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Spatially controlled cell growth using patterned biomaterials

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ABSTRACT

Development of functional tissues often requires spatially controlled growth of cells over 2D surfaces or 3D substrates to maintain their distinct cellular functions; particularly it is essential for culturing anchorage-dependent cells. In this regard, development of new surfaces/substrates with superior surface properties that could control the cell behavior is of great important and extremely necessary for functional tissue engineering as well as to study how the cells spatially recognize and interact with synthetic material systems. Surface patterning is an approach to modify the surface of biomaterials, either chemically or topographically. Both the approaches are well demonstrated in manipulating cell behaviors such as shape, size, orientation, migration, proliferation, and differentiation. In this article, we review various commonly employed methodologies for use in patterning of biomaterial surfaces/substrates and their suitability in controlling cell behaviors.

Keywords: Biomaterials; microfabrication; patterning; spatial cell growth; tissue engineering.

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Contents

1. Introduction

Spatial control of cellular microenvironment is a key to control cell fate and subsequent tissue function *in vitro*, and to develop novel cellular substrates for use in the development of tissue alternates [**1-3**]. The development of new tissue replacements depends to a large extent on the ability to direct or regulate cell behaviors such as attachment, spreading, proliferation, migration, and differentiation. Cells frequently interact with their surrounding microenvironment to maintain their physiological and metabolic activities and subsequent tissue organization. Cellular microenvironment is comprised of a complex mixture of extracellular matrix (ECM) molecules, soluble and non-soluble biochemical factors, and multiple cell types. Microenvironment where the cells reside is therefore critical for controlling cell fate and function, and thus spatial control of cellular microenvironment can effectively regulate cell behaviors such as attachment, spreading, proliferation, migration, and differentiation under laboratory conditions.

Control of cell-substrate interactions often requires stern control over the surface properties of biomaterial substrate, because the cultured cells initial response to the substrate mainly depends upon its surface properties rather than its bulk properties. It is known that cells in our body are arranged in distinct patterns during their development and these cellular patterns are organized by spatial and temporal environmental cues over many length scales. The modification of the surface of a biomaterial by distinct patterning can thus be used to mimic the native cellular environment. It should also be noted that most cellular components and biological structures possess length scales that range from a few tens of nanometers to a few centimeters. Therefore, patterning biomaterials with features on similar length scales can be used to regulate cell behaviors. Micro- and nanofabrication technologies offer the capability to design a well-defined chemical composition and topology of the material substrate, suitable to control cell–substrate, cell–cell, and cell–soluble factor interactions [**4, 5**]. In this article, we review some methodological and technical aspects of surface patterning of biomaterials suitable for spatially controlled cell behaviors.

2. Spatially controlled cell growth utilizing chemical patterning

Chemical patterning refers to a process of modifying a biomaterial substrate with patterns of different chemistries. The simple example of a commonly used patterning material is self assembled monolayers (SAMs) of organic macromolecules [**6-9**]. SAMs are versatile molecular assemblies that are formed spontaneously by the adsorption of a surfactant with a specific affinity of its one end of molecule (called "head group") to a substrate. SAMs also consist of a tail with a specific functional group at the other end of the same molecules (called "tail group"). SAMs are known to influence cell attachment and other function [**10-12**]. The preparation of SAMs is a 'bottom-up' approach that facilitates the assembly of unidirectional, ultra-thin layers on a solid surface using the appropriate chemicals by the spontaneous organization of their constitutes via covalent bonding (called chemisorption) or non-covalent bonding (called physisorption) [**13-15**]. There are a variety of organic SAMs developed with different functional groups for various biological applications. Among them, silane- and thiol-based SAMs are well characterized systems for the cell and tissue engineering applications. SAMs are generally prepared on a metal (e.g. gold) or hydroxyl-terminated substrates (e.g. silica glass). In this approach, the solid substrate is first cleaned with strong acids and then gently dipped into a solution containing the SAMs precursors under ambient conditions in order to facilitate the self-assembly process. This method has provided a straightforward way to obtain well-ordered monolayers and the SAMs formed by this method are chemically stable [**16**]. In the following sections, the most commonly used methodologies for patterning of SAMs are discussed with illustrated experimental examples.

2.1. Photolithography-based patterning

Photolithography is a microfabrication technique which allows the formation of distinct patterns with desired geometry onto the biomaterial substrate suitable for cell studies. There are three key components involved in this technique, which includes a light source, a photosensitive material (also called photoresist), and a photomask. The light source provides the energy required for the exposure of photoresist or ablation of SAMs over the selected regions of the substrate. Ultraviolet (UV) light has been the source often used for patterning. Photoresists are often made from organic compounds, whose molecular chains are capable of re-organizing or crosslinking upon exposure to energy. A photomask is a solid substrate (planar) usually made of quartz and coated with a thin layer of chrome with desired pattern geometry. Some of the early studies in the use of microfabricated structures and cells were done by using this approach. For example, in the late 1980s, Kleinfield et al., demonstrated that neurons can be spatially cultured onto photolithographically patterned SAMs [**17**]. The processing steps involved in the chemical patterning of a silica glass substrate by conventional photolithography

