Lab on a Chip

Merging drops in a Teflon tube, and transferring fluid between them, illustrated by protein crystallization and drug screening

Electronic Supplementary Information

ESI methods

Merging 51 drops

For Figure 6A, 51 800-nl aqueous drops were engulfed in one super-drop of tetradecane + 2.5% Span80, which – in turn – was engulfed by HFE7500; this train passes (flow 2 ml/h) through LED1/photo-diode1 to yield the blue voltage trace (data collected at 100 Hz). Once the last of the oil passes LED1, flow is increased to 6 ml/h, and water drops merge one after another. Flow is reduced to 2 ml/h when the front of the oil travels 2.5 m to reach LED2/photo-diode2 (which gives the red voltage trace). The two LED/photo-diode systems yield slightly different voltage differences between HFE7500 and oil, so differences were equalized.

Imaging

All images (other than those of cells; below) were collected using a camera (Olympus D7100 DSLR) connected to an epi-fluorescent microscope (Olympus IX53; 1.25X, 4X, 10X, 25X objectives) with translation stage and overhead illuminator (Olympus IX3 with filters) for bright-field images, and LED wavelength-specific sources (CoolLED) for fluorescent images. Image processing, analysis and illustrations were prepared using Matlab and/or CorelDraw. For several images, the PTFE tube was immersed in water to improve contrast with the walls of the tube. All movies were collected at video rates and play in real time.

Assessing cross-contamination

Cross-contamination between drops/trains was assessed using fluorescein and imaging (Figure S3) or using DNA and quantitative PCR (qPCR; Figure S4). In the latter case, drops ± DNA (100 ng/µl of plasmid pcDNA3; Invitrogen) were loaded manually (flow rate 0.05 ml/h) into a 150-µm tube using a rack and pinion (to give the architecture in Figure S4 so that drops contain more accurate volumes than those obtained manually): 30 nl water (drop 1), 30 nl tetradecane + 1% Span80, 200 nl HFE7500, 30 nl water containing DNA (drop 2), 30 nl tetradecane + 1% Span80, 200 nl HFE7500, and so on. After creating a train with 13 aqueous drops, the pump withdrew a further 3 µl HFE7500, and then was stopped for 5 min. Drops were now ejected individually into different Eppendorf tubes (flow rate 0.05 ml/h) containing 4 µl water (with the tip of the150-µm tube just breaking the water-air meniscus, as monitored with a camera attached to a 25x lens). Next, the DNA content of each tube was measured using a kit (Platinum® SYBR® Green One-Step qRT-PCR kit; Life Technologies) according to the manufacturer's instructions for qPCR (i.e., using Platinum Taq polymerase but no reverse transcriptase). PCR (25 µl) was performed using primers "pcDNA3 fw" and "rev" (Table S1), and a Mastercycler® realplex2 (Eppendorf) using temperatures of 95°C for 2 min, then 40 cycles of 95°C for 20 s + 62°C for 20 s + 72°C for 30 s, 72°C for 5 min, and finally those for the pre-programed melting-curve.

Cells

As CO_2 is used to maintain the pH of media during cell growth and Teflon is permeable to the gas, all fluorocarbons and oils used were pre-equilibrated in open vessels with 5% CO_2 in a conventional CO_2 incubator for >30 min; tubes were also placed in the incubator to allow cells to grow.

For Figure 9, NF-κB/Jurkat/GFP™ Reporter cells (System Biosciences, catalogue number TR850A-I) were used and maintained as described by the manufacturer. These cells grow in suspension and were derived by the supplier as follows. The human immunodeficiency virus was used to insert a GFP gene under the control of the minimal cytomegalovirus promoter downstream of four copies of the nuclear factor κB (NF-κB) consensus transcriptional-response element. Positively-transduced cells were selected using fluorescent activated cell sorting (FACS), and clonal populations stably retaining the provirus further selected; finally, a clone demonstrating a low GFP background, and a robust increase in GFP expression upon stimulation with TNFα was chosen. Two sets of 15 trains (Figure 9Ai) were generated in 2 tubes (fluids – 50% FC40 + 50% HFE7500, growth medium ± TNFα, 5-sSt silicone oil + 0.25% AbilEM180); each train contained one 1-µl drop with ~4,000 cells, plus a second 1-µl drop of medium + 20 ng/ml TNFα (PeproTech). An identical set without TNFα provided a control. After merging drops, tubes were plugged (by inserting metal plugs into free ends), placed in a conventional CO_2 incubator for 20 h, bright-field and GFP images of randomly-selected drops collected under water using a camera (AxioCam MRm) attached to a microscope (Zeiss Axioskop 40), and 30 merged drops from 2 tubes ejected into 1 ml PBS. GFP intensity in individual cells was now analyzed using a fluorescence-activated cell sorter (FACSCalibur™, BectonDickinson; GFP expression assessed using the 488-nm argon-ion laser, scattered signal using channel FL-1 and 515-545 nm). Cells grown ± TNFα and treated conventionally in a plate provided a control.

