Generating multiplex gradients of biomolecules for controlling cellular adhesion in parallel microfluidic channels†

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Here we present a microfluidic platform to generate multiplex gradients of biomolecules within parallel microfluidic channels, in which a range of multiplex concentration gradients with different profile shapes are simultaneously produced. Nonlinear polynomial gradients were also generated using this device. The gradient generation principle is based on implementing parallel channels with each providing a different hydrodynamic resistance. The generated biomolecule gradients were then covalently functionalized onto the microchannel surfaces. Surface gradients along the channel width were a result of covalent attachments of biomolecules to the surface, which remained functional under high shear stresses (50 dyn/cm\textsuperscript{2}). An IgG antibody conjugated to three different fluorescence dyes (FITC, Cy5 and Cy3) was used to demonstrate the resulting multiplex concentration gradients of biomolecules. The device enabled generation of gradients with up to three different biomolecules in each channel with varying concentration profiles. We were also able to produce 2-dimensional gradients in which biomolecules were distributed along the length and width of the channel. To demonstrate the applicability of the developed design, three different multiplex concentration gradients of REDV and KRSR peptides were patterned along the width of three parallel channels and adhesion of primary human umbilical vein endothelial cell (HUVEC) in each channel was subsequently investigated using a single chip.

1 Introduction

Over the past decade, engineering tools have increasingly been applied to design and develop \textit{in vitro} platforms to mimic important aspects of cellular microenvironments. This includes quantitative and reproducible characterization of cellular responses to chemical gradients.\textsuperscript{1} Significant advances in this regard were first made through conventional assays such as the Boyden chamber.\textsuperscript{2} Recently, lab-on-a-chip (LOC) devices have opened new avenues for \textit{in vitro} study of biological samples through creating gradients of biomolecules based on microfluidic principles. Forming multiple simultaneous gradients with highly resistant surface bonds in a simple and straightforward set-up, while maintaining a small device footprint, are important factors in designing microfluidic gradient-based devices for high throughput applications.

Several recent reports have implemented gradient-based microfluidic platforms to study biological phenomena such as cancer cell migration under the influence of well-defined chemokine and growth factor gradients,\textsuperscript{3–5} the quantitative relationship between directional guiding cues and chemotaxis of immune cells,\textsuperscript{6} stem cell differentiation due to spatial and temporal distribution of cytokines and growth factors,\textsuperscript{7} angiogenesis,\textsuperscript{8} selective cell adhesion and spreading\textsuperscript{9–11} and micro-organism response to surrounding chemical gradients.\textsuperscript{12}

Diffusion and convection are the two principle methods used to generate gradients of biomolecules in microfluidic devices.\textsuperscript{14,13–15} The first systematic microfluidic-based gradient device was introduced by Jeon \textit{et al}.\textsuperscript{16} This tree-like design repetitively mixes and splits solutions of different concentrations, until several laminar flows, each possessing a percentage of the initial concentration, converge within a single channel. A stable gradient is then generated through diffusion between converging laminar flows perpendicular to the flow direction. Subsequently, Whitesides \textit{et al}.\textsuperscript{16–18} reported on different configurations of this basic design to create gradients in microfluidic channels. Theoretical modelling and simulation of this design and similar devices are also available in the literature.\textsuperscript{19–22} Several studies have since modified this design to create more precise, rapid and well-controlled gradient profiles.\textsuperscript{4,23–27} However, the tree-like platform contains a series of microfluidic channels specifically dedicated to gradient generation which results in a big device footprint—thus minimizing the space available for experiments.\textsuperscript{25} Irinia \textit{et al}.\textsuperscript{28} also reported a microfluidic design to generate desired non-linear concentration gradient profiles based on parallel linear shape barriers on the flow direction. Recently
Selimovic et al. proposed a simpler design using asymmetric channels with two inlets to address some limitations of the tree-like design and successfully generated gradients of one biomolecule.

The tree-like design was implemented to generate multiplex gradients of two different biomolecules. Multiplex concentration profiles of biomolecules was also reported using a scanning microfluidic probe in open volumes.

