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A versatile method of patterning proteins and cells

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Abstract:	Substrate and cell patterning are widely used techniques in cell biology to study cell-to-cell and cell-to-substrate interactions. Conventional patterning techniques work well only with simple shapes, small areas and selected bio-materials. This article describes a method to distribute cell suspensions as well as substrate solutions into complex, long, closed (dead-end) polydimethylsiloxane (PDMS) microchannels using negative pressure. This method enables researchers to pattern multiple substrates including fibronectin, collagen, antibodies (Sal-1), poly-D-lysine (PDL), and laminin. Patterning of substrates allows one to indirectly pattern a variety of cells. We have tested C2C12 myoblasts, the PC12 neuronal cell line, embryonic rat cortical neurons, and amphibian retinal neurons. In addition, we demonstrate that this technique can directly pattern fibroblasts in microfluidic channels via brief application of a low vacuum on cell suspensions. The low vacuum does not significantly decrease cell viability as shown by live/dead cell assays. Modifications are discussed for application of the method to different cell and substrate types. This technique allows researchers to pattern cells and proteins in specific patterns without the need for exotic materials or equipment and can be done in any laboratory with a vacuum.
Author Comments:	Dear Editor, This submission has two first authors and two corresponding authors as follows

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Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

September 19, 2016

Editor, Journal of Visualized Experiments

Dear Editor,

We are submitting a manuscript entitled "A versatile method of patterning proteins and cells" co-authored with Derek Yip, Cheul Cho, Bonnie Firestein, and Ellen Townes-Anderson for publication in the Journal of Visualized Experiments.

Patterning proteins and cells is becoming an important tool for biologists to understand how the microenvironment of a cell affects its growth and behaviors. For example, specific spatial control of proteins allows researchers to examine the effects of specific guidance cues on axonal extension in neurons while control over the geometry of cell adhesion onto a substrate has also provided important insights in biology. The problem with current techniques to pattern proteins and cells however, is the amount of specialized materials, cell substrates, equipment, and techniques needed to accurately pattern proteins and cells on a specific surface.

In this report, we describe a method of patterning proteins and cells with poly-dimethyl-siloxane (PDMS) microfluidic channels using simple and readily available materials and tools that biological labs can apply to their specific area of interest. By taking advantage of the gas permeability property of PDMS, we utilized a low pressure vacuum (commonly available as the house vacuum in most labs) that pulled solution into the PDMS microfluidic channel allowing for complete filling of the channels with either a protein or cell solution. Removal of the PDMS microfluidic channel then left the proteins or cells in specific areas of the cell culture dish. This method works with all substrates and protein solutions that we have tested so far, as well as many different cell types which we detail in this report. Thus, the audience for this particular biological technique may be wide ranging because of its ease and flexibility.

Please let us know if you have questions or concerns.

Sincerely,

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TITLE:
A versatile method of patterning proteins and cells

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KEYWORDS:
Microfluidic; cell pattern; substrate pattern; cell culture; neuron; myoblast; fibroblast; vacuum; soft lithography; PDMS; Microchannel;

SHORT ABSTRACT:
This report describes a simple, easy to perform technique, using low pressure vacuum, to fill microfluidic channels with cells and substrates for biological research.

LONG ABSTRACT:
Substrate and cell patterning techniques are widely used in cell biology to study cell-to-cell and cell-to-substrate interactions. Conventional patterning techniques work well only with simple shapes, small areas and selected bio-materials. This article describes a method to distribute cell suspensions as well as substrate solutions into complex, long, closed (dead-end) polydimethylsiloxane (PDMS) microchannels using negative pressure. This method enables researchers to pattern multiple substrates including fibronectin, collagen, antibodies (Sal-1), poly-D-lysine (PDL), and laminin. Patterning of substrates allows one to indirectly pattern a variety of cells. We have tested C2C12 myoblasts, the PC12 neuronal cell line, embryonic rat cortical neurons, and amphibian retinal neurons. In addition, we demonstrate that this technique can directly pattern fibroblasts in microfluidic channels via brief application of a low vacuum on cell suspensions. The low vacuum does not significantly decrease cell viability as shown by cell viability assays. Modifications are discussed for application of the method to different cell and substrate types. This technique allows researchers to pattern cells and proteins in specific patterns without the need for exotic materials or equipment and can be done in any laboratory with a vacuum.

INTRODUCTION:
In tissue engineering and biosensing, the ability to control the spatial organization of proteins and cells on a micron scale, has become increasingly important over the last four decades[1-3]. Precise spatial organization of proteins and cells has allowed researchers to examine the interaction between cells and substrates containing similar or different types of cells, to guide cell growth, and to immobilize biomolecules for the fabrication of biosensors[4-9].

Current methods of patterning proteins include photopatterning and microcontact printing. Photopatterning utilizes light sensitive material which is crosslinked upon exposure to ultra violet (UV) light. UV light directed at a photomask (consisting of transparent areas with darker regions to prevent UV light transmission) causes crosslinking in specific regions which can then be used for subsequent attachment of biomaterials or cells[10,11]. While this scheme is very accurate and allows for precise control of the topography of the culture surface, it is limited to UV-sensitive biomolecules that can be patterned by UV radiation[12]. Microcontact printing is another popular method of patterning specific proteins[13,14]. In this method, a poly-dimethyl

siloxane (PDMS) stamp is treated with a variety of surface modification reagents before being soaked in a solution of the chosen biomolecular substrate. It is then gently pressed onto a glass coverslip or other surface thus "stamping" the biomolecule onto the culture surface. However, stamping is limited to the type of material that can be transferred as well as the wettability of biomolecules to the surface of the PDMS stamp[15].

Direct patterning of cells can be more difficult and relies on complex methods such as switchable substrates, stencil based methods, or patterning with specific cell adhesion molecules[16,17]. These methods are limited in their ability to pattern cells due to the lack of compatible cell adhesion substrates, incompatibility of the process to work with sensitive biological cells and constraints, inconsistency in reproducing the patterning, and complexity of the procedure. For example, with switchable substrates, custom substrates need to be designed for every cell type, to switch their adherence to specific cell types without degradation upon exposure to the UV light and heat used in process[17-20]. Stencil based patterning methods are versatile in their ability to pattern cells; however, it is difficult to manufacture PDMS stencils at the appropriate thicknesses for use[16,21]. Direct injection of cells into PDMS microfluidic channels have some advantages such as: 1) ease in fabrication of microfluidic channels and 2) suitability for many different cells and substrates. However, the prevalent issue of air bubble capture during the injection process due to the hydrophobicity of PDMS without the use of plasma cleaning, or other methods to decrease air bubbles, makes it difficult to consistently create patterned cells on glass or plastic surfaces[21].

This work expands upon capillary micromolding[22-26] and reports a method to inject protein and cell suspensions into microchannels. The method used here demonstrates the patterning of substrates and both direct and indirect patterning of specific cell types. This technique overcomes the high hydrophobicity of PDMS and eliminates the presence of bubbles during injection of either substrates or cells by taking advantage of the gas permeability of PDMS[27]. This paper demonstrates the use of the technique with several different substrates and cell types. The article also highlights the fabrication of molds for soft lithography using conventional photolithography as well as a simple and low-cost adhesive tape method useful in resource limited settings[28,29].

PROTOCOL:

Note: Please consult all relevant material safety data sheets (MSDS) before use. Some of the chemicals used in this protocol are toxic and carcinogenic. Please use all appropriate safety practices (fume hood, glovebox) and personal protective equipment (safety glasses, gloves, lab coat, full length pants, closed-toe shoes) when using toxic or acid/base materials.

1. Fabrication of master molds for soft lithography using photolithography

1.1) Draw the layout of the microchannel using a computer-aided design (CAD) drawing tool.

