Electronic Supplementary Material (ESI) for Analyst.

Supplementary information for: Multiplexed microfluidic chip for cell co-culture

Craig Watson[1], **Chao Liu**[1], **Ali Ansari**[1], **Helen C. Miranda**[2], **Rodrigo A Somoza**[3,4], **and Samuel E. Senyo**[1,✉]

[1]Department of Biomedical Engineering, Case Western Reserve University, Cleveland OH USA
[2]Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, OH, USA
[3]Department of Biology, Skeletal Research Center, Case Western Reserve University, Cleveland, OH, USA
[4]CWRU Center for Multimodal Evaluation of Engineered Cartilage, Cleveland, OH, USA

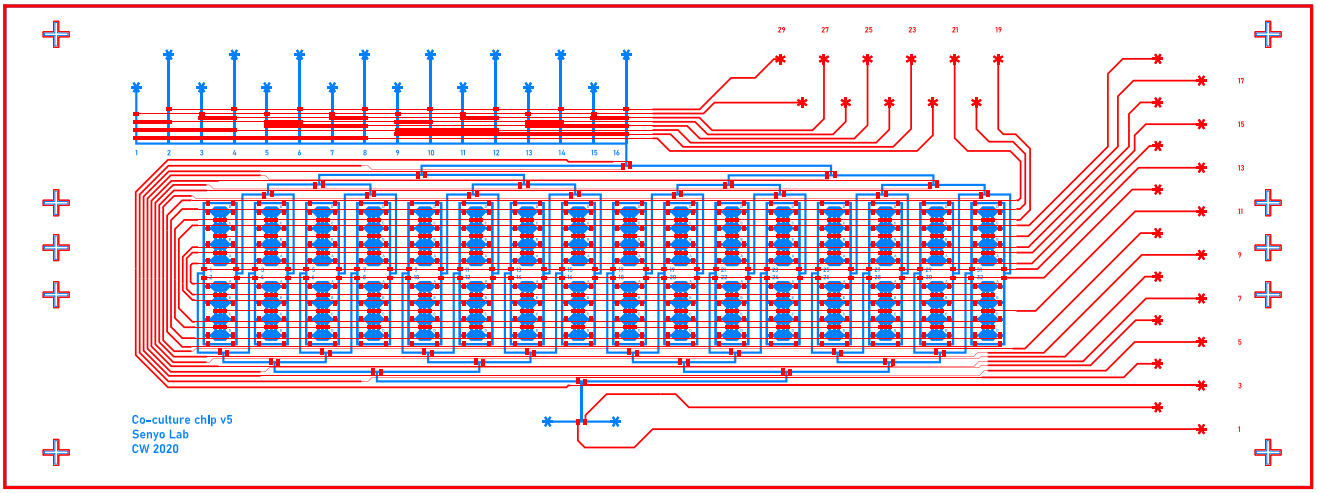
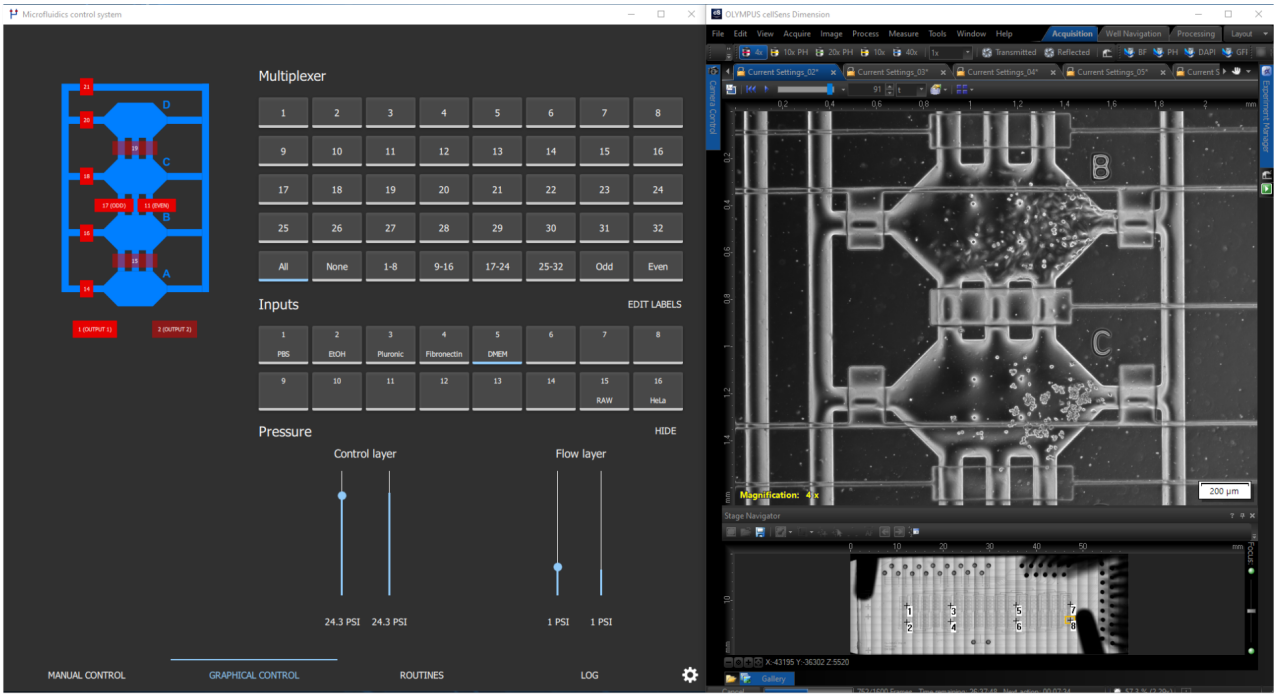


