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Steady-state cell-free gene expression with microfluidic chemostats

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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.

Key Words

microfluidics, cell-free, synthetic biology, steady-state gene expression

1. Introduction

One of the enduring challenges in synthetic biology today is the overwhelming difficulty of predictive forward-engineering, despite major efforts to characterise, standardise, and mathematically model synthetic biological parts and systems ***(1)***. Even if parts such as promoters and regulators are initially well-characterised, combining them together into larger subsystems typically changes the context of the parts as well as the host cell, resulting in diminished predictive accuracy and in some cases a loss of the original function altogether. Functional designs are therefore usually developed not in a purely rational manner, but require rounds of empirical design-build-test cycles. While this approach can certainly yield functional designs, it is preferable to ultimately develop more efficient and rational ways of engineering gene circuits.

Within synthetic biology, the adoption of cell-free systems has become increasingly widespread ***(2)***. From an engineering perspective, they behave as a very simplified 'host cell', providing a constant and controllable environment in which to build synthetic gene networks. Cell-free systems are thus well-suited for rational, bottom-up engineering of biomolecular systems ***(3,4)***. Furthermore, the functionality of cell-free systems can be expanded by inclusion of additional components ***(5)***, and provide a system for quantitative analysis including mRNA and protein concentrations ***(6,7)***. A second key benefit is that their ease of preparation and scalability also accelerates design-build-test cycles, resulting in their adoption as an efficient rapid prototyping platform. Both lysate ***(8,9)*** and recombinant ***(10)*** cell-free reaction systems can now be readily generated using standard laboratory equipment at reasonably low costs.

Microfluidics have allowed these benefits of cell-free synthetic biology to be more fully realised ***(11)***. By increasing the throughput, lowering reagent consumption, and providing control and quantitative monitoring of thousands of reactions in parallel, they have enabled precise characterisation of cell-free gene circuits both in integrated chips ***(12)*** as well as in encapsulated droplets ***(13,14)***.

Batch cell-free reactions typically run to chemical equilibrium as substrates are exhausted, reaction products accumulate, and enzymatic machinery degrades. To maintain a more life-like non-equilibrium steady state, large-scale continuous exchange or continuous flow reactors have been used to feed the reaction with small molecules and wash away products through ultrafiltration membranes ***(15)***. At the microfluidic level, microchemostat devices have been developed which replenish not only substrates but also the enzymatic machinery, while at the same time diluting away reaction products ***(16,17)***. These microchemostats enable long-term steady state reactions, and

also allow for the investigation of biologically-relevant dynamical behaviours such as oscillations ***(16,17)*** and pattern formation ***(18)***.

In this chapter, we describe the entire process of designing, fabricating, and operating a microfluidic chemostat device. The chip we chose as an example is a revised and simplified version of the microchemostat presented in Niederholtmeyer et al. 2013 ***(16)***, and is shown in Figure 1.

The operation of the device first involves selecting an input solution using the multiplexer unit, which is directed to one of eight separate reactor rings. Each reactor contains four output ports, located at specific positions around the ring. Opening these ports exchanges a fixed fraction of the reactor volume, with the exact fraction depending on the position of the port. The placement of these ports allows the reactor to be loaded with a reaction of fixed composition, and importantly also allows a dilution step to occur which preserves this composition. In between dilution steps, the reaction is mixed using a peristaltic pump. Full details are given in Section 3.5.

We describe the photolithographic steps required to print the chip design on a chrome mask, and subsequently transfer it onto silicon wafers. Once fabricated, these silicon molds can be used for multiple rounds of soft lithography where they are used to cast PDMS devices. Finally, the hardware required for operating the chip is described, and a standard experiment outlined. Related protocols are available in the literature ***(19,20)***.

2. Materials

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.