For Figure S5B, HEK-293 reporter cells (NF-kB/293/GFP-Luc™ Transcriptional Reporter Cell Line; System Biosciences, catalogue number TR860A-I), were used; they were maintained as indicated by the manufacturer, and seeded on to Cytodex beads (Cytodex® 1 micro-carrier beads; Sigma Aldrich; ~20,000 cells/1 cm^2 micro-carrier surface), where they grow attached to the surface. These cells were derived by the supplier as follows: a lentiviral vector was used to insert a GFP gene (plus a luciferase reporter, but luciferase levels were not assessed here) under the control of the minimal cytomegalovirus promoter downstream of four copies of the NF-κB consensus transcriptional-response element, and positively-transduced cells that responded robustly to stimulation with TNFα selected as described above for the analogous Jurkat reporter line used in Figure 9. Trains were generated (fluids – 50% FC40 + 50% HFE7500, growth medium ± TNFα, 5-cSt silicone oil + 0.25% AbilEM180); each train contained one 1-µl drop with cells on Cytodex beads (as above), plus a second 1-µl drop medium + 20 ng/ml TNFα (PeproTech). An identical set without TNFα provided a control. After merging drops, tubes were plugged, incubated for 20 h (as above), and bright-field and GFP images of selected drops collected (as for Figure 9Bi).

For Figure S5C, Hela cells were maintained in DMEM high-glucose medium (Life Techologies) + 5% FBS, seeded on to Cytodex beads, grown overnight, and serum-starved (18 h) to accumulate cells in G1 phase in medium containing 0.5% FBS. Subsequently, 15 trains each composed of a 1-µl drop containing ~30-40 microcarrier beads with the cells plus another 1-µl drop containing either TNFα (20 ng/ml; PeproTech) or medium only were loaded and merged, and sealed tubes incubated for 2.5 h – all as described for Jurkat cells. After incubation, merged drops from 5 similar trains were all ejected into 500 µl Direct-zol™ to lyse cells, and total RNA isolated using the Direct-zol™ MiniPrep kit (ZymoResearch). Levels of mRNA encoded by a gene responding to TNFα – TNFAIP2 – were now assessed using qRT-PCR as outlined above with primers "TNFAIP2 fw" and "rev" (Table S1). Results are averages given by the 3 sets of 5 trains. Three sets of RNA extracted from cells grown and treated conventionally on plates provided a control.

For Figure S6, standard Jurkat cells (a T-lymphocyte line which grows in suspension) were cultured routinely in RPMI 1640 (Sigma Aldrich) containing 10% FBS (Sigma Aldrich). Prior to incorporating cells into a 560-µm tube, the tube was filled with HFE7500, immediately loaded (flow rate 2 ml/h) successively with: 1 µl containing ~2,000 cells in RPMI 1640, 200 nl silicone oil (5 cSt) + 0.25% AbilEM180, 1 µl RPMI 1640 plus a drug (or DMSO as a control). Drops had merged in the first trains to be loaded prior to loading the last train, and flow was increased to 5 ml/h to ensure merging in all trains. Drugs (final concentrations given after merging drops) were 100 µM DRB (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; Sigma Aldrich) – an inhibitor of RNA polymerase II – or ionomycin (1 µg/ml) + 62.5 ng/ml PMA – (phorbol-12-myristate-13-acetate; Sigma Aldrich) – inflammatory activators.[45] The free end of the tube was now inserted into a second syringe to pressurise the system, and the now-sealed tube placed in a CO_2 incubator for 4 h at 37°C. Next, merged drops were ejected individually into 12 µl "CellsDirect resuspension and lysis buffer" (CellsDirect™; Life Technologies), and the mixture incubated (10 min; 75°C) to lyse cells. Changes in levels of c-MYC and IL2 mRNAs were assessed (25-µl reactions) using qRT-PCR ("Platinum® SYBR® Green One-Step qRT-PCR" kit; Mastercycler® realplex2, Eppendorf) and the ΔΔCt method,[46] primers ("c-MYC fw" and "rev", or "IL2 fw" and "rev"; Table S1), and the following temperatures – 50°C for 20 min, 95°C for 5 min, 40 cycles at 95°C for 15 s + 60°C for 30 sec, and finally the pre-programed cycle used for melting-curve analysis. All qRT-PCR runs contained a control lacking reverse transcriptase to assess amplification of genomic DNA, and expression levels were normalized relative to those of 5S rRNA (assessed using primers "5SrRNA fw" and "rev"; Table S1). Cells grown and treated conventionally provided a control.

