A microfluidic platform for studying the effects of small temperature gradients in an incubator environment

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Studies on the effects of variations in temperature and mild temperature gradients on cells, gels, and scaffolds are important from the viewpoint of biological function. Small differences in temperature are known to elicit significant variations in cell behavior and individual protein reactivity. For the study of thermal effects and gradients *in vitro*, it is important to develop microfluidic platforms which are capable of controlling temperature gradients in an environment which mimics the range of physiological conditions. In the present paper, such a microfluidic thermal gradient system (μTGS) system is proposed which can create and maintain a thermal gradient throughout a cell-seeded gel matrix using the hot and cold water supply integrated in the system in the form of a countercurrent heat exchanger. It is found that a uniform temperature gradient can be created and maintained in the device even inside a high temperature and high humidity environment of an incubator. With the help of a hot and cold circuit controlled from outside the incubator the temperature gradient can be regulated. A numerical simulation of the device demonstrates the thermal feature of the chip. Cell viability and activity under a thermal gradient are examined by placing human breast cancer cells in the device. © *2008 American Institute of Physics.* [DOI: 10.1063/1.2988313]

I. INTRODUCTION

Microfluidics has grown in recent years to encompass microanalysis, sensing, biological assays, genomics tools, and microelectromechanical systems (MEMS) technology.[1] In particular, the domain of biological sciences has quickly adopted microfluidic techniques in processes such as protein crystallization,[2–4] manipulation and examination of single as well as multiple cell systems,[5–7] cell sorting,[8] DNA amplification with a polymerase chain reaction (PCR),[9,10] and many others. Specifically the assays and experiments involving cells and extracellular matrix (ECM) are important from the viewpoint of understanding biological processes *in vitro*, which mimic *in vivo* geometric dimensions such as cell layers and capillaries to a greater degree than the more traditional *in vitro* techniques.

Biological assays and chemical lab-on-chip studies in areas like genomics,[11] proteomics,[12] catalyst optimization,[13,14] and cell migration[15] are largely carried out with chemical activities as the major manipulable factors. This is because the chemical activities are known to be the key in major cellular and extracellular biological activities such as protein synthesis, nucleic acid tran-

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scription, and signal transduction, for example. Biophysical parameters such as fluid shear stress,[16] substrate or matrix stiffness,[17,18] and electrophoresis[19,20] have also been studied to a considerable extent in biological assays and experiments in microdevices.

By comparison, there have been far fewer studies investigating the effects of temperature on cell or protein function. This might be attributable to the fact that humans, and most homoiothermic animals, are known to regulate their temperature within rather narrow limits. However, it is also recognized that one reason for this close regulation is that protein function is exquisitely sensitive to even small deviations in temperature from 37 °C. In general, thermal effects play a significant role in biology, are common occurrences in disease, and are used in a variety of therapeutic procedures. Yet, relatively few studies have been conducted to examine the effects on cell function of small differences in temperature or thermal gradients. Much remains to be learned about these potentially critical phenomena and their use in regulating biological function.

Perhaps the best known strongly temperature-dependent process is the PCR for DNA amplification. Microfluidic system design for temperature cycling[21] as well as flow type PCR (Refs. 22–25) is a major area of microfluidic study. Many of the thermal applications of these microfluidic devices arise from their mass transfer analog. For example, establishment field gradients like *p*H gradient for isoelectric focusing[25] has given rise to a relatively new area of temperature gradient focusing[26] (TGF) which exploits the fact that electrophoretic mobility is a function of temperature. It is also important to note that many chemical/thermal phenomena are gradient dependent rather than being dependent on the absolute value of species concentration/temperature. Hence a need exists to design devices which can create a specified gradient in the field variable (concentration/electric field/temperature).

The above temperature-dependent applications like the PCR and TGF require steep temperature gradients. For example, the PCR typically cycles between 95 °C, 55 °C, and 72 °C, and TGF utilizes temperature gradients between 95 °C and 55 °C, both working under normal room conditions of temperature and humidity. The most convenient method of attaining this type of temperature gradient is Joule heating.[27,28] An alternative approach is to use a microfluidic device with parallel flow channels to create temperature gradients, as in the study by Mao *et al.*[29] which examined the phase transitions in a phospholipid membrane. However, the assumed one-dimensional temperature gradient in the device is questionable because the temperature gradient of a parallel flow heat exchanger continuously decreases in the flow direction.

