Lab on a Chip

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/loc

Lab on a Chip

RSCPublishing

PAPER

Three-dimensional Cell Manipulation and Patterning using Dielectrophoresis via a Multi-layer Scaffold Structure

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth October 2014,
Accepted ooth October 2014

DOI: 10.1039/xoxxoooooox

www.rsc.org/

H. K. Chu,[a] Z. Huan[ab] J. K. Mills[c] J. Yang[b] and D. Sun*[a]

Cell manipulation is imperative to the areas of cellular biology and tissue engineering, providing them a useful tool for patterning cells into cellular patterns for different analyses and applications. This paper presents a novel approach to perform three-dimensional (3D) cell manipulation and patterning with a multi-layer engineered scaffold. This scaffold structure employed dielectrophoresis as the non-contact mechanism to manipulate cells in the 3D domain. Through establishing electric fields via this multi-layer structure, the cells in the medium became polarized and were attracted towards the interior part of the structure, forming 3D cellular patterns. Experiments were conducted to evaluate the manipulation and the patterning processes with the proposed structure. Results show that with the presence of a voltage input, this multi-layer structure was capable of manipulating different types of biological cells examined through dielectrophoresis, enabling automatic cell patterning in the time-scale of minutes. The effects of the voltage input on the resultant cellular pattern were examined and discussed. Viability test was performed after the patterning operation and the results confirmed that majority of the cells remained viable. After 7 days of culture, 3D cellular patterns were observed through SEM. The results suggest that this scaffold and its automated dielectrophoresis-based patterning mechanism can be used to construct artificial tissues for various tissue engineering applications.

Introduction

Manipulation of biological cells is a fundamental process in the areas of cellular biology and biomedical science. Many cell-based research studies such as cell proliferation and cytotoxicity assays often require manipulating and positioning a large group of cells on a substrate to facilitate data collection, sampling and analysis. Common techniques that have been examined include mechanical, inertial, electrical, optical and magnetic methodologies.[1] In recent years, the ability to manipulate and organize cells into desired patterns has attracted interest from the tissue engineering community for the development of artificial tissues. To construct these artificial tissues in vitro, three-dimensional (3D) porous structures called engineered scaffolds are often utilized to provide a template for cells to adhere and gradually proliferate into functional tissues.[2,3] The design of these scaffolds plays a vital role to the control of the cellular pattern and the overall quality of the tissue substitute.

To date, a number of engineered scaffolds have been proposed by different research groups. [4-11] These scaffolds are usually made of natural or synthetic polymers such as poly lactic glycolic acid (PLGA), polycaprolactone (PCL), and hydrogels with different mechanical properties and geometric structures. In general, scaffolds fabricated with conventional micro-fabrication techniques such as particulate leaching, gas foaming and electrospinning can achieve high level of porosity; however, the pore size, the pore distribution, and the interconnectivity between pores are not easy to be controlled.[2,4,12] On the contrary, scaffolds fabricated using advanced three-dimensional fabrication techniques such as rapid prototyping, laser cutting and micromolding technique provide higher degrees of controllability over a number of design parameters so that a sophisticated scaffold design can be constructed to suit different applications including bone, muscle and cartilage tissue regenerations.[5-7,13] Due to the manufacturing limitations with the fabrication technologies, the dimensional resolution of these scaffolds is generally low and the ability to maximize the density of cells seeded on these scaffolds having relatively larger pores would become critical to assure the quality of the developed tissues.

In this work, a novel scaffold structure constituting multiple layers of bio-compatible materials is fabricated through the laser cutting technology. One unique feature of this structure is to incorporate the technique of dielectrophoresis (DEP) to

Lab on a Chip Accepted Manuscript

manipulate and pattern biological cells in the 3D domain. Under DEP, cells are automatically seeded and patterned within the porous interior part of the structure. The high initial seeding efficiency achieved will be beneficial to the construction of high-quality 3D artificial tissues. In general, dielectrophoresis is one of the non-invasive techniques that utilizes electric fields to manipulate a large amount of biological cells simultaneously.[14] To date, majority of the DEP research were carried out through a microfluidic chip[15] as the platform for applications such as sorting,[16,17] trapping,[18] and transporting[19] different biological cells. The present research aims to extend the DEP manipulation technique beyond the lab-on-a-chip environment and investigates batch manipulation of cells via dielectrophoresis for active seeding and patterning of an engineered scaffold structure.

In our previous work,[20] we first demonstrated the feasibility of utilizing DEP for patterning of cells through a two-layer structure. In the current paper, we extend the proposed DEP mechanism and incorporate into a multi-layer design for constructing multiple layers of three-dimensional (3D) cellular patterns with an electrode-integrated structure. Different process parameters were examined in order to determine their influence on the quality and viability of the 3D cellular patterns. To the best of our knowledge, research work on manipulating cells with a three-dimensional structure via dielectrophoresis has yet to be published and this paper is amongst the first to propose the incorporation of DEP in the design elements of a multi-layer scaffold structure to perform 3D cell manipulation and patterning automatically.

Materials and Methods

Cell patterning mechanism via Dielectrophoresis

The technique of using electric field for particle manipulation, also known as dielectrophoresis (DEP), has been studied extensively since the discovery by Pohl.[21] One key advantage of using dielectrophoresis over other techniques such as magnetic[22-24] and rotational[25] cell patterning is that the cells to be manipulated do not need to be treated with magnetic nanoparticle coating or kept in a stirring medium for a long period of time. When a cell is placed in a non-uniform field, the cell will become polarized and a dielectrophoretic force will be induced onto the cell. Depending on the surrounding medium, the DEP force from a more polarizable medium will direct the cells towards the high electric field regions (positive DEP phenomenon), whereas the DEP force from a less polarizable medium will repel the cells to the low electric field regions (negative DEP phenomenon). The magnitude of the force is specified as[15]:

$$F_{DEP} = 2\pi r^3 \varepsilon_m \,\mathrm{Re}\left[K(\omega)\right]\nabla E_{rms}^2 \qquad (1)$$

where r is the particle radius, ε_m is the permittivity of the medium, ∇ is the Del vector gradient operator, E_{rms} is the root mean square electric field, ω is the frequency of the electric field, and Re[K(ω)] is the real part of the Clausius-Mossotti, CM, factor.

One common practice for utilizing dielectrophoresis for patterning cells is to employ an electrode pair within a microfluidic chip to manipulate or repel the suspended cells towards the bottom side of electrode surface.[15,26-28] For instance, Albrecht *et al.*[29] and Gray *et al.*[30] have successfully applied the positive DEP technique to manipulate cells onto a circular and rectangular pattern, respectively, while Suzuki *et al.*[31] and Ho *et al.*[32,33] have employed the negative DEP technique to construct periodical and radial line patterns with cells. To incorporate dielectrophoresis for patterning cells onto the scaffold structure, we proposed to integrate multiple electrode pairs into the design to manipulate cells in the three-dimensional domain.

