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A Serial Dilution Microfluidic Device for Cytotoxicity Assays

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Abstract— **A novel microfluidic device is presented which creates a linear serial dilution of two input fluid streams. This platform facilitates higher productivity as a component of a high throughput cytotoxicity testing strategy. A modeling solution is presented to create custom linear dilution schemes. The featured device creates a serial dilution of two solutions in the range of 1:9 through 9:1 across nine discrete dilutions. It has been validated to create a highly linear progression of dilutions with an R^2 value of 0.9993. The device functions equivalently over a wide range of flow rates. The standard deviation of dilution values averages 0.76% over six flow rates spanning 0.5 to 16 μl min^{-1}.**

I. Introduction

Procedures used to conduct cytotoxicity screening in conventional molecular and cellular biology labs are effective, but are laborious, time consuming and expensive when faced with the demands of high throughput screening. Certain cell types, reagents, and exploratory compounds are often rare and expensive, making replicates and large scale studies either not possible or prohibitively expensive. Due to the inherent variability in cell behavior and response, replicates are necessary to draw statistically significant conclusions. Studies conducted using methods of cell culture in multiple 96 well plates and manual serial dilutions are time consuming and require the expertise of skilled researchers to conclude meaningful results. Several of these processes have been automated using robotic systems, but these systems are expensive and require significant laboratory space. Therefore, a cytotoxicity screening strategy requiring less time, labor, and expense is necessary to facilitate higher productivity in cell biology studies.

Microfluidic technology addresses the shortcomings of traditional methods. Researchers have developed microfluidic platforms designed to culture cells [1]-[3] and conduct cytotoxicity assays [4]–[5]. A key feature of cytotoxicity assays is the ability to create a serial dilution. Serial dilutions allow researchers to test the dosage dependency response of a particular reagent on cell behavior. Serial dilutions are most commonly created by one of two methods. The first method relies on diffusion [6]-[8], whereas the second relies on fluid partitioning [4], [9]-[12]. While these methods have their advantages, the work presented simultaneously offers the ability to generate a linear serial dilution independent of flow rate. The device separates two fluid streams into nine dilutions and is configured in a streamlined manner, which will serve as a functional component of a high throughput cytotoxicity testing platform.

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II. Methodology

A. Design

This microfluidic device was designed to create nine dilutions of one fluid in another. These dilutions are created independently of the flow rate through the device. The only requirement is that the flow rate and viscosity of each fluid input are the same. The design is composed of two functionally equivalent branch networks, one mirrored and superimposed upon the other in a stacked two layer structure, with the branch ends meeting at common outlets. When flows from channels in both branches are combined, mixtures of the two inputs are created in sequentially increasing dilutions. After the dilution is created, diffusional mixing occurs. Diffusional mixing depends on diffusional distance and time, and therefore at sufficiently low flow rates or long channel lengths, each dilution has time to completely mix before being delivered to its target cell culture. Figure 1 shows the resultant microfluidic channel design and a three dimensional rendering of the interlayer interface.

B. Modeling

An analytical solution was derived to enable the design of a serial dilution microfluidic device incorporating an arbitrary number of dilutions. Modeling began with the assumption that fluid flow on the microscale is within the laminar regime [13]. Given this assumption, a direct analogy can be drawn between fluid flow through microchannels and ideal current flow through electrical resistance networks. The volumetric fluid flow rate is analogous to electrical current, and fluid channel resistance is analogous to electrical resistance. Furthermore, Ohm's Law and Kirchhoff's First Law can be used to design microfluidic networks.

The branched microfluidic channel structure used for the model is shown in Figure 2a, and its electrical resistance circuit equivalency is shown in Figure 2b. Note that only

