Microfluidic Device-Assisted Etching of p-HEMA for Cell or Protein Patterning

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DOI 10.1002/btpr.2576
Published online November 11, 2017 in Wiley Online Library (wileyonlinelibrary.com)

The construction of biomaterials with which to limit the growth of cells or to limit the adsorption of proteins is essential for understanding biological phenomena. Here, we describe a novel method to simply and easily create thin layers of poly (2-hydroxyethyl methacrylate) (p-HEMA) for protein and cellular patterning via etching with ethanol and microfluidic devices. First, a cell culture surface or glass coverslip is coated with p-HEMA. Next, a polydimethylsiloxane (PDMS) microfluidic is placed onto the p-HEMA surface, and ethanol is aspirated through the device. The PDMS device is removed, and the p-HEMA surface is ready for protein adsorption or cell plating. This method allows for the fabrication of 0.3 μm thin layers of p-HEMA, which can be etched to 10 μm wide channels. Furthermore, it creates regions of differential protein adhesion, as shown by Coomassie staining and fluorescent labeling, and cell adhesion, as demonstrated by C2C12 myoblast growth. This method is simple, versatile, and allows biologists and bioengineers to manipulate regions for cell culture adhesion and growth. © 2017 American Institute of Chemical Engineers *Biotechnol. Prog.*, 34:243–248, 2018
Keywords: microfluidics, cell patterning, protein patterning, p-HEMA, PDMS

Introduction

The development and subsequent interaction between distinct cell types plays a role in neuronal development and repair. For example, during development, growth factors, molecular gradients, and chemotactic agents are released from target tissues to enhance survival and growth of neurons from the retina to the tectum[1,2] and in the peripheral nervous system.[3,4] A major hurdle in both studying these processes *in vitro* and producing biomaterials to aid in regeneration is the creation of biomaterials with regions where cells or proteins will not adhere. Multiple techniques have been used to create such substrates for cellular growth. For example, nanoimprint lithography of water-stable and patterned protein films[5,6] and hybrid multilayer films produced by layer-by-layer technology[7] allow for regions on biomaterials where cells adhere and grow and other areas devoid of cells. For distinct biological applications, polydimethylsiloxane (PDMS) microfluidic devices have been used to pattern 2-methacryloyloxyethyl phosphorylcholine for examining C2C12 myoblast fusion and calcium transients,[8] and more recently, amphibian retinal neurons have been patterned to study regional effects on photoreceptor varicosity formation.[9] Although a number of techniques can create regions of cell adhesion and repulsion with very high fidelity to the designed patterns, complex materials and methods to create these regions limit their usefulness. Therefore, the creation of a method to simply and easily pattern cells and proteins is of great importance to advancing biological studies of cellular function, responses, and interactions.

Historically, poly(2-hydroxyethyl methacrylate) (p-HEMA) has been used to create regions of decreased protein and cell adhesion of spheroid cultures, and in specific, rat hepatocytes.[10–12] The fabrication of p-HEMA-coated regions with microfluidic devices has been used for creation of walls and

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molds that allow for single cell isolation of cells and other cell types.[13,14] While very useful for studies of single cells, this fabrication type has several limitations. For example, the ability to fabricate specific areas for cell adhesion and growth is limited by the use of closed channels. Currently, p-HEMA coating can only be fabricated in areas contiguous with the inlet. Isolated areas of p-HEMA may be difficult to fabricate with this method. In addition, p-HEMA fabrication schemes create "tall" 5 μm barriers, which may interfere with additional application of PDMS-coated devices. Therefore, methods to create thin p-HEMA-coated areas are essential for the study of single cells using both cellular patterning and signal detection with devices, such as microelectrode arrays or nanofibers.

Here, we describe a simple method to pattern p-HEMA using negative patterning. This method differs from that recently described by Ye and colleagues[14] in that our technique involves negative micropatterning while the other method provides positive micropatterning. Furthermore, we can pattern the p-HEMA an order of magnitude thinner that the previously reported method. Our novel method increases the fidelity of cell or protein patterning using microfluidic devices and provides a simple, versatile method of patterning cells or proteins onto glass or cell culture surfaces. This method can easily be applied using thin microfluidic channels or via the use of microfluidic devices created with scotch tape fabrication.