1.2) Print the layout on a blank mask plate using laser mask writer.

1.3) Rinse a 4-inch silicon wafer with acetone followed by isopropanol and dry with a nitrogen air gun to ensure that no solvent is left on the wafer.

1.4) Dehydrate the wafer by baking it on a hot plate at 200 °C for 5 min.

1.5) Select a negative photoresist designed for the desired height of the microchannels. For example, use negative photoresist SU-8 50 to obtain microchannels with a height of 50 µm.

1.6) Allow the wafer to cool to room temperature, and then align the wafer on the chuck of a spin coater.

1.7) Dispense 4 mL (1 mL/inch diameter of the wafer) of negative photoresist onto the center of the wafer.

1.8) Spin coat the wafer with a two-step spin cycle using a spinner. First, apply a spread cycle of 500 rpm with an acceleration of 100 rpm/s for 10 s. Then apply a spin cycle of 2000 rpm with an acceleration of 300 rpm/s^2 for 30 s to obtain a 50 µm coating of photoresist on a wafer.

1.9) Soft bake the wafer in two steps on a hot plate according to the manufacturer's directions. For a 50 µm coating of photoresist, first pre-bake the wafer at 65 °C for 6 min and then immediately ramp up the hot plate temperature to post-bake the wafer at 95 °C for 20 min.

1.10) Allow the wafer to cool to room temperature and then load the wafer onto the lithography stage.

1.11) Align the mask on the wafer using mask aligner and expose the wafer to UV light (as per the manufacturer's instructions) at an intensity of 1.5 mW/cm^2 for 146 s to apply the total UV dose of 220 mJ/cm^2 required for a 50 µm thick layer of photoresist.

1.12) Apply post exposure bake to the wafer in two steps on a hot plate. For a 50-µm coating of photoresist, first pre-bake the wafer at 65 °C for 1 min and immediately ramp up the hot plate temperature to post-bake it at 95 °C for 5 min.

1.13) Develop the wafer in by submerging the wafer in SU-8 developer and gently shaking until the features become clear on the wafer. Develop the wafer for ~6 min for the 50-µm thick photoresist.

1.14) Rinse off excess developer with isopropanol and ethanol, then dry the wafer with a nitrogen air gun.

1.15) Hard bake the silicon wafer on a hot plate at 150 °C for 15 min.

1.16) Place the wafer in a desiccator with 25 µL of the silanizing agent tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane.

Note: The silanizing agent is corrosive, thus should be used in an acid/base fume hood, with appropriate personal protective equipment (safety glasses, gloves, lab coat, full length pants, closed-toe shoes).

1.17) Apply the vacuum for 5 min and expose the wafer to the vapors of silane for 30 min without releasing the vacuum.

1.18) Transfer the wafer into a suitably sized petri dish.

2. Fabrication of master molds for soft lithography using adhesive tape

2.1) Draw the layout of the microchannel using a CAD drawing tool and print the drawing on white paper.

2.2) Clean a glass slide large enough to accommodate the design with isopropanol and then dry the slide with an air gun.

2.3) Attach adhesive tape onto the cleaned glass slide. Be careful not to trap any air bubbles.

2.4) Tape the sides of the glass slide onto the paper with the design, with the tape side up.

2.5) Use a scalpel to cut the tape on the glass slide using the paper design as a reference and then peel off tape from unwanted areas of the glass slide.

2.6) Rinse the glass slide with isopropanol and then dry with an air gun. Bake the glass slide in an oven at 65 °C for 30 min.

2.7) Gently roll over the tape with a rubber roller to remove air bubbles and allow the slide to cool to room temperature.

3. Soft lithography fabrication of the PDMS devices

3.1) Mix PDMS elastomer and its curing agent in a ratio of 10:1 (w:w), stir the mixture vigorously, and then degas the mixture by placing it into a vacuum chamber until no air bubbles appear in the mixture.

3.2) Pour the mixture onto a silanized silicon mold or adhesive tape mold in a petri dish to obtain a ~ 2 mm thick layer of PDMS and degas until all air bubbles disappear.

3.3) Cure the PDMS in an oven at 65 °C for 2 h.

3.4) Use a scalpel to cut the PDMS layer at least 5 mm away from the features and then peel the cured PDMS off the wafer using tweezers.

3.5) Punch a single inlet hole with a 1 mm biopsy punch anywhere on the microchannel, preferably at the end of a network of microchannels as seen in Figure 2a and 4b.

3.6) Clean the PDMS cast with adhesive tape to remove dust particles adsorbed onto the device. Sterilize the PDMS microchannel device (PDMS cast) by rinsing with a solution of 70% ethanol followed by sterile deionized (DI) water, and expose to UV for 30 min.

3.7) Keep the sterile PDMS device in a sterile petri dish until use.

4. Substrate patterning

4.1) Place the sterile PDMS cast using tweezers onto a sterile glass coverslip and apply gentle pressure using the tip of the tweezers.

Note: The PDMS cast makes a conformal but reversible seal with the glass coverslip forming microchannels without use of any adhesive.

4.2) Place a droplet (20-40 µL) of the substrate solution on the inlet completely covering the inlet hole. Pattern Poly-D-Lysine (PDL) by placing a 20 µL droplet of PDL in sodium tetraborate buffer on the inlet of the microchannels.

4.3) Put the petri dish in a vacuum of ~254 mmHgA (equivalent to house vacuum in biological laboratories) for 10 min, and observe air coming out of the channel in the form of bubbles.

4.4) Release the vacuum and observe the substrate solution flowing into the microchannel.

4.5) Incubate the dish at the appropriate conditions for substrate adherence. See Tables 1 and 2 for incubation conditions (these vary depending on the substrate). For PDL patterning, incubate for 1h at 37 °C.

4.6) Carefully peel off the PDMS stamp using sterile tweezers and wash the pattern on glass coverslip thrice with DI water.

4.7) Add a 1% bovine serum albumin (BSA) solution to the petri dish to cover the dish or the glass coverslip and incubate overnight at 37 °C.

Note: This will reduce non-specific adherence in uncoated regions.

4.8) Aspirate off the 1% BSA solution, and the patterned substrate is now ready to use.

5. Indirect patterning of cells

5.1) Prepare the cell suspension in serum-free culture medium. The cell type and cell density are listed in Table 1.

5.2) Add the cell suspension to the patterned petri dish and completely submerge the patterned region in cell suspension.

5.3) Incubate the petri dish at 37 °C in an incubator for 15 min to allow cells to attach to the patterned substrate.

5.4) Aspirate the excess cell suspension from the petri dish and wash the patterned cells three times with phosphate buffered saline (PBS) while gently shaking for 10 s to remove unattached cells.

5.5) Add the appropriate culture medium to the patterned cell cultures and incubate the patterned cells at 37 °C in a CO_2 incubator.

6. Direct patterning of cells

Note: This technique is an alternative to the indirect cell patterning described in step 5. However, unlike in step 5, in this technique cells are patterned on tissue culture surfaces with or without substrate coating.

6.1) Sterilize the microfluidic device by rinsing with a solution of 70% of ethanol followed by DI water.

6.2) Soak the device overnight in a solution of 1% BSA to prevent the cell adhesion to the PDMS.

6.3) Dry the device at room temperature and attach it to the bottom of the tissue culture-treated petri dish.

6.4) Prepare the cell suspension in serum-free culture media. The cell type and cell density are listed in Table 2.

6.5) Place a droplet (4-8 µL) of the cell suspension, enough to fill the microchannels, on the inlet completely covering the inlet hole.

6.6) Put the petri dish in a vacuum for 10 min and observe air coming out of the channel in the form of bubbles. Release the vacuum and observe the cell suspension flowing into the microchannel.

