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Corresponding Author:	Wei Li Texas Tech University Lubbock, Texas UNITED STATES
Corresponding Author's Institution:	Texas Tech University
Corresponding Author E-Mail:	wei.li@ttu.edu
Order of Authors:	Celine R. Garcia
	Zhenya Ding
	Hilario C. Garza
	Wei Li
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TITLE:

Design and Development of a Three-Dimensionally Printed Microscope Mask Alignment Adapter for the Fabrication of Multilayer Microfluidic Devices

AUTHORS AND AFFILIATIONS:

Celine R. Garcia[1*], Zhenya Ding[1*], Hilario C. Garza[1], Wei Li[1]

[1]Department of Chemical Engineering, Texas Tech University, Lubbock, TX, USA

*These authors contributed equally.

Email addresses of co-authors:

Celine R. Garcia	(celine.r.garcia@ttu.edu)
Zhenya Ding	(zhenya.ding@ttu.edu)
Hilario C. Garza	(chris.garza40@yahoo.com)
Wei Li	(wei.li@ttu.edu)

Corresponding author:

Wei Li (wei.li@ttu.edu)

KEYWORDS:

3D printing, photolithography, microfluidics, chemical engineering, multilayer microfluidic device, soft lithography

SUMMARY:

This project allows small laboratories to develop an easy-to-use platform for the fabrication of precise multilayer microfluidic devices. The platform consists of a three-dimensionally printed microscope mask alignment adapter using which multilayer microfluidic devices with alignment errors of <10 µm were achieved.

ABSTRACT:

This project aims to develop an easy-to-use and cost-effective platform for the fabrication of precise, multilayer microfluidic devices, which typically can only be achieved using costly equipment in a clean room setting. The key part of the platform is a three dimensionally (3D) printed microscope mask alignment adapter (MMAA) compatible with regular optical microscopes and ultraviolet (UV) light exposure systems. The overall process of creating the device has been vastly simplified because of the work done to optimize the device design. The process entails finding the proper dimensions for the equipment available in the laboratory and 3D-printing the MMAA with the optimized specifications. Experimental results show that the optimized MMAA designed and manufactured by 3D printing performs well with a common microscope and light exposure system. Using a master mold prepared by the 3D-printed MMAA, the resulting microfluidic devices with multilayered structures contain alignment errors of <10 µm, which is sufficient for common microchips. Although human error through transportation of the device to the UV light exposure system can cause larger fabrication errors, the minimal errors

achieved in this study are attainable with practice and care. Furthermore, the MMAA can be customized to fit any microscope and UV exposure system by making changes to the modeling file in the 3D printing system. This project provides smaller laboratories with a useful research tool as it only requires the use of equipment that is typically already available to laboratories that produce and use microfluidic devices. The following detailed protocol outlines the design and 3D printing process for the MMAA. In addition, the steps for procuring a multilayer master mold using the MMAA and producing poly(dimethylsiloxane) (PDMS) microfluidic chips is also described herein.

INTRODUCTION:

A well-developed and promising field in engineering research is microfabrication because of the vast expanse of applications employing microfluidic platforms. Microfabrication is a process wherein structures are produced with µm- or smaller-sized features using different chemical compounds. As microfluidic research has developed over the last 30 years, soft lithography has become the most popular microfabrication technique with which to produce microchips made from poly(dimethylsiloxane) (PDMS) or similar substances. These microchips have been widely used for the miniaturization of common laboratory practices[1–4] and have become powerful research tools for engineers to mimic reaction processes[5–7], study reaction mechanisms, and mimic organs found in the human body in vitro (e.g., organ-on-a-chip)[8–10]. However, as the complexity of the application increases, it is typical that a more complex microfluidic device design allows for better replication of the real-life system it is intended to imitate.

The basic soft lithography procedure involves coating a substrate with a photoresist substance and placing a photomask over the coated substrate before subjecting the substrate to UV light[11]. The photomask has transparent regions that mimic the desired pattern of the microfluidic device channels. When subjecting the coated substrate to UV light, the transparent regions allow the UV light to penetrate through the photomask, causing the photoresist to be crosslinked. After the exposure step, the un-crosslinked photoresist is washed away using a developer, leaving solid structures with the intended pattern. As the complexity of the microfluidic devices becomes greater, they require multiple-layer construction with extremely precise dimensions. The process of multilayer microfabrication is much more difficult compared to single-layer microfabrication.

Multilayer microfabrication requires precise alignment of the first layer features with the designs on the second mask. Normally, this process is performed using a commercial mask aligner, which is expensive and requires training to operate the machinery. Thus, the process of multilayer microfabrication is typically unattainable for smaller laboratories that lack the funds or time for such endeavors. While several other custom-built mask aligners have been developed, these systems often require the purchase and assembly of many different parts and can still be quite complex[12–14]. This is not only expensive for smaller laboratories, but also requires time and training to build, understand, and use the system. The mask aligner detailed in this paper sought to alleviate these issues as there is no need for the purchase of additional equipment, only requiring equipment that is typically already present in laboratories that produce and use microfluidic devices. In addition, the mask aligner is fabricated by 3D printing, which with the recent advancement of 3D printing technology, has become readily available to most laboratories and universities at an affordable cost.

The protocol detailed in this paper aims to create a cost-effective and easy-operation alternative mask aligner. The mask aligner detailed herein can make multilayer microfabrication feasible for research laboratories without conventional fabrication facilities. Using the microscope mask alignment adapter (MMAA), functional microchips with complex features can be achieved using a regular UV light source, optical microscope, and common laboratory equipment. The results show that the MMAA performs well with an example system using an upright microscope and a UV light-exposure box. The MMAA produced using the 3D printing process was used to acquire a bilayer master mold of a herringbone microfluidic device with minimal alignment errors. Using the master mold fabricated with a 3D-printed MMAA, microfluidic devices were prepared with multilayered structures containing alignment errors of <10 µm. The alignment error of <10 µm is minimal enough to not hinder the application of the microfluidic device.

In addition, the successful alignment of a four-layer master mold produced using the MMAA was confirmed, and alignment errors were determined to be <10 µm. The functionality of the microfluidic device and minimal alignment errors validate the successful application of the MMAA in creating multilayer microfluidic devices. The MMAA can be customized to fit any microscope and UV exposure system by making minor changes to the file in the 3D printer. The following protocol outlines the steps necessary to fine-tune the MMAA to fit the equipment available in each laboratory and 3D-print the MMAA with the required specifications. In addition, the protocol details how to develop a multilayer master mold using the system and subsequently produce PDMS microfluidic devices using the master mold. Generation of the master mold and microfluidic chips then allows the user to test the effectiveness of the system.

PROTOCOL:

1. Designing the MMAA

1.1. Obtain the dimensions of the tray of the available UV light emission system to be the upper bound for the dimensions of the wafer holder (or UV exposure unit) shown in **Figure 1**. As shown in **Figure 2A**, measure the diameter (d) of the inner circular rim, the inner height (h) of the UV light emission system's tray, the total width (w), and length (l) of the tray.

NOTE: As an example, the available UV light exposure system had inner tray dimensions of 5 inch (") x 5" x 0.25" with a 4" circular cut-out. The dimensions of the MMAA were then designed to be no greater than the inner tray dimensions to fit properly and sit flat within the tray of the system as shown in **Figure 2B.** See **Figure 3** for the 3D-printed pieces of the MMAA: photoresist-coated silicon wafer and a fastener to fix the setup to the microscope.

1.2. Measure the length between the screws on the available upright microscope stage that hold the slide holder in place. Additionally, measure the width of the screws. Apply these dimensions to customize the magnetic holder (**Figure 1**) to fit the available microscope to allow for easy and precise fixation of the MMAA to the microscope (**Figure 4A**).

1.3. Using an available computer design application, customize the wafer holder and magnetic microscope fastener to fit within the measured dimensions. Design the height, width, and length of the wafer holder to be no greater than the height (h), width (w), and length (l) of the UV light emission system's tray. In addition, include the circular cut-out at the bottom of the wafer holder with the same diameter (d) as the UV light emission system's tray. Generate STL or CAD files for the two pieces of the MMAA to be used for 3D printing of the device (see **Supplemental Material**).

2. 3D Printing the MMAA

2.1. Upload the generated STL or CAD files to the available 3D-printing software. 3D-Print the two pieces of the MMAA by following the appropriate procedure for the 3D process and printer used. Complete the pieces by following any required post-printing steps (e.g., removal of support material, removal of uncured resin, additional washing or curing steps). Alternatively, use an available 3D printing facility to have the designed pieces printed and completed elsewhere.

2.2. Ensure the wafer holder fits well and sits flat inside the tray of the available UV light exposure system (**Figure 2B**). Additionally, ensure that the microscope fastener is attached to the microscope stage and can be moved easily using the knobs that control the x- and y- positions of the microscope stage (**Figure 4A**).

2.3. Once the pieces have been finalized, insert and fix the magnets into the wafer holder and microscope fastener (**Figure 3A**), using super glue or any other fixing substance. Allow for the glue to dry before testing the system.

NOTE: If desired, a protype piece can first be printed using a Fused Deposition Modeling (FDM) 3D printer to save resources and money[15]. This protype can then be assessed for accurate fit to the available equipment, and the design can then be modified, if needed. The final device can then be printed using a more accurate process (e.g., Stereolithography) for better precision. The final device can also be printed with a translucent finish for optimal use under the microscope.

3. Experimental testing of the MMAA

3.1. Design and printing of the microfluidic device photomasks with alignment markers

3.1.1. Use a computer design application to design photomasks for the desired bilayer microfluidic device.

3.1.2. Include additional structures on the side of the microfluidic device channel structures that will act as alignment markers (closer towards the edge of the photomask/master mold) as shown in **Figure 5A,B**. Ensure there is one alignment marker on each side of the microfluidic device (for a total of at least four). In addition, ensure the photomask contains a straight edge that can align perfectly with the straight edge of the silicon wafer.

NOTE: The higher intricacy of the alignment marker structure will allow for greater alignment accuracy of the additional layers. At the least, a simple cross structure with measurements of 1 mm x 1 mm should be used (**Figure 6A**). An example of the alignment markers can be seen in the corners and bottom middle edge of **Figure 5A,B**, which depict the first- and second-layer photomasks used to generate a double-layer master mold. Alternatively, the photomasks can be printed by a commercial vendor or by other accessible facilities.