Fig. S1. Complete chip design schematic, with flow layer in blue and control layer in red.

A



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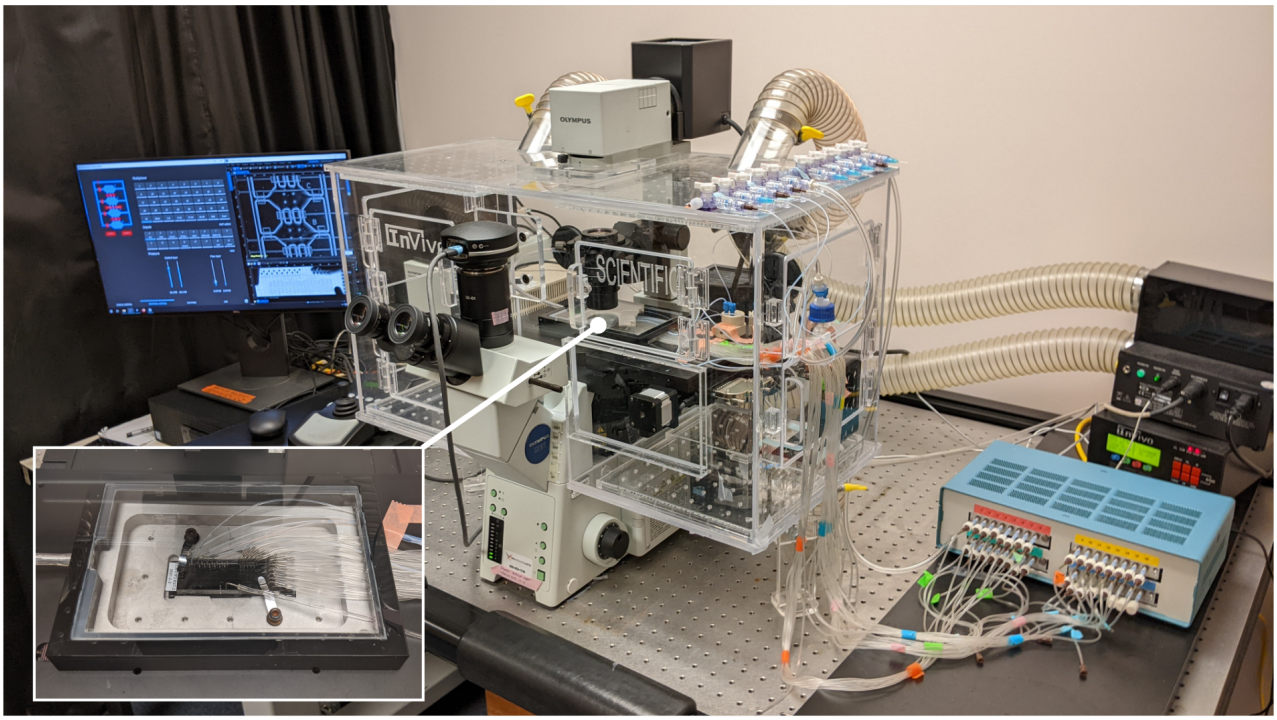

