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Multi-Step Variable Height Photolithography for Valved Multilayer Microfluidic Devices

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Multi-Step Variable Height Photolithography for Valved Multilayer Microfluidic Devices

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SHORT ABSTRACT:
Multilayer microfluidic devices often involve the fabrication of master molds with complex geometries for functionality. This article presents a complete protocol for multi-step photolithography with valves and variable height features tunable to any application. As a demonstration, we fabricate a microfluidic droplet generator capable of producing hydrogel beads.

LONG ABSTRACT:
Microfluidic systems have enabled powerful new approaches to high-throughput biochemical and biological analysis. However, there remains a barrier to entry for non-specialists who would benefit greatly from the ability to develop their own microfluidic devices to address research questions. Particularly lacking has been the open dissemination of protocols related to photolithography, a key step in the development of a replica mold for the manufacture of polydimethylsiloxane (PDMS) devices. While the fabrication of single height silicon masters has been explored extensively in literature, fabrication steps for more complicated photolithography features necessary for many interesting device functionalities (such as feature rounding to make valve structures, multi-height single-mold patterning, or high aspect ratio definition) are often not explicitly outlined.

Here, we provide a complete protocol for making multilayer microfluidic devices with valves and complex multi-height geometries, tunable for any application. These fabrication procedures are presented in the context of a microfluidic hydrogel bead synthesizer and demonstrate the production of droplets containing polyethylene glycol (PEG diacrylate) and a photoinitiator that can be polymerized into solid beads. This protocol and accompanying discussion provide a foundation of design principles and fabrication methods that enables development of a wide variety of microfluidic devices. The details included here should allow non-specialists to design and fabricate novel devices, thereby bringing a host of recently developed technologies to their most exciting applications in biological laboratories.

INTRODUCTION:

For the past 15 years, microfluidics as a field has undergone rapid growth, with an explosion of new technologies enabling the manipulation of fluids at the micrometer scale[1]. Microfluidic systems are attractive platforms for wet laboratory functionality because the small volumes have the potential to realize increased speed and sensitivity while at the same time dramatically increasing throughput and reducing cost by leveraging economies of scale[2,3]. Multilayer microfluidic systems have made particularly significant impacts in high-throughput biochemical analysis applications such as single cell analysis[4-6], single molecule analysis (*e.g.* digital PCR[7]), protein crystallography[8], transcription factor binding assays[9,10], and cellular screening[11].

A central goal of microfluidics has been the development of "lab on a chip" devices capable of performing complex fluidic manipulations within a single device for total biochemical analysis[12]. The development of multi-layer soft lithography techniques has helped realize this goal by enabling creation of on-chip valves, mixers, and pumps for actively controlling fluids within small volumes[13-15]. Despite their advantages and demonstrated applications, many of these microfluidic technologies remain largely unharnessed by non-specialist users. Widespread adoption has been challenging in part due to limited access to microfabrication facilities, but also due to inadequate communication of fabrication techniques. This is especially true for multilayer microfluidic devices featuring structures for valves or complex geometries: the paucity of detailed, practical information about important design parameters and fabrication techniques often deters new researchers from embarking on projects involving the design and creation of these devices.

This article aims to address this knowledge gap by presenting a complete protocol for making multilayer microfluidic devices with valves and variable height features, starting from design parameters and moving through all fabrication steps. By focusing on the initial photolithography steps of fabrication, this protocol complements other microfluidics protocols[16] that describe downstream steps of casting devices from molds and running specific experiments.

Microfluidic devices with monolithic on-chip valves are composed of two layers: a "flow" layer, where the fluid of interest is manipulated in microchannels, and a "control" layer, where microchannels containing air or water can selectively modulate fluid flow in the flow layer[14].

These two layers are each fabricated on a separate silicon molding master, which is subsequently used for polydimethylsiloxane (PDMS) replica molding in a process called "soft lithography[17]." To form a multilayer device, each of the PDMS layers are cast on their respective molding masters and then aligned to one another, thereby forming a composite PDMS device with channels in each layer. Valves are formed at locations where flow and control channels cross one another and are separated by only a thin membrane; pressurization of the control channel deflects this membrane to occlude the flow channel and locally displace the fluid (**Figure 1**).

Active on-chip valves can be fabricated in multiple ways, depending on the desired final application. Valves can be configured in either a "push down" or "push up" geometry, depending on whether the control layer is above or below the flow layer (**Figure 1**)[15]. "Push up" geometries allow for lower closing pressures and higher device stability against delamination, while "push down" geometries allow for the flow channels to be in direct contact with the bonded substrate, conferring the advantage of selective functionalization or patterning of the substrate surface for later functionality[18,19].

Valves can also be either intentionally leaky "sieve" valves or fully sealable, depending on the cross-sectional profile of the flow channel. Sieve valves are useful for trapping beads, cells or other macroanalytes[1], and are fabricated via the use of typical negative photoresists (*i.e.* SU-8 series), which have rectangular profiles. When a control channel is pressurized over these valve regions, the PDMS membrane between the control and flow layer deflects isotropically into the rectangular profile of the valve without sealing the corners, permitting fluid flow but trapping macro scale particles (**Figure 1**). Conversely, fully-sealable microfluidic valves are fabricated by including a small patch of rounded photoresist at valve locations. With this geometry, pressurization of the control channel deflects the membrane against the rounded flow layer to completely seal the channel, halting fluid flow. Rounded profiles in the flow layer are generated via the melting and reflow of positive photoresist (*e.g.* AZ50 XT or SPR 220) after typical photolithography steps. We have previously demonstrated that post-reflow heights of valve regions depend on chosen feature dimensions[21]. This protocol demonstrates the fabrication of both valve geometries within a bead synthesis device.

[Insert Figure 1 here]

Devices can also include complex passive features such as chaotic mixers[13] and on-chip resistors[20] that require features of multiple different heights within a single flow layer. To achieve a variable height flow layer, different groups have employed many methods including printed circuit board etching[22], multilayer PDMS relief alignment[23], or multi-step photolithography[24]. Our group has found multi-step photolithography on a single molding master to be an effective and reproducible method. To do this, a simple photolithography technique of building thick channels of negative photoresist (*e.g.* SU-8 series photoresists) in layers without development in between application of each layer is employed. Each layer is spun in negative photoresist according to its thickness using manufacturer instructions[25] on the silicon master. Features of this height are then patterned onto the layer using a specific

transparency mask (**Figure 2**) affixed to a glass mask plate and aligned to the previously spun layer before exposure. In multi-step photolithography, precise alignment between layers is critical in forming a complete variable height flow channel. After alignment, each layer is subjected to a thickness-dependent post-exposure bake. Without development, the next layer is similarly patterned. In this way, tall features can be built up on a single flow wafer layer-by-layer via the use of multiple masks. By skipping development between each step, previous photoresist layers can be used to generate composite height features (*i.e.* two 25 µm layers can make a 50 µm feature)[24]. Additionally, channel floor features such as chaotic mixer herringbone grooves[13] can be made using layers with previously exposed features. A final development step completes the process, creating a single flow wafer with features of variable height (**Figure 3**).

Here, a complete protocol for multi-step photolithography that includes examples of all procedures necessary to fabricate on-chip valves and flow channels with multiple heights is provided. This fabrication protocol is presented in the context of a multi-layer microfluidic bead synthesizer that requires valves and variable-height features for its functionality. This device includes T-junctions for generating water droplets in an oil sheath, on-chip resistors to modulate flow rates through controlling Poiseuille resistance, a chaotic mixer for homogenizing droplet components, and both fully sealing and sieve valves to enable automated workflows involving multiple reagent inputs. Using multi-step photolithography, these features are each fabricated on a different layer according to height or photoresist; the following layers are constructed in this protocol: (1) Flow Round valve layer (55 µm, AZ50 XT) (2) Flow Low layer (55 µm, SU-8 2050) (3) Flow High layer (85 µm, SU-8 2025, 30 µm additive height), and (4) Herringbone Grooves (125 µm, SU-8 2025, 40 µm additive height) (**Figure 3**).

Hydrogel beads can be used for a variety of applications including selective surface functionalization for downstream assays, drug encapsulation, radiotracing and imaging assays, and cell incorporation; we previously used a more complex version of these devices to produce spectrally encoded PEG hydrogel beads containing lanthanide nanophosphors[20]. The designs discussed here are included in **Additional Resources** for any lab to use in their research efforts if desired. We anticipate that this protocol will provide an open resource for specialists and non-specialists alike interested in making multi-layer microfluidic devices with valves or complex geometries to lower the barrier to entry in microfluidics and increase the chances of fabrication success.

PROTOCOL:

1. Multi-Layer Device Design

Note: Features of different heights and/or photoresists must be added sequentially 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 printed on their own mask (**Figure 4**).

1.1. Download a computer-assisted design (CAD) drafting program (*e.g. AutoCAD Educational Version*).

1.2. Define the 4” wafer area by drawing a 4” circle. Wafer designs (**Figure 4**, **Additional Resources**) are provided as an example.

1.3. Inside the 4” wafer outline, place device borders using 300 µm polyline rectangles. Use these device borders for alignment during photolithography.

1.4. Create different layers for each different height or photoresist needed for the final design (*i.e.* flow round, flow low, flow high, and control in the design) using the Layers panel.

