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## Steady-state cell-free gene expression with microfluidic chemostats

Nadanai Laohakunakorn<sup>1</sup>, Barbora Lavickova<sup>1</sup>, Zoe Swank<sup>1</sup>, Julie Laurent<sup>1</sup>, [Sebastian Maerkl](#)<sup>1</sup>

<sup>1</sup>EPFL - EPF Lausanne

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Maerkl Lab



Sebastian Maerkl  
EPFL - EPF Lausanne



### ABSTRACT

Cell-free synthetic biology offers an approach to building and testing gene circuits in a simplified environment free from the complexity of a living cell. Recent advances in microfluidic devices allowed cell-free reactions to run under non-equilibrium, steady state conditions enabling the implementation of dynamic gene regulatory circuits in vitro. In this chapter we present a detailed protocol to fabricate a microfluidic chemostat device which enables such an operation, detailing essential steps in photolithography, soft lithography, and hardware setup.

Laohakunakorn\_final.pdf Rchemostat\_V2.cif Rchemostat\_V2.dwg Rchemostat\_V2.dxf

### MATERIALS TEXT

The photolithography steps were carried out in a Class 100 clean room at EPFL. Soft lithography was done in a dedicated space in a standard wet lab. Specialised machines, consumables, and chemicals are listed below.

#### 1. Photolithography machines

- VPG200 photoresist laser writer (Heidelberg Instruments Mikrotechnik GmbH)
- HMR900 mask processor (Hamatech APE GmbH)
- Optispin SB20 spin coater and VB20 hotplate (ATMsse GmbH)
- MJB4 mask aligner (Süss MicroTec AG)
- Tepla 300 plasma stripper (PVA Tepla AG)
- LSM250 spin coater and HP200 hotplate (Sawatec AG)
- AccuPlate thermal accumulator and hot plate system (Detlef Gestigkeit)

#### 1. Photolithography consumables

- AZ 9260 positive photoresist (MicroChemicals GmbH)
- GM1070-SU8 negative photoresist (Gersteltec)
- 1-methoxy-2-propyl-acetate (PGMEA) developer (Sigma)
- AZ 400K developer (Merck)
- AZ 351B developer (Merck)
- Cr01 chrome etchant (Technic)
- Hexa-methyl-disilazane (HMDS) primer (Technic)
- Silicon wafers, diameter 100±0.5 mm, thickness 525±25 µm, P-type (boron-doped), resistivity 0.1-100 Ωcm (Siegert)
- SLM5 5" blank chrome mask (Nanofilm)

#### 1. Soft lithography machines

- ARE-250 centrifugal mixer (Thinky)
- SCS G3P-8 spin coater (Specialty Coating Systems Inc.)
- Schmidt Press manual hole puncher and 21-gauge (OD 0.04") pins (Technical Innovations, Inc.)
- Diener Femto 40 kHz low pressure plasma oven with O<sub>2</sub> supply (Diener electronic GmbH + Co. KG)
- Universal Oven UF110, 108L (Mettmert)
- SZX10 dissection microscope with DF PLANO 1.25x objective and KL 1500 LCD light source (Olympus)

#### 1. Soft lithography consumables

- Trimethylchlorosilane (Sigma)
- Sylgard 184 polydimethylsiloxane (PDMS) elastomer and curing agent (Dow Corning)

- Glass slides 76x26x1 mm 631-1550 (VWR)

#### 1. **Microfluidic hardware**

- 12-station aluminium pneumatic manifold with 24V 3-way normally-open solenoid valves (S10MM-31-24-2/A Pneumadyne)
- Polycarbonate manual luer manifold (Cole-Parmer)
- Custom relay circuit board (*see* **Note 1**)
- Type 10, 2-60 psi and 2-25 psi pressure regulators (Marsh Bellofram)
- 0.1-3 bar pressure gauge (Riegler & Co. KG)

#### 1. **Microfluidic connectors**

- Synflex 1201-M06 polyethylene (PE) tubing, OD 6 mm ID 4mm (Eaton)
- PE-LD tubing OD 1/8" ID 1/16" (Tuyau)
- Tygon tubing, OD 0.06" ID 0.02" (Cole-Parmer)
- FEP tubing, OD 1/16" ID 1/32" (Upchurch)
- PEEK tubing, OD 1/32" ID 0.18 mm (Vici)
- Luer stubs 12 mm, 23 and 20 ga
- Male-to-male and 1/16" barb to male luer adaptors
- Stainless steel connecting pins OD 0.65 mm ID 0.35 mm, 8 mm (Unimed)
- Brass Series G pneumatic fittings (Serto AG)
- Blue Series pneumatic fittings (Riegler & Co. KG)