Fig. S2. Hardware and software setup **A)** Screenshot of the software in operation. Left: the Qt-based GUI with graphical control showing the live state of the chip and buttons to select multiplexer channel, input, and pressures for the control and flow layers. Some of the most commonly-used multiplexer configurations are provided, while any others are set manually by actuating individual valves (in the manual control screen, not shown). Right: microscope control software (Olympus CellSens) shown here acquiring time-lapse images. **B)** Hardware setup including microscope with heated enclosure and stage-top chamber (inset) for temperature and CO_2 control, respectively.

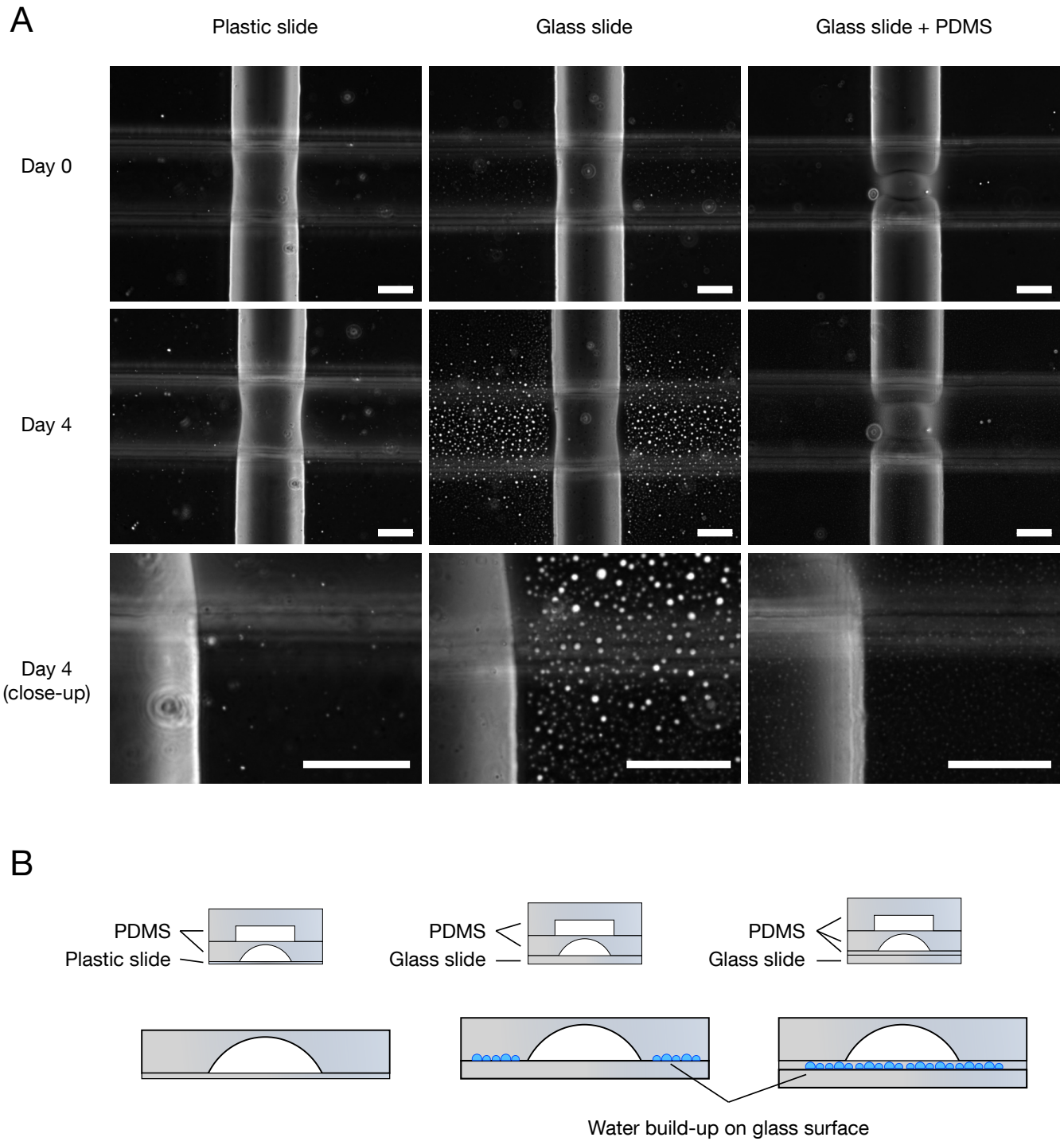

Fig. S3. A) Phase contrast images of a flow channel (vertical) and control channel assembled onto the three different substrates. Water slowly diffuses out of the channels and accumulates on