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TITLE:

Simple Lithography-Free Single Cell Micropatterning Using Laser-Cut Stencils

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KEYWORDS:

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SUMMARY:

This protocol introduces a lithography-free micropatterning method that is simple and accessible to those with a limited bioengineering background. This method utilizes customized laser-cut stencils to micropattern extracellular matrix proteins in a shape of interest for modulating cell morphologies. The procedure for micropatterning is demonstrated using induced pluripotent stem cell derived cardiomyocytes.

ABSTRACT:

Micropatterning techniques have been widely used in cell biology to study effects of controlling cell shape and size on cell fate determination at single cell resolution. Current state-of-the-art single cell micropatterning techniques involve soft lithography and micro-contact printing, which is a powerful technology, but requires trained engineering skills and certain facility support in microfabrication. These limitations require a more accessible technique. Here, we describe a

simple alternative lithography-free method: stencil-based single cell patterning. We provide step-by-step procedures including stencil design, polyacrylamide hydrogel fabrication, stencil-based protein incorporation, and cell plating and culture. This simple method can be used to pattern an array of as many as 2,000 cells. We demonstrate the patterning of cardiomyocytes derived from single human induced pluripotent stem cells (hiPSC) with distinct cell shapes, from a 1:1 square to a 7:1 adult cardiomyocyte-like rectangle. This stencil-based single cell patterning is lithography-free, technically robust, convenient, inexpensive, and most importantly accessible to those with a limited bioengineering background.

INTRODUCTION:

The advent of hiPSCs and the subsequent development of protocols for their directed differentiation into different cell types have made it possible to study development and disease at a molecular and patient-specific level, specifically using patient-derived iPSC cardiomyocytes (iPSC-CMs) to model cardiomyopathies[1,2]. However, a major limitation to studying development and physiology using the iPSC system and other in vitro models is the absence of a structured microenvironment. In situ, cells are subjected to the constraints of the extracellular matrix (ECM), as well as neighboring cells. The particular biochemical composition and stiffness of these microenvironments dictate the spatial distribution of cells as well as factors available for engaging in cell adhesion. This, in turn, influences intracellular signaling pathways, gene expression, and cell fate determination. For example, micropatterned iPSC-CM in an adult-like rod shape has a significantly better contractile ability, calcium flow, mitochondrial organization, electrophysiology, and transverse-tubule formation[3]. Thus, the properties of the microenvironment are integral in the regulation of cellular functions.

Previous micropatterning techniques heavily relied on photolithography (**Figure 1A**). In this technique, a layer of photosensitive polymer, or photoresist, is spun on a flat substrate from solution to form a thin film about 1 µm thick. Next, ultraviolet (UV) light is applied onto the photoresist through a mask containing the desired pattern. Exposure to ultraviolet (UV) light chemically alters the photoresist by modifying its solubility in its respective developer solution, transferring the desired pattern from the mask onto the substrate. Many micropatterning methods incorporate photolithography, as it confers nanometer to micrometer-level control over the design of the cell patterns. However, the spinning of the photoresist is highly sensitive to impurities, because the smallest dust particles will disrupt the spreading of the solution into a thin film. Photolithography must therefore be carried out in uncontaminated facilities, which are costly to maintain and require special expertise to utilize. In addition, the chemicals used in photolithography are often toxic to cells and can denature important biomolecules. Thus, photolithography poses significant obstacles to the fabrication of micropatterns for convenient biological applications.

In 1994, Whitesides and colleagues[4] overcame some of the challenges associated with photolithography by pioneering a collection of techniques called soft lithography. In soft lithography, a microstructured surface made with polydimethylsiloxane (PDMS), a transparent, rubber-like material, is used to generate a pattern of ECM proteins[4]. Common soft lithographic techniques include microcontact printing and microfluidic patterning. In microcontact printing,

currently the most popular soft lithographic method, a PDMS stamp coated with ECM proteins transfers the material onto a surface at the areas contacted by the stamp (**Figure 1B**). In microfluidic patterning, microstructures are designed on a PDMS surface such that when the stamp is pressed to a substrate, a network of microchannels, through which fluids can be delivered to desired areas, is created (**Figure 1C**)[5]. Soft lithography offers several benefits over photolithography. Once a master wafer is microfabricated, the PDMS stamps can easily be replicated without further employment of clean-room facilities. In addition, the absence of organic solvents in the process of soft lithography allows for utilization of polymeric materials such as polystyrene, typically used in cell culture. Finally, micropatterning using soft lithographic methods is not restricted to flat surfaces. Thus, soft lithography increases the accessibility and functionality of micropattern fabrication over photolithography[6]. However, soft lithography has significant drawbacks. For example, an initial etching step, using photolithography, is still required to microfabricate the stamp. In addition, micropatterning using a PDMS stamp is subject to variations in the quality of protein transfer onto the substrate[6]. Avoiding these discrepancies requires optimization and consistency in the pressure applied to the PDMS stamp during protein transfer, otherwise deformation and distortion of the feature sizes of the PDMS molds can occur[6]. There is also a major concern of repeatedly using the PDMS due to small molecule absorption[7].

To avoid using soft photolithography and PDMS stamps, we describe a stencil-based, lithography-free single cell micropatterning method that overcomes many of the obstacles associated with photolithography and soft lithography. In this method, a polyacrylamide hydrogel is used as a substrate for stencil-based ECM protein incorporation, allowing for selective plating of single hiPSC-CMs. This technique is highly compatible with polymeric materials used in classic cell culture conditions. Moreover, with proper cleaning and maintenance, the stencils are reusable and resistant to degradation and protein absorption during the microfabrication process. Finally, the patterning process is technically robust, inexpensive, customizable, and accessible to those with no specialized bioengineering skills. This stencil-based micropatterning technique has been broadly utilized in our recent publications modeling varied cardiomyopathies[8–10].

PROTOCOL:

1. Fabrication of negative pattern polyimide-based stencils

1.1. Generate a pattern (**Figure 2A**) in .dxf format using computer-aided design software (e.g., AutoCAD, SolidWorks, Onshape, Adobe Illustrator).

1.1.1. Generate a circle (diameter = 22 mm) to delineate the border of the stencil.

1.1.2. Draw a solid-filled shape or the pattern desired.

1.1.3. Include a chiral letter (e.g., R) to mark the front side of the stencils.

NOTE: As an example, an array of squares and rectangles is generated to pattern iPSC-CMs at single-cell and cell-pair levels. The design files are available (see **Supplemental Files**). The microfabrication resolution limit is ~10 µm.

1.2. Submit the design to a microfabrication company to laser-cut polyimide film and fabricate stencils (**Figure 2B**,**C**).

2. Preparation of sulfo-SANPAH aliquots

NOTE: The protocol is modified from Fischer et al.[11].

2.1. Use a moisture-resistant cardboard sample storage box (e.g., 100 well, 10 x 10 spaces for centrifuge tubes, dimensions: 13.4 cm x 13.4 cm x 5.1 cm). Label it "name/date/sulfo-SANPAH 40 µL/reconstituted with 1,200 µL of PBS".

2.2. Label 50 microcentrifuge tubes (polypropylene, 1.5 mL) with an 'S' on the lid.

2.3. Take 4 mL of anhydrous, extra dry dimethyl sulfoxide (DMSO) and sterile filter the solution using a membrane filter unit (pore size = 0.22 µm) into a sterile 15 mL conical tube.

