Conformal nanopatterning of extracellular matrix proteins onto topographically complex surfaces

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Our Patterning on Topography (PoT) printing technique enables fibronectin, laminin and other proteins to be applied to biomaterial surfaces in complex geometries that are inaccessible using traditional soft lithography techniques. Engineering combinatorial surfaces that integrate topographical and biochemical micropatterns enhances control of the biotic-abiotic interface. Here, we used this method to understand cardiomyocyte response to competing physical and chemical cues in the microenvironment.

The nano- and microscale patterning of biomaterial surfaces has enabled the development of advanced systems to control cell structure and function. Specifically, engineering topographical, chemical and/or mechanical cues in defined geometries can directly regulate cell adhesion, morphology, cytoskeletal organization and cell-cell interactions. The technology to do this is based primarily on photolithographic techniques to create nano- or micropatterned masters (typically silicon wafers) that are then replica molded to make topographically patterned surfaces in other materials such as hydrogels and elastomers. These are used directly for cell culture or are formed into stamps and microfluidic systems to pattern extracellular matrix (ECM) proteins, growth factors and other bioactive molecules onto surfaces1. Researchers have shown that these nanometer- and micrometer-scale patterns of topography and biochemistry can each align cells, organize anisotropic tissue sheets and modulate gene expression profiles2,3. There is also evidence of the synergistic effect of combining these patterned cues into an integrated surface, such as for the enhanced alignment of neurons4 and endothelial cells5. However, to date, the ability to independently engineer microtopography and patterned chemistry into hierarchically structured surfaces has been limited due to the technical challenge of chemical patterning onto rough surfaces.

Here we report the Patterning on Topography (PoT) printing technique, which is able to directly transfer ECM proteins in defined geometries from a smooth release surface onto a microtopographically complex surface while substantially maintaining pattern fidelity (Fig. 1a and Online Methods). Briefly, thermally sensitive poly(N-isopropylacrylamide) (PIPAAm) is spin-coated onto glass coverslips (Fig. 1a, step 1, and Supplementary Fig. 1); then an ECM protein is patterned onto the PIPAAm using microcontact printing (µCP) with a polydimethylsiloxane (PDMS) stamp (Fig. 1a, step 2). Next, a topographically patterned surface is brought into contact with the ECM patterned PIPAAm-coated coverslip (Fig. 1a, step 3), submerged in distilled water at 40 °C and slowly cooled to room temperature. As the PIPAAm transitions through its lower critical solution temperature at ~35°C, the PIPAAm swells and pushes the patterned ECM protein as an ~5-nm thick layer6,7 onto the adjacent, topographically patterned surface where it adheres due to hydrophobic interactions (Fig. 1a, step 4). As the PIPAAm continues to swell, it eventually dissolves (Fig. 1a, step 5) and the PoT-printed surface can be used for cell seeding and culture (Fig. 1a, step 6).

The unique capabilities of PoT printing to pattern ECM proteins on topographically patterned surfaces are clearly demonstrated when compared to standard µCP and protein coatings adsorbed from solution. To show this, we used PDMS either spin coated onto glass coverslips as a flat control surface or cast against A4 paper, 150-grit sandpaper or 220-grit sandpaper. These surfaces were chosen because the heterogeneous distribution of feature width, depth and morphology enabled us to simultaneously evaluate the ability to pattern a wide range of microscale feature dimensions. We examined the full range of test surfaces and used confocal imaging and three-dimensional (3D) rendering to evaluate PoT printing fidelity (Fig. 1b and Supplementary Fig. 2). As expected, the spin-coated PDMS surface could be patterned with PoT or µCP, with no discernible difference. In comparison, even the A4 paper was rough enough to present challenges to µCP, resulting in a collapse of the line pattern and gaps in pattern transfer that caused a loss of fidelity. Results were worse on the rougher 220- and 150-grit sandpaper surfaces, with fibronectin (FN) transferred in patches and large gaps on the order of hundreds of micrometers. In contrast, the PoT-printed surfaces had well-transferred and conformal FN lines that maintained pattern fidelity and followed surface contours, even on the sandpaper surfaces (Fig. 1b and Supplementary Fig. 2).

