# Survey of gradient generation using microfluidics and application to cellular differentiation.

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## Abstract

**Microfluidics has shown promise in mimicking a cellular environment that is more physiologically relevant than standard cell culture techniques. Furthermore, using microchannels in a no-flow setup also seems to allow both autocrine and paracrine signaling. One of the emerging applications of microfluidics is in the field of stem cells. Embryonic Stem Cells (ESCs) have the ability to differentiate into every cell type in the body given the right conditions such as growth factors, temperature, support cells, extracellular matrix, as well as cell-cell signaling. Microfluidics can be applied to the field of stem cells to determine which conditions allow for optimum differentiation. In this paper, different methods will be surveyed for generating growth factor gradients using microchannels and their application to stem cell differentiation will be discussed.**

## Introduction

Growth factors are known to affect the differentiation of stem cells in a cell culture environment [1]. Cellular response to growth factors is complex, with different concentrations of the same growth factor leading to different cell lineages and multiple growth factors often acting at once on a single cell. Specific combinations and concentrations of growth factors are difficult to test with current cell culture techniques. Current methods isolate cell lines, then culture in a flask, or large well plate, and apply known concentrations of growth factors [2]. These conditions do not closely simulate *in vivo* conditions and only test over discrete concentrations of growth factors.

Microfluidic channels provide an improved solution to the current culture techniques. Microfluidic channels can be manufactured to control the concentrations of substances within the channel causing the *in vitro* environment to more closely mimic *in vivo* conditions [3]. One important aspect of *in vivo* conditions is cell signaling, either autocrine or paracine [4]. Therefore it is advantageous to create a culture system that will allow for these signaling methods while maintaining a growth factor concentration gradient formation. There are a number of methods which can be employed to construct a concentration gradient generator that allows for cell signaling based on the same principles [2]. The principles and techniques used to create this new culture platform will be explained along with explanations of the successes of each method.

## Theory

Diffusion is the dominant mechanism that allows a concentration gradient to form over the length of the microchannel with a source-sink configuration [2]. To ensure that diffusion is the dominant mechanism, convective fluid forces must not be present. In order to stop convection, the source-sink configuration will be connected to the microfluidic channel through a high resistance pathway.

There are several reasons why a high resistance pathway is implemented in the microchannel construct. First, the pathway will work to minimize the amount of convective fluid flow [4]. Also, if gradient generation is needed to last over a number of days, replenishing of the source and sink will be needed to ensure cell viability. The high resistance pathway will ensure the stability of the gradient as the source and sink are replenished [3].

Different high resistance pathways can be used depending on whether a 3-D or 2-D cell structure is to be maintained [3,4]. For the 3-D cell culture, Matrigel was used. For the 2-D cell cultures, polyester membranes and agarose were used. Cell viability within the microchannels was also examined.

## Methods and Materials

### *Device Construction*

Fabrication of the cell culture channels was done using standard photo and soft lithography techniques [5, 6]. Briefly, SU-8 photoresist was spun onto a silicon wafer and exposed to ultraviolet (UV) light through a photomask. An SU-8 specific chemical developer dissolved away unexposed photoresist, leaving only the features defined by the photomask. The silicon wafer and the remaining SU-8 acted as a mold onto which poly(dimethylsiloxane) (PDMS) was poured. The silicone elastomer was combined in a 10:1 base:curing agent ratio, allowed to degas for 20 minutes, poured onto the previously formed mold, and cured for 2 hours at 95 degrees Celsius.

Two different channel designs were used for experimentation: a straight channel and a T-channel. The straight channel (Fig.1a) was 2mm long, 0.3mm wide and 0.15mm tall with ports 0.8mm in diameter and 0.25mm tall. The T-channel was made by addition of a cross-channel measuring 2mm long, 0.1mm wide, and 0.15mm tall to the straight channel (Fig. 1b). Also, a second SU-8 mold was fabricated and cast with PDMS to create large reservoirs to hold the source and sink solutions on top of the cell channel ports (Fig. 1c).

### *Device Integration*

3-D technique

After the PDMS was cured a straight channel was removed from the SU-8 mold and immediately placed onto a microscope slide. The second layer of PDMS with reservoirs was place on top of the first. Matrigel, stored at 4° C, was flowed into the channel using a cooled micropipette tip and then placed on a hot plate at 37° for 10 minutes to gel [7]. A small volume (approx. 50 µl) of 10 kiloDalton (kDa) Dextran labeled with Texas Red was placed in the source reservoir and a small volume (approx. 300 µl) of distilled water was placed in the sink reservoir.