Smaller temperature gradients may also have physiological or pathophysiological significance. It is well known that temperature gradients exist within the body, which can be essential in specific cellular or organ functions. For example, mammalian sperm cells use the small gradient of the order of 2 °C for navigation in the female genital tract.[30] By analogy to cell migration under chemical gradient known as chemotaxis,[31–33] such migration under thermal gradient has been termed thermotaxis. Chemotaxis has been studied in microfluidic devices for neutrophils[34] and numerous other cell types. Thermotaxis can also be observed in complete organisms that migrate under a temperature gradient for food or survival. The model organism Caenorhabditis elegans shows thermotaxis under smaller temperature gradients[35–37] (<10 °C). Embryonic development is also found to be influenced by temperature gradients over space and time using microfluidic devices[38] where the development of a Drosophila melanogaster embryo was studied under the co-flow of a hot and cold stream. The temperature gradient used here was also mild. The effect of temperature on cell function under normal and therapeutic conditions is well known. For example, Dayank *et al.*[39] reviewed the cytotoxicity of natural killer (NK) cells against tumor cell targets during hyperthermia. They showed that cytotoxicity becomes important at temperatures above 39.5 °C. They found that the process also involves MHC Class I and heat shock protein HSP70. HSP70 is also found to interfere in the cell apoptosis under thermal stress.[40]

Thermal gradients can also arise due to regional differences in the rate of energy utilization—temperatures rise in exercising muscle or due to a local inflammatory response. Thermal gradients also exist at the body surface for cooling, and are regulated by variations in superficial blood flow. In tumors, temperatures tend to be elevated relative to surrounding, noncancerous tissues. Finally,

therapeutic processes such as hyperthermia and hypothermia are commonly used to treat a variety of diseases. In each of these cases, thermal gradients exist, but we know relatively little about their effect on cell or tissue function.

Thus, while it is clear that thermal gradients and small variations in temperature can exert an important influence on cells and proteins, these effects have rarely been studied relative to the extensive literature on chemical gradients. This is due, in part, to the greater prevalence and importance of chemical gradients, but it also likely stems from the relative scarcity of *in vitro* microscale systems capable of producing and maintaining known thermal gradients in the range of *in vivo* conditions in health and disease. Our work was motivated by a need for microfluidic systems capable of creating small temperature gradients typical of those that naturally occur in tissues and organisms in a standard controlled environment like that inside an incubator. Here we introduce a new microfluidic thermal gradient system (μTGS) that provides for close control over temperature gradients within a region containing three-dimensional (3D) matrix material with or without seeded cells. This system provides a means of growing cells *in vitro* under tight thermal control and to visualize their behavior. Applications of this μTGS range from comparative studies of small temperature variations associated with localized inflammation, to heat exchange from the body surface, to gradients generated by localized heating.

II. SYSTEM DEVELOPMENT

To create the μTGS with an objective of using it inside an incubator, first the platform is chosen and a conceptual design is created. Based on simple calculations a nominal size of the μTGS was determined. This was numerically simulated using commercial software and finally, fabricated with the chosen material. These steps are described in more detail below.

A. Conceptualization

One important requirement of the μTGS was to avoid the need for Joule heating, which requires a metallic heating element and a heat sink, and use a counterflow heat exchanger instead. Unlike a previous design[29] this will ensure a more uniform temperature gradient along the length of the device. For initial design the μTGS dimensions were chosen to be a cylinder 30 mm in diameter and 10 mm in height. Two parallel U-shaped tubes were proposed as shown in Fig. 1 for carrying heating and cooling water in order to create the temperature gradient. To keep the prototype simple a flat rectangular channel with cylindrical ends for sample injection was chosen [Fig. 1(g)] with dimensions $8\times2\times0.2$ mm^3; the tubing for water flow was 2 mm inside diameter. Two considerations are important in choosing the tubing dimension—the heat transfer coefficient from the tube to the μTGS (which is $\sim$1000 W/m^2 K in the present case) and the total system pressure drop to be supplied by the pumps.

B. Fabrication of the devices

The device shown in Fig. 1(c) was fabricated by soft lithography.[41] This technique uses a master pattern of the chip created on a silicon wafer using the standard photolithographic technique used for fabrication of microelectronic devices. After sylanization of the patterned wafer, polydimethylsiloxane [(PDMS), from Dow Corning Corporation] is used to mold the device on the silicon wafer pattern in two layers. First a thin layer (about 3 mm thick) PDMS was poured in the mold and this first layer of the device was cured in the oven at 80 °C for 2 h [shown in Fig. 1(b)]. Subsequently, a perforated bridge, containing the two U-tube segments hanging from it, was placed over the thin device in such a way that the tubes touch the thin device at the proper place (parallel and equidistant from the channel on both the sides) as shown in Fig. 1(b). Then a second layer of PDMS was poured such that the tubes are submerged in it. The whole assembly was carefully placed in the oven and cured. The two-layer fabrication technique ensures that the channel formation is undisturbed by the heat exchanger fabrication. After curing the devices [Fig. 1(c)] were cut out from the wafer. 2 mm holes were punched on both sides of the channel to allow injection of cells and media for experiment [Fig. 1(d)]. The device was subsequently autoclaved