2.4. Prepare a liquid nitrogen bath and cover with the lid to minimize the evaporation of the liquid nitrogen.

2.5. Dissolve 50 mg of sulfo-SANPAH in 2 mL of the filtered DMSO.

2.6. Vortex well to fully dissolve the sulfo-SANPAH in the DMSO.

2.7. Distribute 40 µL aliquots of the sulfo-SANPAH solution into sterile 1.5 mL tubes.

2.8. Transfer the tubes into the box.

2.9. Flash-freeze the tubes in liquid nitrogen for 5 min.

2.10. Store the aliquots at -80 °C.

NOTE: The aliquots can be used for up to 6 months without diminished efficiency. The stock sulfo-SANPAH concentration is 25 mg/mL or 50.77 mM.

3. Sterilization of tools

3.1. Autoclave two forceps, 24 glass coverslips (22 x 22 mm, No. 1 or No. 1.5), one razor blade, and three lint-free absorbent wipes.

NOTE: To make 12 hydrogel constructs, 24 glass coverslips are needed. There should be two coverslips per construct. It is advisable to prepare some spare coverslips.

3.2. Place two pieces of paraffin film (4 x 4 inch) in an ethanol resistant plastic box (e.g., a 1,000 µL pipette tip box) and sterilize them in fresh 70% ethanol for at least 15 min. Afterwards place each piece of paraffin film in a sterile 15 cm Petri dish and dry completely under sterile conditions.

4. Preparation of polyacrylamide hydrogel precursor solution

NOTE: The protocol is modified from Lee et al.[12].

4.1. Dissolve 125 mg of aminoethyl methacrylate (AEM) in 36.25 mL of deionized water in a 50 mL polypropylene centrifuge tube by vortexing.

4.2. To prepare 50 mL of 10 kPa polyacrylamide precursor solution (8–0.15–15 mM), mix 10 mL of 40% acrylamide solution, 3.75 mL of 2% bis-acrylamide solution, and 36.25 mL of the AEM solution prepared in step 4.1 in a new 50 mL polypropylene centrifuge tube.

NOTE: The final concentration of precursor solution is 8% acrylamide, 0.15% bis-acrylamide, and 15 mM AEM, which was measured to have ~10 kPa stiffness by atomic force microscopy. According to the tissue applications of interest, the stiffness of the hydrogel can be altered by changing the ratio of the 40% acrylamide to the 2% bis-acrylamide solution. For example, 8% acrylamide–0.08% bis-acrylamide solution yields 3 kPa hydrogels; 8% acrylamide–0.48% bis-acrylamide solution yields 38 kPa hydrogels; 8% acrylamide–0.48% bis-acrylamide solution yields 60 kPa hydrogels. It is advisable to confirm the stiffness of hydrogels made from the precursor solutions with altered ratios.

5. Preparation of photoinitiator solution

5.1. For 1 mL of 5% photoinitiator solution, first dissolve 50 mg of 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone powder in 700 µL of 100% ethanol in a 1.5 mL polypropylene centrifuge tube with lid.

NOTE: 2-Hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone is not soluble in water. Make sure to fully dissolve the powder in ethanol by vortexing.

5.2. After completely dissolving the 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone in ethanol by vortexing, add 300 µL of phosphate-buffered saline (PBS) for a final volume of 1 mL.

NOTE: The final concentration of 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone solution is 5%. The foiled 5% photoinitiator solution is good for 4 weeks at 4 °C.

6. Preparation of basement membrane matrix protein solution

NOTE: Basement membrane matrix (**Table of Materials**) is a temperature-sensitive material. Make sure to work on ice with chilled pipette tips and tubes as well as ice-cold solutions. A new stock of basement membrane matrix should be thawed slowly on ice at 4 °C overnight.

6.1. Prepare 200 µL of basement membrane matrix protein solution per hydrogel construct. For 12 constructs, 2.4 mL of basement membrane matrix protein solution are needed. Prepare 10% extra solution (e.g., 2.6 mL).

6.2. For every new batch/lot of basement membrane matrix protein solution, optimize the dilution ratio. For the first time, test dilution ratios of 1:10, 1:20, 1:40 to find the dilution ratio that yields the best qualities for cell patterning.

NOTE: If the basement membrane matrix protein solution is too viscous, it will form a gel on top of the stencil, and it is more likely to peel off when removing the stencil. If the protein solution is too fluid, it will penetrate through the pattern holes of the stencils, which will result in poor quality patterning.

6.3. Dilute basement membrane matrix protein stock solution on ice with ice-cold Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) media or 1x PBS to the optimized dilution ratio above. For example, dilute 120 µL of the stock solution in 2.4 mL of DMEM/F12 media (1:20) and keep on ice for later use.

7. Hydrogel fabrication

7.1. Place a UV bench lamp (365 nm, 4 mW/cm^2) in a biological safety hood.

7.2. Take the two 15 cm Petri dishes with the sterilized and dried paraffin films from step 3.2. If the paraffin films are not completely dry, use a vacuum aspiration system to remove any residual liquid. Place six autoclaved coverslips from step 3.1 on each paraffin film using the autoclaved forceps.

7.3. Prepare UV-crosslinkable polyacrylamide precursor solution by diluting 5% photoinitiator solution (step 5.2) with polyacrylamide precursor solution (step 4.2) in a 50 mL polypropylene centrifuge tube. For 5 mL of UV-crosslinkable polyacrylamide precursor solution, add 50 µL of 5% photoinitiator solution to 5 mL of polyacrylamide precursor solution.

7.4. Filter the precursor solution from step 7.3 using a 50 mL filter unit with a 0.22 µm pore size for sterilization.

NOTE: Always prepare fresh precursor solution.

7.5. Dispense 200 µL of polyacrylamide precursor solution from step 7.4 on each 22 x 22 mm glass cover slip in the paraffin filmed Petri dish and carefully cover with another 22 x 22 mm glass coverslip on top to sandwich the solution in between (**Figure 3A**).

NOTE: Paraffin film is used to avoid spilling and to confine the precursor solution in between the coverslips.

7.6. Place the coverslip-containing Petri dish from step 7.5 under the UV bench lamp and photopolymerize the polyacrylamide hydrogels for 5 min (**Figure 3B**).

NOTE: To protect from UV radiation, cover the lamp with aluminum foil and wear UV protection goggles.

7.7. Carefully detach one cover slip off from the other using a razor blade by leverage action (**Figure 3C**).

NOTE: The hydrogel will usually stick to the top coverslip. Pay extra attention when detaching the coverslip from the soft hydrogel, because it is likely to break.

7.8. Fill a disposable polypropylene reservoir with PBS. Transfer the coverslips in a PBS-containing reservoir to rinse the hydrogels in PBS for 5 min. After rinsing, place the hydrogel construct back on the paraffin film in the Petri dish.

8. Protein conjugation

8.1. Prepare an ice bucket and precool PBS.

NOTE: For six hydrogels, 1,200 µL of cold PBS is needed.

8.2. Take out the sulfo-SANPAH aliquot (1 vial = 6 gels).

NOTE: If necessary, the amount of sulfo-SANPAH used can be reduced after optimization.

