RESEARCH ARTICLE

A novel 3D printing method for cell alignment and differentiation

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Abstract: The application of bioprinting allows precision deposition of biological materials for bioengineering applications. Here we propose a 2 stage methodology for bioprinting using a back pressure-driven, automated robotic dispensing system. This apparatus can prepare topographic guidance features for cell orientation and then bioprint cells directly onto them. Topographic guidance features generate cues that influence adhered cell morphology and phenotype. The robotic dispensing system was modified to include a sharpened stylus that etched on a polystyrene surface. The same computer-aided design (CAD) software was used for both precision control of etching and bioink deposition. Various etched groove patterns such as linear, concentric circles, and sinusoidal wave patterns were possible. Fibroblasts and mesenchymal stem cells (MSC) were able to sense the grooves, as shown by their elongation and orientation in the direction of the features. The orientated MSCs displayed indications of lineage commitment as detected by fluorescence-activated cell sorting (FACS) analysis. A 2% gelatin bioink was then used to dispense cells onto the etched features using identical, programmed co-ordinates. The bioink allows the cells to contact sense the pattern while containing their deposition within the printed pattern.

Keywords: bioprinting, surface guidance, automated robotic deposition, precision etching, bioink, stem cells

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1. Introduction

Robotic deposition has a promising potential in biomaterial engineering for automated and reproducible patterning for cell delivery. Its application has given tissue engineers a method for cellular deposition with control up to the micrometer level[12]. Additionally, cell adhering surfaces can be modified to influence the orientation and morphology of the attached cells[3]. Cues can be introduced to the surface of a cell binding material that attracts and coordinates the attachment of the cell anchoring integrin proteins[4,5]. The shape adopted by the cell through the interaction with the surface cues feeds back to the cell nucleus via integrin receptors, which in turn affects the gene expression profile of the adhered cell[6,7]. This can have the effect of influencing cellular behavior, such as stimulating the switch in smooth
mature muscle cells from secretory to contractile phenotype. However, when using multi- or pluripotent cells, the effect can aid the routing of cell differentiation towards desired cell types.

Such exploitation of cell orientation by a modified surface has been defined as “Topographic guidance” or “Contact guidance”. With regards to regenerative bioengineering, alignment of cells and extracellular matrix is a structural feature of several tissues and organs such as corneal stroma, vasculature, tendons, bones, and skeletal muscles. It may also be useful in the culture and study of neurons.

As mentioned, the patterned cues can affect the phenotype of seeded cells. For example, mesenchymal stem cells (MSCs) seeded on electrospun-aligned silk fibroin cast on a rotating mandrel demonstrated enhanced differentiation into ligament fibroblasts. Jiang et al. found that the seeding of human MSCs (hMSCs) on aligned polycaprolactone fibers enhanced the differentiation towards neuronal lineage in the presence of the induction factor retinoic acid. Finally, MSCs have been found to respond to aligning features alone to differentiate towards a cardiomyocyte lineage. Li et al. created aligned channels by direct femto-laser machining of poly(lactic acid)-co-(ε-caprolactone) (PLLA-PCL) electrospun fibrous mats, and subsequently hMSCs were cultured onto these scaffolds. The cells elongated and aligned in the direction of the channel, and also displayed signs of lineage commitment towards cardiomyocytes, as demonstrated by quantitative Real-time polymerase chain reaction (qRT-PCR).

The surface modification through the introduction of cell aligning channels or grooves is widely known as an effective method for the topographic control of cells. Cells have been found to align on grooves as shallow as 150 nm; however, the deeper the groove, the more effective they are for cell alignment, with the effect being noticeable down to depths of approximately 25 μm. The reverse is true for channel width; as the width increases, the degree of cellular alignment decreases.