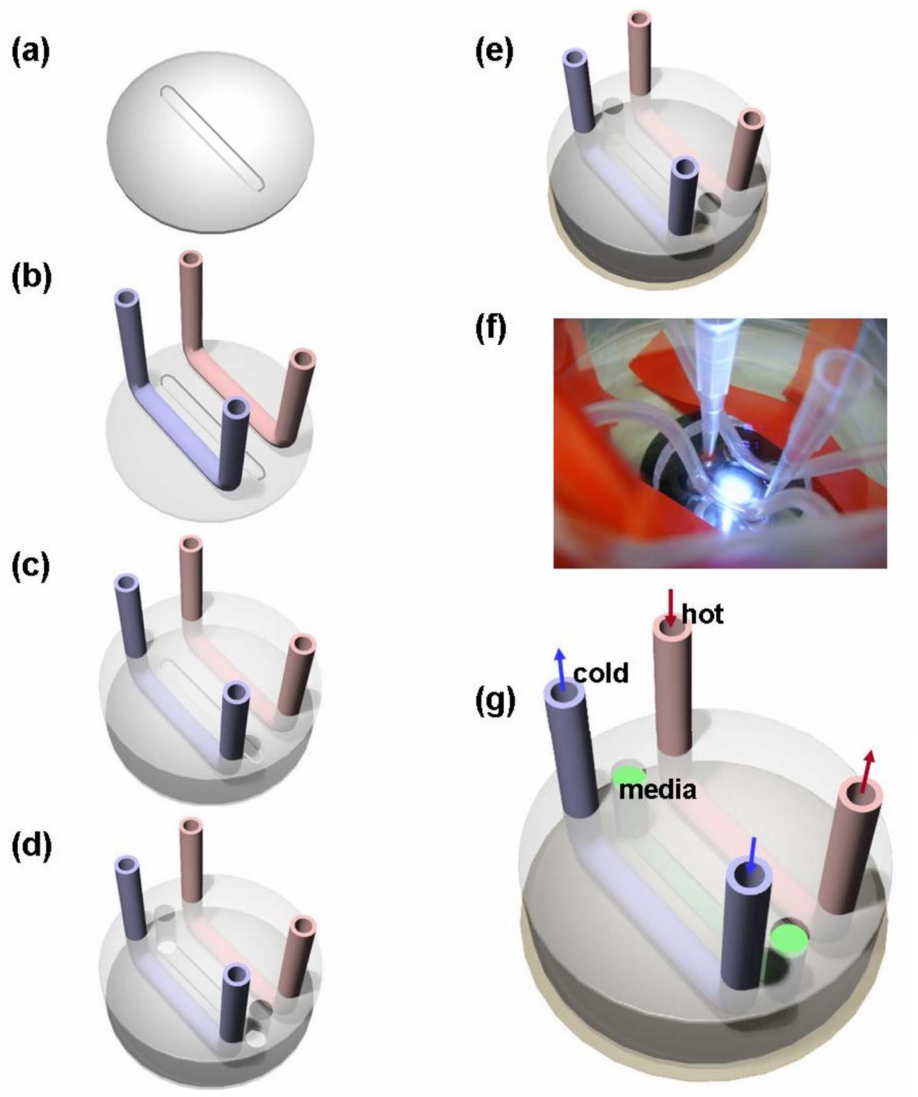


FIG. 1. The proposed microfluidic chip. (a) Molded first layer with microfluidic channel. (b) Two tubes are located on the first layer and (c) the second layer is molded with tubes. (d) After curing, inlets/outlets are punched and (e) the coverglass is bonded by oxygen plasma. (f) Photograph of the chip while running an experiment. (g) After cell seeding and culture, cold and hot water are supplied to generate the thermal gradient.

and dried, and the PDMS surface was rendered hydrophilic by treating with oxygen plasma (Plasma cleaner, Harrick Scientific, NY). Glass coverslips of 170 μm thickness were plasma bonded to them to form the closed channel. On the glass coverslip two thermocouples (0.25 mm diameter, Teflon-coated Chromel Alumel, Omega Engineering, Inc., Stamford, CT) were bonded with adhesive midway between the hot and cold flow channels. The accuracy of the thermocouples as quoted by the supplier is 0.3 °C.