1.4.1. Design features of a particular desired height on the corresponding layer. The example design shows 4 different active layers, each with its own color (**Figure 4**).

Note: Device borders, global text, and the wafer outline should be made on their own layer (*i.e.* 1-Negative in the designs), which, later, will appear on all layers for global alignment. Features of different photoresist (such as fully sealable valves that must be fabricated with positive resist) must appear on different layers, regardless of height.

1.5. Using closed zero-width polylines, design device features within device borders.

1.5.1. Consider design parameters in **Table 1** to increase chances of successful fabrication.

1.5.2. For each height, select that layer in the Layers panel and add all features of that height.

1.6. Ready designs for transparency film printing using the Basic Mask File (**Additional Resources**) where each 4” wafer circle is inserted within a 5” rectangular border. Each layer will be printed on a separate transparency film for sequential addition of each photoresist layer.

Note: This Basic Mask File represents the final designs used for printing.

1.6.1. To complete design, turn all Layers off **except** 1-Negative and the AZ50 XT valve layer. Copy the entire wafer with the active layer (*i.e.* valves) and global features (*i.e*. device borders).

1.6.2. Open the Basic Mask File and paste this design into the rectangle entitled AZ50 XT valves. Use the outer wafer border for alignment and subsequently delete it after pasting.

1.6.3. Repeat for the rest of the layers (*e.g.* in the example design: flow square low, flow square high, and control). Example transparency files are provided (**Additional Resources**).

1.6.4. Send files to a commercial printing company (*e.g.* FineLine Imaging) for printing on transparency film. Use 32,000 DPI for printing >10 µm features and up to 50,000 DPI for smaller features. If features less than 7 µm are needed, order a Chrome Mask instead of a transparency film.

[Place Table 1 here]

2. Preparing a Wafer for Photolithography

Note: These steps additionally appear in table-format in **Table 2**.

2.1. In cleanroom or designated clean area, clean and dehydrate a 4" test-grade silicon wafer (single-side polished).

2.1.1. Rinse the wafer well with methanol.

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.

2.1.2. Blow dry with N_2 or compressed air.

2.1.3. Bake on an aluminum hotplate at 95 ^oC for 10 minutes to fully evaporate solvent.

2.2. Fabricate a uniform 5 µm thick layer of SU-8 2005 to improve adhesion for subsequent photoresist layers.

2.2.1. Place the cleaned wafer on a spin coater, turn on the vacuum to affix it to the spin chuck, and blow away dust with N_2 or compressed air.

2.2.2. Apply 1-2 mL of SU-8 2005 negative photoresist in center of wafer and spin as follows: spread: 500 rpm, 10 s, 133 rpm/s acceleration; cast: 3000 rpm, 40 s, 266 rpm/s acceleration.

2.2.3. Remove wafer and soft bake by switching wafer between two hotplates set at 65 ^oC and 95 ^oC according to the following program: 65 ^oC: 2 min, 95 ^oC: 3 min, 65 ^oC: 2 min.

2.2.4. Allow wafer to cool to RT.

2.2.5. Place wafer in the chuck of a UV mask aligner and expose without a mask ('flood exposure') for a total energy deposition of 124 mJ (here, 20 s at ~6.2 mW/cm^2 lamp intensity). If available, select hard contact mode to achieve a 300 µm wafer: mask separation.

2.2.6. Remove wafer and post-exposure bake by switching the wafer between two hotplates set at 65^oC and 95^oC as follows: 65 ^oC: 2 min, 95 ^oC: 4 min, 65 ^oC: 2 min.

Fabricating Rounded Valves

2.3. Use online AZ50 XT valve predictor resource[26] to plan spin speeds for desired valve dimensions and heights.

Note: The following steps will deposit a 55 µm layer of positive photoresist for valve definition

and reflow rounding.

2.4. Place the wafer on a spin coater, turn on vacuum to affix it to the spin chuck, and blow away dust with N_2 or compressed air.

2.5. Apply 2-3 mL of AZ50 XT positive photoresist to center of the wafer. Spin as follows: spread: 200 rpm, 10s, 133 rpm/s acceleration; cast: 12000 rpm, 40 s, 266 rpm/s acceleration; Snap spin to remove edge bead: 3400 rpm, 1 s, 3400 rpm/s acceleration.

2.6. In a 5" petri dish, lay down the wafer carefully and let relax for 20 minutes.

2.7. Soft bake the wafer on a hotplate: 65 °C – 112 °C, 22 min, 450 °C/h ramping speed.

2.8. Remove the wafer and let rest overnight at RT in a petri dish for ambient rehydration.

2.9. Tape Flow Round transparency mask to 5" glass plate print-side down (closest to wafer) and load into mask positioner of the UV mask aligner. Expose the wafer to 930 mJ of UV in 6 cycles (*e.g.,* 6 cycles of 25 s at ~6.2 mW/cm^2 lamp intensity, 30 s wait time between exposures).

2.10. Develop wafer immediately by immersing in a stirred bath of 25 mL of AZ500k 1:3 Developer in 6" glass dish for 3-5 minutes or until bath turns purple and features emerge.

2.10.1. Remove the wafer and rinse well with DI water.

2.10.2. Assess pre-reflow height using a profilometer (stylus force of 10.5 mg).

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.

2.11. Reflow hard bake the wafer to melt and round valve features as follows: 65 °C - 190 °C, 15 hours, 10 °C/h ramping speed.

2.12. Let the wafer cool to RT. Assess post-reflow height using a profilometer (stylus force of 10.5 mg). Heights of 55 µm ± 2 µm should be expected for this device geometry.

3. Fabricating Variable Height Features in Tandem

3.1. Proceed to variable height fabrication with the developed wafer with Flow Low, Flow High and Herringbone Mixer transparencies of the Bead Synthesizer design.

3.2. To adjust protocol for the designs, use manufacture data sheets[25] to determine exposure energy, spin speeds and bake time parameters, allowing for ± 5% tolerance.

Note: This protocol fabricates a 55 µm tall Flow Low layer using SU-8 2050 negative photoresist spun over the valve features.

3.3. Place the cleaned wafer on spin coater, turn on the vacuum to affix it to the spin chuck, and blow away dust with N_2 or compressed air.

3.3.1. Apply 1-2 mL of SU-8 2050 negative photoresist to center of the wafer and spin as follows: spread: 500 rpm, 10s, 133 rpm/s acceleration; cast: 3000 rpm, 40 s, 266 rpm/s acceleration. Spin photoresist over developed valve features.

3.4. Carefully place the spun wafer in 5" petri dish and let relax for 20 minutes on flat surface or until any streaking patterns fade.

3.5. Remove the wafer and soft bake by placing on two hotplates set at 65 ^oC and 95 ^oC as follows: 65 ^oC: 2 min, 95 ^oC: 8 min, 65 ^oC: 2 min.

3.6. Allow the wafer to cool to RT.

3.7. Tape the Flow Low transparency mask to a quartz 5" glass plate print-side down (closest to the wafer) and load into the mask positioner of the UV mask aligner.

3.8. Place the wafer in UV mask aligner chuck and, using microscope eyepiece or camera, carefully align new Flow Low layer features to Flow Round valve layer features. Begin by aligning horizontal, vertical and tilt axes of device borders to the device border features on mask. Next, align cross-hair features between layers. Finally, confirm that valve features intersect Flow Low features where applicable.

3.9. Expose to 170 mJ UV deposition (28 s at ~6.2 mW/cm^2).

3.10. Remove the wafer and post-exposure bake by switching between two hotplates set at 65 ^oC and 95 ^oC as follows: 65 ^oC: 2 min, 95 ^oC: 9 min, 65 ^oC: 2 min.

3.11. Without developing, allow the wafer to cool to RT and proceed to fabrication of Flow High layer. This Flow High layer will add 30 µm of photoresist to the undeveloped 55 µm photoresist layer to yield 85 µm features in previously unexposed locations.

3.12. Repeat steps 3.3 to 3.10 using SU-8 2025 and the Flow High layer mask with these modifications for the spin coat settings: spread: 500 rpm, 10s, 133 rpm/s^2 acceleration; cast: 3500 rpm, 40 s, 266 rpm/s^2 acceleration

3.12.1. Expose to 198 mJ UV deposition (32 s at ~6.2 mW/cm^2).

3.13. Without developing, allow the wafer to cool to RT and proceed to fabrication of Chaotic Mixer Herringbone layer. Final features in this layer will have a total height of 125 µm: 55 µm

from the Flow Low layer, 30 µm from the Flow Square layer, and 40 µm from this Chaotic Mixer Herringbone layer (see **Figure 3**) and include 35 µm herringbone grooves.

3.14. Repeat steps 3.3 to 3.10 using SU-8 2025 and the Herringbone layer mask with the following modifications, ensuring that herringbone grooves are completely within Flow High channel outlines.

3.14.1. Use the following soft bake program: 65 ^{o}C: 2 min, 95 ^{o}C: 7 min, 65 ^{o}C: 2 min.

3.14.2. Expose to 148 mJ UV deposition (24 s at ~6.2 mW/cm^2).

3.15. After all layers have been completed, develop by immersing the wafer in a stirred bath of 25 mL of SU-8 developer in a 6" glass dish for 3.5 min or until features clearly emerge. Check that features have clear, defined feature boundaries using a stereoscope.

