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Finite Element modelling and design of a Concentration Gradient Generating Bioreactor: Application to biological pattern formation and toxicology

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Abstract

This paper describes the use of a microfluidic gradient maker for the toxicological analysis of some conventional biomolecules such as hydrogen peroxide and a local anaesthetic, lidocaine on different cell cultures, human endothelial cells and myoblasts, respectively. The microfluidic device was designed and simulated using COMSOL Multiphysics® and the concentration gradient in the microfluidic network was analysed through a fluid-dynamic and mass-transport study. Subsequently the device was fabricated with soft lithography, casting PDMS in a master to obtain channels about 250 µm deep. Hydrogen peroxide was tested on human endothelial cells, while lidocaine was tested on C2C12 myoblasts and an analysis was performed using propidium iodide staining followed by an imaging processing routine to obtain quantitative dose–response profiles in the gradient maker. The results show that the Gradient Maker (GM) bioreactor is a more sensitive method for detection of cell toxicity, and compared with testing of drug toxicity using

microwells with individual cell cultures, allows one shot testing with a single cell culture exposed to a large number of concentrations. Moreover, the Gradient Maker was also modelled in order to realise biological pattern formation using two morphogenes acting as activator and inhibitor with varying diffusion rates.

Keywords: Microfluidics; Gerier-Meinhardt Model; Concentration Gradient Maker; Anaesthetics; Cell testing

Introduction

The microfluidic device proposed by Whitesides for generating concentration gradients through laminar flow [McDonald JC et al,2000] has been extensively used by several researchers for generating large concentration gradients over small length scales. Basically, the Gradient Maker (GM) is a microfluidic device consisting of a series of channels connected to a chamber where the gradient is formed. An essential requisite of the GM system is the presence of a laminar flow with a very small Reynolds number in order to prevent convective mixing. A wide range of biological studies and applications have been performed with this type of gradient generating systems [Glawdel T et al, 2009; Atencia J et al, 2009;Toh YC et al, 2009].

One of the main topics in which these systems are usually used are studies of chemiotactic gradients that can be used to guide cell migration. These devices are highly effective for analysis of cell behaviour in a simple two dimensional environment. This type of study can help to understand how specific gradients, controlled in space and time, are able to steer cell fate and function. Moreover it is possible to distinguish studies in which immobilised gradients are created on an adhesive surface [Adams DN et al, 2005], or steady state gradients of soluble molecules, such as a morphogenes [Lander AD et al, 2007] or chemokines [Saadi W et al,2007], are established with a continuous perfusion of a specific solution.

Since these systems have the ability to generate large concentration gradients in just few millimetres, dose-response curves can be easily constructed with a single experiment and using just a single cell culture (Tirella et al, 2008). Toxicity tests are easily implemented with this novel system, giving the possibility to run a single “one-shot” test instead of the classical protocols, that are usually based on a multi concentration set of experiments.

Alan Turing in his seminal paper “The chemical basis of morphogenesis” [Turing A et al, 1952], showed that steady state chemical patterns can be generated in two component system due to different diffusion rates of the reacting chemical substances. Since then, several specific models for pattern formation using reaction-diffusion systems have been developed. For examples, Gierer and Meinhardt proposed a model for biological pattern formation in which two morphogenes acted as activator and inhibitor [Meinhardt H, 2008; Gierer A. and Meinhardt H., 1972]

Here we describe the design and realisation of a gradient generating microfluidic bioreactor using multiphysics finite element modelling in particular to test the toxicity effect of molecules on different cell lines. Moreover using the FEM analysis we modelled the GM bioreactor in order to realise biological patterns using the Gierer and Meinhardt (G&M) theory [Meinhardt H, 2008].

The GM bioreactor was used to analyse the toxicological effect of hydrogen peroxide on human endothelial cells, and the toxicity of a local anaesthetic namely bupivacaine and lidocaine, which is known to induce apoptosis in striated muscle fibres in vitro and in vivo [Zink W and Graf BM, 2004 ; Irwin W et al, 2002] on myoblasts cells.

Our aim was to demonstrate the feasibility of using the GM system as an alternative to early stage screening of drugs, and to critically assess its efficiency in single shot testing compared with classic microwell analyses. In the GM a single cell culture is exposed to a range of drug concentrations, so that it may also be possible to discern the protective effects of less damaged or healthy cells on damaged ones, or even sympathetic toxification. Cell toxicity was analysed following the application of a steady state well-defined concentration gradient for 6 h.

Furthermore, we show how the GM can be used, at least in theory, to produce patterns based on reaction-diffusion of morphogenes.

Materials and Methods

Design and realization of the Gradient Maker Chamber

The microfluidic GM circuit is based on the geometry originally proposed by Whitesides [Li Jeon N et al, 2002; Jiang X et al, 2005] with two inputs and with 6 final channels coming out as affluents

after flowing through a “serpentine” shaped mixer (fig.1a). The presence of 6 channels allows a smoother resolution of concentration gradient to be obtained. The topology of the GM is composed of two parts: a serpentine shaped circuit and a cell treatment chamber. The development of the concentration gradient is possible if there is a completely developed laminar flow inside the treatment chamber and the fluids at the exit of the microchannels are perfectly mixed. For this last reason, the treatment chamber and the acute angles were minimized. The GM Bioreactor is made of Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, Usa) and is obtained by Soft-molding of a silicon master. The master was realized by lithographic process as reported in [Vozzi et al, 2003] using EPON-SU8-100 (Microchem, Newton, Ma, USA) as photoresist. PDMS is prepared by mixing the monomer and catalyser in a 10:1 w/w ratio. The mixture is degassed and casted on the silicon master and, then, degassed again to improve filling of the microchannels. The mould is finally baked in an oven for four hours at 65° C and detached from the wafer after cooling. The GM bioreactor also has 3 silicone tubes of 1 mm inner diameter glued directly in the mold. To ensure complete sealing between the PDMS circuit and the plexiglass, the device was clamped with a Teflon frame (Fig. 1b). A mini peristaltic pump (P720, Instech, Plymouth, PA, USA) was connected to the two inlet tubes which in turn were connected to two reservoirs. The output silicone tube instead was connected to a beaker.

FEM Modelling

Prior to the modeling in COMSOL (COMSOL AB, Stockholm, Sweden), basic geometric features of the GM system were established such that the complete microfluidic circuit fitted inside a standard silicon wafer, and the cell culture chamber had same area as 3.14 cm² microwell plate.

The microfluidic circuit was designed using COMSOL, and following dimensions were chosen:

- microchannel width: 1.6 mm;
- width of the cell culture chamber: 14.6 mm;
- length of the cell culture chamber: 20 mm.

The microfluidic GM network system was analyzed using finite element modeling in order to determine the efficiency of the 6 microchannels and estimate the concentration gradients in the cell chamber. Two application modes of COMSOL Software were used: Fluid Dynamics and Convection and Diffusion, the ruling mode was the fluid dynamic regime.

The first mode determined the motion and the velocity profile present inside the system using the Navier–Stokes equations for an isothermal incompressible fluid; the second mode allowed calculation of the concentration gradient using the convection-diffusion equation using the velocity calculated from the Navier–Stokes equations as an input. Since the microchannels were 250 μm deep (this is the range obtained with the soft lithography technique) the device was approximated to a 2D system. In fact the microchannels had an infinitesimal height along the z axis in comparison with the characteristic dimensions along the other axes.