Materials and Methods

Microfluidic channel fabrication

Microfluidic devices were fabricated as previously described.[9,14] Briefly, a 50 μm thin layer of photoresist SU-8 50 (MicroChem, USA) was spin-coated onto a 3 inch silicon wafer (University wafers, USA) and exposed to 365 nm UV light through a transparency photo-mask after pre-exposure bake on hotplate. Unexposed photoresist was removed by developing the wafer in propylene glycol monomethyl ether acetate (PGMEA, Sigma Aldrich, USA) after post-exposure bake on a hotplate. The wafer with patterned photoresist was rinsed with isopropanol, dried by blowing nitrogen gas and exposed to (Tridecafluoro-1,1,2,2-Tetrahydrooctyl)-1-Trichlorosilane vapors (United Chemical Technology, Inc., USA) under vacuum. This was necessary to prevent sticking of the polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Inc., USA) to the mold during soft-lithographic replication. PDMS was then vigorously mixed at a 10:1 ratio of polymer to catalyst, poured onto the surface of the mold, placed into a vacuum chamber for 30 min to remove bubbles, cured at 60°C for at least 2 h, and cut from the mold. Inlets and outlets were then punched from the mold using a 1 mm biopsy punch (Tedpella, USA)

Scotch tape microfluidic devices were fabricated as previously described.[15] Briefly, glass slides were cleaned with isopropyl alcohol (IPA) and dried with an airgun. Scotch tape was then applied to the surface of the glass slide and rolled with a rubber roller to remove air bubbles. Device design was printed onto regular paper and placed underneath glass slide as reference. A clean scalpel blade was used to cut the excess scotch tape from glass slide. IPA was then used to carefully clean excess adhesive from the glass slide, and the device was placed in a 65°C oven for 2 h to improve adhesion. Uncured PDMS was then poured over the master, exposed to vacuum for 30 min to remove bubbles, and cured in a 65°C oven for 2 h. PDMS microfluidic devices were then cut from the scotch tape masters, 1 mm inlet and outlets punched into the devices with a biopsy punch, devices cleaned with scotch tape, and devices were sterilized with 70% ethanol and UV exposure prior to use.

p-HEMA patterning

Poly (2-hydroxyethyl methacrylate; p-HEMA, SIGMA, USA) was dissolved in 95% ethanol shaking overnight at room temperature and used to coat either 22 mm No. 1 square coverslips or six-well plates. The coverslips were first briefly flamed and subsequently placed flat individually into six-well plates. Plates with and without coverslips were then briefly coated with 500 μl of p-HEMA. Excess p-HEMA was aspirated off of the surface of the dish and allowed to dry in the hood with the cover off for at least 1 h. Clean and sterile microfluidic channels were then attached to the surface of the p-HEMA-coated plates or coverslips. A 50 μl drop of 70% ethanol (EtOH) was placed onto the inlet of the microfluidic channel and immediately aspirated from the outlet of the microfluidic channel. 50 μl of sterile deionized (DI) H_2O was applied to the inlet of the microfluidic channel and immediately aspirated from the outlet of the microfluidic channel. The PDMS microfluidic channel was then removed from the surface of the plate with sterile tweezers. The plates were washed three times with DI H_2O (Figure 1).

Characterization of p-HEMA patterning

Patterned p-HEMA samples were sputter-coated with gold/palladium and imaged via scanning electron microscopy (SEM). SEM images were taken with a Zeiss Sigma scanning electron microscope with Oxford EDS. Thickness of patterned p-HEMA samples were measured using a profilometer (Dektak3ST Surface Profilometer).

Patterned p-HEMA glass coverslips were incubated in phosphate buffered saline (PBS) at 37°C for 7 days. p-HEMA-coated glass coverslips were imaged after 1 day and 7 days of incubation at 40× magnification using an EVOS FL microscope. Channels size was measured in ImageJ. Statistics were performed in Graphpad Prism 6.0.