8.3. Add 1,200 µL of cold PBS into a vial of sulfo-SANPAH aliquot and mix by pipetting up and down.

8.4. Dispense 200 µL of sulfo-SANPAH on the paraffin film in the Petri dish and cover with the hydrogel-coverslip composite with hydrogel contacting sulfo-SANPAH (**Figure 3D**).

NOTE: Sulfo-SANPAH is very susceptible to water due to hydrolysis. Freshly thaw from -80 °C right before usage. Do not reuse or refreeze any leftovers.

8.5. Expose the hydrogel to the UV lamp at 365 nm, 4 mW/cm^2 for 5 min to activate sulfo-SANPAH.

NOTE: After exposure, the sulfo-SANPAH will change from orange to brown (**Figure 3E**). If the hydrogel turns brown, it indicates successful activation of the phenyl azide group of sulfo-

SANPAH and incorporation of sulfo-SANPAH onto the hydrogel. Directly proceed with steps 8.6−8.9 within a maximum of 10 min, because the activity of sulfo-SANPAH will decline.

8.6. Replenish a disposable polypropylene reservoir with fresh PBS. After sulfo-SANPAH activation, transfer the activated hydrogels and quickly rinse the hydrogels in a PBS reservoir to remove unbound sulfo-SANPAH.

NOTE: A long wash can result in low protein conjugation efficiency.

8.7. After a quick rinse, place the hydrogel-attached coverslips on the paraffin film in the Petri dishes and carefully dab the hydrogels with sterile lint-free wipes.

NOTE: The remaining PBS on top of the hydrogel will result in leakage of the basement membrane matrix protein solution through the stencil, and drying too extensively will lead to rupture of the hydrogel upon removal of the stencil due to too high adherence to the stencil.

8.8. Place the stencil on top of the hydrogels and dab with autoclaved lint-free wipes to ensure tight sealing between the stencil and the hydrogel (**Figure 3F**).

8.9. Carefully dispense 200 µL of the diluted basement membrane matrix protein solution prepared from step 6.3 (**Figure 3G**) on top. Leave overnight in the incubator (37 °C).

NOTE: When the stencil-hydrogel contact is tight and the basement membrane matrix protein solution concentration is optimal, the basement membrane matrix protein solution will be sequestered and remain on top of the stencil (**Figure 3H**). Incubation time can be reduced upon optimization. Theoretically, it can be reduced down to 30 min–2 h.

8.10. The next day, transfer the hydrogels to a 6 well plate and fully immerse them in PBS.

NOTE: It is recommended to peel off the stencil with PBS immersion over the coverslip to prevent tearing and sticking of the hydrogel to the stencil.

8.11. Carefully remove the stencil using autoclaved tweezers without tearing the hydrogel. Resuspend well with DMEM, return the patterned hydrogels to the incubator, and leave them overnight to test for any contamination.

8.12. If the media remains clear, the hydrogels are ready to be used for cell plating. Optimize the cell plating density and incubation time to allow adhesion of the cells for respective applications.

NOTE: For single cell patterning of hiPSC-CMs, seed and incubate overnight. The following cell numbers are recommended for seeding: 30,000, 60,000, and 90,000 cells/well. Seeding too many cells leads to more than one cell per pattern. A cell strainer is recommended to strain out non-single cells before cell seeding.

8.13. The next day, observe cell attachment and spreading, and change the media to get rid of detached cells.

9. Cleaning of the used stencils

9.1. To remove residual matrix proteins on the used stencils, submerge the stencils in enzyme solution (**Table of Materials**) for 10 min at 37 °C.

NOTE: The enzyme should be selected depending on the matrix proteins used. For collagen-rich matrix protein solution, use collagenase. A 10–15 min ultrasonication in enzyme solution is also recommended.

9.2. Aspirate the solution and replenish with 10% bleach. Incubate for 10 min at room temperature.

9.3. Rinse 3x with PBS.

9.4. Store in 70% ethanol at 4 °C until use.

REPRESENTATIVE RESULTS:

Fabrication of stencils containing an array of squares or rectangles has been demonstrated (**Figure 4A**). Following this protocol, we obtained patterned matrix protein islands (**Figure 4B** and **Figure 5A**) and cells (**Figure 4C**). Suboptimal matrix protein solution concentration led to suboptimal patterning (**Figure 5B**). It is critical to use the front side of the stencil. If the back side of the stencil is used, the size of the matrix protein islands increases due to the direction of the laser cutting (**Figure 6A**,**B**). While the width of the rectangular patterns did not significantly change (**Figure 6C**), the height significantly increased when using the back side of the stencil, leading to a reduction in the intended aspect ratio (**Figure 6D**,**E**). We applied stencil-based patterning to silicon elastomer substrates (**Figure 7**). The patterned cardiomyocytes on silicon elastomer substrates were visualized by immunocytochemistry of cardiac troponin T at low magnification (**Figure 7A**) and sarcomeric protein α-actinin at high magnification (**Figure 7B**). The stencil-based patterning could also be applied to pattern two cardiomyocytes side-by-side on hydrogels with different stiffnesses. As a demonstration, we show the cardiomyocyte patterning on physiologically-relevant stiffness (**Figure 8A**) and pathologically-relevant stiffness (**Figure 8B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of three gold-standard micropatterning strategies. (**A**) Photolithography. (**B**) Microcontact printing. (**C**) Microfluidic patterning.

Figure 2: Workflow of designing stencils. (**A**) The computer-assisted design of the stencil. (**B**) A representative photograph of a laser-cut polyimide stencil. (**C**) A representative microscopic image of a stencil. The yellow area represents the polyimide stencil, while the light blue area represents an array of laser-cut holes.

Figure 3: Photographs of hydrogel fabrication steps. (**A**) Polyacrylamide hydrogel fabrication by sandwiching the precursor solution in between two coverslips. (**B**) UV photo-crosslinking of polyacrylamide hydrogels under the UV bench lamp. The UV lamp is covered with aluminum foil for user protection. (**C**) The photo-crosslinked hydrogel is retrieved by detaching a coverslip on one side. (**D**) The hydrogel construct is in contact with sulfo-SANPAH solution to modify the hydrogel for ECM protein incorporation. Before UV activation, the sulfo-SANPAH is orange. (**E**) After UV activation, sulfo-SANPAH turns brown, indicating that it is activated and incorporated onto the hydrogel surface. (**F**) After dabbing the hydrogel with sterile lint-free wipes to remove excess water, the stencil is placed on the hydrogel. (**G**) Basement membrane matrix protein solution is pipetted over the stencil-hydrogel constructs. (**H**) If the stencil-hydrogel contact is successfully tight, the basement membrane matrix protein solution will stay on top and will not seep through.

Figure 4: Micropatterning of basement membrane matrix proteins and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) with different rectangular shapes. (**A**) Microscopic images of stencils containing laser-cut rectangles with different aspect ratios (1:1, 4:1, 11:1). (**B**) Representative images of immunostained matrix protein islands on hydrogel substrates. (**C**) Representative images of hiPSC-CMs stained for nuclei (cyan) and cardiac troponin T (magenta). Scale bar = 20 µm for panels B and C.

Figure 5: The ECM protein concentration plays an essential role in generating proper patterns. (**A**) Representative image of an optimal concentration with very homogenous distribution of basement membrane matrix proteins in the single patterns. (**B**) Suboptimal matrix protein patterns due to low concentration, which caused leakage of matrix protein outside the patterns and low amounts of matrix proteins within the patterns as determined by wheat germ agglutinin staining. Scale bar = 20 µm for panels A and B.