To carry out the simulation the following constants and boundary conditions were imposed:

- the temperature was set to 37 °C and the fluid was assumed to be water because the viscosity and density of the culture medium are almost identical to that of water;
- the two input concentrations C1 and C2 were initially set at C1 = 0 and C2 = 1 mM to enable the effects of the drug diffusion coefficient on the concentration profile to be evaluated;
- since the exact value will depend on the experimental parameters, various simulations were carried out using different values of D (diffusion coefficient) ranging from 10^{-5} to 10^{-10} m^2/s ;
- the flow rate was fixed at 170 $\mu\text{l}/\text{min}$ to reduce the number of experimental variables. It was shown to be adequate to ensure the formation of a smooth gradient at all the values

of D tested. Lower flow rates give rise to flat concentration profiles (or near zero concentration gradients) while higher rates could lead to cell damage from shear stress;

- the fluid dynamic boundary conditions imposed within COMSOL were no-slip at all walls and zero pressure or resistance to flow at the outlet of the device.

The equations used for fluid dynamic analysis of the system were the following:

$$\rho \frac{\partial u}{\partial t} + \rho(u \cdot \nabla)u + \nabla p - \eta \nabla^2 u = 0 \quad (1)$$

$$\nabla \cdot u = 0 \quad (2)$$

where u is the velocity vector, ρ the fluid density, η the dynamic viscosity and p the pressure. The first equation is the momentum balance, and the second is the equation of continuity for incompressible fluids.

The equation used for concentration analysis was:

$$\frac{\partial C}{\partial t} + \nabla \cdot (-D \nabla C) = -u \cdot \nabla C \quad (3)$$

where C is the concentration, and u is the velocity vector calculated in the Fluid Dynamic mode.

The number of elements of mesh was around 200.000, obviously increasing them the results become more precise.

Gierer and Meinhardt reaction-diffusion model.

The possibility to generate new patterns with previously described and tested microfluidic devices was also evaluated, using a reaction-diffusion model proposed by Gierer et al. [Gierer A. and Meinhardt H, 1972]. Gierer and Meinhardt (G&M) modeled the reaction between an activator and an inhibitor, and show how short range activation and long range inhibition are able to explain the effect of morphogenes and also the formation of morphogenetic patterns. The pattern topology is

essentially determined by ranges of activation and inhibition, where the range is the mean distance between production and decay (or removal) of respective species. In this analysis the lateral inhibition model considered and expressed with two equations:

$$\frac{\partial a}{\partial t} = \rho \frac{a^2}{b} - \mu a + D_a \frac{\partial^2 a}{\partial x^2} \quad (4)$$

$$\frac{\partial b}{\partial t} = \rho' a^2 - \nu b + D_b \frac{\partial^2 b}{\partial x^2} \quad (5)$$

Where a is the activator and b is inhibitor respectively, ρ and ρ' are production rates of a and b respectively, while μ and ν are consumption rates of a and b respectively.

In order to realise patterns with a specific topology it is necessary to respect some condition expressed by the G&M model, moreover initial conditions represent a significant role for the stability of the pattern generated.

To model the activator-inhibitor model proposed by G&M, two Convection-Diffusion Analyses were performed and combined with the Mass Balance Module using Comsol Multiphysics.

Initially we modeled a spatial 2D environment having the same dimensions as the treatment area of the GM bioreactor.

To carry out the simulation the following constants and boundary conditions were imposed:

- the temperature was set to 37 °C and the fluid was assumed to be water because the viscosity and density of the culture medium are almost identical to that of water;
- the two input concentrations C_1 and C_2 were initially set at $C_1 = 10^{-6}$ M and $C_2 = 0$ M;
- the initial condition of variable a is set as a controlled uniform constant a_0 that are equal to the input concentrations previously reported (C_1 and C_2). The reaction rate R_a is defined as described by G&M:

$$R_a = \rho \cdot \frac{a^2}{b} - \mu \cdot a \quad (6)$$

- the initial condition of variable b is set as a constant b_0 equal to 10^{-5} mM, while the reaction R_b is written in the subdomain expression as:

$$R_b = \rho' \cdot a^2 - \nu \cdot b \quad (7)$$

- An isotropic diffusion was assumed and selected values were 10^{-9} for the activator (D_a), and 10^{-7} for the inhibitor (D_b);
- the flow rate was fixed at 170 $\mu\text{l}/\text{min}$ with boundary conditions as in the previous model;
- the value of ρ , ρ' , μ , and ν were fixed to have a single or periodic pattern in the treatment area. For the single pattern $\rho=6 \cdot 10^{-5}$, $\rho'=2 \cdot 10^{-4}$, $\mu=10^{-5}$, and $\nu=10^{-4}$ [moles/secs]; for the periodic pattern $\rho=6 \cdot 10^{-3}$, $\rho'=2 \cdot 10^{-2}$, $\mu=10^{-3}$, and $\nu=10^{-2}$ [moles/secs]

When the activator has reached the treatment area reaction with the inhibitor (supposed uniformly distributed in the treatment zone at a concentration b_0) starts. Initial concentration, a_0 of the activator determined the pattern generation for the lateral-inhibition model proposed by G&M. We also modeled this system using Microfluidics Analysis of MEMS Module, combining two Convection-Diffusion Modules (one for activator, one for inhibitor), as it enables a pseudo 2D analysis to be performed on microfluidic device. The Subdomain Settings were the same as the previous model, with reaction rates R_a and R_b described by G&M. Using the stationary solver a pseudo-stationary state in which a single pattern is generated was obtained. The number of elements of mesh was equal to the previous simulations.

Image analysis setup

To demonstrate the generation of a concentration gradient inside the GM bioreactor and to estimate it in the treatment area, we used two dyes (red and blue) for each inlet. As the dyes flow along the serpentine they are repeatedly mixed and produce a coloured gradient in the treatment area, going gradually from blue to red.

Using a digital camera with 640x400 pixel resolution (DTA, Italy), a movie of the experiment was acquired and its frames were extracted every 30 seconds. The image acquisition was focused on the cell treatment area. The frames were filtered to remove the noise and to improve the contrast of the picture without changing the colour gradient properties.

Filtering was necessary to reduce the noise due to discontinuity of the light source and to shadows caused by the bioreactor frame.

RGB Images were decomposed in 3 matrices each one corresponding to Red, Blue and Green channels, using Matlab Image Processing Toolbox (The Math- Works Inc., USA). To obtain the concentration profile in the treatment area, the image matrix was reduced to a vector, where each point was the mean of each matrix column.

The values obtained were plotted in a semilog space for Red and Blue matrices. The experiment was performed at different peristaltic pump velocities.

