3D printed molds for PDMS-based microfluidics tests on neurons

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This document is represented by a resource in the NRP VAS

Important links

Lab-On-a-chip project page
See initial work by Tibi and Daniel

R&D reports cited

- PDMS Curing Onto 3D Printing Resin
- BIOCOMPATIBILITY OF THE 3D PRINTING PLASTIC SubG+ (MakerJuice Labs)
- R&D - Exploring the potential of DLP 3D printing for microfluidics prototyping

Main collaborators

Tyler (initiator of the project)
Daniel (maker)
Tibi (facilitator)

Goal

To make a master mold (positive relief) using the 3D prototyping technique that SENSORICA has developed, and compare the generated replicas with PDMS replicas fabricated by soft lithography with a standard silicon substrate.
We'll use a CAD file design that we need to make into a format accessible to B9Creator.
The prototypes (molds for PDMS) will be characterized and tested with standard silicon moulds in a side-by-side manner.
Then we will see if neurons are as viable inside the devices as fabricated by your method as by standard soft lithography.
The resin is likely to have some cytotoxic effects in these neurons, we anticipate that after a few PDMS replicas are made, the remaining resin will be negligible on future PDMS replicas.

This project is in synergy with the 3D printer project, and more precisely the Micro 3D printer project.

Introduction/Significance

One of the major factors that creates a bottleneck between the number of people using microfluidic technology and the number using the technology to answer biological questions is the bio-compatibility of the materials being used. Even though PDMS-based devices are widely cited as being readily bio-compatible, one must be very particular in the preparation and handling to ensure cells are happy within their environment.

Of the range of potential cellular models of study, they can all be broadly classified into 2 main categories: cell-lines and primary cells. Cell lines are necessarily able to continue dividing to expand their numbers. As a result, they are generally more robust to challenging environments, however they also represent more poorly the original cell types from which they were isolated. Thus, they are limited in the depth of biological conclusions they can provide. Primary cells are

dissected and dissociated from an embryo or animal, and as a result they directly reflect the biology in question. However, since the drastic change from life in vivo to life in vitro is so drastic and occurs in one generation, primary cells are less robust to changes in their environment, and are generally harder to keep happy. One of the major struggles in making microfluidic devices available to study biology is having them compatible with primary cells. Therefore, a reasonable standard to measure the bio-compatibility of a new technique of microfabrication will be to test the viability with primary cells.

There are plenty of potential primary cells to study, and the relative interest that each will receive from researchers will depend almost entirely on their field of study. Testing all primary cell types individually would be a time and cost-intensive path to pursue. A more reasonable approach is to choose a primary cell type that is less robust than most, such that any conclusions made with this type of primary cell could be reasonably extrapolated to most other cell types. For this reason, we choose to work with primary neurons from the embryonic spinal cord from rats. These neurons grow long axons in culture, and have pronounced growth cones at the axon tip. Furthermore, a lot can be concluded about the health of the neuron by the size and morphology of the growth cone, which are known to ‘collapse’ and retract when their environment is not optimal. Therefore, primary neurons would make for an excellent gold-standard to assess the viability of primary neurons within a microfluidic device.

Info

Use the B9Creator for larger structures

A high definition DLP 3D printer (B9Creator) was already used by Tibi and Daniel to fabricate microfluidic devices. See announcement.

From Daniel

If we need to print a mold, the channels become walls and the B9Creator works with a video projector thus square pixels.

1- The smallest pixel it can produce is 50µm large and when you want to print a 50µm wall, it represents one row of pixels.. Now the polymer will not polymerize well with only one row of pixels because the light is not intense enough, it takes a minimum of 2 adjacent rows of pixels where the light they shoot overlaps and becomes strong enough to polymerize the resin. (it would work if you want to print channels directly, print a positive from wich you will create a mold)

2- And most important (either positive or negative), the wall will not print out with round curves because the projector shoots a grid (matrix) of square pixels, it's as if you try build a rounded wall with cubes in Minecraft, or with square Legos... This will create unwanted turbulances in the fluids.

We can set the z resolution (layer thickness) anywhere between ~10µm and 100µm, the stepper motor and main screw configuration allows for ~6µm steps.

I don't think the B9 (or any 3D printer out there) can do the job for 50µm wide channels, it starts to have acceptable results on channels 200µm and wider.

From Tyler
To be compatible with the B9, I will make some changes to the design to account for these technical limitations: 1) I will increase the minimum channel width to 200um, so long as all proportions are scaled accordingly, the mixing should still be alright. My only concerns with this are that by raising the volume effectively 4x (assuming same height of 50um), I may be surpassing the threshold where the Reynold's number is no longer sufficiently small for all flow to be laminar. The other concern is for the neurons: we know shear forces have a negative influence, and an increase in the channel width will translate to an increase in flow-rate, but this is an experimental question that I can control for easily enough in my experiments. 2) To account for the issue of not being able to print curves in the channels, I will replace the curves with straight edges; some published versions of this 'premixer gradient generator' have this feature. If, as I expect, the volume is sufficiently low that we're still in the microfluidic regime, then the flow will be laminar and the straight-edge channels shouldn't cause problems with turbulence.

The main purpose of the experiment I want to do is to test first-off whether the devices fabricated in this way allow my neurons to survive. If for any of the above reasons the microfluidic mixing isn't working perfectly and the concentration gradient isn't well established, then this will be a finding independent of their survival and general health. If we find that the neurons are healthy but I can't generate a concentration gradient because of the channel width being too large, then an alternative would be to use a lower channel width with a multiple step replication: I can 3D print a channel instead of a wall (negative relief) and make a secondary mold from PDMS with positive features, and I can then make my devices from the PDMS mold. I've done this previously had success so I'm confident it will work, so long as I'm sure to space all the channels far enough apart from each other that no walls are below the 200 um limit.

It will take me some time to make the necessary changes to the device, so it's very unlikely I'll pass by tomorrow. It's more likely to be early next week, but I will be sure to give advances enough warning.

Use the Micro 3D printer

Open the Micro 3D printer main doc.

The Idea is to polymerize a substrate with a perfect (liquid-like surface), then dip it in photopolymer to draw (polymerize) the walls with a microfiber delivering UV (365µm wavelength) from an LED. At that wavelength, there are some very interesting polymers widely available.

