, R. J. & Jenkinson, H. F. Streptococcus adherence and colonization. *Microbiology and molecular biology reviews : MMBR.* **73** (3), 407-450, Table of Contents, doi:10.1128/MMBR.00014-09, (2009).
40 Hope, C. K., Clements, D. & Wilson, M. Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling. *Journal of applied microbiology.* **93** (3), 448-455 (2002).
41 Adams, H. *et al.* Development of a laboratory model to assess the removal of biofilm from interproximal spaces by powered tooth brushing. *American journal of dentistry.* **15 Spec No** 12B-17B (2002).
42 Ledder, R. G. & McBain, A. J. An in vitro comparison of dentifrice formulations in three distinct oral microbiotas. *Archives of oral biology.* **57** (2), 139-147, doi:10.1016/j.archoralbio.2011.08.004, (2012).

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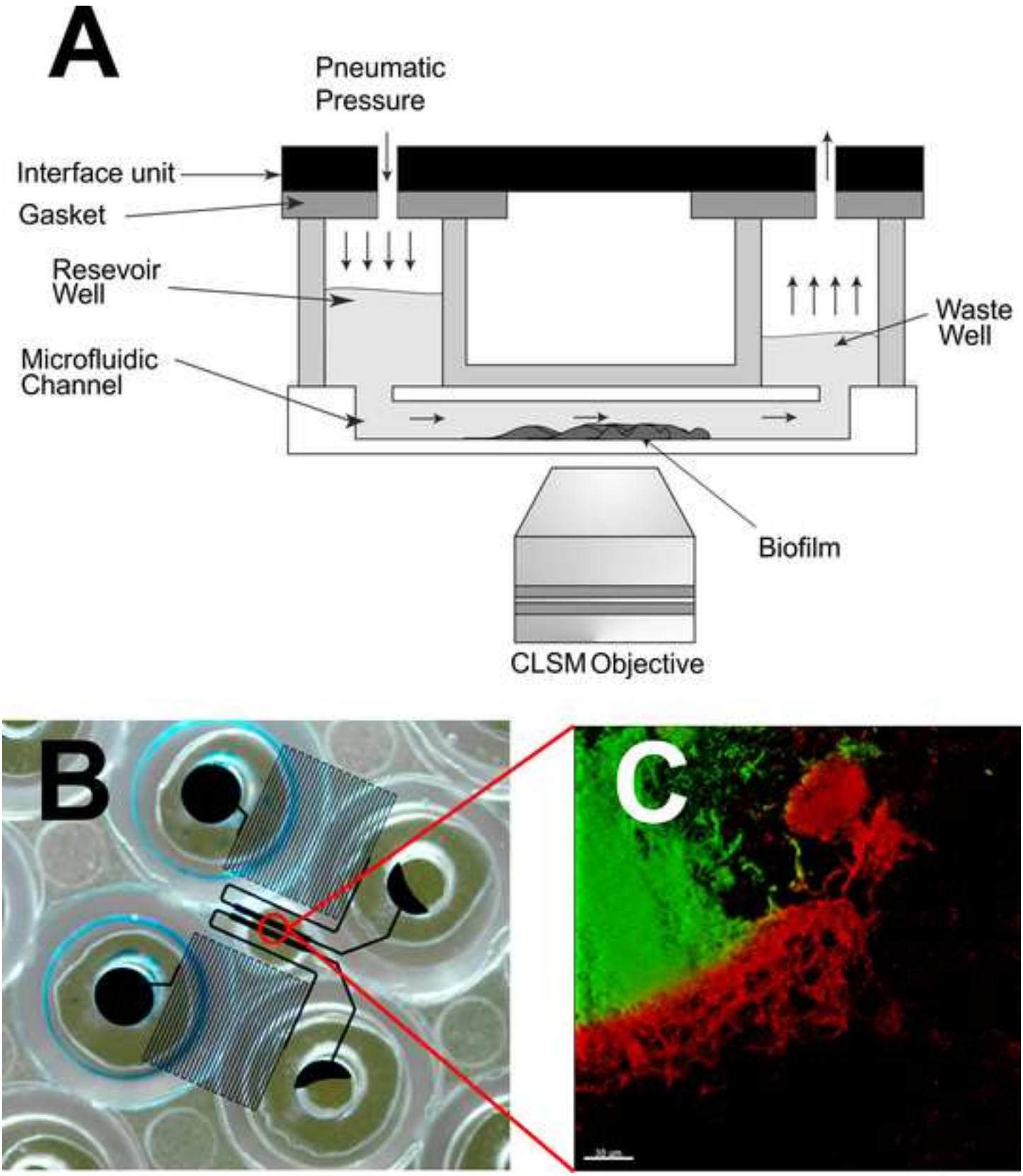

