FISH 'N' CHIPS – A SINGLE CELL GENOMIC ANALYZER FOR THE HUMAN MICROBIOME

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ABSTRACT

Uncultivable microorganisms likely play significant roles in the ecology within the human body, with subtle but important implications for human health. Focusing on the oral microbiome, we are developing a processor for targeted isolation of individual microbial cells, facilitating whole-genome analysis without the need for isolation of pure cultures. The processor consists of three microfluidic modules: identification based on 16S rRNA fluorescence in situ hybridization (FISH), fluorescence-based sorting, and encapsulation of individual selected cells into small droplets for whole-genome amplification. We present here a technique for performing microscale FISH and flow cytometry, as a prelude to single cell sorting.

KEYWORDS: Bacteria, saliva, metagenomics, microbial ecology, sequencing

INTRODUCTION

Human beings are host to an amazing diversity of microbial species. The human body possesses a multitude of vastly different environments, such as the mouth, stomach, gut, mucosa, and skin, each of which is host to different populations of microbes. Major shifts in each of these populations may occur over the course of an individual's lifetime as a result of natural developmental stages, diet and lifestyle choices, and external influences, and it is expected that the composition of this "microbiome" might be indicative a variety of otherwise undetected pathologies, as well as contribute to the onset and progression of some diseases.

Efforts to characterize the full diversity of microbial species in the human body are underway, but these efforts are complicated by the observation that many microbes are resistant to growth in pure culture. Metagenomic techniques can untangle genomes in simple communities consisting of a few bacterial species, but microbial communities as diverse as those found in the mouth or gut are largely intractable by established techniques. We thus seek a targeted approach to selectively isolate individual bacterial cells of interest from a complex mixture, including rare and/or uncultivable phylotypes, and perform genome amplification and sequencing without the biases introduced by culture.

Fluorescence in situ hybridization (FISH) presents a convenient and powerful way to identify or label specific bacteria, on the basis of nucleic acid sequence information, commonly the sequence of 16S rRNA which has long been used for determining phylogenetic relationships between bacteria [1]. 16S rRNA sequence is routinely gathered by metagenomic experiments with complex human and environmental samples, and provides evidence of the existence of rare or novel phylotypes that have never been isolated in pure form. Simultaneously, the 16S rRNA can itself be used as a target for hybridization with a fluorescently labeled oligonucleotide probe. FISH thus offers the possibility to selectively label target cells, with molecular specificity, without the need for prior isolation of the cells (*e.g.* to raise antibodies for labeling). FISH combined with fluorescence-activated cell sorting presents the opportunity for isolating individual cells. Current sequencing methodologies require nanograms to micrograms of DNA, and thus the genomic DNA from a single cell must be amplified; this can be accomplished with relatively uniform coverage using multiple displacement amplification (MDA) using Phi29 polymerase; this procedure is prone to artifacts when starting with very small amounts of template (*e.g.* from a single cell), and reactions with a single cell are best performed in a small volume.

We are developing a microfluidic processor with three modules, capable of performing FISH (for identification of microbial cells), fluorescence-based flow cytometry and cell sorting (for selection of labeled cells), and encapsulation of selected cells in nanoliter droplets (for single-cell genome amplification). The microfluidic approach is advantageous compared to conventional scale processing (*e.g.* microcentrifuge tubes and bench-scale FACS) when dealing with small or precious samples, where sample loss during operations or between discrete steps is unacceptable.

EXPERIMENTAL

Custom microfluidic chips were fabricated from quartz by Caliper Life Sciences, using standard photolithography and wet chemical etching, with a channel depth of 30 μm. Polyacrylamide gel membrane structures were photopatterned within the microchannels using a UV laser, as described previously [2]. Chips were mounted in a custom Delrin holder with aluminum compression frame, and electric fields were applied using a custom-built miniaturized multichannel power supply [3]. Microscopic observations were made using an Olympus IX71 microscope with Andor Clara interline CCD camera, and on-chip flow cytometry was performed using a home-built setup with a 488 nm laser and PMTs for detection of forward scatter and fluorescence, as described previously [4]. *E. coli* K12 was grown aerobically using standard liquid culture protocols. *Lactobacillus acidophilus* (ATCC 4356) and *Streptococcus mutans* (ATCC 700610) were grown in small volume liquid culture using anaerobic pouches. Cells were harvested during log-phase growth and fixed by incubation overnight with 4% paraformaldehyde. Fixed, Gram-positive cells (*L. acidophilus* and *S. mutans*) were permeabilized by incubation with lysozyme for 30-60 minutes prior to hybridization. FISH hybridizations were performed using probes listed in Table 1, labeled at either the 5' or 3' end with Cy3 or AlexaFluor 488.