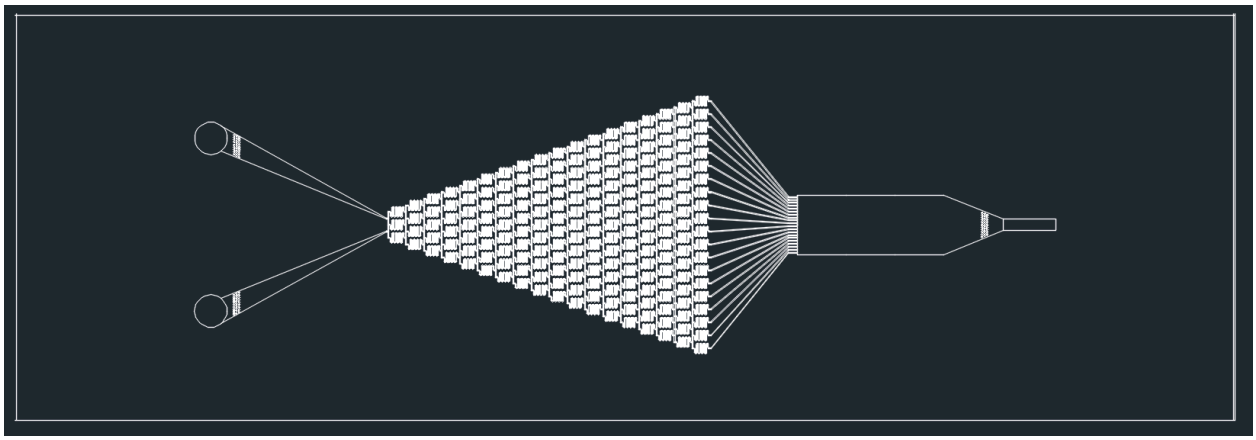
Experimental plan

1. Make appropriate changes to CAD files to be suitable for printing on the b9creator.
    a. Ensure minimum feature (wall) width is 200 microns.
2. 3D print the positive-relief mould.
3. Produce several negative-relief replicas using soft-lithography in PDMS using 3D printed master.
4. Produce several negative-relief replicas using soft-lithograpy in PDMS using silicon master.
5. Create devices as usual using plama to bond PDMS and glass. Load all devices with dissected and dissociated neurons from the same preparation.
    a. Prepare several glass coverslips as a positive control.
    b. For the 3D printed master group, test two sub-conditions:
        i. Neurons cultured within a fluidic flow
        ii. Neurons cultured with no net directional flow
        @/ This will let us know whether the increased flow rate from the larger channels has a damaging influence.
6. Culture neurons under all conditions for 48 hours, then fix them in paraformaldehyde and image them on an inverted microscope.
7. Prepare several representative images of neurons in each condition. (Should not need a quantitative analysis for the time being.)

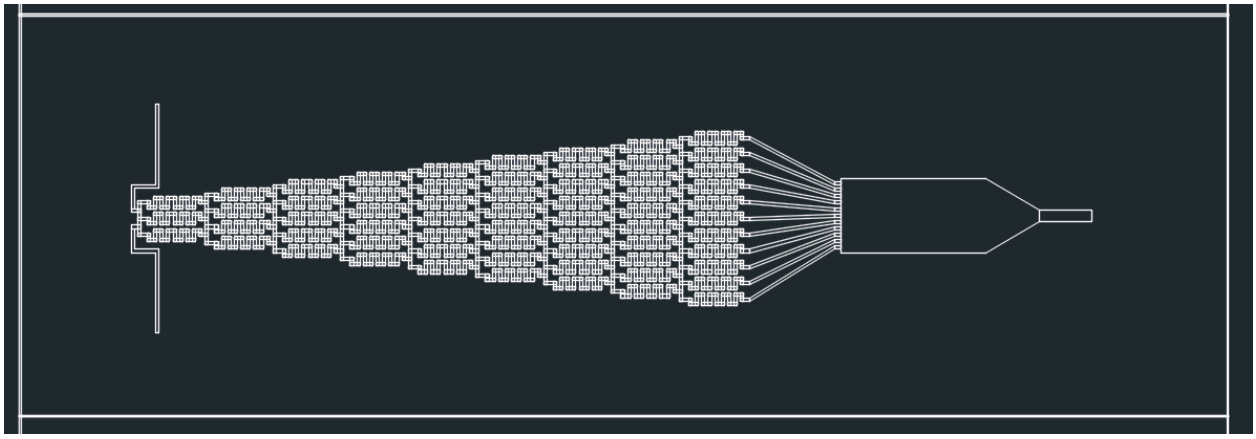
Progress

Adapting CAD files to be compatible with b9creator

The original :

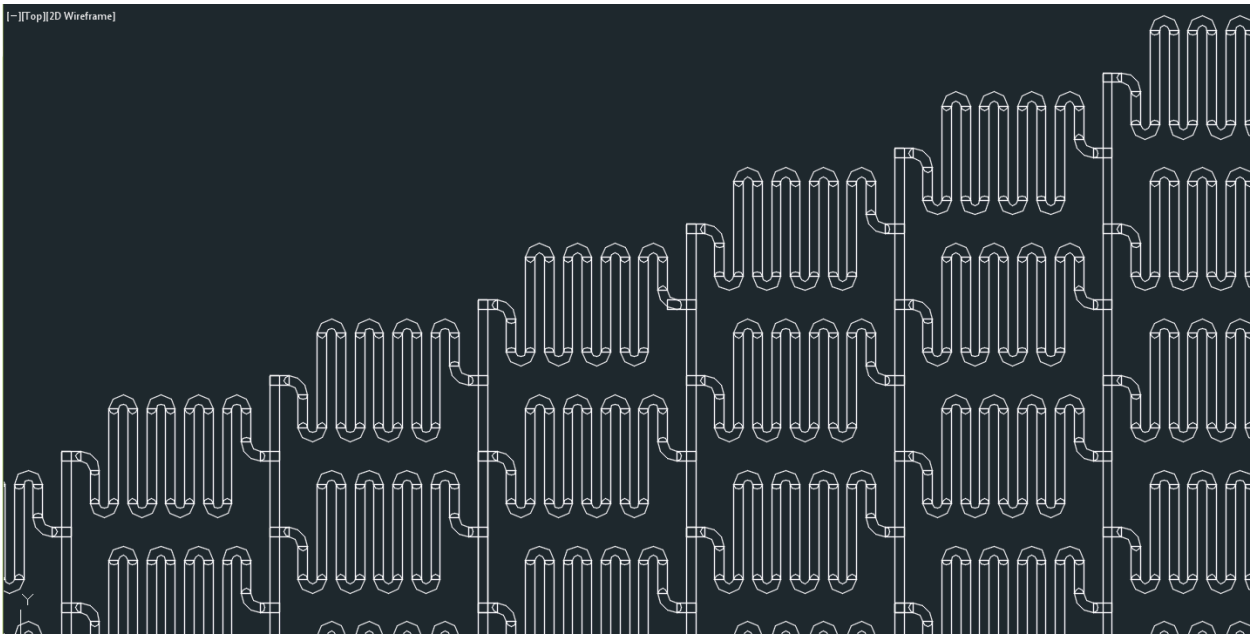


The version modified to be consistent with b9's constraints:

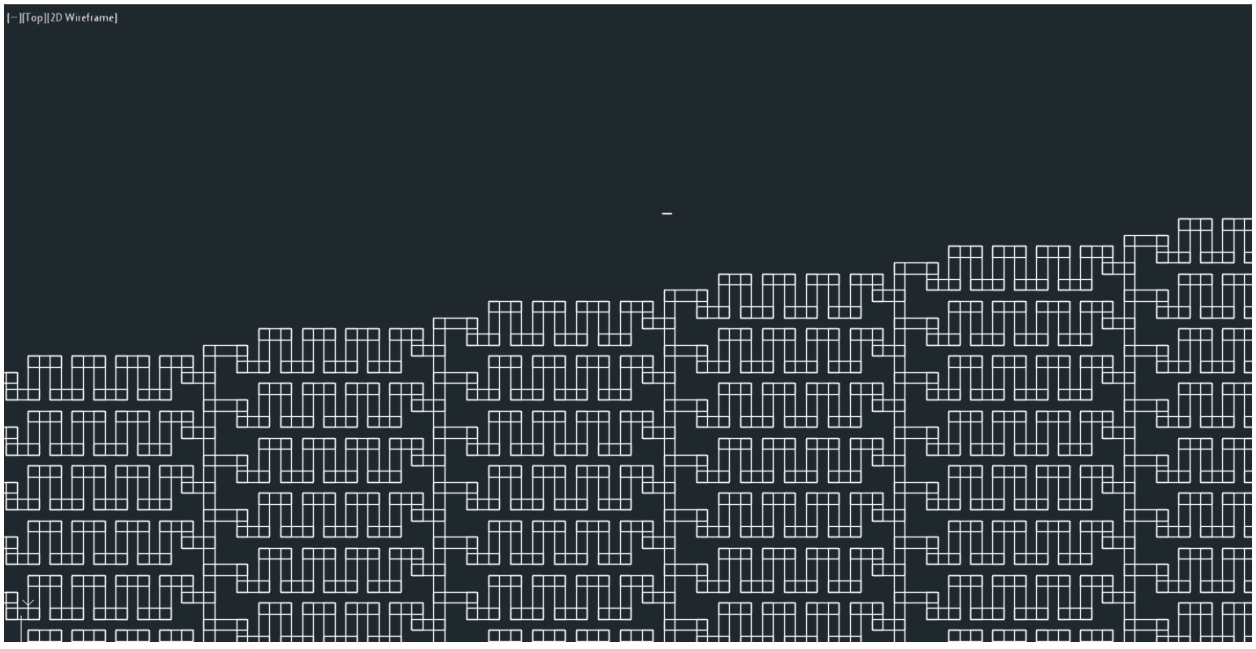


Note: The rectangle surrounding the design has the dimensions of a standard glass microscopy slide (75mm X 25mm). I leave these on the design so it is easy to cut the PDMS to the appropriate size.