Figure 6: Stencil side effect. (**A**) Basement membrane matrix protein patterns when the front side of the stencil was used. (**B**) Basement membrane matrix protein patterns when the back side of the stencil was used. Quantification of (**C**) width, (**D**) height, (**E**) aspect ratio of rectangular basement membrane matrix protein islands. Graph is plotted mean ± SEM. Statistics are calculated by unpaired student T test. n.s. = not significant. **** = $p < 0.0001$. Scale bar = 20 µm.

Figure 7: Stencil-based patterning on silicon elastomer substrate. (**A**) A representative image of single cell patterned cardiomyocytes on silicon elastomer substrates stained by the cardiac marker, cardiac troponin T (cTnT), at low magnification. Scale bar = 300 µm. (**B**) A representative single-cell-patterned cardiomyocyte stained by sarcomeric protein α-actinin at high magnification. Scale bar = 10 µm.

Figure 8: Micropatterning of hiPSC-CMs on polyacrylamide hydrogels with different stiffnesses. (**A**) 10 kPa, (**B**) 60 kPa.

Table 1: Troubleshooting guidelines. Frequent problems, possible reasons, and potential solutions are discussed.

DISCUSSION:

We describe a lithography-free stencil-based micropatterning method that enables effective patterning of adherent cells. In this protocol, we demonstrate patterning of hiPSC-CMs in different length-to-width ratios by micropatterning basement membrane matrix protein islands on polyacrylamide hydrogels with physiologically- or pathologically-relevant tissue stiffnesses or silicon-based elastomer substrates. This method is relatively simple and highly accessible to any researchers, including those who have little background in photolithography and microfabrication techniques. The described method circumvents significant challenges associated with generating stamps using soft lithography and transferring proteins from stamp molds to substrates of interest involved in the microcontact printing technique. This protocol provides a workflow to 1) create custom-designed stencils with an area and shape of interest in a cost-effective manner; 2) fabricate a hydrogel substrate with the stiffness of interest; and 3) efficiently conjugate ECM proteins on the hydrogel substrate.

Traditional soft lithography methods have been used for patterning various shapes of ECM proteins including triangles and stars on substrates of different materials such as silicon-based materials and glass coverslips[13,14]. In this study, we only demonstrate rectangular patterns given the physiologically- and pathologically-relevant shape of cardiomyocytes. We believe stencil-based patterning of other complex shapes can work as long as the smallest feature of the shape does not go beyond the resolution (~10 µm). With regards to substrate materials, we demonstrate the stencil-based patterning can be applied on hydrogel (**Figures 2**–**6**, **8**) or silicon-based elastomer substrate (**Figure 7**). The presented method does not work on substrates with minimal adhesiveness to polyimide-materials such as tissue culture plastics and glass coverslips because the tight sealing between the polyimide-based stencil and these substrates is not achieved. To enable tight sealing, further surface modification is required. Lastly, we show that the method can be used to reliably produce cell-pair patterns on hydrogels with different stiffnesses (i.e., 10 kPa, 60 kPa) (**Figure 8**).

In the protocol, the most critical step is optimization of ECM protein concentration (protocol section 6). The goal of this step is to find the optimal concentration of ECM protein that is viscous enough to inhibit the protein solution to seep through the holes of the stencil, but not too concentrated, to avoid gelation (**Figure 5**). This stencil-based method does not restrict the type of ECM protein. Although viscous ECM protein solution is more likely to stay on the top of the stencil, a less viscous protein can also be used. One requirement is making sure that the surfaces of the stencil and PA hydrogel are dry enough to form a relative stable hydrophobic interface. In this demonstration, basement membrane matrix protein was used because it is viscous and suitable for culturing hiPSC-CMs. However, other ECM proteins can likely be used (e.g., fibronectin, gelatin, collagen, laminin).

In addition to optimization of the ECM protein solution for each given application, generating a proper seal between the stencil and the hydrogel surface is crucial to obtain a precise pattern with the ECM protein. Because excessive drying can lead to rupturing of the hydrogel, it is essential to not overdry the stencil. These steps (i.e., placing the stencil on the hydrogel as well

as gently pealing it off afterwards) require some practice. Establishing an optimal handling procedure for each given condition will also avoid having the hydrogel stick to the stencils. With this optimization, submicron scale laser burns caused by flaws in the laser cutting will not substantially affect the technique in regards to hydrogel damage.

Another important step is to use the front of the stencil when placing the stencil on the hydrogel substrate. We found that use of each side has an effect on the substrate, presumably due to laser cutting (**Figure 6**). One recommendation when patterning cells in specific size or shape for the first time is to generate an optimization stencil. Stencil-design features do not necessarily match the final patterned cell sizes. We recommend generating an optimization stencil that contains a gradient of feature sizes. We prepared a table listing potential problems and possible solutions to help with troubleshooting (**Table 1**).

It is not required to use the same microfabrication company mentioned in this manuscript. The keys to precise generation of the stencil are the beam diameter of the laser and the thickness of the stencil film, in our case, polyimide (50 μm). We believe a laser lab equipped with an appropriate laser could generate the stencil with a few steps, optimizing laser intensity, alignment, and other variables. Although we have only worked with one company, there are many available companies who perform precision laser cutting.

A limitation to this method is resolution. While photolithography can achieve nanoscale resolution, stencil-based patterning resolution is limited to the resolution of the laser-cutting of the polyimide stencils, which to date is usually on the scale of a few microns (~10 μm). Nevertheless, given that the average cell size is over 10 μm, the resolution is generally applicable to any single cell patterning.

In summary, this method provides versatile platforms for studying cell-ECM interactions, examining cell structure-function correlation, and many other applications. We believe this protocol will benefit many biomedical researchers and facilitate single cell studies.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Burridge, P. W. et al. Chemically Defined and Small Molecule-Based Generation of Human Cardiomyocytes. *Nature Methods*. **11** (8), 855–860 (2014).
2. Sayed, N., Liu, C., Wu, J. C. Translation of Human-Induced Pluripotent Stem Cells: From Clinical Trial in a Dish to Precision Medicine. *Journal of the American College of Cardiology*. **67** (18), 2161–2176 (2016).
3. Ribeiro, A. J. S. et al. Contractility of single cardiomyocytes differentiated from pluripotent stem cells depends on physiological shape and substrate stiffness. *Proceedings of the National Academy of Sciences*. **112** (41), 12705–12710 (2015).
4. Kumar, A., Biebuyck, H. A., Whitesides, G. M. Patterning Self-Assembled Monolayers: Applications in Materials Science. *Langmuir*. **10** (5), 1498–1511 (1994).
5. Wilbur, J. L., Kumar, A., Kim, E., Whitesides, G. M. Microfabrication by microcontact printing of self-assembled monolayers. *Advanced Materials*. **6** (7–8), 600–604 (1994).
6. Théry, M. Micropatterning as a tool to decipher cell morphogenesis and functions. *Journal of Cell Science*. **123** (24), 4201–4213 (2010).
7. Toepke, M. W., Beebe, D. J. PDMS absorption of small molecules and consequences in microfluidic applications. *Lab on a Chip*. **6** (12), 1484–1486 (2006).
8. Seeger, T. et al. A Premature Termination Codon Mutation in MYBPC3 Causes Hypertrophic Cardiomyopathy via Chronic Activation of Nonsense-Mediated Decay. *Circulation*. **139** (6), 799–811 (2019).
9. Lee, J. et al. Activation of PDGF pathway links LMNA mutation to dilated cardiomyopathy. *Nature*. **572** (7769), 335–340 (2019).
10. Wu, H. et al. Modelling diastolic dysfunction in induced pluripotent stem cell-derived cardiomyocytes from hypertrophic cardiomyopathy patients. *European Heart Journal*. **40** (45), 3685–3695 (2019).
11. Fischer, R. S., Myers, K. A., Gardel, M. L., Waterman, C. M. Stiffness-controlled three-dimensional extracellular matrices for high-resolution imaging of cell behavior. *Nature Protocols*. **7** (11), 2056–2066 (2012).
12. Lee, S., Stanton, A. E., Tong, X., Yang, F. Hydrogels with enhanced protein conjugation efficiency reveal stiffness-induced YAP localization in stem cells depends on biochemical cues. *Biomaterials*. **202**, 26–34 (2019).
13. Théry, M., Pépin, A., Dressaire, E., Chen, Y., Bornens, M. Cell distribution of stress fibres in response to the geometry of the adhesive environment. *Cell Motility*. **63** (6), 341–355 (2006).
14. Kilian, K. A., Bugarija, B., Lahn, B. T., Mrksich, M. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proceedings of the National Academy of Sciences*. **107** (11), 4872–4877 (2010).