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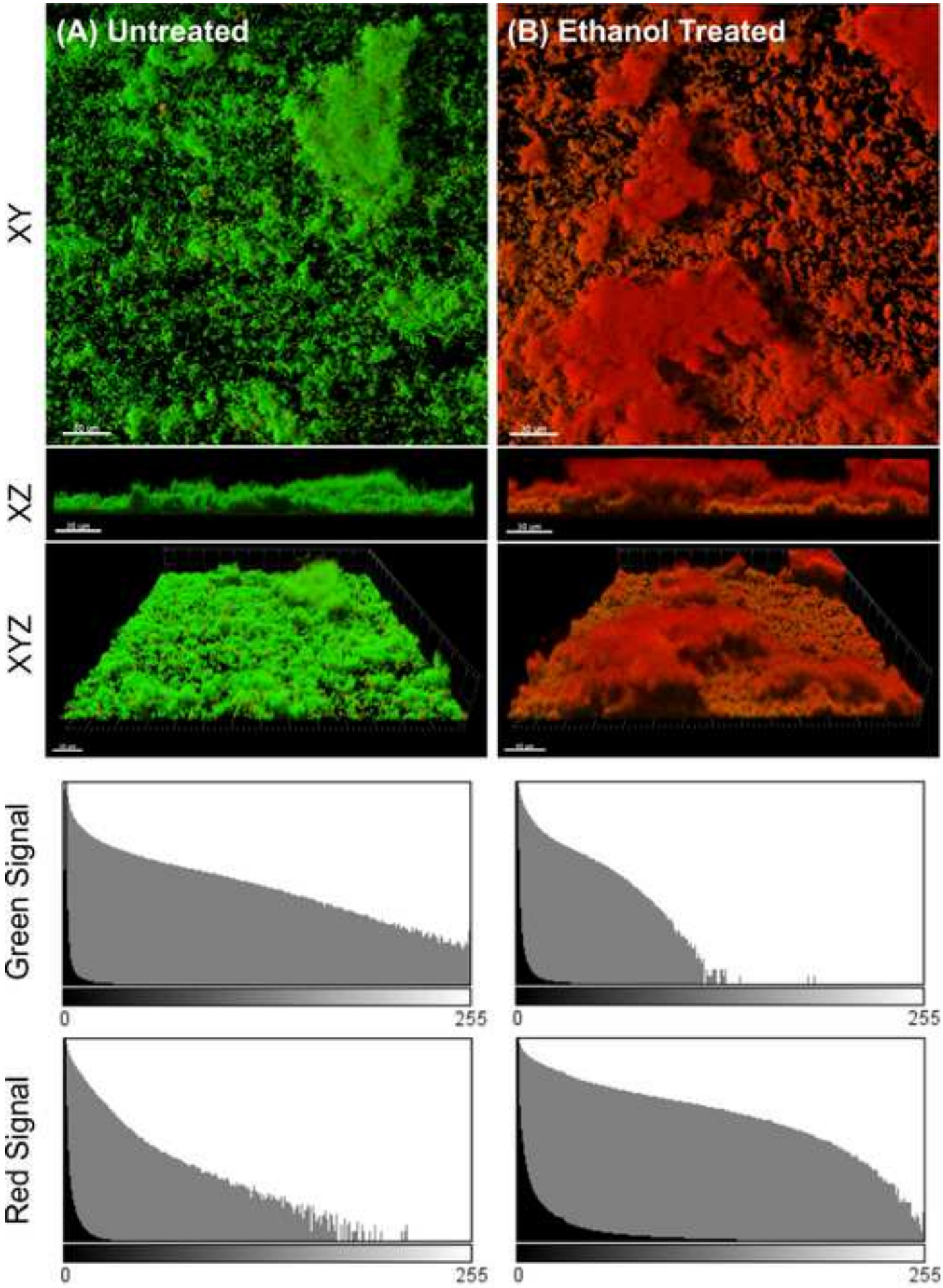