Close up on the details of the 'premixing channels'. In the original, the channel width is 50um, with 50um gaps between adjacent channels:

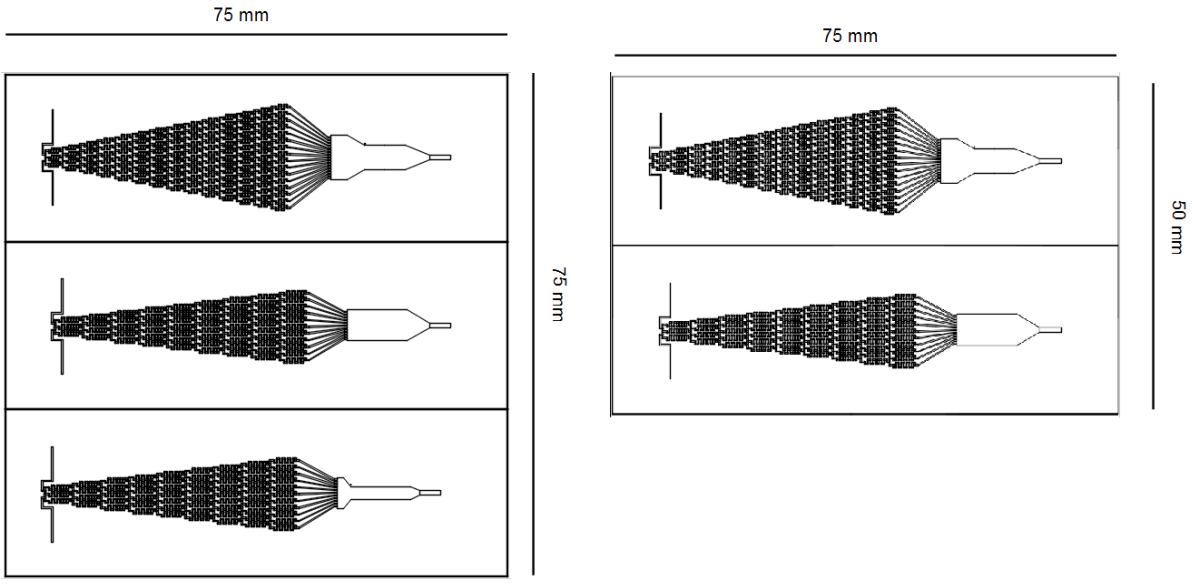


In the updated model, the channel widths have been increased to 200um, along with the spaces between the channels. In addition, all curves and rounded edges in the model have been replaced by straight edges, to be consistent with the square pixels to be printed by the b9creator:



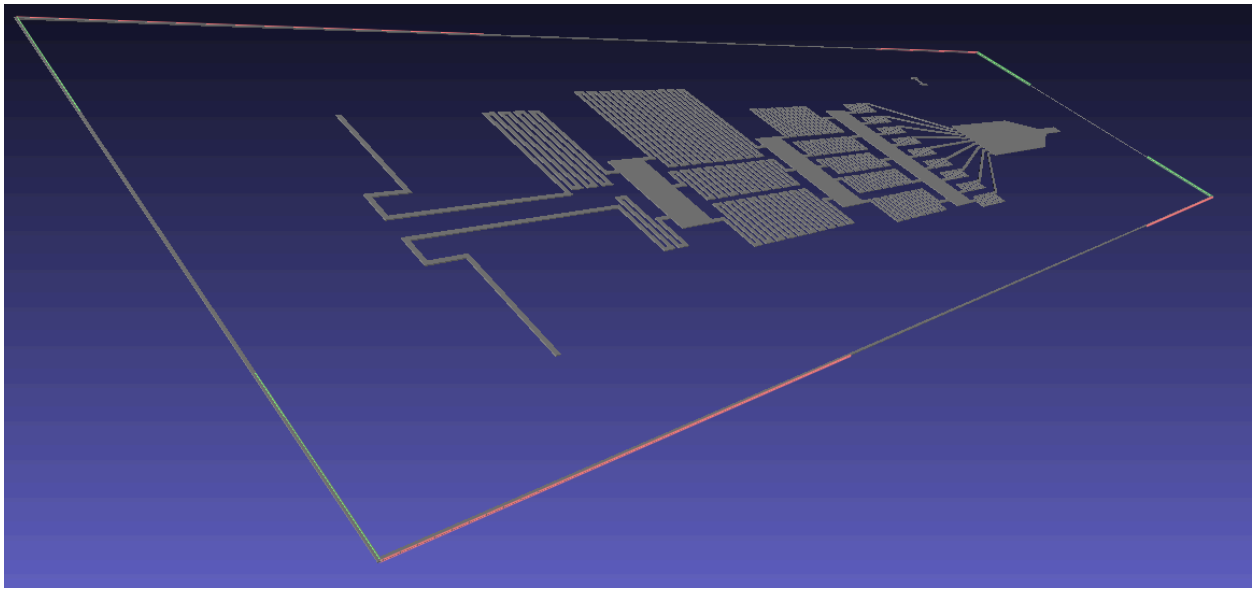
Since I don't know exactly how the mixing will behave with the new flow rate, I propose to try several designs simultaneously. From what I have read about the b9Creator, it seems that different pixel settings allow for different maximum printable areas. If we use the 100 um pixel