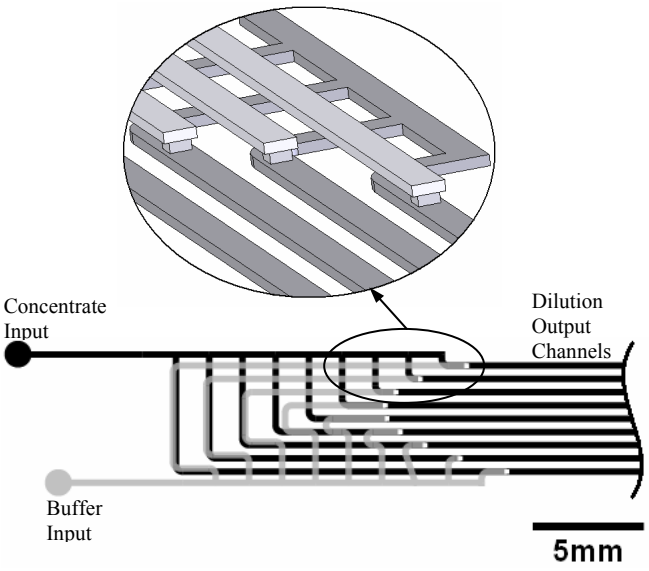

Fig. 1. One dimensional configuration of the two layer serial dilution microfluidic device. The bottom channel network is shown in black and the upper channel network is shown in grey. The interlayer access ports are shown in white. The expanded view shows where the upper channel network deposits fluid into the lower network through the via ports.

one of two layers is shown here. The two layers are functionally equivalent. The key variable of the system, R_k, determines the relative ratios of fluids that are combined from the top and bottom layers. All bridge resistors, R_b, have a normalized value of 1. The value N is the total number of dilutions desired, which is also the number of branches in the network. Solving for the branch resistor values is an iterative process.

The current, I_k, through each branch resistor, R_k, will depend on the total number of serial dilutions:

$$I_k = \frac{k}{\sum_{m=1}^{N} m} \tag{1}$$

The total downstream equivalent resistance from a branch node, R_T is expressed as:

$$R_T = \frac{R_T R_{k-1}}{R_T + R_{k-1}} + 1 \tag{2}$$

The proportion of current going through R_T and R_k is known and the voltage across each must be the same, thus:

$$I_k R_k = \frac{\sum_{m=1}^{k-1} m}{\sum_{m=1}^{N} m} R_T \tag{3}$$

Solving for R_k and simplifying terms gives:

$$R_k = \frac{\sum_{m=1}^{k-1} m}{k} R_T \tag{4}$$

By iterating between equations (2) through (4) for the total number of branch resistances, all R_k values can be calculated. Using this model, it is possible to create any number of discrete dilutions, ranging from 0% to 100%. An electrical resistance network was created in P-Spice (MicroSim, Version 6.3) and used to verify the theoretical model, resulting in full agreement.

Resistance values, R_k, are equated to fluidic channel resistance, expressed by equation 5 [13].

$$R = \frac{12\mu L}{wh^3}\left[1 - \frac{h}{w}\left(\frac{192}{\pi^5}\sum_{n=1,3,5}^{\infty}\frac{1}{n^5}\tanh\left(\frac{n\pi w}{2h}\right)\right)\right]^{-1} \tag{5}$$

Assuming equivalent viscosity μ between the two fluids, channel dimensions, L, w, and h are solved. Holding the channel width and height constant throughout the design leaves the channel length as the sole variable used to adjust channel resistance. As such, the channel length values are adjusted in direct proportion to the normalized resistance values. The two mirrored and superimposed manifolds have the same branch channel lengths, but are configured differently to allow the upper manifold to align with the lower manifold at the interlayer through holes. After the manifold channels meet, the downstream resistance is arbitrary, provided that each downstream channel resistance is the same. The channel dimensions chosen were based on the desired compactness of the design and the desired cell culture area in each channel. In the device shown in Figure 3, the channel dimensions are 100 μm high, 300 μm wide, and the entire device volume is approximately 10 μl.

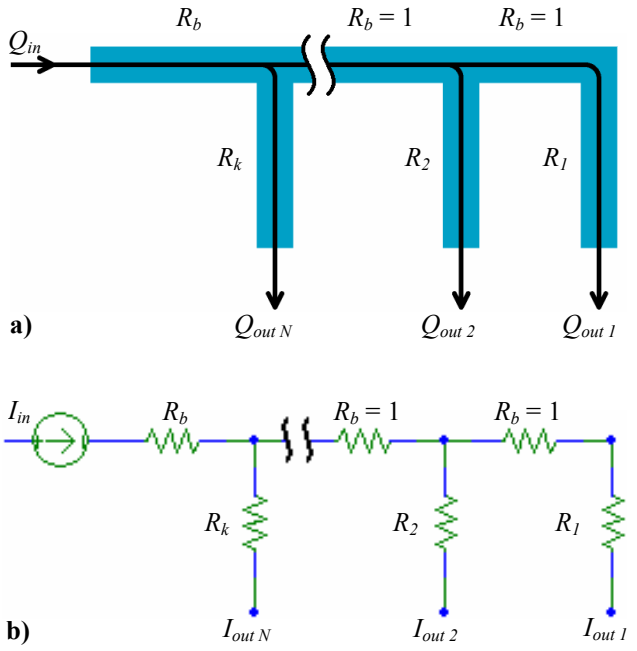

Fig. 2. a) Branch structure of fluidic channel network necessary to create desired flow volumes through each channel, and b) Electrical resistance circuit equivalency.