3.2. Creation of the bilayer master mold using the MMAA (photolithography)

3.2.1. Using standard photolithography techniques and the photoresist manufacturer's instructions, create the first layer of the master mold using the first layer photomask[16]. Use a 4" silicon wafer with the appropriate photoresist (i.e., SU-8) to create the desired layer thickness. Ensure the first layer thickness is greater than the subsequent layers for easy identification of the alignment markers.

3.2.2. Use a light-colored marker pen (e.g., gold) to color the first layer's alignment markers on all four sides.

3.2.3. Using the photoresist manufacturer's instructions, initiate the second layer of the master mold by spin-coating the photoresist onto the wafer and performing the soft bake[16]. Insert the coated wafer into the wafer holder of the MMAA (**Figure 3B**) and fix the coated wafer to the MMAA using tape.

3.2.4. Attach the wafer holder to the available upright microscope using the magnetic microscope fastener (**Figure 4A**). Move the position of the MMAA using the x- and y-direction knobs of the microscope stage until one of the colored alignment markers on the wafer is in view through the microscope lens.

3.2.5. Insert the second-layer photomask into the wafer holder, on top of the coated wafer (**Figure 3C**). Ensure that the first-layer, colored alignment markers can be partially seen through the alignment markers on the photomask. Ensure that the straight edge of the photomask is superimposed with the straight edge of the silicon wafer to avoid any rotational error.

3.2.6. Attach the photomask to a scissor lift (also known as a support jack) through one of the side cut-outs (**Figure 4B**) with tape. Use the scissor lift to adjust the z-direction position of the photomask until it lies right above the coated wafer (**Figure 3C**).

NOTE: The scissor lift will then allow for fine adjustment of the z-position of the photomask, as the scissor lift can be used to move the position of the attached photomask in the z-direction.

3.2.7. While keeping the photomask still, look through the microscope lens and identify the first-layer, colored alignment markers beneath the alignment markers of the photomask. Use the x- and y-direction knobs of the microscope stage to move the position of the MMAA (**Figure 4D**).

Adjust the position of the MMAA until the alignment marker on the photomask is superimposed with the colored alignment marker on the first layer (**Figure 6A,B**) by observing the position of the alignment markers through the microscope lens.

3.2.8. Carefully apply a slight force to the photomask and use tape to secure the photomask in place on top of the coated wafer. Detach the photomask from the scissor lift. Ensure all four alignment markers on the photomask are in alignment with the four alignment markers on the first layer.

3.2.9. Once the alignment is achieved, carefully detach the wafer holder from the microscope stage. Insert the glass top plate on top of the wafer and photomask to decrease the gap between the two pieces (**Figure 1**). Place the entire wafer holder into the available UV light exposure system as shown in **Figure 4E**. Expose the second layer for the appropriate time and light intensity as described in the photoresist manufacturer's instructions[16].

3.2.10. Remove the wafer holder from the UV light exposure system. Remove the coated wafer from the wafer holder and detach the photomask from the wafer. Complete the processing of the second layer (e.g., post-bake, developing, and rinse and dry) as per the photoresist manufacturer's instructions[16].

NOTE: The exact spin-coating, soft baking, exposing, post-baking, and developing conditions (time, temperature) will vary based on the photoresist being used and the desired layer thickness. The actual conditions and exact photolithography procedure should be based on the photoresist manufacturer's instructions.

3.3. Preparation of a microfluidic device using the master mold (soft lithography)

3.3.1. Retrieve the master mold and secure it in the middle of a 150 mm x 15 mm plastic Petri dish with tape.

3.3.2. Prepare ~15–20 g of PDMS based on the manufacturer's instructions. Place the PDMS in a vacuum chamber or let it rest until free of any bubbles. Pour the PDMS into the Petri dish containing the master mold.

3.3.3. Let the Petri dish with the master mold rest on the countertop until the PDMS is free of any bubbles. Place the Petri dish in an oven at 65 °C until the PDMS is fully cured (at least 3 h).

3.3.4. Cut out the PDMS to reveal the microchannel structures. Cut the PDMS around the microchannel structures into separate microchips and create the inlet and outlet holes for the microfluidic device. Use tape to gently remove any small particulates that may lie on the PDMS surface.

3.3.5. Complete the microchip fabrication by bonding the PDMS chip to the PDMS or a microscope slide by plasma-treating the PDMS chip and the additional substrate.

3.4. Determination of the alignment error

3.4.1. Retrieve the master mold and use the upright microscope to determine the gap distance (alignment error) between the first layer and second layer. Do this by simply measuring the distance by which the second layer is shifted and misaligned from the first layer on the microchannel structures (see **Figure 5D** for an example of a measured gap distance).

3.4.2. Use the upright microscope to determine whether the PDMS chip contains channel walls that are straight with clear device edges. Additionally, check the PDMS chip for any possible defects that may hinder device functionality.

NOTE: The master mold fabrication (sections 3.2 and 3.3) may need to be repeated to achieve a lower alignment error. Repeated practice using the MMAA is shown to enhance the user's ability to create a well-aligned master mold. In addition, images can be obtained by scanning electron microscopy (SEM) (**Figure 7**) to confirm the alignment error.

REPRESENTATIVE RESULTS:

Through the optimization and use of the MMAA (**Figure 1**), multilayer master molds with minimal alignment error were fabricated. The final MMAA was fabricated using the fused filament fabrication (FFF) 3D-printing process (**Figure 2**). The FFF process confers increased accuracy for the desired device dimensions. The MMAA consists of two main pieces (**Figure 3**): the base piece and the custom fastener. The base piece consists of the UV exposure unit, which acts as the wafer holder. The UV exposure unit allows proper alignment of the photomask and the coated silicon wafer. The second piece is the custom fastener that fixes the wafer holder to the platform of the microscope with magnets. The entire setup used to assist in the alignment of the top and bottom layers of the double-layer master mold is depicted in **Figure 4**. This system and the described protocol were used for the alignment of the markers on the photomask with the markers on the initial layer of the master mold (**Figure 6**). The double-layer SU-8 master mold for a microfluidic device with a herringbone pattern was then fabricated and was shown to have a gap distance of <5 µm between the two layers (**Figure 5**).

The two-layer master mold (**Figure 7A**) was then used to fabricate PDMS microchips that can be seen in **Figure 7D**. The SEM images seen in **Figure 7B**,**C** show that the microfluidic device with the herringbone pattern contains clear edges, straight-channel walls, and well-aligned layers, which are essential for proper device functionality. In addition, a four-layer master mold with simple circular features (**Figure 8A**) was created using the MMAA to show successful alignment of a multilayer master mold. Profilometer data (**Figure 8B**) confirms the four distinct layers of the master mold. Measurements taken of the alignment error obtained for multiple four-layer features of differing geometry confirm that the alignment error is no greater than 5% of the designed distance between the layers. From the images of the final device, it is clear that human error during fixation of the mask onto the MMAA before the UV exposure of the second layer increased the gap distance between the two device layers and caused misalignment. However, as the user becomes more familiar with the procedure, the final device can be produced with a resulting alignment error of <10 µm, as confirmed by the depicted results.

FIGURE AND TABLE LEGENDS:

Figure 1: Design of a 3D-printable MMAA for multilayer microfabrication. The illustration depicts the two pieces of the MMAA: the UV exposure unit and the custom microscope fastener. The UV exposure unit houses, in descending order, the glass top plate, which holds the photomask against the wafer; the photomask; and the photoresist-coated wafer. The UV exposure unit is then magnetically attached to the custom microscope fastener, which is attached to the microscope stage, and then allows for proper alignment of the photomask and wafer. Abbreviations: MMAA = microscope mask alignment adapter; UV = ultraviolet.

Figure 2: Customization and 3D printing of an MMAA and post-processing for a fully cured device. (**A**) Photo of the tray of the available UV light emission system showing the necessary measurements needed to customize the MMAA. The user should measure the diameter (d) of the inner circular rim, the inner height (h), the total width (w), and the length (l) of the tray. (**B**) After customization, the MMAA should then sit flat inside the tray as shown here. (**C**) Illustration of the FFF 3D printing process. The FFF process produces structures by layering the 3D-printed filament. The filament is deposited in thin layers, one on top of the next, until the final 3D-printed piece is produced. (**D**) The curing of the final 3D-printed MMAA in the UV curing chamber as part of the post-printing process. Abbreviations: MMAA = microscope mask alignment adapter; UV = ultraviolet; FFF = fused filament fabrication.

Figure 3: 3D-Printed pieces of an MMAA. (**A**) Two pieces were connected by magnets (indicated by red dashed rectangle). (**B**) MMAA containing a silicon wafer coated with a thin layer of photoresist (SU-8). (**C**) MMAA with a photomask over the coated silicon wafer in preparation for the alignment process. Abbreviation: MMAA = microscope mask alignment adapter.

Figure 4: Procedure to use a 3D-printed MMAA for alignment of the photomask. (**A**) After the MMAA has been loaded with the photoresist-coated silicon wafer, the MMAA is then placed on the stage of an upright microscope system and fixed to the stage using the magnetic microscope fastener as shown in the image. (**B**) The photomask is then inserted into the MMAA and attached to the z-direction-adjusting platform, otherwise known as a scissor lift, through one of the sides of the MMAA as shown in the image. (**C**) The scissor lift platform height is then adjusted until the photomask lies right above the coated silicon wafer as shown in the image. From this point onwards, the photomask is not moved until alignment is completed. (**D**) To achieve perfect alignment, the position of the MMAA and hence, of the silicon wafer, on the microscope stage is then adjusted in the x- and y-directions using the microscope's knobs as shown in the image. The x- and y-positions of the silicon wafer are finely adjusted, while the user observes through the microscope lens until the alignment markers on the silicon wafer and the photomask are superimposed. Once this is achieved, the photomask can then be secured to the wafer. (**E**) After alignment is achieved, the MMAA is carefully detached from the microscope stage and placed in the tray of the UV light exposure system. The tray can be closed so that the wafer can be exposed to UV irradiation to cure the photoresist. Abbreviation: MMAA = microscope mask alignment

adapter.

