SMALL MOLECULE GRADIENT GENERATOR FOR MICROFLUIDIC VISCOUS SHEAR-FREE CELL CULTURE
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ABSTRACT
We present a microfluidic device for generating steep gradient interfaces of small molecules by confronting two flows with different chemical concentrations. We separate a gradient generation channel and a cell culture channel by a polyester membrane so that viscous shear stress from the bottom channel flow does not interfere with cells attached on the top of the membrane. We use rhodamine-B (400 Da) and BCECF AM (686 Da, pH-sensitive cell-permeable dye) to characterize the steepness of the diffusional interface. We demonstrate ~50 µm wide steps narrower than 5 cell diameters for < 1kDa molecules and ~140 µm steps for pH gradients across HeLa cell culture with no convective shear applied to the cells.

KEYWORDS: Small Molecules, Gradient, Membrane, Microfluidics, HeLa Cell

INTRODUCTION
In developmental biology, diffusible signal molecules (morphogens) play a key role in inducing and timing proliferation, differentiation, and migration of cells [1, 2]. Controlling and sensing morphogen gradients provides a basis for understanding many patterning/developmental processes. Here we use a novel streamline of two fluids to generate stable and uniform gradient interfaces by confronting one fluid with the other. We then map chemical gradients generated in a bottom chamber across a diffusible membrane onto an overlying cell culture chamber so that we prevent viscous shear stress by the bottom channel flow from disturbing the chemical environment of cultured cells seeded on the membrane in the top channel (Fig. 1A). The device was fabricated from two layers of PDMS and a 10 µm thick polyester membrane [3, 4].

RESULTS AND DISCUSSION
As seen in Fig. 1C, the two flows meet each other in the center of the device, creating a boundary of rhodamine-B parallel with the diagonal of the square bottom chamber. Fig. 1D shows gradient profiles of rhodamine-B by quantifying fluorescence intensities along the x-axis under different flow rates ranging from 0.01 mL/h to 1 ml/h. Since slow flow rates allow longer diffusion time for molecules to mix with the other buffer solution than high flow rates, it is obvious that faster flows produce sharper gradients. The steepest gradient recorded had a 50 µm transition region between the two concentrations.
In order to characterize how the lower chamber gradients mapped across the membrane and onto the upper, cell culture chamber, we used adherent cultures of HeLa cells and BCECF, an indicator the fluoresces only when taken up by cells [5]. Cells in medium suspension were loaded into the top channel, allowed to settle, and then provided nutrient media using continuous flow across the bottom channel overnight (Q$_2$ and Q$_3$=0.1 mL/h). As seen in Fig. 2A, HeLa cells grew and attached on the cell membrane overnight. We then ran a solution of BCECF AM through one port (Q$_3$=1 mL/h) and a solution of media through the other port (Q$_2$=1 mL/h, creating a gradient as in Fig. 1). BCECF AM is non-fluorescent, but is uptaken by viable cells and converted to BCECF, which fluoresces. After 1 hour, a BCECF pattern matching the underlying flow gradient is clearly visible in the cells. The fluorescence intensity between x = -300 µm and x = 0 µm gradually increases with time, indicating that cells uptake more BCECF AM molecules. On the other hand, the fluorescence intensities between x = 0 µm and x = 300 µm remain constant, implying that cells experience a distinct, sharp boundary (approximately 5–10 cell diameters) between the two solutions. This experimental result confirms that sharp gradients generated by the bottom channel are mapped to cells attached on the membrane without subjecting them to convective flow.
obtained in 60 min and 120 min show selective staining of cells and (D) shows a distinct fluorescence boundary between BCECF and media.

Given that BCECF fluorescence is pH-sensitive, we modified the same assay to quantify how sharply gradients of extremely small molecules could be imposed on the cell culture. As in the previous experiment, we loaded cells into the top channel and then seeded them on the membrane. We then flowed DMEM/BCECF AM through the top channel, uniformly loading the cells in the entire chamber with the BCECF indicator (Fig. 3A). After rinsing and filling the top channel with ~1 mL of a PBS buffer solution (pH=7.6) to remove any BCECF AM residue, we flowed DI water (Q₂=1 mL/h, pH=6.6) and an HBSS solution (Q₃=1 mL/h, pH=8.54) through the bottom channel to generate a pH gradient. After 60 minutes, two different zones of pH can be seen in BCECF-loaded cells; the profile reached steady-state at t=120 minutes. The quantified results of Fig. 3C are in good agreement with Fig. 3B and the distinct interface thickness was measured to be ~140 μm. As expected, the interface is much wider than BCECF gradient results due to the higher diffusion constant of the hydroxyl ion compared to BCECF.

Figure 3 Cells were stained by filling the top channel with BCECF AM for 30 min. The top channel was then rinsed and filled with PBS buffer (pH=7.6). The ratio of BCECF fluorescence at 490nm/430nm is linearly proportional to pH. (A) Ratiometric image at t=0 min, (B) ratiometric image at t=120 min, showing pH gradient.

CONCLUSIONS

We demonstrated that our microfluidic device makes it possible to generate steep gradients of small molecules such as rhodamine-B (400 Da), BCECF AM (689 Da) and even hydroxyl ions (~17 Da) over large spans of cell culture by confronting flows. We successfully demonstrated that small molecule gradients could be mapped onto HeLa cells using both BCECF and different pH buffers. We believe our approach could be useful in the study of small molecule gradients in developmental biology.

REFERENCES