Multi-layer scaffold structure design

A multi-layer scaffold structure is proposed for automated manipulation of cells via dielectrophoresis (Fig. 1a). Each layer of the structure mainly serves for two purposes: to provide a platform for cells to adhere, and to use as an electrode for electric field generation. Each layer has a dimension of 15mm × 15mm with a thickness of 500μm. To prevent direct electrical contact between layers, the bottom side of each layer is coated

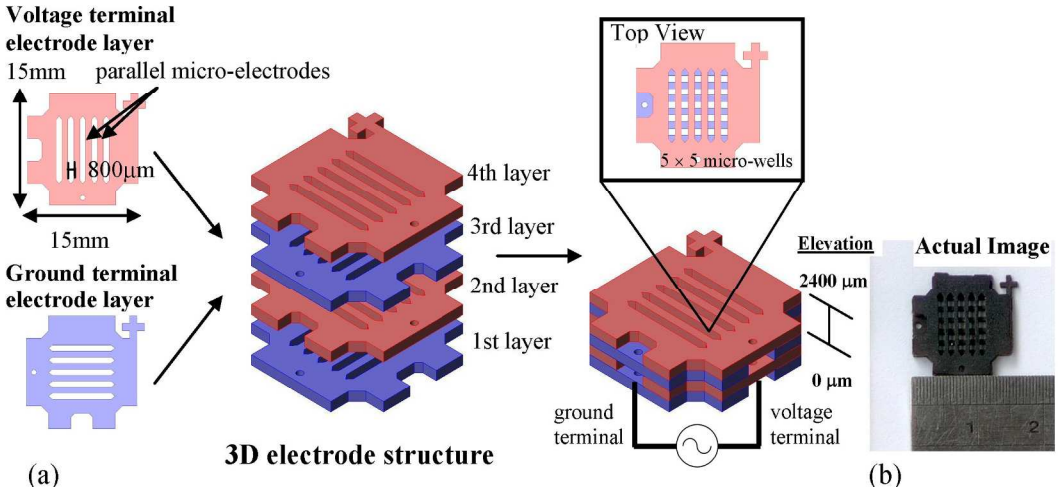

Fig. 1 (a) Schematic and (b) Photograph of the multi-layer scaffold structure for 3D cell manipulation

Lab on a Chip Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

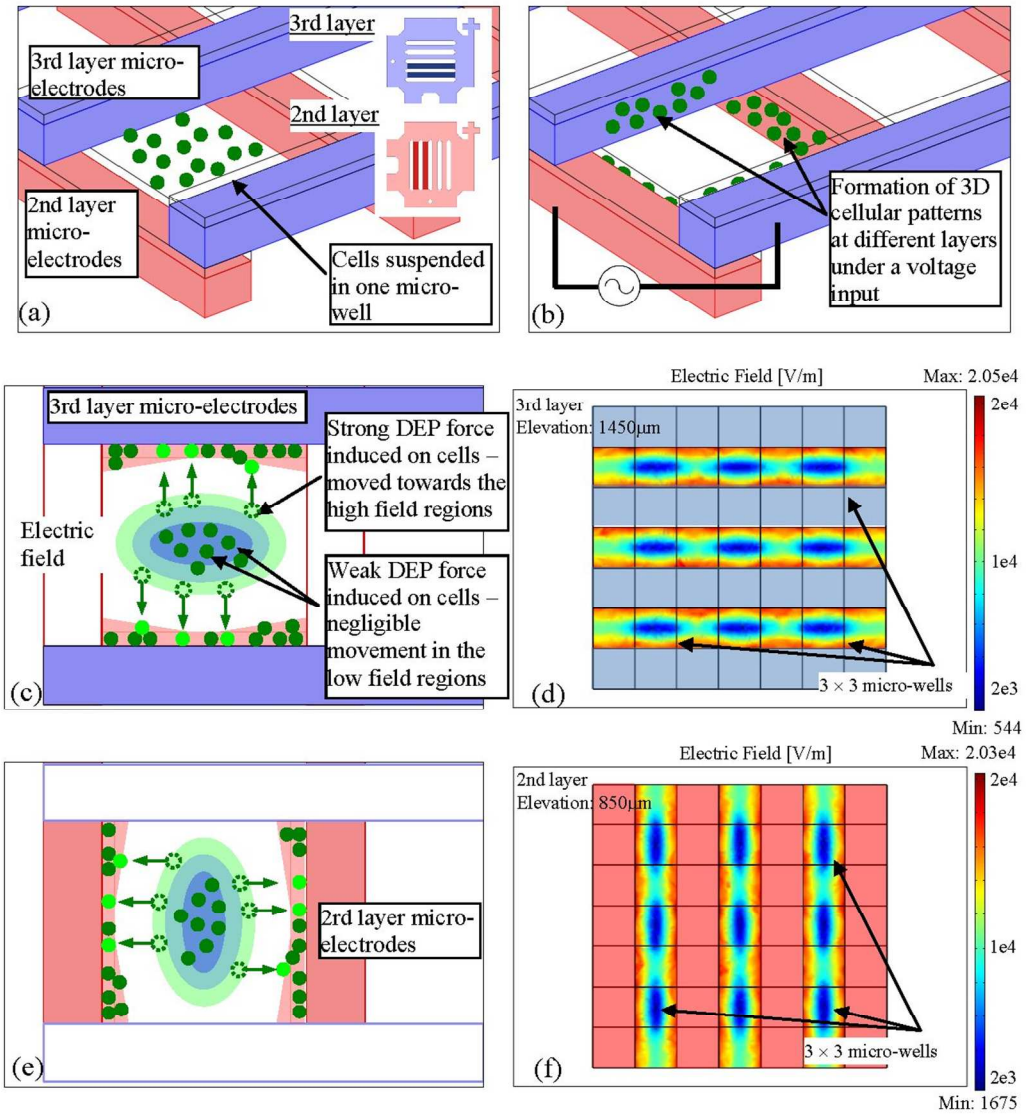

Fig. 2 DEP cell manipulation via the multi-layer scaffold: (a) Cells in one micro-well; (b) Formation of 3D cellular pattern; (c) Cell manipulation at the 3rd layer due to the electric field; (d) electric field simulation of the 3rd layer at 10V voltage input; (e) Cell manipulation at the 2nd layer due to the electric field; (f) electric field simulation of the 2nd layer at 10V voltage input

with a 100μm thick insulating material. Trenches of 800μm wide are cut from each layer to construct multiple parallel micro-electrodes of 800μm wide for cell attachment. To generate the required electric fields in the three-dimensional spatial domain, alternating voltage is supplied to the adjacent layers of the structure. After stacking multiple layers together, a 5×5 array of interconnected rectangular micro-wells (pores) is established within the scaffold structure for cell attachment and nutrient exchange (Fig. 1b).

When a droplet of the cell medium is pipetted onto the scaffold, DEP forces are induced on the cells to construct 3D cellular pattern throughout its multi-layer scaffold body (Fig. 2a and Fig. 2b). To enable effective cell manipulation via positive dielectrophoresis, the strengths of the electric fields along the parallel micro-electrodes must be higher than the surrounding medium. Finite Element Analysis (FEA) software, COMOSL, was used to model the electric field distribution under a voltage supply. Simulation results show that highly non-uniform electric fields can be generated in between the parallel micro-electrodes of each layer through this multi-layer configuration. As shown (Fig. 2c to Fig. 2f), relatively weak DEP forces are induced on biological cells located near the center of the micro-well (regions in blue) and the movement of these cells are negligible. In contrast, stronger DEP forces are induced on the cells located closer to the micro-electrodes. Thus, these cells are polarized and manipulated towards the high electric fields (regions in red), forming cellular patterns along the electrode surfaces at different layers (Fig. 2c and Fig. 2e).