Many gradient dependent biological applications such as cell adhesion or cell migration experiments require bio-functional and stable surface gradients. To produce surface gradients, most devices rely on physical adsorption of biomolecules onto surfaces. Adsorbed biomolecules, however, tend to detach unpredictably and are particularly problematic in microfluidic systems operating under flow. Physical adsorption can also cause random orientation of biomolecules and alter their bio-functionality. To address this limitation, covalent binding using silane solutions are employed. This method creates active groups on microfluidic substrates for subsequent attachment of biomolecules. However, stringent experimental conditions are required that in turn reduce the versatility of the silanization for the development of time effective interfaces in microfluidic devices.

Herein, we introduce a microfluidic platform for generating multiplex concentration gradients of biomolecules based on hydrodynamic resistances. The microfluidic design generates simultaneous multiplex concentration gradients of several biomolecules with varying concentration gradient slopes in up to seven parallel channels. The main advantage of such a device is the ability to perform high throughput gradient-dependent experiments in parallel channels with multiplex gradients in each channel. Furthermore, nonlinear polynomial (parabolic) gradients could also be produced using this design. The other important feature of this design is observing 2-dimensional gradients in which concentration gradients could be produced along both the length (800 μm) and width (600 μm) of the channel.

To produce highly resistant covalently bound surface gradients, amine functional groups were immobilized on the target microchannel surface using micro-contact printing. Micro-contact printing of amino silanes not only drastically reduces the time and effort required for silane patterning of the surface; but also produces a homogenous monolayer of functional amine groups on the microchannel surface. This results in producing covalently bound homogenous surface concentration gradients of biomolecules.

Solutions of IgG antibody conjugated with three different fluorescent dyes were used to demonstrate the produced surface gradients. The antibody attachment to the surface was achieved through chemically stable and robust bonds, which remain stable under high shear stress. Using this device, different multiplex concentration gradients of REDV and KRSR peptides, along the channel length. For a microchannel of length \( L \), hydrodynamic diameter \( D_h \) and cross section area \( A \), the hydrodynamic resistance can be defined as:

\[
R_h = \frac{\Delta P}{Q}
\]

(1)

in which \( Q \) is the flow rate and \( \Delta P \) is the pressure drop along the channel length. For a microchannel of length \( L \), hydrodynamic diameter \( D_h \) and cross section area \( A \), the hydrodynamic resistance can be defined as:

\[
R_h = \frac{f \rho L}{2D_h^2A}
\]

(2)

in which \( \mu \), \( f \) and Re are dynamic viscosity, friction factor and Reynolds number, respectively. Using eqn (1) and assuming the...
flow rate at each channel to be the product of channel cross section area and average velocity, the hydrodynamic resistance of a straight channel with rectangular cross section ($w$: width and $h$: height, assuming $w \sim h$) can be calculated as: 31,32

$$R_h \sim \frac{12 \mu L}{wh^3} \left[ 1 - \frac{h}{w} \left( \frac{192}{\pi} \sum_{n=1,3,5} \frac{1}{n^4 \tanh \left( \frac{n \pi w}{2h} \right)} \right) \right]^{-1} \quad (3)$$

Therefore in straight microchannels (in a laminar, steady and incompressible flow) the hydrodynamic resistance depends only on the microchannel geometry (eqn (3)).

For curved microchannels, different empirical equations have been proposed. For curved microchannels the hydrodynamic resistance becomes more complicated for curved channels. The target channels designed for multiplex gradient generation in this work (Fig. 1a), consist of both curved and straight sections. Therefore simulation of the velocity field was performed to optimize the hydrodynamic resistances in each channel prior to device fabrication, and predicted flow rates were later verified by experiment.

