Design and Fabrication of a Microfluidics Gradient Generator System for High-Throughput Molecular Interaction Studies

by

Guan-Jong Chen

S.B. Biology
Massachusetts Institute of Technology, 2003

SUBMITTED TO THE BIOLOGICAL ENGINEERING DIVISION IN PARTIAL
FULFILLMET OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN TOXICOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
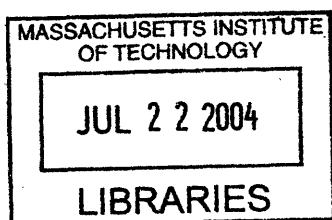
MAY 2004
[June 2004]

Signature of Author: _____

Biological Engineering Division

Certified by: _____

Robert S. Langer, Sc.D.
Germenhausen Professor of Chemical and Biomedical Engineering
Massachusetts Institute of Technology
Harvard-MIT Health Sciences & Technology
Thesis Supervisor

Accepted by: _____

Ram Sasisekharan, Ph.D.
Professor of Bioengineering
Massachusetts Institute of Technology
Chairman, Committee for Graduate Students

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Submitted to the Biological Engineering Division on May 7, 2004 in Partial Fulfillment of the Requirements for the Degree of Master of Science in Toxicology

ABSTRACT

Design and fabrication of a microfluidics system capable of generating reproducible and controlled micro-biochemical environments that can be used as a diagnostic assay and microreactor is important. Here, a simple technique was developed to create a robust microfluidics system capable of generating precise gradients of biochemical properties within its channels. Through this approach, it is possible to create a gradient generator with mammalian cells patterned and seeded under its poly(dimethylsiloxane) (PDMS) channels. Cells that were seeded and patterned under the PDMS channels remained viable and capable of performing intracellular reactions. Using the gradient generator within the PDMS microfluidic device, a gradient of specific and controlled biochemicals can be flowed on seeded cells allowing for high-throughput molecular interaction analysis. The microfluidics system provides a way to study and analyze cell response in the presence of a combination of biochemical signals.

ACKNOWLEDGEMENTS

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INTRODUCTION

Current methods to study cell response in the presence of a combination of biochemical signals are expensive and limited in the number of tests that can be performed simultaneously [1]. Developing a technology that could analyze the behavior of cells in response to many combinations of molecules could lead to advancements in a number of fields ranging from tissue engineering to drug discovery.

To overcome the difficulties associated with analyzing multiple conditions simultaneously, a prototype poly(dimethylsiloxane) (PDMS) microfluidic system (Scheme 1) was designed and fabricated. By placing a concentration gradient generator upstream in the system, this research and previous studies [2] have shown that the concentration gradient generator is able to generate stable concentration gradients through a series of mixing and redistribution of the chemical reagent in the serpentine channels (Fig. 1 & Fig. 2). This feature gives researchers an opportunity to study how cell response/behavior is affected *in vitro* by a gradient of biochemical reagents at various concentrations as the reagents act on the cell (Fig. 3).

The fabricated prototype is capable of generating approximately 10 designated biochemical conditions and could be potentially expanded to test for hundreds or thousands of conditions. In many aspects, micro-devices comprised of microfluidic components hold great promise in the development of improved bioanalytical and

diagnostic devices because they allow for great reduction of sample volumes while increasing the throughput and efficiency of analysis [1].

Developing a technique to pattern and seed cells within the prototype device was an important component of the device. Photolithography is currently one of the most commonly used techniques to pattern within microfluidic channels. It has been used for patterning cells, proteins [3], hydrogels [4, 5, 6], directing the flow of liquids [7, 8], as well as etching [9] and building microstructures [9] within microchannels. Although the technique proves to be successful in controlling the surface properties of the microchannels and is an integral part of many emerging applications [10, 11], it has potential limitations due to cytotoxicity of the photoinitiator [12], the need for specialized equipment, and the difficulty in patterning the surface without modifying the surface topography [5, 6]. Although techniques such as microcontact printing [13], microfluidic patterning [3, 14, 15], micromolding [16-18], and capillary force lithography [19] have served as inexpensive, convenient and scalable tools for patterning surfaces, the merger of soft lithographic patterning approaches and microfluidics has not been realized. As a result, the design and fabrication of the prototype device discussed in this research could be a solution as it provides a simple and direct approach to pattern and seed cells under the microfluidic channels.

Through the design of the prototype device, viable mammalian cells can be seeded under different channels in the prototype device. With the prototype device, one can conduct *in vitro* analysis of cell response as the cells under separate channels interact with

designated biochemical environments generated by the device. Furthermore, previous studies have shown that it is possible to induce fluid mixing [20], direct fluid flow [21] and provide strategies to generate functional microfluidic component such as valves [22] within a microfluidics device. Harnessing these techniques and incorporating them into this device in the future will provide additional means to control the ways that multiple cell types interact with designated biochemical environments within a microfluidics system, which is critical to the development of high throughput analytical devices [23] and multi-step bioreactors [5,6].

The fabricated microfluidic device system could be used to perform tests on cell behavior to various combinations of factors. For example, it may be possible to study the effects of various concentrations of growth factors in combination on stem cell differentiation. So far, most experiments have studied the response of stem cells to individual or limited number of combinations of cytokines. More importantly few studies have rigorously analyzed and optimized the effect of multiple cytokines as well as their interactions at various doses. The device could aid in the performing factorial experiments that could quantitatively analyze the effect of cytokine interactions on stem cell response. Thus, this type of analysis will be helpful in defining differentiation factors, concentration threshold effects on self-renewal and differentiation.

In addition to stem cell research, the PDMS microfluidics device may also provide a simple and direct technique in studying environmental chemicals/mutagens that are detrimental to the human health or in shaping and designing new combinatorial/cocktail

medical treatment for diseases, such as AIDS and cancer. Thus, the fabricated microfluidics gradient generator system has potential for many applications relating to various biomedical and pharmaceutical research. Through this research, it was demonstrated that the system provided an efficient way to conduct study and analysis on chemical/toxin dosage-response and cell apoptosis.

BACKGROUND

Recent advances in micromachining, microelectromechanical systems (MEMS), and microsystem technology have provided a unique opportunity to fabricate miniature biomedical devices for a variety of applications ranging from implantable drug delivery systems and lab-on-a-chip to neural prosthetics [24-26]. These systems offer several major advantages as compared to other devices fabricated using traditional manufacturing techniques. These include: (1) smaller dimensions, (2) lower cost, (3) the ability to incorporate sensing and signal conditioning functions in close proximity or on the same substrate, (4) superior functionality (resolution, sensitivity, etc.), and (5) the ability to provide redundancy and hence enhance the reliability by using numerous miniature devices.

Traditionally, silicon and glass have been the two major materials used in the micromachining and MEMS. These materials show superior mechanical properties which make them suitable for a variety of applications. However, many biomedical devices require alternative soft or polymeric materials such as silicone rubber, polycarbonate, isobornyl acrylate, and polyimide [27]. Polymers are inexpensive and can be used in disposable devices, relaxing stringent sterilization-upon-reuse requirements. In addition, polymers provide a more suitable interface with biological tissue, reducing irritation and scarring. Finally, polymers can exist in either a hard, glassy state or a soft, rubbery state, a variety that is not present in MEMS structures based on silicon or glass.

Most of the activities in the biomedical MEMS (BioMEMS) area in the 1990s were concentrated in the academia, but there is currently a move towards commercialization of many BioMEMS devices and systems. For example, a MEMS-based DNA sequencer (Smart Cycler®) developed by the Cepheid is currently being installed in post offices across the country for Bio-agent detection [28]. Bio-MEMS drug delivery systems are also actively targeted by many investigators and companies as is evident, for example, from current efforts by MicroCHIPS, to commercialize an electronically activated drug delivery microchip, by ChipRx, to introduce systems that integrate silicon and electroactive polymer technologies for controlled delivery [28], and iMEDD, to develop nanoporous membranes and micromachined particles for a variety of drug delivery applications.

Most MEMS fabrication techniques have their roots in the standard manufacturing methods developed for the semiconductor industry [29-31]. Therefore, a clear understanding of these techniques is necessary for anyone starting to embark on a research and development path in the micromachining area. In addition, several MEMS-specific techniques developed for fabricating micromechanical structures have been added to the micromachinist's toolbox over the past 30 years [32-34].

Basic fabrication methods currently used in MEMS and micromachining are the same as those employed by microchip manufacturers over the last four decades. These techniques are: (1) thin-film deposition, (2) lithography, (3) etching, and (4) substrate bonding. Thin films are deposited using various chemical or physical techniques and are used for

masking, isolation, and structural purposes. Subsequent to thin-film deposition, the lithography step is performed in order to transfer the designed pattern onto the substrate. This is basically identical to standard photography and printing techniques. The patterned substrate is etched using various chemicals in the liquid or gas phase. Finally, substrate bonding is used either to integrate multiple functionalities or for packaging purposes. These steps can be repeated numerous times depending on the complexity of the design and process.