C. Numerical simulation

In order to assess the temperature gradient that can be achieved with the proposed chip inside the incubator a three-dimensional simulation was carried out for the geometry shown in Fig. 2 using the finite element method (FEM). Commercial multiphysics enabled software COMSOL 3.3 was used for this purpose. The entire chip was divided into four different zones. In the PDMS chip

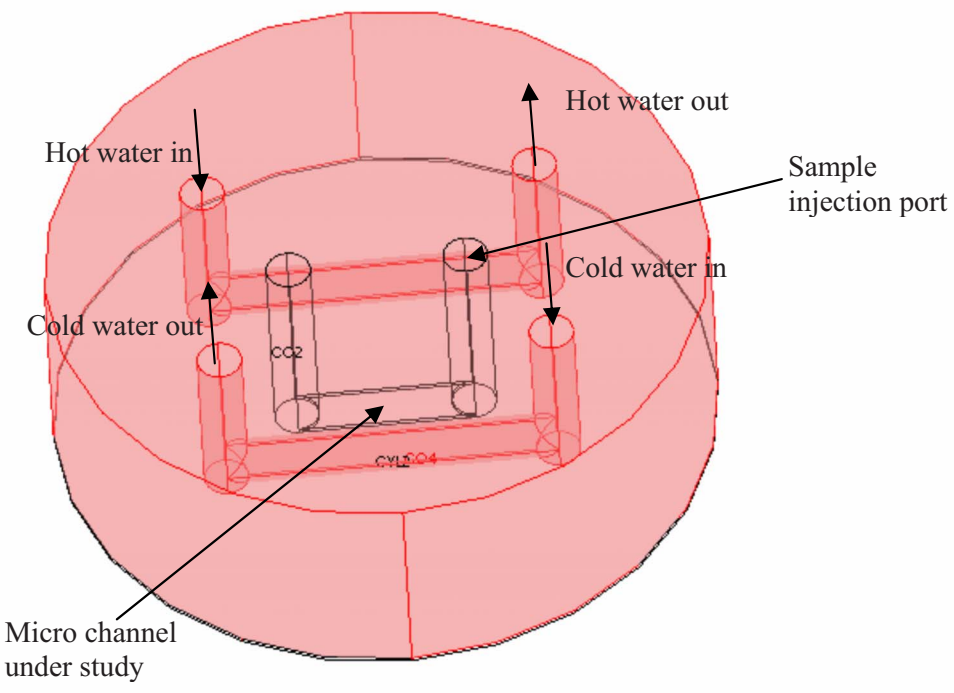


FIG. 2. The three-dimensional solid model of the chip used for simulation. The two ports at the end of the channel are for injecting samples and the two parallel lines are for hot and cold fluid flow. A counterflow arrangement is used for these channels.

and the glass coverslip only conductive heat diffusion was considered. Heat conduction alone is also considered in the microchannel and the punched ports for sample injection containing the liquid (this in an actual experiment will contain a biological fluid or cell medium but here we consider it to be water). This is because the Rayleigh number in this geometry is much below the critical value ($\sim$1707) required for the onset of natural convection. The tubes containing hot and cold fluid in the device are assumed to provide laminar forced convective heat transfer because the Reynolds number here is much below ($100 < \mathrm{Re} < 600$) the critical value for transition to turbulence. The external boundaries of the chip are assumed to exchange heat by natural convection, so the boundary convection coefficient was calculated as the natural convection coefficient for horizontal and vertical surfaces as the case may be. Ambient temperature was assumed to be the incubator temperature of 37 °C.

The computational domain created by the software (shown in Fig. 2) was divided into 123 334 tetrahedral elements and was solved by the biconjugate gradient iterative solver with preconditioning with a convergence criterion for temperature of 10^{-6}. Since the problem is a conjugate heat transfer problem with conduction diffusion coupled to the convection inside the hot and cold channels, the flow and energy equations were solved in a coupled way. The problem was solved by a pseudo steady-state approach to observe physical consistency.

III. EXPERIMENTAL PROCEDURE

A. Experimental setup for creation of gradient

The experimental circuit consists of two microfluidic devices placed in parallel for comparison and repeatability. The devices are placed inside an incubator (Model 3326, Forma Scientific Inc., Ohio) where a standard cell culture atmosphere of 37 °C, 5% CO_2 concentration and saturated humidity is maintained (Fig. 3). The hot and cold water is circulated in closed circuits by two peristaltic pumps (Model 3386, 0.4–85 ml/min, Control Company, Friendswood, Texas). Each