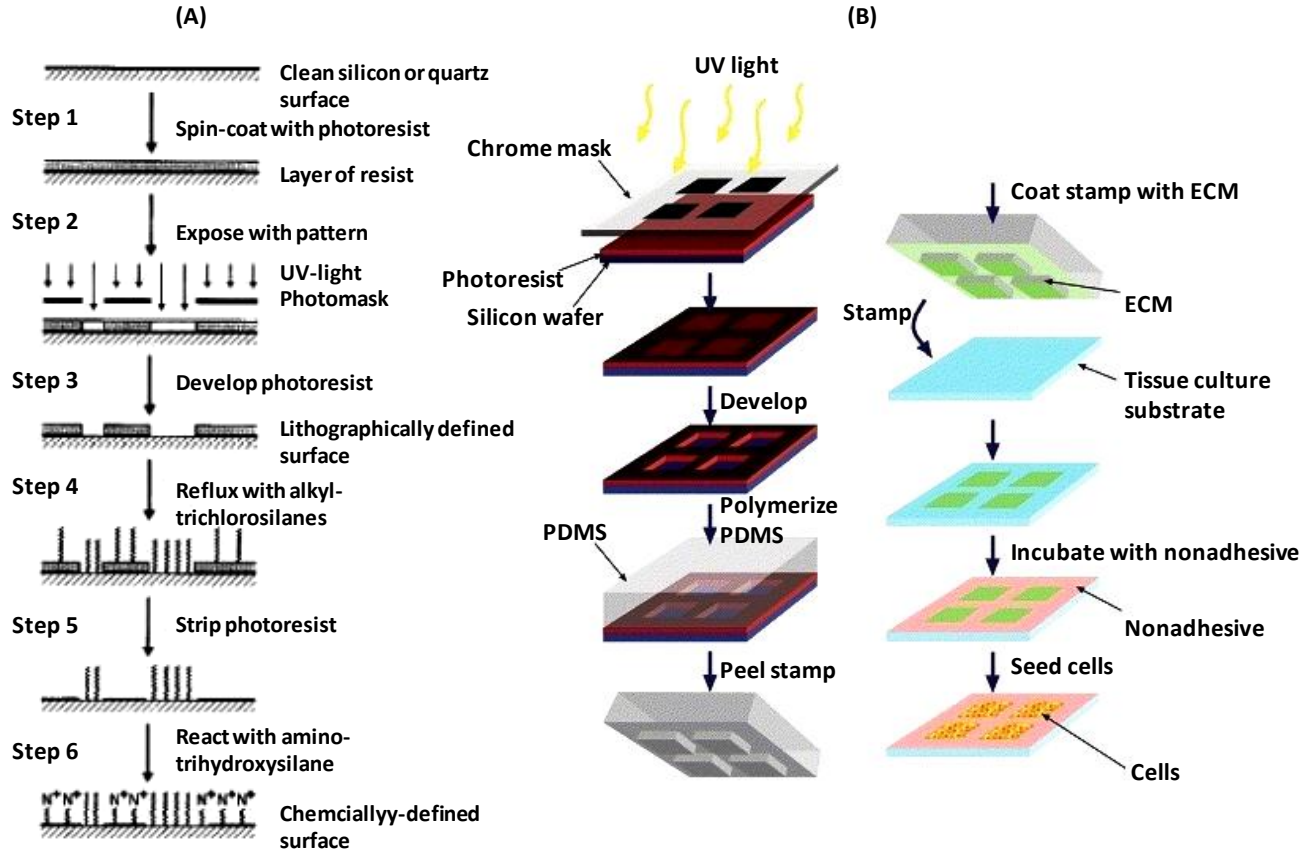

Fig. 1. (A) Schematic of the processing steps involved in chemically patterning a silicon or slicon dioxide (quartz) substrate by photolithography, and **(B)** Schematic of the soft lithography based approach for micropatterning process. PDMS stamps are generated by photolithography (left column) and, using these stamps, ECM proteins are printed onto tissue-culture substrates upon which cells are seeded (right column) [Fig. 1A is adapted with permission from Ref. **17**, and Fig. 1B is adapted with permission from Ref. **19**].

(i.e. photoresist-based) are schematically shown in **Fig. 1A**. The spin-coating with the photoresist was carried out after cleaning the surface of the test substrate (**Fig. 1A, Step 1**). The photoresist-coated substrate was exposed to UV light through the photomask (**Fig. 1A, Step 2**). Note that the light rays were transmitted only through the quartz background, but not through the chrome pattern on the mask. This chemically altered the solubility of the photoresist in certain developer solutions due to the molecular chain re-arrangement. The light exposed part of the photoresist was then solubilized in a developer solution, which yielded a photoresist pattern that corresponds to the image of the pattern designed on the mask (**Fig. 1A, Step 3**). Consequently, the material of interest (e.g., alkylchlorosilane, a cell non-adhesive agent) to be patterned was applied on the photoresist pattern (**Fig. 1A, Step 4**) and then the photoresist was carefully removed (**Fig. 1A, Step 5**), and the open areas (the area other than the cell non-adhesive region) were back-filled with another material of interest (e.g., alkylaminosilane, a cell adhesive agent) (**Fig. 1A, Step 6**). This lithographic process led to chemically-defined substrate with patterns of cell adhesive and non-adhesive regions. By using this photolithographic technique, the authors constructed simplified neuronal patterns with synoptically active neurons *in-vitro*. The study revealed that dissociated neurons can be directed to adhere and grow in high-resolution patterns and the cells underwent normal morphological and physiological development during their culture period of 12 days. Therefore, the photolithographically-patterned biomaterials can be used for studying how cells response to synthetic surfaces with different chemistries.