In this research, lithography is used to generate the designs of the microfluidics channels on silicon wafer masks. Lithography is the technique used to transfer a computer-generated pattern onto a substrate (silicon, glass, etc). This pattern is subsequently used to etch an underlying thin-film (oxide, nitride, etc.) for various purposes (doping, etching, etc.). The starting point subsequent to the creation of the computer layout for a specific fabrication sequence is the generation of a photomask. This involves a sequence of photographic processes (using optical or e-beam pattern generators) which results in a glass plate having the desired pattern in the form of a thin (~100 nm) chromium layer. Following the generation of photomask, the lithography process can proceed. After depositing the desired material on the substrate, the photolithography process starts with spin-coating of the substrate with a photosensitive resist material to a thickness of 0.5–2.5μm, depending on viscosity and spin speed. Following spinning, the substrate is soft-baked (5–30 min at 60–100 °C) in order to remove solvents from the resist and improve adhesion. Subsequently, the mask is aligned to the wafer and the photoresist is exposed to a UV source.

After exposure, the photoresist is developed by washing off the UV-exposed or the unexposed regions, depending on whether the resist material is "positive" or "negative", respectively (this process is similar to the development of photographic film). The resist is subsequently hard-baked (20–30 min at 120–180 °C) in order to further improve the adhesion. The hard-bake step concludes the photolithography sequence by creating the desired pattern on the silicon wafer. Lastly, the underlying thin film is etched away and photoresist is stripped in acetone or other organic removal solvents.

As was mentioned previously, in the biomedical MEMS area, soft materials (polymers and gels) have some unique advantages over silicon and glass. The fabrication of micropatterns and microstructures with soft materials, sometimes called "soft lithography", is based on the technique of molding. Polydimethylsiloxane (PDMS), also known as silicone rubber, is the material of choice for micromolding. PDMS is inexpensive, biocompatible, and has excellent sealing properties, making it very suitable for making microfluidics devices [35]. In addition, it can be easily bonded to itself, allowing the fabrication of multilayer structures. Finally, thin PDMS diaphragms are elastically extensible so that much larger deflections can be achieved than with hard materials.

Fabrication of PDMS structures with micromolding starts with a silicon mold or mask that is fabricated using the previously discussed hard micromachining techniques. Depending on the mold material and topography, a surface treatment is usually recommended in order to facilitate demolding. Subsequent to the micromold fabrication,

a prepolymer solution (usually a mixture of PDMS linear polymer and a crosslinking agent) is poured on top of the mold. For high-aspect-ratio mold topographies, this step may need to be performed under vacuum to remove air bubbles. Once polymerization is completed, the PDMS layer is carefully peeled off. The mold can be reused many times, which reduces the cost considerably. Multilayer soft lithography that combines soft lithography with the capability to bond multiple patterned layers of PDMS has also been used to fabricate monolithic blocks of PDMS with various levels of microchannels [36]. One can also insert a thin PDMS diaphragm in between two microchannels containing layers forming pneumatically actuated valves and peristaltic pumps by applying a positive pressure to the top channel. This results in the deflection of the membrane and blocking of fluid passage in the bottom channel. Extensibility of the PDMS diaphragm is controlled by its thickness, aspect ratio, and the amount of crosslinker in the prepolymer. PDMS bonds well to itself, glass, and silicon nitride. Bonding of PDMS to other materials is facilitated by surface pretreatment with plasma or HCl.

Microfluidics channels made from PDMS are in essence channels with at least one dimension less than 100 µm. These channels are ideal in mimicking the vascular of the body since their dimensions can be greatly controlled and the shear rates can be analyzed. The flow of a fluid through a microfluidc channel can be characterized by the Reynolds number.

PDMS microfluidics can be used to generate gradients of chemicals and proteins inside channels. Microfluidics systems take advantage of the laminar flow of fluids within

narrow channels (<100 μm) to allow for the formation of concentration gradients of soluble factors. In a typical channel which is 50 μm wide with a velocity of 0.6 cm/s, the Reynold's number (Re) is ~ 1. The flow is completely laminar and no turbulence occurs. Therefore, the diffusion of molecules across the width of the channel when multiple flowing streams are combined generates a gradient perpendicular to the direction of fluid flow [37-41]. The shape of these gradients could be directly controlled depending on the properties of the different inlet streams. Within these inlet streams, desired concentration gradients can be obtained through sequential mixing and separating o channels within microfluidics channel. The shape of the gradient could be controlled to form step-like drops of smooth concentration gradients (Fig. 4 & Fig. 5). In general, Re<<2000 flows laminarly. Thus, it can be seen that much larger channel could potentially be developed as required. Because of laminar flow, two fluid streams that flow into a common channel mix only by normal diffusion. As a result, it is possible to have multiple streams entering a tube each with distinct chemical properties [42].

The use of microfluidics may also reduce much of the possible transport limitations that are associated with static cultures. It is know that the oxygen and growth factor concentrations adjacent to cells may be considerably different than those of bulk fluid concentrations. The use of continuous streams allow for faster replenishment of the medium immediate to the cells or the chemicals/drugs tested against the cells, which will allow more control over the environment of the cells.

MATERIALS AND EXPERIMENTAL METHODS

Chemicals and Materials

Poly(dimethylsiloxane) (PDMS) elastomer composed of prepolymer and curing agent was purchased from Essex Chemical Sylgard 184 (Edison, NJ). Ethanol (100%) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Ethanol was diluted with PBS to 70% concentration for the experiment. Rhodamine B (R 6626) and Trypan Blue at 0.4% concentration were also purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Rhodamine B was used at 1mg/mL, diluted with PBS. All cells were obtained from American Tissue Type Collection (Manassas, VA). For cell culture, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), fibronectin (FN), trypsin, and other cell culture reagents were purchased from Gibco Invitrogen Corporation (Carlsbad, CA).

PDMS Mold for Surface Cell Patterning and Microfluidics

To create microfabricated channels, the silicon masters (Fig. 6) were made as described in the background section of this research. PDMS molds and microfluidics channels were fabricated by curing the prepolymer on silicon masters patterned with SU-8 photoresist. The masters used for microfluidics had protruding features with the impression of microfluidcs channels (ranging from 50 μm to 600 μm in width and ~80 μm in height). To cure the PDMS prepolymer, a mixture of 10:1 silicon elastomer and the curing agent was poured on the master and placed at 70°C for 2 hrs. The PDMS mold (Fig. 7) was then peeled from the silicon wafer and cut into narrow strips (~0.3 cm x 3

cm). These strips are capable of forming irreversible seal with other PDMS molds or glass slides after being treated with oxygen plasma.

Cell Seeding and Patterning on Glass Slides and under PDMS Microfluidics Channel

NIH-3T3 murine embryonic fibroblasts were maintained in DMEM supplemented with 10% FBS at 37°C and 5% CO_2 environment. In this research, two types of microfluidics gradient generator systems were made. They were a one-dimensional system and a two-dimensional system. Although the primary focus of this research is on the one-dimensional system, it was nevertheless important to examine the future potential of the device if an extra dimension can be added to the device. The one-dimensional microfluidics gradient generator system was made, tested, and analyzed with cells. For the one-dimensional system, the cell pattern and subsequent adhesion was limited on the substrate (glass slide) to a narrow strip. The microfluidics channel was placed on top of the narrow strip of exposed glass when the system was put together. In this research, a technique was developed to pattern and seed the cells on the narrow strip of exposed glass slide. First, it was necessary to tape the substrate (glass slide) with water-resistant tape and leaving only a thin strip of exposed surface where the cells will eventually adhere. The taped glass slide was then treated with oxygen plasma. The taped glass slide (Fig. 8) were washed with PBS three separate times before FN (96±3% and 95±3% relative to bare glass) were applied on the exposed surface. The taped substrate with FN sat for 15 min in a dish at room temperature as the chosen cell type (NIH-3T3) was trypsinized and spinned down at the same time. The spun down cells were then seeded onto the exposed surface coated with FN and maintained in 37°C and 5% CO_2

environment for one day, which allowed the cells to adhere to the exposed surface of the substrate.

Fabrication of Microfluidics Gradient Generator Systems Containing Cells

As mentioned before, two types of microfluidics gradient generator systems were made. A one-dimensional microfluidics gradient generator system (Fig. 9) was made and analyzed before using a different silicon wafer mask (Fig. 10) to fabricate a two-dimensional microfluidics gradient generator system (Fig. 11) for future study. However, only the one-dimensional system was tested successfully with cells.

Before fabricating a one-dimensional microfluidics gradient generator system containing cells (NIH-3T3), a PDMS mold with microfluidics channel designs must be made before taking out the cell-seeded glass slide from the incubation box. The PDMS mold must also be connected with polyethylene tubes (BD, Franklin Lakes, NJ) in both the inlets and outlets of the microfluidics designs. The surface of the PDMS mold that does not face the glass slide will then be sealed with epoxy glue around the places that the polyethylene tubes have penetrated in order to prevent leakage. As the glue seals, it is necessary to plasma clean the PDMS mold for 15-300 s (60 W, PDC-32G, Harrick Scientific, Ossining, NY). After the plasma treatment of the PDMS mold, the cell-seeded glass slide is taken out from the media. It is critical to remove the tape around the glass slide as quickly as possible without disturbing the adhered cell layer. With the tape removed from the slide, the PDMS microfluidics mold is aligned on the glass slide either manually or after the addition of a drop of anhydrous ethanol (to assist the alignment by delaying

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the irreversible binding) under the microscope. The microfluidics channel has to be aligned exactly on top of the layered cell.