the surface of the glass slides. In the case of PDMS-coated slides, the droplets accumulate below channels and chambers too, introducing artifacts when imaging cells, though the effect is less than with the plasma-treated glass slide. Plastic slides are sufficiently permeable to allow evaporation. In all cases, the water diffusion only affects imaging quality as frequent media replenishment prevents any large changes in osmolarity. Scale bars: 50 µm **B)** Cross-section schematic of water build-up, illustrating where droplets accumulate with the different arrangements of layers in the microfluidic chip.

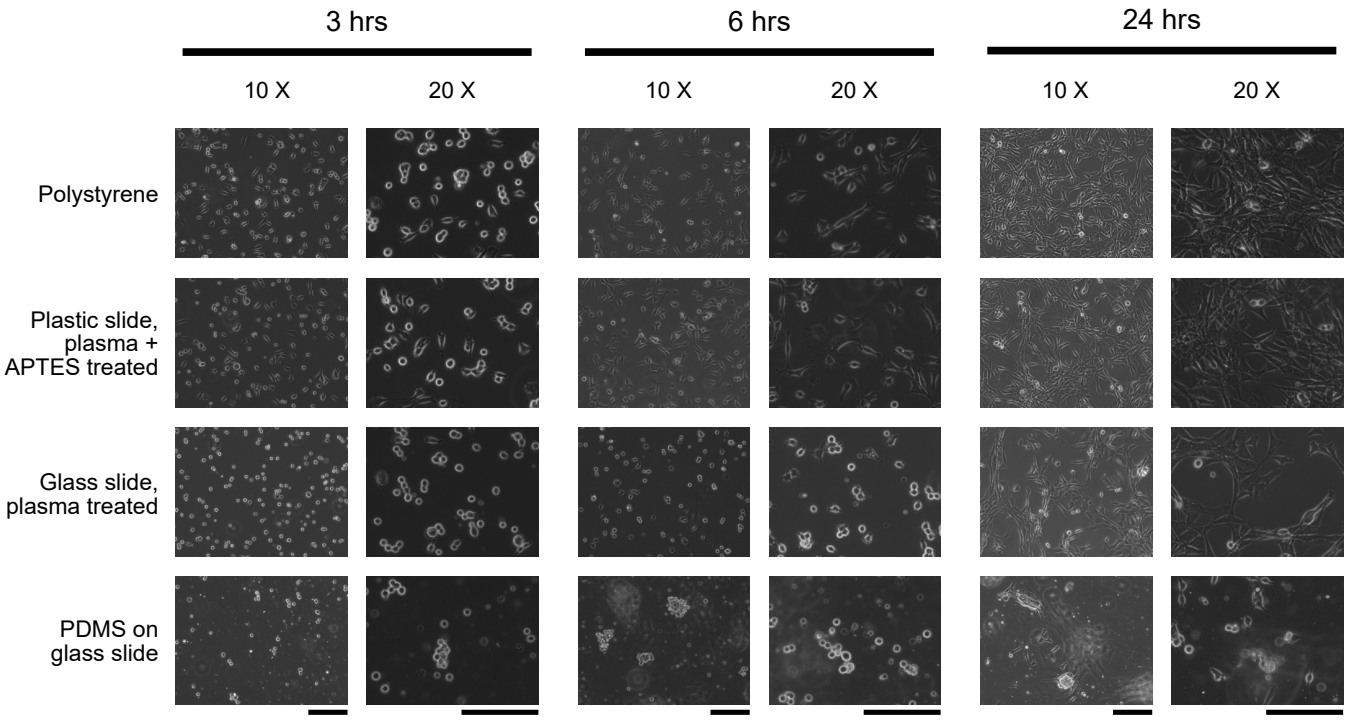

Fig. S4. Cell attachment on different substrates. 3T3 fibroblasts were cultured in a 12-well plate, either directly or plated on the different substrates used in the chip. Substrates were prepared in the same way as in chip fabrication. Scale bars: 200 µm.