2.2. Microcontact printing-based patterning

Microcontact printing (µCP) is a well known technique which allows the transfer of patterns onto biomaterial susbstrates with high spatial resolution suitable for cell studies. This microfabrication technique is one of the most recognized techniques in bioengineering because of its simplicity, flexibility and ability to pattern many biomaterials with feature-sizes down to 1 µm without using any expensive equipment. In addition, this technique can be extended to pattern a non-planar surface that is 3D structures where it is not feasible in conventional photolithography technique.

µCP was initially developed by George Whitesides and his group and it was introduced to pattern SAMs of alkanethiolates onto a gold surface to control cell behavior and for engineering cell shape and function by modulating the surface characteristics [**18**]. The major processing steps involved in the pattern formation by using µCP are schematically shown in **Fig. 1B** [**19**]. This method has been based on the utilization of an elastomeric stamp to print a pattern of the material of interest (cell compatible

compounds, for example) on a solid substrate. There are three major components associated with this technique, namely master, stamp, and ink. The 'master' is a solid substrate (silicon, for example), often created by standard photolithography with a desired geometry with high resolution features that are specific to particular application. The 'stamp' is a soft elastomeric material, frequently created by casting an elastomeric material (for example, polydimethylsiloxane, PDMS) over a pre-designed master). The 'ink' is a functional material chosen to be patterned onto a substrate material. In the patterning process, the stamp is first inked with a solution made from the materials of interest (non- cell compatible compounds, for example) and the stamp is then brought into contact with the surface of the substrate material. For a period of time, the stamp and the substrate remain undisturbed, which ultimately yields a geometrical pattern of the stamp on the substrate material. The result of the stamping process is the formation of patterns on the surface of the substrate material, in those regions where the stamp has come into contact with the substrate. The unstamped regions are then backfilled with non-cell compatible compounds in order to ensure that the resultant patterned surfaces have both cell adhesive and non-adhesive regions, which directs the cells to grow only on the cell adhesive regions. The resultant patterned surface can be used to study the fundamental aspects of cell behavior that can be eventually applied to engineering physiologically functional tissues. This kind of patterned substrates will have a tremendous potential in controlling cell fate and function in order to well understand the basic mechanism underlying the tissue organization.

3. Spatially controlled cell growth utilizing topographical patterning

Topographical patterning is nothing but the physical modification of a biomaterial substrate with distinct textures suitable to control cell growth and function. Modulating topographical features of the biomaterial substrate for specific applications is a key to regulate the cell behavior to control the tissue-specific organization. Cells, in general, have the ability to sense and respond to surface structural features of the substrates where they are cultured [**20**-**24**]. For example, fibroblasts sense the substrate's topography by a sensory element called filopodia and accordingly respond to them [**23**]. Once a suitable site for adhesion is sensed, other cellular activities such as focal adhesions, stress fibers and microtubules are developed, which stabilize the contact between the cells and surfaces. In the early 1960s, Curtis and Varde proposed that cells responded to the microscale topographical environments and their behavior can be controlled by modulating the surface topography [**25**]. This study revealed that the cells are sensitive to the degree of curvature of the surface where they are cultured and they are capable of aligning on a cylindrical glass with a diameter less than 100 μm. On the other hand, nano-featured topography also greatly affects the cell behavior. For example, Yang et al., reported that nanofibers of poly(L-lactic acid) promotes neural cell adhesion, neurite outgrowth and other cellular processes better than their microscale counterparts [**26**], which suggest that topographical features can be used as cues to modulate cell fate and function.

There are a variety of other techniques also employed for patterning of cells on various topographically modified substrates to investigate cell-substrate interactions [**27**-**31**]. These include electron beam lithography [**27**], imprint lithography [**30**], colloidal lithography [**24**], phase separation [**28**], and self assembly [**31**]. The ability to implement these technologies in a manner that is cost-effective, high throughput, and scalable to commercial production of cellular substrates is still a challenge and requires continued efforts. Lithographic techniques, particularly colloidal lithography (CL) and imprint lithography (IL), have recently been shown a great promise in patterning surfaces with a desired geometry, with features less than 100 nm in resolution. In the following sections, the methodology of IL and CL and their efficacy in studying cellular responses, utilizing patterned surfaces, are briefly discussed.