Scaffold fabrication and assembly

A conductive bio-compatible polymer composite is used to fabricate the proposed multi-layer scaffold design. In this work,

Lab on a Chip Accepted Manuscript

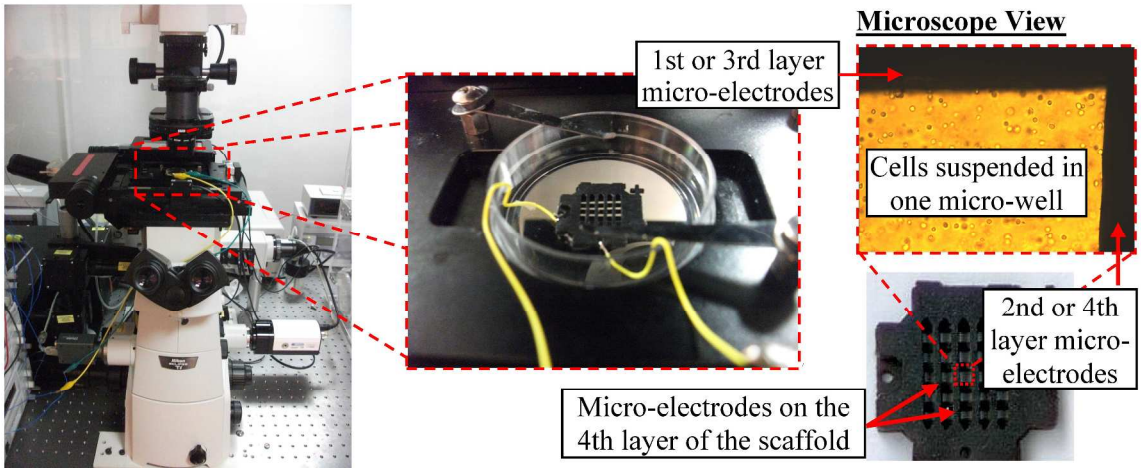

Fig. 3 Multi-layer structure on the Eclipse Ti inverted microscope by Nikon

polydimethylsiloxane (PDMS) elastomer (Sylgard 184, Dow Corning) is selected as the structural material. PDMS is a common biocompatible material for pharmaceutical and bio-medical applications and several research groups have also utilized this material to fabricate 3D scaffolds for culturing different types of biological cells. [34-37] To synthesize this biomaterial, approximately 5 grams of PDMS base were first mixed with the curing agent at a 10:1 ratio and then degassed in a vacuum desiccator to remove the trapped bubbles. To provide electrical conductivity, conductive fillers, carbon black (CB) nanopowders (VXC72R, Cabot), were then added to the mixture in an amount of 30% by weight. Nanosized carbon powder is chosen as it is less toxic than other carbon based conductive fillers such as single-wall or multi-wall nanotubes. [11,38-39] The CB-PDMS paste was shear-mixed thoroughly and sonicated in an ultrasonic bath for 1 hour. Afterwards, the CB-PDMS paste was casted onto a metal mold to form a 500μm-thick 10mm × 10mm sheet. The CB-PDMS sheet was cured in an oven at 70°C for 1 hour. To develop the insulating coating, 1 gram of PDMS was casted onto the sheet, forming an approximately 100μm thick insulating layer on one side of the CB-PDMS sheet. The sheet was then heat again to 70°C for 1 hour to allow the bi-layer sheet to cure completely. The CB-PDMS bi-layer sheet was then cut into an array of scaffold layers using a commercial CO_2 laser cut machine. The machined voltage and ground scaffold layers were finally stacked and bonded together via a plasma bonding machine.

Hardware setup

The entire experiment was carried out through an inverted microscope system (Fig. 3). The multi-layer scaffold structure was first bonded onto the 35mm Petri dish of the stage-top incubator of the system. A functional generator (GAG-809, GW Instek) was connected to an in-house amplifier circuit to amplify the sinusoidal voltage output to a maximum of $60V_{pp}$ at a frequency of 0 to 300kHz. Prior to the experiments, each structure was sterilized in a 70% ethanol solution for 30 minutes and then coated with Poly-L-Lysine (Sigma Aldrich) to enhance the surface affinity for cell attachment.

A low-conductivity DEP buffer (5% sucrose solution) was prepared as the temporary culture medium for DEP manipulation of the various biological cells examined in this work. Similar to the medium used by Ho *et al.*,[32] this medium needs to be less polarizable than the cells and provide the necessary osmotic balance to the cells.[40] Prior to the experiment, the scaffold structure first was rinsed with the DEP buffer and a droplet of the cell containing medium was pipetted to the structure for cell seeding and patterning. Three different types of mammalian cells, namely 3T3, 293FT, and Human Foreskin Fibroblasts (HFF), were considered for the experiments. 3T3 cells are mouse cells derived from mouse embryo tissue while 293FT and HFF are human cells from embryonic kidney and foreskin, respectively. Three-dimensional (3D) models of these cells cultured through the scaffold may be used to facilitate cell-based assays such as drug discovery[41] and transfection studies.[42] The concentration for each cell was adjusted to approximately 1.2×10^6 cells/mL for the seeding experiments.

Results and Discussions

In order to evaluate the process of 3D cell manipulation and patterning via the proposed structure, a series of experiments were conducted to examine the influence of different settings from the voltage source. In this work, the structure requires an input signal that enables efficient cell manipulation via positive dielectrophoresis while maintaining high cell viability. Input voltages in between 5V to 20V at mid-frequency range (50kHz to 300kHz) were examined and the formation of the cellular patterns at different layers as well as their viability were assayed.

Effect of supply voltage input on the multilayer structure

The influence on the voltage frequency for DEP cell manipulation with the proposed structure was first evaluated. According to Equation (1), the input frequency will affect the CM-factor value, where a transition from positive to negative dielectrophoresis could occur at high frequency. From the

Lab on a Chip Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

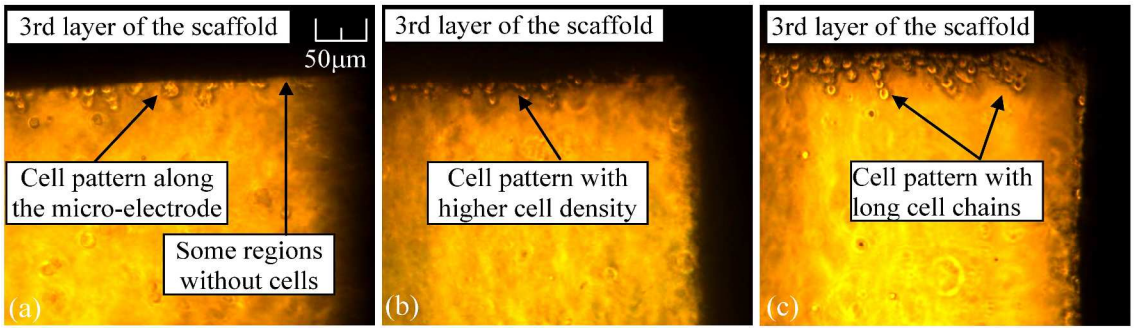

Fig. 4 Formation of the cellular pattern at the 3rd layer under: (a) 5V; (b) 10V; and (c) 20V voltage input