Shorthand notation

The following shorthand notation can be used to describe the creation and evolution of fluidic architectures; it is exemplified first by reference to Figures 1Bi and iii.

1. 150-µm, FC40, tetradecane + 1% Span80, water ± Allura Red dye.
2. ({[red] [ ] })
3. Flow: ->
4. ({ [red] })

Line 1 gives the internal diameter of the tube, and the fluids used as carrier, water, and separating fluid/oil. In line 2, () represents FC40, {} the oil super-drop, and [] one or other of the two water drops engulfed by the oil. After flow in the direction indicated in line 3, line 4 shows there is now only one water drop. Additional details can be included as required (e.g., an air drop can be indicated by <>).

Notes for Electronic Supplementary information

45 A. J. M. Howden, V. Geoghegan, K. Katsch, G. Efstathiou, B. Bhushan, O. Boutureira, B. Thomas, D. C. Trudgian, B. M. Kessler, D. C. Dieterich, B. G. Davis and O. Acuto, *Nat. Methods*, 2013, **10**, 343-346.

46 K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402-408.

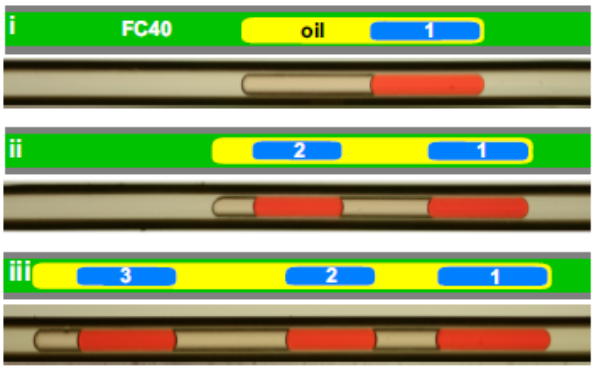

Figure S1. Trains with 1, 2 and 3 carriages. Schematics (with micrographs below) of tubes containing carrier fluid (FC40) surrounding a ~10-nl oil super-drop (tetradecane + 1% Span 80) engulfing 1-3 10-nl aqueous drops (the carriages in a train) containing red dye. A 150-µm tube was loaded by dipping it successively into wells containing: (i) carrier fluid (FC40), water, oil, water, and FC40, (ii) FC40, water, oil, water, oil, and FC40, and (iii) FC40, water, oil, water, oil, water, oil, and FC40. Fluid was drawn into the tube (0.05 ml/h) only when dipped below the surface of the fluid.