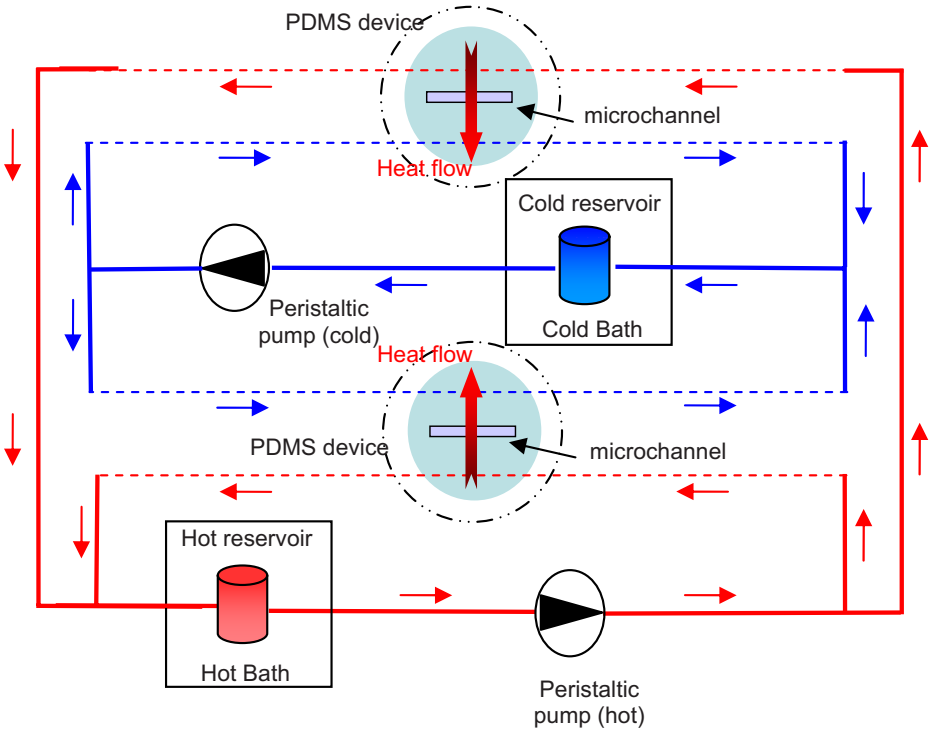


FIG. 3. Sketch of the experimental setup holding two microfluidic devices in parallel creating an identical temperature gradient in both the devices. The broken lines (----) in the diagram indicate the part of the circuit which remains inside the incubator and the solid lines lie outside of it. The heat flow directions and the microchannel locations are also indicated.

line extends outside of the incubator where it bifurcates in a T junction and enters the two devices. After transfer of heat to/from the device, the outlet tube of each line (hot and cold) from the two devices are joined together also by a T junction. The hot line then runs into a hot reservoir which is kept inside a thermostatic bath (E-100, Lauda Dr. R. Wobser, Gmbh, Koenigshofen, Germany). The fluid after getting heated in the reservoir inside the thermostatic bath is pumped by the peristaltic pump to the devices. Similarly, the cold lines from the two devices combine in a T junction and proceed to the cold reservoir which is kept in an ice bath. From the cold reservoir water runs to the cold peristaltic pump which pumps it back to the devices. To maintain balanced condition the two peristaltic pumps are run at the same speed. The temperature gradient is obtained by setting higher temperature in the thermostatic bath which is the variable temperature side while the temperature in the ice bath side remains unchanged and ice melting condition is maintained there. Both lines are insulated using foam insulation up to the incubator door to avoid heat loss/heat gain. The mean temperature of the chip can also be controlled by varying the mass flow rates of the fluids under circulation. The temperature gradient inside the incubator was measured by connecting the thermocouple leads to the meter (HH501DK, Omega Engineering, Inc., Stamford). To ascertain the quality and uniformity of the temperature gradient, a test run of the device outside the incubator was made and thermal images were obtained using an infrared camera (Thermovision A40 M, FLIR Systems Inc.). This provides information about the uniformity of the temperature gradient in the flow direction along the chip. It must be mentioned here that the temperature measurements with both thermocouple and infrared camera give the values on the end glass and not inside the channel. However, due to the small thickness (170 μm) and relatively high conductivity of the glass cover, under steady state the channel will be in thermal equilibrium with the glass and the glass temperature is a fairly close approximation of the channel temperature.

B. Experimental method for testing cell viability

Human metastatic breast cancer cells (cell line MDA-MB-231) were cultured in an L-15 medium supplemented with 10% fetal blood sample (FBS) and penicillin/streptomycin at 37 °C in an air incubator. Cells were detached from the culture dish surface using trypsin after washing with phosphate buffered saline (PBS), and resuspended in medium after centrifugation to a concentration of 1×10^6 cells/ml. Cell suspended medium was then loaded into the microfluidic channel through the punched ports. After 1 h incubation, cell suspension was replaced with medium and kept 24 h in an incubator before applying a thermal gradient.

At the beginning of the experiment the uniformity of the temperature in the devices before the application of the temperature gradient was measured. Subsequently the hot and cold flows were initiated. It takes between 35 and 55 min to establish a steady temperature gradient depending on the range of inlet temperatures. The temperature gradient was monitored every 15 min throughout the course of the experiment and the fluctuation observed was on the order of ± 1 °C. Once the temperature gradient was established, the mean temperature was calculated and the hot bath temperature was adjusted to the requirement.