Protein patterning on p-HEMA-coated surfaces

Six-well plates with patterned p-HEMA were coated with 1 ml of 10% normal goat serum in PBS overnight at 37°C. Goat serum solution was then aspirated off of the surface of the plates, and the plates were washed three times with PBS. Adhered protein was stained with Coomassie blue solution (1% w/v Coomassie Brilliant blue in 50% methanol and 10% glacial acetic acid) for 1 h with shaking at room temperature. Excess Coomassie blue solution was aspirated off, and the plates were washed three times with DI H_2O.p-HEMA coated plates were also coated with 1:100 dilution of AlexaFluor® goat anti-mouse 488 in PBS overnight. Excess fluorescent antibody was aspirated off, and plates were washed three times with PBS. Plates were then imaged with an EVOS FL imaging system under phase contrast and fluorescent imaging.

C2C12 cell patterning

C2C12 cells (ATCC, USA) were passaged and plated at 5×10^4 cells per well in Dulbecco's Modified Eagle Medium (DMEM; Thermofisher, USA) without serum and seeded onto the patterned p-HEMA surfaces. After 15 min, DMEM was aspirated off and replaced with DMEM plus 10% fetal bovine serum

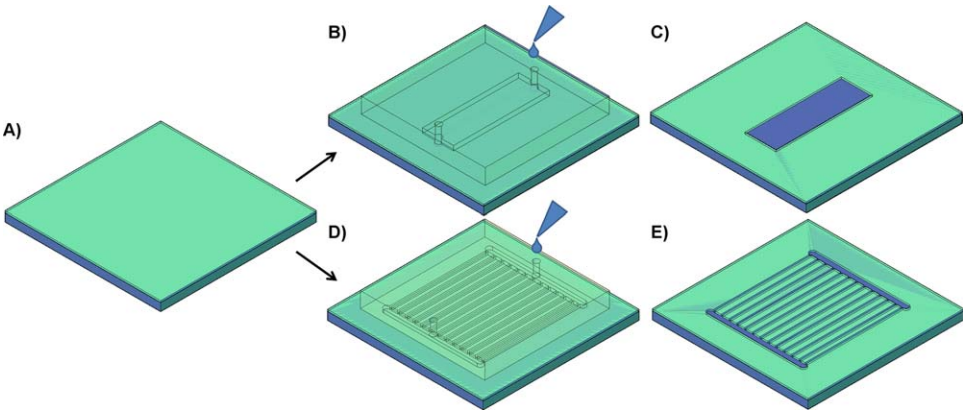

Figure 1. Workflow of p-HEMA patterning with PDMS microfluidic device. (A) Clean and sterile glass coverslip coated with 20 mg/ml p-HEMA. (B) Scotch tape-fabricated microfluidic device or (D) microfabricated microfluidic device is sealed to p-HEMA surface. (C) and (E) p-HEMA surface after ethanol-assisted etching with microfluidic device.

