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Universal Microfluidic Gradient Generator

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Abstract

The study of cellular responses to chemical gradients in vitro would greatly benefit from experimental systems that can generate precise and stable gradients comparable to chemical nonhomogeneities occurring in vivo. Recently, microfluidic devices have been demonstrated for linear gradient generation for biological applications with unmatched accuracy and stability. However, no systematic approach exists at this time for generating other gradients of target spatial configuration. Here we demonstrate experimentally and provide mathematical proof for a systematic approach to generating stable gradients of any profile by the controlled mixing of two starting solutions.

Replication of complex chemical gradients is essential for many in vitro experimental studies in prokaryotic and eukaryotic cells. Investigation of fundamental processes such as the migration of prokaryotic cell toward sources of nutrients,¹ immune cells against intruders,^{2,3} the growth or regeneration of axons toward their connection target,⁴ and the differentiation of embryonic cells in response to morphogens⁵ require precise duplication of the in vivo spatial distribution of various signaling molecules. Such distributions usually occur in nonhomogeneous mediums, under various production, transport, and degradation conditions, and gradients of nonlinear, more or less complex shapes are usually formed.^{6,7} Although linear gradients represent a good first-order approximation for in vivo gradients, and are useful in gaining insights into the biology of cellular responses, growing evidence suggests that a lot of complexity arises and many cellular responses are specific to spatial gradients that are not linear. For example, in bacteria migrating in chemoattractant gradients, the interplay between sensory adaptation and concentration changes due to displacement can lead to variations in chemotactic responses.⁸ Similarly, cancerous cells that typically do not respond to linear chemotactic gradients have been recently shown to migrate in response to nonlinear gradients.⁹ As a result, it is critical for better understanding of cellular polarization, migration, growth, or division responses of cells to biochemical heterogeneities in their microenvironment to be able to consistently replicate chemical concentration profiles comparable to those experienced by cells in vivo. Still, most of the current experimental systems for generating chemical gradients, e.g., Boyden chambers,¹⁰ Dunn chambers,¹¹ or microfluidic gradient generators,¹² only produce linear gradients of soluble biochemical factors, and few alternatives exist for producing gradients of other spatial profiles at scales comparable to cell size. While some gradients that are not linear have been generated using asymmetric flow in symmetric networks,¹³ or serial dilutions schemes,^{14,15}

to our best knowledge no systematic method exists today for replicating complex gradients of target spatial profile starting from two solutions of distinct concentrations.

Here we propose a universal microfluidic approach to generating nonlinear chemical gradients of choice. We restrict the interdiffusion between two adjacent streams of different concentrations by the use of parallel dividers in the direction of flow, and we provide a calculation scheme for the position of all the dividers required for the reproduction of the final spatial concentration profile with chosen accuracy. We demonstrate the generation of gradients of four specific profiles, i.e., power, exponential, error, and cubic root functions, and we prove mathematically that any monotonic gradient can be reproduced using this method starting from two input solutions.

THEORETICAL BACKGROUND

We employed a series of parallel dividers in the longitudinal direction of a microfluidic channel to restrict the diffusion between two initial parallel streams of distinct concentrations and generate a chemical gradient in the direction transversal to the channel. In the absence of the dividers, the unrestricted diffusion between the two streams flowing at low Reynolds number (laminar flow) would result in a characteristic decay profile evolving toward uniform concentration.¹⁶ The presence of dividers restricts the diffusive mixing of the two initial solutions of concentrations C_{00} and C_{01} ($0 < C_{00} < C_{01}$) and leads to the formation of target concentration profiles at the outlet (Figure 1).

At steady state, the target concentration profile represented by a function $f(x)$, where x is the position across the channel, is replicated at the outlet by the juxtaposition of a series of concentrations $C_{N,i}$ ($i=\overline{0, N+1}$), separated by dividers at positions $u_{N,i}$ ($i=\overline{0, N}$), relative to one side of the main channel and in the direction transversal to the channel. After the first level of dividers, one intermediate concentration C_{11} is produced by the mixing of predefined fractions of the two initial solutions in the space between the two dividers at level 1. The process is then repeated at subsequent levels such that at level n and position m of the dividers ($m=\overline{1, n}$), a new concentration $C_{n,m}$ is generated by the complete mixing of solutions of concentrations $C_{n-1,m-1}$ and $C_{n-1,m}$ from the previous level (Figure 1).

