Electronic Supplementary Material (ESI) for Lab on a Chip.
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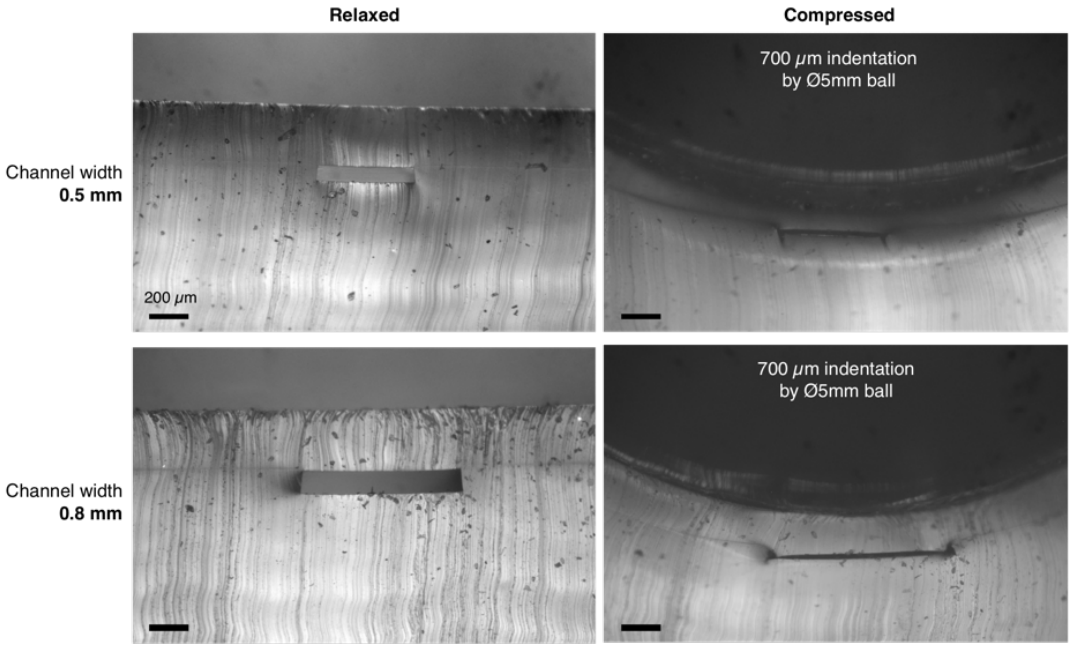
Comprehensive Multiplexed Superfusion System Enables Physiological Emulation in Cell Culture: Exemplification by Persistent Circadian Entrainment

Supplementary Materials

Device design and characterisation



Supplementary Fig. 1 Cross-section images of RPM2 pump channels. Channel occlusion by roller ball compression was tested in channels of 500 µm (wide) x 75 µm (high), and 800 µm (wide) x 125 µm (high). Compression of 700 µm imposed on the channel chip by a roller ball of Ø5 mm was able to fully occlude both channels 300 µm underneath the surface. The channel profile was restored upon compression removal (relaxation).

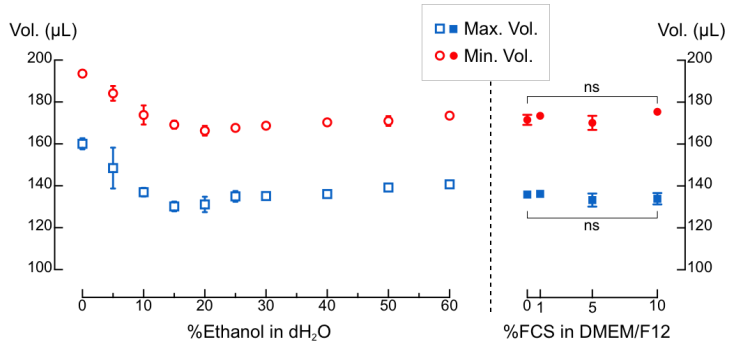
Effect of surface tension in volume fluctuation during superfusion in culture plate

Volume fluctuation in the experiment is an interesting observation. Simplified liquid meniscus shape and column height may be estimated, based on contact angle, surface tension, and liquid density[1]. However, in our perfusion setup, the position (distance to well bottom/side), gauge, and material (stainless steel or PTFE) of the inlet and outlet, and flow rate also played important roles in determining the formation and loss of meniscus. Together, it is impractical to numerically predict the dynamic of retention volume.