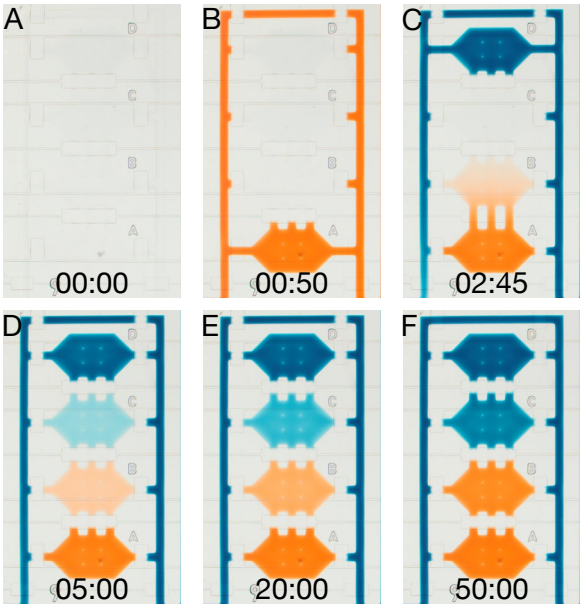

Fig. S5. Validation of media supply protocol using food dye and a 5-minute resupply period; numbers indicate time in minutes:seconds. **A)** All chambers filled with water. **B)** Orange dye perfused through rinse channel then chamber A. **C)** Blue dye perfused through rinse channel then chamber D, while the valve between chambers A and B is opened to allow diffusion. **D-F)** Dye concentration increases in the central two chambers as more media supply cycles are completed.