Cell Cultures

Effect of gradient concentration of H₂O₂ on Human endothelial cells (HUVEC)

Primary endothelial cells from human umbilical cords were used to study the toxicity of hydrogen peroxide. Cells were used from the 2nd to 8th passage and maintained in culture with medium M199 (Sigma-Aldrich, Milan, Italy) containing 10% fetal bovine serum, 1% glutamine, 1% antibiotics (all from Eurobio, Milan, Italy). In addition, the medium also contained 1% endothelial growth factor. The GM was sterilized with H₂O₂ gas plasma and then pretreated with a 1% gelatin (Sigma-Aldrich, Milan, Italy) solution and seeded with a density of 30,000 cells/cm². This is our standard surface of treatment for endothelial cells. HUVEC were cultured for 24h in static conditions in the GM cell chamber to promote cell adhesion after which the dynamic experiment was performed. All cell culture experiments were carried out in a humidified incubator at 37 °C, 5% CO₂ (Hereaus SpA, Milan, Italy). Inlet concentrations were 0.5 mM and 1 mM for 30 minutes of H₂O₂. Experiments were conducted using a flow rate of 170 µl/min. After the H₂O₂ exposure, the GM was disassembled and cells were fixed with 4% paraformaldehyde and stained with DAPI. These data were compared with the same experiment performed in static conditions where the cells were subjected to the two concentrations for 30, 120 and 240 minutes, and then fixed and stained with DAPI.

Effect of gradient concentration of lidocaine on Myoblast cells

C2C12 cells, a mouse myoblast cell line were acquired from LGC Promchem, Teddington, UK. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin– streptomycin mixture, and trypsin/ETDA were all from Lonza, Basel, Switzerland All other chemicals, horse serum (HS), as well as the two drugs, bupivacaine and lidocaine were purchased from Sigma-Aldrich, unless otherwise stated, and were of the highest purity possible.

C2C12 cells were cultured in 75 cm² flasks in DMEM supplemented with 10% FBS (fetal bovine serum), 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

The GM was sterilized with H₂O₂ gas plasma and then pretreated with a 1% gelatin (Sigma-Aldrich, Italy) solution and seeded with a density of 30,000 cells/cm². The two inlet tubes were connected to two reservoirs, one containing the anesthetic in serum free medium, and one with only serum-free medium. Inlet lidocaine concentrations were 0 and 5 mM. Experiments were performed using a flow rate of 170 µl/min and maintaining the device inside the incubator for 6 h. The GM was disassembled and the cells stained with propidium iodide (PI). In order to obtain a positive control, cells were exposed to UV light for 15 min and stained with PI. This serves to estimate an upper limit to the brightness of the cells.

Cell Imaging analysis

Propidium iodide or DAPI stained cells were photographed at a magnification of 100 using a fluorescent microscope (Olympus IX71, Olympus Italia, Milan, Italy). The cell culture area from the microchannel inlets was scanned and recorded using the micrometered microscope stage. The digital images obtained were processed, and a software routine was developed to calculate the average brightness of cells in the image. Owing to the discrete nature of cells, the intensity profile across cell treatment area was non-uniform compared with similar analysis carried out with soluble colored or fluorescent dyes, and presented several troughs and peaks. Therefore the image processing routine involved a first step to identify cells and then brightness (intensity) or luminosity per cell was evaluated along each image. The luminous intensity per cell was averaged using a running average with a window corresponding to 60 X 800 pixels (about 4 cell widths), and the maximum luminosity was set to 1 while the minimum (black) set to 0.

Statistical Analysis

All the cell culture experiments were carried out in triplicate and the results are expressed as means and standard deviations. Statistical analysis was performed using the Student's t-test when comparing between control and experimental groups in static cultures and analysis of covariance – ANOVA and multiple comparison statistics for analysis of the GM data (Matlab Statistics Toolbox, The MathWorks Inc., USA); a p value of less than 0.05 was considered statistically significant.

Tests and Results

FEM Results

We evaluated the velocity field of the fluid and the concentration of the molecule of interest inside the cell culture chamber. As it is possible to see from figure 2a, in the treatment cell chamber the flow presents typical Poiseuille profile. Evaluating the concentration profile for the molecule a , it is possible to note that a linear gradient is generated in the cell treatment area (figure 2b). Initially we evaluated the behavior of flow inside the GM chamber as function of speed of peristaltic pump. From the FEM analysis it is possible to note that Reynolds Number is low enough to guarantee a laminar flow inside the device. This result ensures the generation of a diffusive flux of a chemical species from an higher concentration zone to a lower one.

Image analysis results

The test was performed at different velocity of peristaltic pump. A change of coloured gradient in treatment area was observed already visually, and after imaging processing this result was confirmed as shown in figure 3.

At high fluid velocity there was less diffusion of the molecule, and this phenomenon generated a steeper concentration profile in comparison with the lower fluid velocity, where the diffusion is more marked.

Gerier model results

Initially a static analysis was performed using a Time-Dependent analysis with the Direct UMFPACK linear solver, with stepping time of 0.5 s. Results of the static 2D simulation are shown in figure 4a-b, when a pseudo-stationary state is reached.

As expected, a single non-periodic pattern was obtained when the square root of ratio between diffusive constant and reaction rate is equal to space of reaction (figure 4a). Instead when the value decreases, it is possible to generate a periodic pattern, as described by G&M (figure 4b) [Gierer A and Meinhardt H,1972].

Starting from these results it was also possible to model a pattern with different shapes in the cell chamber with superimposed flow. The same conditions of G&M model in static conditions were applied to the GM, obtaining a pattern with two different shapes along X (across the chamber) and Y (along the chamber parallel to the flow). The presence of a linear gradient of activator, a , across the cell treatment chamber causes a production of a due to auto-catalyses. This is only verified when a certain threshold of a is reached (see equation 4 and 5), as shown in figure in figure 5.

After an initial fast production the activator is removed by the flow imposed, and the lateral inhibition effect, which gives rise to the periodic patterns in figure 4, is damped. Activator is nevertheless still produced autocatalytically in small quantities and is carried across the x-direction of the treatment area. For this reason it was possible to obtain a double gradient among two

directions (figure 5). This pattern is similar to the one described by the G&M model, where the removal process was dominant with respect to the diffusion and the decay rate. The activator gradient in the chamber is obtained imposing an inflow of 17 ml/s, while one inlet has a concentration of 1.2•micromoles/m³ and the other is set to zero. In the chamber the diffusion constant of the activator is equal to 10⁻⁸ m²/s. In order to obtain the reaction a constant value of the inhibitor is imposed in the chamber and equal to 10 micromoles/m³. The constants of the autocatalytic reaction are $\rho = 10^{-5}$, $\mu = 10^{-5}$, $\rho_1 = 10^{-4}$ and $\eta = 10^{-4}$, as proposed by G&M. The model reveals that is established both across the x-axis as well as along y-axis.

Cell experiments

Hydrogen peroxide treatment results

The analysis of luminosity in static conditions showed that it increased as the exposure time increased (fig. 6a) for each concentration. In the GM it was possible to observe the intensity increased linearly as the concentration gradient increased in the cell treatment area (fig.6b). This result is also visible from figure 6c, where the HUVECs stained with Iodide Propidium show a higher intensity in the left side where they were exposed to 1mM with respect to the right side which has a concentration of 0.1 mM.

Lidocaine treatment results

Experiments in the gradient generator were conducted using a flow rate of 170 μ l/min. Firstly, to ensure that the flow of medium in the culture chamber did not damage cells with respect to the static controls, experiments were run without drugs, so that both microfluidic inlets contained

only serum-free culture medium. After 6 h the cells were stained with propidium iodide and the region of interest was photographed with a fluorescence microscope using a 10X objective. In control conditions, PI staining of the cells showed that the luminosity of the cells was constant across the chamber in the region of interest, with low intensity values similar to those shown in Fig. 7 for the drug-free static controls.