C. Fabrication

The microfluidic device was fabricated using the techniques of soft lithography and polydimethylsiloxane (PDMS) replica molding, whose methods are detailed by Whitesides [14]. In brief, micromolding masters were created by spin coating negative tone photoresist (SU-8 2100, Microchem Corp., Newton MA) on a silicon wafer. The photoresist was selectively exposed with ultraviolet light (BlakRay B-100A) through a high resolution transparency containing the channel design. Two masters were created to mold the microfluidic device. The first master had a single layer photoresist structure used to mold the upper manifold channels. The second master contained a two level photoresist structure, with the microchannel pattern on the first level and the through hole pattern built atop. Using the first master, the top channel structure was cast in PDMS (SYLGARD 184 Silicone Elastomer Kit, Dow Corning, Midland, MI). The lower layer is a 300 μm thin film containing the lower channel network and the interlayer through holes. This thin film was made via compression molding [15]. One of the key steps of the compression molding process was laying a fluoropolymer film (3M Scotchpak 1022 Release Liner) over the liquid PDMS on the master. Once the PDMS was cured, the bond between the PDMS and fluoropolymer film facilitated easy handling of the compression molded layer, and the film and PDMS can be easily separated before the next assembly step. The first assembly step was to bond the thin film layer to a glass slide using an oxygen plasma treatment. The top channel structure, also molded into PDMS, was then plasma treated along with the lower layer, wetted with methanol, aligned over the first layer under a stereoscope, and the entire device was baked on a hot plate at 85°C for 80 min to evaporate the methanol. In this final baking step, it was necessary to manually force out any vapor bubbles forming between the two PDMS layers to maintain contact and ensure complete bonding.

D. Operation

Flow was introduced into the microfluidic device via Tygon tubing, 0.51 mm ID x 1.52 mm OD, inserted into access holes in the PDMS cored with a blunt end 16 gauge needle. This tubing was connected to blunt tipped syringes (Becton Dickinson), and flow was regulated using a dual station syringe pump (Pump 11 Pico Plus, Harvard Apparatus).

E. Imaging/Visualization

To demonstrate the ability to create a serial dilution, the microfluidic device was imaged while flowing both food coloring, shown in Figure 3, and fluorescein (Acros Organics) shown in Figure 4b. The food coloring inputs were initially diluted 10:1 in de-ionized water, and the fluorescein was dissolved at 7.5 mg ml^{-1} of acetone. Given the nature of PDMS to swell when exposed to acetone, the fluorescein solution was further diluted at 100:1 in de-ionized water. Color images were taken using a digital camera in macro mode (Sony DSC-F828). Fluorescence images were taken using a monochrome digital camera (Hamamatsu ORCA-ER C4742-80), mounted to an inverted microscope (Olympus IX71), imaged through a FITC filter cube. Images were captured using the IPLab software package (Scanalytics, Inc.) with exposure times set at 150 – 175 msec. The IPLab software was also used to quantify the fluorescence intensity of the fluorescein dilutions flowing through the microchannels.

III. Results

As mentioned, the device was imaged both with food coloring and fluorescein to demonstrate the serial dilution. Figures 3a and 3b show a serial dilution of yellow and blue food coloring, demonstrating the ability to create a color gamut from yellow to blue through different shades of green. Figure 4b shows a fluorescence image of a serial dilution of the fluorescein solution and de-ionized water flowing at 1.0 μl min^{-1} through the device. Figure 4a is a graph of the light intensity measured from the image in 4b. The graph clearly shows the intended linear serial dilution and complete diffusional mixing within channels. The device was also designed to create a consistent linear serial dilution regardless of flow rate. The device was tested using six flow rates, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 μl min^{-1}. This objective was achieved as shown by the data in Table 1. Column one shows the channel numbers, column two shows the intended dilutions, column three shows the actual dilution values and column four shows the standard deviation. An average R^2 value of 0.9994 over the six flow rates demonstrates extreme linearity in the progressing dilution values, and an average standard deviation of 0.76% covering all dilution values and all flow rates proves flow rate independence. The limiting factor on the low end of the flow rate range is evaporation from the channels. The limiting factor in flow rates above the highest value is adequate diffusional mixing at the end of the downstream channels. To increase the flow rate further, longer