Figure 5: Double-layer channel structure created using the MMAA. The double-layer master mold is designed for the production of herringbone microfluidic devices with four parallel channels. (**A**) Image of the first-layer photomask design, which includes the outline for the channels and generates the hollow floor of the microfluidic device. (**B**) Image of the second-layer photomask design, which incorporates the herringbone pattern inside the channels that line the roof of the microfluidic device. (**C**) The inlet structure of the double-layer master mold indicated by red dashed rectangles in (**A**) and (**B**). The image shows minimal gap distance between the two layers. (**D**) A section of the double-layer master mold showing a bend in the channel indicated by green dashed rectangles in (**A**) and (**B**). The gap distance between the two arrows is 5 µm. Scale bars = 100 µm. Abbreviation: MMAA = microscope mask alignment adapter.

Figure 6: Microfabrication results with the MMAA. (**A**) and (**B**) show the alignment of the markers on the photomask. Scale bars = 200 µm. (**C**) and (**D**) are the corresponding images of the markers on the wafer after exposure. Scale bars = 100 µm. Abbreviation: MMAA = microscope mask alignment adapter.

Figure 7: The master mold prepared using the MMAA and the resulting PDMS device made from the master mold. (**A**) Double-layer master mold of herringbone microfluidic device prepared using the MMAA to achieve alignment of layers. (**B**) and (**C**) are SEM images of the herringbone device in different scales with the red arrows pointing at the lower layer. (**D**) PDMS microfluidic device with herringbone pattern made using the double-layer master mold in (**A**). Abbreviations: MMAA = microscope mask alignment adapter; PDMS = poly(dimethylsiloxane); SEM = scanning electron microscopy.

Figure 8: Image and profilometer data of a four-layer master mold created using the MMAA. (**A**) Image of a four-layer master mold created using the MMAA showing successful alignment of the layers. Simple circular features in descending size were chosen to demonstrate the alignment capability of the MMAA. Scale bar = 1,250 µm. (**B**) Profilometer data of the same circular four-layer master mold confirming the presence of the four distinct layers. Abbreviations: MMAA = microscope mask alignment adapter.

DISCUSSION:

The aforementioned protocol outlines the procedure for 3D-printing an MMAA and using the system to create a precise, multilayer, microfluidic device master mold. Although the device is easy to use, there are critical steps within the protocol that require practice and care to ensure proper alignment of the master mold layers. The first critical step is the design of the MMAA. It is essential when designing the MMAA to determine the exact measurements for the device that will allow for a proper fit inside the UV light exposure system. A misalignment of the device can cause uneven UV light exposure, which can create deformities of the master mold features. The second critical step is to take care when aligning the first and second layers of the master mold when using the MMAA. It is imperative after aligning the second-layer photomask with the first-layer alignment markers that the user takes great care when fixing the photomask to the wafer and MMAA. The micron-sized features mean that any small misalignment due to movement of the photomask during fixation can create alignment errors that can render the final PDMS device unusable. Therefore, this step requires accuracy that can be developed with practice using the MMAA. The last critical step is to ensure there is no gap between the photomask and the coated wafer to ensure even UV light exposure. This technique in using the MMAA to create multilayer master molds is limited by the attention to detail and care of the user when following the given protocol as the critical steps above must be followed to ensure well-aligned layers.

Multilayer microfluidic devices are typically difficult to produce with little error unless traditional alignment equipment is available. This equipment is expensive and because of its sensitivity, requires special training and typically a clean room environment that is not always available to smaller laboratories. In addition, previously published custom-built mask aligners typically require the purchase and assembly of many different pieces, which can still render the platforms expensive to produce and difficult to use[12–14]. The significance of the MMAA is that it is an easy-to-fabricate and cost-effective alternative to standard equipment used for multilayer microfluidic device fabrication. Additionally, the MMAA requires no special training for its use, as its application is fairly simple and uses standard laboratory equipment already present in laboratories that regularly produce and use microfluidic devices. This allows small and resource-limited laboratories to produce multilayer microfluidic devices with improved functionality.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Betancourt, T., Brannon-Peppas, L. Micro- and nanofabrication methods in nanotechnological medical and pharmaceutical devices. *International Journal of Nanomedicine*. **1** (4), 483–495 (2006).
2. Wheeler, A. R. et al. Microfluidic device for single-cell analysis. *Analytical Chemistry*. **75** (14), 3581–3586 (2003).
3. Kong, D. S., Carr, P. A., Chen, L., Zhang, S., Jacobson, J. M. Parallel gene synthesis in a microfluidic device. *Nucleic Acids Research*. **35** (8), e61 (2007).
4. Yang, M., Li, C.-W., Yang, J. Cell docking and on-chip monitoring of cellular reactions with a controlled concentration gradient on a microfluidic device. *Analytical Chemistry*. **74** (16), 3991–4001 (2002).
5. Keles, H. et al. Development of a robust and reusable microreactor employing laser based mid-IR chemical imaging for the automated quantification of reaction kinetics. *Organic Process Research & Development*. **21** (11), 1761–1768 (2017).
6. Losey, M. W., Jackman, R. J., Firebaugh, S. L., Schmidt, M. A., Jensen, K. F. Design and

fabrication of microfluidic devices for multiphase mixing and reaction. *Journal of Microelectromechanical Systems*. **11** (6), 709–717 (2002).

7. Kobayashi, J. et al. A microfluidic device for conducting gas-liquid-solid hydrogenation reactions. *Science*. **304** (5675), 1305–1308 (2004).

8. Shuler, M. L. Advances in organ-, body-, and disease-on-a-chip systems. *Lab on a Chip*. **19** (1), 9–10 (2019).

9. Kimura, H., Sakai, Y., Fujii, T. Organ/body-on-a-chip based on microfluidic technology for drug discovery. *Drug Metabolism and Pharmacokinetics*. **33** (1), 43–48 (2018).

10. Lee, H. et al. A pumpless Multi-Organ-on-a-Chip (MOC) combined with a Pharmacokinetic–Pharmacodynamic (PK–PD) model. *Biotechnology and Bioengineering*. **114** (2), 432–443 (2017).

11. Kang, S.-W. Application of soft lithography for nano functional devices. In *Lithography*. Edited by Wang, M., IntechOpen. 403–426 (2010).

12. Challa, P. K., Kartanas, T., Charmet, J., Knowles, T. P. J. Microfluidic devices fabricated using fast wafer-scale LED-lithography patterning. *Biomicrofluidics*. **11**, 014113 (2017).

13. Li, X. et al. Desktop aligner for fabrication of multilayer microfluidic devices. *Review of Scientific Instruments*. **86** (7), 075008 (2015).

14. Pham, Q. L., Tong, N.-A. N., Mathew, A., Voronov, R. S. A compact low-cost low-maintenance open architecture mask aligner for fabrication of multilayer microfluidics devices. *Biomicrofluidics*. **12** (4), 044119 (2018).

15. Ravi, T., Ranganathan, R. Topology and build path optimization for reducing cost in FDM uPrint SE. In *Advances in Additive Manufacturing and Joining*. Edited by Shunmugam, M. S., Kanthababu, M., Springer, Singapore. 189–198 (2019).

16. *SU-8 Permanent Negative Epoxy Photoresist*, <https://kayakuam.com/wp-content/uploads/2020/09/KAM-SU-8-50-100-Datasheet-9.3.20-Final.pdf> (2020).

