**Editorial comments:**

*1. Please upload Tables 1, 2, and 3 as xls/xlsx files.*

Tables 1, 2, and 3 have been uploaded as separate xlsx files.

*2. Please revise the highlighting for the protocol as there is area over 2.75 pages selected. Please reduce this so the videography can occur in a single day.*

We are prepare for the videography using “cooking show” style, where we will prepare samples of wafers stalled after each time-consuming step so that the videography can take place in a single day (even though the actual protocol would require several days). In addition, we have reduced the highlighting in the protocol so that the selected area does not exceed 2.75 pages.

*3. Formatting: Short abstract exceeds 50 words and must be reduced.*

The short abstract has been reduced to 48 words.

*4. Formatting: References – Please include DOI where available.*

The DOI has been included for all possible references.

*5. Length exceeds 2.75 pg of highlighted material and must be reduced accordingly.*

We reduced the length of the highlighted material to 2.75 pages.

*6. Grammar: -1.4.2 – Please use imperative tense or convert to a note.*

This text has been removed.

*7. Grammar: -1.6 – “sequential additional”*

This was a typo and has been edited to read “sequential addition”.

*8. Additional detail is required: -3.7 – Is DI water used?*

This point was clarified to read “Remove the wafer and let rest overnight at RT in a petri dish for ambient rehydration”. No DI water is necessary.

*9. Additional detail is required: -3.9.2 – How is a profilometer used?*

To clarify, the following text was added: “Note: Operate the profilometer according to manufacturer instructions, carefully positioning the force stylus next to a feature channel on the desired layer before profiling. Settings used throughout this protocol were the following: stylus force = 10.5 mg, length = 1000 µm, speed = 200 µm/s, regime = down-up.

*10. Additional detail is required: -4.13, 4.15 – What photoresist is used?*

The photoresist used for these steps (SU-8 2025) is now specified in 4.14.

*11. Additional detail is required: 8.2 – How is pressurization done?*

To clarify, this section now includes the text: “Note: Follow manufacturer instructions for the flow control system of choice. In this work, a custom software-controlled pneumatic system applies pressure to each line using solenoid valves that toggle between 25 psi compressed air (pressurized) and atmospheric pressure (depressurized). Details on this system can be found in Discussion.”

*12. -Section 8 – How are the valves depressurized? Is this performed in the software or manually? Please describe how this is done in each case.*

See previous change, which also addresses this question.

*13. Mylar is trademarked. Please use a generic term.*

‘Mylar film’ has been changed to ‘transparency film’.

*14. Cryotube (Cryo tube) is trademarked. Please use a generic term.*

‘Cryo tube’ has been changed to ‘cryogenic tube’.

*15. -8.8 – Span -Line 556 – AutoCAD*

This has been changed to: “Download a computer-assisted design (CAD) drafting program (*e.g. AutoCAD Educational Version*).”

*16. Discussion: Please discuss the critical steps, significance with respect to alternative methods, and limitations of the protocol.*

To address this comment, we have added the following 2 paragraphs to the Discussion:

“If this protocol is adopted to construct a different microfluidic device than the bead synthesizer presented here, the fabrication of rounded features made from positive photoresists can require significant optimization. We and others have observed that optimizing soft bake temperature, duration, and ramp rate is critical to prevent retention of residual solvent in the resist film via crust formation (which can lead to bubbling of trapped nitrogen gas during exposure). In addition, an overnight rehydration step improves the reproducibility of exposure times required for thick AZ50 XT layers and reduces spatial variability in rates of development across the wafer. Finally, a long (14-15 hour) post exposure bake with a slow ramp rounds rectangular photoresist features to form valves without deforming valve geometries for a wide variety of tested photoresist thicknesses. The major limitation of this protocol is design-to-testing time, which takes ~ 3 days due to these long rehydration and hard bake steps.

Several of the procedures presented here for fabricating layers from negative photoresist include small differences from manufacturer instructions. We suggest a three-step soft bake process that moves wafers between hot plates set at 65C, 95C, and 65C. We have found that gradual warming of wafers reduces the appearance of defects during exposure resulting from rupture of gas bubbles trapped within photoresist via the formation of a “crust” during soft baking. Conversely, gradual cooling of wafers after soft baking can reduce photoresist cracking. Finally, we have found that increasing photoresist relaxation times to ~ 20 minutes reduces small variations in resist height across the wafer.”

**Reviewer #1:**

***Manuscript Summary:****This piece summarizes procedures for lithography to produce rounded and square profile microvalves in an example device for producing crosslinked micro-gel droplets. Some aspects of the methods (fluorinated release agent, multiple resist coating without development) diverge from methods originally established in the field and will be of significant interest to veteran as well as novice practitioners.*

***Major Concerns:****None*

***Minor Concerns:****None, this is a nicely done piece.*

***Additional Comments to Authors:****N/A*

We thank the Reviewer for their kind words and complimentary evaluation! We hope that this manuscript will serve as a useful resource for the community and appreciate the encouragement.