Phase contrast images of the cells were taken before and after applying the thermal gradient to assess the thermal gradient effect. While applying a gradient, two devices were kept in the same incubator without the thermal gradient as control.

IV. RESULTS AND DISCUSSION

The present study proposes the design of a μTGS system using a countercurrent heat exchanger incorporated into a microfluidic platform that can be placed in a standard incubator environment and used for *in vitro* experiments lasting hours to days. As the means of evaluating the device, we first assessed the quality and magnitude of the temperature gradient created in the μTGS outside the incubator with an infrared camera. These thermal images (Fig. 4) confirm that a reasonable and uniform temperature gradient can be created in the device. However, the gradient is likely to change inside the incubator due to heat transfer with the different ambient condition existing there. To assess the effect of ambient temperature and maintainability of temperature gradient, images were taken for two ambient conditions by changing room temperature [Fig. 4(a) for 23 °C ambient and Fig. 4(b) for 28 °C ambient] with the hot water bath maintained at 60 °C. It can be seen from these two figures that with the increase in ambient temperature the temperature gradient in the chip decreases from 6 °C to 2 °C over the specified distance of 10 mm (note the difference in temperature scales). This is expected because the increase in ambient temperature increases the direct convective heat transfer from the ambient to the μTGS surface in the regions where the surface is at a lower temperature than the ambient. The only way to counter this and enhance the temperature gradient is to increase the hot fluid inlet temperature by increasing the temperature of the thermostatic bath. The effect of increasing the temperature of the thermostatic bath is shown in Fig. 4(c) (bath temperature of 70 °C) and Fig. 4(d) (bath temperature of 80 °C), both for an ambient temperature of 19 °C, to establish a gradient of 8 °C and 10 °C, respectively, over the same distance of 10 mm. However, it is also true that by this method (with a constant ice bath temperature) it is difficult to control the mean chip temperature and a method to control the cooling water temperature is required for that.

Numerical solution of the μTGS temperature distribution provides an excellent tool to optimize temperature, and to study the effects of parameters such as hot fluid inlet temperature, ambient temperature, and the geometrical parameters like μTGS dimension, spacing between the hot and cold channels, and heating/cooling fluid flow rates on the temperature gradient as well as the mean chip temperature. Figure 5(a) shows a typical temperature distribution in the chip in the incubator environment. The end temperatures of the glass coverslip for two different ambient temperatures [Fig. 5(b) for 23 °C ambient and Fig. 5(c) for 28 °C ambient] are presented which show close resemblance with the infrared thermographic images presented in Figs. 4(a) and 4(b), respectively (note the different temperature scales in the figures which are adjusted because of the automatic adjustment of the infrared camera to the surrounding temperature). The most important

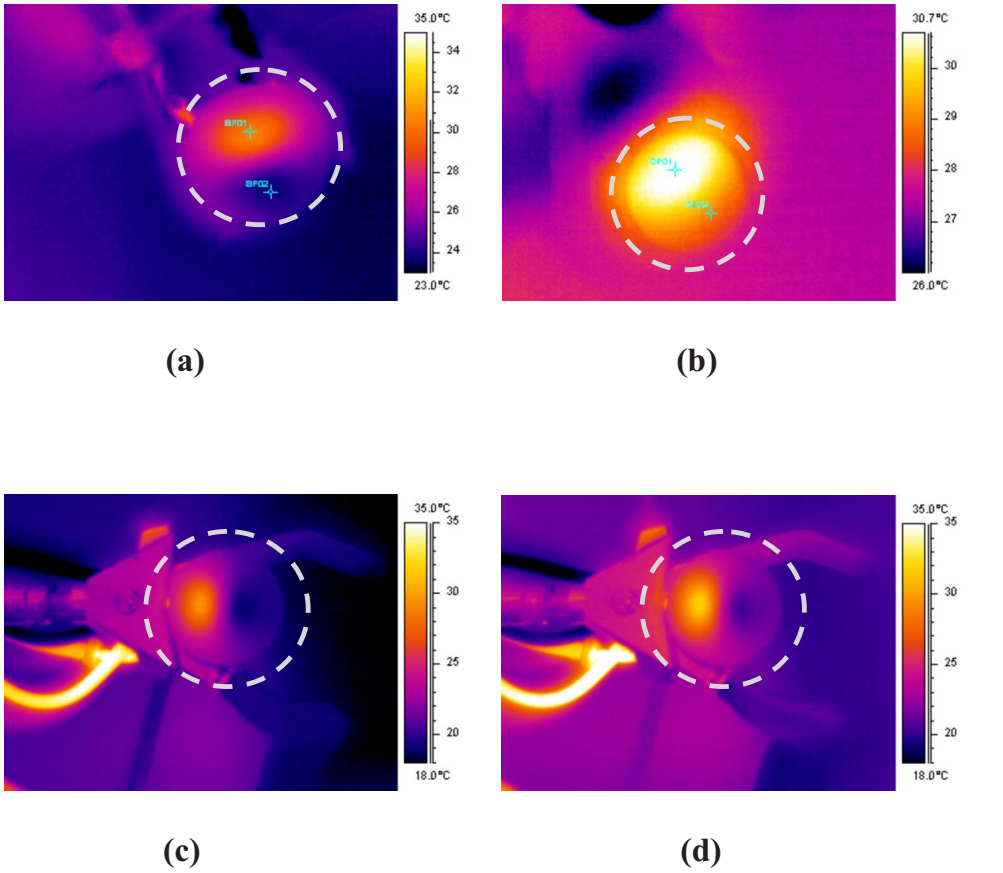
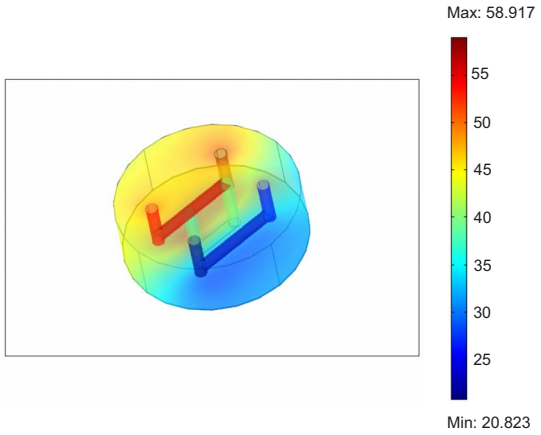