3.1. Imprint lithography-based patterning

Imprint lithography is a technique for patterning biomaterial substrates with topographical features at micro- and nanoscale level. It is a simple and cost-effective technique since it does not require any expensive equipments as conventional lithography techniques. In addition, it is possible to control the spatial distribution of chemical species on the structured surface/substrate. This technique can be applied to pattern 2D or 3D topography of different geometrical patterns on a wide range of biomaterial substrates suitable for cell and tissue engineering. This patterning technique, in principle, replicates topographical patterns by the means of applied pressure and temperature, in which a rigid master (silicon, for example) with topographical features is imprinted onto a polymer resist, that results in a relief replica of the master on the substrate's surface. There are two basic steps involved in this lithographic technique. First is the imprint step, in which a master (also called a 'mold') with custom-designed geometrical pattern is pressed onto a polymer resist (usually in the form of a thin film), layered on a substrate material, and followed by the removal of the master. This step duplicates the topography of the master on the polymer resist. During the process, the polymer resist is heated to a temperature above its glass transition (Tg), because the resist becomes a viscous liquid at this temperature that facilitates the polymer to flow and easily mold into the shape of the master. For example, in order to transfer the pattern from a rigid master onto a poly(methyl methacrylate) (PMMA) resist requires a temperature around 110ºC. The second step is the pattern transfer, where an anisotropic etching process is used to remove the residual resist in the compressed region. This step transfers the thickness of the contrast pattern onto the entire resist, leaving polymer patterns on the substrate material. It should be noted that the process of imprint lithography is fundamentally different from μCP stamping which uses a monolayer of self-assembled molecules. This is because it is more like a physical process rather than a chemical process.

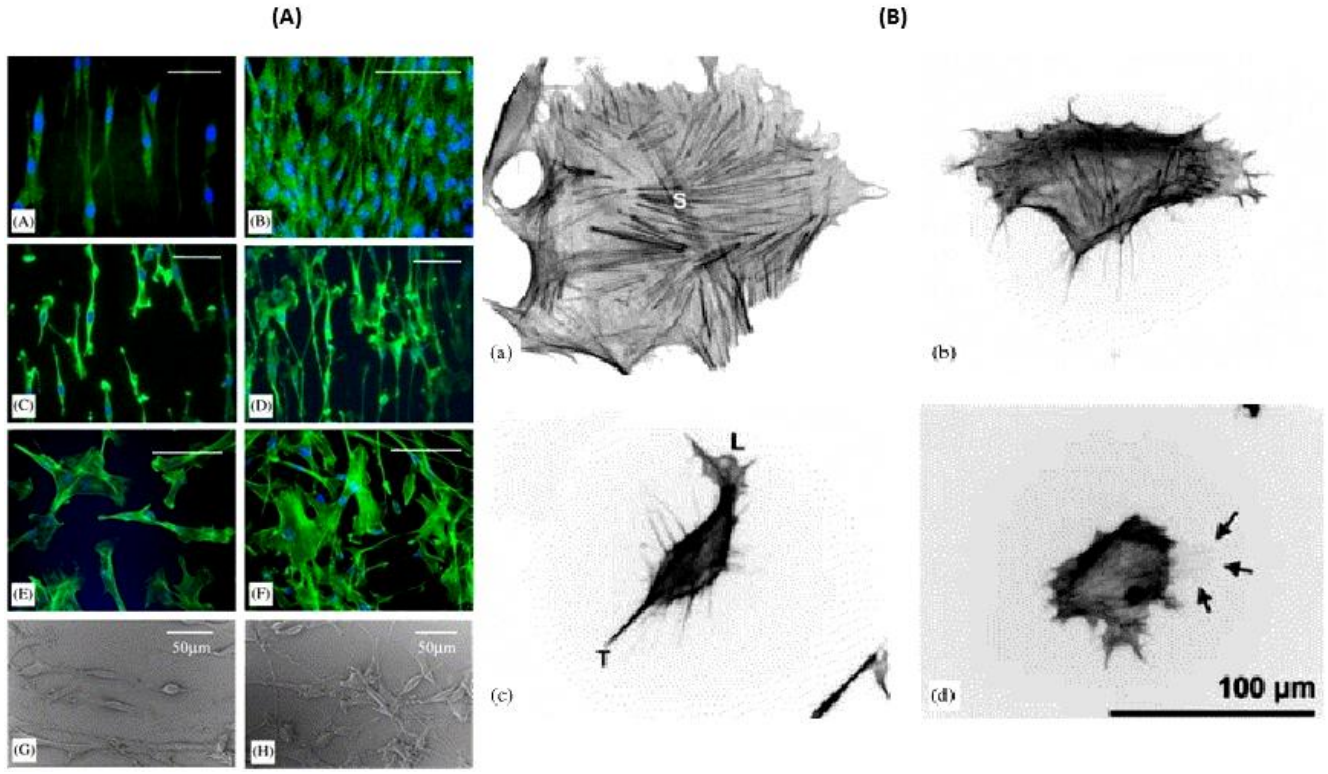

Fig. 2. (A) Confocal micrographs of F-actin stained SMC on **(a)** nano-imprinted PMMA at a low cell density, **(b)** nano-imprinted PMMA at a high cell density, **(c)** nanopatterned PDMS at a low cell density, **(d)** nano-patterned PDMS at a high cell density, **(e)** non-patterned PMMA and **(f)** glass cover slip. SEM micrographs of SMC cultured on **(g)** nano-imprinted gratings on PMMA coated on SiO_2 wafer and **(h)** non-patterned PMMA coated on SiO_2 wafer. Bar = 50 μm for all except (b) Bar = 100 μm, and **(B)** Fluorescent actin staining (images inverted to show filopodia more clearly). (**a** and **b**) Fibroblasts on control, (**c** and **d**) fibroblasts on nano-columns. **(a)** A well spread cell with many stress fibres (s); **(b)** cells becoming well spread, but still with a polarised morphology; **(c)** a rounded cell that is clearly polarised with lamellipodia at the leading edge (L) and a trailing tail (T); **(d)** spreading cell, which is still notably smaller, and has fewer stress fibres that the cells seen in (**a** and **b**) (arrows point to faint filopodia) [Fig. 2A is adapted with permission from Ref. **30**, and Fig. 2B is adapted with permission from Ref. **24**].