For the fabrication of the two-dimensional system, it is very similar to the steps that were used to construct the one-dimensional device. However, a different silicon wafer mask is used (Fig. 10) and no cell was used in the two-dimensional system.

Generating Chemical Concentration Gradient within Microfluidics Channels

Each microfluidics channel has two inlets and one outlet. In order to generate a step-wise gradient of a chemical within the channel, it is necessary to drive the chemical into one inlet, while driving PBS into the other inlet simultaneously (Fig. 12). The fluids were put into B-D 1mL syringe (Becton Dickinson & Co). and were driven through the channels using SP200i syringe pump (World Precision Instruments, Sarasota, FL) that was connected to the device using polyethylene tubes that were attached to the inlets. The flow rate of both the chemical and PBS is maintained between 1-5 µL/min throughout the experiment. As the chemical and PBS flow through the serpentine design of the microfluidics channel, a step-wise gradient is generated. The experiments involving cells and microfluidics were performed on an Axiovert 200 microscope (Zeiss, Germany) with an environmental chamber designed to maintain the temperature at 37° and 5% CO_2 (Fig. 13). The resulting chemical gradient and cell patterns were directly examined under the microscope (Fig. 14).

Cell Apoptosis Analysis within Channels

The fabricated microfluidics gradient generator system has potential for many applications relating to various biomedical and pharmaceutical research. In this research, the applications that were demonstrated with the system are analysis of chemical/toxin dosage-response and cell apoptosis. In order to achieve the analytical purpose, the cells (NIH-3T3) were patterned within the system with the method described previously. Before the systems were built, additional experiments were conducted without cells to demonstrate that the system can indeed generate step-wise gradients.

In experiments that involved cells, the device that contained cells was first analyzed under Axiovert 200 microscope (Zeiss, Germany) with the environmental chamber. The system was then connected to the two 1mL syringes that were mounted on the SP200i syringe pump (World Precision Instruments, Sarasota, FL) through the system's polyethylene tubes. One of the syringes contained 100% PBS, while the other contained Rhodamine B (R 6626) diluted with 70% ethanol at concentration of 1 mg/ml. Rhodamine B served as a tracer dye while 70% ethanol killed the cells within approximately 5-7 minutes. With the micofludics serpentine channel design, a step-wise gradient of the Rodamine B was generated. The flow rate for both the chemical and PBS was maintained between 1-5 μL/min throughout the experiment.

To analyze cell apoptosis, the two original syringes were disconnected from the system and replaced with two syringes containing Trypan Blue at 0.4% concentration. Trypan Blue stained dead cells but not live cells. Trypan Blue was pushed through the system

for approximately 1-2 minutes. Then, the syringes were disconnected again and changed to two syringes that contained PBS, which served as quick wash within the system for 1 minute. In the end, one can examine under the microscope which cells were stained by Trypan Blue and correlated the results with the different chemical dosages that were generated within the microfluidics system.

RESULTS AND DISCUSSION

Before using the one-dimensional microfluidics system to conduct experiments with cells and analyze cells *in vitro*, a one-dimensional microfluidics gradient generator system without cells was built to demonstrate that the system can generate step-wise gradient consistently. Generation of the gradient is important to the dosage-response analyses that were conducted in this research.

The one-dimensional microfluidics gradient generator system was fabricated using the methods described previously. The PDMS mold was plasma treated in order to form an irreversible seal with the glass slide substrate (Fig. 15). When the system was placed under the microscope, its two inlets were connected to two 1mL syringes. One of the syringes contained a concentrated tracer blue dye, while the other contained PBS. The flow rates of both substances were maintained between 1-5 µL/min throughout the experiment. Both the tracer dye and the PBS were injected into the system simultaneously. Within 5 minutes, a step-wise gradient was generated in the system. The results demonstrated that it was feasible to generate concentration gradient of any chemical within the device in a short amount of time (Fig. 16). The generation of the gradient is important to dosage-response analysis because it allows the cells to interact with different concentrations of biochemicals at various doses. As a result, the step gradients generated by the system provides the precision and efficiency in study and analysis that are often lacked in other analytical tools at the same cost.

Once the feasibility of the device to generate concentration gradient was established, a one-dimensional microfluidics gradient generator system with cells was built (Fig. 17). Through the fabrication process, it was discovered that a cell suspension of 5×10^7 cells/mL was the optimal to form cellular monolayers or arrays on the exposed substrate in the growth media. Concentrations of $<1 \times 10^7$ cells/mL did not form confluent cell layers while concentrations $>1 \times 10^8$ cells/ml clogged the channels. Furthermore, once the cells adhered in the channels, the cells did not stain for Trypan Blue dyes, which indicate that they remained viable. These results indicate that the cells could be patterned within microfludics channeld with high confluency and with high precision. The cells have been maintained within the microfluidics channels for 24 hrs indicating that they can be maintained for durations that are relevant for bioanalytical and biosensing applications.

As the one-dimensional microfluidics system containing cells was built, it was placed under the microscope within the environmental chamber immediately in order to keep the cells viable. One of the system's inlets was then connected to a 1 mL syringe containing Rhodamine B/ethanol mixture while the other is connected to the other 1 mL syringe with PBS. With this experimental set-up, the patterned NIH-3T3 cells were in contact with a step-wise concentration gradient of Rhodamine B/ethanol mixture at the flow rate between 1-5 μL/min throughout the experiment. This served as a dosage-response analysis test of the chemical to the patterned cells (Fig. 18). The ability to perform cellular reactions within microfluidics channels has been proposed as a method of fabricating biosensors [5], improved systems to study cellular behavior [43, 44], and

microreactors for biochemical synthesis [6]. The application proposed here is important to mammalian cells because the device is potentially able to detect toxins, pathogens, or capable of performing chemical reactions with fast response times as engineered.

To analyze the chemical dosage that induced toxicity in the cells, the cells in the system were stained with Trypan Blue. Live cells have intact plasma membrane and do not stain by the dye. However, the cells that underwent apoptosis had compromised plasma membranes. The dead cells took up the dye and appeared blue. The dead cells were stained as the Trypan Blue was injected into the system through the two syringes (Fig. 19). Within approximately 1-2 minutes, the system was washed with PBS and analyzed. The cells that encountered the two highest concentration of Rhodamine B/ethanol mixture within the gradient that was generated were stained by Trypan Blue dye (Fig. 20), which yielded the same results with the experiments that were conducted outside of the microfluidics system. The result indicated that the designed system in this research could be used as a technique that can develop and fabricate stable microfluidics channels with precise control over not only the spatial patterning of the cells but the control of the dosage concentration of the chemical that the cells are in contact with.

The results of the one-dimensional system led to the fabrication of the two-dimensional system for future study. It is important to research a way in which the device can be multiplied not only in the amount of cells that it can analyze at a time but also in the dimensions that the cells can be analyzed. By adding extra dimensions into the system, the system provides a way to simulate an actual biochemical environment, where cells

interact with chemicals and growth factors from various directions. For example, if the device is used to help study stem cell differentiation, it is not only important to study the effects of various concentrations of growth factors in combination on the stem cells, but it is also critical that the stem cells are tested in a multi-dimensional environment that simulate the actual environment where the stem cells were harvested. Thus, a two-dimensional microfluidics concentration gradient generator system without cells was made (Fig. 21) in order to test the feasibility of generating multiple chemical concentration gradients in multiple dimensions within the device.

The two-dimensional system had four inlets. Each dimension of the system possessed two of the four inlets. Through the two sets of inlets, two different, independent chemical concentration gradients were generated within the system, which demonstrated that it is possible to have multiple chemical reactions in different dimensions occur within the system simultaneously. In the experiment, a concentration gradient of Trypan Blue and a concentration gradient of Rhodamine B were generated perpendicularly to each other without random mixture between the channels (Fig. 22). As mentioned previously, adding extra dimensions into the system provides a way to simulate an actual biochemical environment, where cells interact with chemicals and growth factors from different directions. Although the current system can only generate independent concentration gradients in two dimensions, it is certainly feasible to add extra dimensions to the current system in order to simulate the actual cellular environment within the system and to provide a better analytical tool to advance studies, such as stem cell differentiation.

To examine the potential of the device to analyze how cell response/behavior is affected *in vitro* by two or more biochemical reagents at various concentrations and dimensions as the reagents interact molecularly and act on the cell, it is important to fabricate a two-dimensional microfluidics concentration gradient generator system with cells in the future. Through the fabrication process, it was discovered that the cells have a more difficult time adhering to PDMS mold than to the exposed glass substrate. The fabricated two-dimensional systems were often less stable than its one-dimensional counterparts. In future studies, it is necessary to devise a technique to pattern and seed cells on PDMS molds with efficiency and consistency. Furthermore, it is also critical to analyze the original design of the silicon mask that served as the basis of the fabrication of the two-dimensional microfluidics system. In order to place cells in the two-dimensional system, it could be possible that a new design needs to be made on the silicon mask rather than devising a technique to seed and pattern cells on PDMS molds.

In conclusion, the research presented a technique and a device that are applicable to many soft lithographic methods and can be used to create robust and sophisticated microchannels with precise control over the spatial properties of the substrate. Thorough this approach, it is possible to pattern and seed mammalian cells under channeled poly(dimethylsiloxane) (PDMS) molds. Cells that are patterned under the channels remained viable and capable of performing intracellular reactions. Through the gradient generator within the PDMS microfluidic device and laminar flow, multiple specific and

controlled spatial depositions of chemicals onto individual patterns of cells can be achieved and analyzed for various studies.