Table 1. Sequences of oligonucleotide probes used for microfluidic 16S rRNA FISH

Probe name	Sequence	Specificity
EUB338	GCTGCCTCCCGTAGGAGT	Most bacteria (positive control) [1]
NON338	ACTCCTACGGGAGGCAGC	Nonsense probe (negative control) [5]
Lab158	GGTATTAGCA(C/T)CTGTTTCCA	*Lactobacillus/Enterococcus* group [6]
Eco681	CATTTCACCGCTACACCT	*E. coli* [7]
MUT590	ACTCCAGACTTTCCTGAC	*S. mutans* [8]

RESULTS AND DISCUSSION

We have developed a glass microfluidic device and protocol for electrokinetic manipulation of bacterial cells and oligonucleotide probes for on-chip fluorescence *in situ* hybridization (FISH). The glass microchip has dimension of 22x37 mm, and the channel layout is shown in Figure 1A. Three thin, nanoporous polymer membranes, shown in Figure 1B, are photopatterned in the microchannels using a UV laser. Two of these membranes are high concentration, highly crosslinked polyacrylamide, (45% T, 12% C), forming a chamber where cells and FISH probe can be concentrated incubated together. Cell washing is performed at the third, low-concentration membrane (10% T, 2.5% C), which readily permits probes to pass through while cells are retained. To perform on-chip FISH, fixed bacterial cells are pipetted into the cell reservoir and concentrated against the two loading membranes under an electric field of 20 V/cm in a hybridization buffer containing 400 mM NaCl and 20 mM Tris-HCl (pH 9.2). By alternating the field between these two membranes during the loading process, the cells can be concentrated into the chamber without cells sticking to the membranes. Oligonucleotide probes (15 ng/μL) are then introduced electrophoretically into the chamber. A 30-min incubation is conducted while cells and probes are gently moved back and forth between these two membranes. After that, cells are moved to the low-concentration membrane for washing under an electric field of 20 V/cm. The buffer in the channels is exchanged with a washing buffer (50 mM NaCl, 20 mM Tris-HCl, 0.1% bovine serum albumin, pH 9.2) using the electric field. The low ionic strength buffer permits a high-stringency wash, and helps with declumping of cells that tend to aggregate during the high salt incubation. The entire process is performed at 46 ^{o}C and completed in about 2 hr.

On-chip FISH has been tested with cultured *E.coli* K12 (Gram negative) and *L. acidophilus* and *S. mutans* (Gram-positive) cells. As illustrated in Figure 1C and 1D, Cy3-labeled 16S probe Eco681 successfully hybridized to *E. coli*, while Cy3-labeled negative control probe NON338 gave no fluorescence after washing. The orally relevant Gram-positives *S. mutans* and *L. acidophilus*, have also been successfully loaded and hybridized on chip using probes MUT590 and Lab158, although the thicker cell wall of Gram-positive cells generally requires an additional permeabilization with lysozyme and/or proteases to improve probe penetration prior to loading into the chip. Hybridizations with mixtures of bacteria, *e.g. E. coli* and *L. acidophilus* have been performed to show selective labeling. Experiments with the gram-negative oral pathogen *Porphyromonas gingivalis* are underway, and experiments with bacteria isolated from saliva are planned, including rare phylotypes identified in ribosomal panels of saliva.

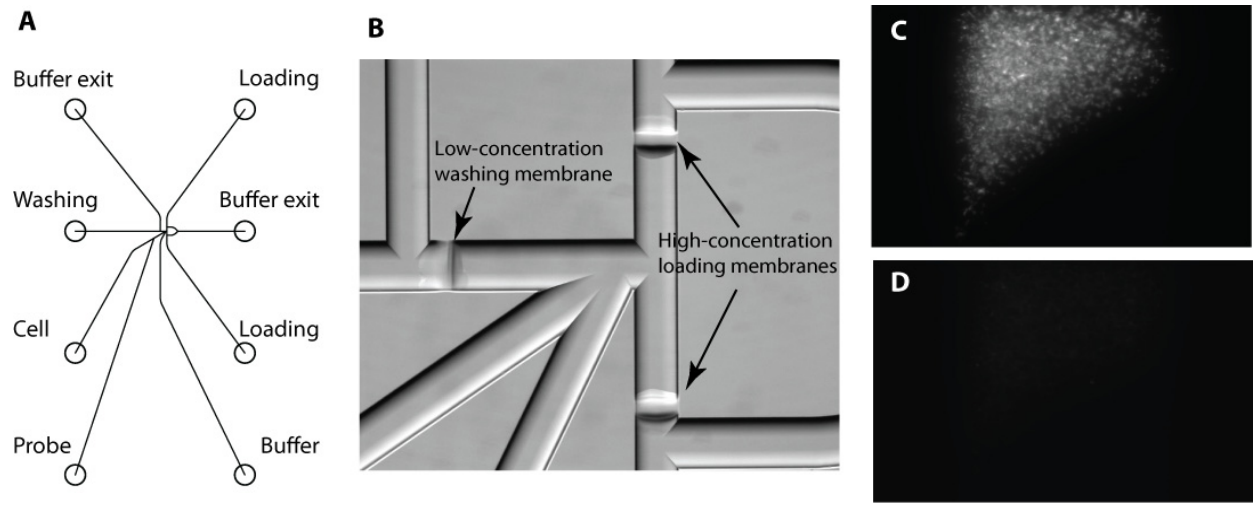

Figure 1. (A) The mask design of the glass microchip for fluorescence in-situ hybridization. (B) The locations of three thin, polyacrylamide membranes for cell and probe concentration and washing. (C) *E.coli* cells labeled with Eco681-Cy3 probes in the microchannel. (D) No fluorescence signal observed in the negative control using NON338-Cy3 probes.

