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 cutter31. 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 molecules27. 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 themselves25. When the PDMS is then exposed to atmospheric pressure, the PDMS bulk material and microchannels retain a negative pressure for some time25,30. 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 cutter31. 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.”