Cells can be directly seeded onto a patterned surface; however, when more than 1 cell line are seeded and their spatial distribution is important, then the deposition of the cells require more accurate control. We have previously demonstrated how cells can be bioprinted with relative precision using robotic dispensing system, delivering viable cells within a hydrogel bioink. When cell-containing growth medium is deposited, the printed trace spreads out due to its low viscosity (at approximately 0.94 cP). The bioink consists of a biocompatible polymer, at a concentration that confers for a viscosity able to retard the collapse and spreading of the printed trace. However, the concentration must not be too high, as not to hamper the sedimentation of the cells onto the etched grooves. The hydrogel bioink chosen here was 2% gelatin in Dulbecco’s modified eagle’s medium, for which we have recorded a viscosity greater than 200 cP (data not shown).

Many cell types can be orientated and influenced by such topographical guidance. In this study, we show the orientation and elongation of both fibroblasts and MSCs by the robot-etched grooves. As an example of the phenotypic modulation induced by grooves, we demonstrate evidence of MSC differentiation towards cardiomyocytes, following elongated alignment on the etched surface. Furthermore, we assess whether cells bioprinted with the dispenser are able to sense the grooves and alter their shape to respond to them. Hence, we aim to demonstrate a dual role for the bioprinting apparatus to:

- Be used for the modification of the cell seeding surface by etching topographical guidance channels,
- Subsequent bioprinting of MSCs onto the guiding features in a gelatin bioink that allows the cells to sense the grooves,
- The dispenser will use identically programmed co-ordinates for the etching and dispensing, so that the cells are placed onto the grooves,
- The viscosity of the bioink should contain the cells’ spread for discrete patterns of cellularization.

This dual application of the dispenser system has the potential to increase its usefulness for the production of more complex cell arrangements for scaffold design in bioengineering.

2. Material and Methods

2.1 Materials

Gelatin from the porcine skin (Bloom 300, type A) and phosphate buffer saline (PBS) of pH 7.2 were purchased from Sigma-Aldrich. Human dermal fibroblasts (HDF) and human mesenchymal stem cells (hMSC) were supplied by Lonza, and red fluorescent protein expressing rat mesenchymal stem cells (RFP-MSC) were supplied by Cyagen Biosciences Incorporation.

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Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin streptomycin solution were purchased from Invitrogen.

2.2 Robotic Dispensing System

The Janome 2300N pressure-controlled robotic dispensing system consists of a computer control, robotic XYZ table, and a pressure-driven syringe mechanism (Figure 1). Programming was done with the help of a specialized JR-C points software (manufacturer supplied) to define the specifications for both dispensing and etching. Original programs (data points for generating various shapes) were created in compatible programming languages (such as DXF, Gerber data, etc. in ASCII format) by using point job commands to create the lines. Hence, the software controlled the X-Y-Z geometry and the deposition rate. Bioink-Printing was performed using 0.05 MPa back pressure, 5 mm/sec writing speed from a 25 mL syringe and a 30 gauge needle (inner diameter 250 micron).

Figure 1. (A) The Janome 2300N pressure controlled robotic dispensing system. (B) Printing arm with customized etching stylus attachment. (C) Bioprinting with backpressure driven syringe containing 2% gelatin (in growth medium) bioink containing cells.

2.3 Patterning and Characterization of a Polystyrene Surface

The substrate was patterned using a desktop dispensing robot (SANEI-TECH, Japan) and manufacturer supplied software. Modifications were made to the robot to allow for ablation patterning. Specifically, the dispensing head was replaced by a sharpened stylus in order to cut the substrate surface (Figure 1).

Since both the polystyrene tissue culture plates and polystyrene films were used, all surfaces were plasma-treated and coated with fetal bovine serum to produce a standardized surface treatment between experiments. Plasma treatment, using a Femto Science system, preceded using conditions of 150 W, 30 sccm of oxygen for 10 minutes in a plasma system (Femto Science). FBS was then coated on the surface and incubated for 2 hours before washing thrice in PBS.