Next, we used PoT to pattern ECM protein lines onto microridges with defined geometries to determine the limits of the technique. Test surfaces with 20-µm-wide, 20-µm-spaced microridges demonstrated that we could PoT print up to 37 µm deep, a depth-to-width aspect ratio of 1.85 (Supplementary Fig. 3). The FN lines were conformal to the ridge tops, the bottoms of the trenches and the vertical sidewalls, even on undercuts. Further, we could PoT print on deeper, 48-µm microridges with a 2.4 aspect ratio corresponding to nearly 500% strain of...
the FN; however, there were gaps in the FN on the sidewalls (see Supplementary Note for further discussion on PoT limitations). Varying feature dimensions demonstrated PoT printing on microridges from 2 µm wide, 2 µm spaced and 2.5 µm deep up to 200 µm wide, 200 µm spaced and 36 µm deep (Supplementary Fig. 4), and we expect that smaller (sub-micron) and larger features can also be patterned based on the sandpaper results (Fig. 1b and Supplementary Fig. 2). We also validated that other ECM proteins, including laminin and collagen type IV, could be PoT printed and that multiple proteins could be combined, such as FN and laminin lines orthogonal to each other and 45° to the microridges (Supplementary Fig. 5). Although we refer to PoT as a nanopatterning technique because the transferred ECM proteins are ~5 nm thick,6,7, we verified that PoT can also print conformal FN lines 100–500 nm wide and 5 µm spaced on 15-µm deep microridges (Supplementary Fig. 6). Finally, control experiments confirmed (i) that µCP of the FN lines using a PDMS stamp with a low Young’s modulus (E ~50 kPa) to better conform to the microridges failed to achieve results comparable to PoT (Supplementary Fig. 7) and (ii) that FN lines could also be PoT printed on stiffer photoresist microridges (Supplementary Fig. 8; see Supplementary Note for further discussion on µCP techniques compared to PoT).

Next, we used PoT printing to investigate cell response to surfaces presenting orthogonal topographic and chemical guidance cues. This is important for applications such as cardiac tissue engineering, where cardiomyocytes (CMs) can be engineered into anisotropic two-dimensional (2D) tissues using surfaces with either microridges8 or micropatterned9,10 FN lines. But what happens when CMs are presented with both alignment cues? Control 20-µm-wide, 20-µm-spaced microridges uniformly coated with FN and seeded with chick CMs resulted in cell alignment parallel to the microtopography (Fig. 2a). In contrast, CMs seeded on microridges with orthogonal PoT printed FN lines followed the FN lines despite having to traverse relatively large topographic features (Fig. 2b,c and Supplementary Fig. 9). Notably, the CMs were able to spread and deform around the microridges (Fig. 2d–i) while displaying a characteristic sarcomeric structure (Supplementary Fig. 10). To quantify this difference in CM response, we analyzed actin alignment relative to the microridges and FN lines (Fig. 2j). On the uniformly FN-coated surface, CMs aligned in parallel with the underlying microridges (Fig. 2j). However, on the PoT-printed surface with 5-µm microridges, CMs aligned with the underlying FN lines (Fig. 2k). As
the depth increased, CM alignment became bimodal, with cells on the ridge tops continuing to align to the FN lines and cells in the trenches shifting alignment 90° to the microtopography (Fig. 2i and Supplementary Fig. 9). This demonstrated that CM response was depth dependent, and cross-sectional views suggested that adhesion to the ridge sidewalls influenced this process (Fig. 2i). When vertical sidewalls were not present and changes in height were more gradual, such as for FN lines PoT printed on a PDMS replica of a 220-grit sandpaper, CM alignment followed the protein pattern (Supplementary Fig. 11). Control experiments confirmed that stability in culture and cell response to PoT-printed FN lines were similar to standard µCP FN lines (Supplementary Fig. 12).