Figure 1

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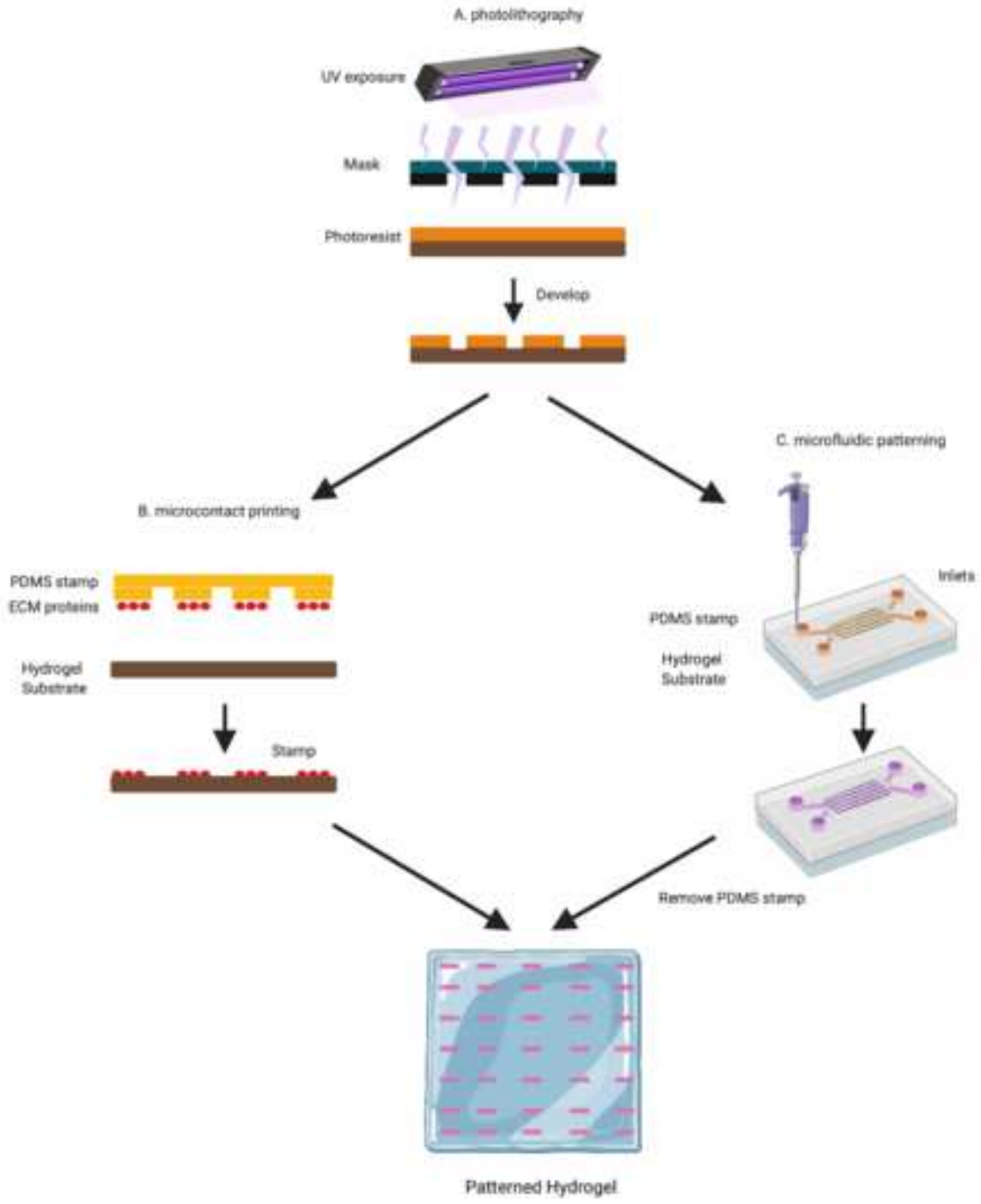