Different patterns are generated by entering coordinate points into the JR-C points software. Patterns in a variety of forms were obtained for this study, e.g. linear, S-shaped, and circle. Linear patterns with the spacing of 50, 100, 250, 500, and 1000 μm were produced. The depth of each groove can also be varied easily using the Z-height of the system. In this test, grooves set at 40, 80, and 170 μm were cut into the surface of 1 mm thick polystyrene (PS) sheets.

In order to view the cross-section of the patterned surface (especially for characterizing the depth of the sample), the patterned sample was first scored along the back of the samples to introduce a crack. Force was then applied to produce a controlled stress fracture across the sample. The exposed cross-section can then be analyzed accurately. In order to visualize the depth of the etching on the sample, an optical microscope was used (Olympus IX71, Japan). For quantitative measurement of the features (e.g. such as in measuring the depth of the grooves), ImageJ software was used.

2.4 Preparation of Cell-containing Gelatin Bioink

Bioink solutions were prepared by dissolving gelatin at 2% in DMEM. The solutions were heated and stirred at 60°C for 2 hours to aid solubilization. After cooling, the fibroblasts and the MSCs were suspended at concentration of 5 × 10⁶ cells mL⁻¹ within the bioink. The cellularized bioinks were extruded into thin lines via a 30-gauge needle onto a polystyrene film surface, following a pre-programmed deposition pattern within a 10 cm diameter tissue culture dish. After a 1 hour incubation, 10 mL growth media was added and the cells were incubated for 24 hours before viewing. Cell viability was assessed by observing and recording the presence and density of the green fluorescence protein (GFP) expressing cells by fluores-
cence microscopy.

2.5 Evaluation of Patterned Surface for Cell Alignment

The cells were seeded with a density of $3 \times 10^5$ cells/cm² and left to grow for 2 days. For an evaluation of cell orientation, the samples are stained with fluorescein diacetate (FDA) and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) nuclear stain. The cells are then observed under a fluorescence microscope (Olympus IX71, Japan).

2.6 Cardiac Expression Study for Aligned Cells on a Patterned Surface

Polystyrene films (2 × 2 cm) were patterned linear grooves with 100 µm spacing and were seeded with hMSC at $3 \times 10^5$ cells/cm².

For FACS, hMSCs were seeded for 7 days on the polystyrene surfaces before intracellular staining for CD29 (Abcam) and GATA4 (Abcam) by the following procedure:

Cells were fixed with 4% paraformaldehyde for 30 minutes, then permeabilized with 0.25% triton X 100 for 10 minutes and washed with PBS, then blocked with 0.5% bovine serum albumin (BSA) for 30 minutes, all performed at 4°C. Cells were then incubated with primary antibodies for 30 minutes, followed by the FITC labeled secondary antibody for 30 minutes. Cells were finally re-suspended in PBS and analyzed for marker expression with the Millipore GUAVA easycyte HT flow cytometer.

3. Results

The back pressure-assisted dispenser from the robotic dispensing unit could be easily substituted from the printing arm and replaced with a sharp needle that could etch distinct grooves into polystyrene sheets (Figure 1(A), (B), (C)). The apparatus could then be programmed to print complex patterns by creating straight lines, and sinusoidal S-shaped and circular patterns on the polystyrene sheets with X-Y axis control to approximately 50 µm as shown in Figure 2(A–F). Following the etching, the dispensing unit

![Image](image_url)

**Figure 2.** Patterns etched into polystyrene films presented at x4 (A, B, C) and x10 (D, E, F) magnification. The robot could be programmed to etch linear (A and D), S-shaped or waveform (B and E) and concentric circles (C and F). The robotic dispensing system etched a pattern of aligned grooves into the polystyrene surface (G), then printed the bioink directly to the grooves following identical coordinates (H).
can be returned to the printing arm of the robotic dispensing unit, and the bioink can be delivered, following the etched pattern (Figure 2(G) and (H)). The Z-axis programming also allowed precision in creating the depth of the etched groove down to 180 µm (Table 1 and Figure 3). This allows for some optimization in the groove depth for different cell types. Furthermore, the depth of the groove can determine whether the cells within the grooves can make contact with the cells between and within the neighboring grooves. A shallow groove can produce a more continuous sheet of cells whereas the deeper grooves create more distinct lines of cells.