6.7) Incubate the petri dish in an incubator to promote cell adhesion as per the incubation conditions (depend on type of cell patterned) mentioned in Table 2. Add PBS to the petri dish submerging the PDMS device.

6.8) Peel the PDMS carefully off the petri dish with tweezers and wash the pattern with PBS. Add cell culture media to the petri dish and place the cell culture in the incubator.

REPRESENTATIVE RESULTS:

This method allows the patterning of proteins and indirect patterning of cells using dead-end microfluidic channels with dimensions as small as 10 µm and equipment available in almost all biological laboratories once the master mold is made. This technique can be utilized with PDMS microfluidic channels created using traditional soft photolithography, or with PDMS microfluidic channels created with adhesive tape fabrication (Figure 1)[28,29]. We have previously demonstrated the use of this procedure in indirect patterning of neuronal cells for evaluation of axon guidance in photoreceptors[4,30], and have now demonstrated its use in several additional cell types of varying origins. Embryonic rat cortical neurons patterned on the poly-D-Lysine (PDL) and laminin surfaces demonstrate general adherence to the patterned areas and growth along the PDL and laminin stripes (Figure 3A). C2C12 myoblast cell lines also adhere to the patterned area and demonstrate fusion into multinucleated myotubes (Figure 3B). We have also adapted this technique for direct cell patterning as seen with fibroblasts in Figure 4. As previously reported, this direct cell patterning method showed no adverse effect on cell survival[30]. The variables that lead to successful direct or indirect patterning of stated cell types are listed in Table 1 and 2. Thus in the future, researchers may utilize this technique to pattern cells and discover phenomena related to their specific patterning.

[Figure 1 here]

Figure 1: Fabrication of mold by soft lithography. Left: Photolithography process. SU-8 is spin coated onto a silicon wafer, exposed to UV radiation, and developed to remove excess SU-8. Right: Adhesive tape fabrication process. Adhesive tape is attached to a clean glass slide, cut with a scalpel, and excess tape is carefully removed. PDMS cast is then fabricated from each mold using soft lithography.

[Figure 2 here]

Figure 2: Example of a PDMS device for substrate patterning. a) PDMS microfluidic cast with inlet used for microfluidic patterning of substrates. Inlet was punched out at one end of microchannels. b) Microfluidic channel as seen under phase contrast microscopy. Microfluidic channels are 15 mm in length, 20 µm wide, and separated by 200 µm. Figure 2 is reproduced with permission from IOP Publishing. All rights reserved[30].

[Figure 3 here]

Figure 3: Indirect cell patterning. a) Vacuum assisted protein patterning process. PDMS microfluidic channel device is attached to a petri dish, substrate solution is loaded into inlet, vacuum is briefly applied to chamber and released, PDMS device is removed, pattern is washed, and ready to use. b) Embryonic cortical rat neurons seeded onto a coverslip with a patterned PDL and laminin substrate. c) C2C12 myoblast cell lines grown on patterned PDL and laminin. Figures 3a and 3c are reproduced with permission from IOP Publishing. All rights reserved[30].

[Figure 4 here]

Figure 4: Direct cell patterning. a-c) Fibroblasts patterned via direct cell patterning. a) Microfluidic channel device fabricated using adhesive tape lithography. b) Microfluidic channels after filling with fibroblast cell suspension using vacuum assisted patterning technique. c) Fibroblasts after removal of microfluidic device. d) Calcein-AM staining of fibroblasts after microfluidic patterning. e) Phase contrast image of fibroblasts after microfluidic patterning. f-g) Fibroblasts stained with phalloidin-TRITC (red) and DAPI (blue). Figure 4 is reproduced with permission from IOP Publishing. All rights reserved[30].

[Table 1 here]

Table 1: Indirect cell patterning conditions. List of incubation conditions such as substrate type, time, temperature, and cell density for typical cell types. Researchers should optimize the incubation conditions for their own model substrates and cell type. Sal-1 is the antibody substrate used to grow Salamander retinal neurons.

[Table 2 here]

Table 2: Direct cell patterning conditions. List of incubation conditions such as temperature, time, and cell density for typical cell types. Researchers should optimize the incubation conditions for their own model cell type.

DISCUSSION:

While conventional photolithography is a well-established technique for the creation of molds for soft lithography, the equipment, materials, and skills necessary to use conventional photolithography are not readily available to most laboratories. For laboratories without access to these resources, we have presented adhesive tape fabrication as a method of creating molds with relatively simple features for microfluidic devices. This method allows any laboratory to create and utilize microfluidic devices for research purposes with readily available tools. The adhesive tape method may be improved with a low-cost desktop vinyl cutter[31]. Desktop vinyl cutters may increase reproducibility, resolution, as well as layout complexity. Each of these options, conventional photolithography, desktop cutters, or adhesive tape fabrication have different capabilities and limitations and researchers must carefully consider which method would be sufficient for their needs.

In cured PDMS, the long polymeric chains of dimethyl siloxane form a lattice, nanoporous structure which creates empty regions that can be filled with air molecules[27]. This gas permeable property is key to our method of filling microfluidic channels. When placed into a low pressure environment, air molecules are removed from the PDMS bulk material as well as the microchannels themselves[25]. When the PDMS is then exposed to atmospheric pressure, the PDMS bulk material and microchannels retain a negative pressure for some time[25,30]. This negative pressure draws liquid into the microchannels automatically filling the microfluidic channels. This negative pressure continues to draw liquid into the channel until the bulk PDMS equilibrates with atmospheric pressure.

This vacuum-assisted technique allows for patterning of specific proteins and substrates onto a growth substrate. In this study, we were able to pattern several different substrates and cell types both directly and indirectly (Table 1 and 2). However, the experimenter may need to test different incubation times, cell densities, or seeding media for their particular cell type. These variables likely relate to the innate ability of either the cell or substrate to adhere to the surface it is being placed upon. For example, here C2C12 cells required the use of serum-free media for the seeding media before culturing the cells in DMEM with 2% horse serum. Additionally, conditions may vary based upon whether a glass coverslip or plastic petri dish is being patterned. Here, both worked well to allow for specific patterning of various cell types.

One of the initial concerns we had while utilizing this technique was how exposure to vacuum might affect cell viability. This study and previous studies, however, should alleviate those concerns as we have shown that vacuum has little to no effect on a variety of cells[30]. Wang *et al.* previously utilized degassed irreversibly sealed microfluidic chambers to load HeLa cells[32]. Other studies have used vacuum-assisted cell seeding for a variety of cell types including human stem cells, adherent and non-adherent cells, and human-hamster hybrid cell line (A_L) cells to load into microfluidic channels[33]. In addition, other researchers have subjected cells to much greater vacuum pressures in comparison to this current study with little to no discernible effect on the cells[33]. Bubbles formed during the removal of air from the microchannel tend to congregate on the surface of the droplet of the suspension at the inlet. Often these bubbles do not rupture due to the surface tension of the suspension. We have not observed a noticeable decrease in cell viability due to bubbles. In addition, the experiment with Calcein-AM does not suggest a significant decrease in cell viability. Because of this, we have not thoroughly examined cell death specifically due to bubbles.

Previous attempts to pattern substrates or cells were often plagued by problems such as the formation of air bubbles in the microfluidic devices. The formation of air bubbles made it difficult to inject liquids easily and efficiently without the use of equipment or materials that are not available in most laboratories. For example, previous methods utilized the use of plasma treatment or corona treatment to decrease the hydrophobicity of PDMS microchannels[15,34]. While effective, plasma and corona treaters are not readily available in most laboratories. In this protocol, we demonstrate the ability to pattern cells and substrates simply using common laboratory vacuums. Using the adhesive tape fabrication technique, the

methodology can be utilized to create PDMS microfluidic channels to pattern substrates or cells in nearly any laboratory.