#### 1. **Microscope hardware**

- Ti2 Eclipse Inverted Microscope (Nikon)
- Objectives: CFI Achrom 4x NA 0.1 (Nikon); CFI S Plan Fluor 20x NA 0.45 ELWD DIC N1 (Nikon)
- Filters: F36-504 mCherry HC filter set (Semrock); FITC (Nikon)
- Microscope enclosure and heater (Okolab)
- Sola SM II Light Engine (Lumencor)
- Orca-Flash 4.0 V3 Digital CMOS Camera (Hamamatsu)

#### 1. **Software**

- AutoCAD2019 (Autodesk)
- CleWin (WieWeb)
- LabView 2018 (National Instruments)
- Matlab 2019 (Mathworks)

#### 1. **Experimental reagents**

- TX-TL cell-free extract, ribosomes and energy solution, prepared as in (13)
- DNA template, prepared as in (14)

### Design of Microfluidic Devices

- 1 Design the device (see Note 2) on AutoCAD 2019 or other software with similar functionality. A specific example is shown in Figure 1, and other designs are available on our webpage (see Note 3). Export the final design as a .dxf file.
- 2 Using CleWin, convert the designs to a machine-compatible .cif file ready for photomask fabrication.
- 3 During curing, the PDMS layers will differentially shrink, with the thicker flow layer shrinking more than the thinner control layer, which remains attached to the rigid mold. Thus, it is crucial to enlarge the entire flow layer design by 1.5%. This can be done in CleWin during the conversion.

### Mask Fabrication

- 4 Expose chrome masks with the VPG200 laser writer, using a 20 mm write lens (see Note 4) and 48% intensity. Make sure the polarity and mirroring of the mask is correct (see Note 5).
- 5 Next, process the exposed masks using the HMR900 mask processor. This involves the following automated steps:

- 6 First purge the machine with DI water.
- 7 Then develop for 100 s with a diluted developer mixture (AZ 351B:DI water in the ratio 1:3.75), and rinse with DI water.
- 8 Etch through the chrome layer for 60 s using the Cr01 etchant, and rinse.
- 9 Finally strip the photoresist using the AZ 400K developer for 35 s, followed by a final rinse and drying with CO<sub>2</sub>. The completed masks should be completely dry before use.

#### Flow Mold Fabrication

- 10 Prime a clean Si wafer with HMDS (see Note 6) for 10 s in vacuum, using the VB20 hotplate.
- 11 Transfer the wafer onto the Optispin SB20 spin coater and dispense a few ml of positive resist AZ9260 onto the centre of the wafer, taking care to avoid bubbles (see Notes 7, 8).
- 12 Spin coat at 920 rpm for 100 s, followed by 60 s relaxation at 0 rpm. This deposits a 14- $\mu$ m layer of photoresist on the surface of the wafer.
- 13 When the spin coating has finished, immediately transfer the wafer to a preheated hotplate, and 'softbake' for 6 minutes exactly at 115°C.
- 14 Transfer the wafer to an opaque storage box and allow it to rehydrate for a minimum of 1 hour (see Note 9).
- 15 Load the appropriate chrome mask onto the MJB4 mask aligner, and expose for 2 cycles at 18 s per cycle, with a waiting time of 10-15 s between each cycle, using the Hg-i line (365 nm) at 20 mW/cm<sup>2</sup> (see Notes 10, 11). Use the following parameters: expose type = hard, alignment gap = 30, WEC type = cont, N<sub>2</sub> purge = NO, WEC-offset = OFF.
- 16 Develop immediately (maximal waiting time is 1 hour) by transferring the wafer to a bath of diluted AZ 400K developer (1:3 developer:DI water). Develop face-up, and gently agitate the wafer in the bath for 10 minutes (see Note 12).
- 17 Rinse with DI water, then carefully but rapidly dry the wafer with N<sub>2</sub>, and inspect features under a microscope. If photoresist residues remain, develop further until all the residues are removed and repeat the cleaning and drying.
- 18 Finally transfer the wafer to the AccuPlate hotplate, and carry out a 'reflow' bake using the following program to round off features (see Note 13): 1 hr ramp up to 170°C, 2 hrs at 170°C, 1 hr ramp down to room temperature

#### Control Mold Fabrication

- 19 Clean an Si wafer with 2.45 GHz O<sub>2</sub> plasma in the Tepla 300 Plasma Stripper, using 500 W for 7 min and 400 ml/min of O<sub>2</sub>.
- 20 Transfer the wafer onto the LSM250 spin coater, and dispense a few ml of negative resist GM1070-SU8 onto the centre of the wafer, taking care to avoid bubbles.