It was found, when cells were seeded on the etched surfaces, that the grooves printed close together caused cells both within and between grooves to align. An inter-groove distance of 50 and 100 µm led to the best cell alignment. When greater distances were applied (250 µm to 1 mm), the cells were not oriented in the direction of the etching (Figure 4). For this study, a gap

Table 1. Displays the depth programmed into the robotic dispenser as a Z-axis co-ordinate compared to the obtained depth etched into the polystyrene surface.

<table>
<thead>
<tr>
<th>Programmed Depth (µm)</th>
<th>Etched groove depth (µm)</th>
</tr>
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<tbody>
<tr>
<td>40</td>
<td>35±7</td>
</tr>
<tr>
<td>90</td>
<td>81±6</td>
</tr>
<tr>
<td>180</td>
<td>175±3</td>
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Figure 3. (A, B, C) Control of etched groove depth through. The grooves cut into polystyrene are presented from above and (D, E, F) cross-section, with the expected and quantified depth displayed in Table 1. The programmed groove depths are 40 (A and D), 90 (B and E) and 180 µm (C and F).

Figure 4. Linear patterns were etched into polystyrene films with inter-groove spacing of 50, 100, 250, 500 and 1000 µm for (A), (B), (C), (D) and (E) respectively. hMSCs were seeded onto the films to observe stem cell alignment. The cells were visualized using FDA and DAPI staining.
distance between the grooves of 100 µm was applied.

As mentioned in the introduction, the purpose of pre-etching grooves before cell seeding was to allow the promotion of cell anisotropy and the regulation of stem cell differentiation. In Figure 5, it can be clearly seen that HDF cells (labeled with Calcein AM and DAPI) have adopted a stretched morphology and are aligning in the direction of the etched grooves on a polystyrene culture plate (Figure 5(A)). In comparison, the HDF cells on unmarked polystyrene appear more dendritic and rhomboid (Figure 5(B)), hence the grooves do have cell orientating effects.

Figure 5. Human dermal fibroblasts were elongated and aligned on the etched grooves following direct seeding in tissue culture medium onto the (A) etched polystyrene (B) compared to seeding on unpatterned polystyrene. The fibroblasts were stained with fluorescein diacetate (FDA) and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) for visualization.

Further subsequent experiments were performed on hMSCs. It has been found previously that the elongated alignment of human MSCs promoted the adoption of a more cardiovascular-like phenotype [18-23]. Here, FACS was performed on hMSC seeded on an etched surface of the linear and S-shaped grooves and compared to those cultured on an unmarked plate. The cells were tested for the expression within the population of the undifferentiated stem cell marker CD29 and the cardiomyocyte marker GATA4, to demonstrate relative stemness/lineage commitment (Figure 6). It was found that after 7 days, the linear groove-aligned MSCs had significantly reduced labelling for CD29 at 39% compared to 92% for cells on the un-patterned surface. Both the S-shaped and the linear grooves demonstrated a higher percentage of GATA4 positive cells at 91% and 62% respectively, whereas the control cells presented only 11% GATA4 positive cells. Hence, the FACS results do indicate that the etched alignment of polystyrene can promote differentiation towards a cardiomyocyte phenotype.

The work thus far demonstrated that the automated dispenser unit can be modified to introduce an etching attachment that can produce cell aligning grooves.