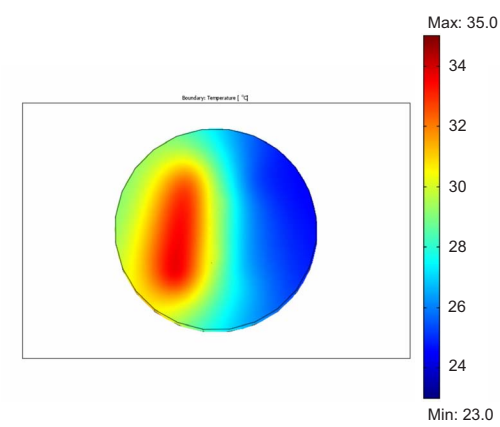
FIG. 4. Infrared thermographic images of the chip with temperature gradients: (a) ambient 230 °C and hot bath temperature 600 °C; (b) ambient 280 °C and hot bath temperature 600 °C; (c) ambient 190 °C and hot bath temperature 700 °C; (d) ambient 190 °C and hot bath temperature 800 °C.

feature of these simulations is a fairly uniform temperature gradient across the microchannel over the entire channel length due to the countercurrent arrangement of the heating and cooling channels. This is not achievable either by parallel flow arrangement[26] or by Joule heating where mean system temperature increases rapidly with Ohmic dissipation. A comparison between the simulation and experimental variation of temperature gradient [Fig. 5(d)] shows close agreement between them. The discrepancy at the higher end of the hot bath temperature is mainly due to the high humidity (which has not been considered in the simulation) of the ambient air inside the incubator giving a higher heat transfer rate when the device is at an elevated temperature. However, the difference is small and the simplicity of the analysis is attractive for design purposes.

We examined cell viability and the effect of thermal gradients with human metastatic breast cancer cell line MDA-MB-231. Three different conditions were tested. Figure 6 shows the cells before and after applying a thermal gradient. In the medium range (b), healthy cells cover the whole channel without any detectable influence of the thermal gradient. In the cold range (a), cell density is less than that of medium range but still shows no thermal gradient effect. In the hot range (c), cells viability is low, and differences are seen associated with the thermal gradient. Some cells grow on the surface only on the cooler (left) side when the cells on the hotter (right) side could not survive. It should be mentioned here that the temperature bar in this figure indicates the temperature difference between the thermocouples which are about 10 mm apart. Hence, the real temperature difference across the channel is of the order ~2 °C. The experiments were carried out for 12 h and the device showed quite remarkable cell viability under a steady temperature gradient.