2.4 Computational Fluid Dynamics (CFD) analysis

Full scale simulation of the experimental setup was performed using a 3D model of the microfluidic design. The governing equations used for computational fluid dynamic (CFD) analysis are as follows:

\[
\nabla[(\eta(\nabla u + (\nabla u)^T)) + \rho(u, \nabla u) + \nabla p = 0 \quad \quad \text{(4)}
\]

\[
\nabla \cdot u = 0 \quad \quad \text{(5)}
\]

Eqn (4) and (5) represent the Navier–Stokes and continuity equations, respectively, assuming incompressible steady state conditions. $u$ is the velocity vector, $p$ is pressure, $\eta$ is the dynamic viscosity and $\rho$ is liquid density. CFD analysis was performed using COMSOL (COMSOL Inc., Burlington, MA) software. Simulations were performed using a 12-core computer with 24GB of memory running on a Linux operating system. The geometrical model was generated in AutoCAD and imported into COMSOL interface. Mesh density was designed to be finer.
at the areas targeted for gradient generation. Iterations were performed until the residual sums for velocity components reached values $< 10^{-3}$.

The fluid inside the channels was assumed to be Newtonian, with the physical properties of water. The applied boundary conditions were as follows:

1. Inlet boundary conditions:

$$u_i = u_0, \ i = 1, 2, 3, \ldots, 7$$

2. Wall boundary conditions:

$$u_w = 0$$

3. Outlet boundary conditions:

$$P_o = 0$$

in which $u_0$ is equal to $Q/A$, where $Q$ is the applied flow rate and $A$ is the cross section of the inlet channels. Inlets and outlets were specified as the entire opening at the beginning and end of each geometry, as opposed to the experimental platform in which holes are in the channel roof.

### 2.5 Rapid generation of covalent surface gradients

#### 2.5.1 Micro-contact printing of amine groups.
Detailed information about covalent surface functionalization using micro-contact printing protocol has been explained elsewhere. Briefly, flat PDMS stamps were used to micro-contact print APTES onto the glass substrates in the areas which form the target microchannels. The PDMS stamp was plasma treated for 1 min using oxygen plasma (60 s, 200 W, 200 mTorr $O_2$). It was then covered with 10 μl of 2% APTES solution in ethanol at room temperature. To avoid evaporation of the APTES solution, a cover slip was placed on the stamp for 1 min. After rinsing with 70% ethanol solution for 15 s and drying under nitrogen gas for 30 s, the stamp was gently brought into contact with the predetermined areas on glass substrate for 5 s. Immediately after printing the APTES solution, the plasma treated PDMS substrate (μFN) was brought into contact with the micro-contact printed glass substrate to form the microfluidic device (Fig. 1b).

The device was then placed in an oven at 100°C for 60 min to ensure that the covalent binding of APTES with glass proceeded through condensation of hydrogen bonded silanol groups.

#### 2.5.2 Device assembly and covalent surface functionalization.
Glass substrates were placed in piranha (H$_2$O : H$_2$SO$_4$, 1 : 3 v/v) solution for 10 min, rinsed extensively with deionized water, and dried under a nitrogen stream. Two substrates forming the microfluidic device (PDMS and glass) were plasma treated followed by micro-contact printing of (APTES) onto the glass substrate (Fig. 1b). Immediately after microcontact printing, the glass and PDMS substrates were attached to form an irreversible microfluidic device. The platform was then placed in an oven at 100°C for 60 min. $N$-hydroxysuccinimide (NHS) was used along with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to crosslink the biomolecules to the amino-silanized patterned surface. EDC (2 mg ml$^{-1}$) and NHS (5 mg ml$^{-1}$), dissolved in PBS, were incubated over the main channel for 20 min to activate the printed amine groups on the glass side of the microfluidic channel. After creating active amine groups on the surface, laminar flow streams of biomolecules were passed through the device to create solution gradients followed by covalent attachment of the biomolecules onto the channel surface.

For multiplex gradient generation, Cy3 conjugated IgG was introduced from inlets 1, 5 and 7, Cy5 conjugated IgG from 3rd and 6th inlets and FITC conjugated IgG from 2nd and 5th inlets. Concentration of antibody solutions was 10 μg ml$^{-1}$ except for the 7th inlet which was 20 μg ml$^{-1}$. In addition, outlet of the 3rd target channel was pressurized to block the flow through this channel and create the highest hydrodynamic resistance at this channel. The aforementioned laminar flow streams were applied at a flow rate of 1 mm s$^{-1}$ for 60 min. The channels were then rinsed with PBS at a flow rate of 10 cm s$^{-1}$ (50 dyn/cm$^2$) at each inlet channel.