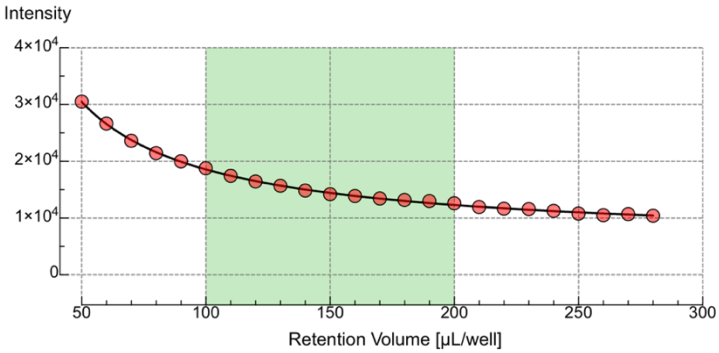
To experimentally test the effect of surface tension on the range of medium volume in our perfusion system, we constitute 0-60% ethanol in water to obtain a spectrum of solutions of decreasing surface tension (27.9 to 72.6 mN/m) without significantly altering the viscosity based on previous report[2]. We then subjected these solutions to perfusion cycle in Fig.5d in a 96-well format. The minimum volume (Min. Vol.) is recorded when the meniscus to the outlet breaks and the maximum volume (Max. Vol.) is recorded when the liquid surface re-establish contact with the outlet. As shown in Supplementary Fig. 2 (left), the average retention volume dropped from 176.8±2.6 µL (in pure water, γ=72.6 mN/m) to 148.7±3.6 µL (in 20% ethanol, γ=42.9 mN/m) and stayed relatively stable despite of further decrease in surface tension. Notably, the range of volume fluctuation remained unchanged at 34.5±2.2 µL regardless of surface tension shift. We then proceeded to measure the volume fluctuation using culture medium (DMEM/F12) with increasing concentrations of FCS, representing perfusion medium and complete growth medium. The surface tension of culture broth/medium has been previously reported in the range of 40-50 mN/m[3]. As shown in Supplementary Fig. 2 (right), despite of an increase in FCS content, the retention volume limits for the medium

are largely unchanged going from Min. 134.8±1.4 µL to Max. 172.7±2.3 µL, corresponding to a similar measurement in the ethanol standard with surface tension ~40 mN/m on the left. Meanwhile, to probe the phenomenon in different perfusion format, volume fluctuation in a 24-well was also explored. As expected, for an averaged 1 mL retention, a Min-Max volume difference of 224.2±4.5 µL was recorded, which (after considering the well bottom surface area) corresponds to a similar ~1.1 mm liquid height change that gives the volume difference of 38.9 µL if in a 96-well format. Therefore, the observation and understanding of volume fluctuation may be applicable to different well/perfusion formats with predictable patterns.

Collectively, while many factors are at play to affect the dynamics of the volume fluctuation in our perfusion system, the current setup can still be a practical and robust approach to multi-well perfusion with acceptable precision and accuracy, especially if we operate within the recommended pragmatic perfusion protocol as highlighted in Fig. 5g to obtain ⩽5% drug concentration variation. Meanwhile, with the fluctuation period depending on ΔQ (=outflow-inflow), researchers may adjust the fluctuation frequency to match and simulate artery pulsing or breathing rhythm for additional layer of emulation capacity in perfusion.



Supplementary Fig. 2 Upper and lower volume limits during fluctuation cycles in 96-well perfusion format. Left: various ethanol concentrations diluted in water. Right: DMEM/F12 culture medium with various biologically relevant FCS concentrations. Volumes were calculated through dividing the weight of liquid by respective solution density as per formulation. Three independent measurements were averaged in each condition. Two-way ANOVA (0% - 10% FCS).