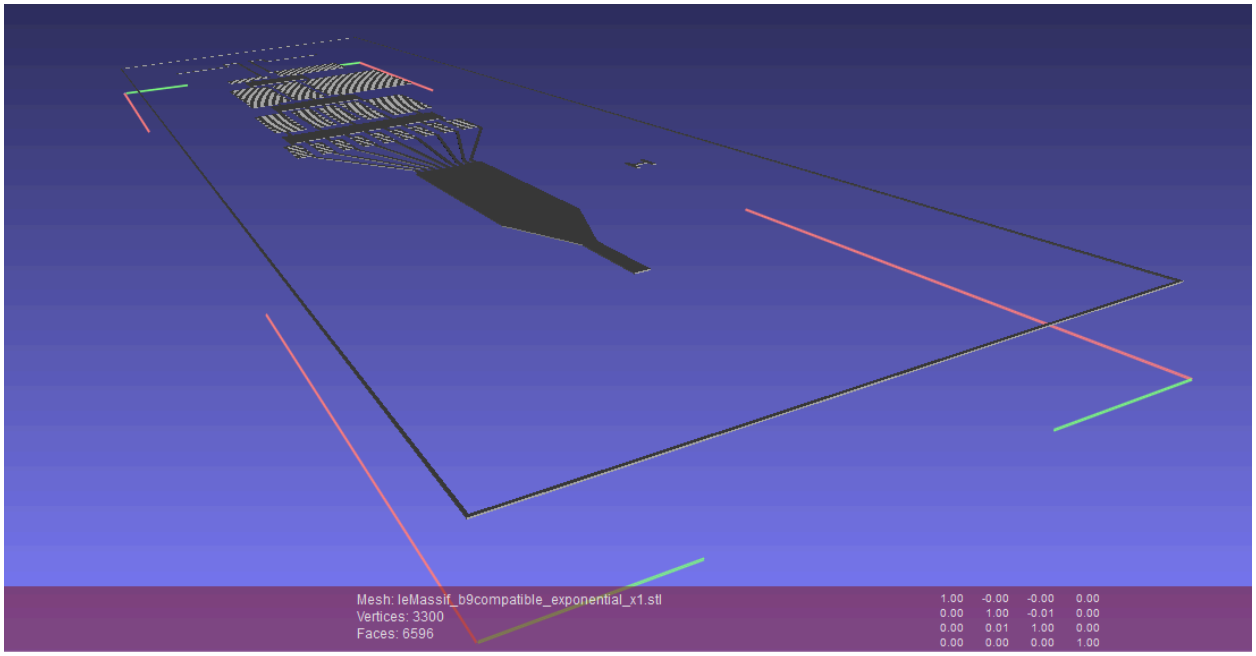
setting, then it should be possible to print 3 designs at once (left). With the 75um pixel setting, we should be able to print 2 designs at once. Unfortunately , the design is too long to print at 50um pixel resolution, so it would have to be changed entirely.



3D models compatible with b9creator

I’ve played around (a.k.a. struggled) a bit with the STLOUT command in autocad but have managed to output the 3D CAD files as *.stl, which should be compatible with the b9creator. Images are included below.

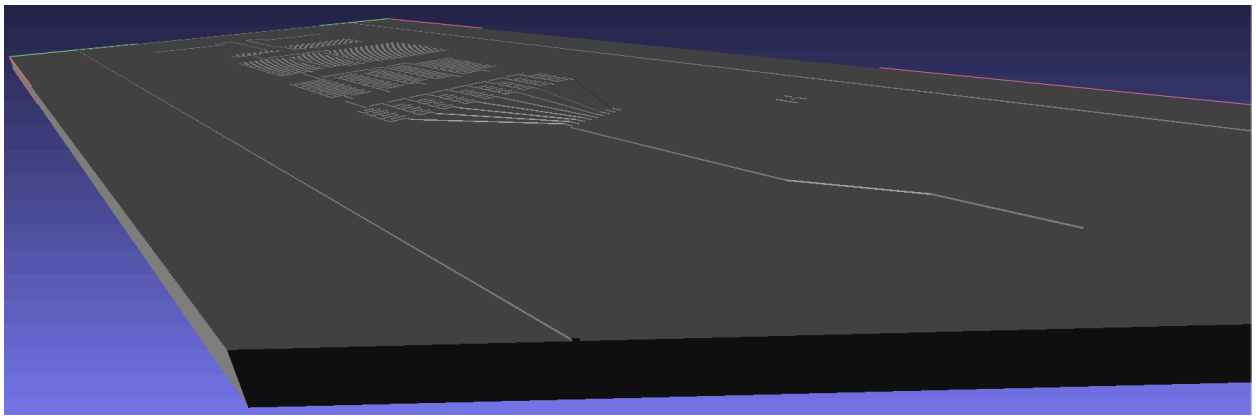




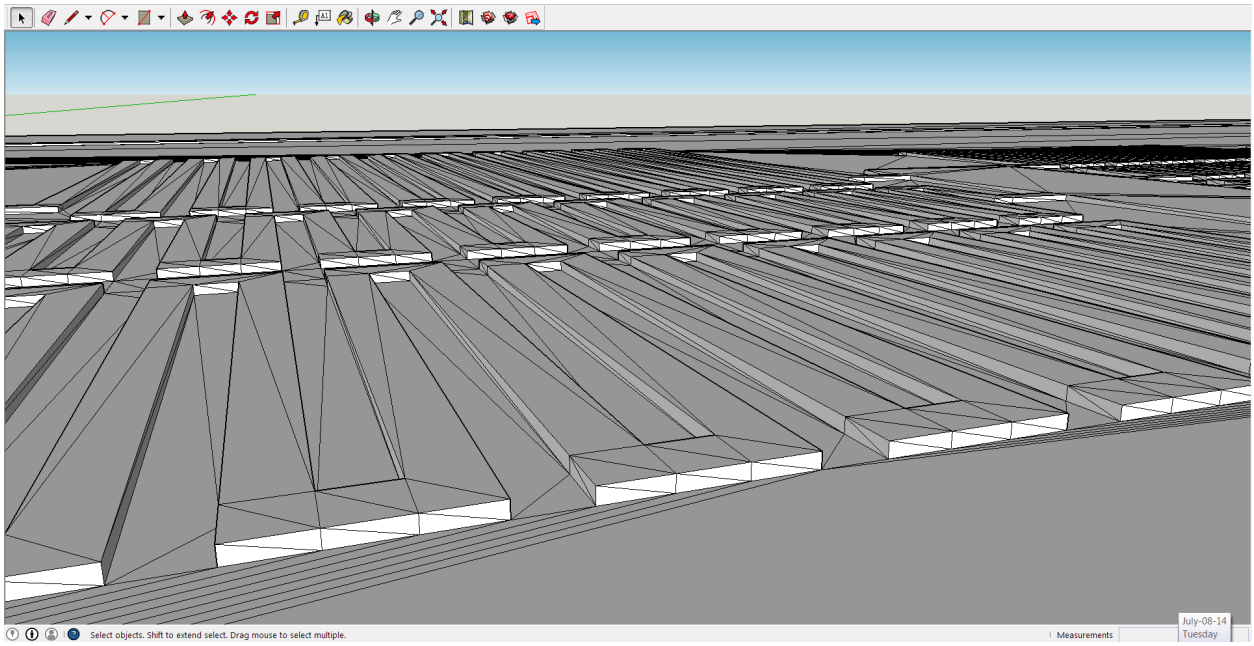
The files: '**leMassif_b9compatible_exponential_x1.stl**' and **'leMassif_b9compatible_exponential_x1(inverted).stl'** are uploaded to the same folder as this document (Lab-on-a-chip)

I've tested the outputted files in another program (MeshLab v1.3.3) to be sure they are complete. Both files I uploaded are mirror images of the same device design (a variation of the premixer gradient generator from the previous images). **Both stl files contain all the features that they should, and have the appropriate height**.

After taking another look at the McGill Collaboration images, I changed the design to include a base. I don't know what thickness is ideal so I figured 1mm seemed appropriate. In this model, 50 um features sit atop a 1000 um base.