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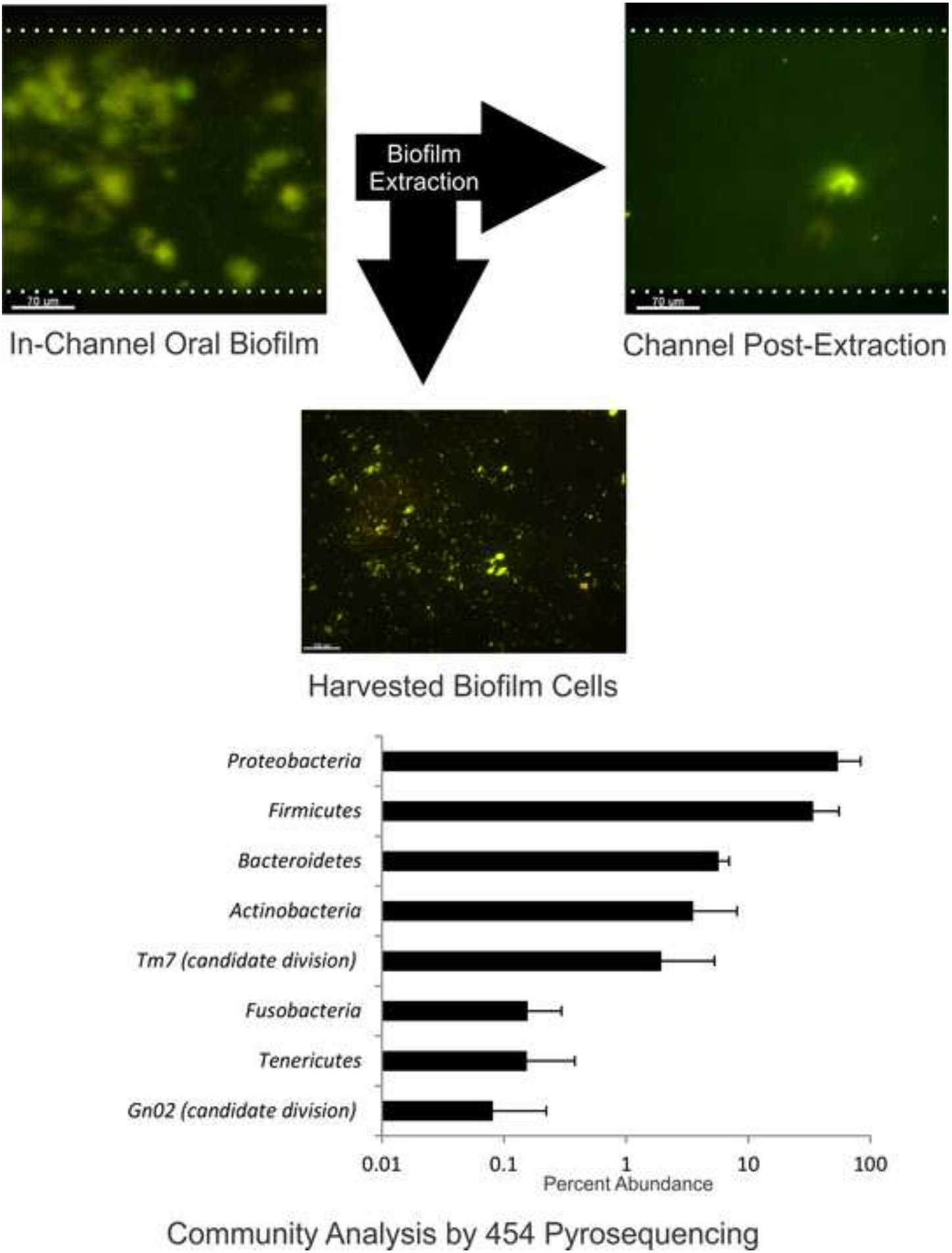