As demonstrated in this research, the fabricated microfluidic device system could be used to perform tests on cell response/behavior to various combinations of factors. The system present the possibilities to study the effects of various concentrations of growth factors in combination on stem cell differentiation. The device could aid in the performing factorial experiments that could quantitatively analyze the effect of cytokine interactions on stem cell response. Thus, this type of analysis will be helpful in defining differentiation factors, concentration threshold effects on self-renewal and differentiation etc.

Besides stem cell research, the PDMS microfluidic device may potentially be pertinent in studying environmental chemicals/mutagens that are detrimental to the human health or in shaping and designing new combinational/cocktail medical treatments or innovative drug delivery approaches for diseases, such as AIDS and cancer. For example, the device can potentially be leveraged as a cost-effective mean to shorten many lengthy clinical trials conducted by various cutting-edge drug developers and pharmaceutical companies. The device may also apply to studying soft tissue/cartilage regeneration, abnormal bone growth, and tissue engineering application in general as complex microfluidics devices have been previously designed for the use of creating vascular networks *in vitro* [48, 49]. In addition, the cells patterned within the system can be used to generate cell-based biosensors and bioreactors that are capable of enzymatic reactions. Thus, the device

presented here has the potential to revolutionize the way that much scientific and medical

research will be conducted in the future.
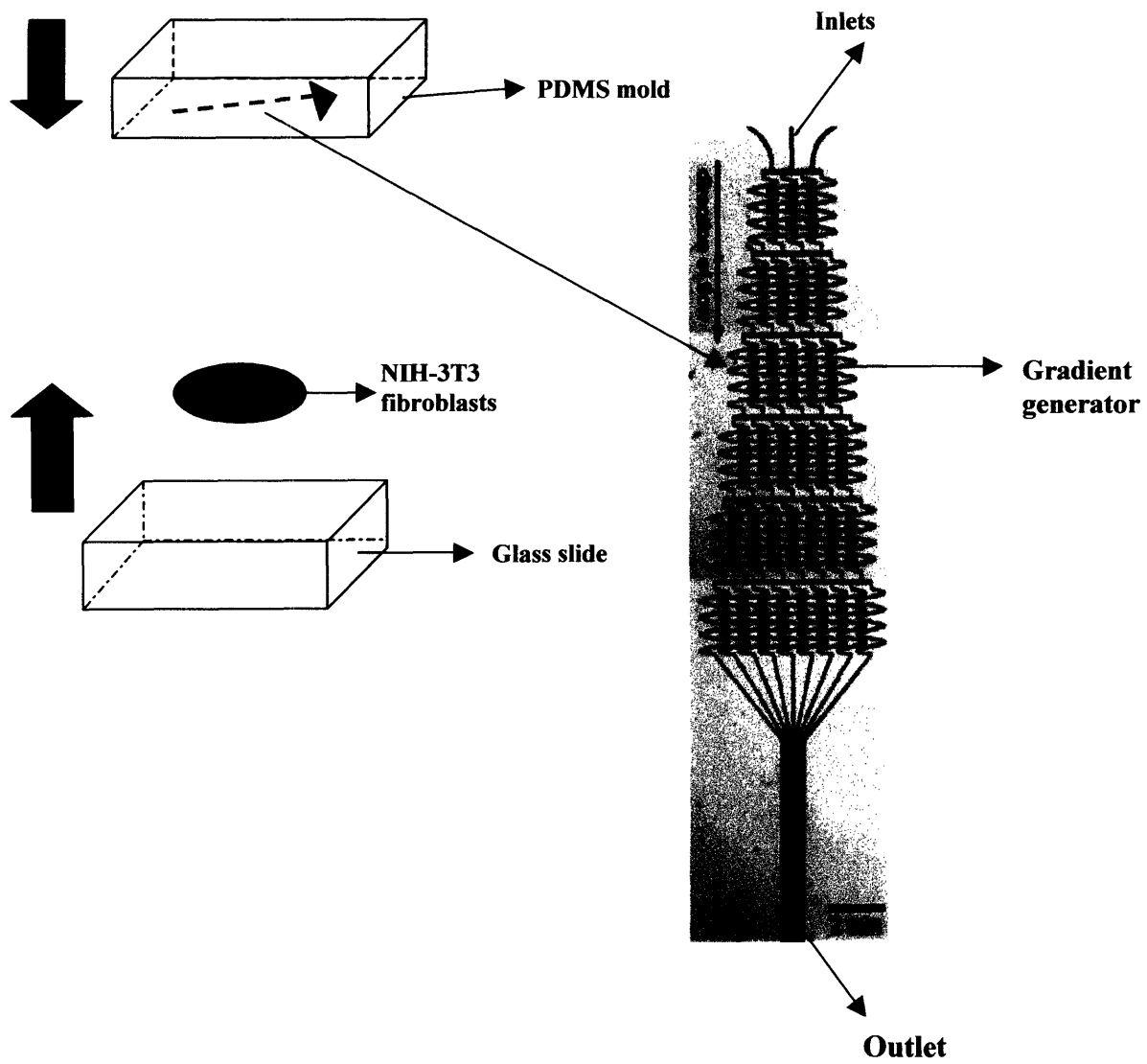
Scheme 1. Schematic drawings of the microfluidics gradient generator.

Guan-Jong Chen – Scheme 1
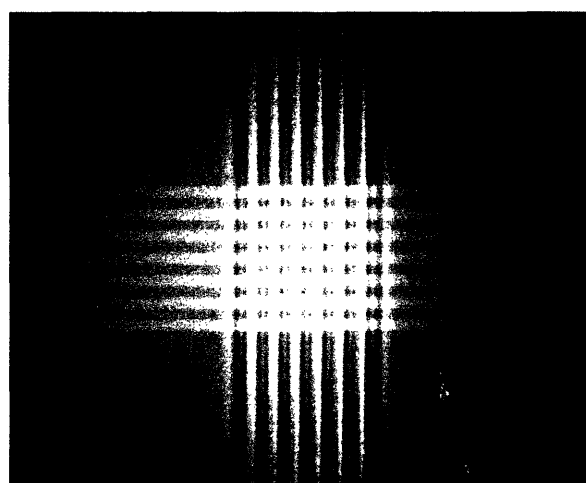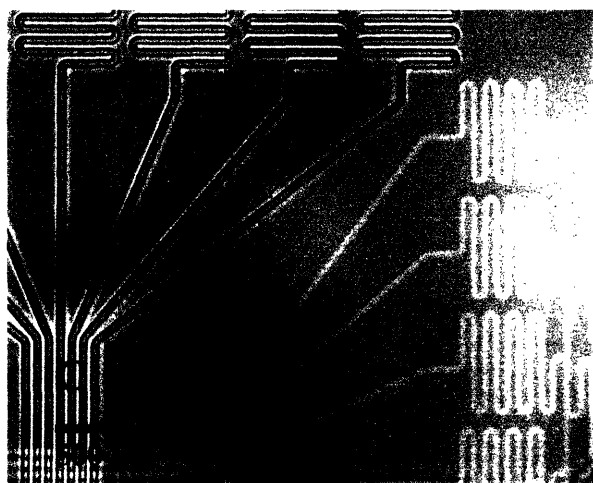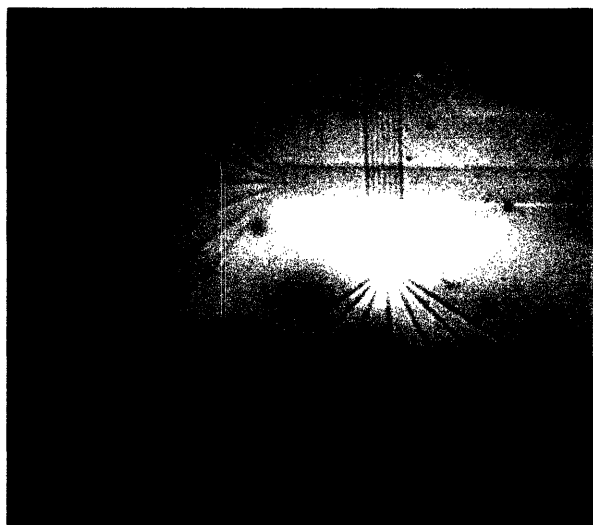
Figure 1. Micrographs of microfluidic channels (A) The device is consisted channels that are separated and irreversibly sealed through oxygen plasma treatment. The dimension of the serpentine meanders is 80 μm high and 50 μm in width. (B) - (D) The two channels are aligned perpendicularly to each other.