Finally, we used PoT-printed FN circles to produce spatially variable guidance cues where the circle edge changed from being parallel to orthogonal to the underlying microridges at a π/2 interval. To do this, we patterned an array of 100-µm-diameter FN circles on 20-µm-wide, 20-µm-spaced and 5-µm-tall microridges (Fig. 3a). As expected, the FN circles conformally coated the ridge tops and trench bottoms, and the seeded CMs formed a cardiac microtissue that covered the entire surface (Fig. 3b). The microtissue was ~15 µm thick, with distinct differences in CM alignment based on vertical position (Fig. 3c). Analysis of actin alignment (Fig. 3d and Supplementary Fig. 13) showed that CMs in the grooves were aligned to the microridges, those above the microridges were aligned to the edge of the FN circle, and those above that appeared completely isotropic, with no apparent guidance from the aligned CMs below. Increasing the diameter of the FN circle from 100 µm to 250 or 500 µm (Supplementary Figs. 14 and 15) showed that CM alignment to the edge of the FN circle extended ~100 µm from the periphery. Although this is a basic example of combining spatially variable microtopographical and chemical cues, it clearly demonstrates (i) that in our system patterned FN over-rides topography in terms of CM alignment and (ii) that CM alignment to either cue depends on direct physical contact, and there is limited ability of CMs to propagate the alignment to neighboring cells.

PoT provides a methodology to probe how cells respond to competing topographical and chemical cues and to engineer surfaces that maximize a given behavior, such as organization of CM into an aligned cardiac tissue. Looking forward, PoT printing enables improved control over the cellular microenvironment, which will be useful for understanding basic cell-surface interactions as well as engineering more advanced biomaterial surfaces where topography and chemistry can be independently optimized.
**ONLINE METHODS**

**Fabrication of microtopographically modified surfaces.** Polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning) was mixed in a 10:1 base resin-to-curing agent ratio, degassed and then used to create the different microtopographically modified surfaces in this study. Smooth control surfaces were fabricated by spin-coating the PDMS on 25-mm-diameter glass coverslips at 4,000 r.p.m. and cured at 65 °C for 4 h to create ~15-µm-thick films. Surfaces with random microtopographies were fabricated by creating PDMS replicas (negatives) of A4 printer paper and 220-grit and 150-grit sandpaper. To do this, a 4 × 4–cm square of the paper was placed into the bottom of a 10-cm petri dish and PDMS was poured on top to an approximate thickness of 0.5 cm. The PDMS was cured at 65 °C for 4 h, peeled off and then cut into 1 × 1–cm squares. Surfaces with microridge topographies were fabricated by using photolithography according to previously published methods. The materials used for photolithography varied depending on the photoresist layer thickness. For photoresist layers with a thickness between 2 and 10 µm, SPR 220.3 photoresist (Microchem) was spin-coated onto glass wafers. Thicker photoresist layers between 10 and 40 µm were prepared by spin-coating SU-8 2015 photoresist (Microchem) onto silicon wafers. SU-8 2050 photoresist (Microchem) was spin-coated onto silicon wafers to generate photoresist layers thicker than 40 µm. All photoresist layers were exposed to UV light through a transparency photomask and then developed using SU8 developer or SPR developer. The PDMS substrates were sonicated in 50% ethanol for 30 min and dried at 40 °C for 1 h, rinsed two times with distilled water and then dried under nitrogen. The PDMS substrates to be patterned were UV/ozone treated for 15 min, rinsed two times with distilled water and then dried under nitrogen. For µCP, PDMS stamps with microridges 20 µm wide, 20 µm spaced and 2 µm tall were fabricated using photolithography as described above for the microtopographically modified surfaces. PDMS stamps were sonicated in 50% ethanol for 30 min and dried by nitrogen, incubated with FN solution for 1 h, rinsed two times with distilled water and then dried under nitrogen. The µCP of laminin (LAM, Invitrogen) and collagen type IV (COL IV, Sigma) were performed similarly except both were diluted to working concentrations of 100 µg/mL in distilled water. The PDMS substrates to be patterned were UV/ozone treated for 15 min and then brought into conformal contact with the PDMS stamps for 5 min. Gentle pressure by lightly tapping the back of the PDMS stamps with tweezers was used to ensure contact.