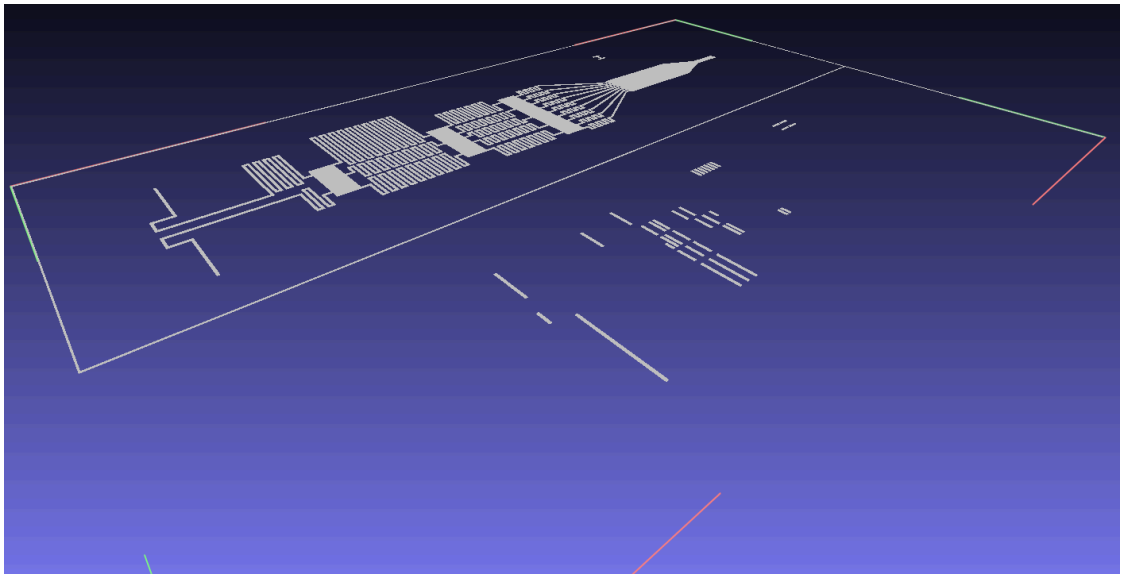


I double-checked to make sure the designs would be compatible with SketchUp, since that (I think) is the software used in the SENSORICA lab. It seems to look alright. Are these lines normal? The file is called **"leMassif_b9compatible_exponential_x1_wBase1mm.stl"**



Challenges

- **File conversion**: the microfluidic design is fairly complex, containing many individual elements. This lead to trouble when trying to output the dwg file as stl. I used the STLOUT command in autoCAD (2015) and found that pieces of the model were being neglected in the stl file (see example below). I found that this occurred because I was trying to create a model with two microfluidic designs. When I removed one of the designs, the model was exported correctly. So, **it seems the issue was having too many elements in the model.**

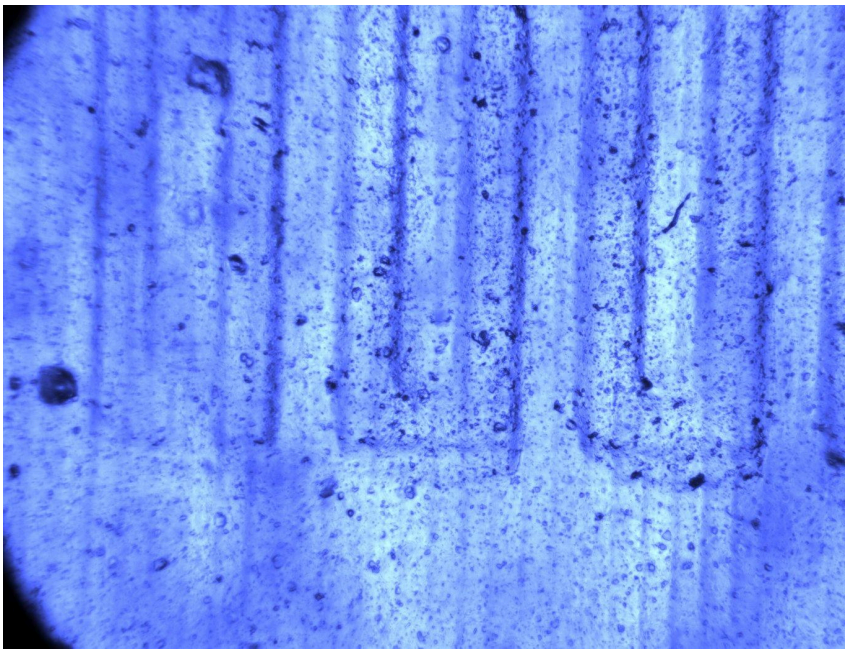


Printed prototype

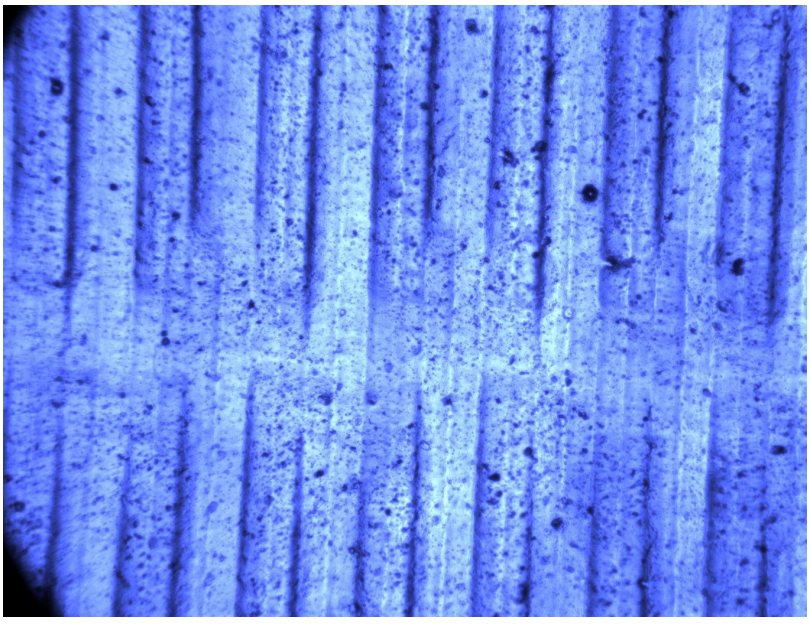
We made a first replica with a height of 50um channels.
We examined the features with a stereomicroscope and they were replicated well (all features were present). However, we were a bit concerned by how close the walls are to one another. Also, there is a clear topography on the flat areas of the chip, but I don't know what influence this will have until I make PDMS based devices and test them.
My first experiments will be to make PDMS replicas and test the flow and mixing with food coloring. I will then post images of this to see how well the fluid is constrained in the channels, and if (as our concern) there is exchange between channels where the resulting walls aren't well sealed. After this initial test, I'll be able to test with neurons in a PDMS replica (assuming the flow within the device allows it at this point).
Daniel and I discussed a few alternatives for the fabrication. In one case, we would print the walls to a height of 100um instead of 50, resulting in 100um channels. Alternatively, the entire pattern could be scaled down in size such that the minimum wall size is 150um instead of 200, using the 75um pixel resolution of the b9. This would reduce the risk of fluid exchange between channels.

Photomicrographs of first mould

I started out by taking a few images on a Leica upright bright field microscope with a 5x objective. I was under the impression that the height of the surface imperfections were on the same order of magnitude as the feature (wall) height of 50 microns. It was difficult to find a focal plane where I could easily see the channels, in absence of other surface topographies.



