## Final Mother Machine protocol

The following represents the functional protocol after having solved each of the issues mentioned above. Issue discussions and their solutions are highlighted directly in the protocol to understand the context they are in:

### Layer 1: Cell Trenches

1. **Substrate Pre-Treatment**

   (a) Place a new 76.2 mm / 3" Si wafer into a crystallising dish filled with fresh acetone. Place the entire crystallising dish into a sonicator water bath so that all of the acetone is underneath the water line. Sonicate at high power for 5 min.

   (b) Sequentially rinse the wafer with streams of methyl alcohol (MeOH), isopropyl alcohol (IPA) and $dH_2O$ ($\sim$10 seconds per solvent).

   (c) Spin wafer 1 minute at 3000 rpm to dry.

   (d) Dehydrate wafer 2 minutes on a hot plate set to 200°C.

   [**Issue 2b**: Triangularly-shaped wafer segments without photoresist.

   **Solution**: Thorough wafer cleaning before coating. These non-covered areas of wafer occur when the wafer is not appropriately cleaned. Small contaminants will then act as an obstacle to the spinning SU8, leaving a "shadow" of non-coated area behind them. Thorough cleaning of the wafer eliminates this risk. A common Si wafer cleaning reagent is the "Piranha solution", a mixture of sulfuric acid and hydrogen peroxide. Though a powerful cleaning agent, it is one of the most dangerous solutions available and avoided in our lab. The steps shown represent a good compromise between safety, time invested and surface cleanliness.]

   (e) Go to plasma cleaner and expose to plasma for 30 sec, 100% power, $O_2$ 0.3 mBar.

   [**Issue 4**: SU8/feature-Si wafer bonding issues.

   **Solution**: Prime the Si wafer surface through plasma oxidation. Initially unaware of the concept of wafer priming, I attempted to bond SU8 2001 directly to the Si wafer and found the small features literally rolling down the wafer after development. This happens most likely because the silicon on the wafers requires freestanding hydroxyl groups to form covalent bonds with the SU8. Piranha solution is a very strong oxidising agent and would have hydroxylated the Si surface. By changing the cleaning procedure, the surface has inadvertently been rendered less reactive. Some protocols in the literature used an adhesion promoter

while others used plasma oxidation. I found the latter to be a lot easier to use and performed at least 30 sec of plasma oxidation at maximal power and 0.3 mBar $O_2$. Note: larger features do not appear to have this problem to the same extend, and one can often "get away" without priming the Si wafer for feature sizes wider than 25 µm in largest x-y dimension.]

2. **SU8-Coating**

   (a) Check that the spin coater is perfectly balanced.

   [**Issue 2a #1**: Uneven coating of SU8 on Si wafer.

   **Solution**: Use a balanced centrifuge. A spin coater has to stand on a balanced surface for even centrifugation to occur (**Figure 3.4a**). Unfortunately, among the many spin coaters in the Nanoscience cleanrooms, only the SU8 spin coater did not stand on a leveling platform. One has to find ways to temporarily source a leveling platform for their fabrication process.]

   (b) At spin coater, set spin program to:

      i. Step 1: 500/100/10 (spin speed [RPM] / acceleration [RPM/sec] / time [sec])

      ii. Step 2: 900/300/60 (specific step 2 max. spin speed has to be empirically evaluated)

   (c) Take the cleaned Si wafer and center on top of spin coater chuck.

   [**Issue 2a #2**: Uneven coating of SU8 on Si wafer.

   **Solution**: More precise wafer centering on spin coater chuck.

   Placing and centering the Si wafer on the spin coater is a very manual process in academic labs. Due to the non-transparent nature of silicon, it is very difficult to perfectly center a wafer. Unfortunately for the engineering principle of "poka-yoke" ("mistake-proofing"), only practice primes perfection.]

   (d) When centered, activate chuck vacuum.

   (e) Test how well the Si wafer is centered by briefly running the spin programme without any SU8 and see whether you can see any “wobbling” of the Si wafer. If necessary readjust and check again.