Figure 1

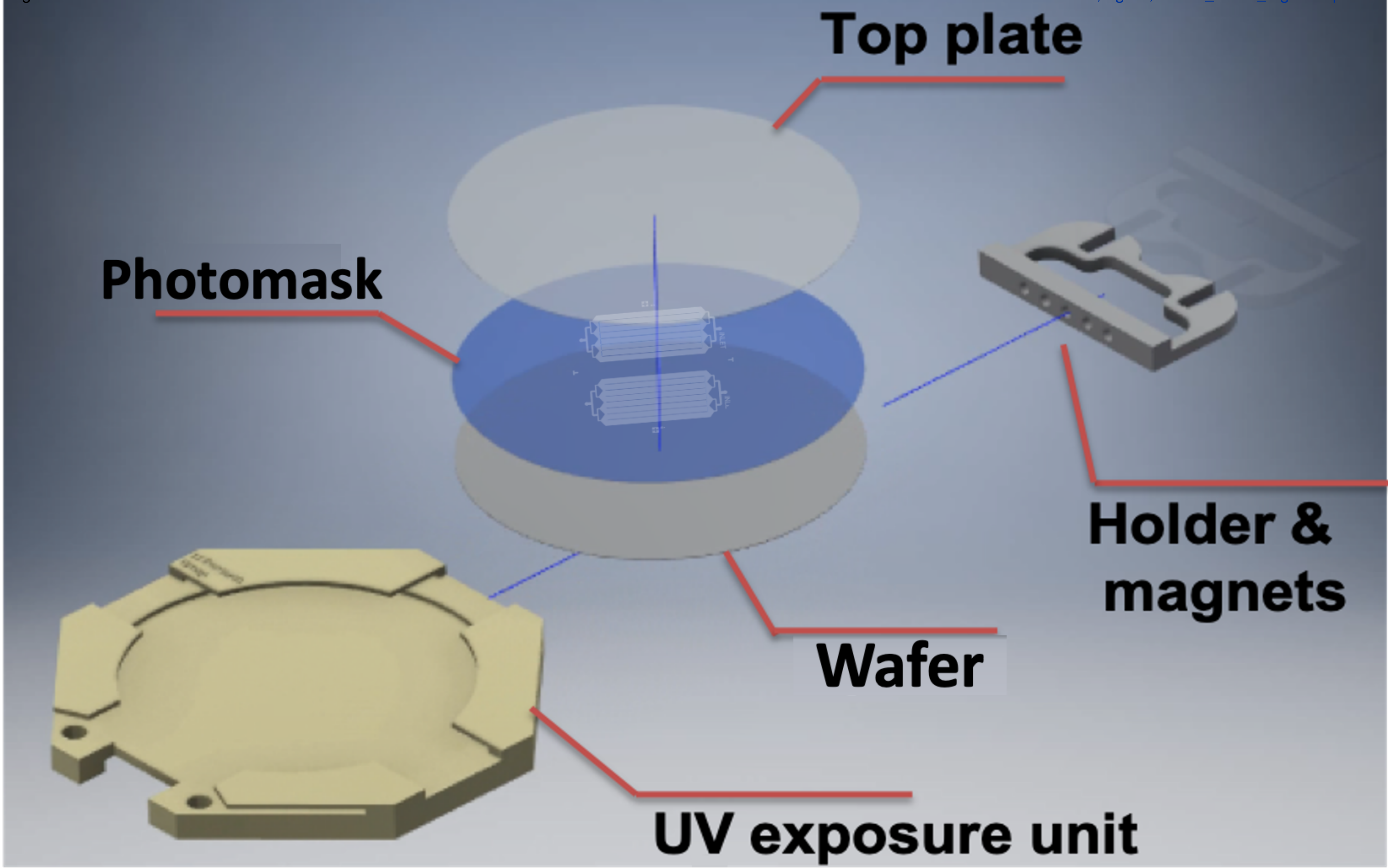


Figure 2

Click here to access/download;Figure;MMAD_JOVE_Figure 2.pdf

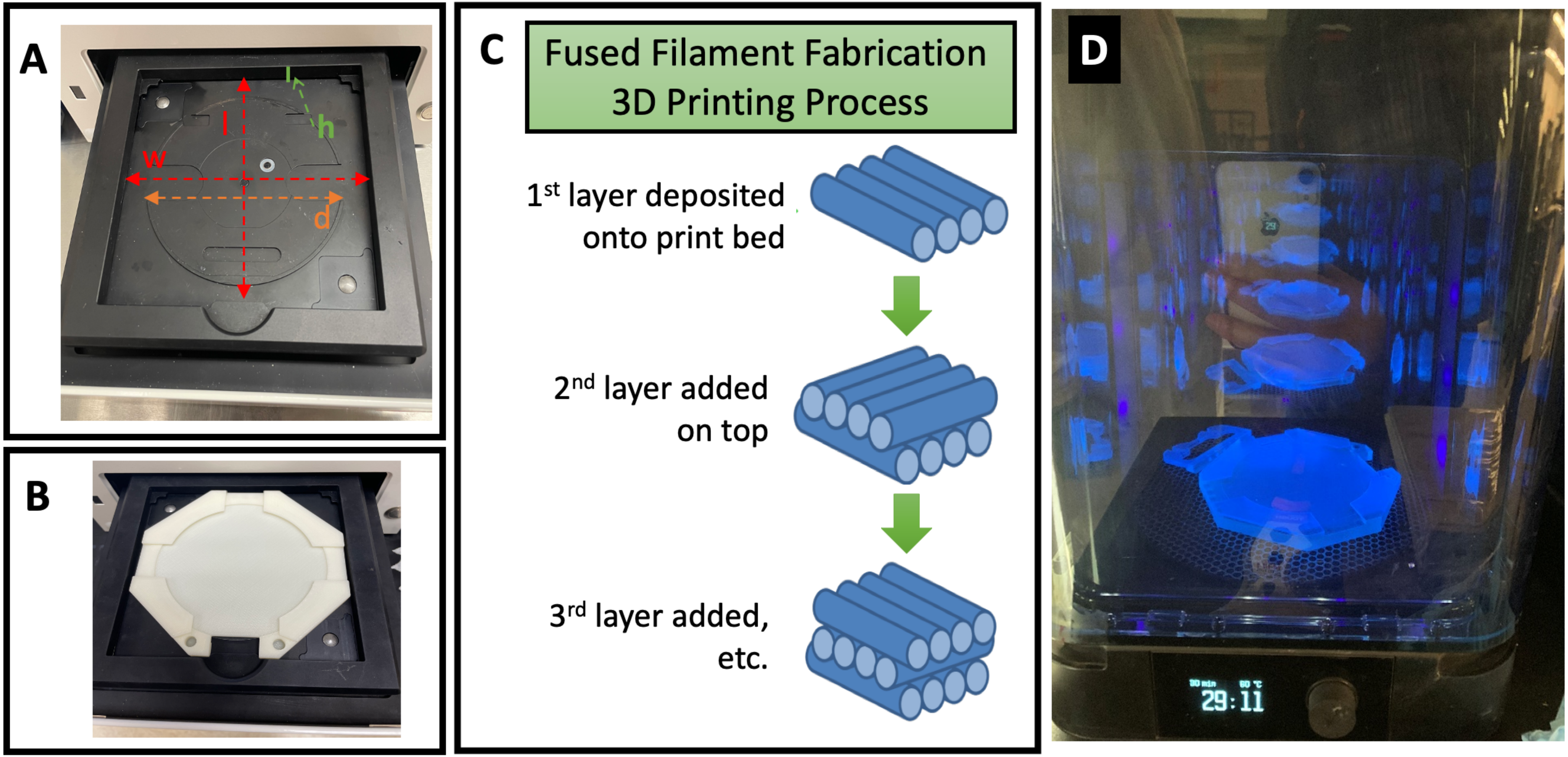


Figure 3

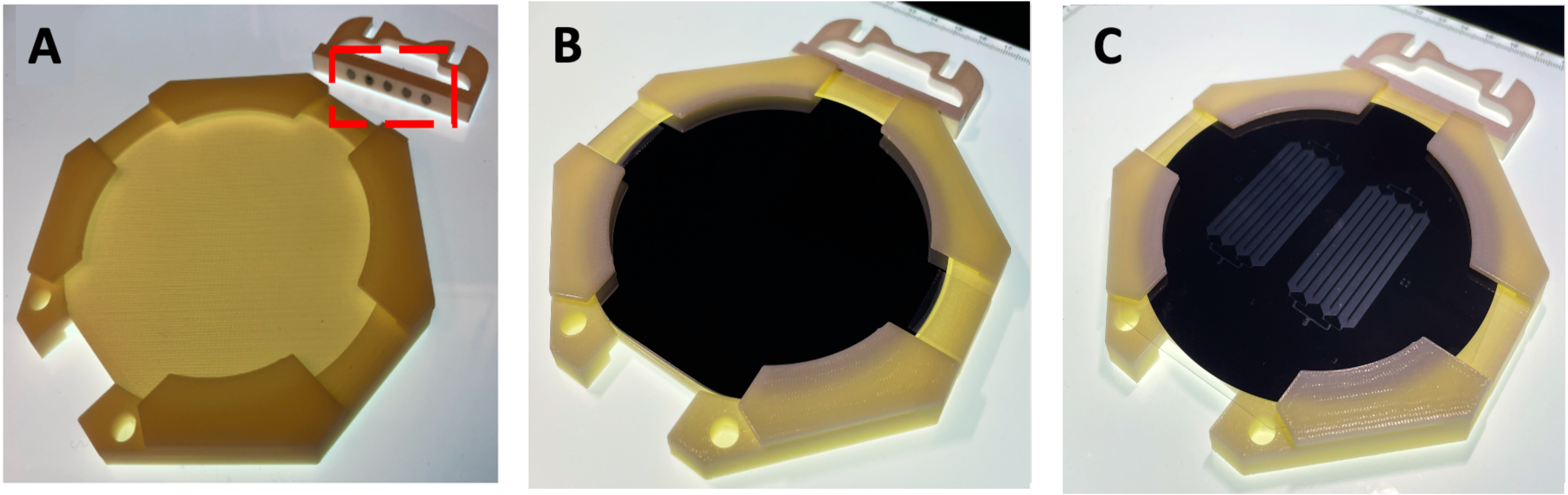
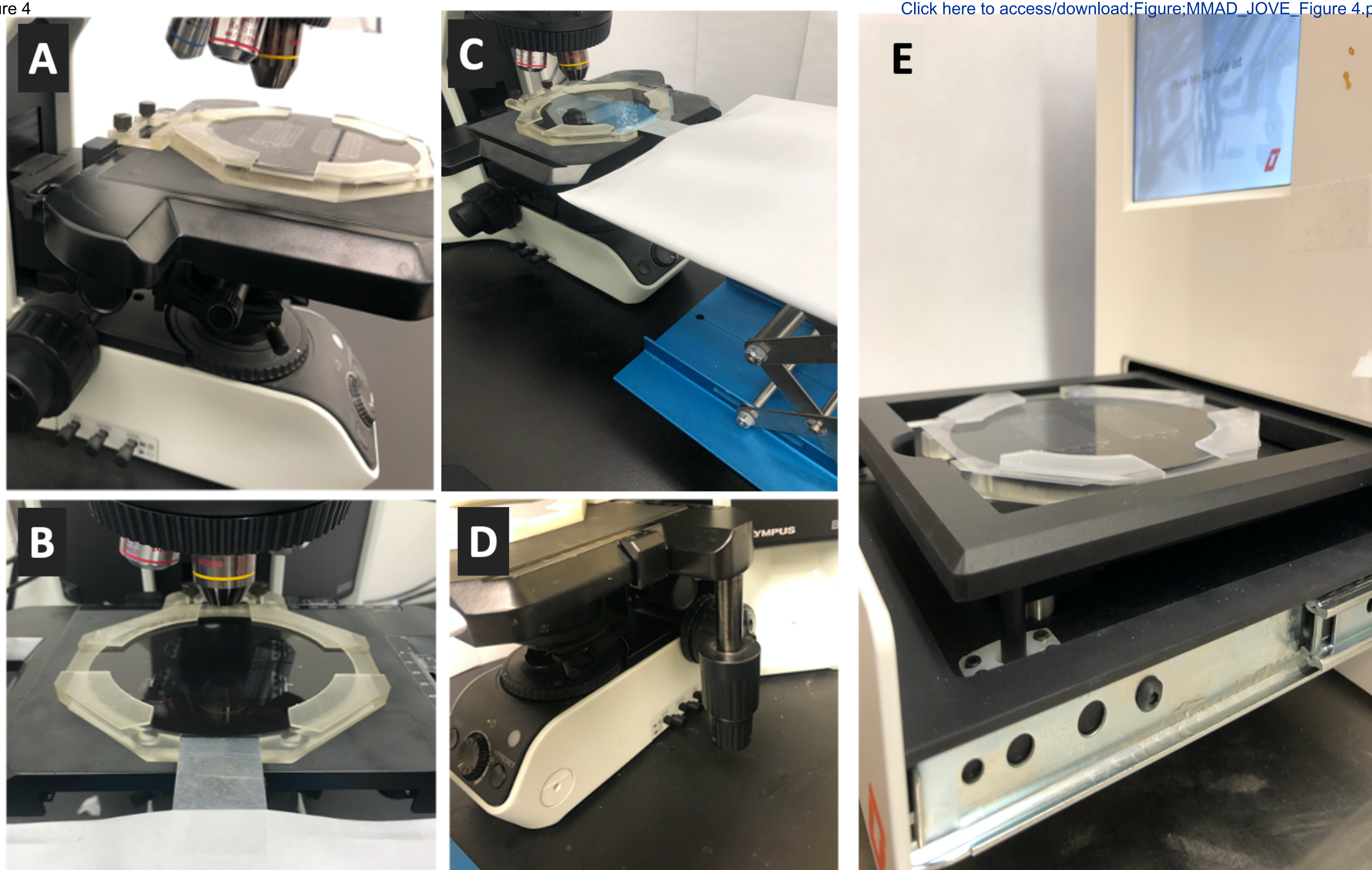