**Uniform coating and microcontact printing (µCP) of ECM proteins.** For uniform protein coatings adsorbed from solution, the PDMS substrates were sonicated in 50% ethanol for 30 min and dried by nitrogen. The ECM protein fibronectin (FN) (Corning, catalog number 356008) was diluted to a concentration of 100 µg/mL in distilled water; 40% of the FN was conjugated to Alexa Fluor 488 fluorescent dye (Life Technologies). A 300-µL droplet of FN solution was incubated on the PDMS substrates for 15 min, rinsed two times with distilled water and then dried under nitrogen. For µCP, PDMS stamps with microridges 20 µm wide, 20 µm spaced and 2 µm tall were fabricated using photolithography as described above for the microtopographically modified surfaces. PDMS stamps were sonicated in 50% ethanol for 30 min and dried by nitrogen, incubated with FN solution for 1 h, rinsed two times with distilled water and then dried under nitrogen. The µCP of laminin (LAM, Invitrogen) and collagen type IV (COL IV, Sigma) were performed similarly except both were diluted to working concentrations of 100 µg/mL in distilled water. The PDMS substrates to be patterned were UV/ozone treated for 15 min and then brought into conformal contact with the PDMS stamps for 5 min. Gentle pressure by lightly tapping the back of the PDMS stamps with tweezers was used to ensure contact.

**Patterning on Topography (PoT) printing of ECM proteins.** The PoT printing process is a modification of surface-initiated assembly where the release of the ECM protein occurs directly onto a microtopographically structured surface. To do this, we first spin-coated 25-mm-diameter glass coverslips with a 10% poly(N-isopropylacrylamide) (PIPAAm, Polysciences, Inc.) in 1-butanol (w/v) solution at 6,000 r.p.m. To PoT print features with a depth-to-width aspect ratio higher than 0.5, we increased the thickness of the PIPAAm layer by spin-coating glass coverslips with a 40% PIPAAm in 1-butanol (w/v) solution at 2,000 r.p.m. PIPAAm layer thickness was determined by first spin-coating PIPAAm at different concentrations and then making a small scratch through the PIPAAm layer with a razor blade. Next, we used atomic force microscopy (AFM, MFP3D, Asylum Research) in air using AC mode with AC160TS-R3 cantilevers (Olympus Corporation) to scan over the scratch. The thickness of the PIPAAm layer was then quantified using the IGOR Pro software environment (WaveMetrics, Inc.). Prior to PoT printing, the PIPAAm-coated coverslips were sterilized using high-intensity UV light for 15 min. Fluorescently labeled FN, LAM and COL IV were then patterned onto the PIPAAm-coated coverslips by µCP with various geometries, including (i) 20-µm-wide, 20-µm-spaced lines; (ii) 100-µm-diameter circles; (iii) 250-µm-diameter circles and 500-µm-diameter circles. Next, PDMS substrates with microtopography on the surface were sterilized using high-intensity UV light for 15 min and placed in conformal contact with the protein-patterned PIPAAm coverslips for 10 min. Making sure to keep the PDMS stamp and PIPAAm surface in contact, the samples were placed within the well of a 6-well plate, 40 °C sterile distilled water was poured onto the stamp and PIPAAm and then the plate was placed in a 37 °C incubator for at least 3 h to allow time for water to enter the channels or spaces and diffuse into the PIPAAm. The plate was then removed from the incubator and placed in the biosafety cabinet at room temperature for 1 h to dissolve the PIPAAm layer and release the ECM patterns onto the PDMS substrates. Once the PIPAAm dissolved, the PDMS and coverslip were no longer attached and the PoT-printed PDMS substrate could then be removed and used for cell culture or analysis. We also tested the ability to PoT print onto very stiff substrates by placing an SPR 220.3 master mold containing 20-µm-wide, 20-µm-spaced and 3-µm-tall microridges into conformal contact with a PIPAAm-coated coverslip patterned with 20-µm-wide and 20-µm-spaced FN lines. For all experiments, PoT-printed substrates that had defects due to poor contact with the PIPAAm-coated coverslip or to improper water penetration within the topography were discarded. At least three samples per condition were used for imaging and cell seeding.