Table

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Table 1. Quantification of biofilm properties of untreated and 70% ethanol treated 2

Parameter/Treatment	Untreated
Average Viability (%)	**80.8** (0.9)
Average Biovolume ($\mu m^3/\mu m^2$)	**17.7** (8.4)
Average Thickness (μm^2)	**23.3** (11.3)
Average Roughness	**0.4** (0.3)

22 h biofilms developed in pooled cell-free saliva (CFS) from an inoculum cell-containing saliva (CCS). Bold

70% EtOH
28.3 (5.8)**
16.1 (3.0)
15.6 (6.4)
0.50 (0.2)

l values are the averages and non-bolded values in brackets are the standard deviations). Data is from at

t-least five images from three microfluidic channels. Significant differences to the untreated control are

highlighted by an asterisk (*: $P < 0.05\%$; ** $P < 0.01\%$).

Excel Spreadsheet- Table of Materials/Equipment
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SUPPLIES AND EQUIPMENT

Falcon 50mL Conical Centrifuge Tubes
Falcon 15mL Conical Centrifuge Tubes
Dithiothreitol (White Crystals or Powder/Electrophoresis), Fisher BioReagents
Sorval ultracentrifuge (SS-34 compatible)
Thermo Scientific SS-34 Rotor
Thermo Scientific Type 1 Reagent Grade Deionized Water
Nalgene Rapid-Flow Filter Units and Bottle Top Filters, PES Membrane, Sterile.
Glycerol
BioFlux microfluidic system
Bioflux 24-channel plate
PBS (Gibco)
LIVE/DEAD stain (Invitrogen)
Confocal Laser Scanning Microscope
Epifluorescence Microscope
Pyrosequencing facilities
Decon SaniHol 70 Ethanol Solution
Ultra Low Temperature Freezer -80°C
Tips (20, 200, and 1000uL)
Single Channel Variable Volume Pipettors (20, 200, 1000uL)

SOFTWARE

Bioflux dedicated software
Imaris
Leica SPE
ImageJ
COMSTAT/COMSTAT 2

AVAILABLE FROM COMPANY	CATALOG NUMBER
Fisher Scientific	14-432-22
Fisher Scientific	14-959-49D
Fisher Scientific	BP172-5
Thermoscientific	Unit-dependent
Thermoscientific	28-020
Thermo Scientific Inc	23-290-065
VWR	73520-986
Thermo Fisher Scientific Inc	NC0542269
Fluxion	Bioflux 200 system
Fluxion	910-0004
Thermo Fisher Scientific Inc	10010023
Invitrogen	L7012
Lecia	SPE or eqivalent system
Multiple choices	Multiple choices
Multiple choices	Multiple choices
Fisher Scientific	04-355-122
Multiple choices	Multiple choices
Multiple choices	Multiple choices
Multiple choices	Multiple choices

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Thanks for spotting this error – corrected to "x g".

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b.Modifications and troubleshooting.
c.Limitations of the technique.
d.Significance of the technique with respect to existing/alternative methods.
e.Future applications or directions after mastering this technique.

Slight changes in the Discussion have been made. We think that each of these elements are touched upon and also addressed the limitations of this (and any) biofilm model system.

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We used Endnote to generate the reference list. We have amended the reference list as best as we can to be in-line with the formatting. We think it now complies – in particular we noticed that the volume listing was not in bold, the year was in the wrong place, and author initials did not have periods after the initials. We noticed that DOI was not available with some papers via Endnote (see Smith, 1964 and Anon., CDC paper).

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