Guan-Jong Chen – Fig. 1

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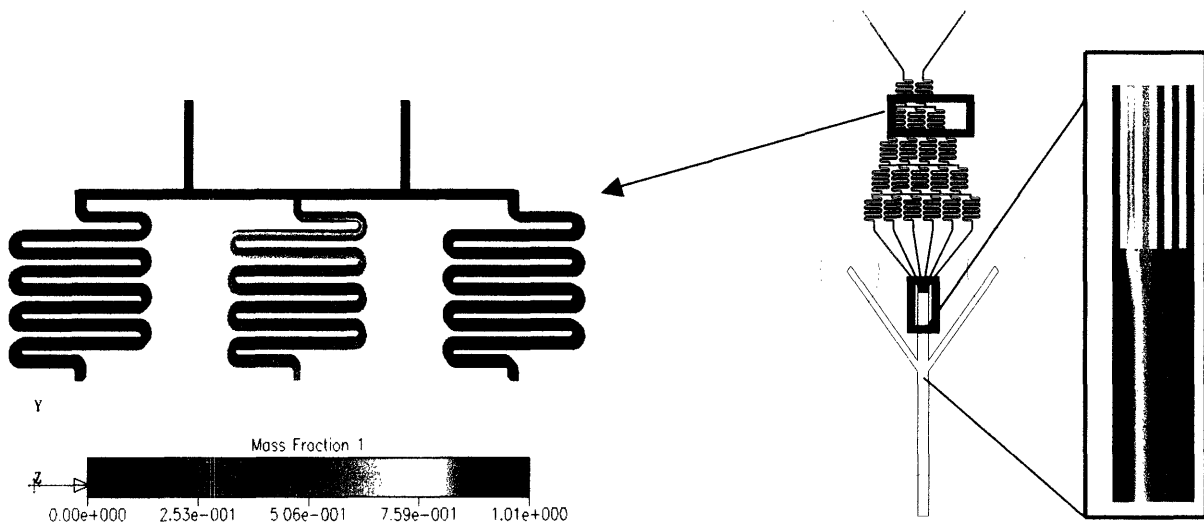
Figure 2. A microfluidic system generates stable concentration gradients of soluble molecules. Linear gradients in concentration spanning 550 μm is created through the serpentine networks.
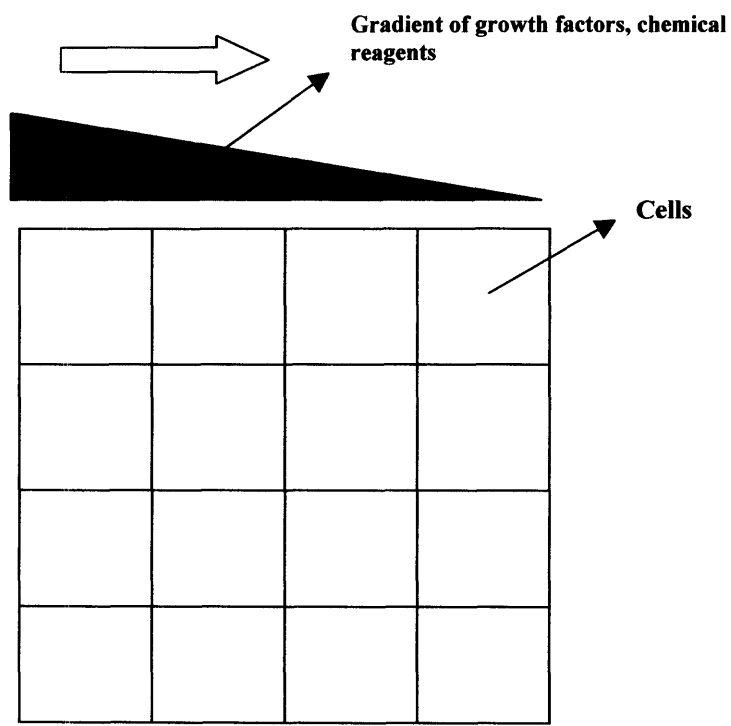
Figure 3. Schematic drawing illustrating the basis of how a complex gradient generator is able to generate multiple biochemical conditions simultaneously and affect multiple, independent cells.

Guan-Jong Chen – Figure 2 & 3

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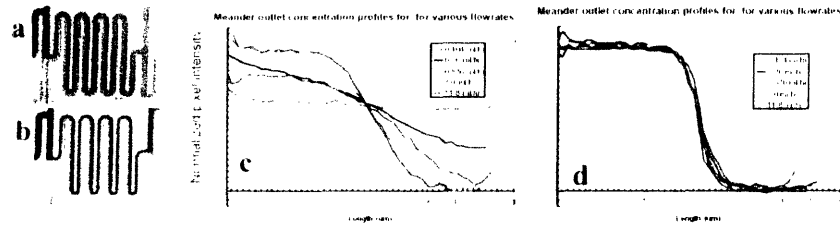
Figure 4. The effects of the flow rates on the mixing properties of tracer dye within each channel were tested. a) – b) are micrographs of meanders in the gradient generator under flow rate of (a) 0.104, (b) 11.8 mL/hr respectively. As it can be see at flow rates of 0.104 mL/h, or lower, the solution leaving each meander is fully mixed. c) – d) quantitative show the inlet and outlet concentration profiles.

Measured main channel concentration profiles for
various flowrates at the inlet

Measured main channel concentration profiles for various
flowrates at 2831 um downstream from the inlet
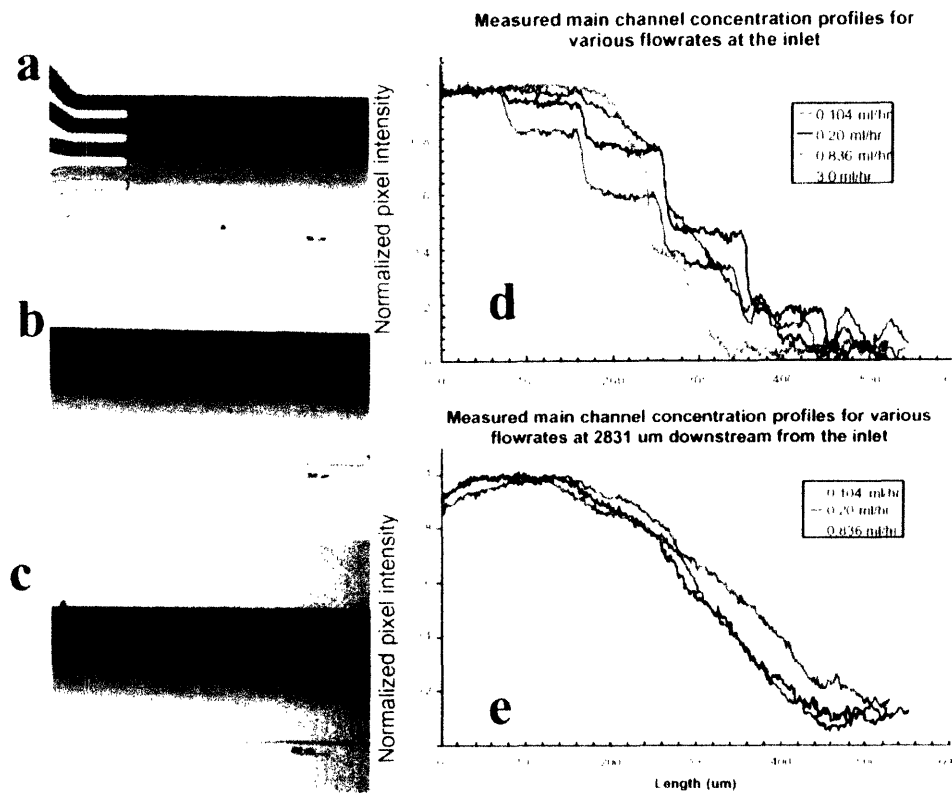
Figure 5. Effects of flow rate on the gradients in the main channel. Linear and complex concentration gradients were formed within the main channel. The shape of the gradients was dependent on the position downstream from the inlet as well as the flow rate. a) – c) are micrographs of the channels at the inlet (a) and at 2.88 mm downstream from the inlet for flow rates of 11.8 mL/hr (b) and 0,104 mL/hr (c). d) – e) represent the graph of concentration vs. position within the main channel at distinct points downstream from the inlet.

Guan-Jong Chen – Fig. 4 & 5
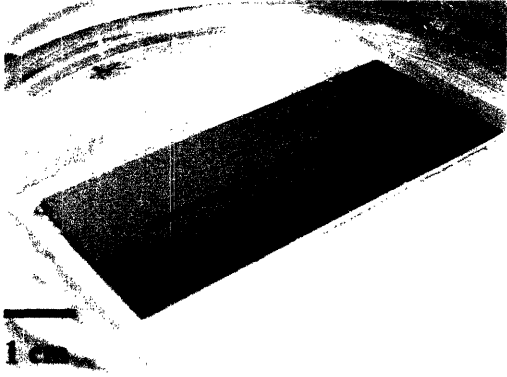
Fig. 6. A photolithography mask with the features of the microfluidics channel is created using AutoCAD. The silicon mask is used to generate a pattern of 80 μm high SU-8 photoresist on silicon wafers using contact photolithography to generate a negative master.
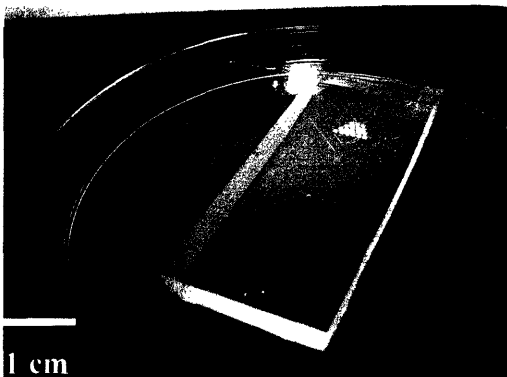

Fig. 7. Positive replicas are fabricated by molding PDMS against the master. The PDMS can form an irreversible seal with either glass or another PDMS mold if treated with oxygen plasma.
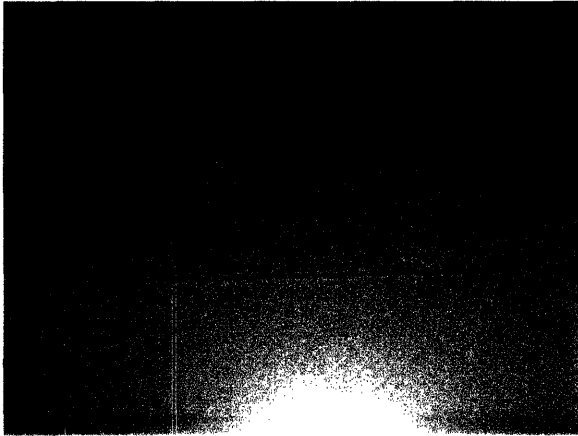
Fig. 8. The taped substrate (glass slide or PDMS mold) leaves only a narrow strip of exposed surface. The exposed surface has two purposes. It serves as the adhesion and pattern sites for the chose cell type. Also, it will potentially be the area in which other microfludics channels will form an irreversible seal to with plasma treatment.