3.16. Hard bake the wafer to stabilize all photoresist features on a hot plate as follows: 65 ^{o}C – 165 ^{o}C, 2 hours 30 min, 120 ^{o}C/h ramping speed.

3.17. Assess feature height in all layers using a profilometer (stylus force of 10.5 mg).

4. Control Wafer Fabrication

4.1. Clean, dehydrate, and fabricate a 5 µm adhesion layer on a new 4" silicon wafer as in Section 4.

4.2. Fabricate a 25 µm Control Layer using SU-8 2025 negative photoresist.

4.3. Place the wafer on a spin coater, turn on vacuum to affix it to the spin chuck, and blow away dust with N_2 or compressed air.

4.4. Apply 1-2 mL of SU-8 2025 negative photoresist in the center of the wafer and spin as follows: spread: 500 rpm, 10s, 133 rpm/s acceleration; cast: 3500 rpm, 40 s, 266 rpm/s acceleration.

4.5. Remove the wafer and soft bake by switching between two hotplates set at 65 ^{o}C and 95 ^{o}C as follows: 65 ^{o}C: 2 min, 95 ^{o}C: 5 min, 65 ^{o}C: 2 min.

4.6. Allow the wafer to cool to RT.

4.7. Align the control transparency mask to a 5" glass plate and load into UV mask aligner.

4.8. Place the wafer in chuck of UV mask aligner and expose to 155 mJ UV deposition (25 s at ~6.2 mW/cm^2 lamp intensity).

4.9. Remove the wafer and post-exposure bake by switching between two hotplates set at

65 °C and 95 °C as follows: 65 °C: 2 min, 95 °C: 6 min, 65 °C: 2 min.

4.10. Develop by immersing the wafer in a stirred bath of 25 mL of SU-8 Developer in 6" glass dish for 1 min or until features emerge. Check features using a stereoscope.

4.11. Hard bake the wafer to stabilize photoresist features as follows: 65 °C - 165 °C, 2 hours 30 min, 120 °C/h ramping speed.

5. Silane Wafer Treatment for Easy PDMS Lift Off

5.1. Place the completed wafers in wafer rack within a bell-jar vacuum desiccator inside a fume hood free of water or water-soluble reagents.

5.2. Under the hood, use a dropper to apply 1 drop of trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl) silane (PFOTS) to a glass slide and place inside the desiccator.

5.3. Close the desiccator lid and apply vacuum for 1 minute.

5.4. After 1 minute, turn off vacuum without re-pressurizing or evacuating bell jar.

5.5. Let the mixture sit for 10 minutes while aerosolized PFOTS coats wafer surface.

5.6. Open the bell jar lid and remove wafer using tweezers. Place into a petri dish for PDMS replica molding. Dispose of silane-coated slides in proper hazardous waste.

Note: Wafers coated with fluorinated silanes can be used hundreds to thousands of times without re-treatment. A sacrificial layer of 1:10 PDMS can be cast on wafers, cured, and discarded after the first silane treatment to remove excess silane groups from wafer surface.

6. PDMS Replica Molding

6.1. Fabricate multilayer microfluidic devices in a "push up" geometry on glass according to existing open-access protocols[16].

Note: A detailed protocol can additionally be found on the website[27].

6.2. By visual inspection, ensure all valves are aligned properly to control lines and all inlets (on both the flow and control layers) are punched fully before proceeding.

7. Production of Hydrogel Beads from Droplets

7.1. Connect tubing (*e.g.,* Tygon) loaded with water to a flow control system (*e.g.* syringe pumps, fluidic controllers, or an open-source solenoid valve array with reservoirs[28]).

7.2. Connect metal pins to tubing and connect to device ports at control line inlets. Pressurize device control lines by setting the flow control system of choice to 25 psi for each line. Ensure that valves close and re-open by inspection under the microscope.

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.

7.3. Prepare custom microfluidic pressure vessels for reagent and oil loading.

7.3.1. Using a push-pin, punch two holes in the top of a cryogenic vial tube, insert capillary PEEK tubing into one hole, and insert a metal pin connected to the tubing into the second hole.

7.3.2. Seal tubing in place with epoxy. Let dry for 1 hour.

7.4. While waiting, in a microcentrifuge tube, suspend 3.9 mg of LAP photoinitiator into 100 µL of DI water ([LAP] = 39 mg/mL) to prepare photoinitiator solution used for polymerizing droplets to hydrogel beads. Protect from light.

7.5. In a second microcentrifuge tube, add 132 µL DI water, 172 µL PEG diacrylate, 12 µL LAP solution, and 85 µL HEPES buffer to make hydrogel droplet solution.

7.6. Transfer the hydrogel droplet solution to the completed cryogenic tube vessel.

Note: Additives for other applications such as nanocrystals, magnetic particles or biological molecules can be included within the HEPES component.

7.7. Connect the tubing of the cryogenic tube vessel to a controllable pressure source and connect the PEEK tubing to the device reagent inlet.

7.8. Prepare 10 mL of light mineral oil with 2% v/v nonionic surfactant (*e.g.* Span 80) and 0.05% EM90 for oil droplet emulsion. Filter using a 0.22 µm syringe filter and load 1 mL into a second cryogenic tube vessel.

7.9. Insert PEEK tubing at the device outlet for collection of droplets.

7.10. Remove air bubbles from the device by pressurizing oil, water, or PEG mixture inlets (4 psi operational pressure). Turn on all valves. Sequentially turn off each valve in a fluid pathway after 1 minute or until air bubbles have permeated through the PDMS device. For instance, to de-bubble herringbone mixers, turn on valves Inlet 1, Mix 1 out, and Mix Waste. Then depressurize Inlet 1, Mix 1 out, and Mix Waste until all bubbles are gone.

7.11. When the device is repressurized after debubbling, depressurize Ro1 oil valve and set oil pressure to 10 psi.

7.12. Set PEG mixture pressure to 9 psi, depressure upstream valves (Inlet 1, Drops 1) and

adjust as necessary to produce droplets of the desired size. Droplet size can be determined via microscopy using a camera with 50 fps or higher.

7.13. When the droplets have stabilized, position a 5 mm spot from a UV light source (*e.g. a UV* spot curing system with liquid light guide (LLG) or a focused UV LED) over the polymerization region of the device and apply 100 mW/cm^2 UV (365 nm) from the UV source.

7.14. Pressurize bead sieve valve to watch polymerized beads collect and ensure that droplets have hardened into beads. Adjust LLG as necessary to achieve full polymerization.

7.15. Depressurize bead sieve valve and collect beads into tube through outlet PEEK tubing.

REPRESENTATIVE RESULTS:

Here, we demonstrate the fabrication of valved, variable height multilayer microfluidic molds by making devices capable of generating poly ethylene glycol (PEG) hydrogel beads from droplets (**Figure 2**). An overview of the complete fabrication process is included in **Figure 3.** Using design elements from previous work, the bead synthesizer employs 4 heights in its flow layer including (1) rounded AZ50 XT valves for laminar flow modulation (55 µm) (2) flow low channels for introducing reagents at higher resistance (55 µm) (3) flow high channels for directing flow at lower resistance to the outlets and mixers (85 µm), and (4) a herringbone advection mixer (125 µm) for mixing the PEG and crosslinker into a homogenous solution (**Figures 4A and 4B**). A table of design parameters and suggested design constraints used in constructing this design is included in **Table 1.** Design files and mask transfer files are included in **Additional Materials**.

This protocol demonstrates rounding flow valves and constructing multiple heights on the same flow wafer via tandem photolithographic steps without development between each step (**Table 2**). A typical post-reflow valve rounding profile from a profilometer and stereoscope images of our valves post-reflow are shown in **Figures 5A and 5B**. Resultant measured fabrication heights from our multi-height photolithography are listed in **Table 3.** Heights were measured at 10 locations per each layer across all devices in a single flow wafer, assessing both device-to-device and across wafer variation. All features observed <2% CV across the wafer. Manufacturer photoresist data sheets suggest typical height variations of ± 5% when constructing multi-layer features, so this tolerance should be taken into account if this protocol is adjusted for a different design.

As with any fabrication process, errors can result at each step if fabrication parameters such as spin speed or exposure are not optimized for the desired pattern height and geometry. Many resources are available for troubleshooting proper exposure and development times, including a website our facility maintains[27]. Deviations from suggested soft and hard bake times, temperatures, and ramp rates may generate cracks, bubbles or incomplete features. Additionally, rehydration of the AZ50 XT positive photoresist prior to exposure is critical. Wafers without proper rehydration may appear cracked or contain bubbles within the valve areas. If this occurs, using a ‘wet box’ (a closed box with a dish containing deionized water) to house

wafers for the overnight rehydration may help. 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 formulations.

As a proof-of-concept, PEG-diacrylate hydrogel droplets were produced from the bead synthesizer device (**Figure 6A**). **Figures 5C and 5D** show representative images of valve operation and closure on the device when pressurizing and depressurizing valves. Common error modes for valve pressurization include: the use of insufficient pressure (valves will not fully close, which can be checked by flowing food dye), inlet tubing insertion (flow will be inhibited if tubing or metal pins are pushed down too far and device delamination may result), and incorporation of dust or fibers during the fabrication process (channels may be occluded or the device may delaminate). To troubleshoot these error modes, users should maintain clean room conditions during fabrication and systematically test each valve before proceeding to a device run. If device delamination presents as a common problem in experiments, this can be remedied by either running devices at lower pressures (<15 psi) or reducing silanization times.