The control of cell behavior can be done by using imprint lithographically patterned biomaterial substrates. Numerous research groups are focusing research and development on these directions. For example, Yim et al., developed a patterned silicon substrate using a polymer thin film made of PMMA (comprised of gratings with a 350 nm line width, 700 nm pitch, and 350 nm depth) by imprint lithography to study the efficacy of the patterned substrates in regulating cell behavior [**30**]. Smooth muscle cells (SMC) were cultured on these patterned substrates and their morphology and concomitant orientation were studied, with respect to their elongation and alignment. This study revealed that a patterned substrate with nano topographical features can effectively direct cell orientation and function (see **Fig. 2A**) [**30**]. The cells cultured on the patterned surfaces showed an elongated morphology and were mostly parallel to one another (**Fig. 2A, a-d**). In contrast, SMC cultured on unpatterned surfaces showed neither elongation nor orientation at both low and high cell densities (see **Fig. 2A** (**e-f**), respectively). The orientation of the cells along the axis of the gratings could be seen more clearly under scanning electron microscope (SEM) (**Fig. 2A, g**), where they were randomly spread on unpatterned surfaces (**Fig. 2A, h**). In addition, the cytoskeleton and nuclei of the cells were also found to elongate and align to the long axis of the cell (see **Fig. 2A, a-b**). The cells were significantly elongated on the patterned substrates compared to the non-patterned substrates. Based on the experimental examples discussed in this section, and others, imprint lithography can be utilized to fabricate topographical patterns of biomaterials in order to study the fundamental aspects of cell studies and to control their cellular behaviors.

3.2. Colloidal lithography-based patterning

Colloidal lithography is a technique to pattern micro-/nanoscale topographical features on biomaterial substrates. This technique utilizes the ability of colloidal particles to self-organize on surfaces via electrostatic forces which makes them suitable as a mask for pattern transfer onto the biomaterial substrates. There are numerous research groups focusing on controlling cell behaviors by using this kind of technique. For example, Dalby et al., utilized the colloidal lithographic technique in order to modify the surface of the PMMA with cylindrical columnar topographical features [**24**]. The efficiency of the patterned biomaterial substrates in promoting cell adhesion and cytoskeleton development was evaluated using fibroblasts. The changes in fibroblasts morphology and their cellular functions in response to a geometrical pattern were studied. The results of this study showed that the cells grown on the geometrical patterns exhibited many peripheral protrusions, whereas these effects are absent in cells on planar surfaces. For example,

fibroblasts produced a higher number of filopodia per µm of cell perimeter than in planar surfaces and an interaction between the filopodia and the nano-columns could often be seen. The results also showed that the number of filopodia significantly increased in fibroblasts cultured on the nanoscale columnar structures compared to the planar surfaces, which indicated a stronger cellular responses and interaction toward patterned substrates. During the initial stage, the fibroblasts that were in contact with the nano-columnar substrates stimulated the formation of cytoskeleton faster than the fibroblasts on the planar control surfaces. However, over a longer period of time, the organization of cytoskeleton became more diffuse and the morphology of fibroblasts appeared more rounded, thicker, and smaller in size. On the other hand, fibroblasts on the planar surfaces had a clearly defined intermediate filament-like structure. Thus, it appears that, rather than adhering and spreading as that of the cells on the planar surfaces, fibroblasts on the columnar structures were more polarized with rounded cell bodies having a higher density of filopodia, with the filopodia probing the nano-structured environment surrounding the cell.

The study clearly demonstrated the efficacy of the colloidal lithographically patterned substrates for regulating the basic cellular functions. The cell behaviors, particularly morphology, cytoskeletal organization, and focal contacts, in relation to topography and planar surfaces were assessed by determining the protein distribution using immunochemistry and confocal microscopy. The observation of filamentous actin clearly showed different cell morphologies between the cells on the nano-columnar surfaces compared to planar surfaces (see **Fig. 2B**) [**24**]. The cells on the planar surfaces appear to spread with signs of many stress fibers formed at the lamellae region (**Fig. 2B, a** and **b**). The cellular growth behavior on the nano-columnar surfaces appeared to spread less (**Fig. 2B**, **c** and **d**) and many of them were highly polarized with areas of dense filopodia extensions that could be observed interacting with the nano-columns (see **Fig. 2B, c**), compared to planar surfaces (see **Fig. 2B, d**). This is of particular interest when considering cell responses to topographical features. This study demonstrated that control of the cellular environment might lead to increased levels of endocytosis and the topographical patterns may be able to alter the cell morphology, growth and subsequent functions. This study, in addition to others, suggested that colloidal lithographically patterned biomaterial substrates can be used as a model substrate to study cell-substrate interactions with respect to topographical changes.