In the toxicity experiments, the two inlets were connected to two reservoirs containing the drug (5 mM Lidocaine) and drug-free medium respectively. After 6 h, the GM was disassembled and the culture chamber photographed over the region of interest, 1 cm from the microfluidic inlets. Using the image processing algorithm an intensity profile across the chamber was obtained. This was converted into a concentration profile by least squares fitting the luminosity data to the concentration gradients obtained from the COMSOL simulation in the region of interest for different values of D . Fig. 8a and 8b show the resulting concentration across the chamber for the two drugs.

The regression values (non-linear R^2) was 0.79. Using this method, the diffusion coefficient for lidocaine was estimated to be $2.1 \pm 0.7 \times 10^6$. To convert the data into dose–response curves, the data in Fig. 8a were replotted as concentration-intensity graphs by transposing the intensity data onto the concentrations. As expected, a straight line is obtained since the intensity profile exactly matches the concentration profile, and the regression coefficient (R^2) between the data and fitted line is >0.98 (figure 8b). To assess the significance of the results, ANCOVA was performed comparing the luminosity profile for the drug-free run in the GM (not shown) with the data in Fig. 8a and 8b. The slope is significantly different from that of the drug-free run with $p < 0.0001$.

Conclusions

We described the use of a microfluidic gradient maker for testing the toxicity of H_2O_2 and lidocaine, respectively and oxidizing agent and an anesthetic. The GM was first designed and simulated using COMSOL Multiphysics to ensure that the geometry of the device allowed laminar flow within microchannels and the establishment of a smooth concentration gradient in the culture chamber within the range of estimated working parameters. HUVEC and C2C12 myoblasts were seeded in the culture chamber of the device and the substances were introduced in to one of the two inlets of the GM, while medium was pumped through the second inlet to produce a steady state concentration gradient in the culture zone.

After a fixed time of exposure to the molecule of interest the cells were stained with propidium iodide or DAPI and an analysis was performed using an imaging processing routine to obtain a profile of the luminous intensity per cell across the chamber. The results show that the GM is a sensitive method for detection of cell toxicity, and allows one shot testing with a single cell culture exposed to a large number of concentrations.

The efficiency of the GM in toxicity analysis was found to depend on two main factors: the effects of shear on cells, which depends very much on cell origin and nature, and the diffusion coefficient of the drug or toxin, which depends on its solubility in the medium (Tirella et al, 2008).

We also demonstrated using theoretical modeling, and equation of reaction and diffusion as described by Gerier and Meinhardt that with the GM bioreactor it is possible to generate different patterns in the treatment area, changing variables such as the fluid velocity, initial conditions and diffusion constant of activator and inhibitor molecules within the device.

In a first approach we modeled the device with the chemical interactions, supposing a uniform distribution of an inhibitor in the treatment zone with a linear gradient for the activator. A static

periodic pattern, similar to that described by G&M, can be obtained varying the reaction and diffusion constants. Using the same parameters as the static periodic model, we then superimposed a flow on the system, and obtained a single activator pattern with two different gradients along and across the treatment zone, while the inhibitor is consumed throughout the chamber. Starting from these results, we are now studying new systems to generate stable and unstable, but controlled, patterns in order to induce different chemical signaling to cells in culture using a dynamic bioinspired environment.

References

Adams DN, Kao EY, Hypolite CL, Distefano MD, Hu WS, Letourneau PC. (2005) Growth cones turn and migrate up an immobilized gradient of the laminin IKVAV peptide. *J Neurobiol.* 2005 Jan;62(1):134-47.

Atencia J, Morrow J, Locascio LE. (2009) The microfluidic palette: a diffusive gradient generator with spatio-temporal control. *Lab Chip.* 2009 Sep 21;9(18):2707-14.

Glawdel T, Elbuker C, Lee LE, Ren CL. (2009) Microfluidic system with integrated electroosmotic pumps, concentration gradient generator and fish cell line (RTgill-W1)-towards water toxicity testing. *Lab Chip.* 2009 Nov 21;9(22):3243-50.

Irwin W, Fontaine E, Agnolucci L, Penzo D, Betto R, Bortolotto S, Reggiani C, Salviati G, Bernardi P. (2002) Bupivacaine myotoxicity is mediated by mitochondria. *J Biol Chem.* 2002 Apr 5;277(14):12221-7.

Jiang X, Xu Q, Dertinger SK, Stroock AD, Fu TM, Whitesides GM. (2005) A general method for patterning gradients of biomolecules on surfaces using microfluidic networks. *Anal Chem.* 2005 Apr 15;77(8):2338-47.

Lander AD, Nie Q, Wan FY. (2007) Membrane-associated non-receptors and morphogen gradients. *Bull Math Biol.* 2007 Jan;69(1):33-54.

Li Jeon N, Baskaran H, Dertinger SK, Whitesides GM, Van de Water L, Toner M. (2002) Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat Biotechnol.* 2002 Aug;20(8):826-30.

McDonald JC, Duffy DC, Anderson JR, Chiu DT, Wu H, Schueller OJ, Whitesides GM, (2000) (2000), Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis.* 2000 Jan;21(1):27-40. Review.

Gierer A, Meinhardt H. A theory of biological pattern formation. *Kybernetik.* 1972 Dec;12(1):30-9

Meinhardt H. (2008) Models of biological pattern formation: from elementary steps to the organization of embryonic axes. *Curr Top Dev Biol.* 2008;81:1-63. Review.

Saadi W, Rhee SW, Lin F, Vahidi B, Chung BG, Jeon NL. (2007) Generation of stable concentration gradients in 2D and 3D environments using a microfluidic ladder chamber. *Biomed Microdevices.* 2007 Oct;9(5):627-35.

Tirella A, Marano M, Vozzi F, Ahluwalia A., A microfluidic gradient maker for toxicity testing of bupivacaine and lidocaine, *Toxicol In Vitro*. 2008 Dec;22(8):1957-64.

Toh YC, Lim TC, Tai D, Xiao G, van Noort D, Yu H. (2009) A microfluidic 3D hepatocyte chip for drug toxicity testing. *Lab Chip*. 2009 Jul 21;9(14):2026-35.

Turing A, (1952) The Chemical Basis of Morphogenesis, *Phil. Trans. R. Soc. London B* 237 pp 37-72 (1952)

Vozzi G, Flaim C, Ahluwalia A, Bhatia S, (2003) Fabrication of PLGA scaffolds using soft lithography and microsyringe deposition", *Biomaterials*. 2003 Jun;24(14):2533-2540.

Zink W, Graf BM. (2004) Local anesthetic myotoxicity. *Reg Anesth Pain Med*. 2004 Jul-Aug;29(4):333-40. Review.

Figure Legend

Figure 1: (a) scheme of Gradient Maker bioreactor (b) the system all assembled

Figure 2: (a) 2D profile of velocity (b) 3D-gradient concentration in the Gradient Maker bioreactor

Figure 3: Imaging elaboration of gradient in the cell treatment area using red and blue dyes. (a) Picture of cell treatment area; (b) selection of a part of this pic; (c) extraction of red and blue channel of images, (d) determination of gradient concentration of normalised intensity of red and blue dyes in the selected area (blue line: experimental data; red line: modelised linear gradient).

Figure 4: (a) A single static pattern produced using the G&M Model in the gradient maker bioreactor (figure 5a); (b) a periodic pattern, as described by G&M, produced in the gradient maker bioreactor (figure 5b).