Figure 6A shows the bead synthesizer device in operation producing hydrogel droplets in an oil emulsion at the T junction droplet generator and **Figure 6B** shows beads trapped by the on-chip sieve valve. If polymerization is not successful, beads will pass through a sieve valve. If this occurs, UV source intensity and height from the device can be modified to improve polymerization. Hydrogel beads were produced at flow rates of 10 psi (oil) and 9 psi (reagent mixture) using ~100 mW/cm^2 power over a polymerization area 5 mm in diameter (**Figures 6C and 6D**). Resultant beads measured 52.6 ± 1.6 µm (mean ± std). Sizes were analyzed for 2992 beads using a Hough Transform (MATLAB) in bright field images (**Figures 6C and 6D**) with an imposed size filter of ± 3 std (28 outliers, 0.94% of beads). Our entire hardware setup for droplet production is shown in **Figure 7**.

Table 1: Design Parameters and Suggestions.
Design considerations to avoid common pitfalls during the CAD design process of microfluidic devices.

Table 2: Multi-Step Photolithography Parameters.
A table format of all of the photolithography steps with applicable parameters including spin speed, soft bake times, exposure energies and hard bake times.

Table 3: Profilometer Heights Post-Fabrication.
Total feature height post-fabrication for each of the layers fabricated via multi-step photolithography.

Figure 1: Multilayer Microfluidic Valve Geometries.
Typical “push up” device architectures for sieve and fully sealable valves before (top) and after (bottom) pressurization.

Figure 2: Overview of fabrication process.
A schematic indicating steps involved in multilayer device fabrication from design to device testing.

Figure 3: Schematic of multi-step photolithography.
Overview of valve rounding and variable height feature fabrication in photolithography for the creation of a multilayer microfluidic device. Included here are steps for the fabrication of the bead synthesizer device.

Figure 4: Bead Synthesizer Device Design and Images.
(A) CAD Design of the Bead Synthesizer device with layers indicated by different colors (B) Image of the PDMS multilayer Bead Synthesizer device. Control lines appear in orange, flow channels appear in blue and green.

Figure 5: Valve profiles and images.
(A) Representative valve height profile as assessed by a profilometer (B) Representative images of valves and surrounding channels in the silicon molding master flow wafer (C, D) Images of final valve operation on the multilayer PDMS device.

Figure 6: Production of PEG-diacrylate hydrogel droplets.
(A) Image of hydrogel droplet production at the T-junction droplet generator (B) Image of beads post-polymerization trapped at the sieve valve when the valve is pressurized (C) Brightfield image of hydrogel beads produced in the bead synthesizer (4X) (D) Size distribution of hydrogel beads.

Figure 7: Device operation setup.
Image with annotation of all hardware required for device operation.

DISCUSSION:
This work demonstrates a complete multi-step photolithography protocol for a multilayer microfluidic device with valves and variable height geometry that can be tuned for any application with simple modifications to fabrication parameters based on our online tool[26] and manufacturer instructions[25]. This protocol is intended to demystify multilayer photolithography for researchers wishing to construct microfluidic devices beyond simple, passive one-layer molds.

Multilayer microfluidic devices enable the pursuit of advanced functionality within a single soft polymer device for 'lab on chip' applications ranging from single cell analysis to high-throughput biochemical assays[1]. The bead synthesizer device constructed here demonstrates multiple heights with a single flow layer and both fully-sealable and sieve valve geometries to produce hydrogel droplets that can be polymerized to polymer beads. The presence of valves allows for automated, programmable control over chip function that can be easily implemented with any scripting language (*i.e.* MATLAB, LABVIEW, or Python) that interfaces with a lab's flow

control modules (*i.e.* syringe pumps and/or pressure regulators). In this demonstration, custom MATLAB software (code provided here[28]) communicates with Modbus-controlled solenoid valve arrays in a custom pneumatic setup[28] and a MFCS flow control system to pressurize fluids on the device, but many other options for flow control are commercially available. Designs and additional suggestions are included to help maximize success for first-time microfluidic engineers.

The fabrication steps shown here should prove generally adaptable to a wide array of photolithographic patterning for microfluidic devices. In one of the steps (Flow High), 30 µm features have been added to the lower photoresist layer (Flow Low, 55 µm) to create a composite height of 85 µm. This step can be used to make thick features without the need for more viscous photoresists (which are more difficult to work with). In another step (Herringbone), features have been fabricated on top of a previously crosslinked channel, creating patterned grooves on the bottom of a microchannel when replica molded into PDMS. This step can be adapted by users to create complex geometries including well-within-well architectures, if desired.

The success of this fabrication process is demonstrated by using the bead synthesizer device to produce polymer beads from PEG-diacrylate droplets. Exploring different flow pressures can modulate the droplet regime to produce beads of different sizes. Furthermore, additives can be included easily within the polymer mixture. These hydrogel beads can be used for a variety of purposes, including spectral encoding through the incorporation of fluorescent or luminescent phosphors, drug delivery, or cellular assays via surface functionalization.

If this protocol is adopted to construct a different microfluidic device than the bead synthesizer presented here, it should be noted certain steps are critical to achieve a high chance of success on the first fabrication attempt. 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)[29]. 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.

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 ^{o}C, 95 ^{o}C, and 65 ^{o}C. 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.

Due to the flexibility of this fabrication protocol, we expect it will have broad utility for different devices across disciplines. While alternatives such as 3D printing, glass etching and embossing can also achieve microfluidic fabrication, lithographic patterning can achieve more complex functionality, such as valving, that other methods have not yet achieved at scale. The major limitation of this protocol is design-to-testing time, which takes ~3 days (mainly due to steps required to fabricate rounded valves).

We hope that open dissemination of protocols in microfluidics, especially relating to complicated photolithography steps, will encourage continued innovation in the field and that this proof-of-concept demonstration will aid in helping users troubleshoot fabrication issues.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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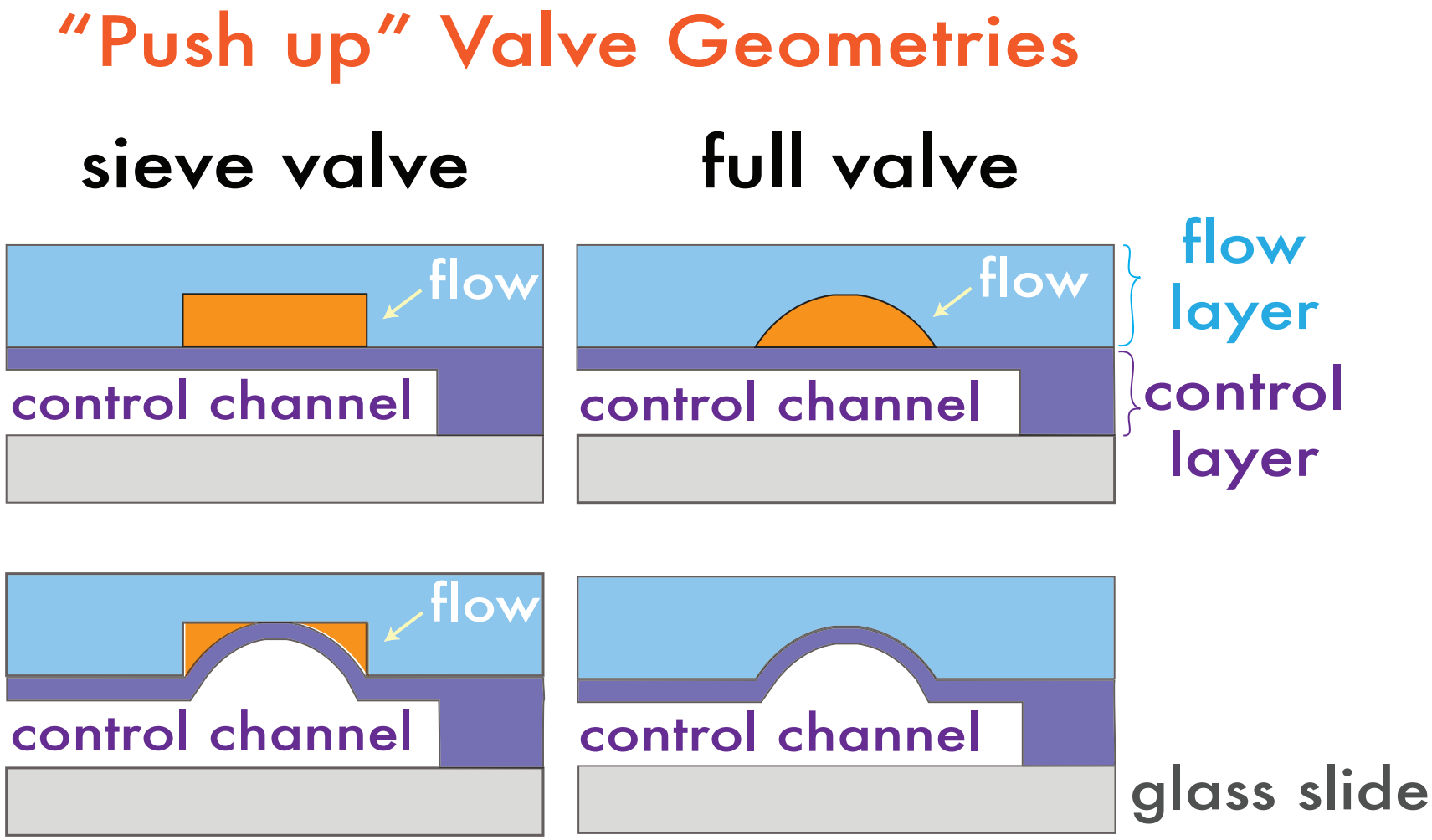