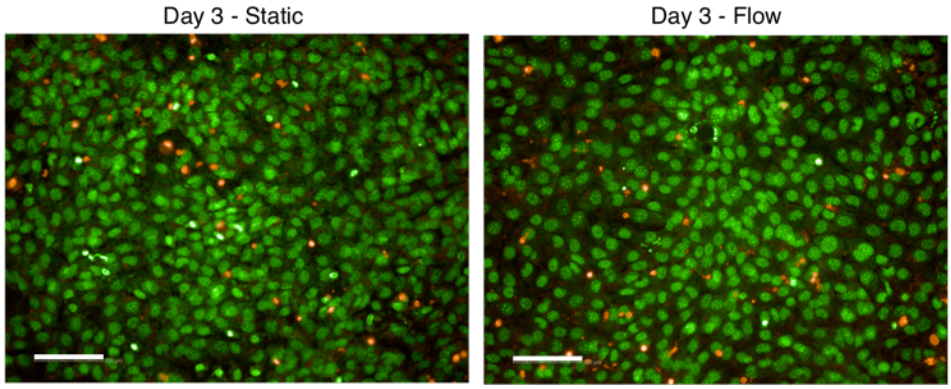


Supplementary Fig. 3 Standard curve of relationship between liquid dye volume in a 96-well and the detected light intensity. 96-well plate with superfusion apparatus installed was placed on a widefield the microscope. Water soluble True-blue dye was diluted to 1% in Milli-Q water. Specific volumes of 50 to 280 µL (at 10µL increment) was added to single wells. Actual volume fluctuation of around 150 µL in superfusion experiment was expected and capped between 100 to 200 µL. Photonic intensity was recorded with a fixed 20 ms exposure time.

Circadian rhythm maintenance with superfusion

For conventional circadian rhythm daily entrainment and monitoring, bioluminescence recording for a period of 2-3 hours/day was lost during the manual cortisol medium exchange due to inevitable operation outside the incubator and imaging platform. There was also an approximately 4 hours recovery of the culture plate (white) from auto-luminescence after ambient light exposure. Hence, the data quality in manual entrainment was considerably

inferior to that in automatic superfused condition, especially on signal-to-noise (S/N) ratio. Therefore, our superfusion-assisted entrainment eliminates the need of daily manual labour whilst also improving the controllability of entrainment and circadian data quality.



Supplementary Fig. 4 Viability of PER2::Luc murine embryonic fibroblast (MEF) was assessed in static and superfused conditions. a) Representative images of acridine orange and ethidium bromide (AO/EB) staining of MEF monolayer culture after 3 days in static or superfused condition. EB (in orange) signifies apoptotic/dead cells while AO (in green) indicates live cells. Scale = 100 μm.

Supplementary Video 1

Superfusion of culture medium at a 200 uL/min flow rate in 24- and 96-well plates. Phenol-red medium was infused into the wells filled with PBS in real time to visualise the medium exchange process. Mechanical stimulation (e.g., shear) on the sample at the bottom of the well was minimised.

Supplementary Video 2

Bioluminescence recording of circadian entrainment on Per2::Luc murine embryonic fibroblast (MEF) monolayer culture using cortisol pulse(s). Plate layout (96-well plate) was shown in Fig. 6a. Briefly, in total twelve wells were superfused with (top right) or without (bottom right) cortisol entrainment, while the static wells were on the left in the same image field.

Reference

1 S. Liu, S. Li and J. Liu, *Eur. Phys. J. E*, 2018, **41**, 1–7.

2 R. Dufour, G. Perry, M. Harnois, Y. Coffinier, V. Thomy, V. Senez and R. Boukherroub, *Colloid Polym. Sci.*, 2013, **291**, 409–415.

3 M. S. Marshall, *J. Infect. Dis.*, 1924, **35**, 526–536.