Figure 4



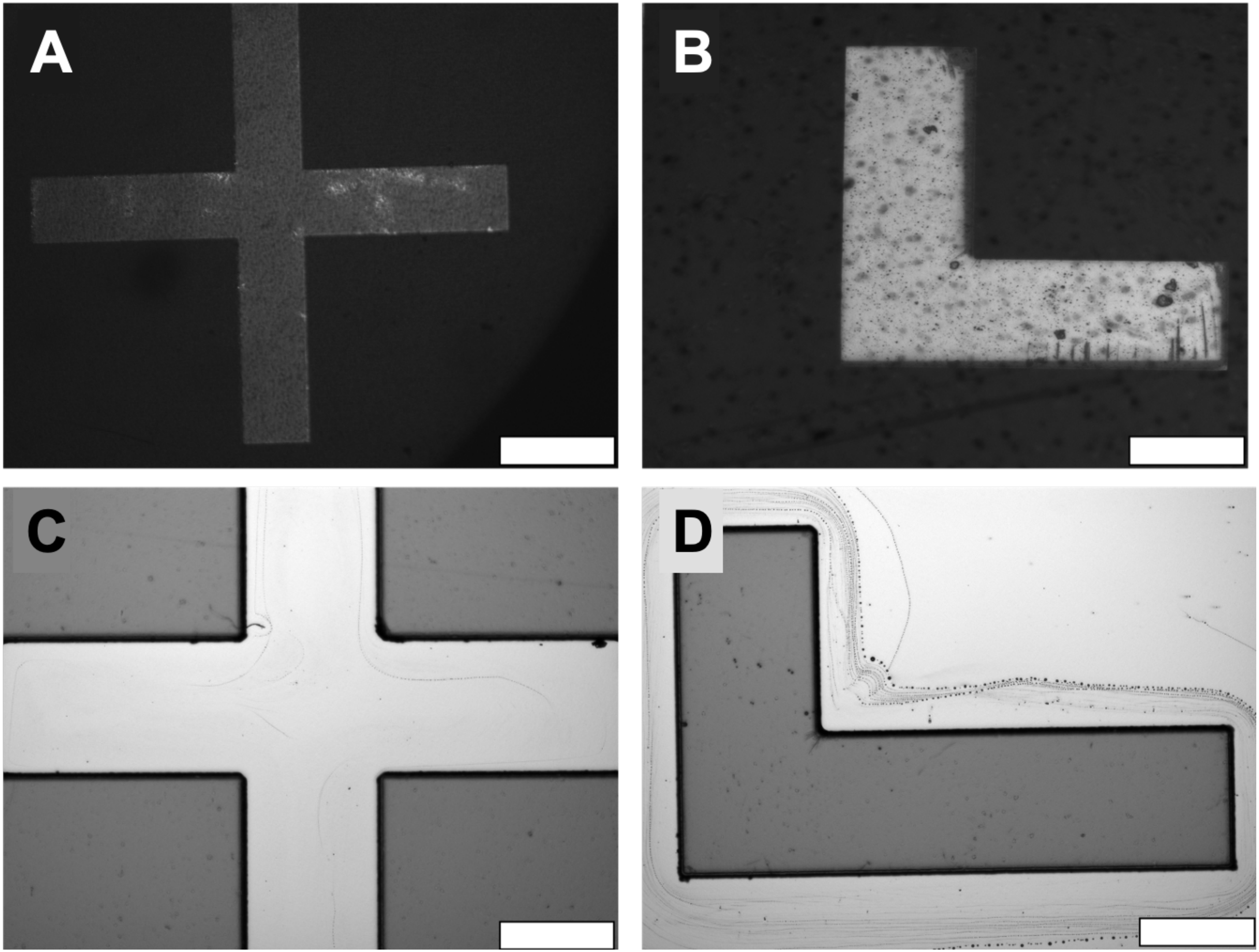

A
B
C
D

Figure 6

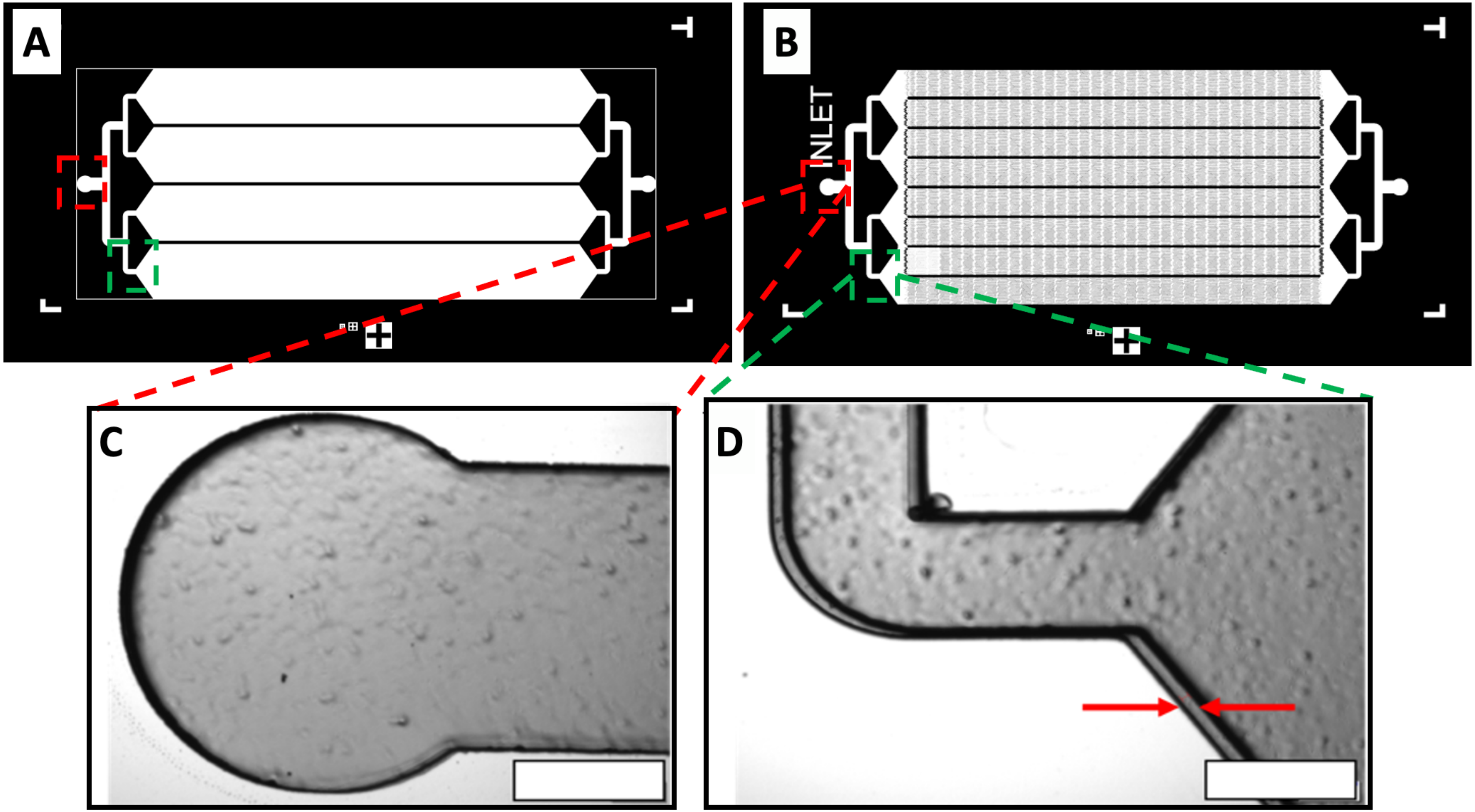


Figure 7

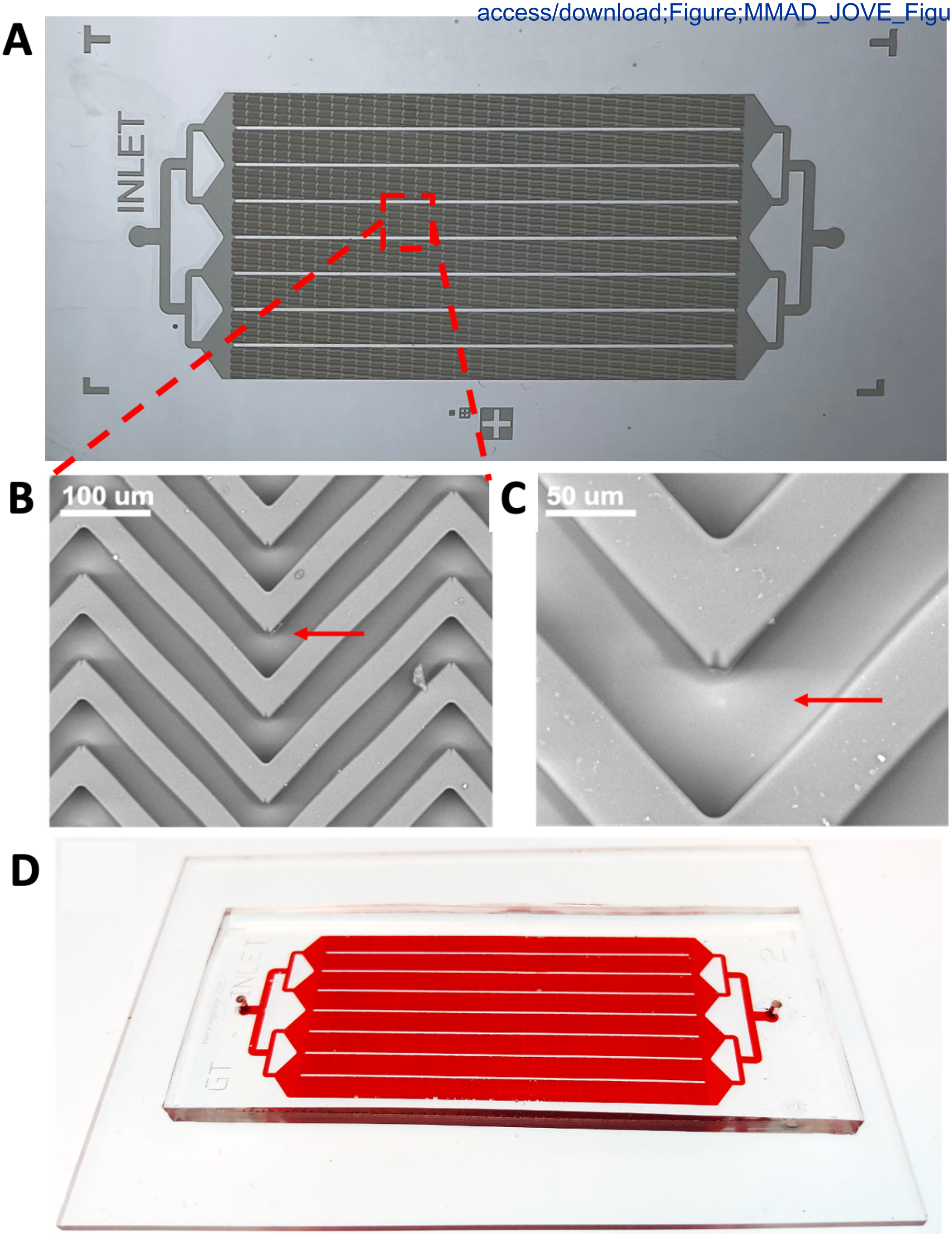
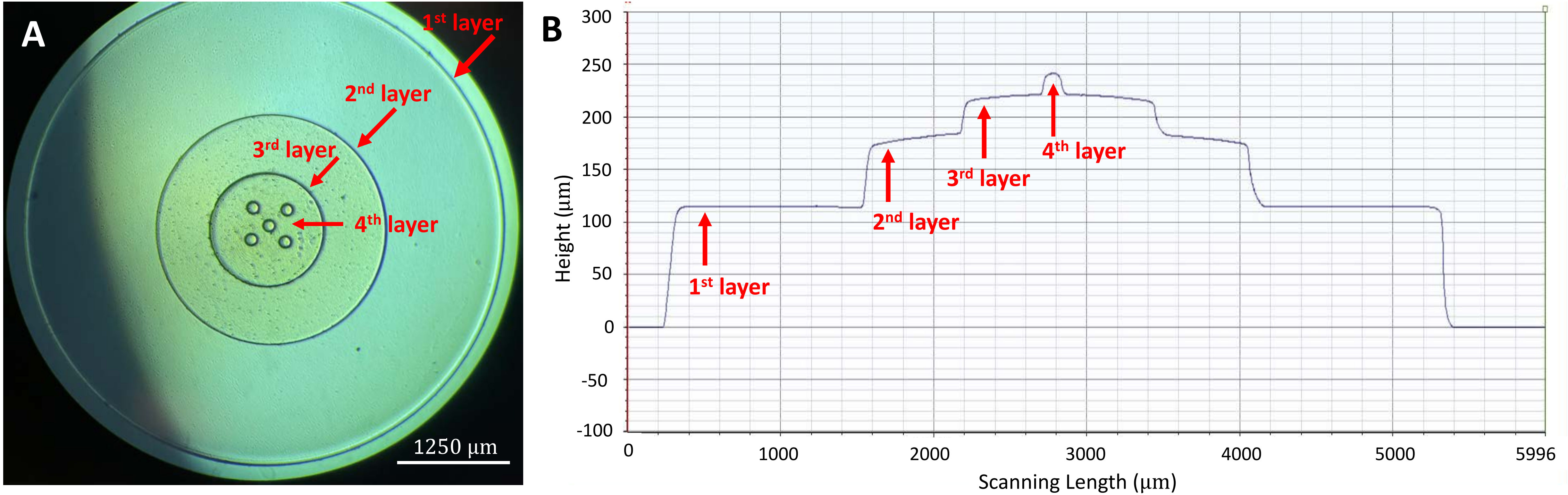


Figure 8

Click here to access/download;Figure;MMAD_JOVE_Figure 8_s.pdf



Name of Material/Equipment	Company	Catalog Number
Acrylonitrile Butadiene Styrene (ABS), 3D Printing Filament		
BX53, Upright Microscope	Olympus	
Form 2, Stereolithography 3D printer	Formlabs	
Advanced Hot Plate Stirrer	VWR	97042-642
Isoproyl Alcohol, 70% (v/v)	VWR	BDH7999-4
Light Colored Marker	Sharpie	
Magnets, 3 mm x 3 mm	WOTOY	
SYLGARD 184 Silicone Elastomer Kit	DOW	4019862
Petri Dish, 150 mm x 15 mm	VWR	25384-326
Printed Photomasks	CAD/Art Services, Inc.	
Aluminum Support Jack - 8" x 8", Scissor Lift	VWR	12620-904
Silicon Wafer	University Wafer	452
Sodium Hydroxide	VWR	
Sonication Bath	Branson	CPX3800H
Spin Coater	Laurell Technologies Corporation	Model WS-650MZ-23NPPB
STRATASYS SR-30	MakerBot Industries, LLC	SR-30
Stratasys uPrint SE 3D Printer	Computer Aided Technology, LLC	
SU-8 50	Kayaku	Y131269 0500L1GL
SU-8 100	Kayaku	Y131273 0500L1GL
SU-8 Developer	Kayaku	Y020100 4000L1PE
Super glue	Gorilla Glue	
Trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane	Sigma-Aldrich	448931-10G
Tape	Scotch	
Form Cure, UV Curing Chamber	Formlabs	FH-CU-01
UV-KUB2, UV Light-Exposure Box	Kloe	UV-KUB2

Comments/Description

Provided by the Texas Tech University 3D printing facility

ASIN #: B075PLVW8W

Dissolvable support material for 3D printing

Point-by-point Response to Editorial and Reviewers' Comments:

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Thank you for the comment. We have made the necessary adjustments in our revised manuscript.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Olympus BX53 microscope

Thank you for the comment. All commercial language has been removed from the revised manuscript.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We appreciate the comment. The protocol has been revised to only include text written in the imperative tense.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Thank you for the comment. We agree that the protocol needed to be revised to better explain the important steps needed for designing and using the MMAA. We have shortened the protocol by adding in necessary citations for the photolithography process and have revised the protocol to better explain the central instructions for design and use of the MMAA.

5. 1.3: As you have highlighted this step, will you be demonstrating this for filming the video? Please provide some details about how to generate the files for the two pieces of MMAA.

Thank you for the comment. This part of the manuscript has been unselected for the purposes of the demonstration as this would be difficult to show in the video. Additionally, the generation of the files is dependent on the software being used and the 3D printing process being used.

6. 2.1: Please give details for the post-printing steps.

We appreciate the comment. The post-printing steps are unique to the 3D printing process that is employed to fabricate the MMAA. Both the fused filament fabrication (FFF) and stereolithography 3D printing techniques were used when printing different versions of the MMAA. In addition, several other printers and processes were used in the early stages of the MMAA development to ensure that the 3D printing technique used did not hinder the functionality of the MMAA. It was determined that any printer or process available to the user would suffice to produce the MMAA as the final product was functional in all cases. Due to this, we sought to leave this part of the protocol open ended to allow room for the user to create the MMAA using whichever 3D printing techniques is available/affordable for them as the 3D printing process used does not hinder the effectiveness of the MMAA.

7. 3.1.2: what sort of structures are you referring to as alignment markers?