experiments, it was found that the scaffold structure operating at 100kHz or lower could have a low seeding efficiency due to a potential voltage short circuit between the layers. During the cell seeding and patterning process, the resistance value across the layers dropped dramatically from around 5MΩ to 100kΩ within 30 seconds. The sudden drop in resistance could be explained by charge buildup across the plasma membrane of the cells at such low voltage frequencies.[43] As a result of charge build up, the cells adhered to the structure surface would form alternative, conductive, short-circuit paths between the layers. For the three types of mammalian cells under examination, experimental results confirmed that a voltage frequency of 100kHz to 300kHz was sufficient to drive these suspended cells in the DEP medium towards the multi-layer structure for pattern formation. A voltage frequency larger than 300kHz was not considered due to the higher rate of transient hole formation in the cell membrane from electroporation, which could reduce cell viability.[44] Since the issue of charge build-up is not prominent at 300kHz, this voltage frequency was selected for the rest of the experiments.

The amplitude of the voltage input is known to have influence on the strength of the electric field, and thus the dielectrophoretic force induced on the cells for the manipulation. Voltage inputs in the range from 5V to 20V were examined. Although a low voltage input (5V) was capable of manipulating a number of nearby cells towards the scaffold body, the time required to construct an extensive 3D cellular pattern was relatively long. Since the induced DEP force induced was weak, the formation of the cellular pattern would heavily rely on the Brownian motion and medium flow to bring more cells closer to the nearby regions for the attachment. The cellular pattern of 3T3 cells created at 5V after 3 minutes of operation had regions that were not covered by cells (Fig. 4a). Such non-uniform cellular pattern might not be suitable for culturing into extensive tissues. In contrast, when a higher voltage (10V) was supplied, the cellular pattern could be formed in a shorter time with higher cell density and better distribution (Fig. 4b). This process was improved further when the voltage supply was further increased to 20V. Under this voltage, cells located farther away from the electrodes were also attracted and adhered to the structure, forming a dense, cellular pattern in one minute. In addition, more cell chains can also be observed (Fig. 4c), indicating the establishment of high electric fields at this voltage. The formation of the cellular patterns under for three specified voltages were recorded and included in the supplementary section. As observed from the experiments, relatively extensive cellular patterns were successfully constructed at each of the four layers of the structure (from the 1st layer to the 4th layer) indicating the ability of utilizing DEP to construct a 3D cellular pattern with the proposed structure.

Characterization of the 3D cellular pattern

The performance on seeding and patterning of cells throughout the entire structure was examined with the motorized platform of the inverted microscope. In order to enhance the contrast between the background and the cellular pattern, the 3T3 cells were stained with MitoTacker Red. The supply voltage was set

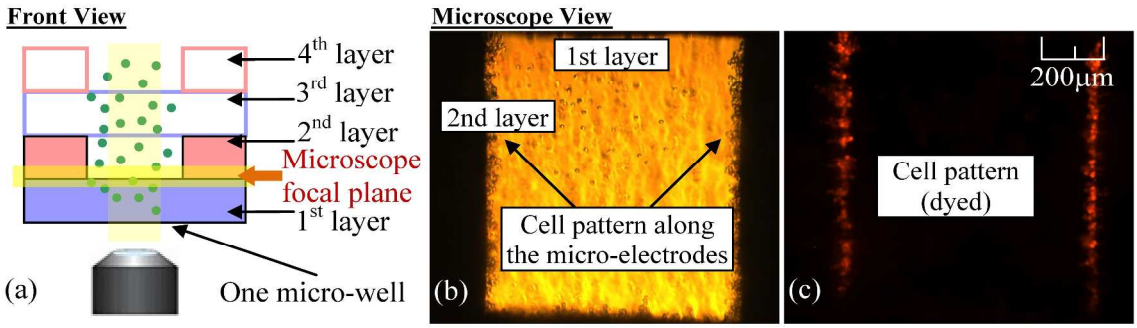

Fig. 5 3D pattern constructed from 3T3 cells: (a) schematic diagram; (b) brightfield image; (c) fluorescent image

Lab on a Chip Accepted Manuscript

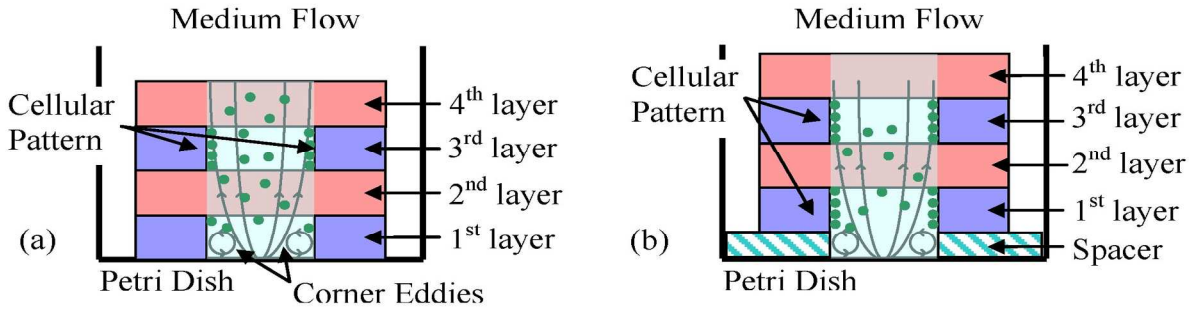

Fig. 6 Medium flow within the structure: (a) original; (b) with the spacer

to 20V and images of the scaffold structure with a 4× objective lens at the elevation near the 1st and 2nd layers were captured (Fig. 5).

As observed through the microscope, similar 3D rectangular patterns were seen in all 25 micro-wells and hence this multi-layer scaffold can be seeded automatically using the DEP mechanism. From the fluorescent images (Fig. 5c), the cellular pattern on the 1st layer (horizontal lines) of the structure was found to have lower cell density as compared to other layers (vertical lines). The reason could be explained by the medium flow condition at different layers. In the current setup, the 1st layer was being bonded onto the surface of the Petri dish; the additional wall shear from this boundary could thus change the flow of the medium surrounding the 1st layer from laminar to turbulent due to corner eddies[45] (Fig. 6a). According to the Stokes' law and Newton resistance law, the drag force acting on the suspended cell is dependent on the medium velocity and it can be evaluated as[45]:

$$F_{Drag} = 6\pi\eta r v \quad (2)$$

where η is the coefficient of the viscosity, r is the radius of the cell and v is the relative velocity of the medium with respect to the cell.

In order for the multi-layer structure to hold and trap the manipulated cells on each electrode surface, the generated dielectrophoretic force must overcome the drag force acting on the cell. As the medium flow in the regions near the 1st layer is turbulent, the relative velocity, v, is higher. Hence, the drag force at this layer is higher than the remaining layers and cells that are attached to the electrode surface can easily be flushed away by the medium flow.