- 21 Spin coat a 40- $\mu$ m layer of photoresist onto the wafer using the following program: 5s/0-500 rpm, 5s/500rpm, 21s/500-1933rpm, 40s/1933rpm, 1s/1933-2933rpm, 1s/2933-1933rpm, 5s/1933rpm, 26s/1933-0rpm.
- 22 When the spin coating has finished, immediately transfer the wafer to the hotplate, and carry out an initial relaxation followed by a softbake using the following program (see Note 14): 30 minutes at 30°C, then 3000 s ramp 30°C to 130°C, 300 s at 130°C, then 3000 s ramp 130°C to 30°C.
- 23 Load the appropriate chrome mask onto the MJB4 mask aligner, and expose for 1 cycle at 16 s, using the Hg-i line (365 nm) at 20 mW/cm<sup>2</sup>. Use the following parameters: expose type = soft, alignment gap = 30, WEC type = cont, N2 purge = NO, WEC-offset = OFF.
- 24 Transfer the wafer to the HP200 hotplate for a post-exposure bake using the following program: 2400 s ramp 30°C to 90°C, 2400 s at 90°C, 2700 s at 60°C, 2700 s at 30°C.
- 25 Transfer the wafer to an opaque storage box and wait from 1 hour to overnight before development.
- 26 Develop by transferring the wafer to a bath of PGMEA developer (see Note 15). Gently agitate the wafer in the bath for 2 minutes before transferring to a bath of new developer for a further 1 minute.
- 27 Rinse with isopropanol. If a reaction is visible (white residues appear) then return wafer to PGMEA for 30 s to 60 s before rinsing with isopropanol again. Let dry naturally.
- 28 Inspect features under a microscope and carefully develop further if needed. Avoid overdevelopment, which can lead to breaking of features.
- 29 Finally transfer to hotplate and carry out a 'hardbake' using the following program: 30 min ramp to 135°C, 2 hrs at 135°C, then 30 min ramp down to room temperature.

#### Silanization of wafers

- 30 Before first use, place wafers inside a sealed box with few drops (0.5 mL) of trimethylchlorosilane and incubate for at least 12 hours. Repeat the silanization before each use for 10 min.

#### Casting and curing of PDMS device

- 31 In two plastic cups, weigh out and add PDMS elastomer and curing agent in a ratio 5:1 (50 g : 10 g) for the flow layer, and 20:1 (20 g : 1 g) for the control layer.
- 32 Defoam the mixture using the ARE-250 centrifugal mixer, by mixing at 2000 rpm for 1 min followed by defoaming at 2200 rpm for 2 min.
- 33 Clean both flow and control wafers using pressurised N<sub>2</sub>.
- 34 Put the flow layer wafer on aluminium foil inside a glass petri dish. Make sure the foil covers the dish and contains the PDMS fully. Pour all of the 5:1 PDMS mixture on top of the wafer, and place the dish inside a vacuum desiccator for 40 min to degas the mixture.
- 35 Put the control layer wafer in the SCS G3P-8 spin coater, and carefully pour a few ml of the 20:1 PDMS onto the centre of the wafer. To coat the wafer, run the following program: Step 0, rpm = 0, disp = 2, ramp = 0.0, dwell = 0; Step 1, rpm = 1420, disp = none, ramp = 20.0, dwell = 35; Step 2, rpm = 100, disp = none, ramp = 20.0, dwell = 1; Step 3, rpm = 100, disp = none, ramp = 1.0, dwell = 0.