Figure 2

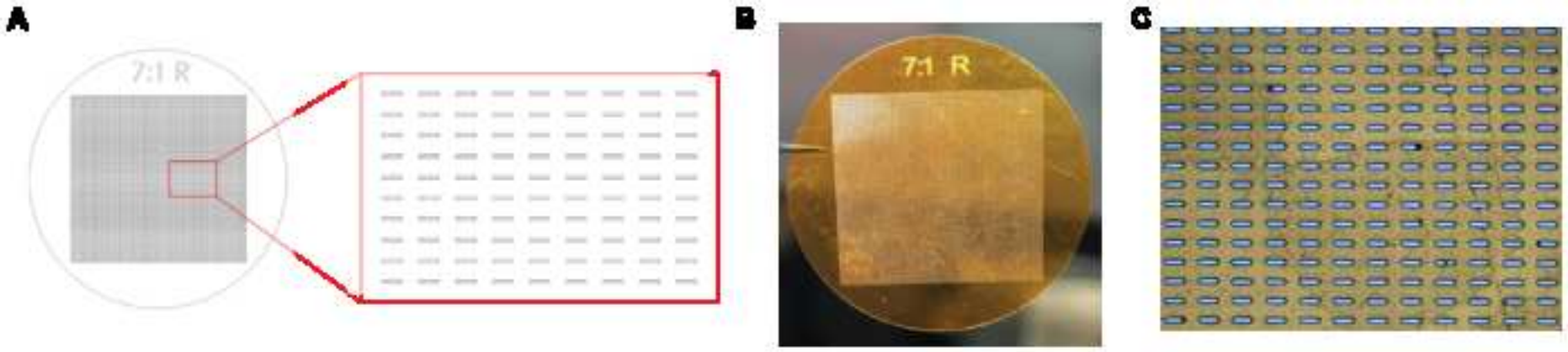

Figure 3

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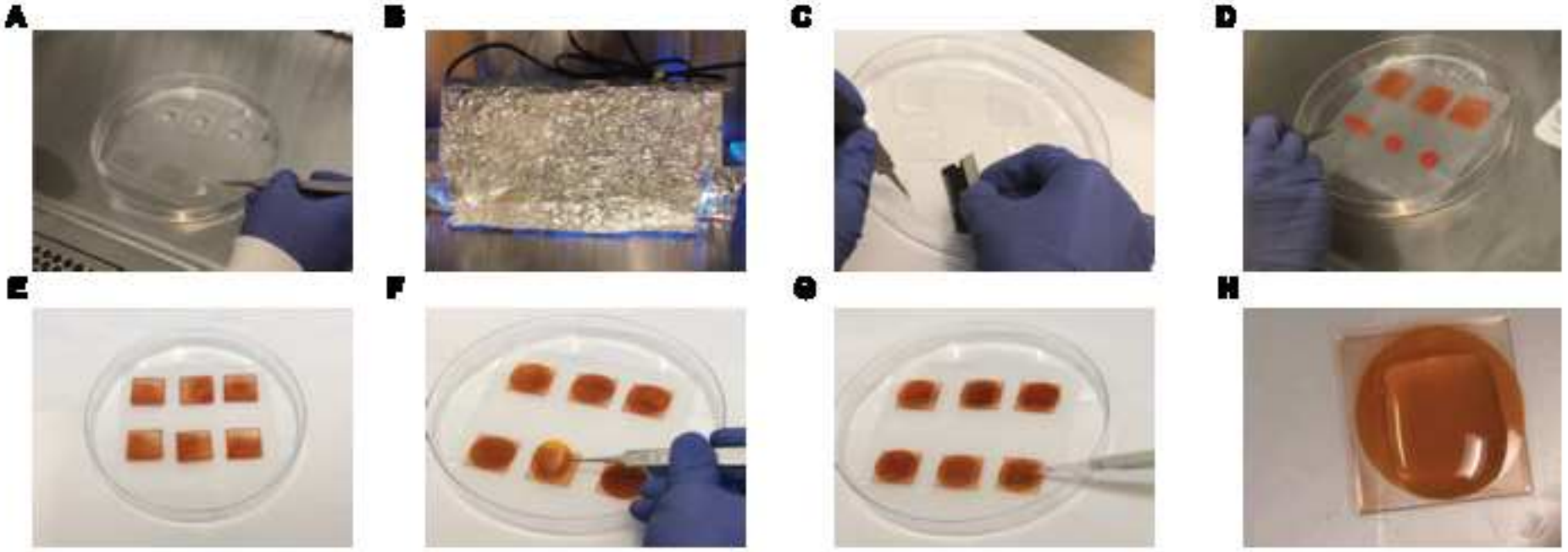

Figure 4

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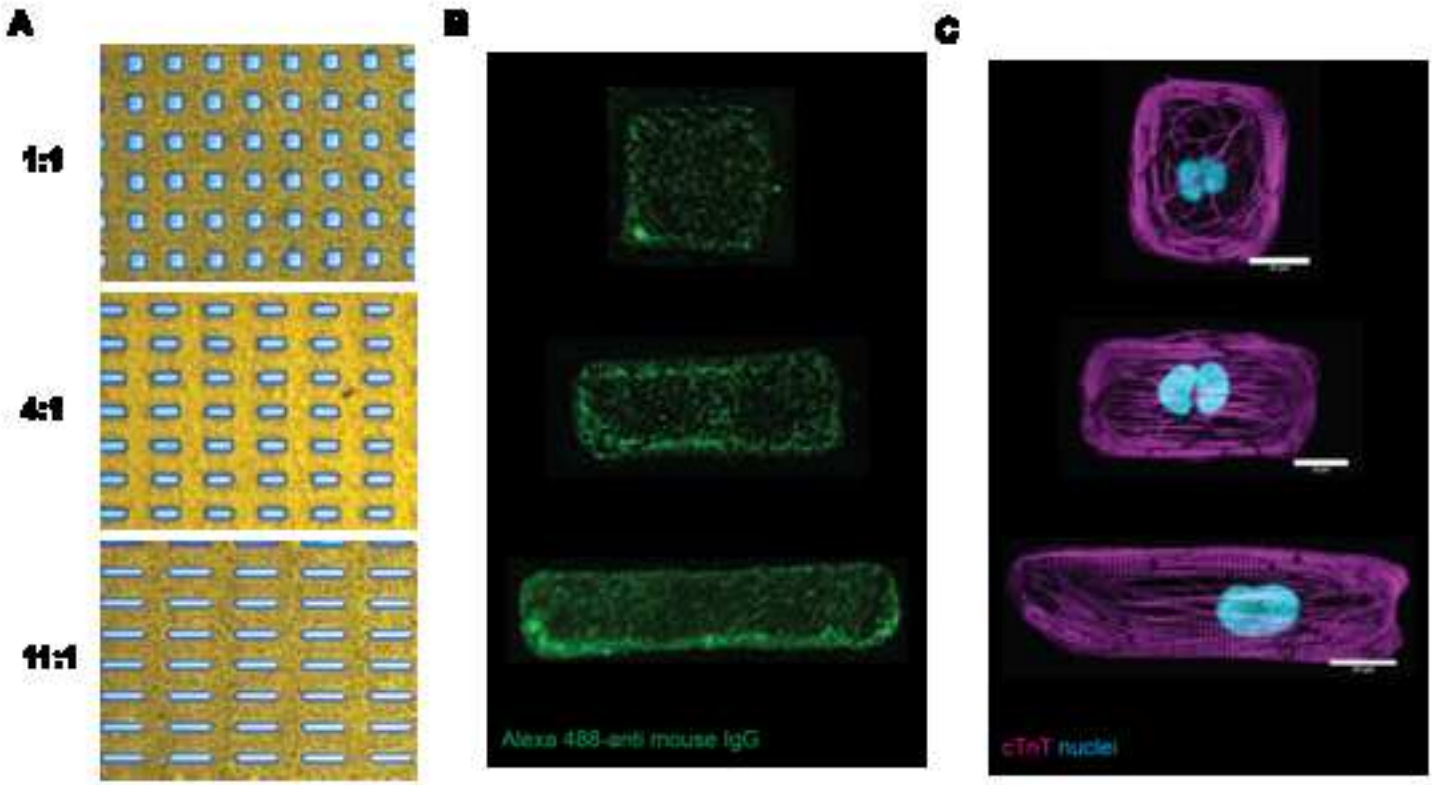