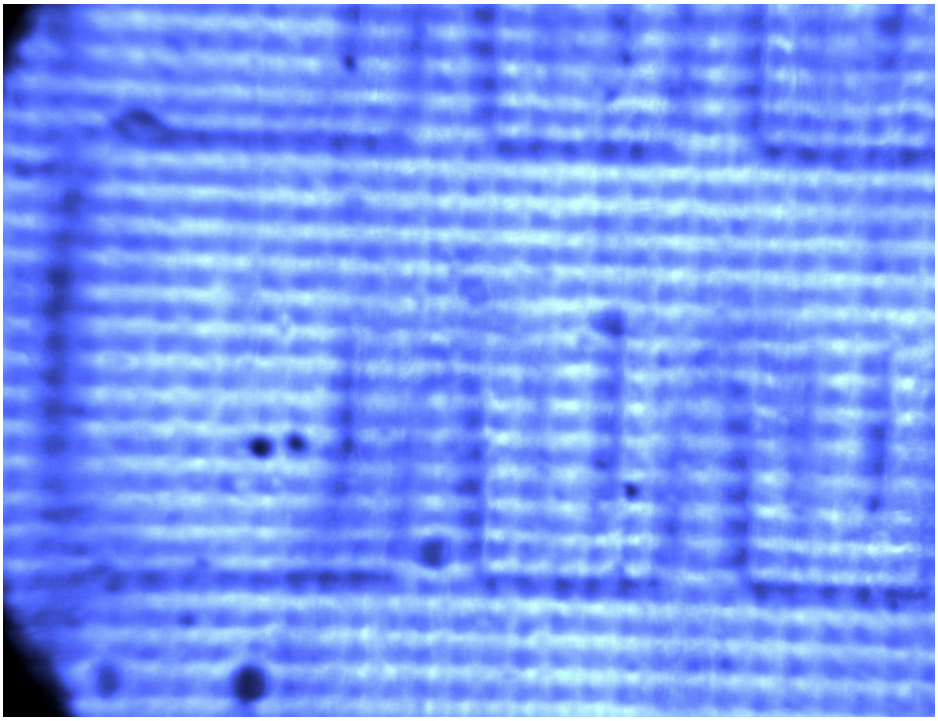
There was

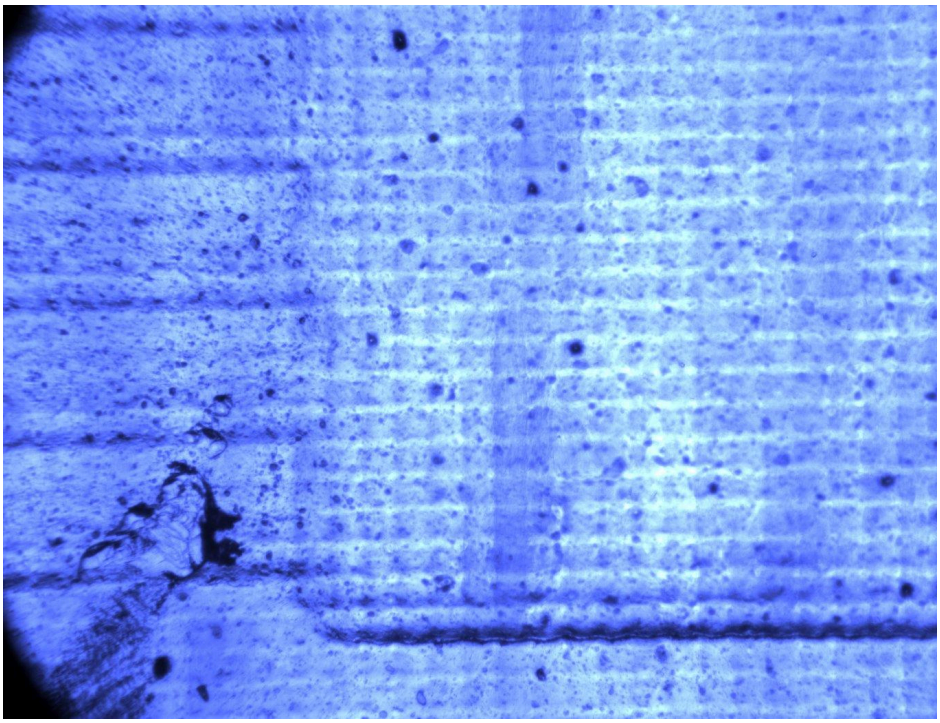


also a strange

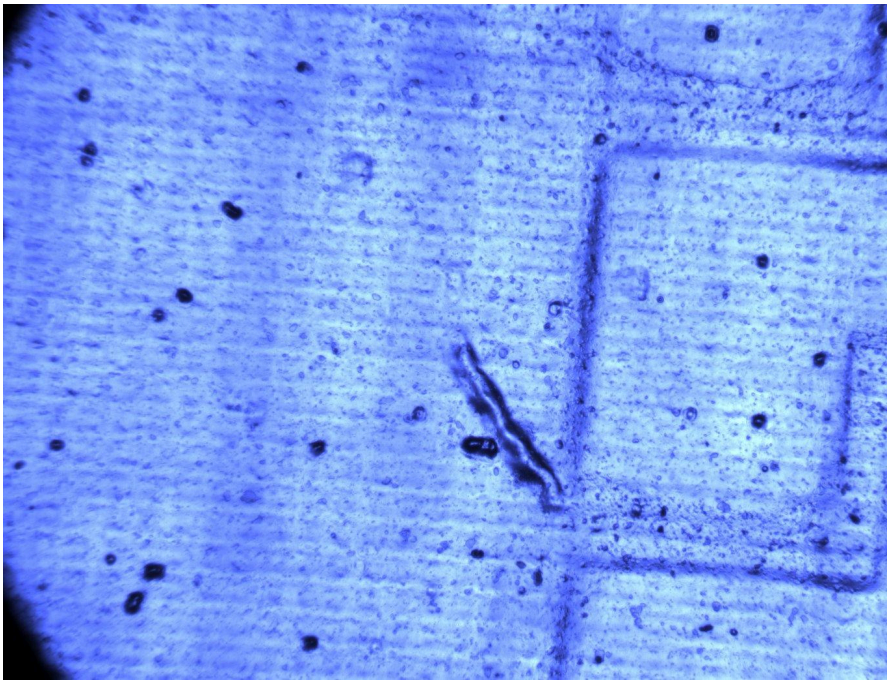
effect where the mixing channels came close to one another. As you can see in the image below, the horizontal channels aren't as well defined as the vertical ones (with respect to the image coordinate system). This makes the imaging blurry in these regions, and will most likely result in discontinuous channels in the PDMS replica.

In the next two images, we can see very clearly the topography on the surface of the mould caused by the pixels of the projector. This isn't necessarily a problem on its own. The regular spaced bumps are definitely smaller than the features (since the focal plane was smaller), but they are clearly on the same scale, and might cause trouble as a result.