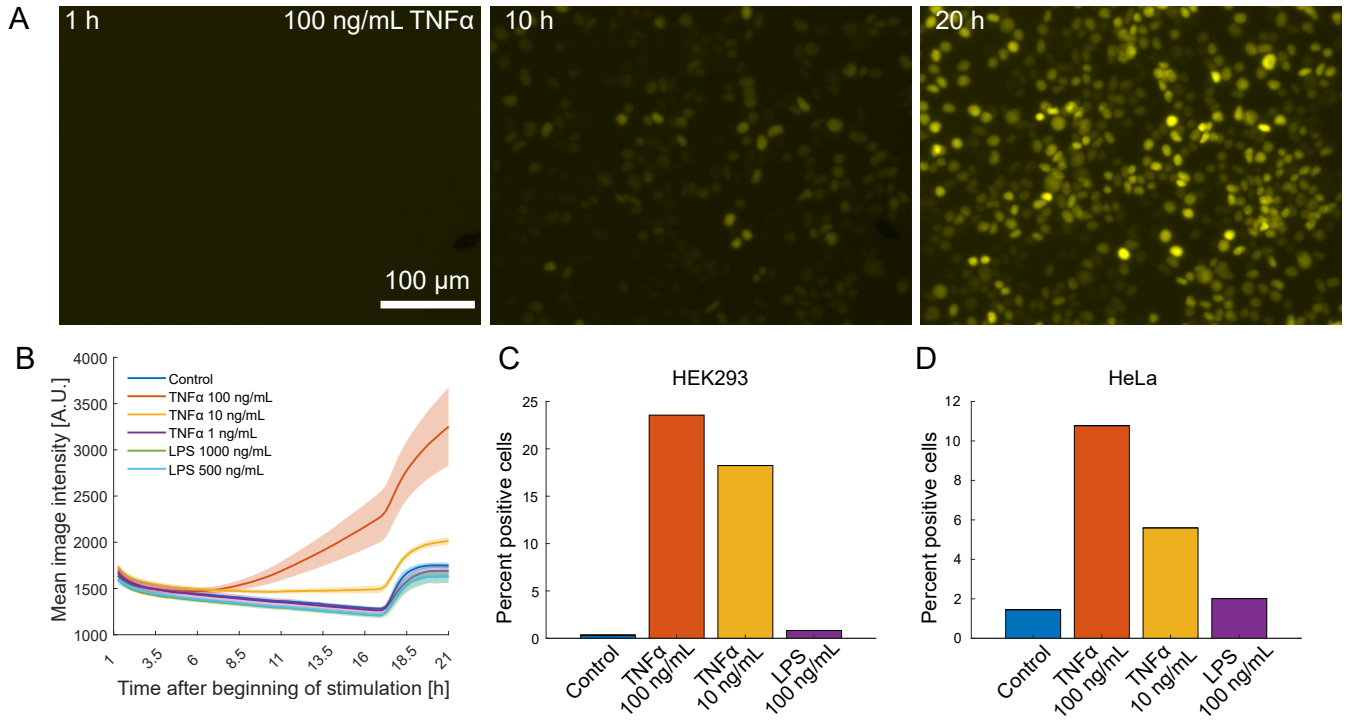

Fig. S6. NFκB mKate2 reporter validation. **A)** HEK293Ta treated with 100 ng/mL TNFα, shown at 1, 10 and 20 hours after stimulation onset. **B)** Mean image intensity (raw, unprocessed images) over time of the transduced HEK293Ta. Images were taken every 15 minutes. Shaded areas indicate standard deviation (1 experiment with 3 images per well, 2-4 wells per condition). **C, D)** Endpoint (22 h) analysis of transduced HEK293Ta and HeLa treated with LPS or TNFα (1 experiment with 3 images per well, 2 wells per condition). Cells in C) and D) were imaged only at the 22h timepoint to prevent photobleaching.