Guan-Jong Chen – Fig. 8

32
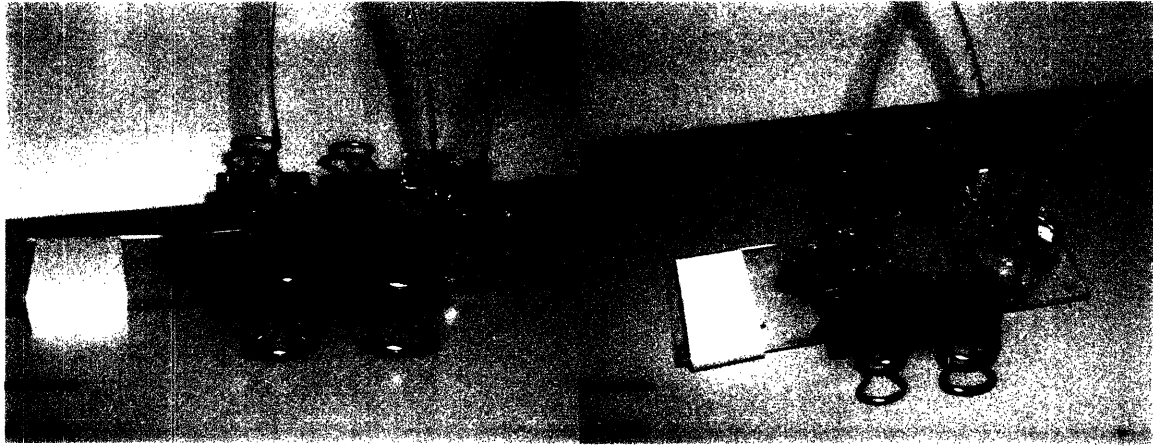
Fig. 9. Prototype of one-dimensional microfluidics gradient generator system without live cells in the device.
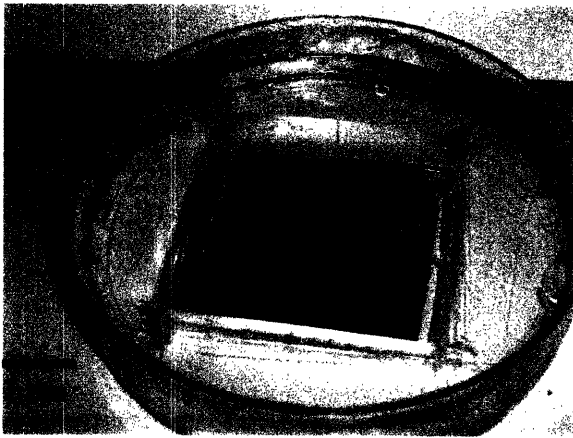
Fig. 10. Silicon photolithography mask with the features of the microfluidics channel is created using AutoCAD. This particular design is used in fabrication of the two-dimensional microfluidics gradient generator system.

Fig. 11. Prototype of two-dimension microfluidics gradient generator system without live cells in the device. The microfluidics channels are facing each other perpendicularly within the system.
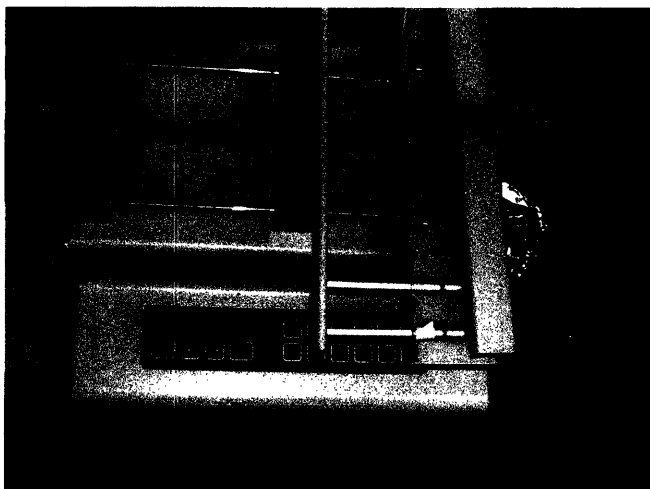
Fig. 12. In order to generate a step-wise gradient within the microfluidics system, the fluids were driven through the channels using SP200i syringe pump (World Precision Instruments, Sarasota, FL) that was connected to the device using polyethylene tubes that were attached to the inlets of the system. The flow rate of both the chemical and PBS is maintained between 1-5 μL/min throughout the experiment.
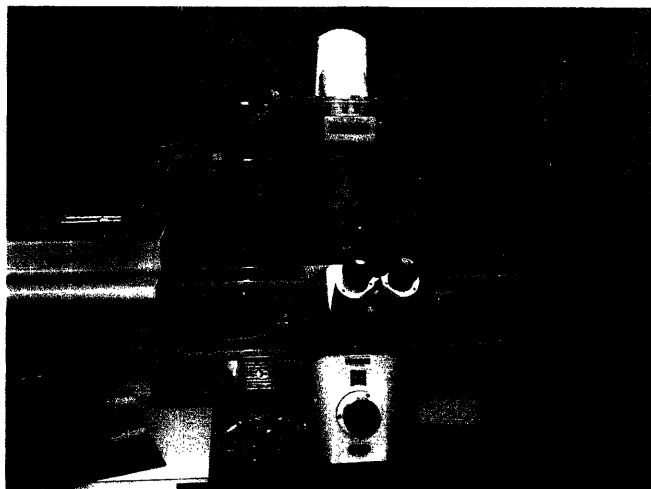

Fig. 13. With experiments involving cells, the cells are kept viable with an Axiovert 200 microscope (Zeiss, Germany) that has an environmental chamber designed to maintain the temperature at 37° and 5% CO_2.