Figure 5

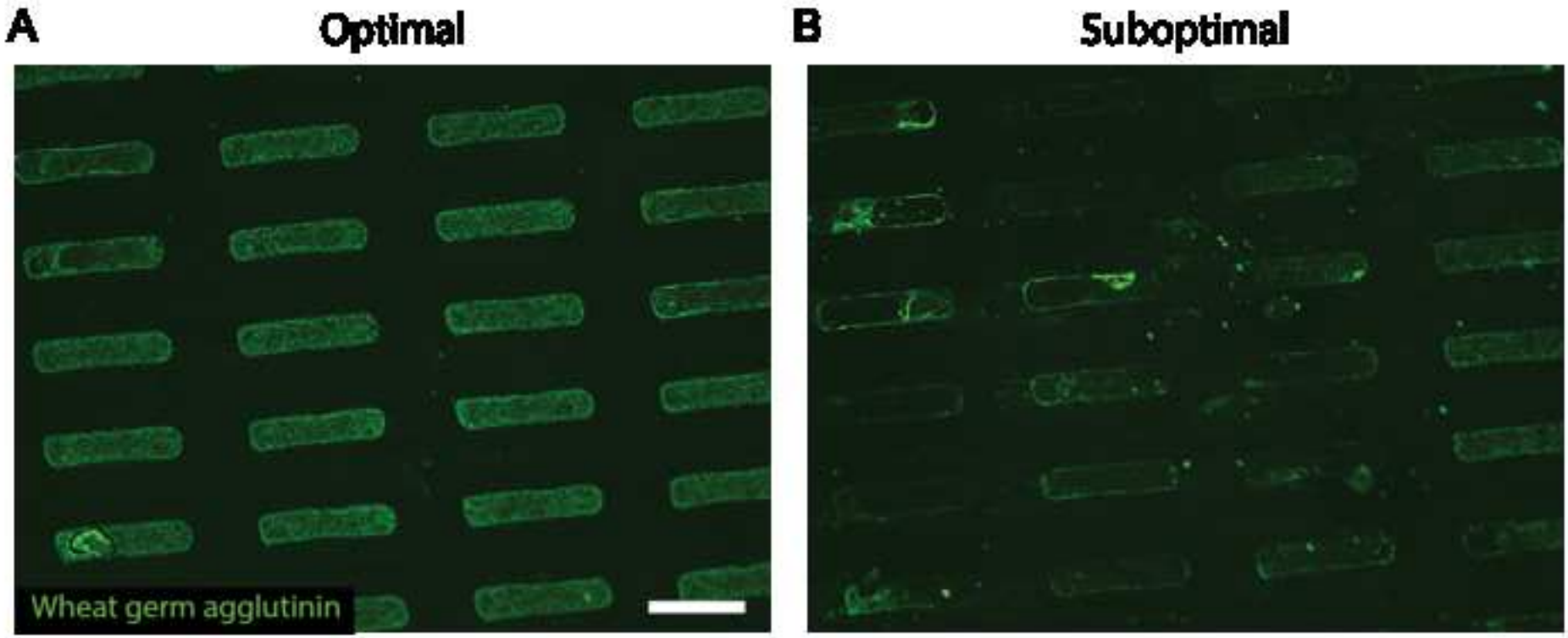

Figure 6

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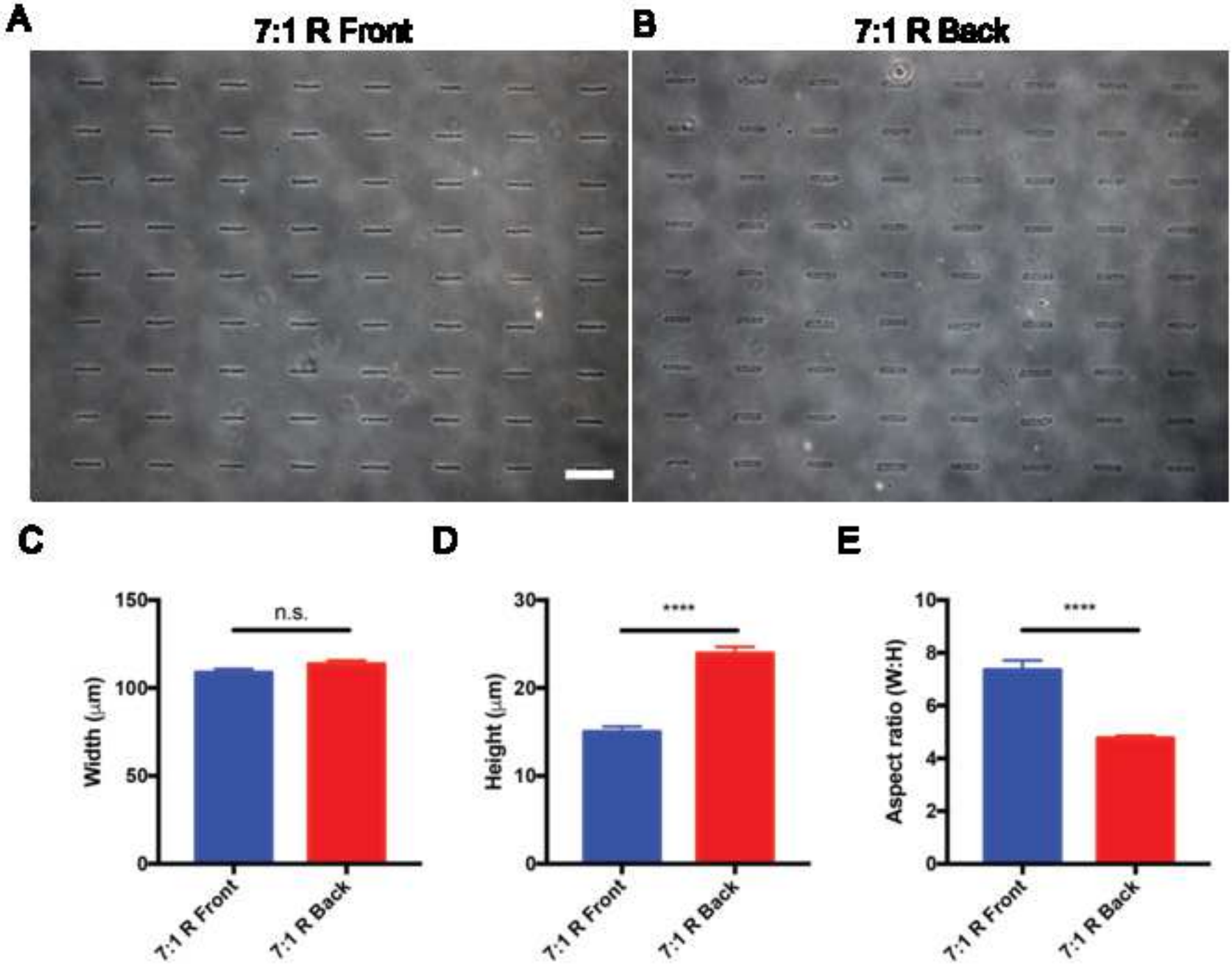