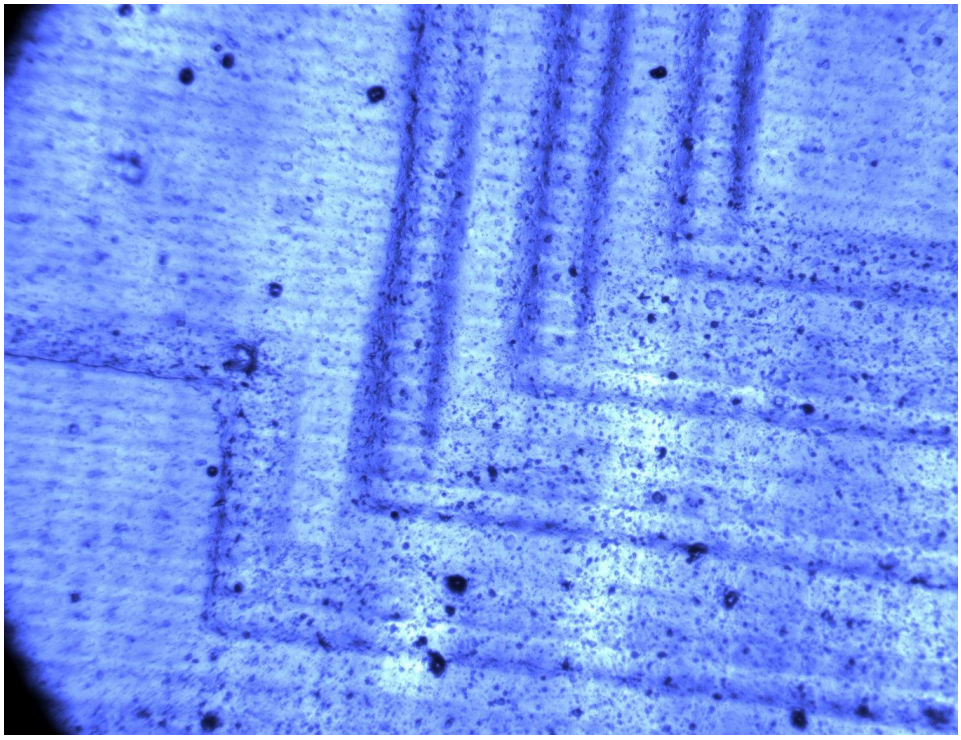




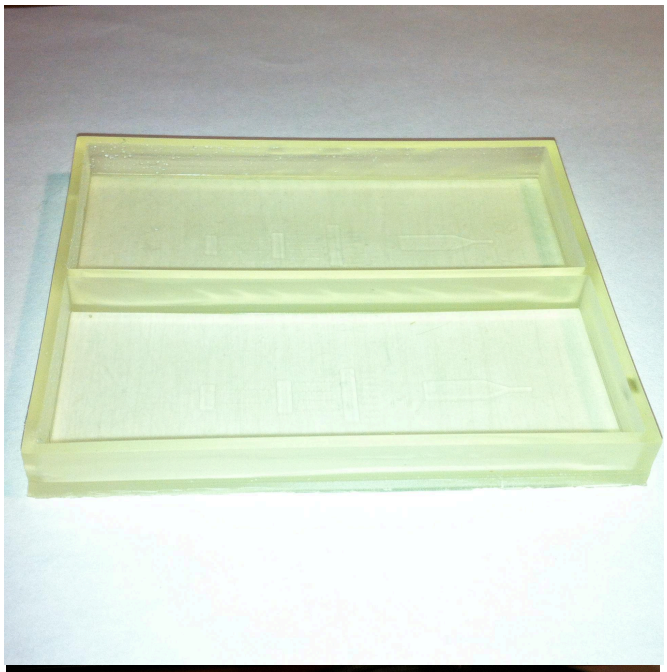
In this image we can see a large fiber that is present on the plastic. Is this normal?



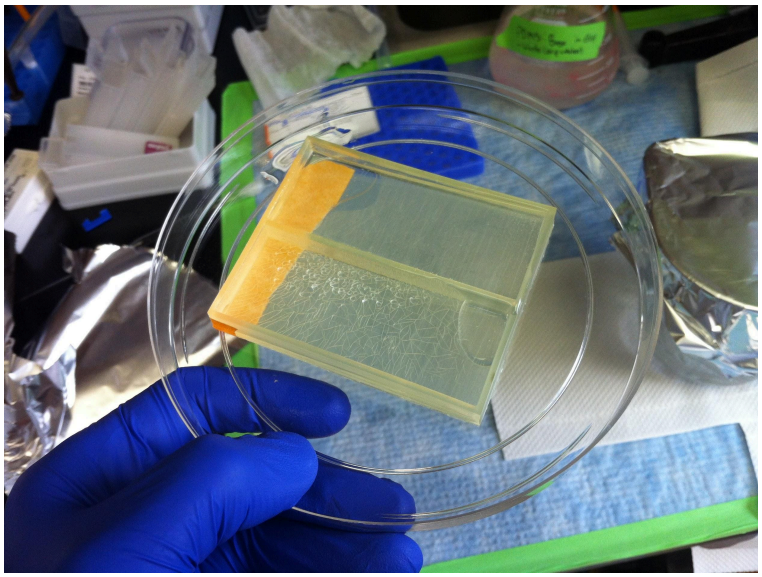
The next image shows a strange phenomena I noticed, but I'm not sure if it's something to worry about. It seems again that the horizontal channels aren't as well defined as the vertical ones, despite the distance between channels being the same in either dimension. Is there any known reason this could result from the printer?



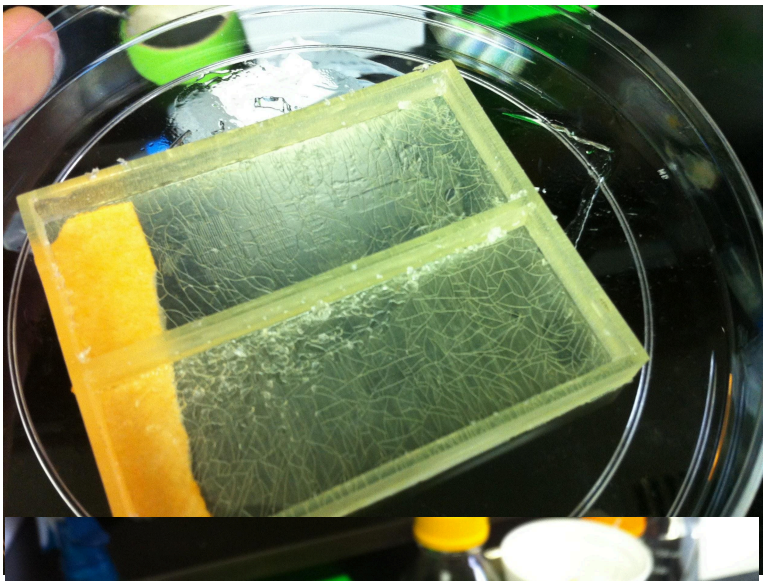
First PDMS replicas



Daniel changed the design to include walls around each device, which would work to keep the PDMS on the mould while curing.

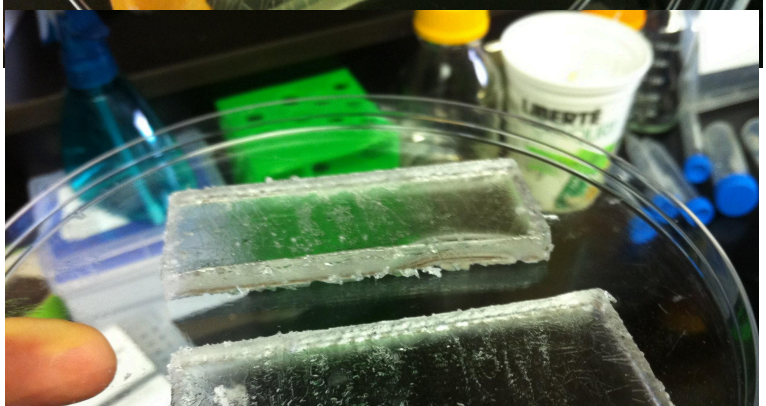


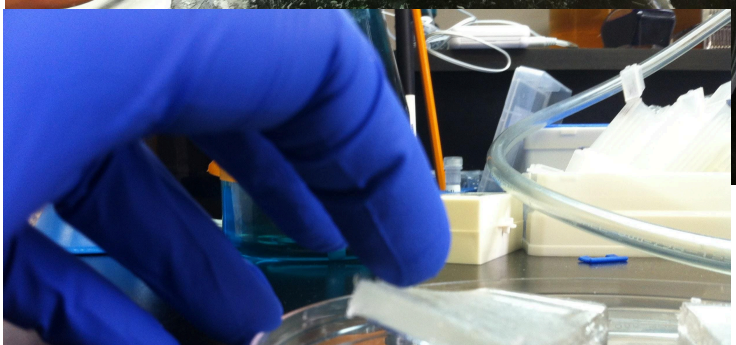
I added the orange tape to stop PDMS from leaking out of the side walls, which had holes in them. I mixed and degassed PDMS to one of the molds, then microwaved for 1 minute. After microwaving, the PDMS wasn't entirely cured and some spilled into the other mold.



While microwaving, the mould cracked, on both the mould containing PDMS and the mould without. After this, I definitely won't be able to generate channels, because the cracks are very deep.