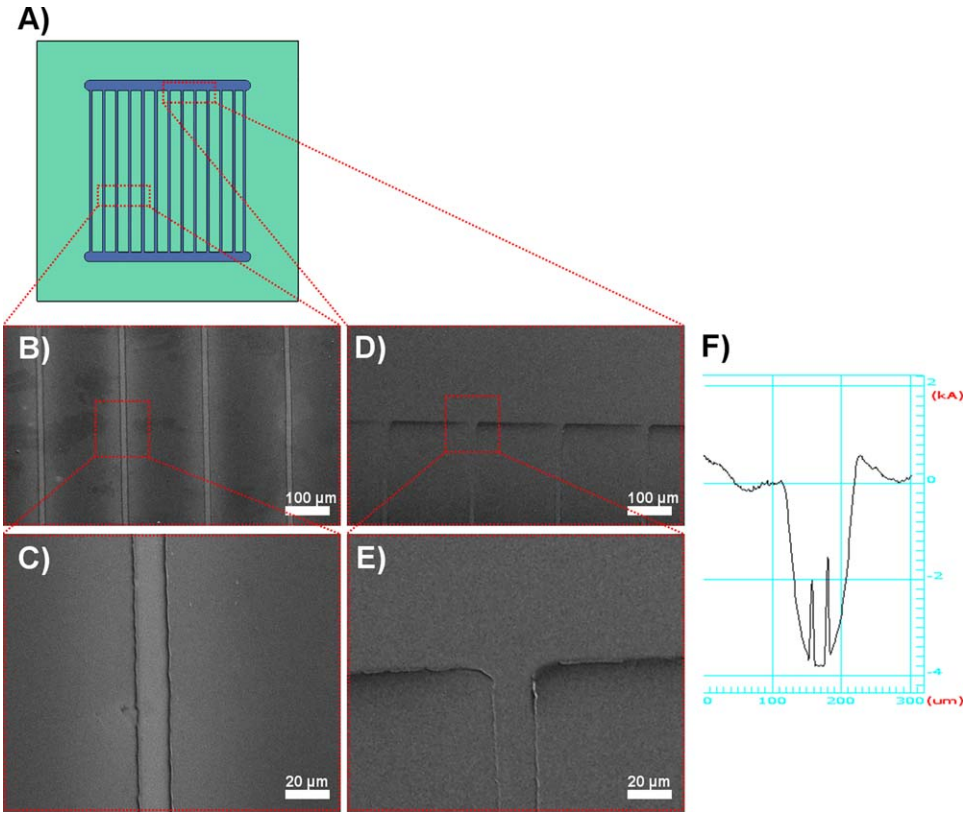

Figure 2. Scanning electron micrograph of p-HEMA pattern. p-HEMA patterned dishes imaged with scanning electron microscopy. (A) Microfluidic design patterned via p-HEMA etching. (B) and (C) Channel region patterned via p-HEMA etching. (D) and (E) Inlet region patterned with p-HEMA etching. (F) Profilometer measurement of p-HEMA etching. Profilometer measurements indicate an average depth of 3 kiloAngstroms (0.3 μm) in etched p-HEMA channels.

(FBS). The next day, DMEM plus FBS was replaced with DMEM plus 2% horse serum (HS). After 6 days, cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (Sigma, USA) and phalloidin-TRITC (Sigma, USA) Fixed cells were imaged with an EVOS FL imaging system with fluorescent imaging. Images were processed using ImageJ.

Results and Discussion

p-HEMA etching

Micropatterning of cells and proteins have proven to be an effective tool in studying how cells develop in response to a variety of cues and to interaction with other cells. Therefore, having more easily accessible methods for specific cell and protein patterning is vital to understanding the mechanisms underlying these biological responses. Here, we demonstrate a method of fabricating thin patterned layers of p-HEMA via etching off a coating of p-HEMA with PDMS microfluidic devices created via traditional soft lithography and scotch tape fabrication.

p-HEMA was chosen as the nonadhesive substrate for our material due to its successful use for the study of rat hepatocyte spheroids, flexibility in use for techniques, such as optical tweezing, and the fact that it is inexpensive, easily