Figure

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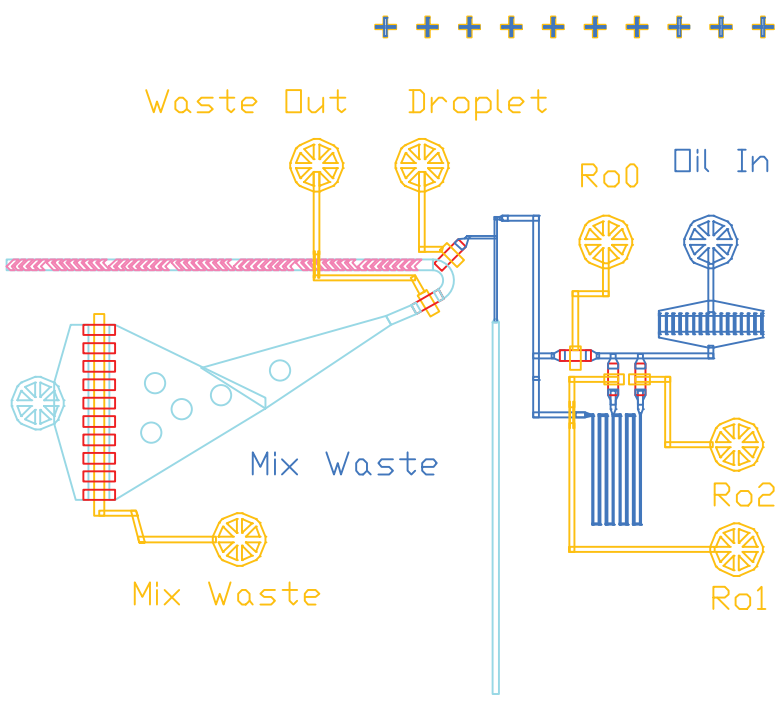
1. Device Design



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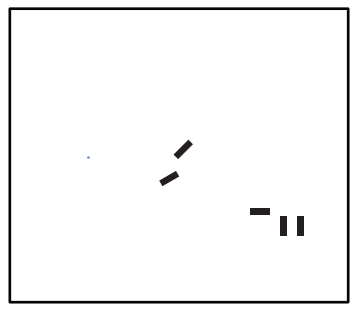
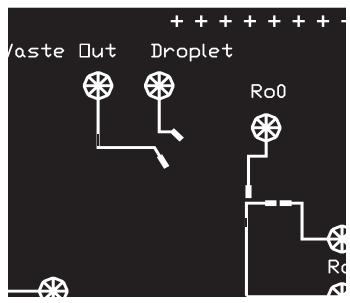