In order to pattern substrates or cells with the vacuum patterning protocol, several steps are critical for successful patterning. First, to fabricate the adhesive tape master, the adhesive tape must be completely attached to the glass slide and free of air bubbles. Air bubbles weaken the bond between the tape and the glass slide and may lead to the tape being peeled off when cured PDMS is peeled off the adhesive tape mold. In addition, bubbles will distort the geometry of the microfluidic channel causing the surface of the channels to be non-planar. To make the PDMS cast from SU-8 mold, the SU-8 mold must be silanized before casting with PDMS. This step is critical in preventing the permanent bonding of PDMS to the SU-8 mold after curing of PDMS. Prior to conformal sealing of the PDMS microfluidic channel to glass coverslips or plastic petri dishes, the PDMS must be cleaned of all dust particles. These particles may prevent the formation of a conformal seal between PDMS and glass and thus prevent the injection of cells or substrates into the microfluidic channel. As stated in the above protocol, cleaning the PDMS channels with adhesive tape is critical prior to adhering the microchannel to glass coverslips or plastic petri dishes. Finally, after placing the device into the vacuum chamber, the vacuum must be gently released to prevent displacement of the droplet of substrate or cell solution from the inlet hole.

If no fluid is observed flowing into the microfluidic channel, there may be a leak in the microfluidic channel which would prevent liquid from flowing into the channel. Remove the PDMS stamp, dry and clean it and then repeat step 4, 5, or 6 of the technique. If the solution does not flow into the microchannel after release of vacuum, ensure that the solution is completely covering the entrance of the microchannel. This can be done by pipetting solution up and down several times while putting it in the inlet hole. If the substrate, after injecting into the microchannel, does not attach to the glass, evaluate and determine the optimal incubation conditions necessary for the substrate used. If removing the PDMS cast from the glass surface or plastic petri dish causes the patterned cells or substrates to be destroyed, use a thinner PDMS cast, submerge the PDMS cast in PBS or media, and slowly and gently peel off the PDMS cast from the glass or plastic surface with tweezers.

Finally, BSA may be critical in decreasing nonspecific adhesion of cells to either the unpatterned glass or plastic surface. BSA is typically used as a blocking reagent in western blotting, immunostaining, and other molecular biology techniques. Other researchers have also utilized it for indirect or direct patterning protocols to decrease nonspecific cell adhesion[35-37]. We found that incubation of the substrate with BSA was necessary for most cell types except for salamander retinal cells. This may be because of the more specialized nature of the substrate (an antibody called Sal-1) that was used as well as the general lack of cell adhesion by this cell type[38]. In our hands, BSA also did not noticeably decrease the adherence of cells to the patterned areas and mainly served to decrease nonspecific adherence.

While this method is designed to be versatile and flexible in its application to different substrate and cell types, there are several limitations to this protocol and the compatible cells

and substrate. Because of the very nature of patterning cells onto a surface, the cell types that this technique can be applied to are limited to those which are amenable to any patterning protocol such as those cells that are able to survive with more limited cell to cell contact as well as those that can survive at lower cell seeding concentrations. For example, one potential application is the patterning of yeast cells for atomic force microscopy (AFM)[39]. While we have tested many different cell substrates, other substrates may not work with this particular technique such as those that form three-dimensional gels. At this time, we have not examined if gels properly form and stay on the surface of the glass coverslip or plastic petri dish after patterning. This technique is capable of patterning complex shapes and large areas. However, it cannot pattern an array of individual separated shapes without interconnecting microchannels. In the absence of interconnected microchannels, each individual shape would require its own inlet making the technique cumbersome. While we did not notice any non-uniformity in patterning protein, the distribution of cells patterned inside the microchannel may be non-uniform. This non-uniformity may be due to the random distribution of cells in cell suspensions, geometry and dimensions of the microchannels, and viscosity of the cell suspension.

In the future, this technique may be applied to other types of substrates and cells. For example, three-dimensional gels such as basement membrane matrix or collagen scaffolds may potentially be patterned and formed using PDMS microfluidic channels. By injecting a hydrogel into the microfluidic device, the hydrogel may take on the shape of the microfluidic device and be able to form complex patterns without the use of 3-D printers or other technologies. These would allow for rapid creation of structured scaffolds with readily available tools and microfluidic devices.

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DISCLOSURES:
The authors declare no competing financial interests.

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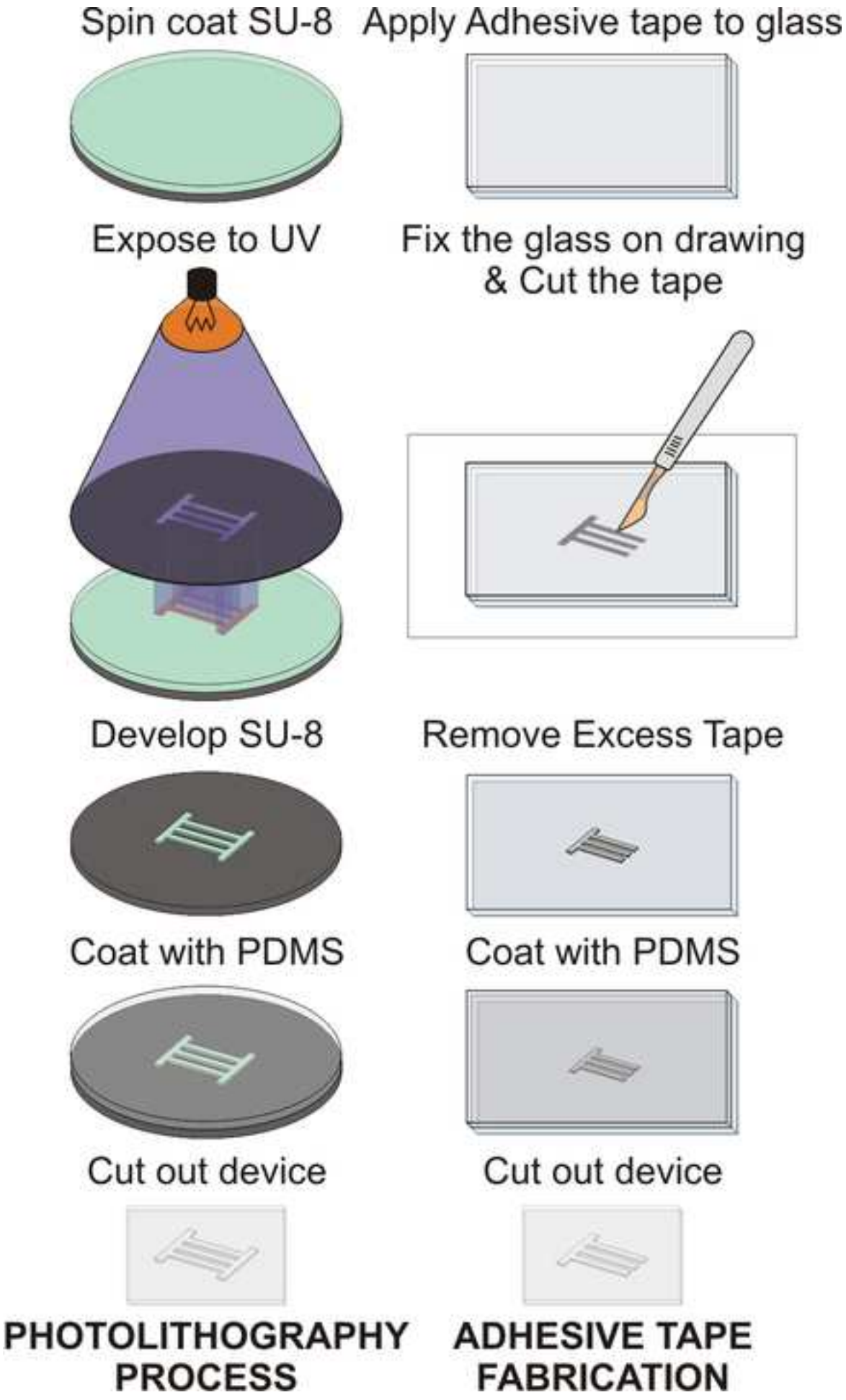