2.1. 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)

2.2. 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)

2.3. 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_2 supply (Diener electronic GmbH + Co. KG)
- Universal Oven UF110, 108L (Memmert)
- SZX10 dissection microscope with DF PLANO 1.25x objective and KL 1500 LCD light source (Olympus)

2.4. Soft lithography consumables

- Trimethylchlorosilane (Sigma)
- Sylgard 184 polydimethylsiloxane (PDMS) elastomer and curing agent (Dow Corning)
- Glass slides 76x26x1 mm 631-1550 (VWR)

2.5. 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)

2.6. 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)

2.7. Microscope hardware

- Ti2 Eclipse Inverted Microscope (Nikon)
- Objectives: CFI Achro 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)

2.8. Software

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

2.9. Experimental reagents

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

3. Methods

3.1. Design of microfluidic devices

3.1.1. 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.

3.1.2. Using CleWin, convert the designs to a machine-compatible .cif file ready for photomask fabrication.

3.1.3. 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.

3.2. Photolithography for mask and wafer fabrication

3.2.1. Mask fabrication

3.2.1.1. 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**).

3.2.1.2. Next, process the exposed masks using the HMR900 mask processor. This involves the following automated steps:

3.2.1.3. First purge the machine with DI water.

3.2.1.4. 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.

3.2.1.5. Etch through the chrome layer for 60 s using the Cr01 etchant, and rinse.

3.2.1.6. Finally strip the photoresist using the AZ 400K developer for 35 s, followed by a final rinse and drying with CO_2. The completed masks should be completely dry before use.

3.2.2. Flow mold fabrication

3.2.2.1. Prime a clean Si wafer with HMDS (*see* **Note 6**) for 10 s in vacuum, using the VB20 hotplate.

3.2.2.2. 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**).

3.2.2.3. Spin coat at 920 rpm for 100 s, followed by 60 s relaxation at 0 rpm. This deposits a 14-µm layer of photoresist on the surface of the wafer.

3.2.2.4. When the spin coating has finished, immediately transfer the wafer to a preheated hotplate, and 'softbake' for 6 minutes exactly at 115°C.

3.2.2.5. Transfer the wafer to an opaque storage box and allow it to rehydrate for a minimum of 1 hour (*see* **Note 9**).

3.2.2.6. 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^2 (*see* **Notes 10, 11**). Use the following parameters: expose type = hard, alignment gap = 30, WEC type = cont, N2 purge = NO, WEC-offset = OFF.

3.2.2.7. 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**).

3.2.2.8. Rinse with DI water, then carefully but rapidly dry the wafer with N_2, and inspect features under a microscope. If photoresist residues remain,

develop further until all the residues are removed and repeat the cleaning and drying.

3.2.2.9. 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

3.2.3. Control mold fabrication

3.2.3.1. Clean an Si wafer with 2.45 GHz O_2 plasma in the Tepla 300 Plasma Stripper, using 500 W for 7 min and 400 ml/min of O_2.

3.2.3.2. 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.

3.2.3.3. Spin coat a 40-µm 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.

3.2.3.4. 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.

3.2.3.5. 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^2. Use the following parameters: expose type = soft, alignment gap = 30, WEC type = cont, N2 purge = NO, WEC-offset = OFF.

3.2.3.6. 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.

3.2.3.7. Transfer the wafer to an opaque storage box and wait from 1 hour to overnight before development.

3.2.3.8. 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.

3.2.3.9. 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.

3.2.3.10. Inspect features under a microscope and carefully develop further if needed. Avoid overdevelopment, which can lead to breaking of features.

3.2.3.11. 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.

3.3. Soft lithography for device fabrication

3.3.1. Silanization of wafers

3.3.1.1. 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.

3.3.2. Casting and curing of PDMS devices

3.3.2.1. 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.

3.3.2.2. 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.

3.3.2.3. Clean both flow and control wafers using pressurised N_2.

3.3.2.4. 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.

3.3.2.5. 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.

3.3.2.6. After coating, the PDMS layer will be uneven due to the high 40-µm 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.

3.3.2.7. 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**).

3.3.2.8. 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.

3.3.2.9. 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**).

3.3.2.10. Put the aligned devices back into the oven at 80°C and bake for a minimum of 1 hour 30 minutes.

3.3.2.11. Cut the multilayer devices off the wafer using a scalpel.

3.3.2.12. Using the hole puncher, punch through all the channel inlets.

3.3.2.13. 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.