To resolve that issue without significantly increasing the input voltage, a 1mm thick PDMS spacer could be placed in between the structure and the Petri dish to lift up the structure from the bottom surface, thereby reducing the medium flow rate at the 1st layer (Fig. 6b). The experiments were repeated and the constructed cellular pattern on the structure with a spacer (Fig. 7). Since the boundary effect from the Petri dish surface on the 1st layer becomes negligible, the DEP force generated from the structure is capable of trapping the cells and constructing extensive cellular patterns onto the 1st layer.

Biocompatibility of the scaffold for cell culture

The multi-layer scaffold structure was used to provide a suitable environment for the cells to grow and proliferate in three dimensions. Hence, it is important that the scaffold material does not release any substances that are toxic to the cells and the adhered cells can remain attached to the material surface over time. PDMS itself is a biocompatible material, but the addition of the carbon black nanoparticles may affect the material properties for in-vitro cultivation. In order to determine the potential effect from these nanoparticles, calcein AM cell viability assay was performed to assess the cells on the scaffold materials. To prepare the specimens, a droplet of 3T3 cell medium was pipetted onto a CB-PDMS material and cultured for 3 days and the results were compared with the cells that were seeded on a PDMS material. Since the CB-PDMS is not transparent, mitotracker red was used to label the cells for fluorescent microscopy. Results from the cytotoxicity assay show that both PDMS and CB-PDMS materials are suitable for

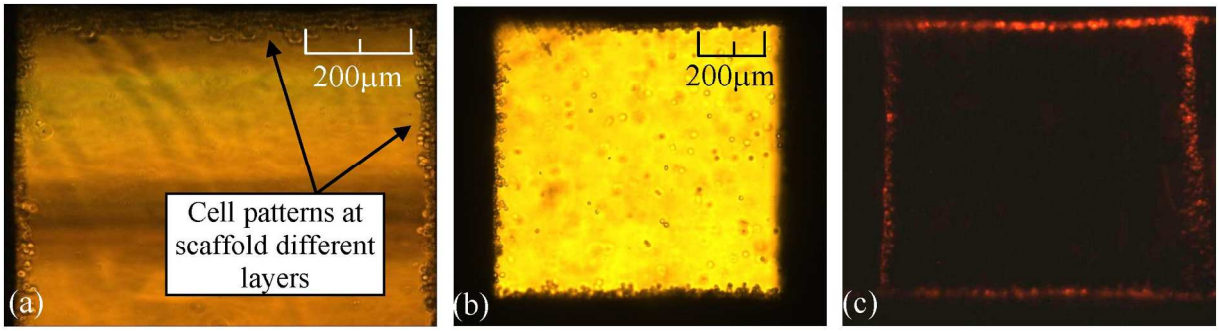

Fig. 7 3D pattern constructed from the structure with a spacer: (a) brightfield image (10×); (b) brightfield image (4×); (c) fluorescent image (4×)

Lab on a Chip Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

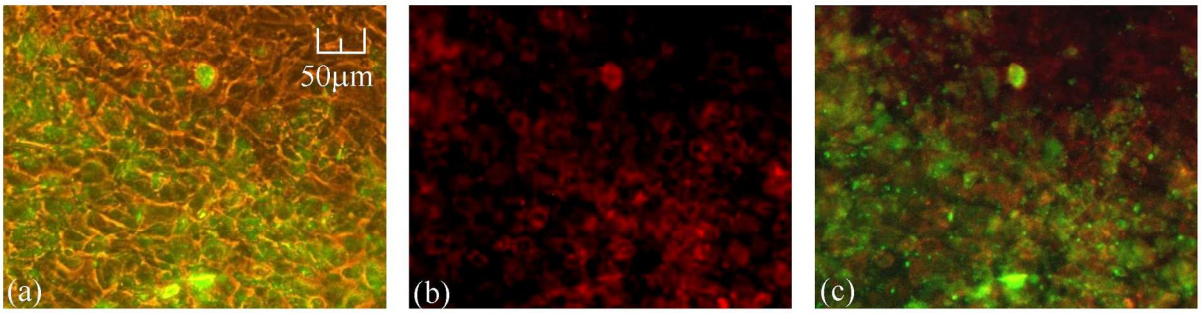

Fig. 8 3T3 cells cultured on PDMS after 3 days: (a) cell viability (green) overlaid on a brightfield image; (b) fluorescent image with 3T3 cells in red; (c) overlaid fluorescent image

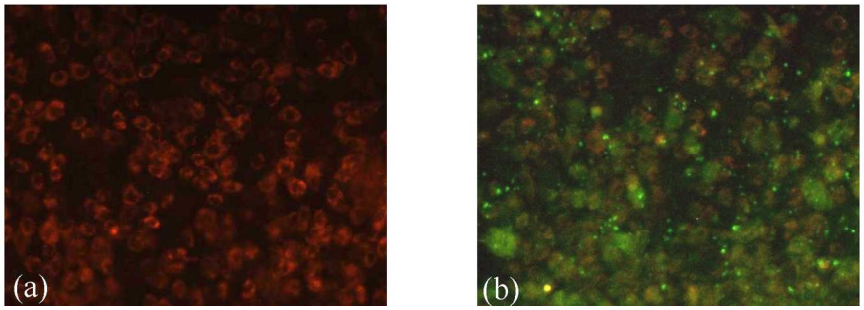

Fig. 9 3T3 cells cultured on CB-PDMS after 3 days: (a) fluorescent image with 3T3 cells in red; (c) overlaid fluorescent image

cell culture as they do not pose a hazard to the cells. Majority of the cells on the material surface remain viable (as shown in green) after 3 days of culture as observed from the images (Fig. 8 and Fig 9).

Assessment of the cell viability after DEP cell patterning

The proposed structure was tested with different voltage inputs to determine their influence on the cell viability after DEP manipulation. A higher voltage input can reduce the time for cellular pattern formation, but this could decrease the viability rate as a tradeoff. Several research papers have reported that using electric fields above 40kV/m can lead to problems such as joule heating and cell destruction.[14,46] Hence, the electric fields generated from the structure were simulated through COMSOL and the maximum electric fields at different elevations of the structure are summarized in Table 1. The simulation result shows that operating the structure at 10V is relatively non-destructive to the cells. In contrast, voltage of 20V or above may not be suitable for cell patterning as the strengths of the generated electric fields could exceed 50kV/m in most of the regions, posing serious threat to the survival of the cells.

To evaluate the viability experimentally, trypan blue was used to detect the dead cells after DEP manipulation. Droplets of the 3T3 cell containing medium were added to four scaffolds and the input voltage on each was set to 0V(control), 5V, 10V, and 20V, respectively for 30 minutes. Two sets of trials were conducted for each voltage input to help evaluate the repeatability of the results. The cells patterned within the structure were dissociated from the structure for assay. The collected cell medium was dyed with 0.4% trypan blue for 5 minutes and a small droplet was dispensed onto a glass slide for inspection.