Guan-Jong Chen – Fig. 12 & 13
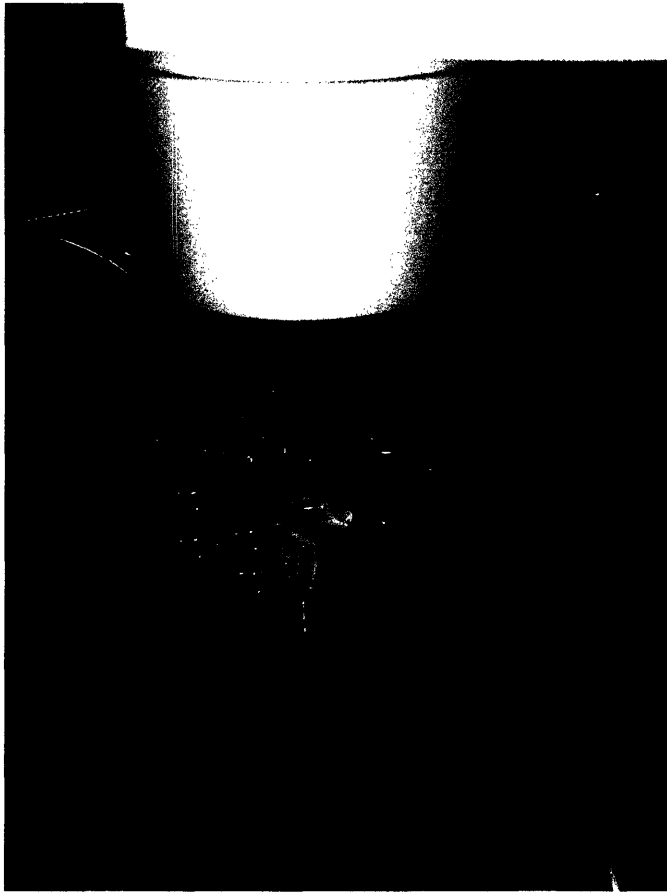
Fig. 14. The microfluidics gradient generator system is placed under the microscope within the environmental chamber in order to keep the cells viable while desired chemical gradient is generated within the system.
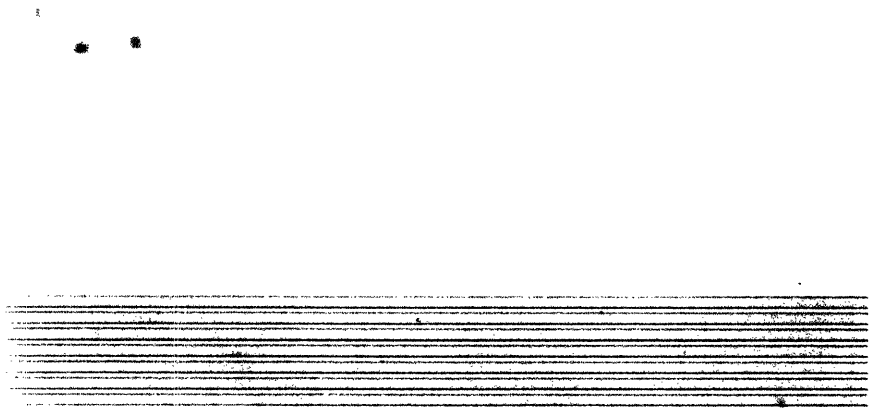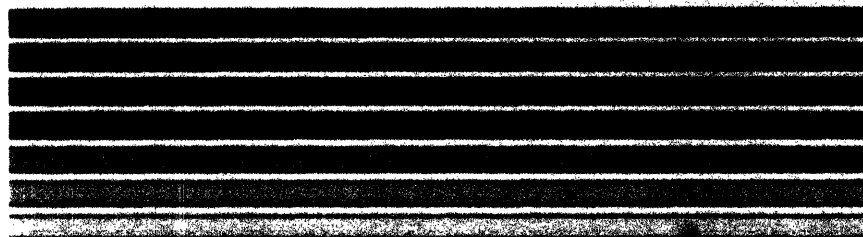
500 μm
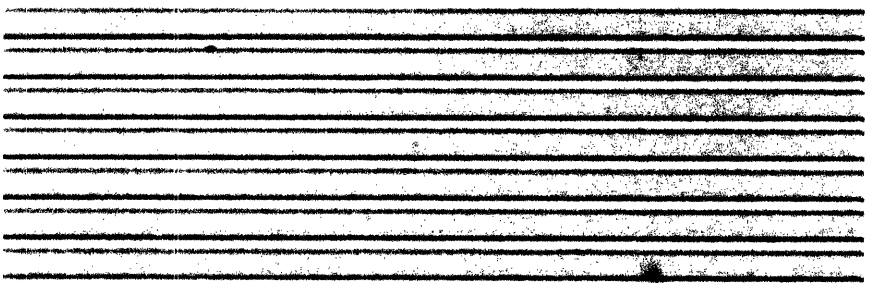
200 μm

Fig. 15. Microfluidcs channels of the system without cells under the Axiovert 200 microscope (Zeiss, Germany).
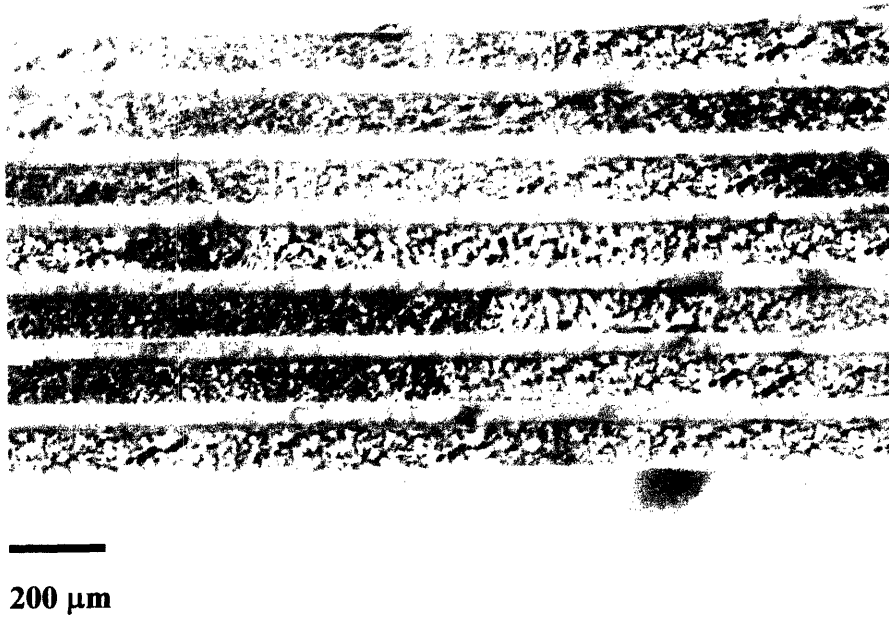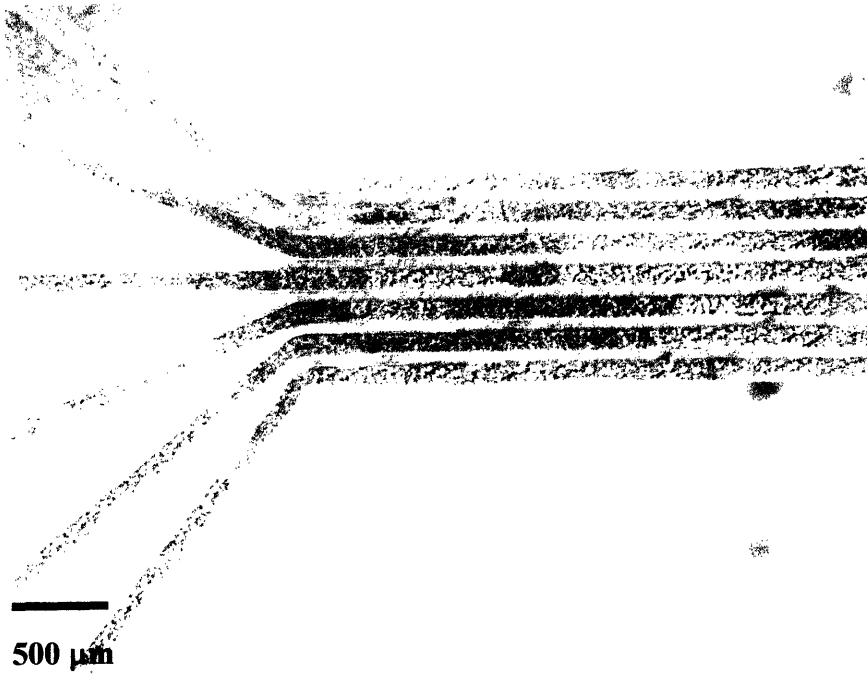
500 μm

200 μm

Fig. 16. A perfect step-wise gradient of the tracer dye is formed within the system as one inlet is injected with the tracer dye and the other with PBS simultaneously. The process takes within 5 minutes from the time that the chemicals are injected within the system.
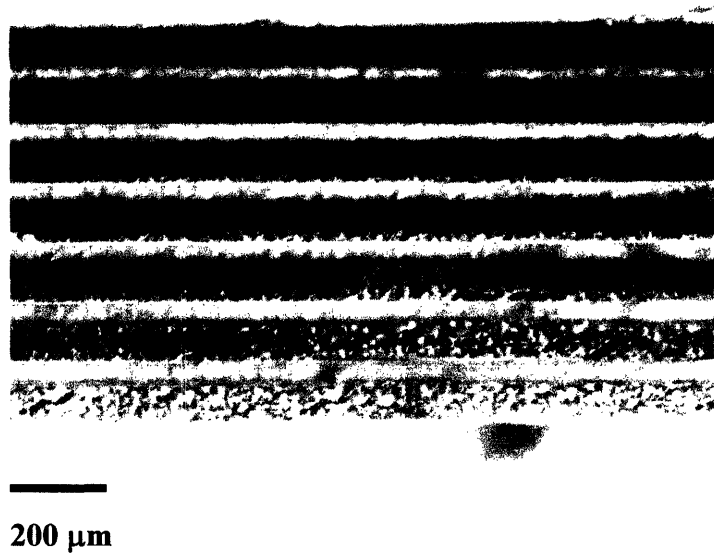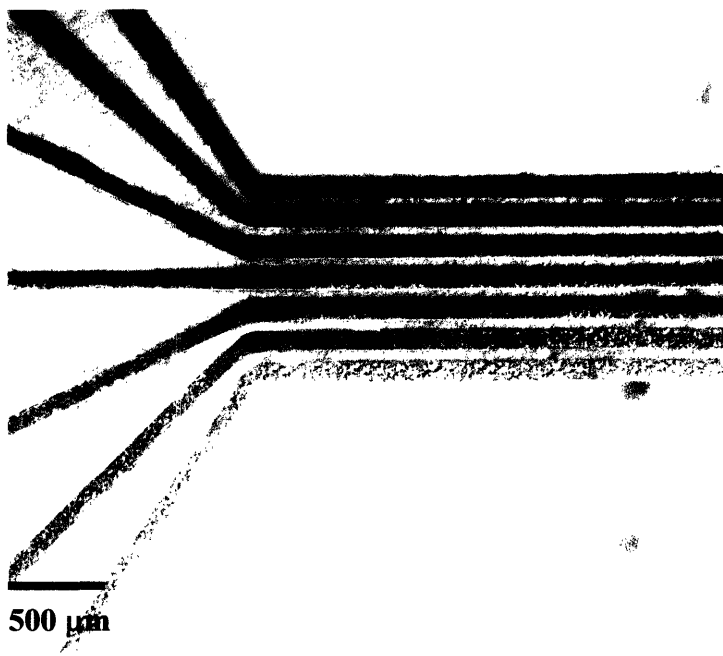
Guan-Jong Chen – Fig. 16

