Materials and Methods

1. Growth conditions for cultured microbes

A single colony of DH5alpha *E. coli* cells was inoculated in 5 mL LB broth and incubated at 37 ^{o}C, 250 rpm until OD 600 reached 1. The cell concentration is about 10^{9} /mL. The harvested *E. coli* cells were washed once with PBS (phosphate-buffered saline, pH 7.4) and fixed with 1% paraformaldehyde in PBS for 16 hours at 4 ^{o}C. The fixed cells were washed twice with PBS and stored in PBS containing 50% ethanol at -20 °C.

For strain RCH2, enrichment cultures for nitrate-reducing bacteria were initiated in the dark under anaerobic conditions in sterile, sealed pressure tubes amended with bicarbonate-buffered circum-neutral freshwater basal medium under a headspace of 80% N_2-20% CO_2 with acetate as the sole electron donor and nitrate as the sole electron acceptor. After multiple transfers in this medium, a single colony (pure culture) of strain RCH2 was obtained by the agar shake tube method two weeks after inoculation. For strain RCH1, enrichments were initiated in defined LS4D media for sulfate reducing bacteria containing 60 mM lactate as the electron donor and 40mM sulfate as the electron acceptor. Periodic transfers of positive enrichments as identified by microscopy, visual turbidity or change in color were made into fresh LS4D media. After several transfers, pure cultures of isolates were obtained from the enrichments by the agar shake tube method. Both strains were cultured in the dark in their respective media used for isolations at 30°C or 37°C for further experiments. Cultured RCH1and RCH2 were washed once with PBS and fixed with 1% paraformaldehyde in PBS for 16 hours at 4°C. The fixed cells were washed twice with PBS and stored in PBS containing 50% ethanol at -20 °C.

2. Instrumentation

The microchip was sandwiched between an aluminum frame (on the bottom) and a Delrin manifold (on the top) with integrated O-ring seals for microchip reservoirs similar to those described previously [1]. 80-μL wells were created by this manifold on each reservoir of the microchip for sample and buffer loading. To control the temperature of the microchip, a resistive heating element and an RTD probe (Omega, Stamford, CT) were bonded to the backside of the aluminum plate, and the temperature was controlled by a custom-built temperature controller. The temperature difference between the microchip surface and the temperature probe on the aluminum plate was calibrated by attaching a thermocouple to the top surface of the microchip. Visualization of on-chip FISH was performed by placing the device on the stage of an inverted microscope (IX-71, Olympus, Melville, NY), using a cooled interline CCD camera (Andor Clara, South Windsor, CT, USA) under the illumination of a mercury arc lamp with a high-speed shutter (Sutter SmartShutter, Novato, CA, USA). A miniaturized programmable high-voltage power supply with eight independent outputs controlled by a home-made LabVIEW program (National Instruments, Austin, TX) were employed to provide and control high voltages to the microchip [1].

The on-chip flow cytometry was conducted on a laser induced fluorescence detection system which was developed previously in our lab [2]. The signals were digitized at 14-bit resolution by a USB data acquisition module (NI USB-6009, National Instruments) into a laptop computer using a LabVIEW control program (National Instruments). Peak information is extracted from the raw data using LabVIEW and converted into a binary file format for FlowJo software (Tree Star, Ashland, OR), where the histograms of the flow cytometry results are plotted and analyzed.

3. Off-chip FISH and flow cytometry

To validate the on-chip results, the same Hanford samples were tested using conventional FISH-FC methods. First, the cells were incubated with 70 μL of hybridization buffer (20mM Tris-HCl, pH 7.4, 900mM NaCl and 0.01% SDS) containing 5 ng/μL PSM G probes labeled with Alexa Fluor 488. After hybridization at 46 °C for 2 hours, the cells were incubated with 50 μL of washing buffer (318mM NaCl, 20mM Tris-HCl, pH 7.4, and 0.01% SDS) at 46 °C for 15 minutes followed by rinsing with DI water. 2 μL of samples was dropped onto a slide for microscopic imaging. Flow cytometry was performed on a BD FACSAria II flow cytometer (BD Biosciences, Sparks, MD) using a Sapphire™ 488-20 laser (13mW, Coherent Inc., Santa Clara, CA) and a 70-μm nozzle orifice under 'purity' sort mode.

Discussion

1. Optimization of on-chip FISH

Several issues arose during translation of conventional FISH in a test tube to FISH on a chip with electrokinetic forces. First, clumping and adsorption of cells was addressed. When concentrating microbial cells into a nanoliter-scale FISH chamber, very high cell densities can be achieved, comparable to a pellet achieved during centrifugation. Certain cell types tended to stick to each other, or to the surfaces of the channel walls and the gel membranes. This leads to inefficient mass transfer of probes during hybridization and washing, incomplete recovery of cells, and non-single-cell detection in flow cytometry. The small length scale of the microchannel and corresponding low Reynolds number for fluid motion prevents efficient physical agitation (e.g. vortexing, ultrasonication) to disrupt clumps as would be possible in a

conventional microcentrifuge tube (the chip could be sonicated, but at this length scale, viscous forces effectively damp fluid motion). To overcome problems with cell clumping and adsorption, we adopted the following preventative measures: First, channel walls are coated with a hydrophilic layer of linear polyacrylamide, and low levels of BSA and SDS are added into the hybridization and washing buffers [3]. Second, during cell loading, hybridization, and wash steps, the direction of the electric field is changed periodically, which keeps microbial cells moving back and forth between two gel membranes without pushing against the gel membranes for a prolonged period. Third, the NaCl concentration of the hybridization buffers is reduced to 400 mM and the pH is increased to 9.2. Reduced ionic strength (*vs.* the standard 900 mM NaCl commonly used for microbial FISH) as well as increased pH of the buffer helps prevent clumping of microbial cells [4-6]. The salt concentration is further reduced to 50 mM during washing, which assists in declumping cells that may have clumped during the hybridization, and also serves to increase stringency during the wash step.

The stringency of on-chip FISH also needs to be addressed. In conventional FISH experiments, the stringency is controlled by adjusting the concentration of formamide in the hybridization buffer while keeping the temperature at 46 ^{o}C, and salt concentration constant (usually 900 mM NaCl). When transferring this process to the microchip, we decided to control the FISH stringency by changing the temperature according to different target cells, as precise control of temperature is easy to realize and adjust quickly on the chip. Formamide was eliminated due to its potential toxicity, and the necessity of creating a dedicated formamide waste stream. Standard correlations between formamide, temperature, and salt concentration were consulted in translating FISH to lower salt conditions, although empirical testing of stringency is still advisable.

The electrokinetic forces used to drive cell motion require consideration. Although most cells have a negative surface charge, it is well known that different species of microbial cells have different electrokinetic mobilities [7], which may vary with cell age or growth conditions, as well as cell type. This could lead to biases when analyzing mixtures, as some cells may enter the hybridization chamber more quickly than others. By designing the FISH chamber close to the cell reservoir, we did not observe severe loading bias. It was also anticipated that use of an electric field might aid the hybridization process, in the same way that an electric field enables transformation of cells during electroporation. However, relatively low electric fields (<20 V/cm) turned out to be most useful in our device, compared to the much higher electric fields typically used for electroporation, and thus it is unlikely that the electric field significantly impacts the rate of hybridization.

Reference

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