**Reviewer #2:**

***Manuscript Summary:****This manuscript describes a protocol for producing molds with multiple feature heights for multi-layer soft lithography which is used in the fabrication of microfluidic devices with integrated valves. In order to illustrate the fabrication of a wide variety of microfluidic elements, the authors present a device capable of producing hydrogel beads by encapsulating droplets of UV-curable PEG in an oil carrier fluid. This device thus implements microfluidic valves, a chaotic mixer, a droplet generator, and multiple-height flow molds. The protocol described in this manuscript includes a set of extremely useful fabrication techniques and will fill a gap in the literature, which often prevents labs from using these kinds of microfluidic devices.*

*I think this is valuable manuscript that would be very useful for the JOVE audience who might be interested in microfluidic device fabrication but I have a few comments that I would like the authors to address before publication. There are countless published protocols in the supporting information of microfluidic manuscripts that describe similar fabrication procedures. Typically there is agreement between most of the steps in these protocols but from time to time there are subtle differences. In some cases, these differences, including baking times and spin speeds etc, are inconsequential and other times these differences can seem contradictory. Additionally, soft lithography protocols often deviate from protocols described in the data sheets and manuals provided by the reagent manufacturers. In the submitted manuscript, the authors have the opportunity to dissect some of these discrepancies by providing a bit more detail to support the choice of fabrication parameters. Additionally I think some of the steps could be clarified and emphasis should be placed on the critical steps. Please see my comments below.*

We appreciate both the Reviewer’s kind words and their very careful, thorough reading of the manuscript! In response to these criticisms, we have made significant edits to the text and feel that the protocol will be more useful to the community as a result. The content of these changes is detailed below in line-by-line responses.

***Major Concerns:***

*Line 200- steps 1.6.1 through 1.6.3 are a little confusing. Why are we transferring these layers into another file? I think it would help to provide some motivation explaining the purpose of these steps.*

We agree that the specifics of the mask design could be potentially confusing to those unfamiliar with photolithography. To clarify this process, we have included the following text at the beginning of Section 1, Multi-Layer Device Design: “Note: Features of different heights/and or photoresists must be sequentially added to the wafer during different fabrication steps to create final composite features. Therefore, designs for each separate height and photoresist to be included on a wafer must be designed and printed on their own mask (**Figure 4**).” In addition, we have added a note for clarification within Step 1.6 that reads: “This Basic Mask File represents the final designs used for printing”. We hope that the added text and direct reference to a Figure showing example mask designs and how they can be added sequentially to produce composite height channels will reduce confusion.

*Line 215- I think it would be helpful to add a little information about the wafer specifications. For example, P-Type test grade wafers. Thickness 475-575 um. Etc. This might help a newcomer figure out what kind of wafers to buy.*

This has been edited to read “In cleanroom or designated clean area, clean and dehydrate a 4” test-grade silicon wafer, single-side polished.”

*Line 215- Wafer cleaning can be a very critical step depending on the original surface quality of the wafer. Some photo-resist protocols (i.e. SU-8) recommend piranha cleaning or reactive ion-etching. Other protocols published in the literature use acetone/IPA/DI water washes. Additionally, many resources suggest dehydrating wafers by baking at 120-140C after cleaning. In my experience, a high-temperature dehydrating step can improve resist adhesion. It might be worth commenting on the cleaning step a bit more to help the reader understand the importance of this step, or at least to help reconcile the existing literature.*

We agree with the Reviewer that cleaning and dehydration at the beginning of the fabrication process can be critical. However, we have found that these factors become less important when a single SU-8 2005 layer is spun across the entire device and exposed prior to deposition of subsequent layers, as the SU-8 2005 layer promotes strong adhesion. To clarify this point, we have added the following text within point 2.1.1: “Note: No further cleaning steps are needed if using the SU-8 adhesion layer described below. Other adhesion layers that deviate from this protocol (e.g. HMDS) often require more thorough cleaning such as Piranha etching.” In addition, we have edited point 2.1.3 to read “Bake on an aluminum hotplate at 95 oC for 10 minutes to **fully** evaporate solvent.”

*Line 235- step 2.2.3, and many of the subsequent baking steps, note three-step baking, at 65-95-65. However, the SU-8 2005 datasheet only mentions a single baking step at 95C and the SU-8 2050 datasheet refers to a two-step process: 65C-95C. If the protocol described here deviates from the manufacturer's manual the authors should explain why.*

We have added a paragraph to the Discussion providing a rationale for why we deviate slightly from the SU-8 manufacturer’s instructions here. The text now reads: “Several of the procedures presented here for fabricating layers from negative photoresist include small differences from manufacturer instructions. We suggest a three-step soft bake process that moves wafers between hot plates set at 65 oC , 95 oC , and 65 oC . We have found that gradual warming of wafers reduces the appearance of defects during exposure resulting from rupture of gas bubbles trapped within photoresist via the formation of a “crust” during soft baking. Conversely, gradual cooling of wafers after soft baking can reduce photoresist cracking. Finally, we have found that increasing photoresist relaxation times to ~ 20 minutes reduces small variations in resist height across the wafer.”