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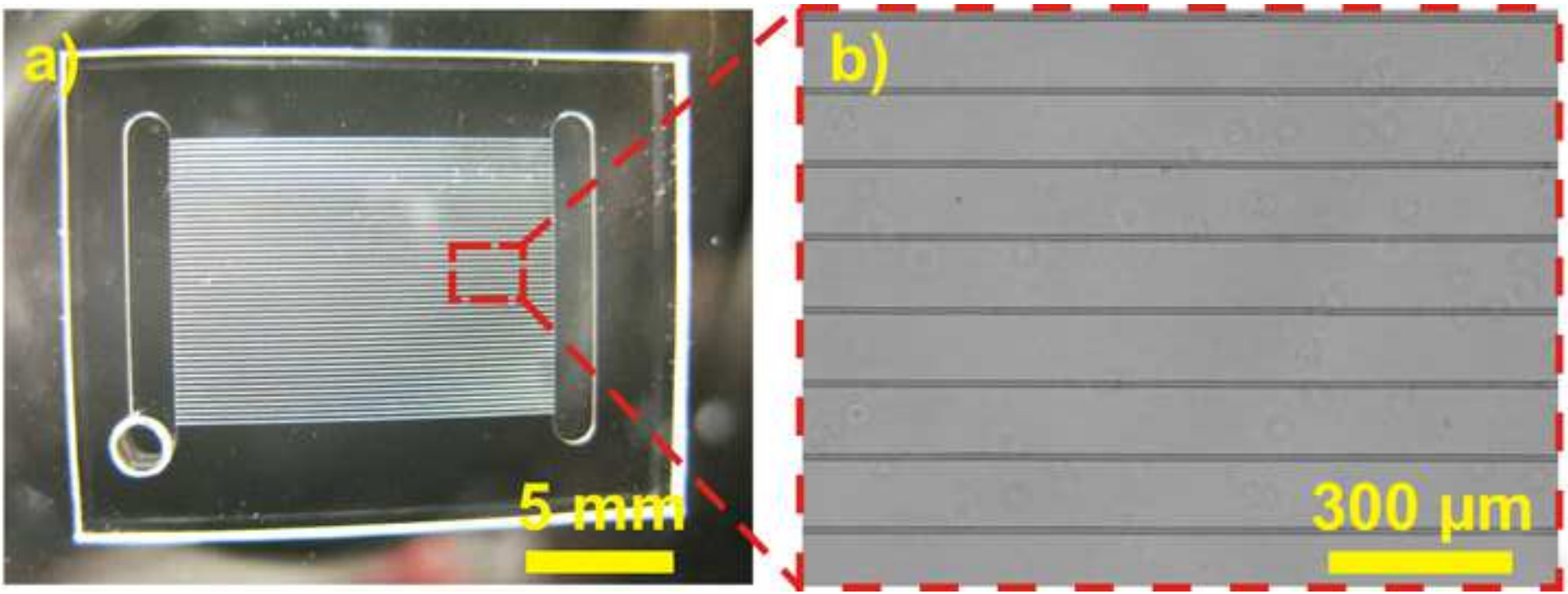

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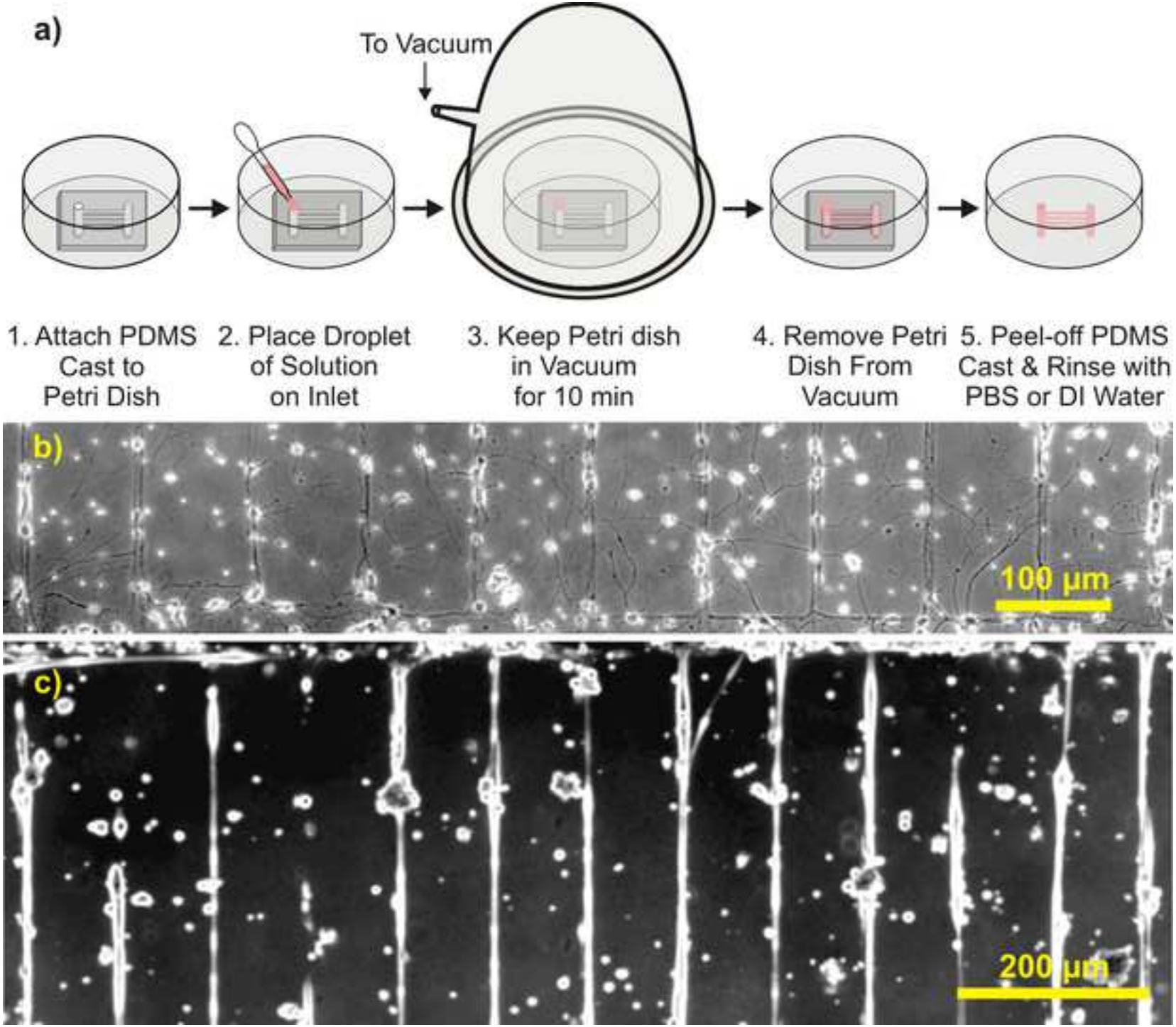