3.3.3. Bonding of PDMS devices to a glass slide

3.3.3.1. Clean glass slides using pressurised N_2.

3.3.3.2. Remove any residual dust from the slide and feature surface of the PDMS device using Scotch tape (*see* **Note 18**).

3.3.3.3. Switch on the Femto plasma oven and place the slide and PDMS device bonding-side-up.

3.3.3.4. Pump out the chamber for at least 15 minutes to ensure a clean vacuum environment.

3.3.3.5. Switch on the O_2 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**)).

3.3.3.6. 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.

3.3.3.7. The completed devices can finally be stored at room temperature until use (*see* **Note 21**).

3.4. Hardware setup

Air pressure is supplied to the setup using polyethylene (PE) tubing connected directly to the laboratory compressed air supply. A schematic of the setup's pneumatic connections is shown in Figure 2.

3.4.1. Regulation of control layer pressure

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

3.4.1.2. 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).

3.4.1.3. 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**).

3.4.2. Regulation of flow layer pressure

3.4.2.1. Connect the other branch of the input air supply to a regulator, and connect the regulated supply to the manual luer manifold.

3.4.2.2. Adjust the pressure as required (typically ~0.3 bar).

3.5. Device Operation

3.5.1. Filling control lines

3.5.1.1. Lower the control manifold pressure to around ~10 psi.

3.5.1.2. Using the PC software, close all the control line valves.

3.5.1.3. Fill each Tygon line with deionised water (*see* **Note 23**) through the connecting pin, using a syringe attached to a luer stub.

3.5.1.4. Connect each line to the appropriate control channel inlet.

3.5.1.5. 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.

3.5.1.6. Visually inspect all the valves to check that they actuate fully.

3.5.2. Filling flow lines

3.5.2.1. Make sure the appropriate manual manifold valve is closed.

3.5.2.2. 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.

3.5.2.3. Attach a syringe to the luer stub, and carefully draw up the required reagent into the tubing. Make sure there are no bubbles.

3.5.2.4. Attach the connector pin to the appropriate flow inlet, before removing the syringe and attaching the luer stub to the manual manifold.

3.5.2.5. 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.

3.5.2.6. 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.

3.5.2.7. 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)***.

3.5.3. Cell-free expression

3.5.3.1. The device can be characterised as shown in Figure 3.

3.5.3.2. A typical experimental program is shown in Figure 4. First switch on the environmental chamber to 29˚C.

3.5.3.3. Load each reactor with cell-free extract, energy solution, and DNA in the ratio 40%, 40%, 20%.

3.5.3.4. The reactor contents are mixed by actuating the four multi-function valves sequentially at a frequency of 20 Hz.

3.5.3.5. 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.

3.5.3.6. The dilution rate can be varied by adjusting the interval between dilution steps.

3.5.3.7. Image the resulting fluorescence using the microscope setup. Software for the analysis, example images, and full documentation can be found online (*see* **Note 24**).

4. Notes

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).
2. Excellent guidance is available e.g. ***(22)***
3. Designs for microfluidic devices are available online at http://lbnc.epfl.ch/microfluidic_designs.html.
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.
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.
6. HMDS priming enhances photoresist adhesion. Alternatively the wafer can also be treated with O_2 plasma or thermally dehydrated.
7. Pouring directly from the bottle introduces fewer bubbles than using a plastic pipettor.

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.
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).
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.
11. For 15 µm AZ9260, the recommended dose is 580 mJ/cm^2 for i-line exposure and 660 mJ/cm^2 for broadband.
12. The recommended development time is around 45 s per µm of AZ9260.
13. Rounded features are crucial for the flow layer as it allows valves to close completely.
14. The variable here is the ramp time, which depends on the specific type of SU8 used
15. It is highly recommended to develop the wafer upside down. Prepare two baths of PGMEA.
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.
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.