   (f) Use a glass pipette to dispense SU8-2001 onto the Si wafer. 70-80% of the surface should be covered.

   [**Issue 1**: Expensive and difficult-to-source, specialised SU8 2000 series.

   **Solution**: Use standard SU8, like SU8-2100, and dilute with cyclopentanone.

Most cleanrooms provide a set of standard photoresists, including SU8-2100 and SU-2025. SU8 is the name of the actual molecule that undergoes UV cross-linkage, and the higher the product number the more SU8 molecules are in solution. In turn, the photoresist is more viscous, leading to larger heights after spin coating. In order to reduce the height, our objective is to reduce the density of SU8 molecules in solution. Most SU8 providers use cyclopentanone as the solvent for SU8 photoresists. This means that instead of spending up to 800 GBP for a 500 ml bottle of highly specialised SU8, one can simply dilute standard solutions with cyclopentanone.]

(g) Run spin protocol

(h) Edge removal: Use a swab soaked with SU8 developer to remove the SU8 coat at the edges of the Si wafer.

3. **Soft Baking**

   Bake/Place the coated wafer on prepared hot plates: 1 min at 65°C, 1 min at 95°C, and 1 min at 65°C.

4. **UV Exposure/Photolithography**

   (a) Load and prepare the chrome mask and wafer in the mask aligner

   [**Issue 3**: Finished features of wrong size and shape.

   **Solution #1**: Use a functional, highly accurate mask aligner.

   Our Nanoscience Centre houses two mask aligners: SUSS MicroTec MJB4, a small and manual aligner which does not have an hourly cost associated with its use, and SUSS MicroTec MA/BA6, a much bigger aligner that has many automated features and currently costs 50 GBP/h (**Figure 3.4d & e**). To save cost and because I saw both aligners being used in Mother Machine protocols in the literature, I exclusively used the MJB4 aligner for at least 1 year. Both aligners have to press the coated, soft-baked Si wafer against the chrome mask, either through a "hard contact" or "vacuum" mode. However, I discovered that the mechanics of the MJB4 are not functional, and that our MJB4 is not configured to the same level as the one in the Harvard Center for Nanoscience (referenced by many publications). It appears that wafer-mask compression issues have caused exposure light to bleed into the photoresist, resulting in more resist cross-linking and larger/"blobbier" features. Step 1 to the solution for this problem was to switch to the high-end MA/BA6 mask aligner.]