Thank you for the comment. The alignment markers are typically used in standard photolithography processes and are small shapes/structures added to each layer's photomask that allow the user to then align the previous photoresist layer with the next layer's photomask (see Figure 6A-B). In our process one of the alignment markers used was a small cross on the side of the silicon wafer (see Figure 5), however the intricacy of the alignment marker can be enhanced in order to increase the accuracy of the layer's alignment. This was briefly mentioned in the note after section 3.1, however further detail about this feature has been added to the revised manuscript to clarify this point. In addition, the photomasks used to create the double layer herringbone master mold described in the paper have been added to Figure 6. Figure 6A and 6B can now be referenced to for a better illustration of the alignment markers that we have described in the protocol and the note after section 3.1.2 has been amended to also reference the reader to Figure 6A and 6B.

8. 3.1.3: if you print the photomasks through other facilities, will this protocol remain user-friendly for small labs?

We appreciate the comment. This process would still be user-friendly for small labs that already procure microfluidic devices as this is not a large expense and would typically be employed by labs who already perform microfabrication.

9. 3.2.1.3, 3.2.1.5, 3.2.3.2: How do you perform the soft bake and the post-exposure bake?

Thank you for the comment. The soft bake and post-exposure bake entail heating of the coated wafer at a certain temperature and time. This is explained in the manufacturer's instruction and the exact time and temperature will vary based on the photoresist being used and the desired layer thickness. The manufacturer's instructions have now been cited in the protocol to be referenced to for these details.

10. 3.2.16: How much time is appropriate?

Thanks for the comment. This time is indicated by the manufacturer's instruction for the exact photoresist being used and the exact thickness of the layers desired by the user. The manufacturer's instructions have now been cited in the protocol to be referenced to for these details.

11. As you are using SEM in Fig 7, please consider adding a note in the protocol that SEM can be used for this purpose.

We appreciate the comment. We have added a note within the protocol to include that SEM can be used to confirm the alignment error (see the note after step 3.4.2).

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in

3-6 paragraphs with citations:
a) Any modifications and troubleshooting of the technique
b) Any limitations of the technique
c) The significance with respect to existing methods

Thank you for the comment. The discussion has been revised to cover the details noted above.

Reviewers' comments:

Reviewer #1:
Journal: Journal of Visualized Experiments
Title: Designing, developing, and testing a multi-layer mask alignment Adaptor (MMAA)

General Comments:
This manuscript describes the design, fabrication, and test of a multi-layer mask alignment device (MMAA) as an easy-to-fabricate and cost-effective alternative to the traditional alignment equipment. The authors claim that their method requires no special training for its use as its application is fairly simple and uses standard laboratory equipment, thus providing a useful tool for small labs. The authors demonstrated that the 3D printed MMAA was useful in fabricating multi-layer microfluidic devices with a fabrication error of less than ten micrometers only using regular UV light source, optical microscope, and common lab equipment.
The reviewer agrees with the importance of the development of an easy-to-use platform for the fabrication of precisely aligned multilayer microfluidic devices without using expensive clean-room-based settings. While the design seems logical and properly executed, the reviewer does not believe that the readers will easily understand and trace the whole procedure described in the presented manuscript and figures. The description of representative results and figure legends lack details and contains ambiguity. The overall flow of the manuscript is short of key explanations that may not be friendly to readers who are unfamiliar with the topic. The reviewer suggests that the manuscript should undergo a major revision before publication.