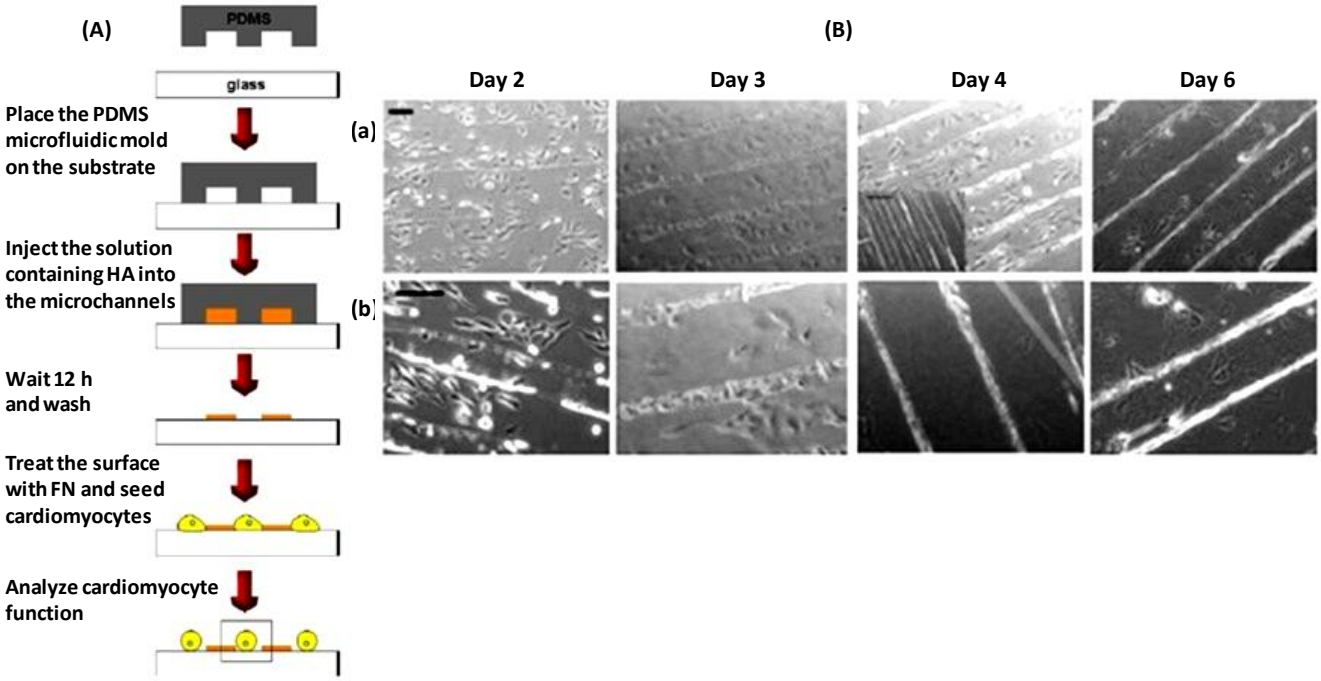

Fig. 3. (A) Schematic diagram of the approach used to fabricate cardiomyocyte organoids, and **(B)** Progression of cardiac organoid formation on HA patterned surfaces. **(a)** Images taken at 100 x. Day 4 inset image taken at 40 x illustrates several millimeter-long cardiac organoids. **(b)** Images taken at 200 x. Scale bars (a, b) 100 µm. Inset scale bar 1 mm [Adapted with permission from Ref. **35**].

4. Spatially controlled cell growth utilizing 3D patterning

In tissue engineering, the goal of a hierarchical organization of cells to promote the *in vitro* development of functional tissue may benefit from the spatially controlled placement of cells in specific locations on a cellular substrate. Most tissue engineering approaches in current use involve random seeding of cells within porous polymer structures. While this has yielded better understanding of the fundamental cell behavior and tissue development, generation of complex tissue structures may require strict control over the localization of behavior of multiple cell types in 3D. Moreover, cells cultured onto 3D substrates behave more physiologically compared to the cells cultured onto 2D surfaces [**32-34**], which eventually leads to the

concept of 3D cell patterning. There are a few methodologies that can be employed to develop 3D cell patterning, which are discussed in the following sections.

4.1. Microfluidics-based patterning

Microfluidic is a technique which allows for patterning of 3D structures suitable for controlling cellular functions. This patterning technique is related to microcontact printing. Instead of stamping a PDMS mold having a relief pattern of the master, microfluidic network is stamped onto a substrate. In this method, the microchannels are used to deliver fluids to selected areas of a substrate and the substrate is exposed to the flow, resulting in patterning of the material. This method is frequently used to pattern multiple components on a single substrate and allows a directed delivery of cells and soluble factors onto the substrate; thereby it has significant implications for the fields of cell biology and cell-based assay. Unlike conventional *in vitro* cell culture methods, microfluidics can provide miniature and complex structures mimicking the *in vivo* cellular environment, which is one of the merits of this technique. Among the numerous types of biomaterials, hydrogels are a particularly attractive for use in cell and tissue engineering owning to their flexible functional properties. For example, they are biocompatible, degradable in a controlled manner, possesses adequate mechanical properties, flexibility in designing, feasible to surface modification and functionalization. The use of hydrogels in microfluidic system plays a critical role as well in controlling cell behavior. In an interesting study, Khademhosseini et al., demonstrated the feasibility of fabricating contractile cardiac organoids via microfluidic patterning of hyaluronic acid (HA) [**35**]. HA micropatterns served as inductive templates for organoid assembly. A schematic representation for fabricating cardiomyocyte organoids using a microfluidic patterning is shown in **Fig. 3A**. In this approach, a PDMS microfluidic mould was placed on a glass substrate, and HA solution was injected into the microchannels (having 100 μm in width and 60 μm in height). Once the cell-repulsive HA regions were formed, the PDMS mould was removed, and the resulting substrate was treated with fibronectin (FN) to generate cell-adhesive regions. The resultant patterns had both cell-adhesive and non-adhesive regions, which could be used for controlling cell behavior *in-vitro*. The efficacy of the microfluidic pattern was tested by culturing primary cardiomyocytes onto the pre-designed microlanes. It was found that cardiomyocytes elongated and aligned along the pattern direction attaching preferentially to the glass substrate and the interface between HA patterns and glass substrate. After 3 days in culture, the linearly aligned myocytes detached from the substrate and formed contractile cardiac organoids (see **Fig. 3B**). This study demonstrated that this kind of microfluidic patterning can be utilized to construct cardiac tissue models *in-vitro*.