Figure 5: Activator pattern obtained by the G&M model simulation in flow conditions.

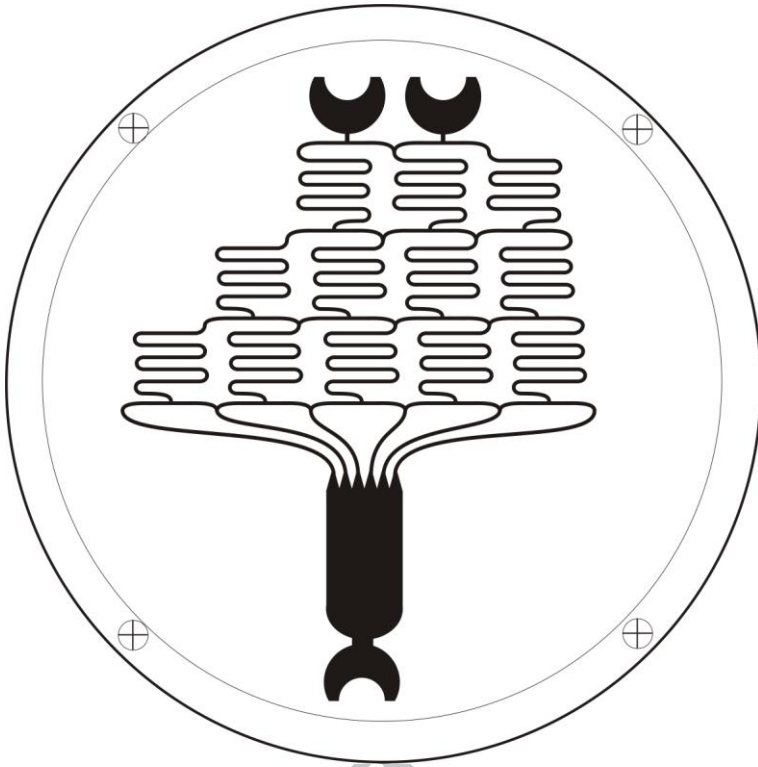
Figure 6: (a) Cell luminosity in static conditions as function of exposure time, (b) HUVEC cell luminosity in dynamic conditions across the GM cell chamber, (c) Fluorescent intensity of DAPI labeled HUVEC exposed to Hydrogen Peroxide gradient across GM cell chamber.

Figure 7: Propidium iodide staining of undifferentiated C2C12 cells in the cell treatment area in comparison with the drug-free static control

Figure 8: (a) Concentration profile across the chamber after 6 hours exposure to lidocaine. The graph was obtained by fitting the processed data to the concentration profile, as calculated by the FEM model. (b) Brightness as function of lidocaine concentration.

Figure 1: (a) scheme of Gradient Maker bioreactor (b) the system all assembled

(a)



(b)

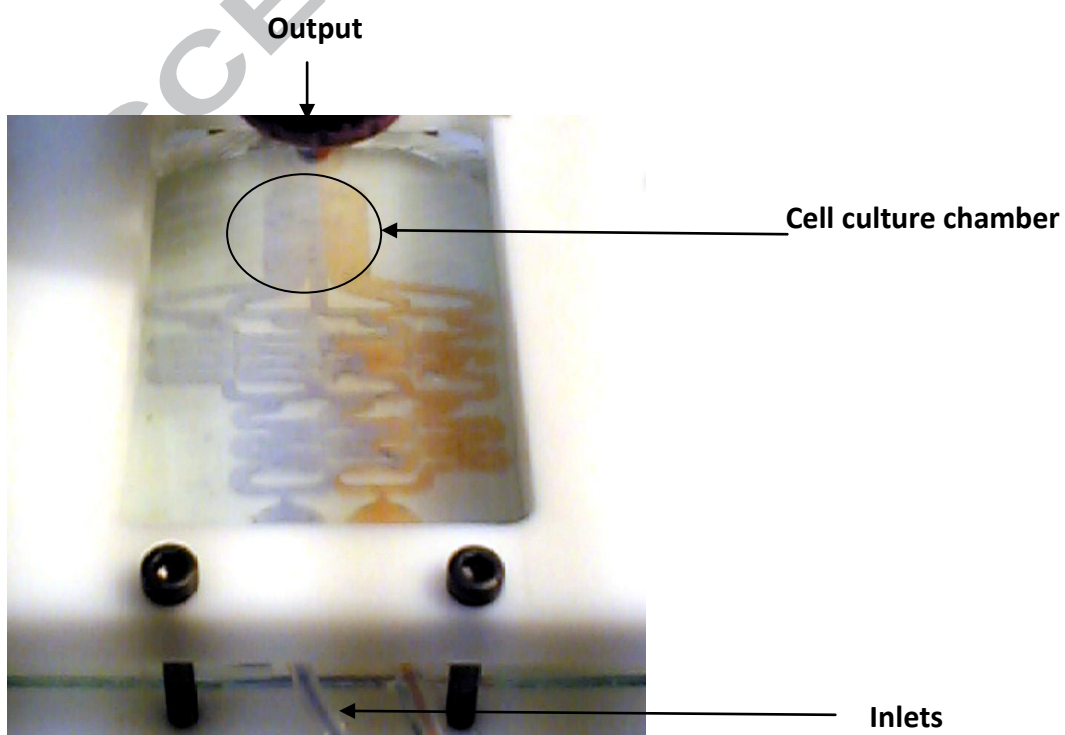
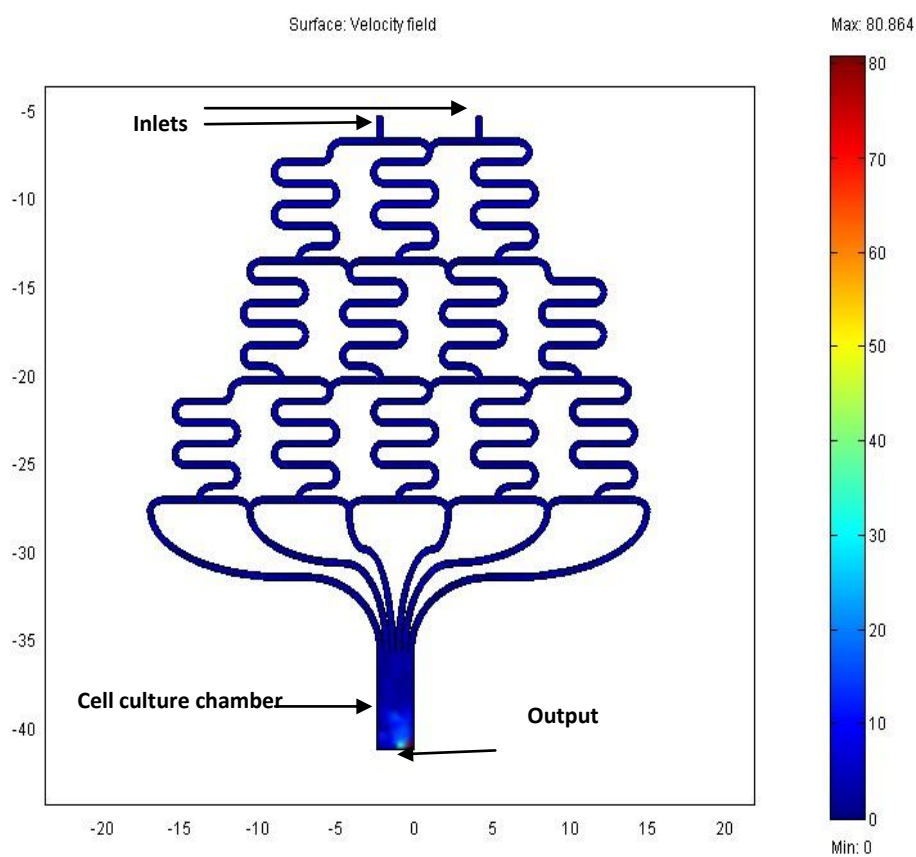