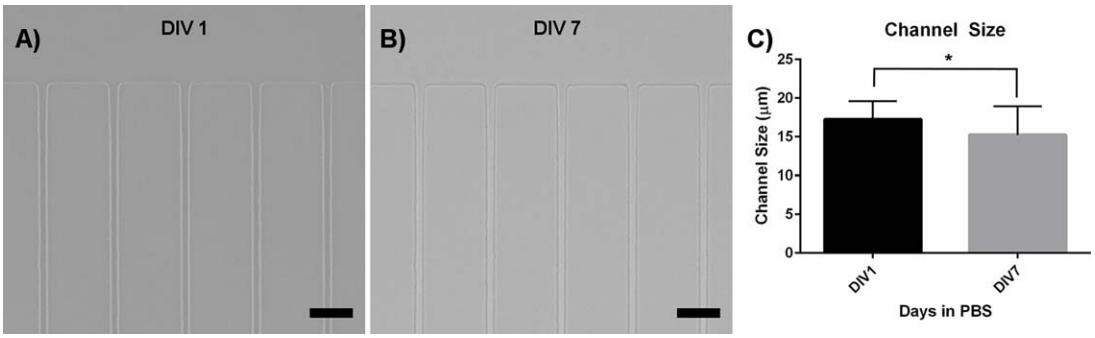

Figure 3. Swelling of p-HEMA patterning in PBS (A) Brightfield image of pHEMA-coated and patterned glass coverslips after 1 day in phosphate-buffered saline (PBS) and (B) 7 days in PBS. Scale bar = 100 μm. (C) Measurement of p-HEMA channel size after 1 day and 7 days in PBS. Bars = standard error. *$P < 0.05$ as determined by Student's *t*-test. $n = 51$ measurements per time point.

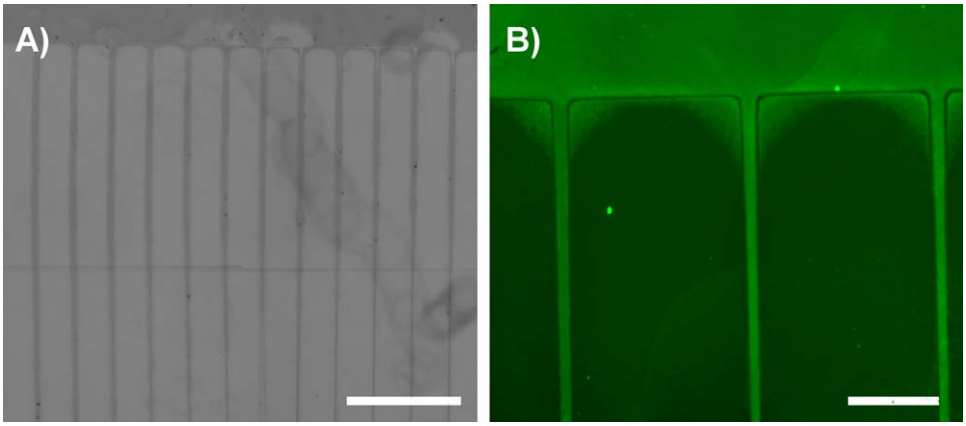

Figure 4. Protein patterning on p-HEMA surface. (A) Coomassie blue-stained p-HEMA-coated plate after overnight incubation with 10% normal goat serum in phosphate-buffered saline. Scale bar = 500 μm. (B) Fluorescent images of p-HEMA patterned surface after overnight incubation with AlexaFluor® goat anti-mouse 488, which marks areas where protein is present. Scale bar = 100 μm.

available, and biocompatible.[10–12,16] In our study, we first coated glass coverslips or a six-well cell culture plate with a thin layer of p-HEMA (Figure 1). This created an initial nonadhesive substrate to prevent protein or cell adhesion. By taking advantage of the solubility of p-HEMA in ethanol, p-HEMA was then etched from the surface of the cell culture plate or coverslip by aspiration of ethanol from the microfluidic channel. In addition, the low surface tension of ethanol (~22.10 mN/m) allows for easy aspiration from the microfluidic channel using a normal house vacuum (~254 mmHg). Normally, the dissolution rate of p-HEMA is extremely slow, whereby approximately 8 h are needed for a 20 mg/ml solution to fully dissolve. However, due to the small amount of p-HEMA (~20 ng of material based on an initial p-HEMA thickness of 0.3 μm) underneath each microfluidic channel, the p-HEMA is easily and quickly dissolved in 50 μl of 70% EtOH. After etching of the p-HEMA surface, the PDMS microfluidic or scotch tape device is removed from the surface and is quickly washed with H_2O. The cell culture surface is now ready to use for subsequent protein or cell adhesion studies.