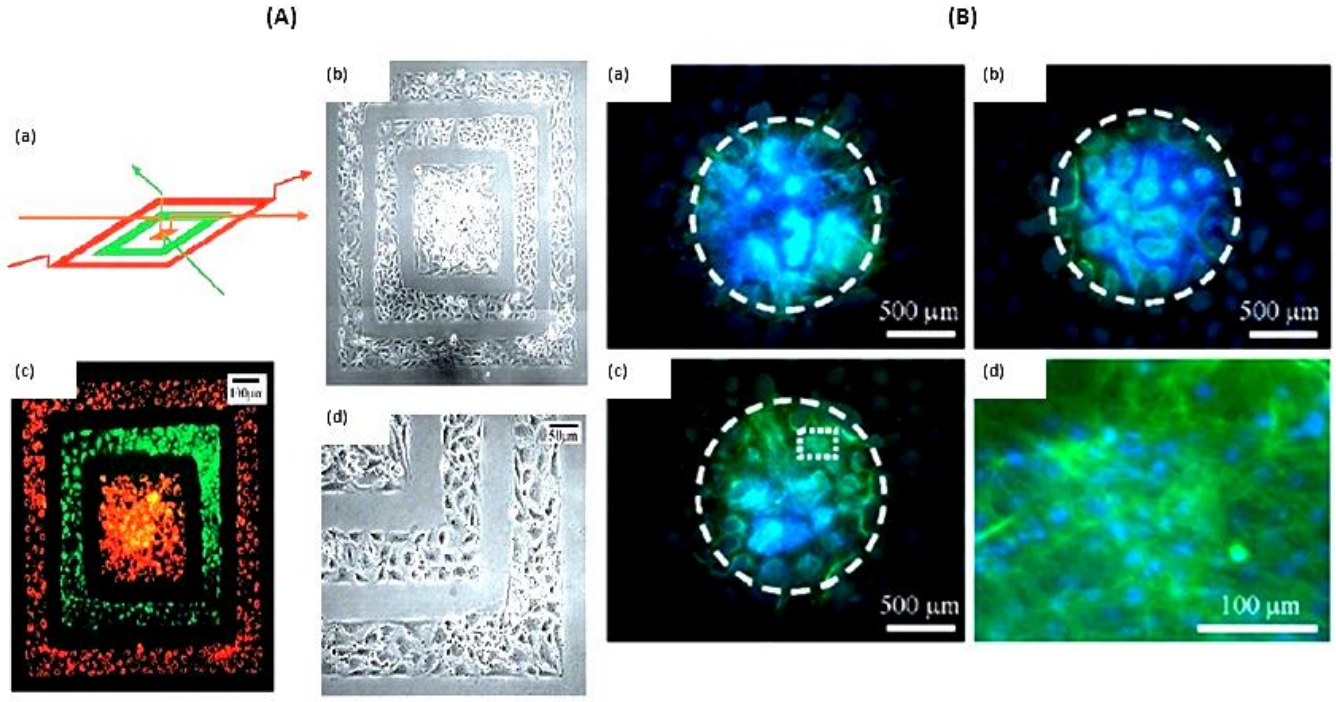

Fig. 4. (A) Fluorescence **(B)** and phase-contrast **(C and D)** pictures of two cell types deposited on a tissue culture dish in a concentric square pattern by using the 3D stamp depicted in A. The cells that appear green are human bladder cancer cells (ECVs) labeled with 5-chloromethylfluorescein diacetate (CMFDA); the cells that appear red are bovine adrenal capillary endothelial cells (BCEs) labeled with DiI-conjugated acetylated low density lipoprotein. Suspensions of cells (≈ 5 million cells per ml) were introduced into the three sets of channels and were allowed to sediment and attach to the surface of the tissue culture dish. These cells were cultured with the stamp in place for ≈24 hr to grow and spread into a confluent layer. The pictures were taken immediately after the PDMS stamp was removed; these cells were immersed under media and were alive. An expanded view of the lower right corner of C is shown in D. **(B)** Different regions of a hydroxyapatite scaffold patterned with osteoblasts using a single agarose stamp with 1000 µm diameter circular features. Images **(a)–(c)** show an area on the top surface of the same scaffold that was patterned during the same stamping event. Dashed white lines indicate areas patterned with cells. **(d)** Higher magnification of the area within the white box in **(c)**. Actin was stained bright green with phalloidin and DNA stained bright blue with Hoescht 33342. The dark blue/grey features in the unpatterned background of the images in **(a), (b), and (c)** are artifacts of fluorescence microscopy, resulting from light reflected from the white hydroxyapatite scaffolds. Images were acquired 24 h after patterning [Fig. 4A is adapted with permission from Ref. **39**, and Fig. 4B is adapted with permission from Ref. **40**].