(a)



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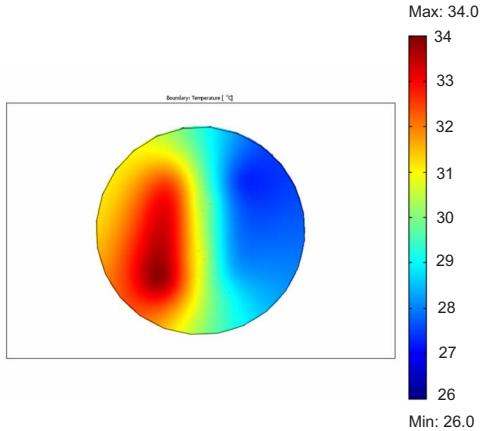
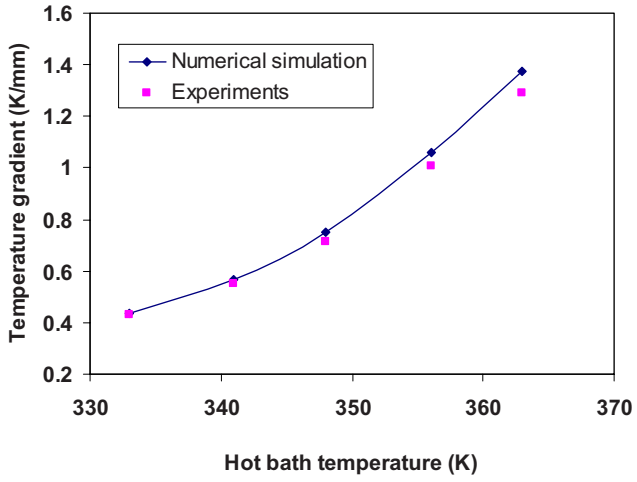


(c)



(d)

FIG. 5. Results of numerical simulation for the device. (a) Typical temperature distribution in the chip in the incubator environment (370 °C ambient and bath at 900 °C); (b) end glass temperature at ambient 230 °C and hot bath temperature 600 °C; and (c) ambient 280 °C and hot bath temperature 600 °C. (d) Comparison of experiments with simulation.

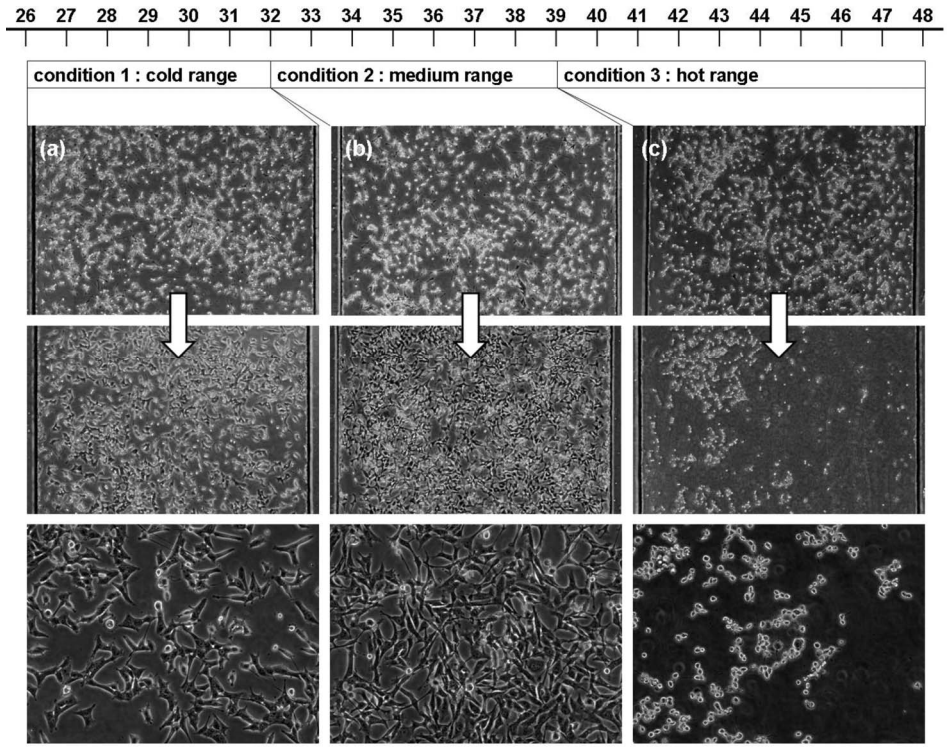


FIG. 6. Applied thermal gradient and cell response. Top: before applying thermal gradient; middle: after 12 h of thermal gradient. The bottom figures are a magnified view of cells in the center of the channel. (a) Cells under condition 1 range between 26–32 °C, (b) cells under condition 2 range between 32–39 °C, and (c) cells under condition 3 range between 39–48 °C.

V. CONCLUSIONS

The design of a μTGS is presented which is capable of creating a mild temperature gradient that realistically mimics many physiological/biological conditions. The μTGS is designed to work within an incubator environment which is crucial for cytological or tissue engineering applications. The system employs a countercurrent heat exchanger to create a uniform temperature gradient, confirmed by infrared thermographic measurement. 3D numerical simulation is found to be an appropriate tool to optimize the design parameters for specific applications. Finally viability of breast cancer cells is demonstrated in the microfluidic device. This system can also be an important tool for thermotaxis studies in other areas involving bacteria and small organisms.

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