### 2.6 Fluorescence microscopy

Generated concentration gradient profiles at each target channel were captured using an inverted fluorescence microscope (Nikon TE 2000-U). IgG Antibody conjugated with Cy3, Cy5 and FITC fluorescent dyes were observed through appropriate filters. All images were recorded using a CCD camera (Photometrics CoolSNAP HQ2) coupled to an optical microscope. Images were analyzed using ImageJ (MacBiophotonics) software.

#### 2.7. Cell culture and cell adhesion onto multiplex peptide gradients

Cell adhesion experiments were conducted using primary human umbilical vein endothelial cells (HUVEC). Cells were cultured in 200 (M-200-500) media with low serum growth supplement (LSSG), and 1% PS. Cells were grown in a humidified incubator at 37°C and supplemented with 5% CO$_2$ for 5 days and then trypsinized with 0.25% trypsin-EDTA (Gibco, USA) and used in the experiments. Cell suspensions were centrifuged at 200g and then re-suspended in media to a concentration of $1 \times 10^5$ cells ml$^{-1}$.

Three different multiplex surface gradient profiles of REDV and KRSR peptides were produced in three of the target channels to investigate HUVECs adhesion to different gradient profiles under flow conditions. After generating surface gradients, outlets of the target channels were used as inlets to ensure that an equal number of cells with equal flow rates are passed through each channel. Target channel surfaces were washed by flowing sterile PBS. BSA was then passed through the channels and incubated for 30 min to avoid nonspecific cell adhesion. Cells were then introduced into the channels at a flow rate of 0.25 μl min$^{-1}$ for 40 min. Finally, channel surfaces were rinsed by flowing cell media.

### 3 Results and discussion

#### 3.1 Gradient generation principle and CFD analysis

Concentration gradients of biomolecules were generated within parallel channels by varying hydrodynamic resistances. First, laminar flows containing solutions of different biomolecules are introduced through seven inlet channels. These inlet channels...
merge to compose a main channel. The main channel then feeds seven separate parallel channels labelled as “target channels” in which gradients are generated. In this design, hydrodynamic resistances were generated through embedded curves and differences in the lengths of channels. Figure S2† shows the effect of curved sections embedded in the design. In this figure, simulation results for flow rates in the proposed design are compared to a design consisting of straight target channels while maintaining the same length.

Imposed differences in hydrodynamic resistances of target channels result in different flow rates through each target channel. This provides control over the distribution of biomolecules into the target channels. To enhance the distribution of biomolecules, one or several target channels are pressurized to impose the highest possible hydrodynamic resistance. This generates a much higher velocity component perpendicular to the flow direction (in Y direction) facing the blocked channel. As such, no gradients are generated in the blocked channel. Interestingly, hydrodynamic resistances of target channels can be adjusted to produce 2-dimensional gradients in the main channel prior to entering the target channels in both X and Y directions.

It is well acknowledged that diffusion of biomolecules between different laminar flow streams occurs within the main channel at the onset of the gradient generation process. It is important to outline however, that this effect is later offset by advection forces (attributed to the action of Y-component of the velocity vector), which is the dominant part of the design that leads to several different gradient profiles on the same device.

To produce surface concentration gradients, functionalization is not affected by increasing flow rates up to 3 mm s⁻¹. The biomolecules are covalently attached to the surface through activated amine groups, thus yielding gradients of biomolecules in each target channel. Depending on the inlets, the biomolecule concentration, and associated flow rates, gradients of 1–3 biomolecules of choice with different concentration profiles can be generated in the target channels.

Fig. 1c shows simulation results for the velocity field in which an average flow rate of 1 mm s⁻¹ was applied at each of the inlets. The outlet of the 3rd target channel has been pressurized to stop the flow through this channel. This figure illustrates how the velocity in the main channel is altered as the flow approaches the target channels. Fig. 1d represents the Y-component of the velocity vector. After entering the target channels, the velocity profile changes into the well-known parabolic shape as expected. Further from the target channels, the Y-component of the velocity vector is zero and as the flow moves towards the target channels, it becomes more pronounced. In this region, Y-velocity varies depending on each channel it is facing, which in turn changes the flow direction, thus orienting the flow to the target channels (see ESI† Movie 1).