- 36 After coating, the PDMS layer will be uneven due to the high 40- $\mu\text{m}$  features. Place the wafer on aluminium foil in a second petri dish, cover to protect from dust, and set aside on the bench for 40 minutes.
- 37 Then bake both flow and control wafers in an oven at 80°C. The flow layer is baked for 20 minutes, and the control layer for 25 minutes. Timings for this step must be exact (see Note 16).
- 38 Remove the wafers from the oven. Using a sharp scalpel, cut out each design from the flow layer, and immediately place on top of the corresponding control layer region, roughly aligning the two layers.
- 39 Once all the devices have been roughly aligned in this way, transfer the control wafer to a stereo dissection microscope, and align the two layers by manually lifting off and carefully placing the top layer in its precise position (see Note 17).
- 40 Put the aligned devices back into the oven at 80°C and bake for a minimum of 1 hour 30 minutes.
- 41 Cut the multilayer devices off the wafer using a scalpel.
- 42 Using the hole puncher, punch through all the channel inlets.
- 43 Protect the PDMS surfaces from dust using Scotch tape. The completed PDMS devices can now be stored in a clean petri dish until the next step.

#### Bonding of PDMS devices to a glass slide

- 44 Clean glass slides using pressurised N<sub>2</sub>.
- 45 Remove any residual dust from the slide and feature surface of the PDMS device using Scotch tape (see Note 18).
- 46 Switch on the Femto plasma oven and place the slide and PDMS device bonding-side-up.
- 47 Pump out the chamber for at least 15 minutes to ensure a clean vacuum environment.
- 48 Switch on the O<sub>2</sub> for 2 minutes at a flow rate of 25 sccm and 0.1 bar, then apply 30 s of plasma at 100 % power (which corresponds to a plasma of 40 kHz and 100 W (see Note 19)).
- 49 Immediately ventilate the plasma by-products before opening the chamber. Put the PDMS and glass together and manually apply even, moderate pressure for a few seconds (see Note 20). Then, put the bonded device into an oven at 80°C for 1 hour to overnight.
- 50 The completed devices can finally be stored at room temperature until use (see Note 21).

#### Regulation of control layer pressure

- 51 Connect one branch of the input air supply to a regulator, and direct the regulated output supply to the aluminium electric manifold.

- 52 The electric manifold directs air pressure to the chip's control lines. Attach Tygon tubing (ID 0.02") to the manifold using appropriate adaptors as shown in Figure 2. The tubing contains a 23 ga luer stub on one end (used for filling and connecting to the manifold) and a stainless steel connector pin on the other (used for connecting to the chip).
- 53 Plug the electric manifold into the relay board, which links via USB to a PC running control software written in LabVIEW. An example of the code and full documentation can be found online (see Note 22).

#### Regulation of flow layer pressure

- 54 Connect the other branch of the input air supply to a regulator, and connect the regulated supply to the manual luer manifold.
- 55 Adjust the pressure as required (typically ~0.3 bar).

#### Filling control lines

- 56 Lower the control manifold pressure to around ~10 psi.
- 57 Using the PC software, close all the control line valves.
- 58 Fill each Tygon line with deionised water (see Note 23) through the connecting pin, using a syringe attached to a luer stub.
- 59 Connect each line to the appropriate control channel inlet.
- 60 Once all the lines are connected, open all valves. This pressurises the control channels, pushing air into the PDMS and allowing them to fill with water. Wait until the channels are completely filled with water, which can take up to 20 minutes. Slowly raise the pressure up to ~20-30 psi.
- 61 Visually inspect all the valves to check that they actuate fully.

#### Filling flow lines

- 62 Make sure the appropriate manual manifold valve is closed.
- 63 Basic reagents such as buffers and chemicals are held in ID 0.02" Tygon tubing. First assemble the tubing which consists of a length of Tygon, a 23 ga luer stub on one end, and a connector pin on the other.
- 64 Attach a syringe to the luer stub, and carefully draw up the required reagent into the tubing. Make sure there are no bubbles.
- 65 Attach the connector pin to the appropriate flow inlet, before removing the syringe and attaching the luer stub to the manual manifold.

- 66 Make sure valves are in the appropriate configuration on the chip before opening the flow manifold valve, and allowing the reagent to fill into the device. Typically, a pressure of ~0.3 bar is ideal for the flow lines.
- 67 For the cell-free extract, follow the previous steps, but instead draw up the solution into the FEP coil through the PEEK tubing. Attach the PEEK tubing directly into the chip.
- 68 An important requirement for long-term steady-state reactions is that the cell-free extract is separated from energy and DNA solutions. If required, cooling elements can be supplemented to further prevent degradation of the solutions (16,20).