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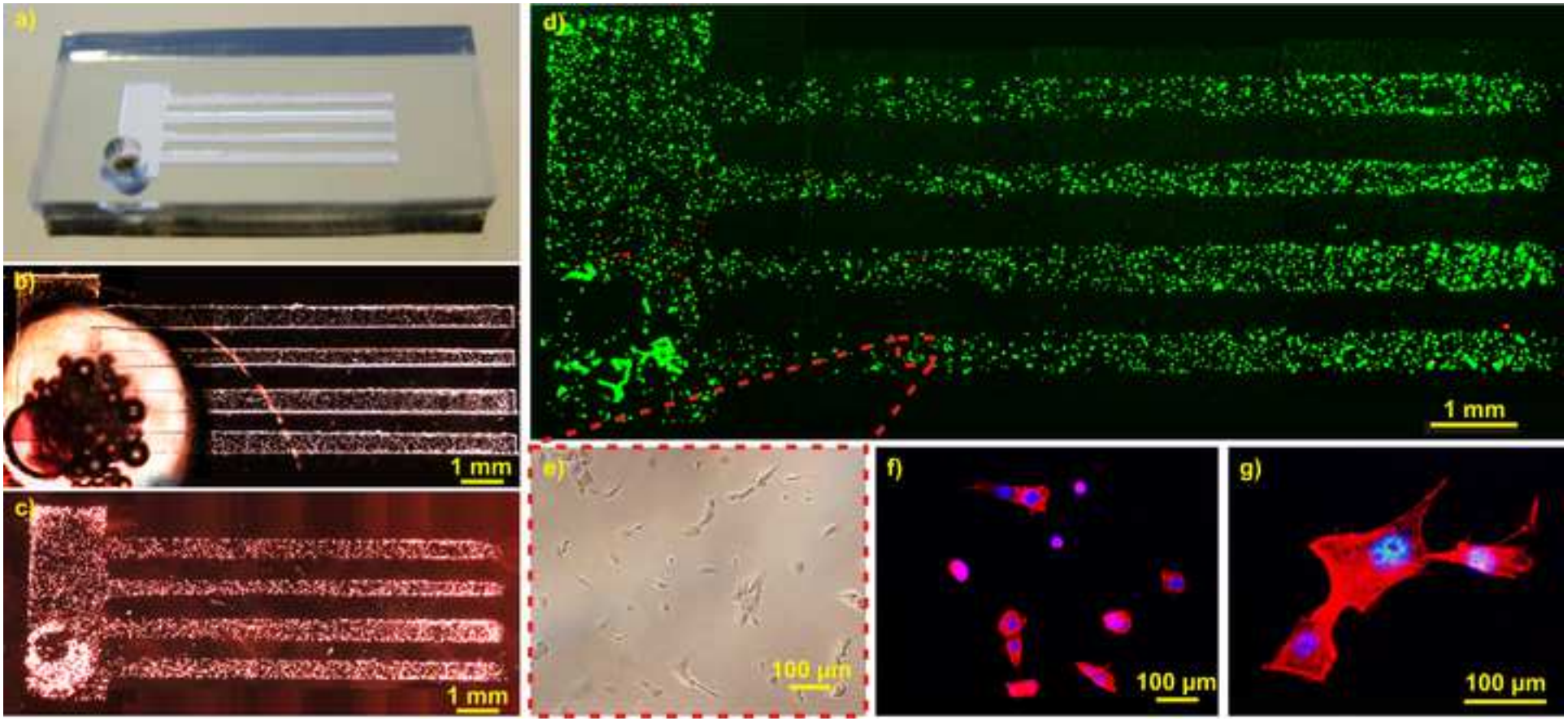

Table 1

Indirect Cell Patterning	Substrate	Protein Incubation Conditions
PC12	Collagen-1	1 h @ 50°C
Fibroblast	Collagen-1	1 h @ 50°C
C2C12	PDL + Laminin	1 h @ 37°C, wash PBS, overnight @ 37°C
Embryonic Rat Cortical Neurons	PDL + Laminin	1 h @ 37°C, wash PBS, overnight @ 37°C
Salamander Retinal Neurons	Sal-1	overnight @ 10°C

Cell Density	Cell Adhesion Duration
5 x 10^6/ mL	1 h @ 37°C
5 x 10^6 / mL	1 h @ 37°C
2 x 10^6 / mL	15 min @ 37°C
2 x 10^6 / mL	15 min @ 37°C
N/A	1 h @ 10°C

Table 2

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Direct Cell Patterning	Substrate	Cell Density
PC12	Collagen-1	6×10^6 / mL
Fibroblast	Collagen-1	6×10^6 / mL
C2C12	PDL + Laminin	2×10^6 / mL
Embryonic Rat Cortical Neurons	PDL + Laminin	2×10^6 / mL
Salamander Retinal Neurons	Sal-1	N/A

Cell Adhesion time
1 h @ 37°C
1 h @ 37°C
15 min @ 37°C
15 min @ 37°C
1 h @ 10°C

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material /Equipment	Company	Catalog #
CorelDRAW X4 CAD Drawing Tools	Corel Corporation, Canada	X4 Version 14.0.0.701
Laser Printer HP	Hewlett Packard, CA	1739629
Bel-Art Dessicator	Fisher Scientific, MA	08-594-16B
Adhesive Scotch Tape	3M Product, MN	Tape 600
PDMS Sylgard 184	Dow Corning, MI	1064291
Petri Dish	Fisher Scientific, MA	08-772-23
Stainless steel Scalpel (#3) with blade (# 11)	Feather Safety Razor Co. Ltd. Japan	2976#11
Tweezers	Ted Pella, CA	5627-07
Glass slides	Fisher Scientific, MA	12-546-2
Glass slides	Fisher Scientific, MA	12-544-4
Rubber Roller	Dick Blick Art Materials, IL	40104-1004
Laser Mask Writer	Heidelberg Instruments, Germany	DWL66fs
EVG Mask Aligner (Photolithography UV exposure tool)	EV Group, Germany	EVG 620T(B)
Spin Coater Headway	Headway Research Inc, TX	PWM32-PS-CB15PL
Photoresists SU-8 50	MicroChem, MA	Y131269
SU-8 Devloper	MicroChem, MA	Y020100
Tridecafluoro-1,1,2,2-Tetrahydrooctyl-1-Trichlorosilane	UCT Specialties, PA	T2492-KG
Isopropanol	Sigma-Aldrich, MO	190764
Ethanol	Sigma-Aldrich, MO	24102
Poly-D-Lysine hydrobromide (PDL)	Sigma-Aldrich, MO	P0899-10MG
Laminin	Sigma-Aldrich, MO	L2020

BSA	Fisher Scientific, MA	BP1605100
C2C12 Myoblast cell lline	ATCC, VA	CRL-1722
PC12 Cell Line	ATCC, VA	CRL-1721
Collagen type 1, rat tail	BD Biosciences	40236
DMEM	GIBCO, MA	11965-084
Horse Serum, heat inactivated	Fisher Scientific, MA	26050-070
Phalloidin-tetramethylrhodamine B isothiocyanate (TRITC)	Sigma-Aldrich, MO	P1951
Calcein-AM live dead cell Assay kit	Invitrogen, MA	L-3224
Biopsy Hole Punch	Ted Pella, CA	15110-10

Description
CAD tool used to draw the layout of the microfluidic device
Used to print the layout of microfluidic device for adhesive tape technique
Used to degass the PDMS mixture
Used to fabricate adhesive tape Master
Casting polymer
Used to keep the mold to cast with PDMS
Used to cut the PDMS
Used to handle the PDMS cast during peeling
Used as surface to pattern the Substrate
Used as surface to pattern the Substrate
Used to attach adhesive tape on glass without trapping air bubbles
Used to fabricate quartz mask used in photolithography fabrication process
Used to expose the photoresist to UV light
Used to spin coat the photoresist on silicon wafer
Negative photoresist used for mold fabrication
Photoresist developer
Coat mold to avoid PDMS adhesion
Cleaning Solvent
Sterilization Solvent
PDL solution is made at 0.1 mg/mL in Sodium Tetraborate Buffer
Laminin aliquoted into 10 µL aliquots and diluted to 20 µg/µL in PBS prior to use

Cell culture
Used to demonstrate C2C12 patterning
Used to demonstrate PC12 patterning
Cell culture
Cell culture
Cell culture
To label cells
Cell viability Assay
Punched hole in PDMS

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Institution:	**Rutgers University, Piscataway, NJ, USA**
Article Title:	**A versatile method of patterning proteins and cells**
Signature:	① ② Date: **09/23/2016**

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Dear Editor,

Thank you for the comments provided by yourself and the reviewers. We believe they will help clarify and strengthen our manuscript for JOVE. We have addressed their concerns below and tracked all changes to the manuscript. Our response to the reviewer's comments are seen below in red text.