FISH is being performed as a labeling technique prior to flow cytometry and sorting. Toward this end, we have designed and tested an integrated microfluidic device which is capable of performing FISH and flow cytometry on a single chip. As shown in Figure 2A and 2B, we utilized an electrokinetic cell focusing method for on-chip flow cytometry. By

adjusting the distribution of the electric field in the channel junction, effective 2D cell focusing sufficient for single-file analysis of cells can be achieved. The flow speed of this method is low compared to that of the hydrodynamic focusing previously used in our lab [4], but is well suited to the small number of cells used in the on-chip FISH (~20 minute analysis time). Electrokinetic focusing is convenient and compatible with up-stream electrokinetic FISH process, eliminating the need for coupled electrokinetic and hydrodynamic control. We conducted a FISH experiment on the microdevice using *E.coli* cells and EUB338-Alexa488 probes, followed by on-chip flow cytometry detected by a laser-induced fluorescence detection system. Figure 2C shows a histogram of fluorescence signal intensity in the FL-1 (Alexa 488) channel, demonstrating the feasibility of performing both processes on a single device. A revised design for the focusing structure is being produced; additional modification of the optical setup and data acquisition hardware and software is planned, allowing for simultaneous collection of multiple fluorescence channels. Our group has previously used optical trapping and deflection as a sorting mechanism [4], which is expected to be compatible with our completely electrokinetic FISH and cytometry process, although testing is still in progress.

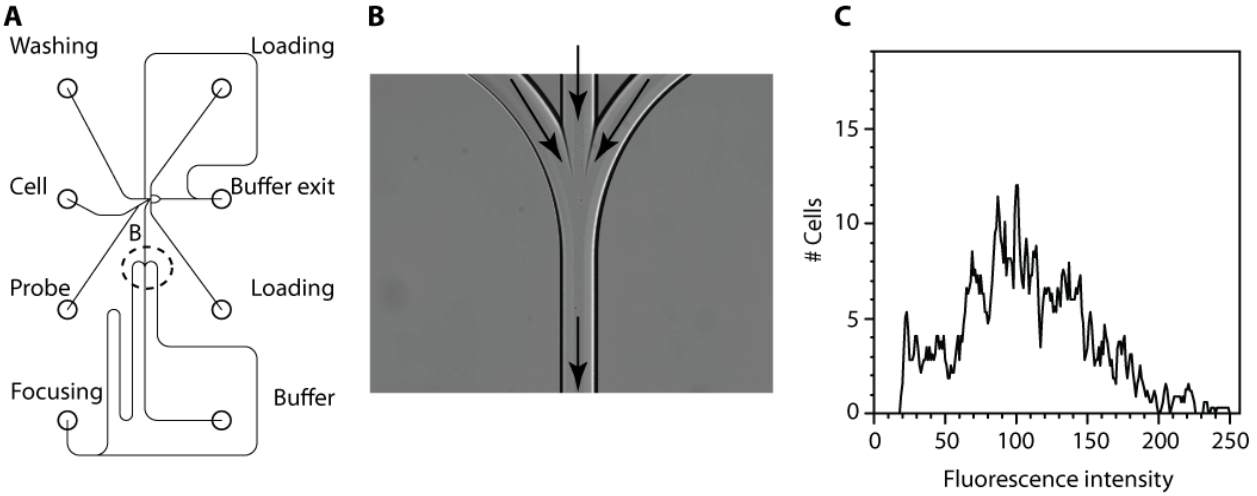

Figure 2. (A) The mask design of the FISH-Flow cytometry microchip. (B) The channel junction for electrokinetic cell focusing. Arrows indicate the direction of current flow in the channels. (C) A histogram of cell counts as a function of fluorescence signal intensity obtained from on-chip FISH and flow cytometry. The *E.coli* cells are labeled with EUB338-Alexa488 probes.

CONCLUSION

We have demonstrated an integrated device capable of performing fluorescence *in situ* hybridization for labeling bacteria on the basis of rRNA sequence, with detection by flow cytometry integrated on the same device, which allows for efficient transfer of small samples. The next step for integration is to use the flow cytometry detection as a basis for sorting single cells, which can be performed using already-developed optical means. Ultimately single cells are to be encapsulated in nanoliter droplets for single-cell whole genome amplification, as a prelude to whole genome sequencing.

ACKNOWLEDGEMENTS

We thank Ron Renzi, Jim Brennan, Dan Yee, and Jim Van deVreugde (Sandia) for engineering support, Phil Hugenholtz (Joint Genome Institute) for discussions of FISH and microbial ecology, and Deepak Saxena and Daniel Malamud (New York University) for helpful discussions and cultures of oral bacteria. This work was funded by the US National Institutes of Health National Institute of Dental and Craniofacial Research (NIH:NIDCR) under grant 1R01DE020891-01A1. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000.

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