Characterization of the p-HEMA surface demonstrates that the p-HEMA coating achieves a thickness of 0.3 μm. In comparison, previous methods for fabrication of p-HEMA micromolded surfaces result in 5 μm thick layers of p-HEMA.[13,14] Measurement of the p-HEMA surface via SEM indicates that p-HEMA etching results in patterned p-HEMA, which adheres closely to the geometry of the microfluidic channel (Figure 2). Swelling of the patterned p-HEMA coating was then assessed. After 1 DIV, the average size of the patterned p-HEMA channels was ~16 μm. After 7 days of incubation in PBS, the p-HEMA coating swelled ~6%, decreasing the average channel size to ~15 μm (Figure 3).

Using a solution of 10% normal goat serum and PBS, we demonstrated that protein adheres only to areas etched out by the ethanol in the microfluidic device as detected by Coomassie blue staining. No Coomassie blue staining is seen in areas not etched by p-HEMA (Figure 4A). This protein patterning is observed regardless of whether glass or polystyrene dishes are used as the initial surface. Consistent with the dye results, immunostaining with fluorescently-labeled goat anti-mouse demonstrates areas where protein adheres to the surface of the p-HEMA-etched dish (Figure 4B). These results are similar to those seen with p-HEMA structures created via direct patterning.[6,14]

After removal of the PDMS device, cells can be immediately patterned on the cell culture or glass coverslips not coated with p-HEMA. To demonstrate the feasibility of cellular patterning, we seeded C2C12 myoblasts onto the material and observed adherence only to regions where p-HEMA has been etched out (Figure 5). The p-HEMA allows for specific alignment and growth of C2C12 myoblasts. p-HEMA