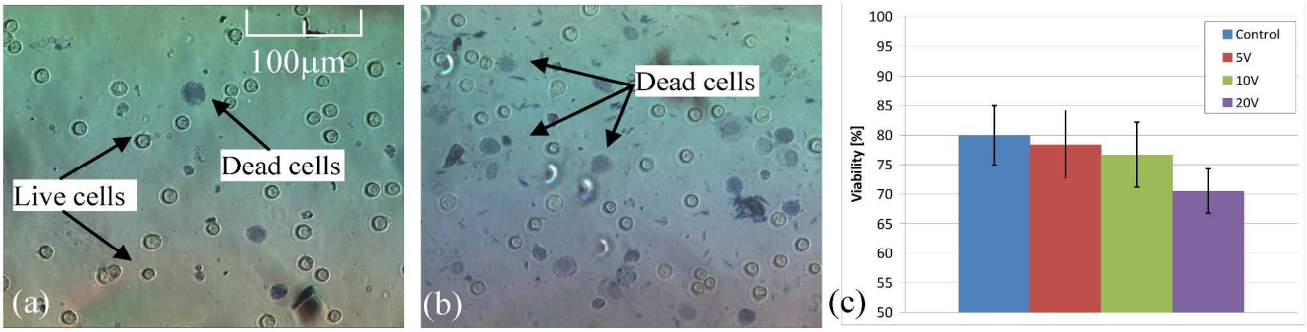

Fig. 10 Viability test on the manipulated cells after (a) 0V input (control); (b) 20V input; (c) Viability of cells at different voltage inputs

Lab on a Chip Accepted Manuscript

Table 1: Maximum Electric Field at Different Elevation of the Scaffold Structure

	Electric Field (kV/m)		
Elevation (μm)	5V	10V	20V
2nd layer			
601	42.6	85.2	170.3
725	12.6	25.2	50.4
850	9.2	18.5	36.9
975	18.7	37.5	74.9
1100	68.7	137.4	274.7
3rd layer			
1201	43.1	86.3	172.6
1325	12.4	24.9	49.8
1450	9.5	18.9	37.8
1575	19.8	39.7	79.4
1700	77.4	154.9	309.7

To quantify the cell viability rate, the average number of dead cells in the dyed cell medium was examined at ten locations for each voltage input. Results obtained from the cell assay at 0V and the 20V are shown (Fig. 10a and Fig. 10b) and the viability rate at different voltage inputs are plotted (Fig. 10c). The viability of cells after 30 minutes of DEP cell patterning with input voltages less than 10V is high. The viability of cells undergone DEP cell patterning at 5V and 10V input is about 78.4±5.6% and 76.7±5.4%, respectively; the viability rate achieved is comparable to the 79.7±5% rate for the control (0V). When the voltage was raised further to 20V, the viability rate drops to 70.5±3.7%. Based on the results, the use of a voltage input at 10V or below will not lead to significant cell damage or death during DEP manipulation and patterning with the proposed structure.

As discussed above, results from COMSOL simulation predicted that, the use of a voltage input 20V and above could cause a significant decrease in the cell viability due to the high electric fields generated. From the experiments, we started to observe stronger detrimental effect on the viability of cells from the DEP fields when the input voltage was increased to 20V. The discrepancy between the simulation prediction and the experiment can be explained by the material properties of the structure used in the simulation. In this work, the structure utilizes a less conductive material as the electrode; voltage drop across the scaffold layers can be expected due to the high internal resistance of the material, and thereby, resulting weaker electric fields to be generated from the structure. However, during the simulation, the ideal scenario was assumed and the input voltage was used as the boundary condition for electric field generation. In addition, it was also reported the viability rate of cells under electric fields might vary between different cell types. [40,47,48] For instance, in the study by Yang *et al.* [47], cell lyses were not observed even when the electric field was raised to above 80kV/m.

Assessment of the cellular pattern after culture

In this work, a set of in-vitro cultivation experiments after DEP cell patterning of 3T3 cells was conducted with our proposed scaffold. After 30 minutes of DEP operation at 10V voltage input, fresh culture medium was supplied to the scaffold and the scaffold was then kept in an incubator at 37°C, 5% CO_2. The medium was replaced every two days for nutrient and metabolic waste exchange. After 7 days of culture, the cells on the scaffold were fixed using 4% paraformaldehyde (PFA) solution. The scaffold was then dried using a critical point dryer and was sputter-coated with a thin deposition of gold on the surface for scanning electron microscope (SEM) imaging. As demonstrated in the images, it can be observed that a significant number of cells were adhered and cultivated on the vertical

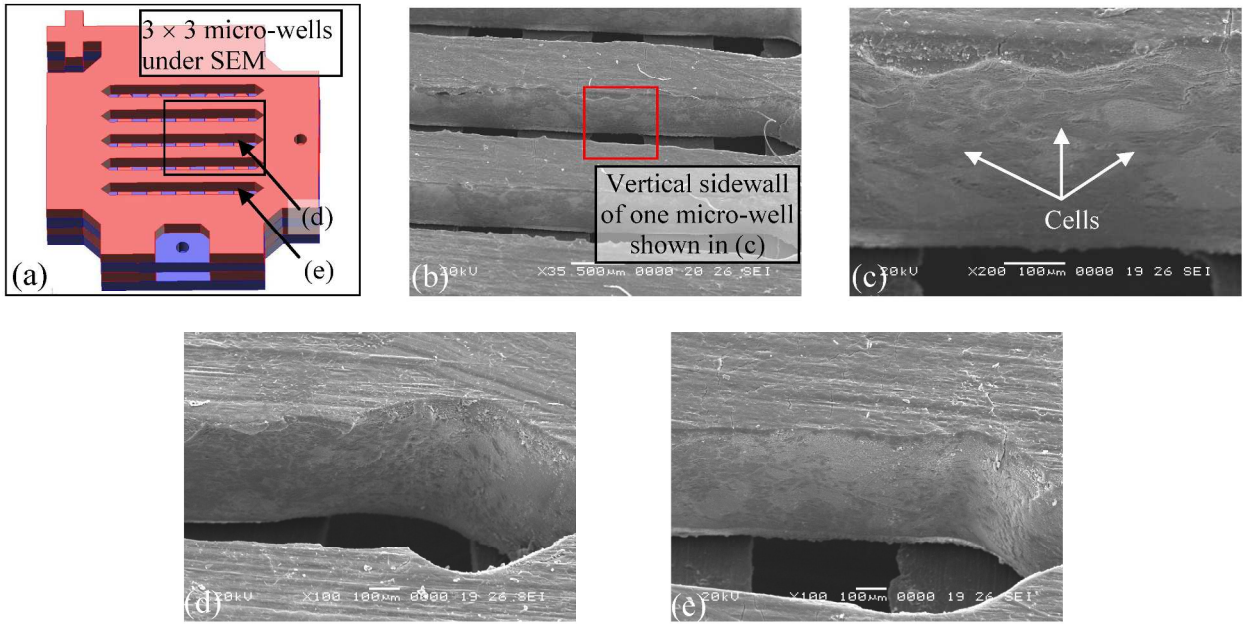

Fig. 11 (a) 3D view of the scaffold; (b) SEM image of the micro-wells after 7 days of culture; (c) 3T3 cells on the vertical sidewall of one micro-well; (d) Micro-well with higher cell density; (e) Outermost micro-well with lower cell density

Lab on a Chip Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

sidewalls of the scaffold (Fig. 11a to Fig. 11c). In addition, a higher initial cell seeding density was observed on the micro-wells near the center regions of the scaffold (Fig. 11d) as compared to the outermost micro-well (Fig. 11e). This may be explained by the smaller amount of cell medium droplet used in the experiment to prevent the medium from over-spilling the scaffold. As a result, a relatively smaller amount of cell medium was being transferred to the outermost micro-well, and hence, a lower initial cell seeding density was observed.