Figure 2: (a) 2D profile of velocity (b) 3D-gradient concentration in the Gradient Maker bioreactor

(a)



(b)

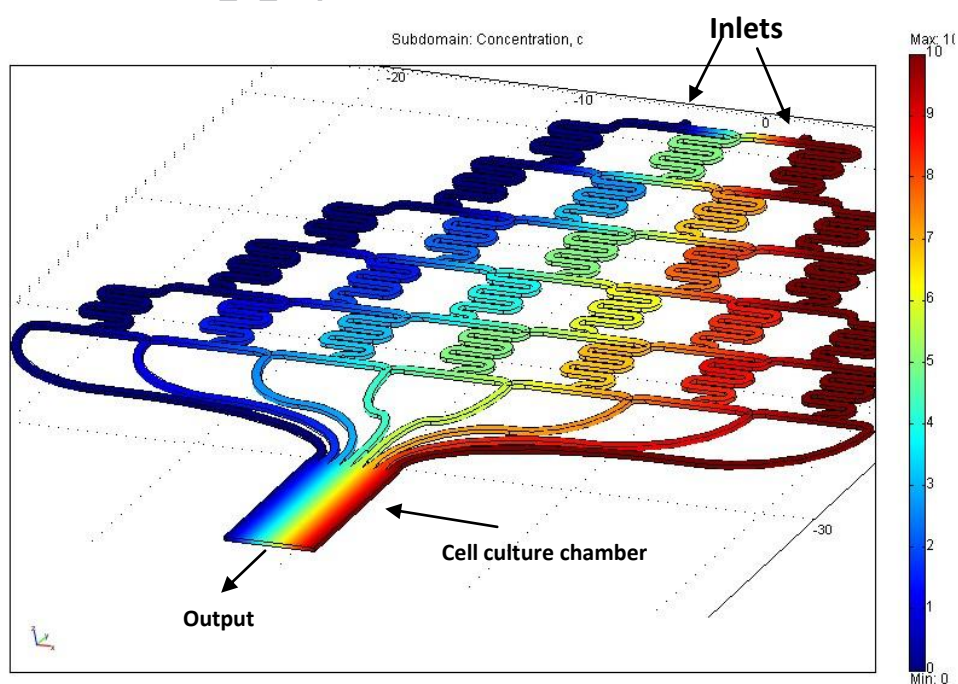


Figure 3: Imaging processing of gradient in the cell treatment area using red and blue dyes

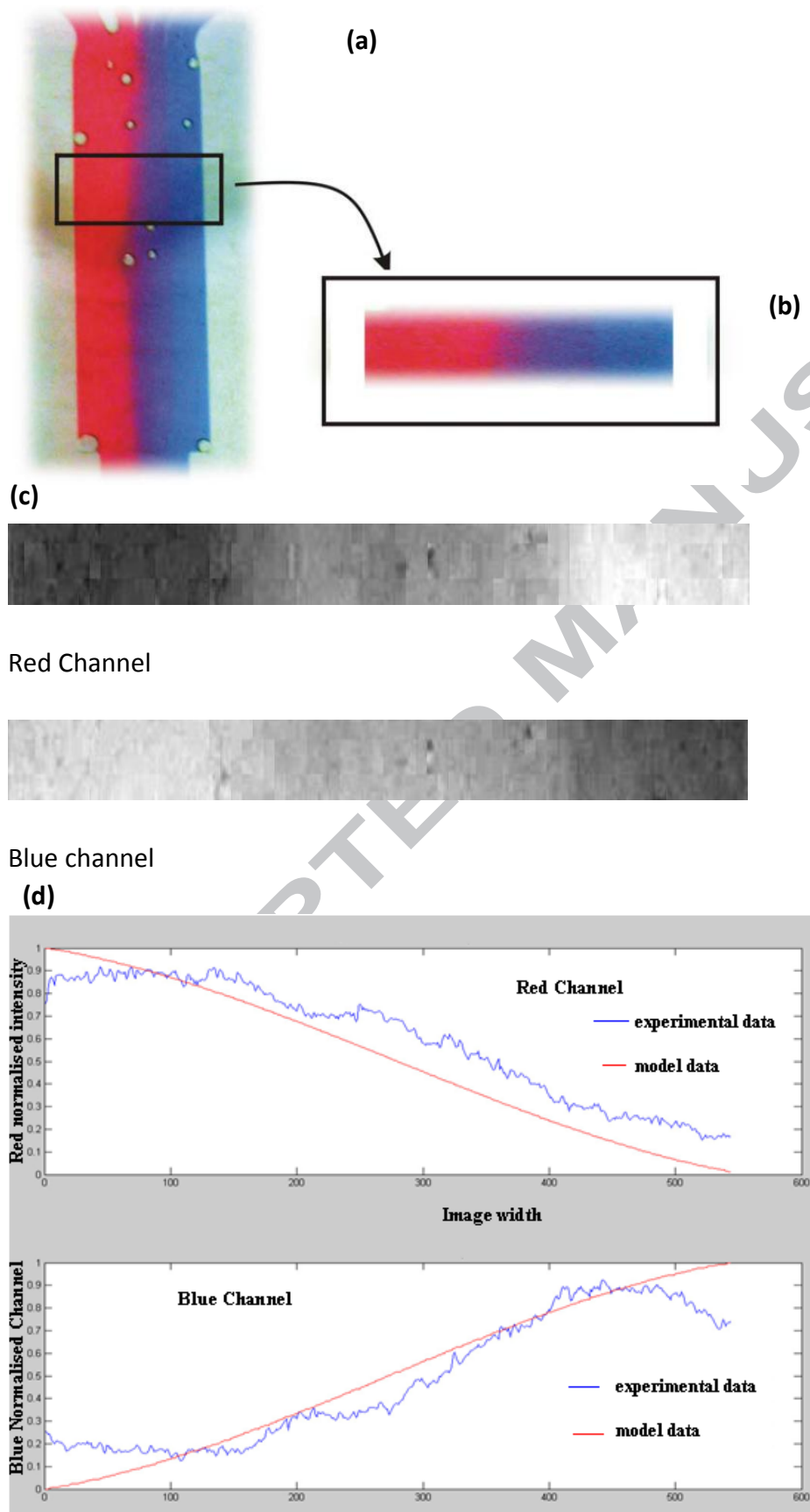


Figure 4: (a) A single static pattern produced using the G&M Model in the gradient maker bioreactor (figure 5a); (b) a periodic pattern, as described by G&M, produced in the gradient maker bioreactor (figure 5b).