In addition, we would like to emphasize that this article has two first authors and two corresponding authors that have contributed equally to this work. These two authors are AB Shrirao and FH Kung.

Editorial comments:
The updated manuscript **55513_R1_100516** is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file. **Please download the .docx file and use this updated version for future revisions.**

• Please ensure that the references appear in the following format: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.
o Please abbreviate all journal titles.
o Please include volume, issue numbers, and DOIs for all references.

All references were updated to ensure correct formatting. 2 references do not have an associated DOI:
Carola, E. Modifying Polydimethylsiloxane (PDMS) surfaces, Institutionen för biologi och kemiteknik, (2007).

Shrirao, A. B. & Perez-Castillejos, R. Chips & tips: simple fabrication of microfluidic devices by replicating scotch-tape masters. Lab Chip. (2010).

• Please use standard abbreviations and symbols for SI Units such as mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

All SI and non-SI units were updated to use standard abbreviations with a single space between the numerical value and unit.

• Please describe centrifuge speeds as "x g" instead of the machine-dependent "rpm" in protocol step 1.8.

Protocol step 1.8 refers to the use of a spin-coater, not a centrifuge. Conventionally, with a spin-coater, "rpm" is the standard terminology used in microfabrication for coating of photoresist on a substrate. The thickness of this coating depends upon speed in rpm.

• Protocol Step 1.9: What is the wait time between pre-bake and post-bake? Is the wafer allowed to cool in between? What is done immediately after pre-bake?

Protocol step 1.9 does not utilize a wait time between pre-bake and post-bake. The wafer is not allowed to cool in between. Step 1.9 has been modified to clarify this point and address the editor's comment. The modified step now reads:
"1.9) Soft bake the wafer in two steps on a hot plate according to the manufacturer's directions. For a 50 µm coating of photoresist, first pre-bake the wafer at 65 °C for 6 min and then immediately ramp up the hot plate temperature to post-bake the wafer at 95 °C for 20 min."

Similarly step 1.12 has been modified to clarify the above point and now reads:
"1.12) Apply post exposure bake to the wafer in two steps on a hot plate. For a 50 µm coating of photoresist, first pre-bake the wafer at 65 °C for 1 min and immediately ramp up the hot plate temperature to post-bake it at 95 °C for 5 min."

• Protocol Step 6.8: Please specify the incubation temperature.

Protocol step 6.8 has been modified to address the editor's comments. The incubation temperature is dependent upon the cell type used as shown in Table 2. The modified step now reads:

"6.8) Incubate the petri dish in an incubator to promote cell adhesion. The incubation conditions depend on type of cell patterned; some examples are listed in Table 2."

Table 2 has also been modified accordingly.

• Please expand the figure legends to adequately describe the figures. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

Figure legends have been updated to include a short title and the descriptions have been modified to improve clarity.

•Length warning: The highlighted material is right at our 2.75 page limit. If material is expanded or added following peer review it may run over.

The authors tried their best to remain under the 2.75 page limit.

•A few minor grammar errors should be correected:
-4.1: "...and apply gentle pressure using [the] tip of the tweezers."
-4.2: "on the inlet of [the] microchannels."

We have addressed these grammatical errors.

•The animal ethics statement at the beginning of the Protocol does not appear to be necessary; ethics statements are only required for protocols that involve animal use.

The animal ethics statement was inserted due to the use of embryonic rat cortical neurons harvested from embryonic rats.

Reviewers' comments:
Reviewer #1:
This article describes a method to distribute cell suspensions as well as substrate solutions into complex, long, closed (dead-end) polydimethylsiloxane (PDMS) microchannels applying vacuum.

Minor Concerns:
The manuscript is interesting and proposed method could be applied some corrections were performed according to recommendation of other reviewers and editors, however the manuscript not well addresses and represents the applicability of cells patterned within some micro patterns, e.g. cells patterned within micro-wells can be very suitable for the investigation of mechanical properties of cells walls or membranes (dependently on cell type) by atomic force microscopy (e.g. Elastic Properties of Chemically Modified Baker's Yeast Cells Studied by AFM. Surface and Interface Analysis, 2011, 43, 1636-1640.), etc. These applicability related aspects should be more clearly indicated in introduction, discussion part and conclusions and supported by corresponding references.

An example of applicability was included and referenced in discussion.

Reviewer #2:

This manuscript describes a interesting method to pattern hydrogels and inherently cells by using a vacuum assisted PDMS stamp. This method is of interest for the community and the manuscript describes thoroughly the protocol. I did appreciate the discussion about the concern of cell viability after being vacuumed and both with the references and the experiments, I was convinced that the filling protocol did work and did not arm cells. I did not catch the interest of the tape vs SU8 molding of PDMS apart for dissemintation of PDMS in non technological labs, which is of interest and should be stated in the final video. Overall, the protocol is correct and details are placed. I recommend for publication.

As per the suggestion of the reviewer, we have inserted a sentence in the introduction clearly highlighting the advantage of the adhesive tape method in non-technological laboratories vs. SU8 mold fabrication using conventional photolithography.

Reviewer #3:
This paper proposes a cell/protein patterning method using simple microchannel and vacuum system.
This paper has demonstrated the simplicity and versatility of its pattering method using various cell types and substrate types.
The vacuum assisted solution injection using hydrophobic PDMS channel can prevent the leaking between stamp and substrate when hydrophilic PDMS channel is used to induce capillary action. However, basic principle for vacuum assisted solution injection is not fully described in the discussion section.

As per the reviewer's suggestion, we have inserted a paragraph on the mechanism behind vacuum assisted solution injection. The paragraph inserted is shown below:

While conventional photolithography is a well-established technique for the creation of master molds for soft lithography, the equipment, materials, and skills necessary to use conventional photolithography are not readily available to most laboratories. For laboratories without access to these resources, we have presented adhesive tape fabrication as a method of creating large, simple molds for microfluidic devices. This method allows any laboratory to create and utilize microfluidic devices for research purposes. The adhesive tape method may be improved with a readily available low-cost desktop vinyl cutter[31]. Desktop vinyl cutters may increase reproducibility, resolution, as well as layout complexity. Each of these options, conventional photolithography, desktop cutters, or adhesive tape fabrication have different capabilities and limitations and researchers must carefully consider which method would be sufficient for their needs.

In cured PDMS, the long polymeric chains of dimethyl siloxane form a lattice, nanoporous structure which creates empty regions that can be filled with air molecules[27]. This gas permeable property is key to our method of filling microfluidic channels. When placed into low pressure environment, air molecules are removed from the PDMS bulk material as well as the microchannels themselves[25]. When the PDMS is then exposed to atmospheric pressure, the PDMS bulk material and microchannels retain a negative pressure for some time[25,30]. This negative pressure draws liquid into the microchannels automatically filling the microfluidic channels. This negative pressure continues to draw liquid into the channel until the bulk PDMS equilibrates with atmospheric pressure.