Conclusions

The work examined three-dimensional cell manipulation and patterning with a novel engineering scaffold structure. This structure incorporates dielectrophoresis as the non-invasive manipulation method to enable automatic seeding and patterning of biological cells with high efficiency. To enable batch manipulation of the cells, the multi-layer structure is supplied with a voltage input to generate electric fields in the three-dimensional domain, polarizing the cells to construct 3D cellular patterns on the structure. Experiments were conducted and the results show that the proposed multi-layer DEP manipulation mechanism is capable of patterning the millimeter-scale structure with high cell density. Different voltage inputs supplied to the structure were examined and the operating range that can seed the structure efficiently with high viability was evaluated. The proposed structure and its automated patterning mechanism could be applied to fabricate large-scale, complex tissues for a variety of tissue engineering applications.

Acknowledgements

This work is supported by a grant from the Research Grant Council of the Hong Kong Administrative Region, China (ref no. CityU 121513).

Notes and references

[a] H. K. Chu. Z. Huan, and D. Sun are with the Department of Mechanical and Biomedical Engineering, City University of Hong Kong, Hong Kong, China. *E-mail:medsun@cityu.edu.hk. Tel: 852-3442-8405.

[b] Z. Huan and J. Yang are with the Department of Precision Machinery and Instrumentation, University of Science and Technology of China, Hefei, Anhui, China.

[c] J. K. Mills is with the Department of Mechanical and Industrial Engineering, University of Toronto, Toronto, Canada M5S 3G8.

Electronic Supplementary Information (ESI) available: [Videos showing the 3D cellular pattern on the scaffold structure at different voltage inputs.]. See DOI: 10.1039/b000000x/

1 M. Li, W. H. Li, J. Zhang, G. Alici, and W. Wen, A review of microfabrication techniques and dielectrophoretic microdevices for particle manipulation and separation, *Journal of Physics D: Applied Physics*, 2014, **47**(6), 063001.

2 A. Seidi, and M. Ramalingam, Protocols for biomaterial scaffold fabrication, in: M. Ramalingam, Z. Haidar, S. Ramakrishna, H. Kobayashi, and Y. Haikel, eds., *Integrated Biomaterials in Tissue Engineering*, Hoboken, NJ: John Wiley & Sons, Inc., 2012, pp. 1-23.

3 C. J. Bettinger, J. T. Borenstein, and R. Langer, Micro- and nanofabricated scaffolds, in: R. Lanza, R. Langer, and J. Vacanti, eds., *Principles of Tissue Engineering 3rd Edition*, Burlington, MA: Elsevier Academic Press, 2007, pp. 359-373.

4 S. Levenberg, J. Rouwkema, M. Macdonald, E. S. Garfein, D. S. Kohane, D. C. Darland, *et al.*, Engineering vascularized skeletal muscle tissue, *nature biotechnology*, 2005, **23**(7), 879-884.

5 F. T. Moutos, L. E. Freed, and F. Guilak, A biomimetic three-dimensional woven composite scaffold for functional tissue engineering of cartilage, *nature materials*, 2007, **6**, 162-167.

6 L. Altomare, N. Gadegaard, L. Visai, M. C. Tanzi, and S. Fare, Biodegradable microgrooved polymeric surfaces obtained by photolithography for skeletal muscle cell orientation and myotube development, *Acta Biomaterialia*, 2010, **6**(6), 1948-1957.

7 N. Thuaksuban, T. Nuntanaranont, W. Pattanachot, S. Suttapreyasri, and L. K. Cheung, Biodegradable polycaprolactone-chitosan three-dimensional scaffolds fabricated by melt stretching and multi-layer deposition for bone tissue engineering: assessment of the physical properties and cellular response, *Biomedical Materials*, 2011, **6**, 015009.

8 A. M. Greiner, B. Richter, and M. Bastmeyer, Micro-Engineered 3D Scaffolds for Cell Culture Studies, *Macromolecular Bioscience*, 2012, **12**, 1301-1314.

9 M. Frydrych, and B. Chen, Large three-dimensional poly(glycerol sebacate)-based scaffold – a freeze-drying preparation approach, *Journal of Materials Chemistry B*, 2013, **1**, 6650-6661.

10 A. Shahini, M. Yazdimamaghani, K. J. Walker, M. A. Eastman, H. Hatami-Marbini, B. J. Smith, *et al.*, 3D conductive nanocomposite scaffold for bone tissue engineering, *International Journal of Nanomedicine*, 2014, **2014**(9), 167-181.

11 A. M. Martins, G. Eng, S. G. Caridade, J. F. Mano, R. I. Reis, and G. Vunjak-Novakovic, Electrically Conductive Chitosan/Carbon Scaffolds for Cardiac Tissue Engineering, *Biomacromolecules*, 2014, **15**, 635-643.

12 M. B. Murphy, and A. G. Mikos, Polymer scaffold fabrication, in: R. Lanza, R. Langer, and J. Vacanti, eds., *Principles of Tissue Engineering 3rd Edition*, Burlington, MA: Elsevier Academic Press, 2007, pp. 309-321.

13 V. L. Tang, and S. N. Bhatia, Three-dimensional tissue fabrication, *Advanced Drug Delivery Review*, 2004, **56**(11), 1635-1647.

14 G. H. Markx, The use of electric field in tissue engineering, *Organogenesis*, 2008, **4**(1), 11-17.

15 R. Pethig, Review Article – Dielectrophoresis: Status of the theory, technology, and applications, *Biomicrofluidics*, 2010, **4**(2), 02811.

16 X. Hu, P. H. Bessette, J. Qian, C. D. Meinhart, P. S. Daugherty, and H. T. Soh, Marker-specific sorting of rare cells using dielectrophoresis, *Proceedings of the National Academy of Sciences*, 2005, **102**(44), 15757-15761.

17 M. Alshareef, N. Metrakos, E. J. Perez, F. Azer, F. Yang, X. Yang, *et al.*, Separation of tumor cells with dielectrophoresis-based microfluidic chip, *Biomicrofluidics*, 2013, **7**(1), 011803.

18 A. Rosenthal, and J. Voldman, Dielectrophohoretic traps for single-particle patterning, *Biophysical Journal*, 2005, **88**(3), 2193-2206.

Lab on a Chip Accepted Manuscript

19 B. G. Hawkin, J. P. Gleghorn, and B. J. Kirby, Dielectrophoresis for cell and particle manipulations, in: J. D. Zahn, ed., *Methods in Bioengineering: Biomicrofabrication and Biomicrofluidics*, Norwood, MA: Artech House, 2009, pp. 133-181.

20 H. K. Chu, Z. Huan, J. K. Mills, J. Yang, and D. Sun, Dielectrophoresis-based automatic 3D cell manipulation and patterning through a micro-electrode integrated multi-layer scaffold, *Proceedings of IEEE International Conference on Intelligent Robots and Systems*, 2014, 2003-2008.