#### Cell-free expression

- 69 The device can be characterised as shown in Figure 3.
- 70 A typical experimental program is shown in Figure 4. First switch on the environmental chamber to 29°C.
- 71 Load each reactor with cell-free extract, energy solution, and DNA in the ratio 40%, 40%, 20%.
- 72 The reactor contents are mixed by actuating the four multi-function valves sequentially at a frequency of 20 Hz.
- 73 Dilution involves flowing cell-free extract, energy solution, and DNA into the reactors in the ratio 8%, 8%, 4%. This corresponds to a 20% dilution of the reactor which preserves the original reaction composition.
- 74 The dilution rate can be varied by adjusting the interval between dilution steps.
- 75 Image the resulting fluorescence using the microscope setup. Software for the analysis, example images, and full documentation can be found online (see Note 24).

#### Notes

- 76 1 A custom relay board is used to control the electric manifold actuation; any appropriate controller can be used in its place, for instance the 24-channel USB24PRMx (EasyDAQ).
- 77 2 Excellent guidance is available e.g. (22)
- 78 3 Designs for microfluidic devices are available online at [http://lbnc.epfl.ch/microfluidic\\_designs.html](http://lbnc.epfl.ch/microfluidic_designs.html).
- 79 4 The 20 mm lens provides the highest write speed, taking ~4 minutes to write a 100x100 mm mask with 2 µm edge resolution, and 1 mm stripe width. Higher resolutions are possible but not necessary for soft lithography.
- 80 5 This is the step most often done incorrectly. The flow layer uses positive-resist AZ, and requires a DARK-mode mask. The control layer uses negative-resist SU8 and requires a CLEAR-mode mask. Finally as the exposure is chrome-side-down, the masks must be MIRRORED AT Y.

- 81 6 HMDS priming enhances photoresist adhesion. Alternatively the wafer can also be treated with O<sub>2</sub> plasma or thermally dehydrated.
- 82 7 Pouring directly from the bottle introduces fewer bubbles than using a plastic pipettor.
- 83 8 Opening the cap to the AZ9260 bottle to allow the release of air bubbles a few minutes before use can also help minimise bubbles.
- 84 9 Homogenous rehydration is important for efficient exposure, and the minimum rehydration time is a function of the photoresist thickness (5 µm, 8 min; 20 µm, 2 hrs).
- 85 10 The mercury lamp contains spectral lines at 365, 405, and 436 nm. On the MJB4 machine the i-line filter is installed which passes only the 365 nm line. Without the filter, the exposure is broadband. The exposure mode must be taken into account during exposure time calculations.
- 86 11 For 15 µm AZ9260, the recommended dose is 580 mJ/cm<sup>2</sup> for i-line exposure and 660 mJ/cm<sup>2</sup> for broadband.
- 87 12 The recommended development time is around 45 s per µm of AZ9260.
- 88 13 Rounded features are crucial for the flow layer as it allows valves to close completely.
- 89 14 The variable here is the ramp time, which depends on the specific type of SU8 used
- 90 15 It is highly recommended to develop the wafer upside down. Prepare two baths of PGMEA.
- 91 16 The precise timing here is important. The PDMS should set sufficiently so that it is not too sticky, but not so much that the resulting multilayer device does not bond together.
- 92 17 This step requires the most practice. Alignment should be completed as quickly and precisely as possible to ensure optimal bonding. Air bubbles are typically caused by buckling of the PDMS layers, and can be removed by first ensuring the top layer is completely flat, and then with gentle application of pressure. Putting weights on top of the PDMS during subsequent baking can also help.
- 93 18 This step is important as the presence of dust between the glass and PDMS can compromise bonding or render the device non-functional.
- 94 19 Plasma treatment converts methylsiloxane to siloxyl groups on the PDMS surface, enabling its covalent cross-linking to silica-containing glass. There is however an optimum amount of treatment, as over-treating increases the surface roughness of the PDMS and decreases the effective contact area (23).
- 95 20 The binding can be checked by putting the chip against a black piece of paper. Regions which are not bound will show up as bubble-like features.
- 96 21 In our experience, devices can still be functional after 6 months' storage.



97 22 <https://github.com/nadanai263/lbnc-cellfree2>

98 23 Ideally all Tygon control lines should have the same length and the same amount of water. The larger the volume of water in the line, the faster the pressure transfer and valve actuation, due to the incompressibility of water; in practice care must be taken so the water does not get into the electric manifold, so do not fill the lines fully. Finally, make certain that there are no air bubbles where the line connects to the chip.

99 24 <https://github.com/nadanai263/lbnc-cellfreeview>



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