18. This step is important as the presence of dust between the glass and PDMS can compromise bonding or render the device non-functional.
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)***.
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.
21. In our experience, devices can still be functional after 6 months' storage.
22. https://github.com/nadanai263/lbnc-cellfree2
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.
24. https://github.com/nadanai263/lbnc-cellfreeview

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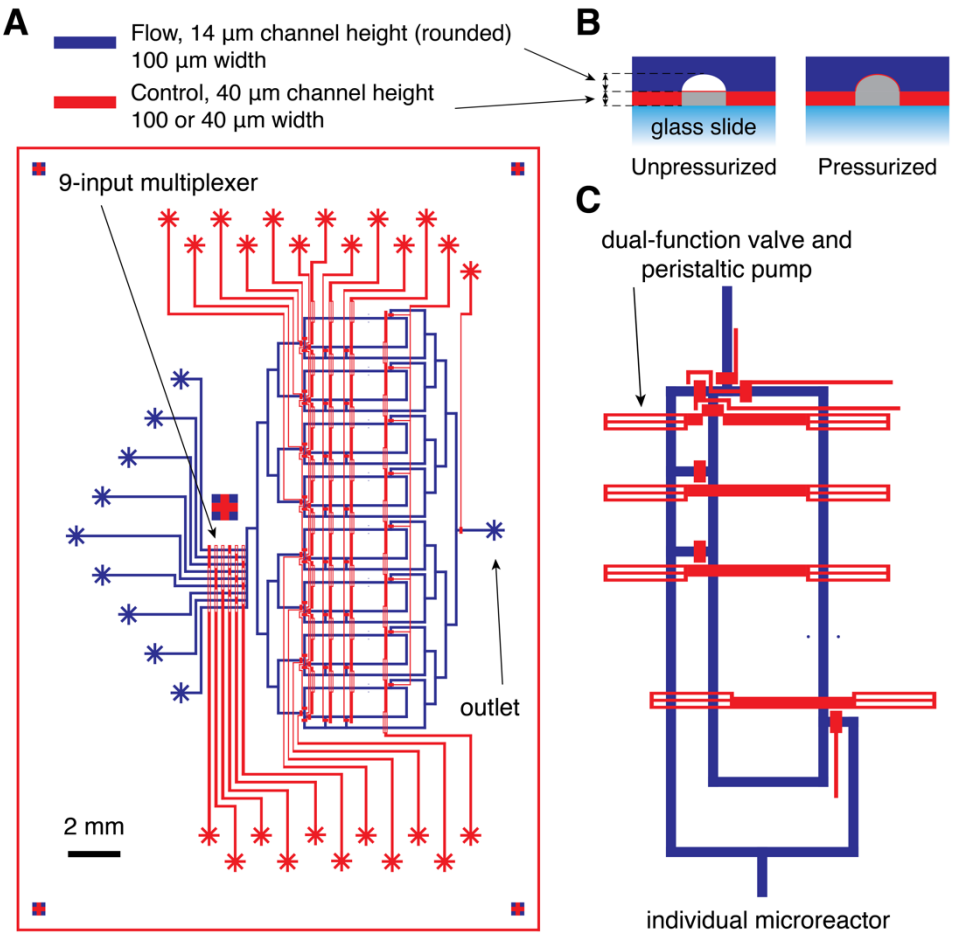
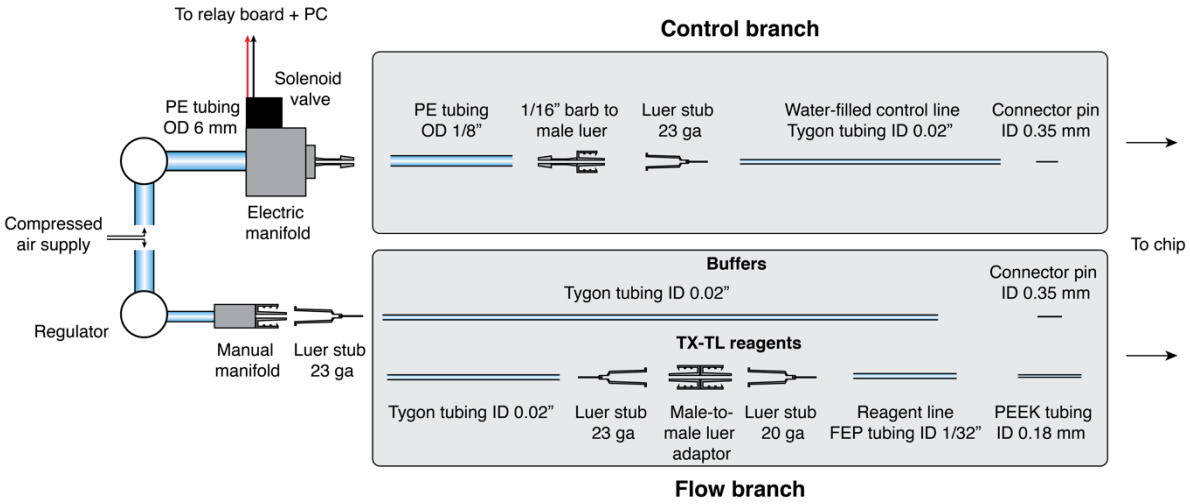
Figure 1