21 H. A. Pohl, *Dielectrophoresis*, Cambridge, UK: Cambridge University Press, 1978.

22 C. Y. Fu, C. Y. Lin, W. C. Chu, and H. Y. Chang, A simple cell patterning method using magnetic particle-containing photosensitive Poly (Ethylene Glycol) hydrogel blocks: A Technical Note, *Tissue Engineering: Part C*, 2011, **17**(8), 871-877.

23 A. M. Bratt-Leal, K. L. Kepple, R. L. Carpenedo, M. T. Cooke, and T. C. McDevitt, Magnetic manipulation and spatial patterning of multi-cellular stem cell aggregates, *Integrative Biology*, 2011, **3**(12), 1224-1232.

24 S. P. Grogan, C. Pauli, P. Chen, J. Du, C. B. Chung, S. D. Kong, *et al.*, In Situ tissue engineering using magnetically guided three-dimensional cell patterning, *Tissue Engineering: Part C*, 2012, **18**(7), 496-506.

25 G. A. Villalona, B. Udelsman, D. R. Duncan, E. McGillicuddy, R. F. Sawh-Martinez, N. Hibino, *et al.*, Cell-seeding techniques in vascular tissue engineering, *Tissue Engineering: Part B*, 2010, **16**(3), 341-350.

26 Z. R. Gagnon, Cellular dielectrophoresis: Applications to the characterization, manipulation, separation and patterning of cells, *Electrophoresis*, 2011, **32**(18), 2466-2487.

27 C. M. Puleo, H. C. Yeh, and T. H. Wang, Applications of MEMS technologies in tissue engineering, *Tissue Engineering*, 2007, **13**(12), 2839-2854.

28 J. Ramon-Azcon, S. Ahadian, R. Obergon, G. Camci-Unal, S. Ostrovidov, V. Hosseiri, *et al.*, Gelatin methacrylate as a promising hydrogel for 3D microscale organization and proliferation of dielectrophoretically patterned cells, *Lab on a Chip*, 2012, **12**(16), 2959-2969.

29 D. R. Albrecht, V. L. Tsang, R. L. Sah, and S. N. Bhatia, Photo- and electropatterning of hydro-gel encapsulated living cell array, *Lab on a Chip*, 2005, **5**(1), 111-118.

30 D. S. Gray, J. L. Tan, J. Voldman, and C. S. Chen, Dielectrophoretic registration of living cells to a microelectrode array, *Biosensors and Bioelectronics*, 2004, **19**(12), 1765-1774.

31 M. Suzuki, T. Yasukawa, H. Shiku, and T. Matsue, Negative dielectrophoretic patterning with colloidal particles and encapsulation into a hydrogel, *Langmuir*, 2007, **23**(7), 4088-4094.

32 C. T. Ho, R. Z. Lin, W. Y. Chang, H. Y. Chang, and C. H. Liu, Rapid heterogeneous liver-cell on-chip patterning via the enhanced field, *Lab on a Chip*, 2006, **6**(6), 724-734.

33 C. T. Ho, R. Z. Lin, R. J. Chen, C. K. Chin, S. E. Gong, H. Y. Chang, *et al.*, Liver-cell patterning lab chip: mimicking the morphology of liver lobule tissue, *Lab on a Chip*, 2013, **13**(18), 3578-3587.

34 T. Masuda, T. Anada, I. Takahashi, F. Arai, T. Fukuda, and O. Suzuki, Fabrication of PDMS Scaffold with Controlled Configurations, *Proceedings of the 2007 International Symposium on Micro-NanoMechatronics and Human Science*, 2007, 362-367.

35 A. Mata, E. J. Kim, C. A. Boehm, A. J. Fleischman, G. F. Muschler, and S. Roy, A three dimensional scaffold with precise micro-architecture and surface micro-textures, *Biomaterials*, 2009, **30**(27), 4610-4617.

36 E. Pedraza, A. Brady, C. A. Fraker, R. D. Molano, S. Sukert, D. M. Berman, *et al.*, Macroporous Three-Dimensional PDMS Scaffolds for Extrahepatic Islet Transplantation, *Cell Transplantation*, 2013, **22**, 1123-1135

37 A. D. Lantada, H. A. Iniesta, B. P. Sanchez, and J. P. Garcia-Ruiz, Free-Form Rapid Prototyped Porous PDMS Scaffolds Incorporating Growth Factors Promote Chondrogenesis, *Advances in Materials Science and Engineering*, 2014, **2014**, 612976.

38 A. Abarrategi, M. C. Gutierrez, C. Moreno-Vicente, M. J. Hortiguela, V. Ramos, J. L. Lopez-Lacomba, *et al.*, Multiwall carbon nanotube scaffolds for tissue engineering purposes, *biomaterials*, 2007, **29**, 94-102.

39 A. M. Schrand, J. Johnson, L. Dai, S. M. Hussain, J. J. Schlager, L. Zhu, Y. Hong, and E. Osawa, Cytotoxicity and Genotoxicity of Carbon Nanomaterials, in: T. J. Webster ed., *Safety of Nanoparticles,*, Springer Science+Business Media, 2009, pp. 159-187.

40 S. V. Puttaswamy, S. Sivashankar, R. J. Chen, C. K. Chin, H. Y. Chang, and C. H. Liu, Enhanced cell viability and cell adhesion using low conductivity medium for negative dielectrophoretic cell patterning, *Biotechnology Journal*, 2010, **5**(10), 1005-1015.

41 T. Garg, O. Singh, S. Arora, and R. S. R. Murthy, Scaffold: A Novel Carrier for Cell and Drug Delivery, *Critical Reviews in Therapeutic Drug Carrier Systems*, 2012, **29**(1), 1-63.

42 Y. Xie, S. T. Yang, and D. A. Kniss, Three-dimensional Cell-Scaffold Constructs Promote Efficient Gene Transfection: Implications for Cell-Based Gene Therapy, *Tissue Engineering*, 2004, **7**(5), 585-598.

43 D. S. Gray, and C. S. Chen, The electric slide: dielectrophoresis for high precision cell patterning, *Proceedings of the Second Joint EMBS-BMES Conference*, 2002, 1686-1687.

44 M. Kanduser, and D. Miklavcic, Electroporation in Biological Cell and Tissue: An Overview, in: E. Vorobiev, and N. Lebovka, eds., *Electrotechnologies for Extraction from Food Plants and Biomaterials*, New York, NY: Springer Science+Business Media, 2008, pp. 1-37.

45 D. E. Angelescu, *Highly Integrated Microfluidics Design*, Norwood, MA: Artech House, 2011.

46 A. Menachery, and R. Pethig, Controlling cell destruction using dielectrophoretic forces, *Proceedings of the IEEE Nanobiotechnology*, 2005, **52**(4), 145-149.

47 L. Yang, P. P. Banada, A. K. Bhunia, and R. Bashir, Effect of Dielectrophoresis on Growth, Viability, and Immuno-reactivity of Listeria monocytogenes, *Journal of Biological Engineering*, 2008, **2**(6), 1-14.

48 P. Bajaj, D. Marchwiany, C. Durate, and R. Bashir, Patterned Three-Dimensional Encapsulation of Embryonic Stem Cells using Dielectrophoresis and Stereolithography, *Advanced Healthcare Materials*, 2012, **2**, 450-458.

Lab on a Chip Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2014