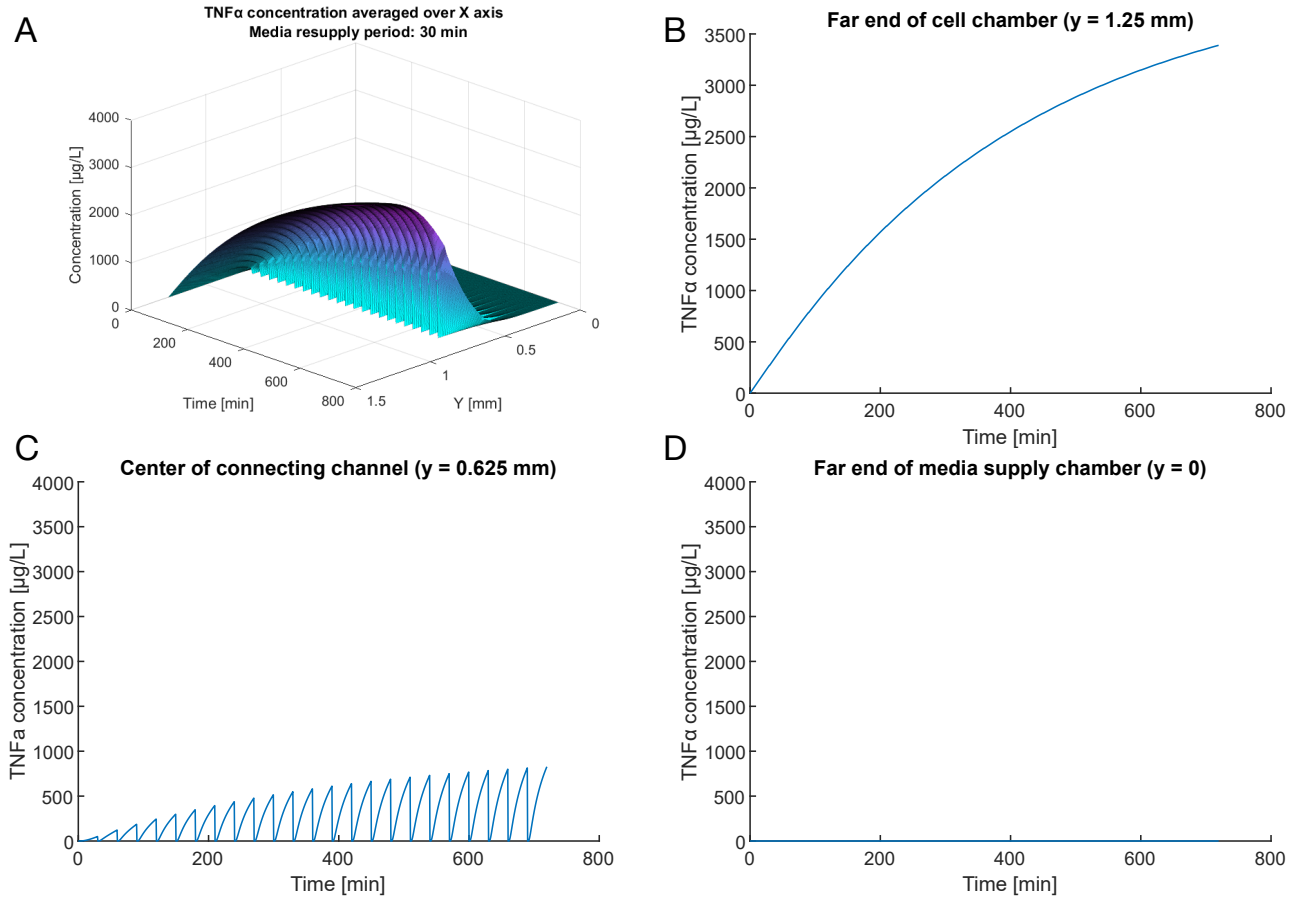

Fig. S7. TNFα secretion and diffusion model. Adapted from the glucose model, with modified secretion and diffusion rates; all other parameters such as chamber geometry and media supply modality were kept identical. **A)** TNFα concentration over time and distance from the media supply chamber, with concentration averaged over the x axis as in the glucose model. **B-D)** Concentration over time at different "slices" of the plot in A (y=1.5 mm, y=0.625 mm and y=0).

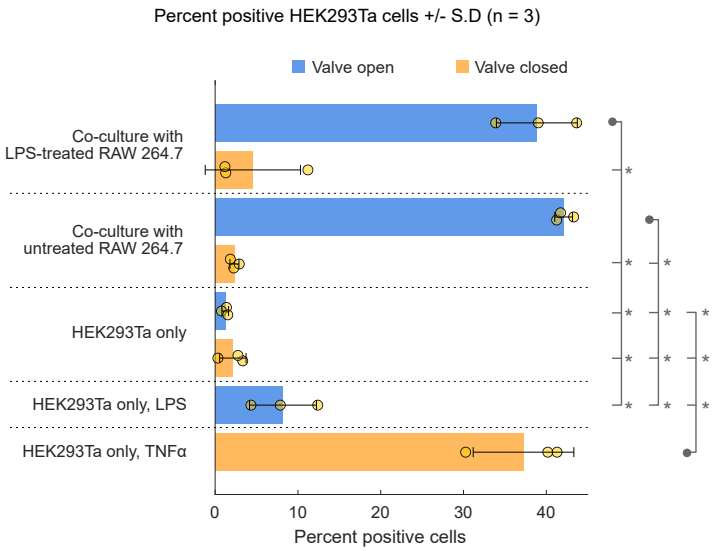

Fig. S8. Detailed results of NFκB co-culture assay, showing statistically significant differences between groups ($p < 0.01$). Comparisons are noted with a gray dot marking the group that is significantly different from all groups on the same line as indicated by tick marks and asterisks. Data including analysis script are provided in supplementary file 4.