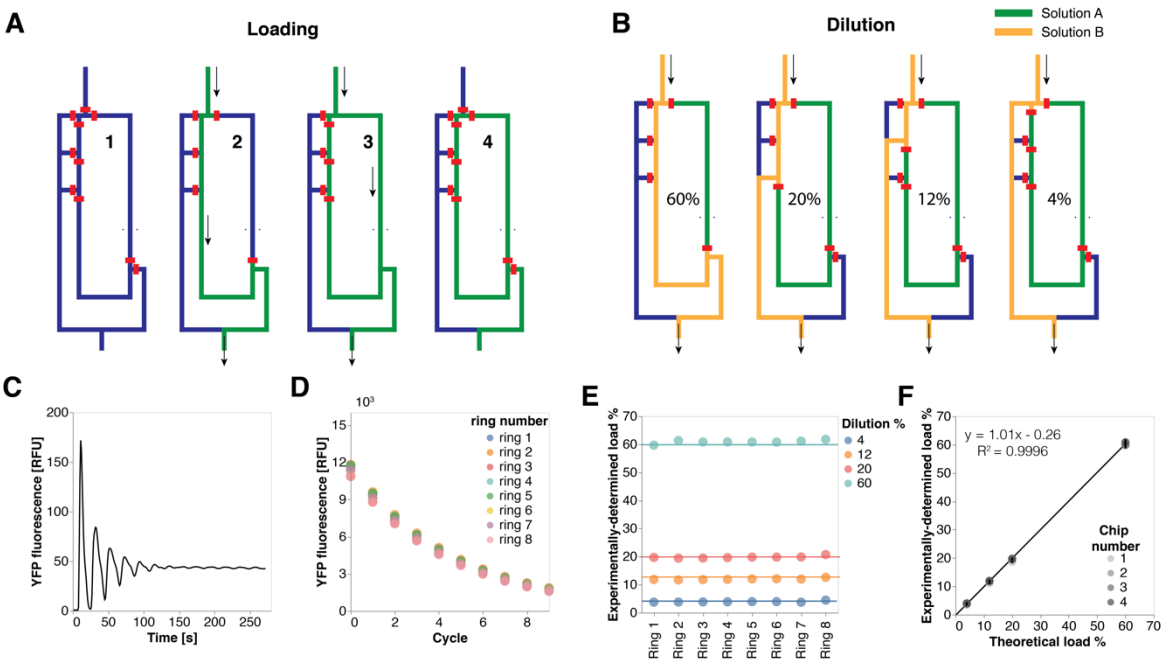
(A) A two-layer microchemostat design consists of a thin control layer sandwiched between a glass slide and a thicker flow layer. **(B)** Applying pressure to channels in the control layer pushes up valves which close off channels in the flow layer. **(C)** The chip contains eight individual chemostat reactors. Four control lines serve as dual-function valves and peristaltic pump. Actuating these lines sequentially mixes the liquid inside the reactors.