We can prove mathematically that, for any chosen output-concentration profile, at least one set of dividers exists such that this particular profile is generated at the output starting from only two solutions at the inlet. For this, we first demonstrate that any set of $n+2$ concentrations at one level n , can be produced from the mixing of $n+1$ solutions from the previous level, $n-1$. In mathematical form, this requires us to prove that for any given set of concentrations and positions of the dividers at level n , $C_{n,i}$ and $u_{n,i}$ with the following restrictions

$$0 \leq C_{n,0} < C_{n,1} \dots < C_{n,n+1} \quad (1)$$

and

$$0 \leq u_{n,0} < u_{n,1} \dots < u_{n,n} \quad (2)$$

there exists at least one set of $C_{n-1,i}$ ($i=\overline{0, n}$) and $u_{n-1,i}$ ($i=\overline{0, n-1}$) that simultaneously verifies the constrain for the position of dividers at two consecutive levels:

$$0 < u_{n,0} < u_{n-1,0} < u_{n,1} < u_{n-1,1} < \dots < u_{n,m-1} < u_{n-1,m-1} < u_{n,m} < u_{n-1,m} < \dots < u_{n-1,n-1} < u_{n,n} \quad (3)$$

and the relation between concentrations at the two consecutive levels

$$0 \leq C_{n,0} \leq C_{n-1,0} \leq C_{n,1} \leq C_{n-1,1} \dots \leq C_{n-1,n} \leq C_{n,n+1} \quad (4)$$

To demonstrate this, we assume flow rates proportional to the width of the spacing between dividers and use the mass conservation equations for fraction mixing between any two dividers:

$$C_{n,m}(u_{n,m}-u_{n,m-1})=C_{n-1,m-1}(u_{n-1,m-1}-u_{n,m-1})+C_{n-1,m}(u_{n,m}-u_{n-1,m-1}) \quad m=\overline{1,n} \quad (5)$$

which we could also rewrite it in the following form:

$$u_{n-1,m-1}=\frac{C_{n,m}(u_{n,m}-u_{n,m-1})-C_{n-1,m}u_{n,m}+C_{n-1,m-1}u_{n,m-1}}{C_{n-1,m-1}-C_{n-1,m}} \quad (6)$$

By plugging $u_{n-1,m-1}$ into the compatibility condition $u_{n,m-1} < u_{n-1,m-1} < u_{n,m}$ we obtain after a couple of reductions

$$C_{n-1,m-1} \cdot (u_{n,m}-u_{n,m-1}) \leq C_{n,m}(u_{n,m}-u_{n,m-1}) \leq C_{n-1,m}(u_{n,m}-u_{n,m-1}) \quad (7)$$

which implies, considering $u_{n,m-1} < u_{n,m}$ that

$$C_{n-1,m-1} \leq C_{n,m} \leq C_{n-1,m} \quad (8)$$

It can be seen immediately that the argument is reversible, and therefore, we obtain the equivalence between eqs 3 and 4. By using mathematical induction principles, we could then further prove that for any series of $C_{N,i}$ ($i=\overline{0,N+1}$) concentrations at the output, a complete set of intermediate concentrations and divider positions can be found for generating these concentrations, starting from two distinct input concentrations.

One interesting remark emerging from the mathematical demonstration is that an infinite number of design solutions satisfying the divider position constrain and mixing equations are possible. These solutions can be systematically represented by the introduction of a set of arbitrary coefficients. We prove this by writing the complementary equations to the mass conservation equations (eq 5) in the following form:

$$C_{n-1,m}=\alpha_{n-1,m}C_{n,m}+(1-\alpha_{n-1,m})C_{n,m+1} \quad (9)$$

where $\alpha_{n,m}$ ($0 < \alpha_{n,m} < 1$) are coefficients at our disposal. This equation contains implicitly the condition from eq 8 and ensures us that we can solve the system at each level obtaining positions for dividers that verify the compatibility condition.

We can also calculate the minimum number of substreams with concentrations $C_{N,\hat{i}}$ which would reconstitute the target concentration profile $f(x)$ with necessary precision. We rely on the fact that the domain of the function $f(x)$ is the compact interval $[C_{00},C_{01}]$, and consequently, $f(x)$ is more than continuous, namely, uniform continuous. Thus, for any we could find a particular N such that for any x_1 and x_2 in the interval $[u_{N,0},u_{N,N}]$, with $|x_1-x_2| < |u_{N,N}-u_{N,0}|/N$, the following $|f(x_1)-f(x_2)| < \epsilon$ is true. In other words, for a particular desired precision of the approximation of a concentration profile, we can determine the

minimum number of microfluidic streams $C_{N,i}$ ($i=0, N+1$) necessary to reconstitute that concentration profile.