*Line 260- For step 3.5 the authors should explain the importance of the "relax" step and why it has to be 20 minutes. I say this not because I think the authors should justify every step but because 20 min is a long waiting step and I wonder if it is necessary.*

As detailed above, we have added the following sentence to the Discussion: “Finally, we have found that increasing photoresist relaxation times to ~ 20 minutes reduces small variations in resist height across the wafer.”

*Line 262- In step 3.6 why must we ramp for this soft bake? Is it absolutely necessary to use a ramping hot plate for this step? Also, it may be useful to clarify -the timing in the ramp step because a ramp at 450C/h starting at 65C would increase the temperature to 230C in 22 min, depending on how the ramping hot plate operates.*

In response to the Reviewer comment, we have included an additional paragraph within the Discussion with details about optimization specific for the AZ50 XT photoresist used to fabricate rounded valves. This paragraph reads: “If this protocol is adopted to construct a different microfluidic device than the bead synthesizer presented here, the fabrication of rounded features made from positive photoresists can require significant optimization. We and others have observed that optimizing soft bake temperature, duration, and ramp rate is critical to prevent retention of residual solvent in the resist film via crust formation (which can lead to bubbling of trapped nitrogen gas during exposure) (see Wanat, S., Plass, R., Sison, E., Zhuang, H., & Lu, P-H. Optimized Thick Film Processing for Bumping Layers. *Proc. SPIE.* 1281-1288 (2003). DOI: 10.1117/12.485181). In addition, an overnight rehydration step improves the reproducibility of exposure times required for thick AZ50 XT layers and reduces spatial variability in rates of development across the wafer. Finally, a long (14-15 hour) post exposure bake with a slow ramp rounds rectangular photoresist features to form valves without deforming valve geometries for a wide variety of tested photoresist thicknesses. The major limitation of this protocol is design-to-testing time, which takes ~ 3 days due to these long rehydration and hard bake steps.”

*Line 264- Step 3.7 An overnight rehydration step makes this protocol quite time consuming. I know that this step can be crucial, but it is heavily dependent on the AZ thickness. Additionally, the newer AZ40XT does not require this step. Since this manuscript might serve as the definitive multi-height mold protocol, I think the authors should provide a little more information about this step and this photoresist, and possibly explore using AZ40XT as an alternative.*

In response to this comment, we have added additional text within the Representative Results suggesting that shorter rehydration times may be possible when fabricating features < 50 µm in height, and have also passed on the suggestion that AZ40 XT may prove an appealing alternative that does not require rehydration. The text now reads: “Shorter rehydration times (~5-6 hours) can be used for AZ50 XT features under 50 µm with similar results, but taller features require overnight rehydration to reduce the chance of feature loss during exposure and development. Newer positive resist alternatives (*e.g.* AZ40XT) may eliminate the need for overnight rehydration; however, we have not tested these newer formulations.”

*Line 278- Step 3.10 is one of the most fundamental elements of valve-based microfluidics and is also possibly the most severe deviation from standard photoresist processing. The ramp rate here is indeed extremely important but I wonder if a 15 hour ramp is completely necessary. Especially since this step adds another day to the protocol time. How critical are the parameters here? The authors should explore the rigidity of this step.*

We have chosen to maintain this long ramp because it provides a robust way to round features with little dependence on feature geometries and additionally makes rounded valves very resistant to the harsh developers used in subsequent steps. We have clarified this in the manuscript by including the following text: “Finally, a long (14-15 hour) post exposure bake with a slow ramp rounds rectangular photoresist features to form valves without deforming valve geometries for a wide variety of tested photoresist thicknesses, and further ensures that valves are not damaged by subsequent development steps. The major limitation of this protocol is design-to-testing time, which takes ~ 3 days due to these long rehydration and hard bake steps.”

***Minor Concerns:***

*Line 319- Step 4.11, should this be "post exposure bake" instead of "hard bake"?*

We thank the Reviewer for finding this typo and have edited the text accordingly.

***Additional Comments to Authors:****I think this is a very important protocol and this manuscript/video will be extremely helpful for the field. For that reason I think the authors should take the opportunity to thoroughly explain some of the details in the protocol. In doing so, users will be able to troubleshoot more effectively and use this manuscript as a starting point to optimize their own customized mold protocols.*

Again, we appreciate the Reviewer’s helpful suggestions and have attempted to implement all proposed changes!