Flow Round

Flow Low

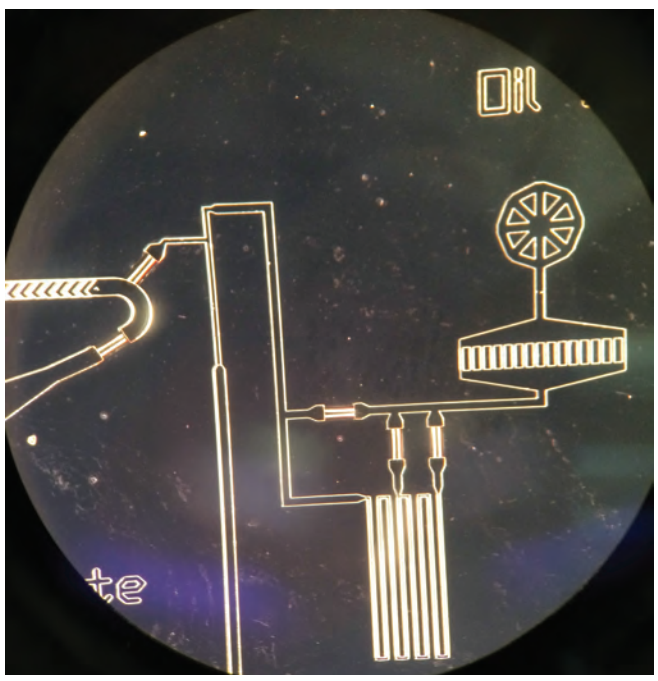
Flow High

Herringbone

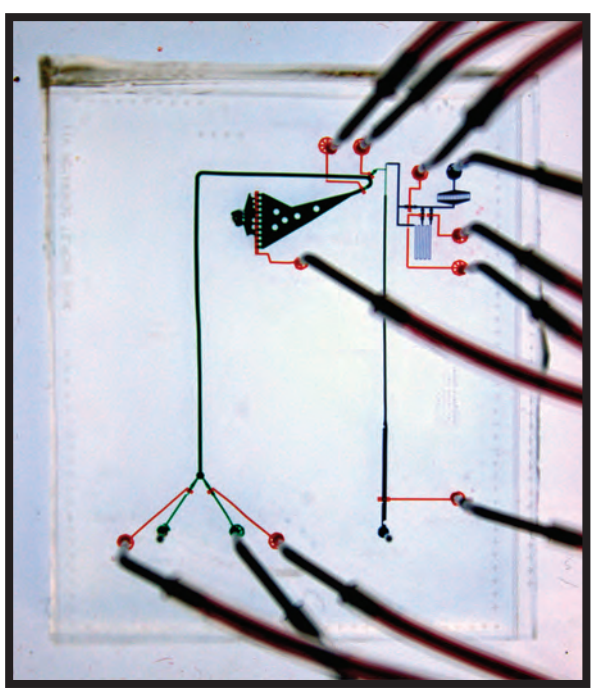


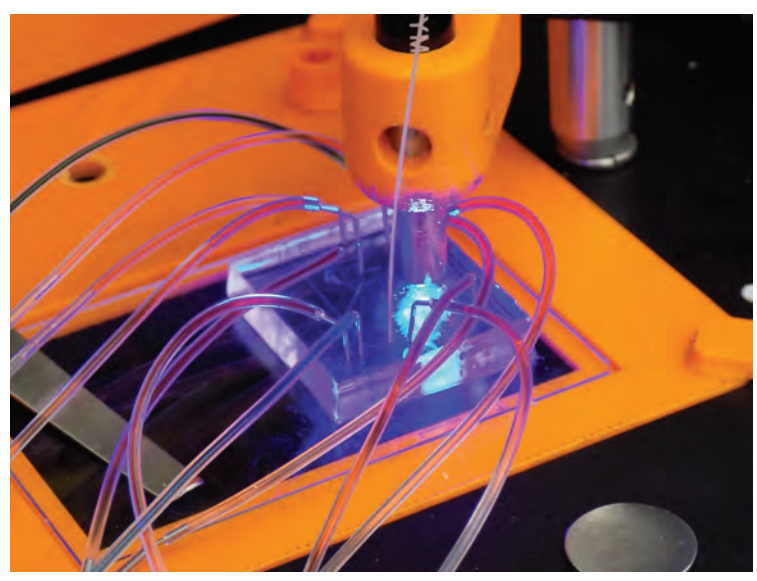
Control

3. Photolithography



4. Device Fabrication, Setup, and Testing





Figure

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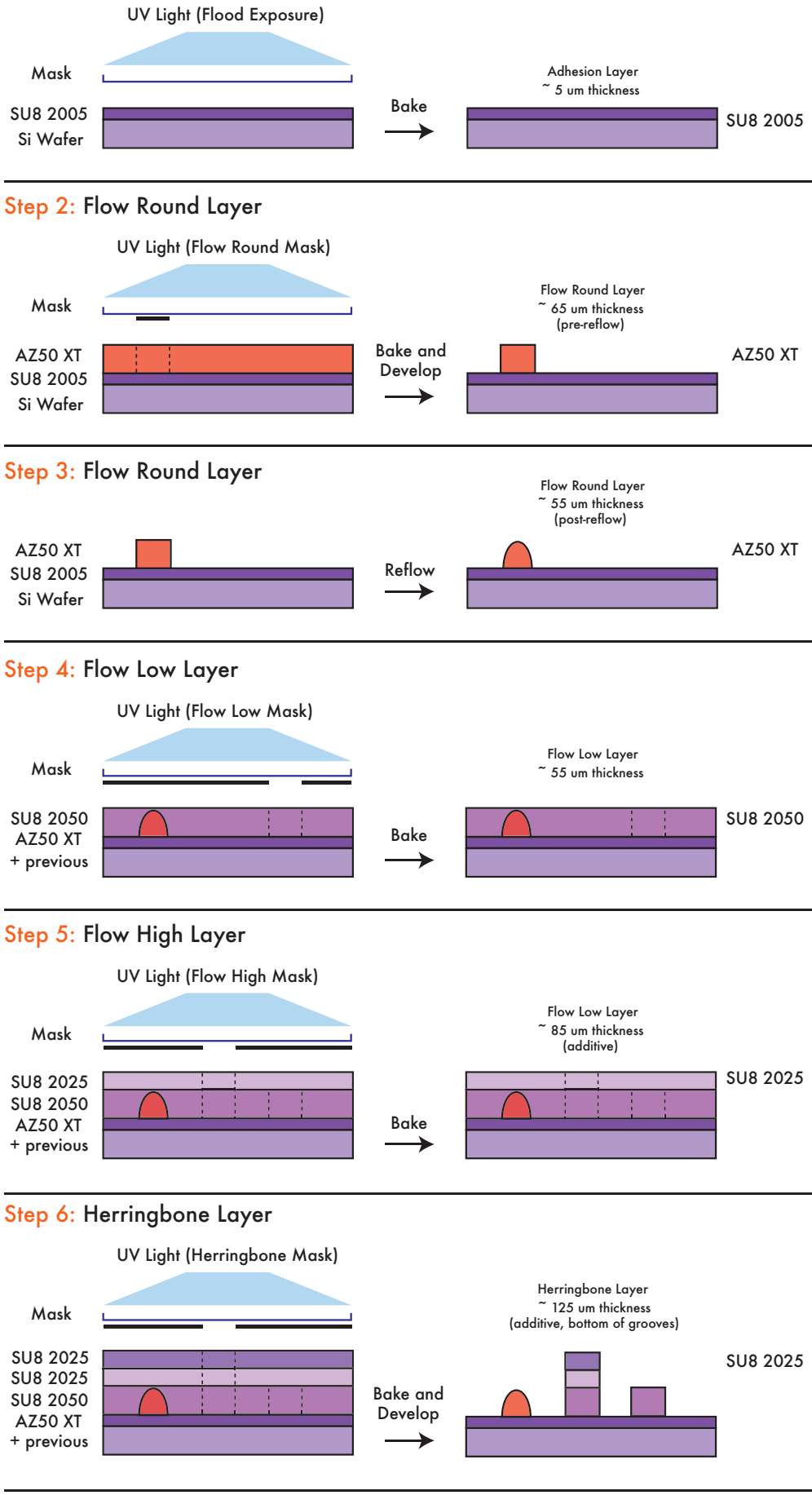

Figure

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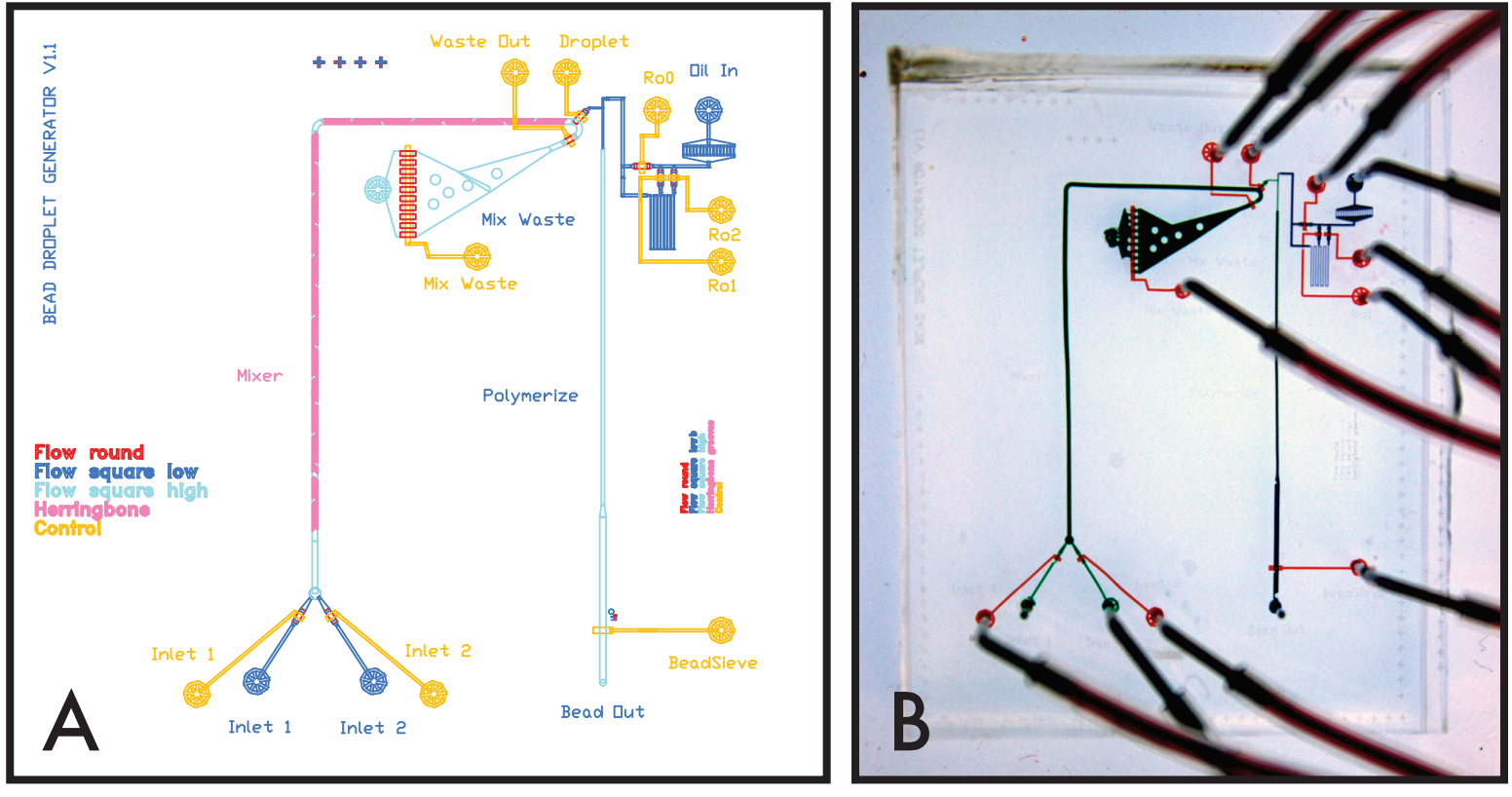

Figure

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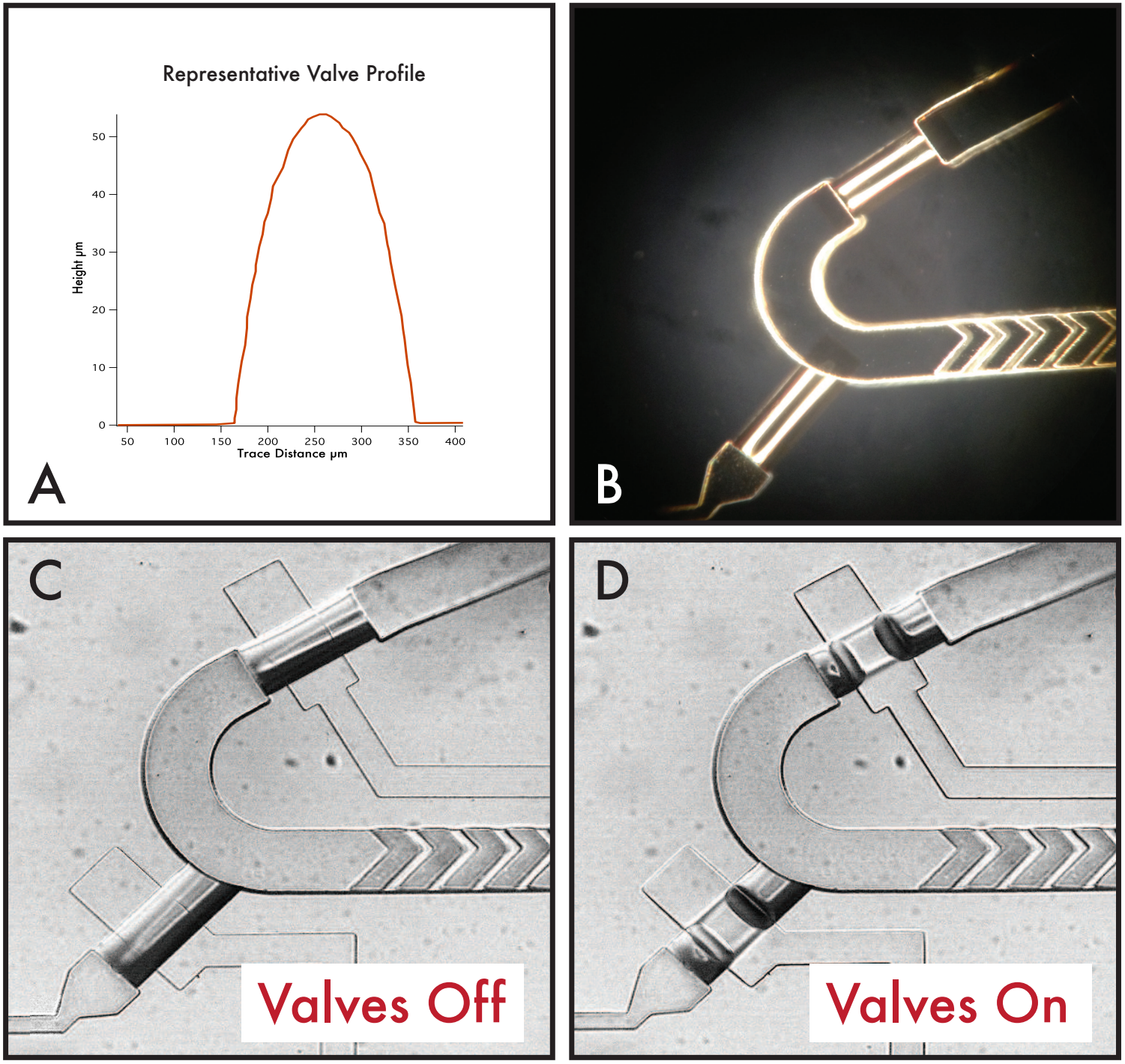

