**Supplementary file**

**Microfabrication protocol of PDMS chips**

Polydimethylsiloxane (PDMS, Sylgard 184 kit, Dowsil), microfluidic assays for 2D (Fig.1, PART1) and 3D (Fig.4B, PART2) layout have been manifactured by well-known soft-lithographic procedures, as described in detail previously, in a clean room facility or foundry, equipped with micro- and nanoscale instrumentations (e-beam lithography, photolithography and thin-film technology, nano-diagnostic tools). NOTE: In the absence of clean room services, there are different companies on the market to order patterned optical masks or masters for PDMS soft-lithography.

Steps can be summarized briefly in the points below:

1. **Fabrication of optical masks.** NOTE: For both 2D and 3D chips two photomasks have been patterned by transferring CAD drawings containing layers respectively of culture chambers and microchannels with alignment marks.
   1. Spin PMMA (AR-P. 679.04, Allresist, Germany) positive electronic resist on chromium photomasks (MB W&A, Germany) at 2000rpm for 60sec.
   2. Pre-bake on a hotplate at 170°C for 5 min.
   3. Expose by 100 kV e-beam lithography. NOTE: E-beam (Raith-Vistec EBPG 5HR system) settings are the following: 650µC/cm2 resist doseand 90nA e-beam current.
   4. Develop in MIBK: IPA = 1:1 (Methyl isobutyl ketone: Propan-2-ol, Carlo Erba reagents) for 30-60sec and rinse in IPA
   5. Place optical mask in a RIE (Reactive Ion Etching) chamber to perform O2 plasma etch for 30 sec.
   6. Remove remaining chromium film in Cr etching acid solution for 2 min.
2. **Fabrication of a microstructured epoxy resin mould, referred to, as ‘’master’’**. NOTE: Both 2D and 3D layout are patterned using two different i-line (365 nm, EV-420 mask-aligner) optical lithography onto the SU-8 3000 series negative sensitive epoxy resins (micro resist technology GmbH, Germany).
   1. Spin SU-8 3005 at 1000 rpm for 60sec to reach a thickness of about 10 microns.
   2. Pre-bake at 95°C for3 min. Choose post-bake temperatures and time according to manifacture datasheet.
   3. Expose the wafer to UV light through optical mask (4-5sec, 12.9mW/cm2, EV-420 double-face contact mask-aligner)
   4. Post-bake at 65°C for 1 min and at 95°C for 2 min.
   5. Develop in PGMEA solution (Propylene glycol monomethyl ether acetate, Sigma Aldrich) and rinse in IPA for 10min.
   6. Hard-bake the wafer at 150°C for 30min.
   7. Spin SU-8 3050 resin to expose cell culture chambers to reach a desired thickness (1000 rpm for 30sec for 2D 100µm-high culture chambers, depicted in Fig.1)
   8. Pre-bake the wafer according to datasheet.
   9. NOTE: For 3D chip layout (Fig.4), repeat steps 2.7 and 2.8 twice to obtain a thickness range of 250-260µm (first spinning step: 2000 rpm for 30sec, second spinning step: 1100 rpm for 30sec)
   10. Expose (17sec,12.9mW/cm2, EV-420 double-face contact mask-aligner).
   11. Execute post-bake of the wafer according to datasheet.
   12. Develop in PGMEA solution and rinse in IPA.
   13. Hard-bake the wafer at 150°C for 1h.
3. **Dry silanization of SU-8 micro-structured master.**
   1. Inside a ventilated chemical fume hood put few drops of the silanizing agent (Trimethylchlorosilane, TMCS, Sigma Aldrich) in a cup and place the O2 plasma pre-treated master over it in a vacuum dessicator. Keep them under vacuum for different hours or overnight. Note: During this process, TMCS evaporates and forms a monolayer on master surfaces to prevent PDMS sticking on its features. Note: Wear safety glasses, gloves and face protection.
   2. Place the wafer on the hotplate in the fume hood 150°C for 10-15 mins to cure and evaporate the excessive silane.
4. **Casting of PDMS liquid solution on microstructured master**
   1. Degas prepolimer solution (10:1 v/v, monomer: curing agent mixing ratio) contained in a plastic cup in a vacuum dessicator for 30 minutes.
   2. Remove any dust on the surface of the silicon wafer by using a nitrogen gun with a 0.45 µm filter.
   3. Pour slowly PDMS solution over the master avoiding bubbles. Leave it under hood for 10 min at room temperature (RT) before baking.
   4. Crosslink PDMS thermally for 1h 30 min at 110 °C on a hotplate. Let it cool.
   5. Detach carefully from the silanized master with a surgical blade and tweezers. Cut polimerized pieces in desired sizes and punch reservoirs (access ports for introducing cells and media) by dermal biopsy tools (Tedpella). Use 6 mm biopsy punches to create wells in the 2D layout, while for 3D layout drill respectively 4mm (media reservoirs) and 1mm-sized holes (gel inlets ) .
5. **PDMS structure irreversible sealing to cell culture substrates by O2 plasma treatment**
   1. PDMS-glass bonding.