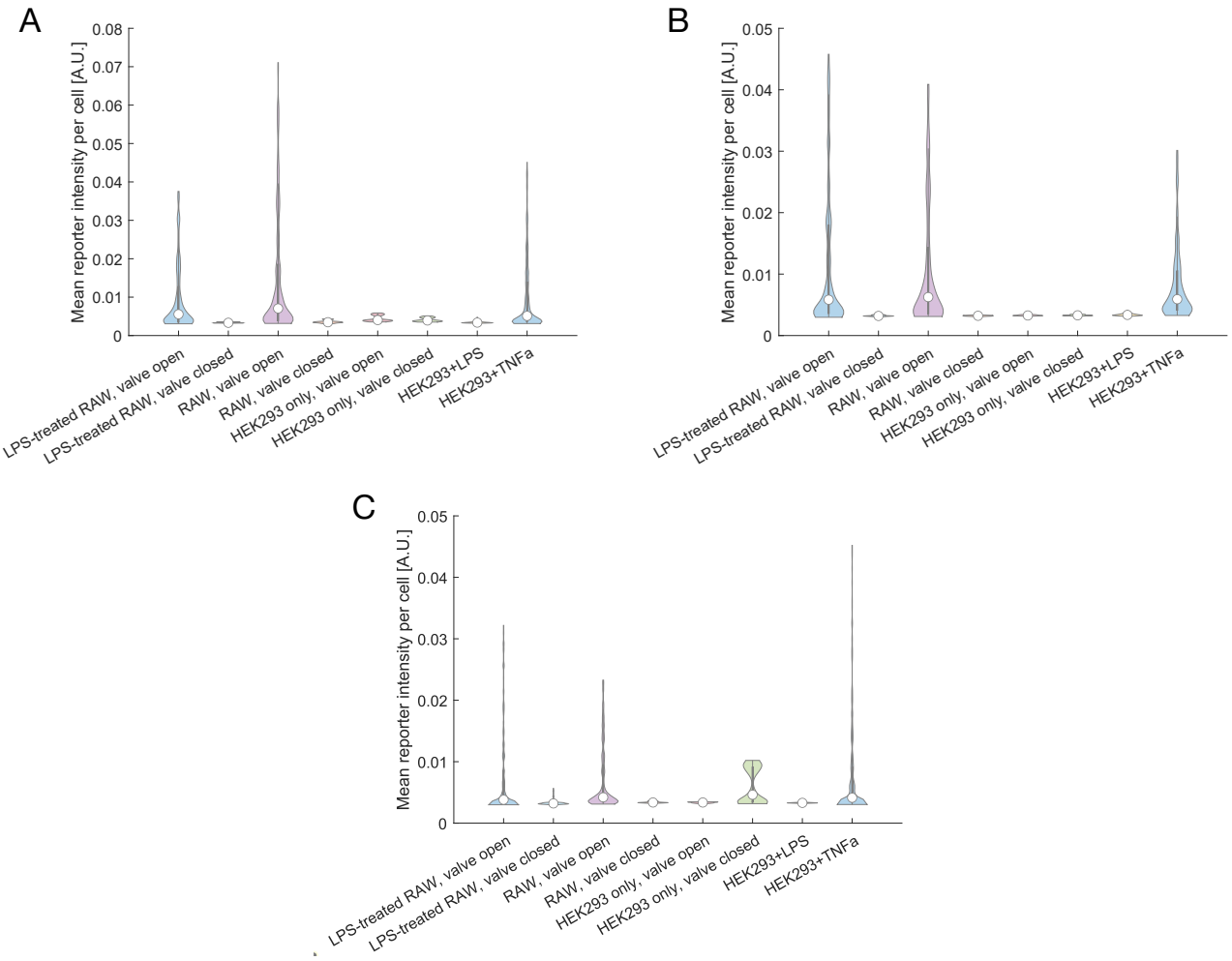

Fig. S9. Distribution of cell intensities in each of the three co-culture experiments (A-C, experiments 1-3 respectively). Outliers (top 10%) were removed to visualize the distributions more clearly. See supplementary file 4 for full data analysis pipeline and results.

Supplementary note 1: Immunocytochemistry protocol

PBS, TX-100 (0.1% in PBS), PFA (paraformaldehyde, 4% in PBS), Stain buffer (0.1% TX-100, 10% goat serum, PBS), primary antibody and secondary antibody solutions are loaded into tygon or PTFE tubing, connected to the chip, and degassed. Then, the reagents are perfused through the culture chamber in the following sequence:

1. PBS, 2 min
2. PFA, 8 min
3. TX-100, 8 min
4. Blocking buffer, 5 min
5. Primary antibody, 15 min
6. Blocking buffer, 2 min
7. Secondary antibody, 10 min
8. (Optional) DAPI stain, 10 min
9. PBS, 2 min

All perfusion steps are done at 2.8 kPa (0.4 psi), through one chamber of all 32 units. Before each step, the reagent is first flushed through the waste channels for 30 seconds at 13.8 kPa (2 psi).