2-D techniques

*Straight Channel*

Two separate techniques were used to create a high resistance barrier between the source and the microchannel: a micropore membrane and an agarose plug. A polyester membrane with 0.8 µm pores was placed over the source port of the microchannel after the channel was place on a microscope slide. After plasma treating the reacting surfaces, the second reservoir layer of PDMS was put over the membrane, sandwiching it in place and irreversibly bonding the device together [8]. The channel and sink were then filled with water, and the fluorescent Dextran was placed in the source (Fig. 2). A second method using an agarose plug in place of the

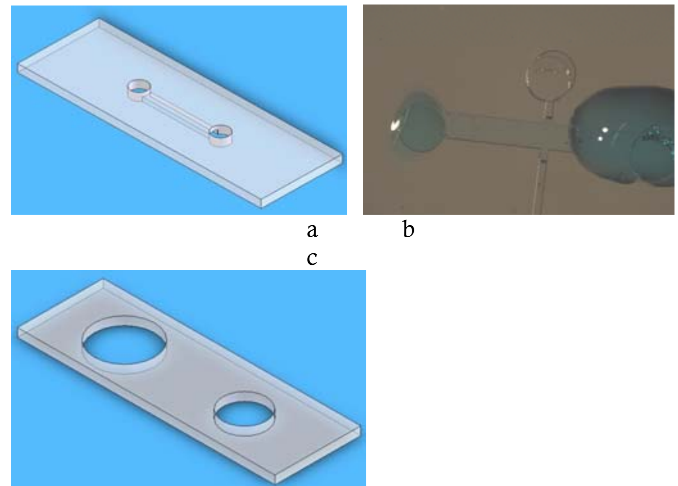



Fig. 1.

a) Straight channel (2 mm long, 0.3 mm wide, 0.15 mm tall)
b) T-channel (straight channel in addition to a cross-channel measuring 2mm long, 0.1 mm wide, 0.15 mm tall)
c) Second layer of PDMS containing the source and the sink This layer is placed on top of the straight channel.

porous membrane was also tried. Agarose (SeaPrep- 2.2% by weight and SeaKem-1.5% and 3.0% by weight) was heated to 100° C and quickly pipetted onto the source port of a straight channel. As the agarose cooled it solidified over the port creating a barrier between the source and the channel, much like a membrane. Once the agarose had cooled to room temperature the reservoir layer of PDMS was placed over the ports and the source and sink were filled with fluorescent Dextran and water respectively (Fig. 3).

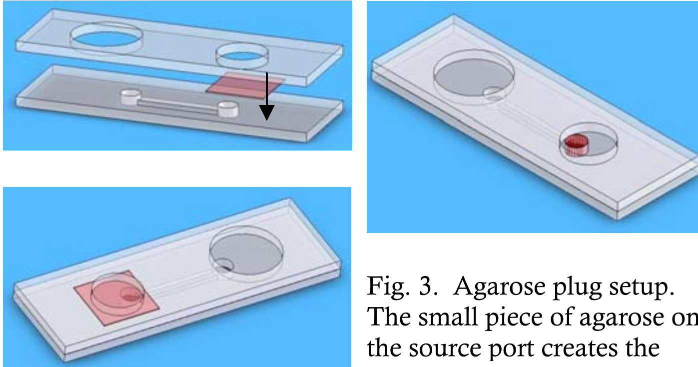


Fig. 2. Membrane setup. The membrane is sandwiched between the two PDMS layers, thereby creating a high resistance barrier.

Fig. 3. Agarose plug setup. The small piece of agarose on the source port creates the

*T-Channel*

Two methods using the T-Channel were tried. For one method, the channels were pre-filled with water. Agarose was then passively pumped from one port to another, filling the channel with agarose and creating a barrier between the source and sink ports (figure 3). The source was then filled with Dextran and the sink with water. For the second method, the channels were empty. A drop of agarose was placed over a cross channel port while a slight vacuum was being applied from the opposite port moving the agarose through the channel, again creating a barrier between the source and sink. The source was then filled with Dextran and the sink with water.

*Imaging*

Gradient formation was characterized using a florescent microscope with a GFP2 filter. Time-lapse pictures were taken every 15 minutes for the first hour and then every hour afterwards. A drop of Dextran was placed next to each channel to serve as a 100% Dextran control for the later image analysis.

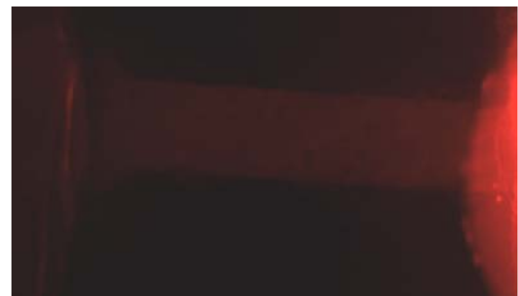
# Results

## 2-D Gradient Results

Membrane

The 2-D gradient formation methods described above were not consistently able to produce a gradient. The main reason for the lack of reproducibility in creating a gradient lies in the inability to determine if a fluid connection has been made between the source and the liquid in the channel. Since the membrane is providing a barrier between these two mediums, any air gap will contain the two mediums and thus not allow the diffusive mixing that is required for gradient formation. Also leakage of the fluid in the channel due to the membrane was a common problem that was encountered. When the membrane was "sandwiched" between two layers of PDMS and a perfect seal was not created, the fluid leaked out of the source and around the membrane into the microchannel.