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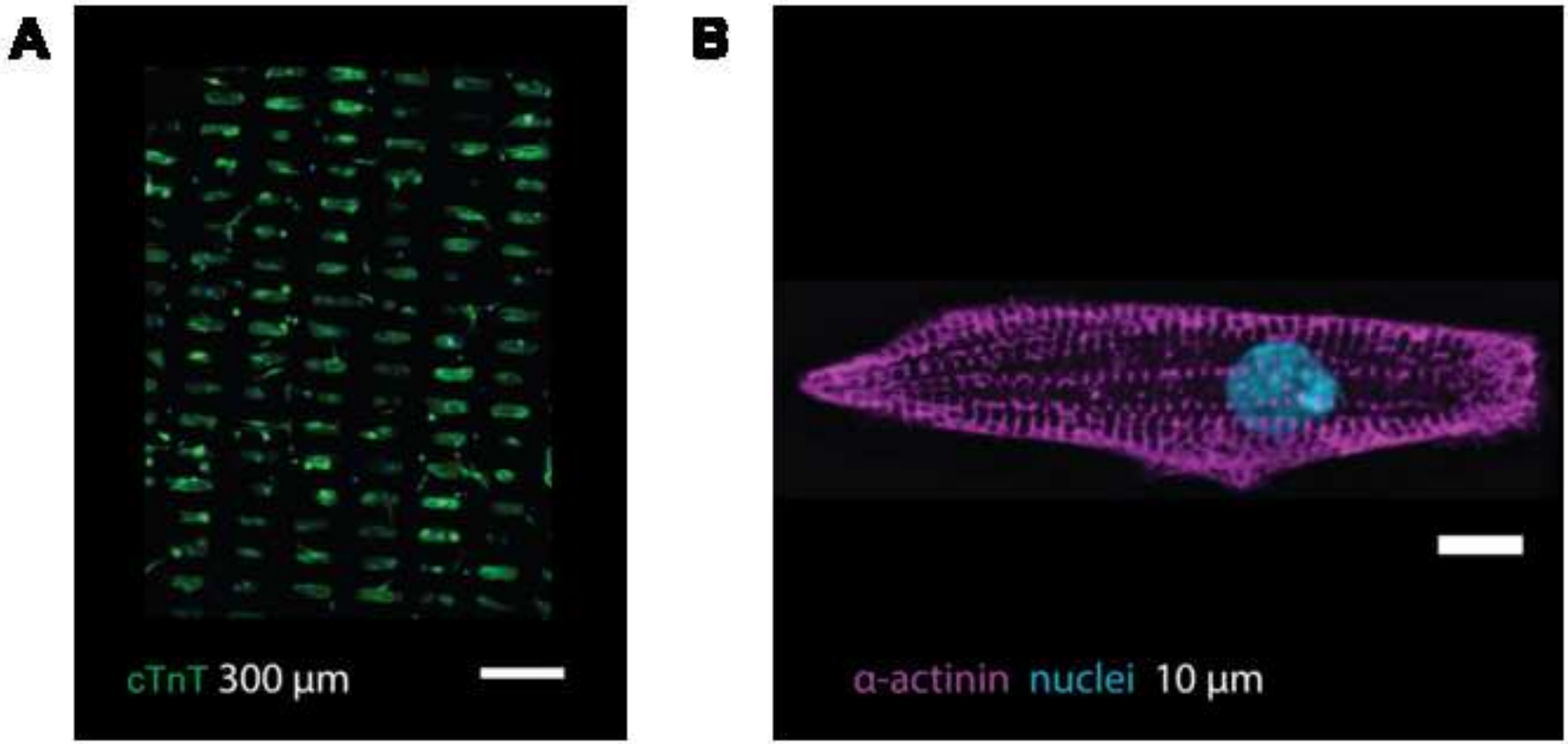

Figure 8

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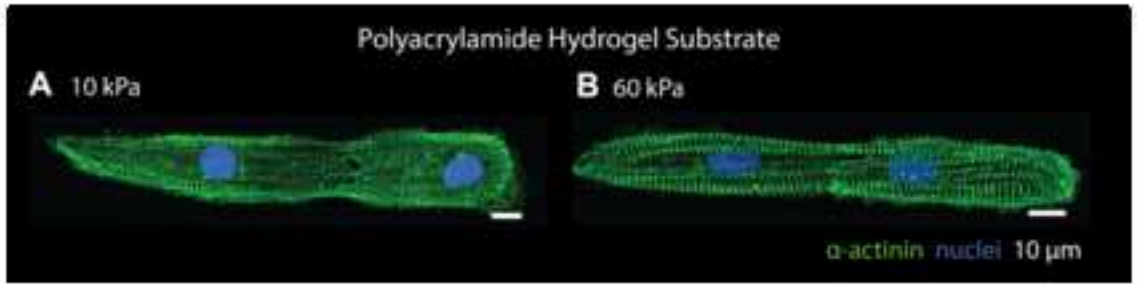

Table

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Step	Problem observed	Possible reason
1.1	The pattern size is bigger than expected	Stencil is flipped
8.1	Hydrogel fractures when peeling stencils	Hydrogel sticks to stencils
8.13	Cell attachment is suboptimal	Degraded sulfo-SANPAH Inefficient protein incorporation Old ECM protein solution
8.13	Cell patterning is suboptimal	Basement membrane matrix protein solution spills over
8.13	More than one cell on one slot	Not in single cell suspension Seeding density not optimal

Solution
Include a chiral letter/shape in the stencil design to indicate the front- and back-side of stencils
Cleaning of stencils Do not dry hydrogel too much before putting stencils
Use new sulfo-SANPAH Prepare new ECM protein solution
Perform immunostaining using fluorophore-conjugated anti mouse IgG antibody or Wheat germ agglutinine to examine the distribution of basement membrane matrix protein solution on hydrogel
Use 40−70 µm cell strainer to have single cell suspension
Optimize the seeding density from 30 k to 90 k per mL

Name of Material/ Equipment	Company
2-Aminoethyl methacrylate hydrochloride (powder)	Sigma-Aldrich
Acrylamide solution 40% (solution)	Sigma-Aldrich
Bench UV lamp 365 nm	UVP
BioFlex culture plate	FLEXCELL INTERNATIONAL CORPORATION, Burlington, NC
Bis-acrylamide solution 2% (solution)	Sigma-Aldrich
Corning cover glasses square, No. 2, W × L 22 mm × 22 mm	Sigma
Irgacure (2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone) (powder)	Sigma-Aldrich
Matrigel	Corning
Methyl sulfoxide, 99.7+%, Extra Dry, AcroSeal, ACROS Organics	Acros Organics
Millex (13mm) filter unit with Durapore Membrane	Millipore
Millipore 50mL Steriflip (0.22 µm)	Fisher Scientific
Stencils	Potomac
Sulfo-SANPAH	ThermoFisher Scientific
TrypLE Select 10x	ThermoFisher Scientific

Catalog Number	Comments/Description
516155	
A-4058-100mL	
UVP 95-0042-07, XX-15L	
	6-well plate with silicon elastomer substrate
M1533-25mL	
CLS285522	
410896	
356231	basement membrane matrix protein solution
326881000	
SLGV013SL	
SCGP00525	
custom design	
22589	
A1217702	Enzyme used for stencil cleaning

Happy Holidays. We thank the editors for thorough and considerate editing directions. We believe we addressed all the comments. Please see below for our point-by-point responses.

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

We used the attached manuscript for revision.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Please note that I have replaced some commercial terms with their respectively generic terms (parafilm by paraffin film, kimwipes by lint-free wipes, Kapton by polyimide, etc.). Please remove or replace other commercial terms in your manuscript.

We believe we removed all commercial language from manuscript.

3. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please see specific comments marked in the attached manuscript.

We added more details to each protocol steps. We believe the revised protocol steps provide enough details for replicating the protocol.

4. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.

We removed all the ™/®/© symbols and sort the materials alphabetically.

Stencil Design File (7:1 Rectangle)

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Supplemental Coding Files
7to1 22mm.DXF

Stencil Design File (1:1 Square)

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Stencil Design File (11:1 Rectangle)

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Supplemental Coding Files

Stencil Design-a single 11_1.DXF