Figure

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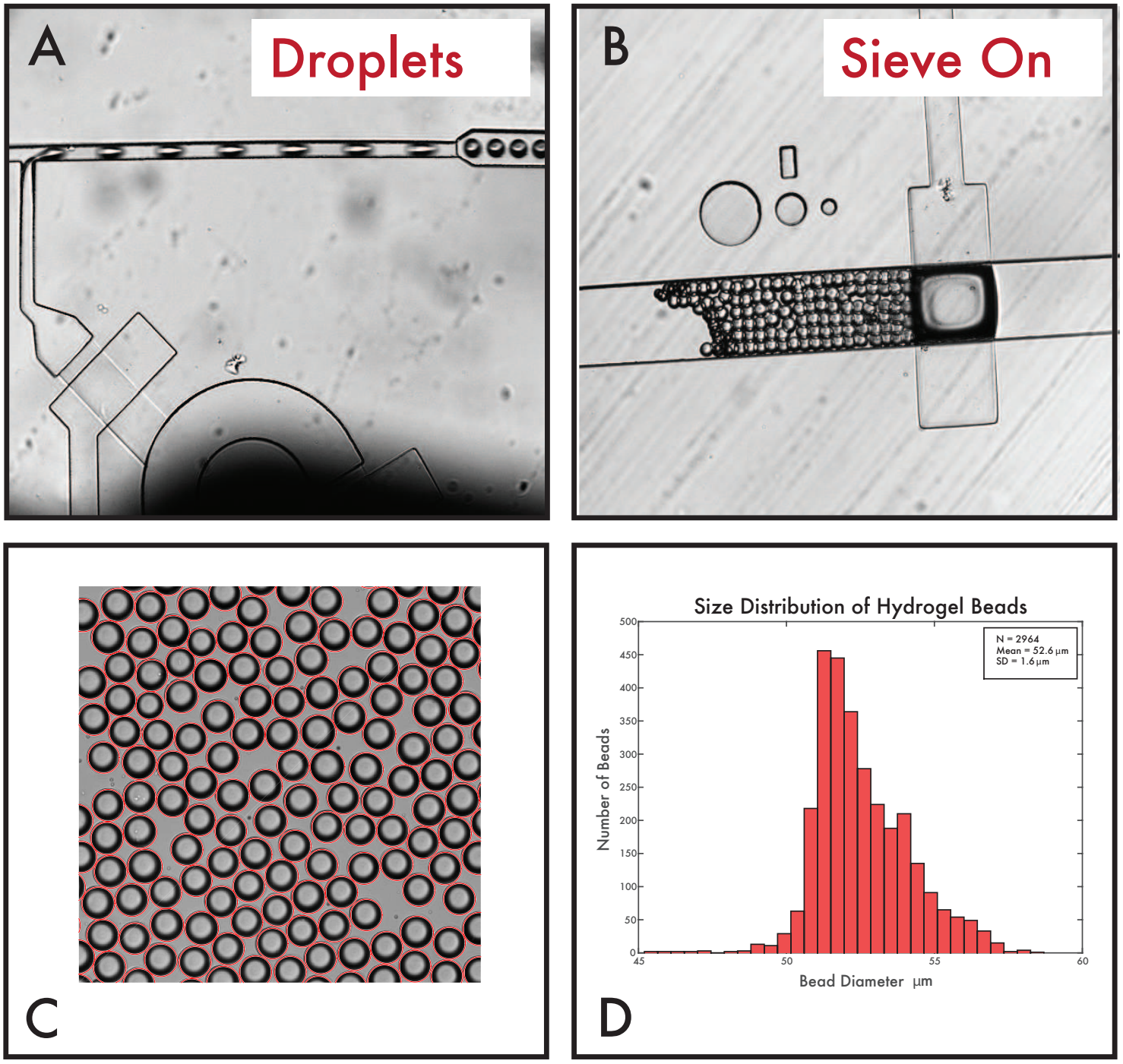

Figure

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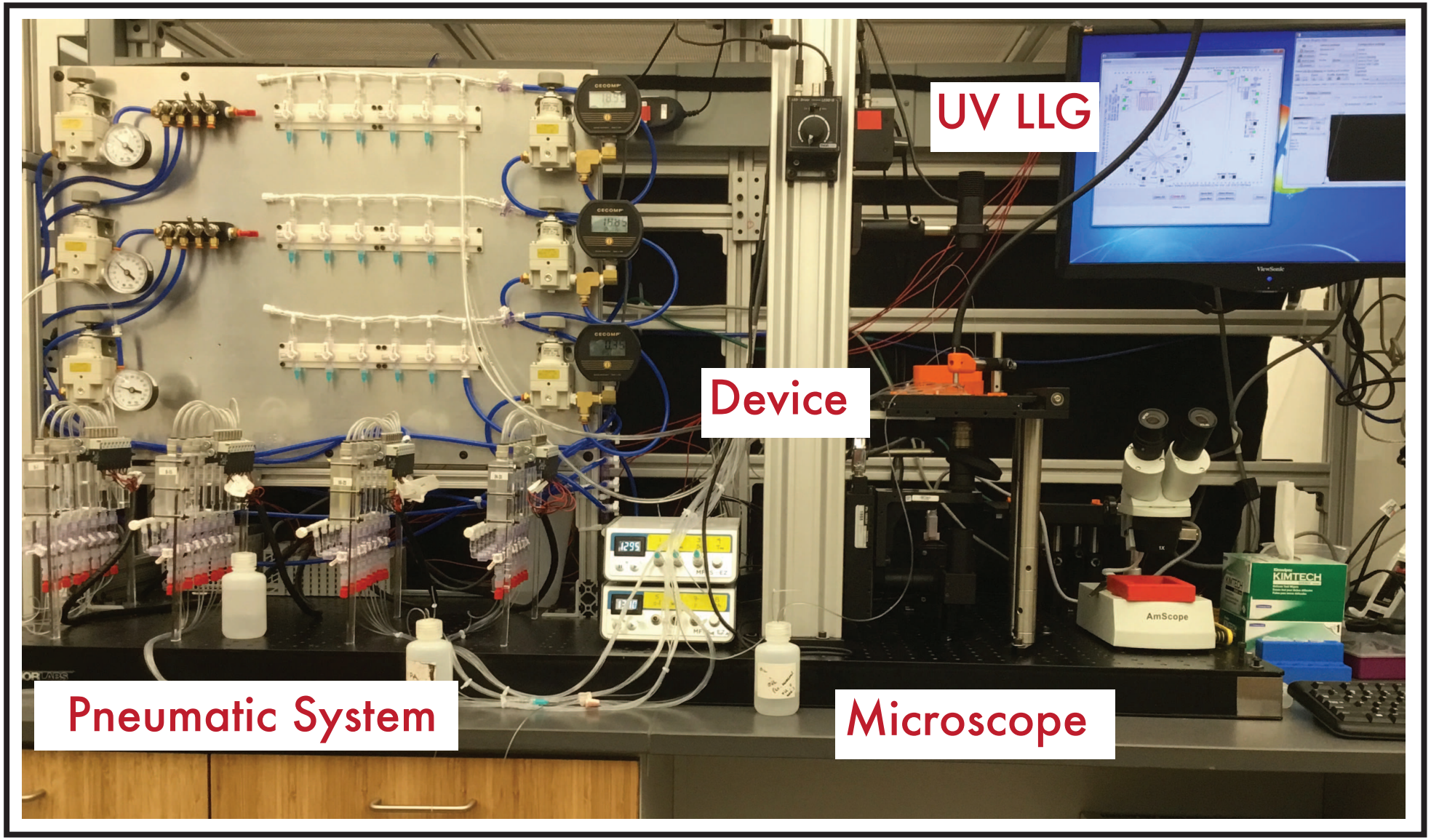

Table

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Design Element
Channel Architecture
Layer to Layer Alignment
Device Spacing and Clearance
Shapes, Lines and Objects
Channel Structural Integrity
Feature-to-Feature Alignment

Parameters and Suggestions
For channels, we use a maximum of 2:1 aspect ratio for SU8 features (which can tolerate high aspect ratio (HAR)) and 1:1 for AZ50xt. While both photoresists can achieve higher aspect ratios, we have found that the PDMS transfer is significantly improved at lower aspect ratios. Additionally, photolithography fabrication difficulty is increased in HAR designs which may become prohibitive for the non-specialist. We also recommend leaving a space between features that is at least half of the
We recommend clear device borders and the inclusion of alignment marks >200 µm (i.e. cross-hairs such as in **Figure 3**) for easy feature alignment.
It is important to have at least 1000 µm between devices and 2000 µm between the wafer border and each device so that devices can be easily cut and transferred from the wafers.
Most printing companies for transparency films use Gerber conversion from your .dwg, .stl, .dmx, or other printing file. This printing format performs best with a single layer Mask file with closed geometrical shapes (i.e. zero width polylines, no full overlap between shapes). To optimize Gerber conversion we also recommend removing constraints and other in-design dimensions before converting as these additional metadata tend to delay the conversion process.
To prevent collapse, in channels greater than 1000 µm in any direction, we normally include circular pillars such that the PDMS membrane does not fall in. We have included these in the MixWaste channel as an example.
We recommend at least 20 µm overlap between features of different layers (i.e. valves and flow channels) such that small mistakes in alignment, exposure or development can be tolerated by still having a complete, connected channel. For valves, we use 50 µm overlap to prevent 'flow gaps' between the rounded profile of the valve and rectangular profile of the surrounding flow channels. We have included these layer-layer overlaps on this design as an example.