Specific comments:
1. Although the title is concise, it can be more specific. It is vague because it does not indicate what application it is intended for or suggest an impactful purpose. The reviewer suggests that the title includes the following keywords; "cost-effective" "3D printed", "Multi-layer microfluidic devices".

Thank you for the comment. We agree with the reviewer that the title should include more detail as to the purpose of the device and the title has been revised accordingly.

2. Abstract should summarize key quantitative outcomes of the developed methods.

Thank you for the comment. The key outcome of the developed method is the generation of the MMAA which allows for the fabrication of multi-layer microfluidic devices. The quantitative data signifying the success of the MMAA was the minimal alignment error achieved when producing the bi-layer herringbone microfluidic device. The alignment error achieved was less than 10 micrometers and this key outcome has been noted in the revised manuscript's abstract to avoid any confusion. In addition, a four layer master mold was also produced and included in the revised manuscript. We were able to determine with the four layer master mold that alignment errors were no greater than 5% of the designed distance between layers and we have added these results to the revised manuscript as well.

3. In section 1 of the protocol, the author describes '1.1. obtain the dimensions of the tray of the available

UV light emission system.' This can be more specific as to which dimensions are required - is it just the inner circular rim?

We appreciate the comment. The dimensions of the tray of the UV light emission system that we are referring to include the diameter of the inner circular rim of the tray, the inner height of the tray (measured from the floor of the tray to the top of the inner lip of the tray), and the maximum inner tray width and length. The MMAA should then be tailored to fit within these measurements to allow for the MMAA to fit within the tray of the UV light emission system and not cause any obstruction that would hinder the functionality of the emission system. We agree with the reviewer that these dimensions should be more specific and have made this adjustment in the revised manuscript. In addition, we have revised Figure 2 to include images 2A and 2B which illustrate the dimensions we are referring to.

4. Section 3.1.2 could be more helpful with a figure to illustrate the markers in more detail.

Thank you for the comment. We agree that a figure showing the alignment markers would be most helpful, and therefore we have added images of the photomasks used to create the double layer herringbone master mold to Figure 6 in the revised manuscript. Figure 6A and 6B can now be referenced to for a better illustration of the alignment markers that we have described in the protocol. In addition, the note after section 3.1.2 has now been amended to also reference the reader to Figure 6A and 6B to give a visual example of the alignment markers we have used.

5. 3.2.1.1 - sub-sub-sub section seems too much. The author could consider using a, b, c for these more detailed instructions instead for more clarity and organization.

Thank you for the comment. We agree with the reviewer that the use of sub-sub-sub sections could be confusing for the reader. We have shortened the protocol by adding in necessary citations for the photolithography process and have revised section 3.2 to better explain the central instructions for use of the MMAA and eliminate the need for the sub-sub-sub sections.

6. Figure 2: The quality of the illustration should be improved. Is it showing the direction of the printed layers? The legend should be more clearly phrased.

Thank you for the comment. The illustration in Figure 2 was intended to show how 3D printed structures are formed using the fused filament fabrication (FFF) 3D printing process. During the FFF process, the 3D printing filament is deposited layer by layer, one layer on top of another to create the desired piece. The illustration was meant to depict this process; however, the quality and figure legend has been improved in the revised manuscript to better illustrate this.

7. Figure 3: The legend describes that the magnets are indicated with red circles, but it is indicated with a red dotted rectangle.

We appreciate the comment. The legend has been updated in the revised manuscript to indicate the magnets are outlined in the red dashed rectangle.

8. Figure 5: The size of the markers on the photomask (A and B) and on the wafer after exposure (C and D) is different. Please explain.

Thank you for the comment. The size of the markers is different between Figure 5A and 5B and 5C and 5D, because the images we're not taken at the same magnification. The size of the scale bar for Figures 5A and 5B is 200 μm, while the size of the scale bar for Figures 5C and 5D is 100 μm. This was an error on our part and has been noted in the Figure 5 description in the revised manuscript.

9. Figure 6: Please show the macro image of the whole channel structure. What is the design for the first and second layers?

We appreciate the comment. We have revised Figure 6 to include images of the photomask files, shown in Figure 6A and 6B, for both the first and second layer to better depict the device design. The first and second layer photomasks represent the design for a herringbone microfluidic device with 4 parallel channels. The first layer, shown in Figure 6A, includes the outline for the channels to allow for the hollow floor of the channels to be produced after PDMS microfluidic chips are rendered from the master mold. In addition, the second layer, shown in Figure 6B, incorporates the herringbone pattern inside the channels which will line the roof of the PDMS device and allow for mixing of the flowing fluid.

10. Figure 7: Please show the image of master mold prepared using the MMAA. Which part of the device was observed by SEM?

Thank you for the comment. We have revised Figure 7 to include the image of the master mold prepared by the MMAA, as seen now in Figure 7A. In addition, we have outlined in the red dashed rectangle the section of the master mold which was observed by SEM. The section imaged was a part of the microfluidic device that contained the herringbone pattern.

11. Although the authors claim that the MMAA is useful to fabricate multi-layer microchannels, the demonstration in the presented work is limited to double-layer master mold, as can be observed in Figure 7. If multi-layer microchannels can be presented, please use photos in elevated view to illustrate the layers clearly.

We appreciate the comment. We agree that it is essential to show a multi-layer structure to demonstrate the alignment capabilities of the MMAA. In the revised manuscript we have included Figure 8 which contains the image and profilometer data for a four layer master mold that was produced using the MMAA. The image in Figure 8A shows the perimeter of the four layers and the profilometer data in Figure 8B confirms the deposition of four distinct layers.

Minor comments:
1. Throughout the manuscript, the spelling of '3D printing' is not standardized, i.e. sometimes spelt as '3-D printing'.

Thank you for the comment. The spelling of ‘3D printing’ has now been standardized in the revised manuscript.

2. There should be more consistency when the author refers to the MMAA. It is termed as Multi-layer Mask Alignment Device. Sometimes it is referred to as MMAA system, sometimes MMAA device and sometimes simply just MMAA. This can also be potentially confusing when the author describes with 'the device…', as there are other devices involved, i.e. Microfluidic device, PDMS device.

Thank you for the comment. We agree with the reviewer that the inconsistency in referencing to the MMAA can be confusing, and we have now made changes to only reference to the device by just the acronym.

3. There are numerous sentences that are too long and hard for readers to understand. Split up the sentences if they involve more than one subject - punctuations can help.

We appreciate the comment. Long sentences have now been modified accordingly in the revised manuscript to improve readability.

Reviewer #2:

Manuscript Summary:

The manuscript presents a cost-effective DYI mask alignment and UV exposure system, that utilizes: a simple microscope and stage, together with a 3D printed wafer holder, and a scissor lift-based mask holder. The publication of such protocol would be beneficial to small laboratories that do not have access to a micro-fabrication facility. However, some concerns need to be addressed prior to publication.

Major Concerns:

-The authors are missing a literature overview of other DYI aligners. Since several similar systems exist already, the authors should discuss how their aligner is better and/or more novel compared to what has already been published:
https://doi.org/10.1063/1.5035282
http://dx.doi.org/10.1063/1.4927197
https://doi.org/10.1063/1.4976690

Thank you for the comment. We agree that a literature overview of other DIY aligners is necessary to explain the advancement of our mask aligner over what has already been published. We have added a brief overview to our revised manuscript to further dictate the novelty of our system and appreciate the reviewer sharing the above journal articles with us as we have added these articles as references.

-The authors do not present much visual evidence of alignment. Fig 6 shows two layers created with the same mask overlayed upon itself, which is fine. However, in a real application, typically different masks are overlayed to make complex multi-height structures. Therefore, the reviewer would prefer to see multi-height structures prepared using this mask aligner. Figure 7 supposedly shows two layers. One layer is the harringbone pattern, but it is not obvious what the second layer is... The red arrow points to something that looks like the "floor" of the device? If that is the case, then why does that even need alignment? Overall, it is strongly recommended to show a multi-height structure that requires the alignment of multiple unique masks.

We appreciate the comment. The herringbone master mold that was prepared in the manuscript has two layers with differing geometries. Figure 6 has been revised to show the first layer and second layer photomasks (Figure 6A and 6B) that were used to create the herringbone master mold. It is essential that the two layers are aligned to create a hollow floor beneath the herringbone pattern that lines the roof of the device. This allows for mixing of the fluid flowing through the microfluidic device, however we agree with the reviewer that a proper description of the herringbone device was not given. We have thus revised both Figure 6 and 7 as well as the figure legends to better illustrate the design of the herringbone device. In addition, we agree that it is essential to show a multi-layer structure to demonstrate the alignment capabilities of the MMAA. In the revised manuscript we have included Figure 8 which contains the image and profilometer data for a four layer master mold that was produced using the MMAA. The image in Figure 8A shows the perimeter of the four layers and the profilometer data in Figure 8B confirms the deposition of four distinct layers.

-The authors should provide part numbers for things such as the scissor lift, since the z-resolution of this device needs to be known since it is critical to quality alignment.

We appreciate the comment. We agree with the reviewer that this information is needed and have added the company and part/catalog number to the revised Table of Materials.

Minor Concerns:
Figure 6 is missing the caption for pane A.

Thank you for the comment. The caption for pane A of Figure 6 has been added in the revised manuscript.

Reviewer #3:
Manuscript Summary:
The authors describe a 3D printed mount system that provides an adapter between the tray in certain low-cost UV exposure systems (e.g., the UV-KUB) and standard microscopes. Using the aligner and the magnetic mount, they were able to perform multilayer SU8 manufacture to a tolerance of <10 um and used it to manufacture a PDMS microfluidic chip with a multi-level herringbone pattern.

Major Comments:
1) The paper is well-written at a prose level and needs essentially no copyediting.

We appreciate the comment.