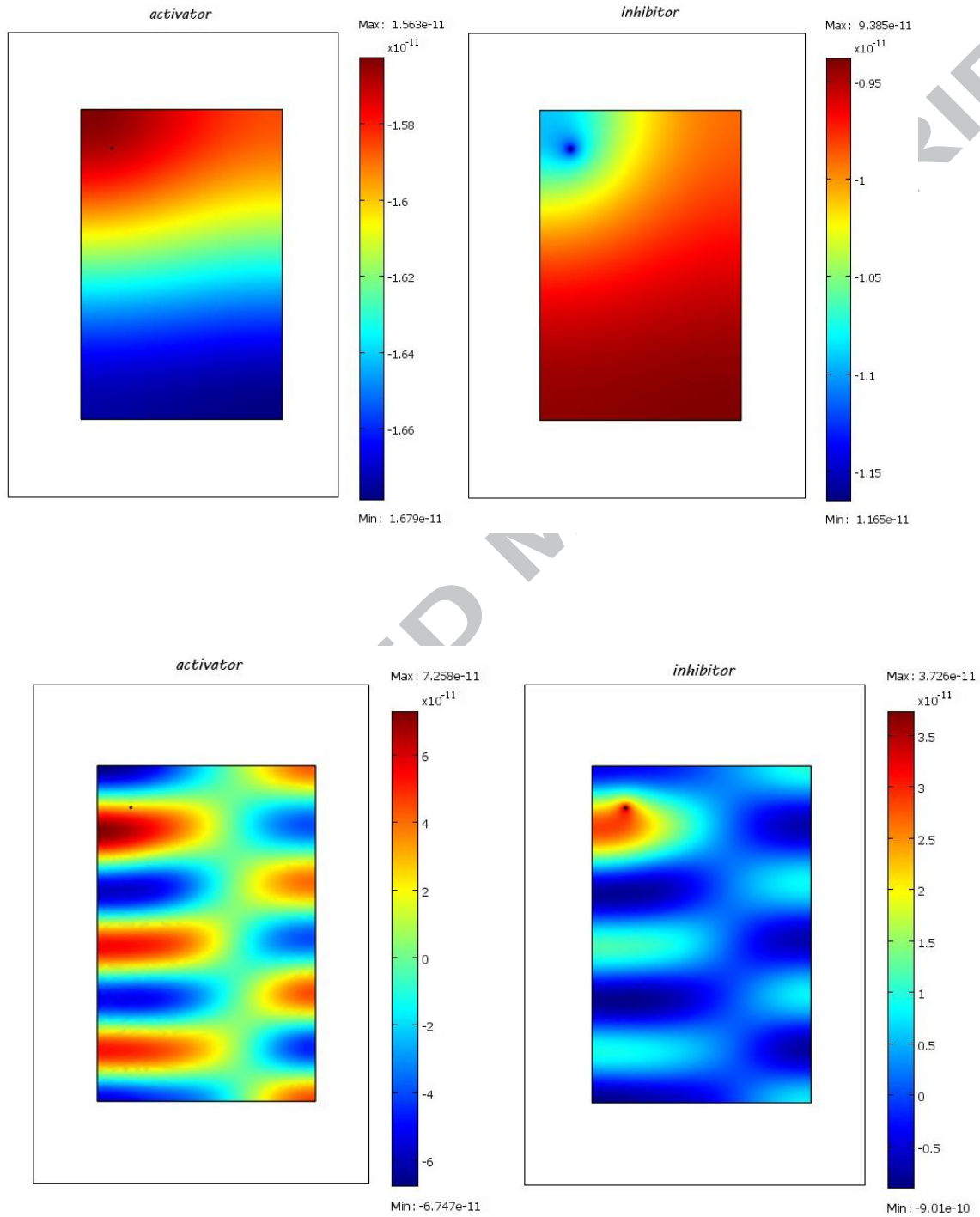


Figure 5: Activator pattern obtained by the G&M model simulation in flow conditions.

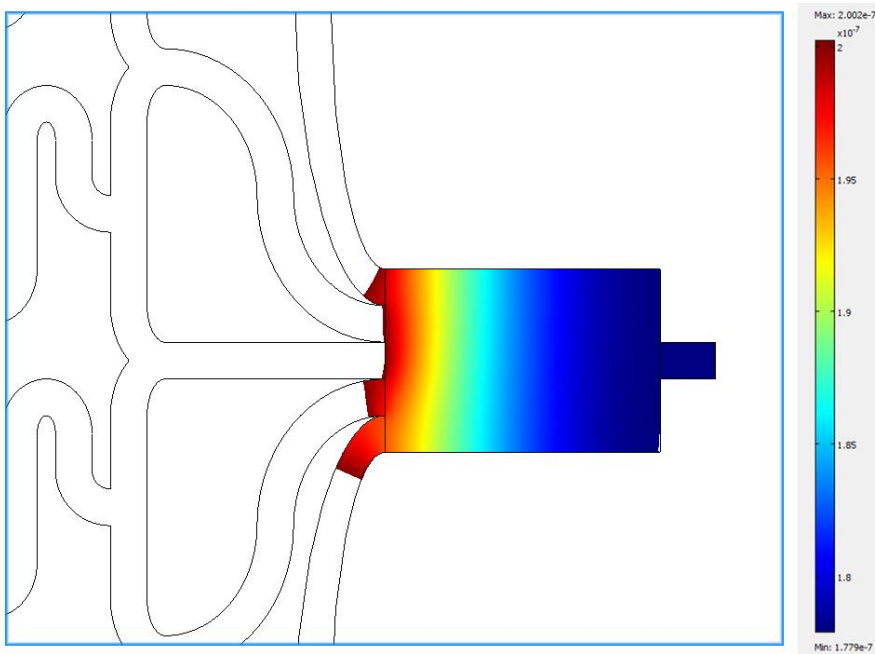
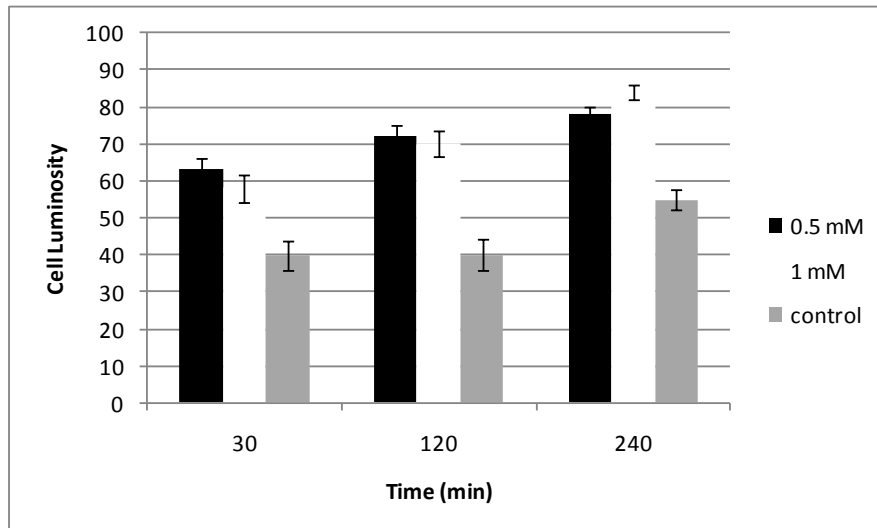
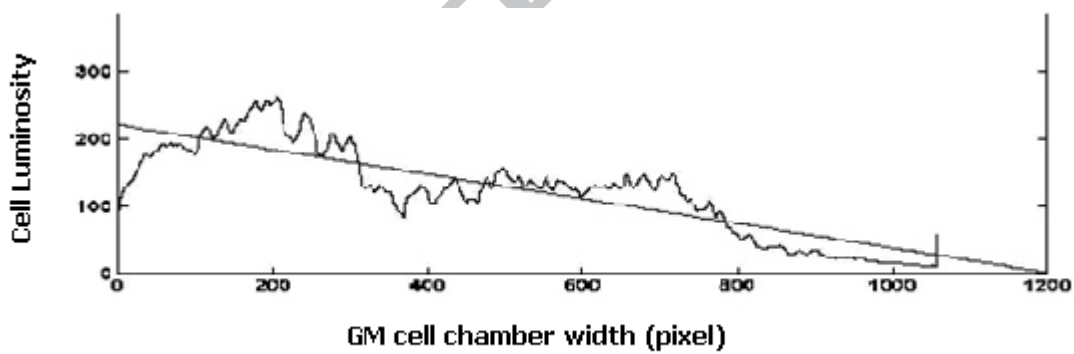