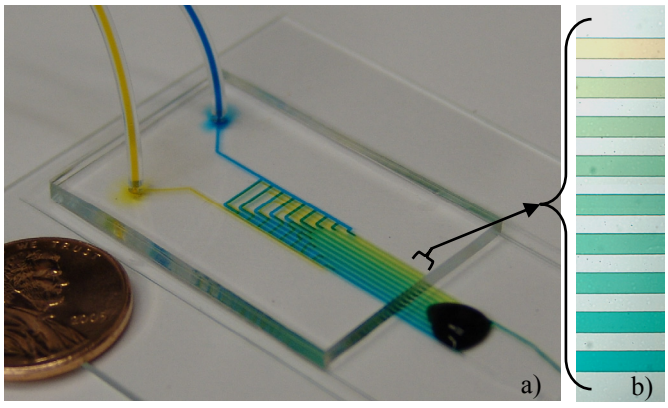

Fig. 3. a) Microfluidic device creating serial dilution of yellow and blue food coloring. b) Magnified image of downstream channels showing a color gamut from yellow to blue through shades of green.

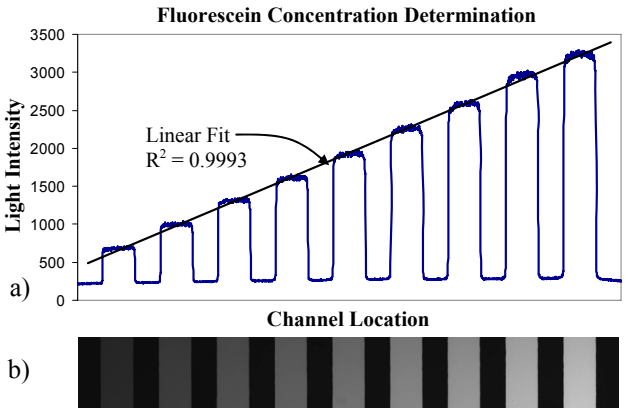

Fig. 4. a) Graph of light intensity over a linear scan across a serial dilution of fluorescein in de-ionized water. b) Fluorescence image of fluorescein serial dilution flowing at 1.0 µl min^{-1}.

downstream mixing channels are required. The device did not create the exact dilutions desired in the lower values. This may be due to slight variation in channel height between the two channel layers, attributable to imprecision in the soft lithography prototyping techniques.

IV. Discussion

Using the modeling equations, the number of dilutions desired can be increased or decreased. However, as more dilutions are desired, the ratio of the lowest branch resistance to the highest branch resistance increases, potentially making the design of the channel network difficult to configure. However, a parallel dilution scheme can be configured by running each output of one network to an input of another serial diluter, allowing a significantly broadened range of dilutions.

This device is currently being tested with human epidermal keratinocytes in the downstream microfluidic channels. Coverage of this material is omitted for brevity, and work to run preliminary cytotoxicity assays is underway. The design discussed in this submission will next be modified to isolate effluent from individual populations of cells exposed to a toxicant serial dilution. This effluent will be analyzed for cytokines, which have been shown to be an indicator of a proinflammatory response of keratinocytes exposed to toxicant solutions.

TABLE I
Actual Serial Dilution Values Averaged Over Six Flow Rates: 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 µL min^{-1}

Channel Number	Target Serial Dilution	Averaged Actual Concentration	Standard Deviation
1	1:9 (10%)	14.0%	0.4%
2	2:8 (20%)	22.9%	0.6%
3	3:7 (30%)	32.4%	0.7%
4	4:6 (40%)	41.7%	0.7%
5	5:5 (50%)	51.1%	0.8%
6	6:4 (60%)	60.9%	1.3%
7	7:3 (70%)	70.7%	1.0%
8	8:2 (80%)	80.3%	0.9%
9	9:1 (90%)	88.9%	0.4%
Average R^2 of all linear fits attached to data for each flow rate = 0.9994			
Average standard deviation spanning all dilutions, flow rates = 0.76%			

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