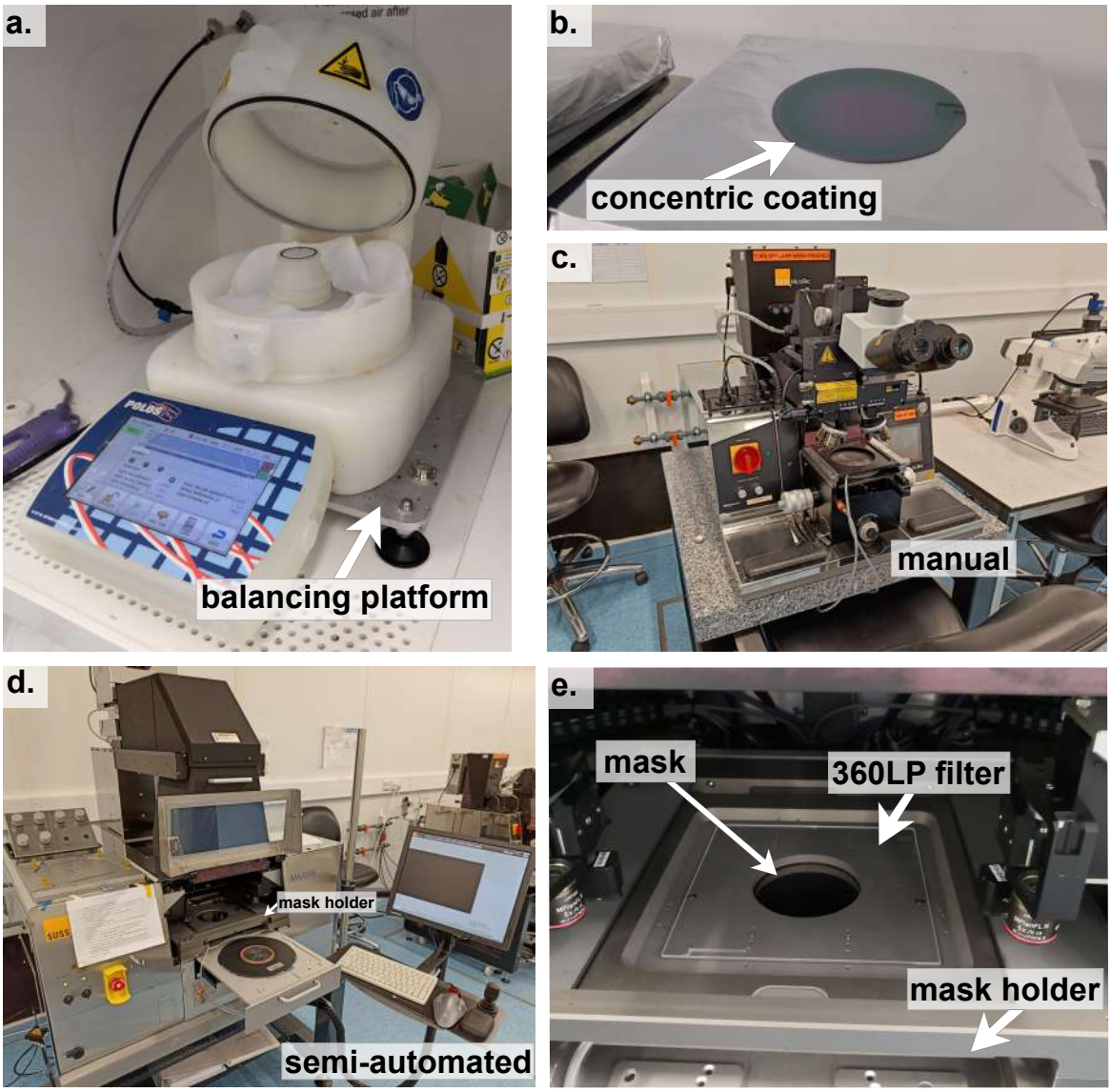


**Figure 3.4** Microfabrication setup solutions. | **a.** Balancing platform for spin coater to avoid spin errors. | **b.** Ideal, concentric SU8-2001 coating on Si wafer. | **c.** Glass filter placement inside MA/BA6 mask aligner just before exposure | **d.** MJB4 mask aligner. | **e.** MA/BA6 mask aligner.

(b) Place a 360nm-LP filter glass (OMEGA Optical cat. no. W2927) on top of the mask holder (just above the holder's hole).

[**Issue 3**: Finished features of wrong size and shape.

**Solution #2**: Use a light filter. Mask aligners can use various UV light sources. Non of them are perfect. To ensure that only light of the correct wavelength is used, and minimise risk of other wavelengths interacting with your mask in

unpredicted ways, resulting in unpredicted photoresist exposure, a filter glass should be used. However, neither of the two mask aligners has an in-built slot for these filters. In fact, the MJB4 UV source chamber is actually smaller than the filter I bought. The MA/BA6 has enough space inside the machine to house the filter glass (**Figure 3.4c**). But, one has to be quick to add the glass just after the alignment procedure, and just before exposure because the glass itself is otherwise a hazard to the machine due to moving parts inside.]

(c) Expose to UV:

i. Choose exposure mode: ConstantDose

ii. Energy: 175 mJ/cm$^2$

[**Issue 3**: Finished features of wrong size and shape.