The algorithm for calculating the positions of the dividers in the channel for a desired output concentration gradient is presented as the logic diagram in Figure 2. The first step is to estimate the number and position of the dividers at the last level for target accuracy. Narrower spacing between dividers is usually considered in the regions of higher gradient steepness. In the following steps, eqs 4 and 7 are repeatedly applied, with arbitrary choices of parameters, to calculate the positions of all the dividers down to level 0 where one divider separates the two initial solutions. Additional practical aspects for the calculation algorithm are related to the thickness and length of dividers. At any level n , the total thickness of the $n+1$ dividers has to be subtracted from the width of the channel before calculating the physical spacing between dividers. Also, the length of the dividers is calculated to match the velocity of the flow streams in the longitudinal direction and the time required for 99% complete mixing by diffusion of the merged streams at each level.¹⁶ This algorithm was implemented in Matlab and verified experimentally for several output gradients.

EXPERIMENTAL METHOD

Standard microfabrication technology was used to fabricate 10- μm -wide dividers in 400- μm -wide and 30- μm -high channels in poly-(dimethylsiloxane) (PDMS, Sylgard 184; Dow Corning, Midland, MI) on glass. Briefly, a 30- μm layer of SU8 (Microchem, Newton, MA) was spun on a silicon wafer and photopatterned according to manufacturer's instructions and using a Mylar mask (Fineline Imaging, Colorado Springs, CO). PDMS was prepared according to the manufacturer's instructions and cast over the developed photoresist mold to create complementary microchannels in PDMS (Figure 3). Through holes, defining the inlets and outlets, were punched using a beveled 25-gauge needle. The bonding surfaces of the PDMS and a regular glass slide (1×3 in., Fisher Scientific, Pittsburgh, PA) were treated with oxygen plasma (150 mTorr, 50 W, 20 s) produced in the parallel plate plasma asher (March Inc., Concord, CA). A good seal between the PDMS and glass was achieved by heating the assembly at 75 °C for 10 min on a hot plate. Before use, devices were primed with distilled water. To generate a gradient, smooth flow (20 $\mu\text{m/s}$) was driven through the device by the height difference between two inlet and one outlet reservoirs.

RESULTS AND DISCUSSION

Concentration profiles during the flow of solutions through the system of dividers were tested in the microfluidic devices using fluorescein isothiocyanate dye and distilled water as starting solutions. The formation of the gradient starts with controlled mixing of the two initial solutions between the two dividers at level 1 and continues all way down to the last level of dividers (Figure 3). Details of the fluorescence distribution across the channel at levels 1, 2, 4, and 8 during the formation of an exponential gradient are shown (Figure 3b), together with the measured fluorescence profiles (Figure 3c). These measurements are compared to the targeted average concentrations by assuming a linear dependence between concentrations and fluorescence intensities.

Four different devices were tested, the equivalent of power, exponential, error, and cubic root functions (Figure 4). Some of these gradients may not occur naturally in vivo, and we choose them mainly to show the flexibility in reproducing both concave and convex functions. Any other monotonic functions defined explicitly or numerically that would be more relevant for in vivo situations than the four chosen could, however, be implemented in a similar manner. For the devices tested, the fluorescence intensity was quantified in

micrographs at 100 μm downstream from the last set of dividers (section AA) and equivalent concentrations were calculated. Comparisons between normalized targeted and the experimental concentration profiles reconstituted from $N=10$ unequal substreams showed differences less than 0.05 between the two. The corresponding root-mean-square deviations between experimental and theoretical results were also relatively small (0.023, 0.051, 0.033, and 0.030 for power, exponential, error, and cubic root functions, respectively). Considering that the precision of gradient replication for the particular choice of divider position for each gradient was calculated on the order of 0.1, we attribute the better precision mainly to the gradient smoothing by diffusion after the last set of dividers.

Two important particularities of the microfluidic devices are worth mentioning regarding the reproduction of nonlinear gradients. First, the configurations presented in this paper rely on the equilibration of the concentrations at the end of the divider sets and, thus, involve some dependence of the gradient on the flow rate. While flow rates lower than a set threshold speed do not affect the outlet concentration profile, flow rates larger than the threshold speed would create gradient profiles closer to the unrestricted mixing. At the highest speed, the two streams would flow next to each other unmixed at the outlet. Configurations where incomplete mixing is used to achieve the output gradients could also be calculated; however, their practical implementation would be very sensitive to the flow rates and more challenging to use. The second particularity of the universal gradient device is that the positions of the external walls of the channel do not have an impact on the output gradient as long as the final gradient measurement is done in a predetermined area correlated to the position of the last set of dividers. For example, in the fabrication of the device for the cubic root gradient, we avoided very close positioning of one divider to the wall, by enlarging the width of the channel. A reference obstacle was added after the last set of posts to define the limits of useful zone for the target gradient (Figure 4d). This “additional divider” did not affect the output gradient or the arrangement of the dividers in the device.