Minor Concerns:
What's the mechanism of liquid flow in closed microchannel while the vacuum is released?

As stated above, we have inserted a paragraph on the mechanism behind vacuum assisted solution injection.

The liquid solution might be injected during the formation of bubbles from air filled channel under the vacuum. And, the formed air bubbles are ruptured in cell suspension during the vacuum. Does bubble rupturing influence cell viability by shear force?

To address this comment, we have included the following text in our discussion:

"Bubbles formed during the removal of air from the microchannels tend to congregate on the surface of the droplet of the suspension on the inlet. Often, these bubbles do not rupture due to the surface tension of the suspension. We have not observed a noticeable decrease in cell viability due to bubbles. In addition, our experiments with Calcein-AM also do not suggest a significant decrease in cell viability. Because of this, we have not thoroughly examined cell death specifically due to bubbles."

As shown in Figure 4, cells are patterned on straight lines but cell population is not uniform on each region, beginning, middle, and end channel. In other word, what is the main factor to produce non-uniform protein patterning during injection of coating solution?

Non-uniform patterning of cells on the straight lines is likely due to the random distribution of cells in the cell suspension. It may also be due to the geometry and physical dimensions of the microchannels. The study of the distribution of cellular density in microchannel is complex and may depend on the geometry and physical dimensions of the microchannels as well as the viscosity of the cell suspension. At this point in time, we do not have any evidence of non-uniform protein patterning during injection. To address this issue in the manuscript, we have inserted the following statements into the discussion:

"While we did not notice any non-uniformity in patterning protein, the distribution of cells patterned inside the microchannel may be non-uniform. This non-uniformity may be due to the random distribution of cells in cell suspensions, geometry and dimensions of the microchannels, and viscosity of the cell suspension."

Reviewer #4:

This is a very neat and straight to the point method that takes advantage of commonly available tools towards studying patterned cells. While it's not groundbreaking by any means it certainly would be useful to many in the field that do not always have access to a cleanroom or when they would like to do rapid prototyping.

Major Concerns:
A cheap craft cutter ($300) could be used to replace the manual master cutting using a scalpel. This can increase the precision and throughput. It would be nice if the authors described this.

As per the reviewer's suggestion, we have included a small paragraph on options that are available to researchers for PDMS mold fabrication including the adhesive tape method using a craft cutter. This paragraph is seen below:

"While conventional photolithography is a well-established technique for the creation of molds for soft lithography, the equipment, materials, and skills necessary to use conventional photolithography are not readily available to most laboratories. For laboratories without access to these resources, we have presented adhesive tape fabrication as a method of creating molds with relatively simple features for microfluidic devices. This method allows any laboratory to create and utilize microfluidic devices for research purposes with readily available tools. The adhesive tape method may be improved with a low-cost desktop vinyl cutter[31]. Desktop vinyl cutters may increase reproducibility, resolution, as well as layout complexity. Each of these options, conventional photolithography, desktop cutters, or adhesive tape fabrication have different capabilities and limitations and researchers must carefully consider which method would be sufficient for their needs."

To whom it may concern,

Editorial comments are presented below in black. Our response is presented below each comment in red. Thank you and Please inform us if there are any questions or concerns.

- **Dr. Anil Shrirao and Dr. Frank Kung**

Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE standard. Please maintain the current formatting throughout the manuscript. The updated manuscript 55513_R0_092816 is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file. Please download the .docx file and use this updated version for future revisions.The file is also attached.

Changes to be made by the Author(s):

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

The manuscript has been edited and proofread thoroughly to ensure no spelling or grammatical errors.

• Please ensure that the references appear in the following format: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.
o Please abbreviate all journal titles.
o Please include volume, issue numbers, and DOIs for all references.

References have been updated to match the guidelines provided.

• Please define all abbreviations at first use, such as CEA, CNRS, BSA.

All abbreviations have been defined at first use.

• Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as CAD drawing software, laser printer, photo transparency, master writer, negative photoresist developer, negative photoresist, dessicator, silanization agent, glass slides, adhesive tape, rubber roller, UV lamp, cell strains, adhesive tape, culture medium, hole punch.

Table of essential supplies have been updated to include all of the items mentioned as well as additional relevant supplies.

• JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent.
o Please replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents.
o Editor has deleted several names such as AUTOCAD, SolidWorks, CorelDraw, Inventor, Scotch tape.
o Please replace the name “scotch tape”, with a generic term such as “adhesive tape”

Manuscript has been edited to remove all commercial sounding language including trademarks and brand names.

• Please add more details to the following 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.

o Step 1.1: Please include the CAD drawing tool you used in the table of materials.
o Step 1.2: Please edit this step to detail the steps you have performed. Optional steps can be mentioned as a note, please avoid the use of the word “or”. Notes will not be part of the filming.
o Step 1.3: Please mention the size of wafer to be used (or that will be during filming, after manuscript acceptance). This will help a reader replicate the exact steps.
o Step 1.5: Please consider using an example of a microchannel and corresponding negative photoresist material to be used, please mention quantity/size and other specifications if appropriate.
o Step 1.7: Please mention the photoresist material you used.
o Step 1.8: Please mention the speed to be used and total duration. Please describe this step along with any equipment used. Was a centrifuge used here?
o Step 1.9: Please provide a reference for temperature and other settings for soft baking, or mention the settings.
o Step 1.11: Please clarify this step. Was a UV lamp used? What were the intensity settings?
o Step 1.12: Please describe the equipment used, and settings such as temperature and time for this.
o Step 1.13: Please add more details to this step. What kind of photoresist developer is used?
o Step 2.7: Please mention the baking temperature.
o Step 3.1: Please restate this as “Mix polydimethylsiloxane (PDMS) elastomer and its curing agent in a ratio of 10:1 (w:w), stir the mixture vigorously, and then degas…”
o Step 3.3: Please avoid the use of the word “or”, instead edit the step to include either “oven” or “hot plate”.
o Step 3.4: Please mention the tool used.
o Step 3.5: Please mention where the holes need to punched.
o Step 3.6: Please mention which device is to be sterilized.
o Step 4.1: Please explain how this is done? Is an adhesive used?
o Step 4.2: What is the composition of the substrate solution used here? While this may vary across users, please provide an example of the solution you used, so that a reader learning this technique can attempt to replicate and learn it.
o Step 4.5: Please mention the substrate used and necessary incubation conditions. Alternatively, this can be added to table 1 if appropriate.
o Step 4.6: Is this done with your hands? Please mention any tools if used.
o Step 6: Is this an alternative to Step 5? Please consider adding a note to indicate that these are different techniques.

All comments have been carefully considered and the protocol has been updated to address the editorial comments provided.

• After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Filmable protocol text has been highlighted in yellow as per the guideline provided. The length of the filmable text is less than 2.75 pages.

• Please expand the figure legends to adequately describe the figures. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

Figure legends have been expanded to include more details and fulfil the requirements as per the editorial comments.

• If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Explicit permission has been obtained and is uploaded along with the revised document. In addition, figure legends include the appropriate citations as required by the editor.

• Please include a figure label (a) on figure 3.

The figure label (a) has been added to figure 3.

• Table 1 appears to be 2 tables, please split the tables and upload each as individual excel files. Please adjust the call-out to the tables in the text accordingly.

Table 1 has been split into 2 tables as suggested and the appropriate call-outs have been updated accordingly.

• Please expand your discussion to cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion has been significantly expanded to include the details requested by the editor.

• Please move the figure permission statement from under disclosures to the correct figure legends.

Figure permission statements are now included in the correct figure legend and removed from the disclosures.

Figure Reuse permission