      1. Put PDMS chips (with patterned side facing up) along with the glass slides inside the RIE chamber (Oxford Plasmalab 80 plus system) at the following RIE settings: 20W (RF power), flux of 60 standard cc/minute, 800-mTorr (pressure), and 30 sec (time). Note: Optimize process parameters according to your plasma cleaner machine.
      2. Bring into conformal contact the treated surfaces of the glass slide and PDMS device (channels side faced down) with each other at a clean bench. Check under an optical microscope the quality of bonding. Note: PDMS can be mounted on glass microscope slides (1mm thick), glass coverslips (170µm thick) and optical bottom 6-multiwells.
      3. Bake on a hotplate at 80-90 °C for 2 h, to improve adhesion strength. To restore hydrophobicity in 3D culture chips, keep for more than 4 hours at same temperature. Store chips in sterile Petri dishes.
   2. PDMS-plastic bonding.
      1. Inside the fume hood, put few drops of the silanizing agent (3-Aminopropyl) triethoxysilane APTES, Sigma Aldrich) in a cup and place the O2 plasma pre-treated polymer coverslips (Ibidi, Germany) over it in a vacuum dessicator. Keep them under vacuum for 1-2 hours.
      2. Put into contact the silanized coverslip and pre-oxidized PDMS piece to assemble final device. Keep them in an oven or on a hotplate at 60-65°C for some hours.
6. **Compatibility with the automated acquisition software for high-content microscopy.** Note: Markers for aligning PDMS chips are defined by laser writing system (Trotec Speedy 100 laser cut with 35W maximum laser power).
   * 1. **Holder Fabrication**. In order to load and write quickly the markers on your glass or plastic substrate, use the laser cutter to realize a holder for the microscope slides (12 in our example). Use as material to fabricate holder Plexiglas 5mm thick (50cm x 30cm foil). Note: Alternatively, use a 5mm thick Plexiglas foil for the holder’s base and a 2mm thick foil for the holder’s cover.
     2. Cut two rectangles of 25cm x 21 cm (Laser Power 90%, speed 0,40cm/s, air ON, repetition rate 2000 Hz). Reduce power to 60% if you use 2mm thick plexiglass foils.
        1. *Holder base (first rectangle)*, open 8 hexagonal holes to insert nuts 1cm apart from the borders on each side of the rectangle (size of the holes according to you nuts); You’ll have to glue your nuts in the holes once you remove the Plexiglas from the laser cutting machine.
        2. *Holder Cover (second rectangle)*, open six holes to insert bolts in the same positions on the rectangle that you used for the nuts’ seating. Open 12 (6x6) rectangular windows of the same size of your microscope slides or coverslips (25mmx75mm in our case). Take care that these windows are placed at least at 2cm from the holder sides and 1cm distant from each other.
        3. *Holder assembly:* Use 8 bolts to screw together the cover and the base
   1. **Marker definition.** 
      1. Prepare the drawing for the markers to be engraved on the glass slides or coverslips considering their position in the holder you realized in step 6.1, so that they’ll match the ones on the chips. Note: Realize suitable markers in your PDMS chip layout fabrication. In our case we defined two crosses 2mmx2mm in the empty space between chips on the SU-8 master to replicate on the final PDMS chip.
      2. Insert your microscope slides in the rectangular seating in the holder.
      3. Place the holder inside the laser cutting machine, taking care to have it aligned with the machine axis and set the origin of your cut on the upper left holder’s corner.
      4. Engrave the markers with parameter suitable for the material that you are using. Note: Settings for glass slides are: Power 60%, speed 15mm/s, repetition 500Hz; for plastic (COC or polycarbonate) slides are: Power 30%, speed 40mm/s, repetition 500Hz.
      5. If some white powder is deposited during the process, clean it with a soft cloth and Isopropanol after having unmounted the substrates form the holder.
7. **Hydrophilic surface functionalization of chips before starting biological experiments.**
   1. 2D layout (Fig 1A). To insert easily medium in culture chambers, oxygen plasma activate the assembled PDMS/culture substrates in the RIE chamber for 20-30 seconds.
   2. 3D layout (Fig 2A). Optionally perform selective oxidation of central immune chamber ina plasma cleaner for 5-10 seconds. This will make the chamber hydrophilic preventing formation of bubbles during loading. Note: Cover gel ports and media channels reservoirs (e.g, by Kapton tape) to guarantee the correct gel matrix positioning by micropillars array and avoid leakage in adjacent chambers. Gel regions have to be kept hydrophobic.