Our new approach to gradient generation shares with other microfluidic devices the stability of the concentration profile over time. Practically, a steady gradient can be maintained for as long as solutions at the inlet are available. Considering the slow flow rates adequate for most cell-based experiments, average solution consumption as low as 3 $\mu\text{L}/\text{h}$ can be achieved. One important difference between our approach and existent microfluidic systems capable of producing nonlinear gradients is the flexibility in the shape of the output gradients starting from only two inlets. Using the system of dividers, any monotonic gradients can be produced, a significant improvement compared to the multiple inlets networks¹⁷ and asymmetric irrigation of the symmetric microfluidic network that can only generate power series gradients¹³ or the dilutor networks that can only produce dilution series gradients.¹⁴ Nonetheless, a smaller footprint of the gradient generator compared to gradient network devices is achieved through the precise implementation based on calculations. In addition, any design for a new gradient requires only the repositioning of the dividers in the main channel while maintaining the rest of the device unchanged.

Further improvements of the device are possible. While in the present configuration mixing between a set of dividers occurs only by diffusion, convective mixing configurations may be also be implemented, e.g., by incorporating of mixing structures in the walls for chaotic mixing.¹⁸ This would reduce the length of the dividers, especially for the initial levels of the device. Also, several streams of different profiles could be combined by the construction of several devices side by side and merger of the output channels^{15,17} to reproduce more complex functions. Consequently, one could overcome the monotony restriction for the functions that can be implemented because any continuous function can be more or less decomposed into simpler monotonic functions on intervals. However, the number of required inputs would increase with the number of inflection points leading to more complex

designs for the universal gradient devices. Finally, while the present device has been designed for steady concentration profiles, temporal variations of the gradients would be possible by the integration of microstructured valves in the microfluidic design,¹⁹ with applications such as the study of the initiation of neutrophil migration¹⁹ or axonal growth or regeneration.²⁰

CONCLUSION

A systematic approach for designer gradients at microscale is presented. A mathematical apparatus for demonstrating the universal applicability of our approach and for extracting the design details starting from any chosen output is also described. The new microfluidic devices, by precisely replicating biochemical heterogeneities at length scales relevant to cells, has the potential to become an useful tool for studying the effects of microenvironment on biological activities such as cellular polarization, migration, or growth, toward better understanding of these fundamental processes in their in vivo context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Universal gradient generator. The position of the dividers at different levels inside the main channel controls the mixing of the two inlet solutions and determines the concentration gradient at the output. Concentrations at different locations inside the channel are denoted C_{ij} and positions of the dividers with respect to the side of the main channel are denoted u_{ij} , where i is the level and j is the position at one level. Direction of the fluid flow is indicated by the arrow.

Figure 2.

Logic diagram for calculating the position of the dividers in the channel for a desired output concentration gradient. Starting from the last level of dividers, and using an arbitrary set of parameters α , the position of the dividers is calculated backward for all levels toward the inlet of the device.

Figure 3.

Gradient evolution inside the universal gradient generator. (a) Scanning electron micrograph of the main channel and dividers in the microfabricated universal gradient generator. A device for the generation of an exponential gradient is presented. Total length of the device is ~ 6 mm. Scale bar is $500\ \mu\text{m}$. (b) Four different positions, outlined by the square boxes in panel a, at the end of the dividers at level 1 (Lv1), 2 (Lv2), 4 (Lv4), and 8 (Lv8), were fluorescently imaged in order to evaluate the concentration profile. Fluorescence intensity was measured along the dashed lines. (c) The normalized fluorescence intensity from the device (solid line) was compared to the calculated values (red dots) at the corresponding levels. The abrupt drop in fluorescence corresponds to the physical dividers. Images were not corrected for photobleaching during the imaging.

Figure 4.

Resulting microfluidic structures, images of steady-state fluorescent gradients inside the devices, and comparisons between experimental and theoretical normalized concentration profiles for (a) fifth power, (b) exponential, (c) error, and (d) cubic root functions. Fluorescence intensity was measured at level AA for all channels (black line). The targeted output functions are also plotted (red line). Scale bar is 500 μm .