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Step #	Description
A	Dehydration bake
A.1	Rinse wafers well with methanol
A.2	Blow dry with N2 or air
A.3	Bake on hot plate to evaporate solvent
B	**SU-8 2005 layer to improve adhesion**
B.1	Spin SU-8 2005
	Spread
	Cast
B.2	Soft bake
B.3	Expose without mask
B.4	Post-exposure bake
C	**AZ50 XT for flow round**
C.1	Spin AZ50 XT
	Spread (generous pour to avoid bubbles)
	Cast
	Snap
C.2	Let sit on flat surface to allow photoresist relaxation
C.3	Soft bake
C.4	Rehydrate
C.5	Expose
C.6	Develop immediately after exposure
C.7	Hard bake to round features
D	**SU-8 2050 for flow square low**
D.1	Spin SU-8 2050
	Spread
	Cast
D.2	Let sit on flat surface to allow photoresist relaxation
D.3	Soft bake
D.4	Expose
D.5	Post-exposure bake
E	**SU-8 2025 for flow square high**
E.1	Spin SU-8 2025
	Spread
	Cast
E.2	Let sit on flat surface to allow photoresist relaxation

E.3	Soft bake
E.4	Expose
E.5	Post-exposure bake
F	**SU-8 2025 for herringbone grooves**
F.1	Spin SU-8 2025
	Spread
	Cast
F.2	Let sit on flat surface to allow photoresist relaxation
F.3	Soft bake
F.4	Expose
F.5	Post-exposure bake
F.6	Develop
F.8	Hard bake

Parameters
95C for 10 min
500 rpm, 10 s, 133 rpm/s acc
3000 rpm, 40 s, 266 rpm/s acc
65C for 2 min, 95C for 3 min, 65C for 2 min
20 s @ 6.2 mW/cm2 (124 mJ)
65C for 2 min, 95C for 4 min, 65 C for 2 min
Mask: Flow round
200 rpm, 10s, 133 rpm/s accl.
1200 rpm, 40 s, 266 rpm/s accl.
3400 rpm, 1 s, 3456x
20 minutes
65C - 112C, full speed, 22 min, autooff
Overnight
6 x 25 s @ 6.2 mW/cm^2 (930 mJ)
Az400k 1:3 in water, 3 min- 4:30 min, by eye!
65C - 190C, 10C/hour, 15 hours
Mask: Flow square low
500 rpm, 15 s, 133 rpm/s acc
3000 rpm, 40 s, 266 rpm/s acc
20 minutes
65C for 2 min, 95C for 8 min, 65 C for 2 min
28 s @ 6.2 mW/cm^2 (170 mJ)
65C for 2 min, 95C for 9 min, 65 C for 2 min
Mask: Flow square high
500 rpm, 15 s, 133 rpm/s acc
3500 rpm, 40 s, 266 rpm/s acc
10 minutes

65C for 2 min, 95C for 8 min, 65 C for 2 min
32 s @ 6.2 mW/cm^2 (198 mJ)
65C for 2 min, 95C for 9 min, 65 C for 2 min
Mask: Herringbones
500 rpm, 15 s, 133 rpm/s acc
3000 rpm, 40 s, 266 rpm/s acc
10 minutes
65C for 2 min, 95C for 7 min, 65 C for 2 min
24 s @ 6.2 mW/cm^2 (148 mJ)
65C for 2 min, 95C for 7 min, 65 C for 2 min
SU-8 developer, 6:30 minutes
65C-165C at 120C/h, 2.5 h timer, autooff

Notes
Use ~2/3 of wafer size drop on spinner (SU8 2005 is very fluid, i.e. non-viscous, so you need to have enough to spin the wafer homogenously and not get streaks in both of the SU8 2005 steps.
This is slightly overexposed to make sure the full layer is exposed and homogenous.
This is to snap off the super viscous AZ50 that sits around the edges. If you still see some edge inhomogeneity, it will likely come off during the resting step.
Remember to Auto-off!
This step can be replaced with 2-3 hours in a wetbox with water.
30s wait between exposures
Use 1:3 dilution of Az400k gold.
Remember to Auto-off!
Pour carefully like a wine bottle to not get bubbles.
No develop

No develop

Table

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Flow Layer Feature	Feature Height
Rounded Valves (Flow Round)	54.43 µm (1.05 µm std., 1.9% CV)
Flow Channels (Flow Low)	84.22 µm (0.91 µm std., 1.1% CV)
Flow Channels (Flow High)	54.10 µm (1.24 µm std., 2.3% CV)
Herringbone Grooves (Mixer)	124.19 µm (1.89 µm std., 1.5% CV)

Click here to download Excel Spreadsheet- Table of Materials/Equipment Materials BrowerWhiteFordyce Jove

Materials

Name
Mylar Transparency Masks, 5"
5" Quartz Plates
4" Silicon Wafers, Test Grade
SU8 2005, 2025, 2050 photoresist
Az50XT
SU8 Developer
AZ400K 1:3 Developer
Pyrex 150 mm glass dish
Wafer Petri Dishes, 150 mm
Wafer Tweezers
Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (PFOTS)
2" x 3" glass slides
RTV 615 elastomeric base and curing agent PDMS set
Tygon Tubing, 0.02" O.D.
Capillary PEEK tubing, 510 um OD, 125 um ID
Cyro 4 mL tube
Epoxy, 30-minute
Metal Pins, 0.025" OD, .013" ID
Poly(ethylene glycol) diacrylate, Mn 700
Lithium Phenyl(2,4,6-trimethylbenzoyl)phosphinate photoinitator
HEPES
Light mineral oil
Span-80
ABIL EM 90

Equiptment

Name
Mask Aligner
Profilometer
Spin Coater
Vacuum Dessicator, Bell-Jar Style
Oven
UV Spot Curing System with 3 mm LLG option
MFCS Microfluidic Fluid Control System
Automated control scripting
Hotplate

Company	Catalog Number
FineLine Plotting	
United Silica	Custom
University Wafer	452
Microchem	Y111045, Y111069, Y111072
Integrated Micromaterials	AZ50XT-Q
Microchem	Y020100
Integrated Micromaterials	AZ400K1:3-CS
Sigma-Aldrich	CLS3140150-1EA
VWR	25384-326
Electron Microscopy Sciences (EMS)	78410-2W
Sigma-Aldrich	448931-10G
Thomas Scientific	6686K20
Momentive	RTV615-1P
Fischer Scientific	14-171-284
Zeus	Custom
Greiner Bio-One	127279
Permatex	84107
New England Small Tube	NE-1310-02
Sigma-Aldrich	455008-100ML
Tokyo Chemical Industry Co.	L0290
Sigma-Aldrich	H4034-25G
Sigma-Aldrich	330779-1L
Sigma-Aldrich	85548
UPI Chem	420095

Equivalent equiptment or homebuilt setups will work equally as well

Company	Catalog Number
Karl Suss	MA6
KLA-Tencor	Alpha-Step D500
Laurell Technologies	WS-650-23
Bel-Art	420100000
Cole-Palmer	WU-52120-02
Dymax	41015
Fluidgent	MFCS-EZ
MATLAB	
Tory Pines Scientific	HP30

Comments
360 um PEEK is readily available by Idex (catalog number: 1571) We typically synthesize LAP in-house.

Comments
Any spincoater can be used that accepts 100 mm wafers UV LEDs, Xenon Arc Lamps, or other UV sources of the same intensity work equally as well Syringe pumps, custom pneumatics or other control systems can also be used Any hotplate with uniform heating (i.e. aluminum or ceramic plates) will suffice.

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Author(s): Kara Brower, Adam K. White, Polly M. Fordyce

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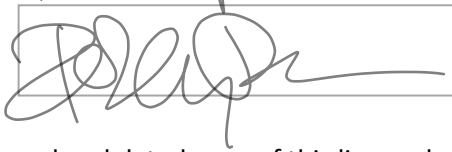
Signature:  Date: 7/26/2016

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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 ^{o}C 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 ^{o}C , 95 ^{o}C , and 65 ^{o}C . 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!

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