After entering the target channels, the Y-velocity decreases inside all seven channels. Fig. 1e represents the overall flow velocity vectors in which the length of the arrows is proportional to the magnitude of the velocity vector. Fig. 1f shows the density and distribution of the streamlines at the entrance of the target channels. It is clear that streamlines are much denser in the channels with lower resistance, leading to higher concentrations of biomolecules in these channels. In addition, the measured experimental results for the flow rate in each of the target channels, corresponds well to the predicted results by simulation (Fig. 1g). Based on the simulation results and experimental measurements, relative hydrodynamic resistances of each target channel can be determined (supporting information eqn S1, eqn S2 and Table S1†). Applying different experimental conditions (flow rate, concentration of biomolecules and hydrodynamic resistances) desired combinations of fixed multiplex gradient profiles in all or some of the target channels can be achieved.

3.2 Multiplex surface gradients

Fig. 2b shows the superimposed fluorescence microscopy image of generated multiplex surface concentration gradients. Cy5, FITC and Cy3 conjugated IgG antibodies were used for this purpose. The normalized fluorescence intensity across the widths of five selected target channels is presented in Fig. 2a–f. It should be noted that the fluorescence intensity of each dye has been normalized independently for each channel. Four target channels (1st, 4th, 5th and 6th) contain patterned gradients of three different antibodies. Magnified superimposed fluorescence microscopy images for the 4th, 5th and 6th channels are shown in Fig. 2g. Fig. 2h and 2i depict the 5th and 6th channels containing FITC-Cy3 and Cy5-Cy3 conjugated IgG, respectively.

Analysis of fluorescence intensities obtained for FITC, Cy5 and Cy3 conjugated antibodies in each of the target channels was performed (Fig. 3). Fluorescence intensities in target channels were calculated from a single image captured for each fluorescence dye in order to be able to compare intensities in different channels. As shown in Fig. 3, different gradient slopes of each antibody can be produced using parallel channels with varying hydrodynamic resistances. Each target channel provides a different maximum concentration of each antibody (shown with fitted polynomial gray lines in Fig. 3). These results clearly indicate the possibility of patterning each of the target channels with multiple biomolecules while obtaining different concentration gradient profiles.

One of the most important advantages of this microfluidic design is the ability to achieve simultaneous multiplex gradient profiles and concentration ranges in separate parallel channels, as opposed to the previously proposed designs in which only one concentration profile could be obtained at a specific time. This is beneficial in performing high throughput gradient dependent experiments where different concentration ranges and profile slopes are required. The multiplex surface gradients, produced via covalent chemical bonds, remain stable under high shear stresses. This provides an important advantage especially for applications where high shear stress is required. The procedure used for forming the irreversibly sealed microfluidic device also underlay operation of the chip at high flow rates.

3.3. Nonlinear and 2-dimensional concentration gradients

Although generating multiplex gradients with different profile slopes and concentrations in target channels would be beneficial, particularly for high throughput applications, nonlinear concentration profiles are often required. The proposed design successfully demonstrates the ability to produce different profile shapes of surface concentration...
gradients. Fig. 4 shows three different gradient profiles obtained for Cy5 conjugated antibody. Fig. 4a was obtained by applying the same hydrodynamic resistances applied in section 3.2.

Different experimental conditions can be applied to produce parabolic shape gradients. Two experiments are presented in which polynomial concentration profiles of Cy5 conjugated IgG were generated. For this purpose, the 2nd, 3rd, 5th and 6th channels were blocked. In the first experiment (Fig. 4b and 4e), the Cy5 conjugated antibody solution was introduced from the 4th inlet; while in the second experiment, it was introduced through the 3rd and 5th inlets (Fig. 4c and 4f). In both experiments, buffer was introduced from the other remaining channels. Results clearly show the possibility of obtaining polynomial (parabolic) gradient profiles by applying simple changes to the experimental conditions.