**Cardiomyocyte isolation and culture.** Chick cardiomyocytes (CMs) were isolated from day 7–8 (HH stages 30–32) White Leghorn embryos and are not considered live vertebrate animals under PHS policy. For each isolation, 7–15 hearts were dissected, cut into small pieces and digested in 3 mL of 0.05% Trypsin-EDTA (Sigma-Aldrich) for 7 min. The supernatant was carefully collected, added to seeding medium (M199 medium, 10% heat-inactivated FBS, 1% penicillin/streptomycin) and filtered through a 40-µm cell strainer to isolate individual cells. The 7-min incubation was repeated three times so that the hearts were completely digested while minimizing cytotoxicity by long exposure to trypsin. The cell solution was then centrifuged at 50g for 10 min and resuspended in 30 mL of seeding medium. Fibroblasts were removed by selective adhesion by pre-plating successively for

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45-min incubations in T75 cell culture flasks. The supernatant, enriched in CMs, was centrifuged at 50g for 10 min then resuspended in seeding medium at 7.5 × 10⁵ cells/mL. PDMS substrates were placed in a 6-well plate, 200 µL of the cell suspension was pipetted on top and incubated for 2 h to allow cells to attach and then the wells were filled with additional seeding medium. On the next day, the medium was replaced with a maintenance medium containing a reduced level of 2% heat-inactivated FBS to minimize fibroblast proliferation.

**Fluorescent staining and imaging.** The CMs and ECM proteins were fluorescently labeled to analyze the surface structure and cell response. The FN (human, Corning, catalog number 356008) was fluorescently labeled before use with fluorescent dyes, either Alexa Fluor 546 maleimide or Alexa Fluor 488 carboxylic acid, succinimidyl ester (Life Technologies), according to published methods. The fluorescently labeled FN was combined with unlabeled FN at a volume concentration of 40%. PDMS surfaces PoT printed with LAM or COL IV were fluorescently stained using monoclonal primary antibodies (Sigma, catalog number L9393 for LAM, C1926 for COL IV) followed by staining using Alexa Fluor 488–conjugated goat anti-mouse secondary antibodies (Life Technologies, catalog number A-11001). The CMs were cultured for 2 days, removed from the incubator, briefly washed with 37 °C PBS and then fixed for 15 min in 4% paraformaldehyde with 0.05% Triton X-100. Next, samples were blocked for 1 h with 5% goat serum and then incubated with 1:200 DAPI, 1:100 primary monoclonal antibody against sarcomeric alpha-actinin (Sigma, catalog number A7811) and 1:60 Phalloidin conjugated to Alexa Fluor 633 (Life Technologies) for 2 h at 37 °C. Samples were subsequently washed three times for 10 min in PBS and then incubated with goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 for 2 h at 37 °C. Finally, samples were washed three times for 10 min in PBS and mounted against No. 1.5 glass coverslips.

The alignment of actin filaments or a Leica SP5 multiphoton microscope with a 25× oil immersion objective. Three coverslips were kept in growth medium without cells. After 3 days in culture, all samples were fixed and cell samples were stained for nuclei and F-actin and imaged as described earlier. C2C12 cells were authenticated and certified mycoplasma-free by the manufacturer.

**Fabrication of low Young’s modulus PDMS stamps for µCP.** To determine the depth-to-width aspect ratio at which µCP is unable to pattern topographical surfaces, we fabricated low Young’s modulus PDMS stamps as described previously. Briefly, stamps with an elastic modulus of ~50 kPa were prepared by mixing Sylgard 184 and Sylgard 527 in a 10:1 ratio (w:w). Sylgard 184 was prepared as described previously while Sylgard 527 was prepared by mixing equal parts of A and B components per manufacturer’s instructions. The 10:1 mixture of Sylgard 184 and Sylgard 527 was then cast over a SPR 220.3 master mold and cured at 65 °C for 4 h. Prior to casting, the SPR 220.3 master mold was silanized (PlusOne Repel-Silane ES, GE Healthcare) in a vacuum desiccator for at least 4 h. After curing, the stamps were peeled off of the master mold, coated with FN as previously described and then µCP orthogonally onto PDMS microridges.

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