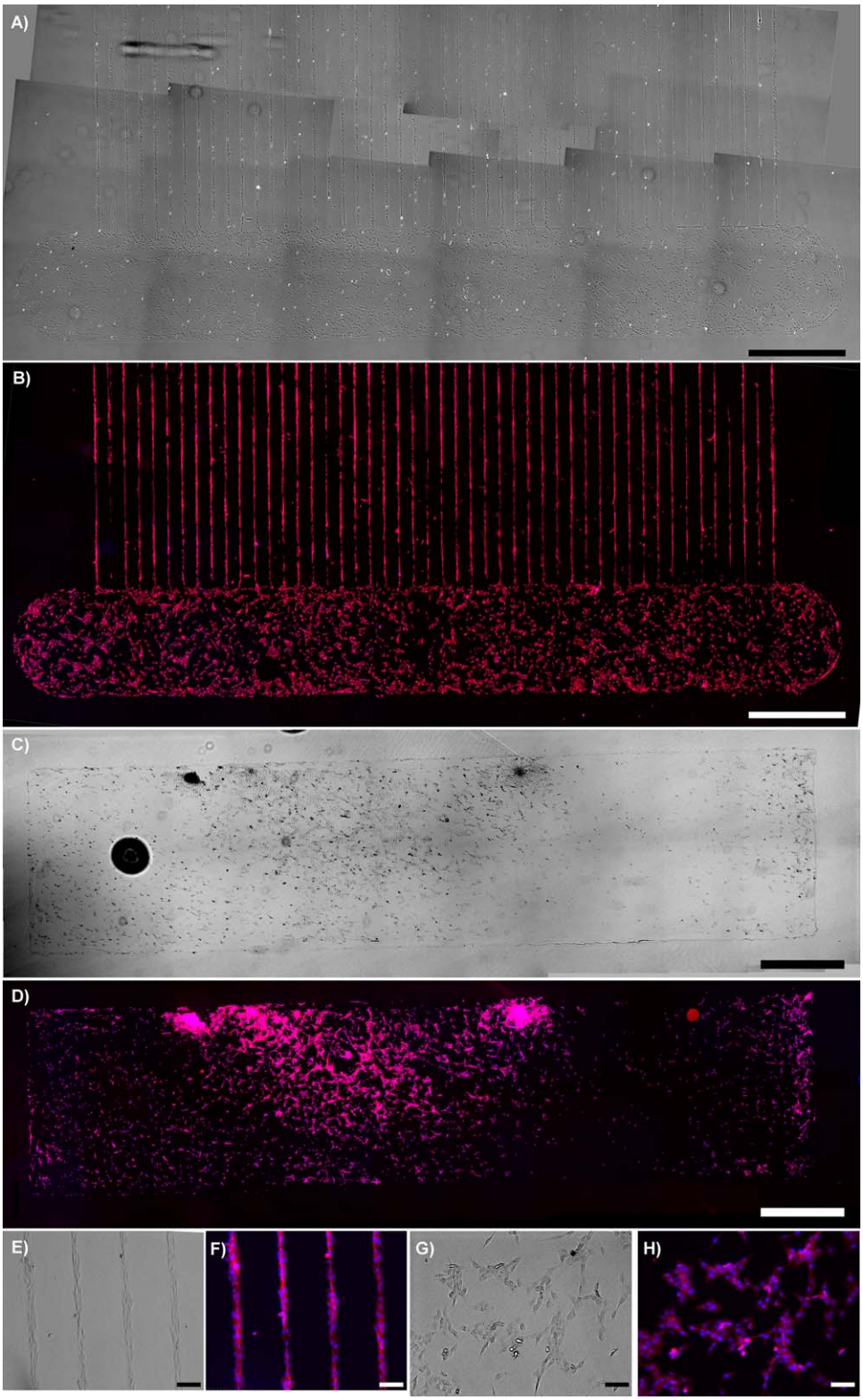

Figure 5. Cellular patterning on p-HEMA etched surface. C2C12 myoblasts patterned with p-HEMA etching. C2C12 myoblasts were imaged under phase contrast (A, C, E, and G) or stained with phalloidin-TRITC, to detect actin in the cells, and Hoechst 3352, to detect nuclei, and imaged with fluorescence microscopy (B, D, F, and H). (A, B, E, and F) C2C12 myoblasts patterned with microfluidic channels. Cells adhere only to areas etched out by ethanol on p-HEMA-coated surfaces. (C, D, G, H) C2C12 myoblasts patterned with scotch tape-created microfluidic devices. Cells adhere only to areas etched out by ethanol on p-HEMA-coated surfaces. Scale bar = 500 μm (A)-(D) or 50 μm (E)-(H). Scale bar = 100 μm.

patterning is achieved with either microfluidic devices or devices created with scotch tape and without the use of any additional cell adhesive molecules.

We anticipate that this technique may be modified to be utilized for the study of other cell types, such as fibroblasts or neurons. Similar techniques have been used with cells,

such as retinal neurons,[9] PC12 cells,[15] and fibroblasts,[15] and thus, it is likely that p-HEMA patterning is compatible with these and other cell types. Viability of C2C12 myoblasts after patterning was not directly examined in this manuscript; however, the high number of adherent cells after 7 DIV suggests that p-HEMA patterning does not have adverse effects on cell viability (Figure 5). In addition, other work from multiple groups employed p-HEMA specifically for its biocompatibility with multiple cellular systems.[13,14,16]

Conclusion

The technique that we report here is a simple and versatile method of patterning cell culture surfaces for proteins or cells. This technique can be further modified to pattern proteins in the microfluidic channels. For example, using the vacuum technique,[15] the microchannels can be filled with poly-D-lysine and laminin to further refine cell and protein patterning since each of these substrates has distinct adherence properties. In the future, additional modification of the p-HEMA surface will be performed to achieve thicker coating[6,14] or for use of copolymers of p-HEMA, such as poly (HPhMA-co-HEMA).[17] The optimization and refinement of our system will increase our understanding of biological phenomena under study and will aid in the development of biomaterials for cellular regeneration after injury.

Acknowledgments

This work was funded in part by NJCSCR Grant #CSCR14IRG005 (to BLF). JDZ is supported in part by NJCSCR Grant #CR16IRG0007. We would like to thank Dr. Anil Shrirao for his help with the profilometer. The authors state that they have no conflict of interest.

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Manuscript received Aug. 14, 2017, and revision received Oct. 16, 2017.