Figure 6: (a) Cell luminosity in static conditions as function of exposure time, (b) HUVEC cell luminosity in dynamic conditions across the GM cell chamber, (c) Fluorescent intensity of DAPI labeled HUVEC exposed to Hydrogen Peroxide gradient across GM cell chamber.

(a)



(b)



(c)

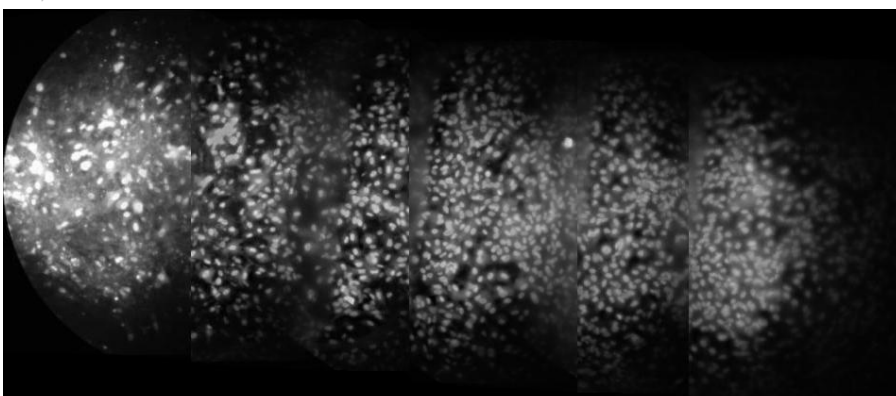


Figure 7: Propidium iodide staining of undifferentiated C2C12 cells in the cell treatment area in comparison with drug-free static control

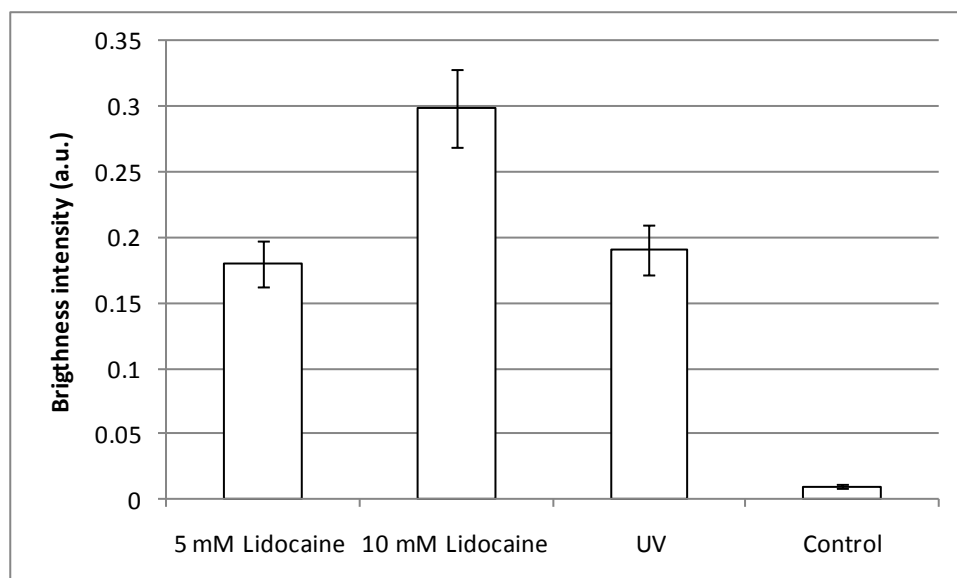
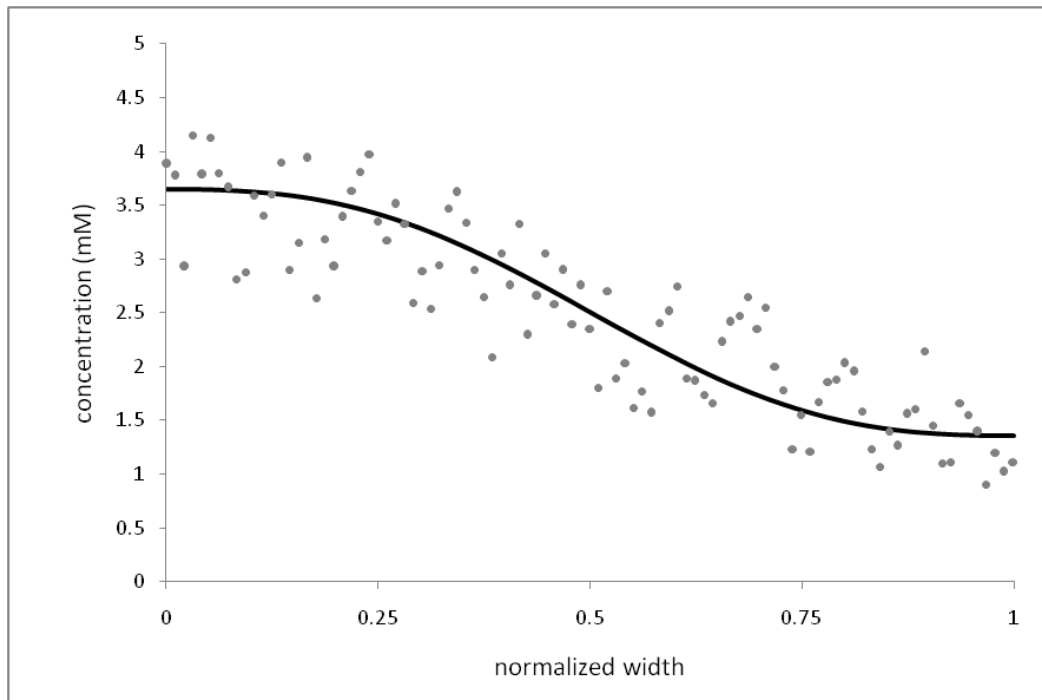


Figure 8: (a) Concentration profile across the chamber after 6 hours exposure to lidocaine. The graph was obtained by fitting the processed data to the concentration profile, as calculated by the FEM model. (b) Brightness as function of lidocaine concentration.

(a)



(b)