**Solution #3**: Use dosage mode. When placing any material in front of a light source, the light exposure on the material can be measured in either power (mW/cm$^2$) or dosage/energy (mJ/cm$^2$). Some experimentalists prefer to state the light source power and the time of exposure at that power in their protocols while others leave even power out and only report the exposure time. For the cross-linking of SU8, both time exposed and light source power matter. However, a UV light source's power output varies enormously over time, and would have to be regularly measured to allow accurate usage of power mode. This has only in the last six months of my PhD happened due to a new, very diligent and very capable cleanroom process coordinator, Lee Robinson. In the absence of careful calculations of the *current* light source power and time, one can use the high-end MA/BA6 mask aligner's inbuilt dosage meter. This allows the machine to measure the complete dose of light a sample has been exposed to and stop automatically at a chosen value, regardless of how long it takes the light source to produce enough energy to reach that value. Since dosage is much more reproducible and not dependent on precise measurements of the light source output before every fabrication procedure, I highly advice using dosage mode.]

5. **Post Exposure Baking**

   Bake/Place the coated wafer on prepared hot plates: 1 min at 65°C, 1 min at 95°C, and 1 min at 65°C.

6. **SU8 Development & Feature Inspection**

   Place the wafer in a container with SU8 developer/PGMEA (with gentle agitation) for 1 min 30 sec and then in container with IPA for at least 10 seconds. If signs of

underdevelopment are seen (white “dust”), place wafer back in SU8 developer for 10 additional seconds (repeat until proper development is achieved).

### Layer 2: Feeding Lanes

After the first layer has been created, I recommend quickly checking the height of the cell trenches in 4 corners of the Si wafer before continuing to fabrication of the feeding lanes (layer 2). Since this layer has never given me any problems and the general procedure is the same, I will abbreviate the protocol for it: SU8-2025 was used at a maximal spin speed of 3,500 RPM, exposed to an energy dose of 280 mJ/cm$^2$, and baking times of 1 min/6 min/1 min. After final development, a hard bake is performed at 150°C for 10 min.

### Results

Initially I have started Mother Machine fabrication with the chrome mask gifted to us by the Paulsson lab, and focused on a "sampler" design. This geometry consists of cell trenches of lengths between 45 - 85 μm with 5 μm increases every 10 trenches, and widths between 1 - 1.5 μm (**Figure 3.3a**). Designs like this enable identification of optimal trench geometries, and are an important step in the prototyping process.

Trenches are designed much longer than they are supposed to be in the final MFD because the feeding lane/second layer has to be built on top of and connected seamlessly to trenches. The final cell trenches therefore heavily depend on the alignment accuracy of the feeding lane mask over the first layer. **Figure 3.5a** shows how a slight misalignment in the y-axis, has made the cell trenches on the top of the feeding lane shorter than initially designed, and the cell trenches on the bottom longer. After implementing all the solutions discussed in the protocol above, layer one features looked excellent with the shortest trench being just under 45 μm in length (i.). After the addition of layer two, the shortest top trenches should be 15 μm but instead are 5 μm long, indicating a location offset in the y-dimension of 10 μm. Location in the x-dimension is optimal, and other geometric features like perpendicularity between trenches and feeding lane are excellent. Interestingly, because this is a sampler design the y-offset actually generates more variety of cell trenches that can be tested. But it would be wrong to state this was by design, and instead this was a fortunate error.

After validating the design with various measurement techniques, and silanization of the finished MFD master (Alfa Aesar™ 1H,1H,2H,2H-Perfluorooctyltrichlorosilane, 97%, Thermo Fisher Scientific cat. no. AAL1660618) it was time to test the finished product. Only cells growing inside the device can verify whether an MFD design works as intended. **Figure 3.5b** shows cells inside the Mother Machine based on the MFD master I have fabricated.