Figure 2



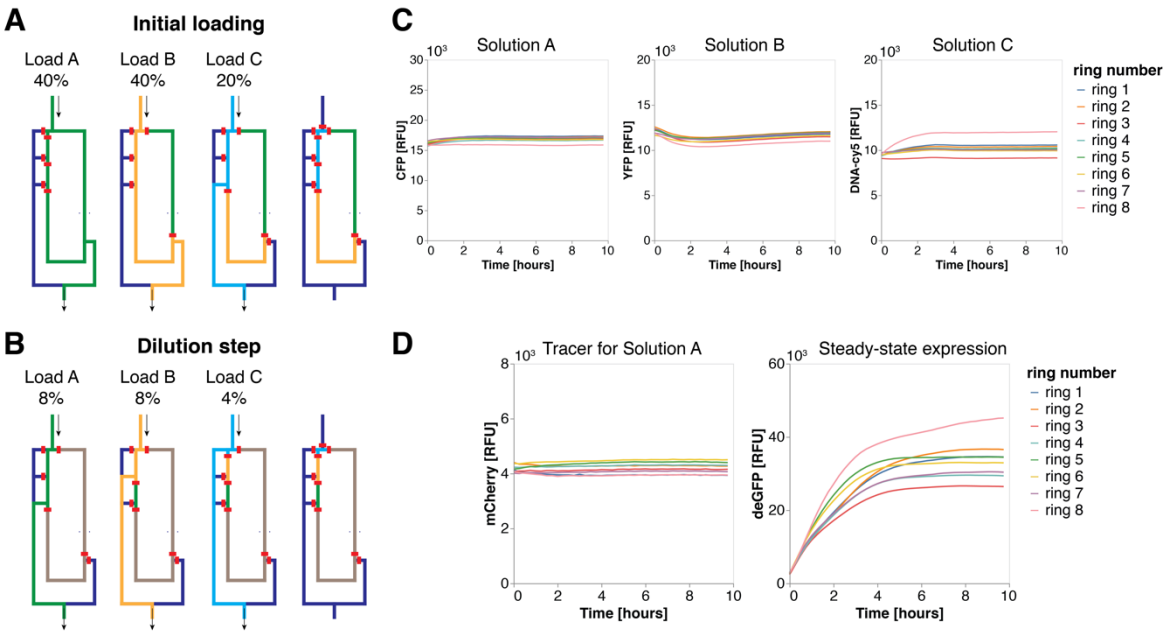
Pneumatic connections for the setup. The compressed air supply is split into two independently regulated branches. Pressure in the control branch is switched using electric valves while the flow branch is controlled manually. Buffers and other input solutions are stored in Tygon tubing, while cell-free (TX-TL) reagents are stored in FEP-PEEK tubing.

Figure 3



Basic operations and characterisation of the chip. **(A)** Initial loading is achieved by flowing an input solution (solution A, green) first through one side of the reactor, then the other. **(B)** Dilution takes place by flushing an input solution (solution B, yellow) through different outlets. The dilution fraction is controlled by the geometric positioning of the outlets and is fixed for a given design. **(C)** After loading 20% of a reactor with YFP, actuating the peristaltic pump at 20 Hz mixes the solution in ~100 s. **(D)** This shows the fluorescence from all eight reactor rings, initially loaded with 20% YFP, and repeatedly diluted with buffer. **(E)** Experimentally determined dilution fraction for each of the eight reactors. **(F)** Experimentally determined load fraction vs theoretical load fraction for four different chips.

Figure 4



Typical experimental operation of the chip. **(A)** The chip is initially loaded with three solutions A-C (green, yellow, blue) in the ratio 40%, 40%, 20%, and **(B)** subsequently diluted with the same solutions in the ratio 8%, 8%, 4%. **(C)** Carrying out this process using aqueous solutions of three different fluorescent tracers demonstrates that steady-state concentrations are maintained over many hours. **(D)** Steady-state cell-free expression can be achieved by adding as the three solutions cell-free lysate (solution A), energy solution (solution B), and DNA template (solution C). The lysate is labelled with an mCherry tracer to assess its concentration (left), while the reaction produces deGFP, which reaches a steady-state concentration when production and dilution rates are equal (right). Here, a dilution step was carried out every 15 minutes.