2) I think that the described MMAA device shows promise and could be useful for a series of applications in microfluidics and MEMS. However, this manuscript feels incomplete, and it is hard to understand exactly how to design, build, and utilize the MMAA system. There are a number of places where the instructions are very cursory (e.g., "customize the wafer holder and magnetic microscope fastener") and don't go into sufficient details to allow researchers to implement the device.

Thank you for the comment. We agree with the reviewer that the protocol needed to be revised to better explain the process needed for designing and using the MMAA. We have made revisions to the protocol to better explain the entire process.

3) The actual benefits of the device are not clearly described in the introduction and abstract. There are a number of strong uses in MEMS and SU8 manufacture, and are all related to making it easier to perform mask alignment. To perform SU8 fab, you need a considerable amount of equipment besides the mask aligner, including a spin-coater, wet bench, and fume hood, and may still require some manner of clean area for processing. This does lower one major cost barrier, however.

Thank you for the comment. We agree that more detail was needed to dictate the importance and uses of the MMAA. The MMAA was designed as a cost-effective research tool for small labs who currently already have the equipment necessary for master mold and microfluidic device fabrication. It eliminates the need for commercial mask aligners or the purchasing and/or assembly of costly and complex custom-built mask aligners. In addition, we were able to determine the MMAA produces multi-layer master molds with minimal alignment errors allowing for the production of functional multi-layer microfluidic devices. To alleviate any confusion we have added a brief overview to the introduction and abstract in our revised manuscript to further dictate the novelty of our system.

4) In contrast, the paper spends a great deal of time explaining how to do the process of photolithography itself, as well as the generation and use of alignment marks, that should be familiar to most people in the field, and for which there are instructions along with the photoresists. Much of pages 4-6 could be compressed with a brief summary and citations (photolithography, SU8 processing, and PDMS pouring/curing). This would free more room for sections such as 3.2.3.4 - 3.2.3.7 (L192-208) which are the central instructions for the paper for the use of the device as a mask aligner.

Thank you for the comment. We agree that the protocol needed to be revised to better explain the important steps needed for alignment of the layers using the MMAA. We have shortened the protocol by adding in necessary citations for the photolithography process and have revised section 3.2 to better explain the central instructions for use of the MMAA.

5) The figures need to be reworked to make the instructions and features of the MMAA clearer. For example, Figure 4 shows photographs of the alignment process, but there aren't annotations sufficient to allow me to understand what's being done in each step. I think you need at least one clear figure showing, qualitatively, what is happening at each step in the alignment process, and another showing the features that are used in designing and using the MMAA. I think you can leave off figure 5 (Or put it in ESI) as it shows alignment marks that are familiar to researchers who have performed SU8 manufacture before. There need to be more detailed figure captions as well that make the steps and contents clearer.

We appreciate the comment. We agree that the figures and figure captions needed to be revised to better illustrate the designing of the MMAA and the alignment process. We have made significant revisions to the figures and figure captions, including figure 4 which we have now edited to give a better and more holistic description of the alignment process. The figure now depicts all important steps of the alignment process in sequential order, and the figure caption also summarizes this process so the user can better follow along with the protocol.

Minor Comments:
1) I'm not sure that the name of the "Multilayer Mask Algning Device" needs the "Multilayer" as a mask aligner is specifically for the purpose of patterning multiple layers on a single wafer. However, it does act as an adapter to allow a microsope to serve as a mask aligner itself, so something like "Microscope Mask Aligning Adapter" might better describe the system (This specific acronym is just a suggestion, so please don't take as binding)

We appreciate the comment and thank you for the suggestion. We agree that the suggested acronym is a better name for the device to describe its function and we have therefore made this change in the revised manuscript.

2) Please make sure that everything shown in a figure is discussed in text. In several of the figures, there are terms that occur only in the figure and do not occur anywhere else in the manuscript. Similarly, make sure terms like "scissor lift" (L202) are described to make sure it is clear how to use it.

Thank you for the comment. We have revised the manuscript to ensure all properties of the figures are now described in the text. In addition, we have also made sure to describe the use of the scissor lift.

3) Please provide full manufacture details in the Materials/equipment section (e.g., what is the make of the sonication bath, what provided the UV curing chamber if it wasn't home-built, and where did you get your photomasks)

We appreciate the comment. The full manufacture detail has now been added to the revised table of material/equipment file.

4) Please be specific about what type of silane you are using. I assume you're using a silane-derivative such as PFOTS, which is standard in microfluidics and colloquially known as "silane." It is important to distinguish that from true Silane (SH4), which is highly toxic and pyrophoric, and has a different set of industrial applications.

Thank you for the comment. We are using trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane. We have made reference to the SU-8 manufacturer's protocol for the lithography process and therefore this note is no longer in the revised manuscript as this is noted in our reference.

5) In L175 and 218, do you mean "compressed nitrogen gas" or "liquid nitrogen"?

Thank you for the comment. The reference to liquid nitrogen was an error as we actually meant compressed nitrogen gas. We would like to thank the reviewer for pointing out this discrepancy. Due to the revision of our protocol this step is no longer in our revised manuscript.

6) You may want to consider, for demonstrating alignment, building a few standard features into the body of the wafer, so you can compare relative offsets with known shapes. The alignment error is qualitative, but it would help if you could show multiple locations across the chip.

We appreciate the comment. We agree with the reviewer that building standard features with known shapes would be helpful to demonstrate the offset produced when using the MMAA. In the revised manuscript we have included Figure 8 which contains the image and profilometer data for a four layer master mold that was produced using the MMAA. For this four layer master mold we chose to build cylindrical shapes with decreasing diameter to better illustrate the alignment capability of the MMAA as it is easier to visually identify the offset between the layers. The image in Figure 8A shows the perimeter of the four layers and the profilometer data in Figure 8B confirms the deposition of four distinct layers.

7) The SEM in figure 7c doesn't show the larger sidewalls, so it's hard to see from that how the pattern is multilevel. Similarly, the device in 7A doesn't show clearly how this makes use of a two-level microfluidic device. The herringbone pattern is presumably for mixing (unsaid in the paper) so you may want to include a mixing demonstration? Alternately, you can find another demonstration, such as using the two-layer system to manufacture microfluidic Vias.

Other potential applications to consider:
* Optofluidic lithography: (S. Chung. et al., Nature Materials (2008)), which is a lab-level system of photodeveloping liquid in a microfluidic channel using a photomask (when a DMD is not available). This does not share the post-processing challenges that standard SU8 lithography has.

* Patterned UV-treatments: (eg electrospinning, some biological collection techniques) a patterned exposure of UV to a surface alters the surface properties for certain applications. This does not require a clean room or the caustic chemicals, and so the MMAA could really help this happen precisely in-lab

* Microfluidic vias (Kartalov 2006) for multilayer microfluidic channel flow.

Thank you for the comment. We agree that it is essential to show a multi-layer structure to demonstrate the alignment capabilities of the MMAA. In the revised manuscript we have included Figure 8 which contains the image and profilometer data for the four layer master mold that was produced using the MMAA to better assess this issue. In addition, we agree with the reviewer that a proper description of the herringbone device was not given. We have thus revised both Figure 6 and 7 as well as the figure legends to better illustrate the design of the herringbone device and it's application.

Final comments:
I apologize if I have misunderstood your explanation or missed some details. However I do not believe I can fully evaluate the strenghts and weaknesses of the proposed methodology until the paper has been rewritten to focus on the design, manufacture, and use details of the specific aligner. I think that the

MMAA really does have value for a number of research labs, and I would like to see how it can be used and more generally adapted.

Thank you for the comments and advice. We greatly appreciate the feedback as it has provided us with many ideas to improve the overall quality of our manuscript. We hope the revisions have made clearer the application of the MMAA.

Reviewer #4:
Manuscript Summary:
This manuscript by Celine R. Garcia and co-authors developed an easy-to-use platform for multilayer photolithography. Overall, this is a solid method and should be useful for the labs that want to fabricate multilayer microfluidic or micro-devices in the lab.

Major Concerns:
My biggest concern is that there is no rotary stage, so the angle is very hard to adjust when aligning the wafer and photomask. It seems that people can only use their hand to rotate wafer or photomask directly to align them. This is very difficult to do. The authors should find way to add a rotary stage in this system to make the angle easier to adjust.

We appreciate the comment. In the revised manuscript we have included an essential step to the process, which can alleviate the need for a rotary stage to keep the cost of the system low for the user. The photomasks we have designed contain a straight edge which aligns perfectly with the straight edge of the silicon wafer. When aligning the photomask to the silicon wafer the straight edges are superimposed to ensure there is no rotational error between layers. However, due to human error when fixing the photomask to the wafer before UV light exposure we believe that any rotational error that may occur would be within approximately 5% of the designed parameters. This step has now been dictated in the protocol of the revised manuscript.

Minor Concerns:
Some minor comments:
1. The authors should draw photomasks in figure 1 to better illustrate this platform.

Thank you for the comment. We agree that the illustration needed to be revised to better represent how the platform is used, and we have revised figure 1 to make this more clear.

2. The current description of aligning second photomask to the wafer is unclear. Based on my understanding, the photomask is lifted by a z-stage so that it does not contact with the wafer, then the wafer is moved by microscope x- and y-stage to align with the photomask? The authors should make this part clearer.

Thank you for the comment. Yes, the aligning of the second photomask is performed by fixing the z-position of the photomask above the wafer and using the x- and y- direction knobs of the microscope stage to then move the wafer and superimpose the alignment markers on the photomask and wafer. We agree with the reviewer that the protocol needed to be revised to better explain the process needed for alignment of the layers using the MMAA. We have made revisions to section 3.2 of the protocol to better explain this process.

3. Quantification results should be added. The authors only used a couple of images to demonstrate the results. They should add more quantification results to indicate the translational error and rotational error.

We appreciate the comment. We agree with the reviewer that further quantification results would be most helpful. A four layer master mold was produced and presented in the revised manuscript. We were able to determine with the four layer master mold, that we have now included in the revised that translational alignment errors were no greater than 5% of the designed distance between layers and we have added these results to the revised manuscript. In addition, the photomasks we have designed contain a straight edge which aligns perfectly with the straight edge of the silicon wafer. When aligning the photomask to the silicon wafer the straight edges are superimposed to ensure there is no rotational error between layers. However, due to human error when fixing the photomask to the wafer before UV light exposure we believe that any rotational error that may occur would also be within approximately 5% of the designed parameters.

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