Distribution of the biomolecules prior to entering the target channels is also of interest. The flow distribution just before entering the target channels can also be manipulated and controlled by adjusting the resistances of these channels. This leads to generation of biomolecule gradients in more than one direction within the main channel (Fig. 4i). We report this observation as 2-dimensional gradient formation. Fig. 4i–j show the resulting 2-dimensional solution gradient of FITC-Cy3 and Cy5-Cy3 conjugated IgG antibody, respectively.

### 3.4 Cell adhesion on multiplex peptide gradients

Three different multiplex surface gradients of REDV and KRSR peptides were generated along the cross section of three target channels. Fig. 2 shows multiplex surface concentration gradients produced in the target channels with different concentrations and different gradient rates. a,c,d, e and f) show fluorescent intensities of patterned gradients of antibodies in target channels. b) Superimposed fluorescence microscopy image, g) magnified fluorescence image of channels 4, 5 and 6. h and i) Magnified fluorescence images of the 5th and 6th channels patterned with FITC-Cy3 and Cy5-Cy3 conjugated IgG antibody, respectively.
channels. HUVEC adhesion onto these respective surfaces was subsequently investigated. Cells adhered across the width of the channels in response to the concentration of REDV peptide. In addition, under the same experimental conditions and parameters, the cells did not respond to the KRSR surface gradients (Fig. 5). HUVEC cells did not also adhere to the surfaces functionalized only with KRSR peptide gradient without the presence of REDV peptide. Moreover, HUVEC adhesion was also investigated on generated REDV surface gradients in the main channel (Fig. 5j–l). Time lapse imaging of the cells for several hours after attachment showed that the cells remained viable (See ESI† Movie 2) and proliferated (See ESI† Movie 3).

It is important to note that cell adhesion density is directly proportional to REDV peptide surface concentration (Fig. 5). Endothelial cells were previously reported to attach onto surfaces functionalized with REDV peptide.37–39 Our results are not only in full accordance with the literature, but also demonstrate the degree of HUVEC adhesion through variation of REDV peptide concentration under flow conditions, as well as the presence of surface gradients of KRSR peptide. The introduced method may also be implemented for selective and controlled cell patterning at specific areas across the channels width.

These results indicate that the microfluidic chip can be implemented to investigate cell adhesion on multiplex peptide surface gradients. The developed design can further be used for in vitro biological applications requiring concentration gradients of multiplex biomolecules such as selective cell adhesion and cell sorting. Furthermore, implementing such a design can be applied to investigations of cellular response to gradients of multiplex stimuli on the surface or in suspension to study migration, differentiation, proliferation or screening against different drug dosages.

4. Conclusion

A novel approach for performing high throughput studies requiring gradients of various chemical agents for cellular analysis is presented. This was addressed by the innovative...
design of a chip in which different microchannels provide different concentrations and varying distributions of multiple biomolecules. Our results show that this microfluidic design provides highly resistant and durable gradients of multiple biomolecules in parallel microfluidic channels, each containing different concentrations and gradient slopes by employing different hydrodynamic resistances. Covalent chemical surface gradients were also produced through micro-contact printing of amine groups onto glass substrates prior to assembling the microfluidic device. These surface concentration gradients remained stable and functional under high shear stresses. To illustrate the versatility of the design, the same IgG antibodies, conjugated to three different fluorescence dyes, were employed. Multiplex gradients were generated simultaneously in parallel microchannels, each possessing a different concentration and gradient slope, thus providing an interface for high throughput analysis of gradient dependent biological processes. To further demonstrate the capabilities of the design, multiplex surface gradients of REDV and KRSR peptides were produced in three parallel channels and HUVECs adhesion was investigated. HUVECs responded favourably to REDV concentration gradients while displaying poor cell adhesion onto KRSR gradients within the same channel. The proposed approach is straightforward, rapid, reliable, cost effective and consumes less reagents. It could facilitate future developments in the field of integrated biosensor or lab-on-a-chip devices. Such technologies are anticipated to become routine analytical tools to study adhesion based cell sorting and patterning, as well as in the development of inexpensive methods for screening clinical, environmental and food samples.

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