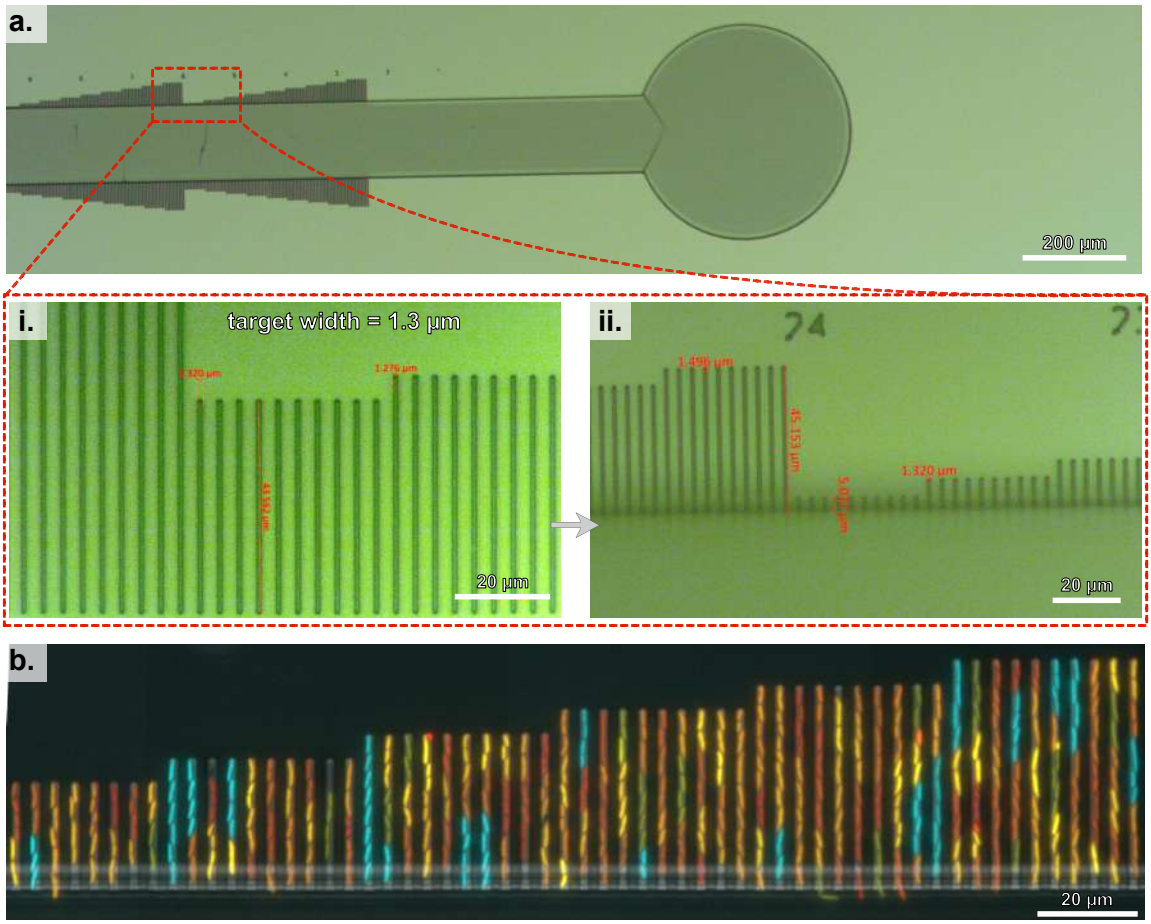


**Figure 3.5** Successful microfabrication of Mother Machine MFDs. | **a.** MFD master of the standard Mother Machine "sampler" design (Paulsson lab). i. Cell trenches imaged after layer one fabrication. ii. Cell trenches imaged after layer two alignment and fabrication. | **b.** Composite image (phase contrast, CFP, YFP, mCherry channels) of *E. coli* cells inside the finished microfluidic device.

A mixture of wild-type *E. coli* MG1655 with genomically-integrated expression cassettes for *SCFP3A*, *mVenus (NB)* and *an mCherry-mKate* fusion is shown for demonstration purposes. The mCherry cells also harbour the SL229 plasmid, the most advanced repressilator developed by Scott Luro, to showcase a genetic circuit in the final device. Cells in the image have already assumed exponential phase/stead-state growth morphology, highlighting their healthy growth. Furthermore, the device exhibits a very short and faint phase contrast halo, facilitating phase contrast based segmentation of the mother cell. This concludes that the device is functional and exhibits ideal characteristics for imaging of healthy *E. coli* cells.