Despite these difficulties, a gradient was formed with the membrane technique as seen in Figure 4. The gradient was characterized using the MATLAB® software package to characterize the luminance of the gradient using the MATLAB® command "rgb2gray". The gradient was then averaged over its "height" (ideally same



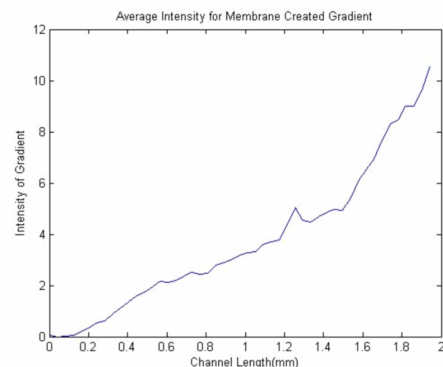
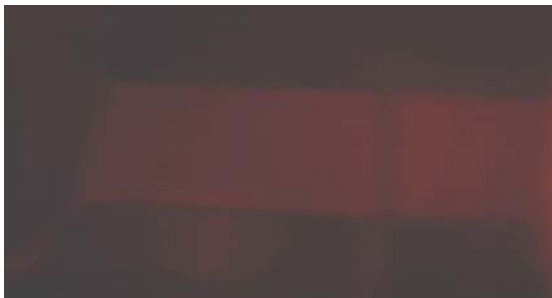



Fig. 4. Gradient formation using the membrane setup. The gradient was characterized using MATLAB and seems to be linear over the length of the channel.

concentration), and the luminance over the length was plotted and averaged over 20 pixels to obtain the Figure 4.

Agarose

The results using the agarose were also mixed. Using the agarose as a plug was difficult to reproduce because of the inability to ensure that a seal has been made at the source port. If a seal was not created then fluid could leak through the port keeping a gradient from forming. Also, there was difficulty in creating this agarose plug because the agarose would cool very quickly making ideal formation difficult. Despite these difficulties, a gradient was formed using the agarose plug as a high resistance barrier, as seen



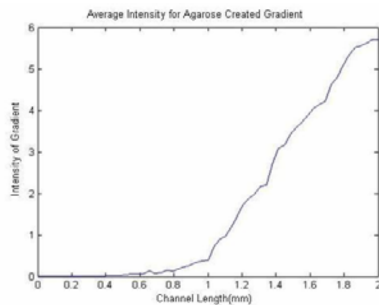


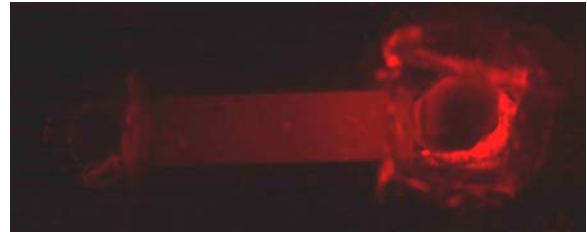

Fig. 5. Gradient formation using the agarose setup. The gradient was characterized using MATLAB and seems to be linear over 1.2 mm of the channel. This image was taken 7.5 hours after the source was filled with labeled Dextran.

in Figure 5. Again, the gradient was characterized using the MATLAB® software explained previously.

<u>3-D Results</u>

Matrigel

The gradient formation using the Matrigel® as the high resistance barrier was successful. The results were highly reproducible due to the relative ease in which the Matrigel® could be placed within the channel and allowed to harden. This created an ideal seal along the entire length of the channel which allowed for highly



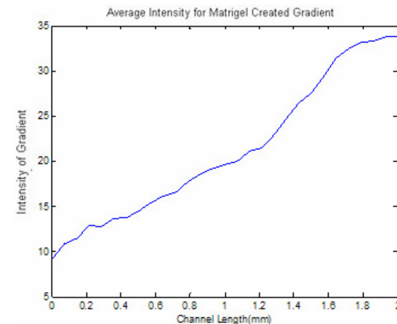



Fig. 6. Gradient formation using Matrigel. The gradient formed over approximately 4 hours. The graph of average intensity values shows that the linear is approximately linear.

reproducible results. A gradient and its corresponding intensity graph can be seen in Figure 6.

## Discussion

A number of different studies have been performed on gradient generation using microfluidics, including 2-D and 3-D techniques. Each method was successful in forming gradients; however, the most successful was the the Matrigel system compared with either the membrane-based or agarose plug methods.

Gradient generation using microfluidics can be applied to the field of stem cells. Stem cells can be differentiated into various cells if presented with the right combination of growth factors. Since microfluidics mimic cellular conditions more closely than standard 2-D cell culture, the incorporation of a gradient generation feature in microfluidics can be groundbreaking in the study of stem cell differentiation.

All the methods discussed in this paper rely on setting up a no-flow system, where the

principle of diffusion only allows for the movement of the growth factor into the channel. Such a setup allows for autocrine and paracrine signaling which are critical in stem cell differentiation.

After considering all the systems tested, the 3-D Matrigel setup will most effectively allow stem cell differentiation to be studied *in vivo*. Cells embedded in a 3-D Matrigel setup will be more representative of how cells are actually organized in the body. The 3-D cell distribution will also allow cell-cell interaction, which can subsequently lead to other cellular processes.

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