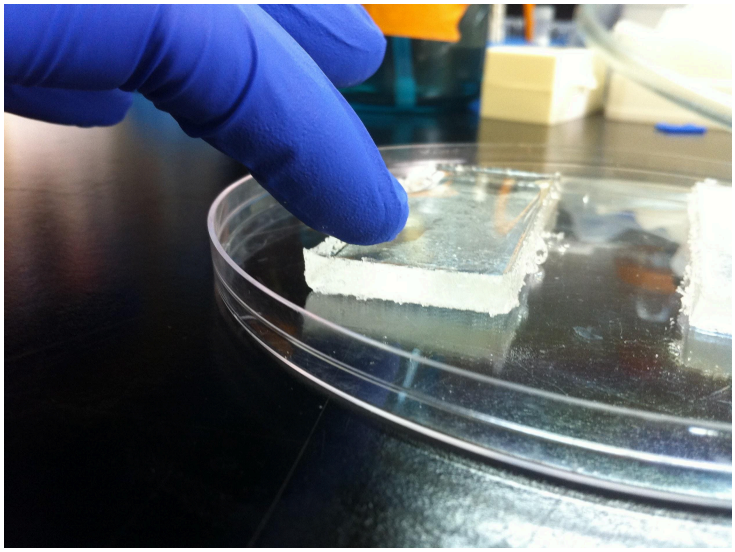
I filled the other mould with degassed PDMS and cured both in the over for a few hours. Usually this is enough for the PDMS to be solid.





I removed both PDMS replicas from the moulds. There were a few issues. The first is that the cracks had PDMS sticking more inside. Generally, the PDMS stuck more to the surface of the mould, as had been noticed previously by Daniel. The surface is definitely not properly cured, despite the surface of the PDMS (exposed to air) was normal. I did a glove test (we can call it this from now on) on the side of the PDMS touching the surface of the

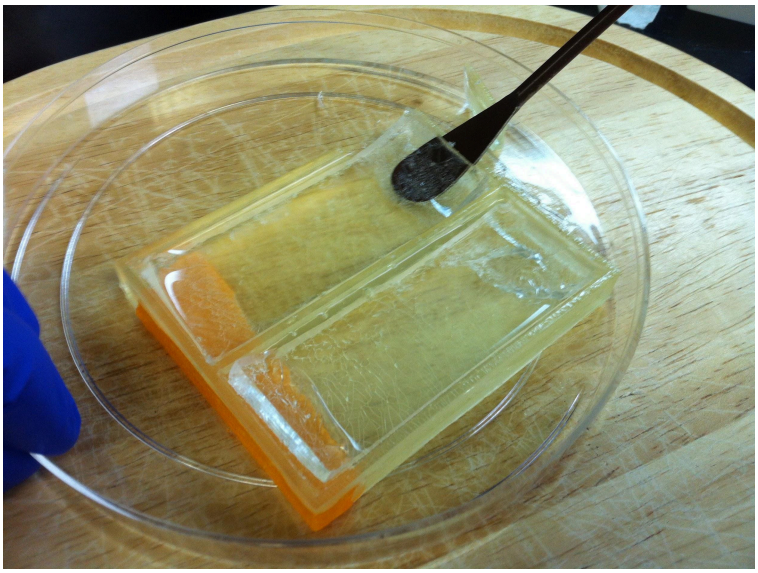
mould. The PDMS is under-cured, and sticks to the glove.



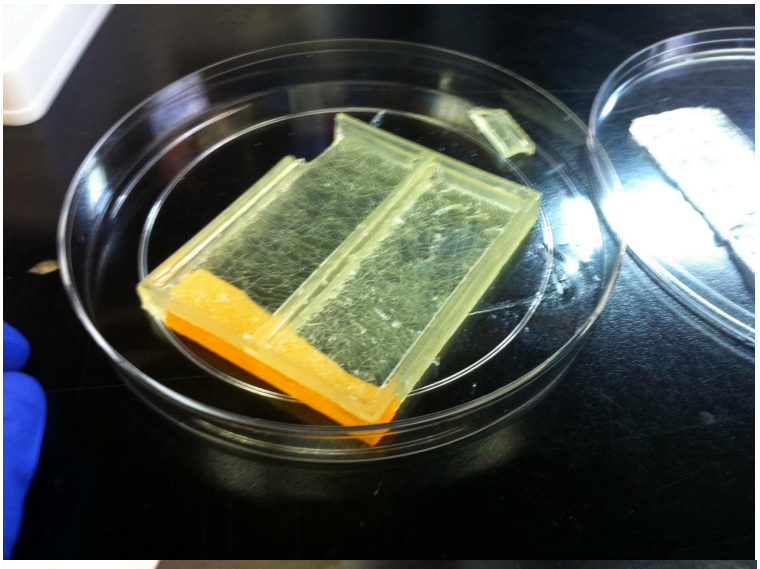
When I flipped the PDMS over and did a glove test on the other side, the glove lifted right off.

We can conclude that the issue isn't from the PDMS itself being undercured, but that it cures poorly at the surface interface, as Daniel found earlier.Unfortunately I didn't find that the microwaved PDMS behaved better in my hands,

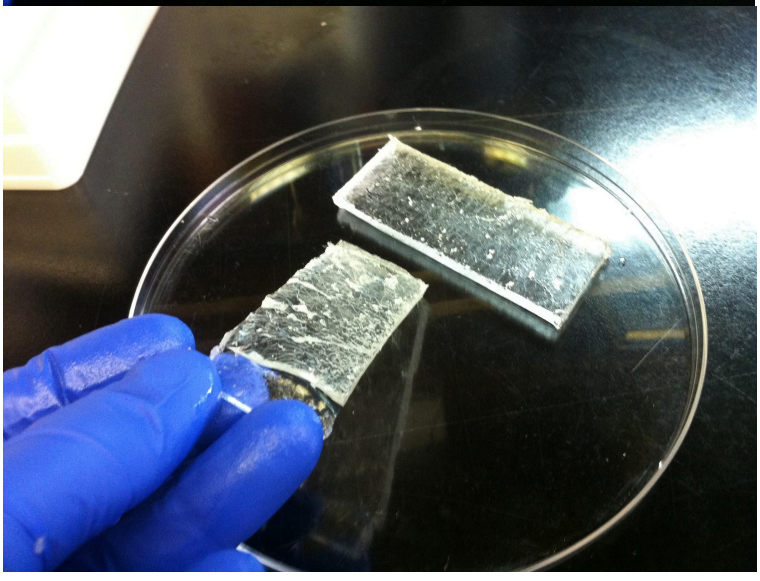
Second Replicas



I adding an increasing amount of curing agent for the next two replicas. In both cases, the PDMS was actually harder to remove from the mold.Usually the PDMS peels off nicely, but here I had to pry it off with force. A surface silane treatment should do the trick, if the surface of the makerjuice resin can be functionalized with plasma.



Despite seeming pretty solid to the touch, the walls broke after I removed the PDMS. This could be resulting from the cracking that happened post-microwaving. Maybe I should continue saving egg shells to add Calcium to the resin as Tibi suggested?



The added curing agent caused too much cross linking within the PDMS, making it brittle. PDMS made to 10:1 base/curing agent doesn't break like this when these kind of forces are applied (in my experience). Therefore, adding more curing agent isn't the solution. The best bet is probably a functionalizd surface.

Next steps:

1. Since the resin may be absorbing the curing agent from the PDMS, I'm attempting using higher proportions of curing agent in the same moulds, to see if the surface in contact with the resin cures better.
2. Create a new mould with higher features to avoid issues with poorly defined walls.