Microfluidic patterning technique also allows for the generation of 3D structures consisting of multiple cell types with all the *in vivo*-like functional properties suitable for 3D tissue engineering [**36-38**]. By this approach, a desired cell type resuspended in an appropriate extracellular matrix component was applied into a microfluidic network. Next, following the contraction of the biopolymer matrix by cells, another layer with a different cell type was applied into the microfluidic network, which can be able to create a tissue assembly with multiple cell types arranged in 3D (z-direction). The 3D topology of the microfluidic network in the stamp makes this technique a versatile one with which multiple cell types can be patterned even in the complex structures. To demonstrate the capability of microfluidic system to generate complex 3D patterns, Chiu et al., developed a two-layer stamp for the deposition of two cell types in a concentric square pattern (see **Fig. 4A**) [**39**]. Bovine adrenal capillary endothelial cells (BCEs) and a human bladder cancer cells (ECVs) were used for patterning. Coating of the channels with a non-cell adhesive agent (bovine serum albumin, BSA) prevented cell attachment to undesired regions. The cell culture data demonstrated the cell viability and spreading of patterned cells only on the channels, which clearly shows that the microfluidic-based 3D cell patterning is an excellent tool for controlling cell functions. Now the direction of research and development drastically changes toward engineering 3D cell patterning with *in vivo*-like microenvironments. The direct patterning of cells on 3D substrate has a unique advantage for engineering physiologically functional tissues.

4.2. Microstamping-based patterning

Microstamping is a simple pattern transfer technique that allows the fabrication of patterned biomaterial substrates with control over topographical features suitable for cell studies. This is technique is quite similar to microcontact printing. In a notable study, Stevens et al., demonstrated a new methodology for generating patterns of osteoblasts with circular shapes (diameters of 200, 700 or 1000 mm) on hydroxyapatite substrates and glass slides using replica stamping [**40**]. The cells (human osteoblasts) were transferred directly from a topographically patterned agarose (hydrogel) stamp onto the surface of hydroxyapatite (a compound rich in bone mineral). The use of a hydrogel for the stamp provided a ‘‘wet’’ surface that kept cells hydrated and maintained cell viability throughout the stamping process. **Fig. 4B** shows spots of osteoblasts patterned on the surface of hydroxyapatite substrates. These patterns were printed with an agarose stamp having 1 mm diameter posts and a pitch of 2.5 mm. The technique transferred material to the surface of the substrate in parallel, making it possible to pattern multiple spots of cells simultaneously. **Fig. 4B** (**a-c**) shows three spots of cells patterned at the same time on the same scaffold, which demonstrates the reproducibility of the pattern transfer on 3D substrates. The viability of patterned cells was also confirmed by imaging the adhesion of cells and spreading the actin cytoskeleton on the surface (see **Fig. 4B, d**). This study suggested that stamping of mammalian cells directly onto tissue engineering scaffolds may find use in controlling the spatial invasion of scaffolds, promoting the hierarchical organization of cells, and in controlling cell–cell interactions. This study demonstrated that this kind of microstamping-based patterning technique can be utilized to direct patterning of cells on 3D substrates and to generate model systems for engineering engineering functional tissues.

5. Conclusion

The experimental examples summarized in this review represent some of the developments of cell patterning by using various surface modification approaches. Cell patterning is an emerging area of applied research and an enabling technology for manipulating cellular assemblies in a controlled fashion. Patterning cells on biomaterials will be of great potential in various biological applications, in particular to engineer tissue constructs or as a tool for understanding the mechanism of how cells respond to synthetic material systems. Although, numerous investigations focused on cell engineering in 2D surfaces, because at present, there is very limited information available on patterning of cells in 3D substrates. Patterning cells in 3D system is more relevant way to characterize the cell behavior and to maintain their normal physiological and metabolic activities *in-vitro*. This is an exciting time to be involved in cell patterning in 3D, with great challenges and also great expectations ahead.

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7. Abbreviations

2D Two-dimensional, **3D** Three-dimensional, **ECM** Extracellular matrix, **SAMs** Self assembled monolayers, **UV** Ultraviolet, **μCP** Microcontact printing, **PDMS** Polydimethylsiloxane, **CL** Colloidal lithography, **IL** Imprint lithography, **Tg** Glass transition, **PMMA** Polymethyl methacrylate, **SMC** Smooth muscle cells, **SEM** Scanning electron microscope, **nm** Nanometer, **μm** Micrometer, **HA** Hyaluronic acid, **FN** Fibronectin, **BCEs** Bovine adrenal capillary endothelial cells, **ECVs** Human bladder cancer cells, **BSA** Bovine serum albumin.

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