Stem cells aligned on these grooves show evidence of differentiation towards a cardiomyocyte phenotype, in particular for the linear pattern since CD29 was greatly reduced and GATA4 dramatically increased (Figure 6). Subsequent experiments examined how bioprinting could be used in conjunction with groove etching. A soluble hydrogel bioink was developed to deliver MSCs within distinct traces on top of the grooves. A low viscosity gelatin hydrogel was selected (2% gelatin dissolved in DMEM growth medium at approximately 4°C) so that the stem cells can gradually sediment as the hydrogel (printed at approximately 24°C) warms to room temperature and is then
incubated at 37°C. The stem cells sense the features and then become stretched and aligned in the direction of the feature. Figure 7(A) demonstrates the high density of cells printed directly on to the etched grooves, as can be viewed by the DAPI stained nuclei. The printed MSCs were then observed to adopt the stretched morphology, aligning along the direction of the etched groove (Figure 7(B)). When the MSCs are deposited in the culture media rather than the bioink, the cells do not form distinct traces; instead the cells adhere both in between and within the grooves, leading to a total confluence of the surface. The cells seeded in this manner still become elongated and align in the direction of the etched features, however, lack the distinct printed trace along the grooves (Figure 7(C)). Figure 7(D) displays non-elongated and randomly aligned stem cells seeded on an unpatterned surface as a control.

Figure 7. Red fluorescent protein (RFP) rat mesenchymal stem cells were bioprinted onto the etched grooves with 100 µm separation using 2% gelatin bioink (A and B), visualized using (A) 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) and (B) RFP fluorescence. The bioprinting was compared to cells seeded in normal growth medium onto (C) etched grooves and (D) unpatterned polystyrene.

4. Discussion

Of these guidance cues, patterning with the grooves has been of considerable focus in the previous literature[6,7]. The technique described here involves the use of etching to control cell alignment on hard polymers, such as polystyrene and polycaprolactone (data not shown). The automated robotic dispenser can etch grooves into hard polymer surface to create complex patterns for effective topographical guidance. The same apparatus can then be used to bioprint MSCs onto these grooves. The printed cells can sense the grooves and respond by elongating and aligning with them. There is the potential of combining both processes in the same printing head to produce cell aligning grooves and simultaneously seed the cells directly on the features as they are generated (Figure 8).

The method described here presents a straightforward and time-efficient method to produce cell aligning features and to also cellularize with relative precision so that etching and bioprinting can both be performed under an hour. Other methods of producing aligning channels and grooves include deep reactive ion etching[26], electron beam lithography[12], direct laser writing[21], femtosecond laser[22], photolithography[23], plasma dry etching[29] etc. (as reviewed by Li and colleagues[7]). These tend to be more time-consuming, involve complex treatments/reactions, do not allow for immediate cellularization, and do not synchronize the surface patterning with that of the bioink deposition.

The benefits of such bioprinting techniques include production of biologically active surfaces, for which different cell types can be arranged without complex surface treatments to select specific cell adhesion[30]. In addition to the studies of cell differentiation and phenotype, the applications of bioprinting include: Creating specific cell-to-cell patterns that mimic in vivo patterns of cellular interaction, such as the neuronal networks[30,31]; tool for facilitating basic biology research on specific cell–cell or cell–ECM interactions[30,21]; cell/tissue bases sensors for chemical, drug, and toxicity testing[33]; and tissue engineering for regenerative medicine, such as the fabrication of 2D cellular organizations that can be stacked into 3D.
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scaffolds and, as demonstrated here, to influence stem cell differentiation on a biomaterial [12, 13, 14, 15, 28–31].

5. Conclusion

A novel 2-stage method has been developed to control the alignment and differentiation of bioprinted stem cells. The first stage involves precision etching of patterned surface grooves, followed by a precision delivery of stem cells into the grooves via bioprinting. Compared to conventional manual cell seeding onto patterned surfaces, which usually leads to uncontrolled distribution of cells, the proposed method is precise, efficient, and of a high seeding quality. Preliminary assessments show that the alignment and differentiation of bioprinted MSCs could be controlled and enhanced by the proposed method.

Conflict of Interest and Funding

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