38

500 μm

200 μm

Fig. 17. NIH-3T3 cells are patterned on the exposed glass substrate and grown over night in media and incubation. Then, it is incorporated into the system as the PDMS mold is plasma treated and forms an irreversible seal with the glass slide as the tape is removed.
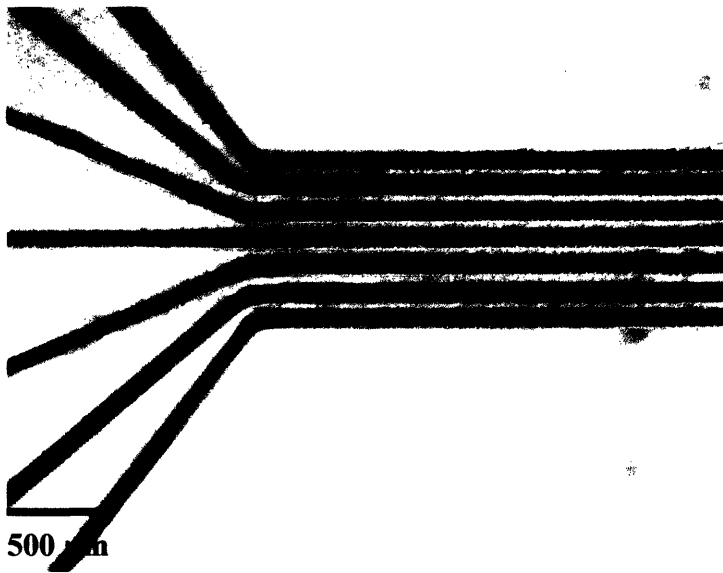
Guan-Jong Chen – Fig. 17

39

500 μm

200 μm

Fig. 18. A step-wise concentration gradient of Rhodamine B/Ethanol mixture at the flow rate between 1-5 μL/min is flowing over the patterned NIH-3T3 cells in the microfluidics system, which served as a dosage-response analysis of the chemical to the patterned cells.
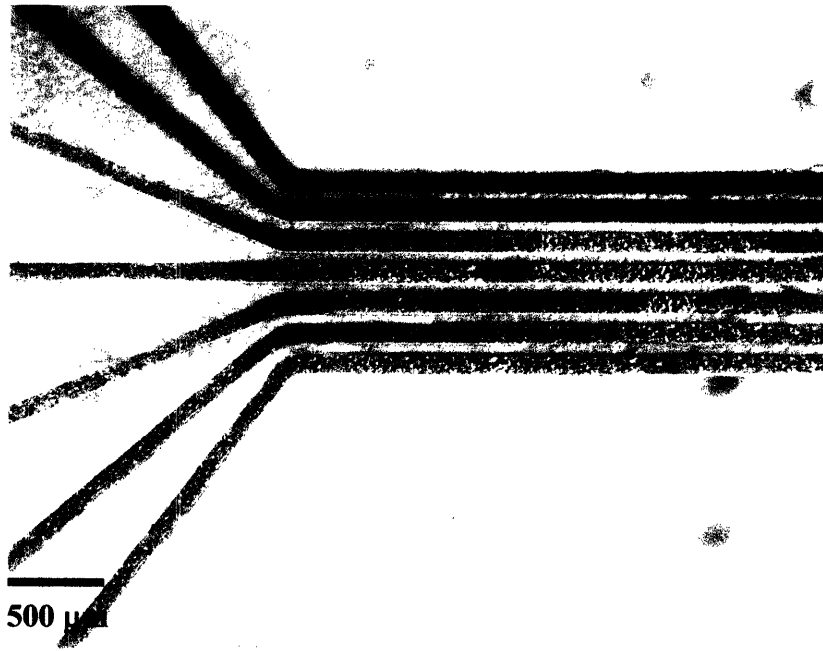
Guan-Jong Chen – Fig. 18

40

500 μm

200 μm

Fig. 19. To analyze at which dosage of the chemical the cells were killed and went through apoptosis, it is necessary to stain the cells in the system with Trypan Blue. The dye runs on top of the cells at the flow rate between 1-5 μL/min for approximately 1-2 minutes.

Guan-Jong Chen - Fig. 19

41

500 μm

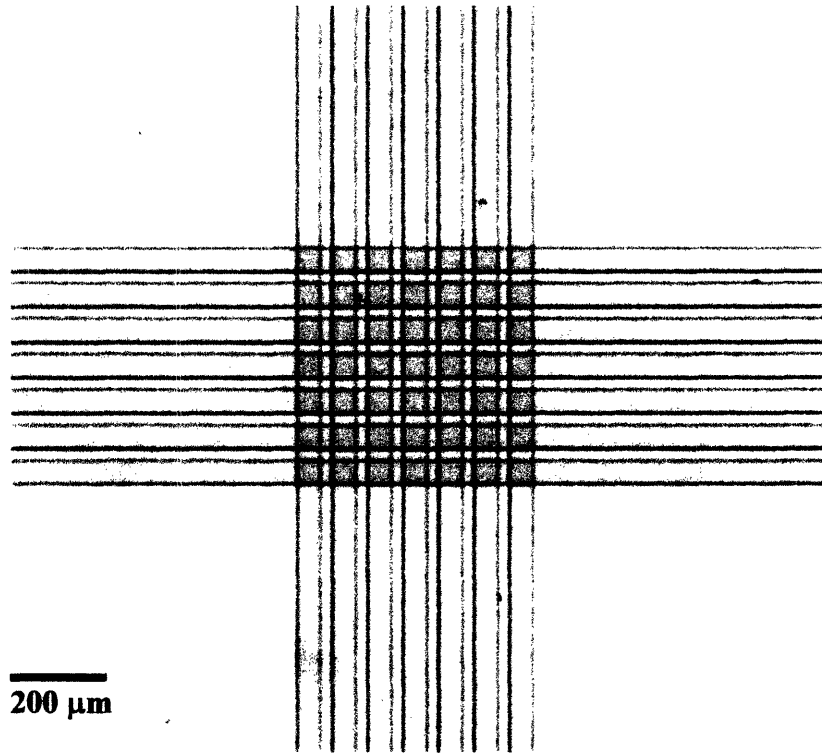**200 μm**

Fig. 20. The cells that encountered the two highest concentration of Rhodamine B/ethanol mixture within the gradient that was generated were stained by Trypan Blue dye. Cells that underwent apoptosis have compromised membrane, and were able to take in the dye.

Guan-Jong Chen – Fig. 20

42

200 μm

Fig. 21. Two-dimensional microfluidics concentration gradient generator system without cells was made and analyzed before a system containing cells was made. The two PDMS slabs with the microfluidics channel design formed irreversible seal after plasma treatment.

200 μm
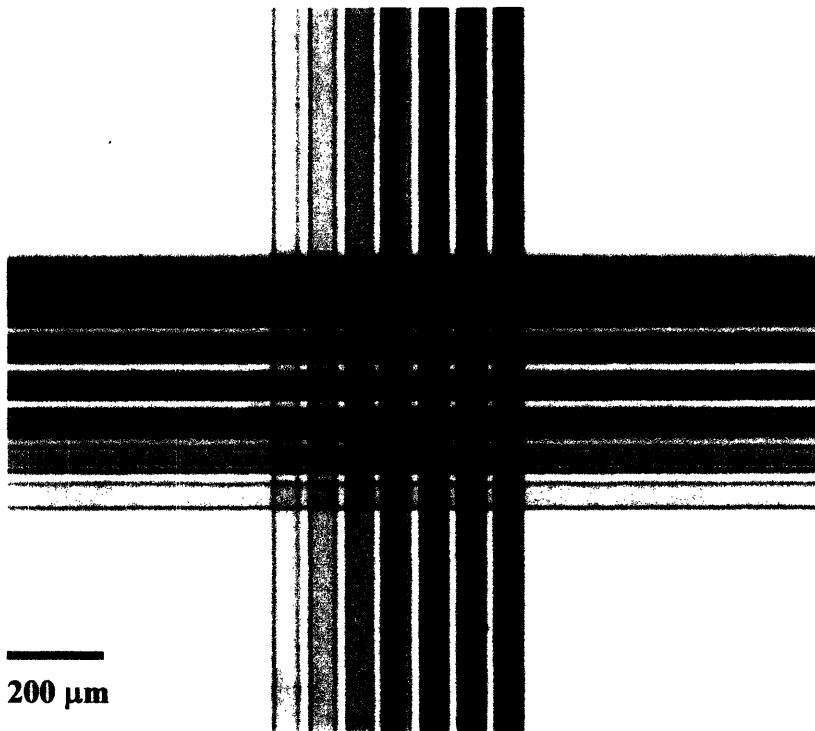
Fig. 22. The two-dimensional microfluidics system without cells was tested to demonstrate it is capable of generating multiple concentration gradients. A concentration gradient of Trypan Blue and Rhodamine B were generated perpendicularly to each other without random mixture between the channels.

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