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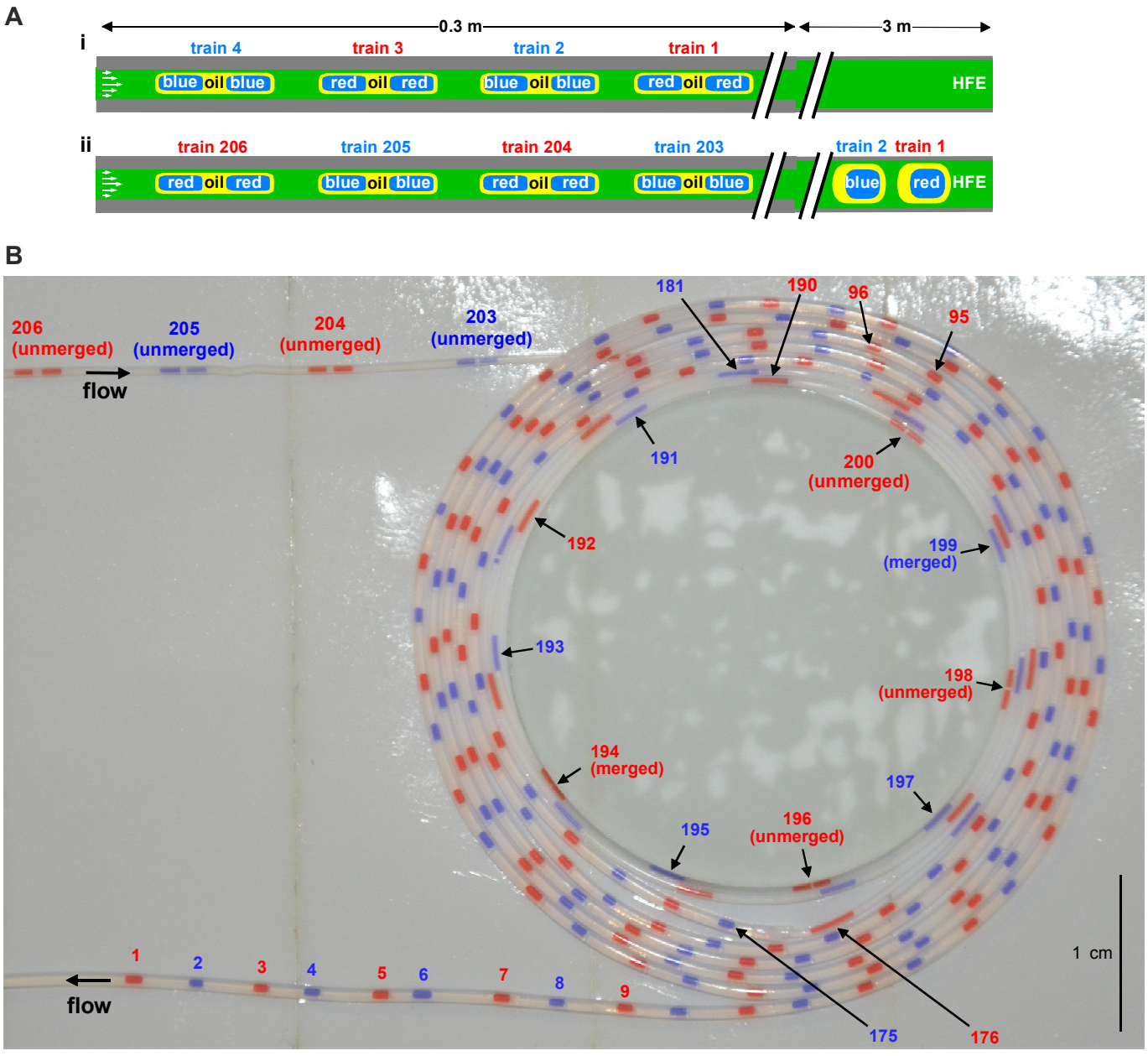

Figure S2. High-throughput demonstration of merging of 206 pairs of drops (fluids – 50% FC40 + 50% HFE7500, water + red or blue dye, tetradecane + 1% Span80). A bipartite tube was made by joining one tube (0.3 m) with a 250-µm bore to another with a 400-µm bore (3 m). All aqueous drops in a train are merged in the thinner segment, and merged drops are "stored" in the wider segment. The wider end was attached to a syringe pump (flow rate of 0.1 ml/h for carrier fluid, 0.08 ml/h for aqueous drops, and 0.06 ml/h for separating fluid; total run-time ~2 h). The narrow end of the tube was dipped successively into appropriate wells in 96-well plates to load 206 trains. Each train contained one oil drop engulfing two 50-nl water drops (each containing the same dye); successive trains contained differently-colored dyes (i.e., red or blue). Results obtained with a second identical tube (with different contents) attached to a second syringe driven by the same syringe pump at the same time are presented in Figure S3.

(A) Fluidic architecture of the first 4 trains in the bipartite tube. (i) Structure after loading the first 4 trains. The first and third trains each contained two water drops + red dye, and the second and fourth trains two water drops + blue dye. Trains loaded subsequently continued this alternating pattern. To demonstrate the indexing potential for different 96-well plates, we loaded trains 95 and 96 with red dye. Then, all odd-numbered trains from 1-95 carried red dye, and all even-numbered ones blue dye; from train 96, all even-numbered trains carried red dye and odd-numbered ones blue dye. Additional indexing could be included by varying volumes of separating fluid and/or drop numbers/colors. (ii) Structures just after loading train 206. Aqueous drops in trains 203-206 in both tubes have not yet merged; however, those in trains 1 and 2 have merged. (B) Bright-field image of the bipartite tube after coiling it around a cylinder. Trains were loaded in the thin end (now at top left); the last train (i.e., 206) has just been loaded, and then the pump was stopped. As a result, the two red drops in trains 206, 204, 202 (which is not visible as it lies under other segments of the tube), and 198 remain unmerged; however, drops in train 196 were just about to merge, whilst those in train 194 have merged. The two blue drops in trains 205 and 203 also remain unmerged (train 201 is also not visible); however, drops in train 199 have merged. [The different dyes cause slight differences in interfacial tension that affect where drops in a train will merge.] Drops in all other trains have merged. Trains 176 and 175 lie on each side of the junction between the two segments with different bores; consequently, the (merged) aqueous drop is longer in train 176 than that in 175. Note the two index trains with red dye (95, 96). Drop architecture remained unchanged during storage at room temperature for 1 day.

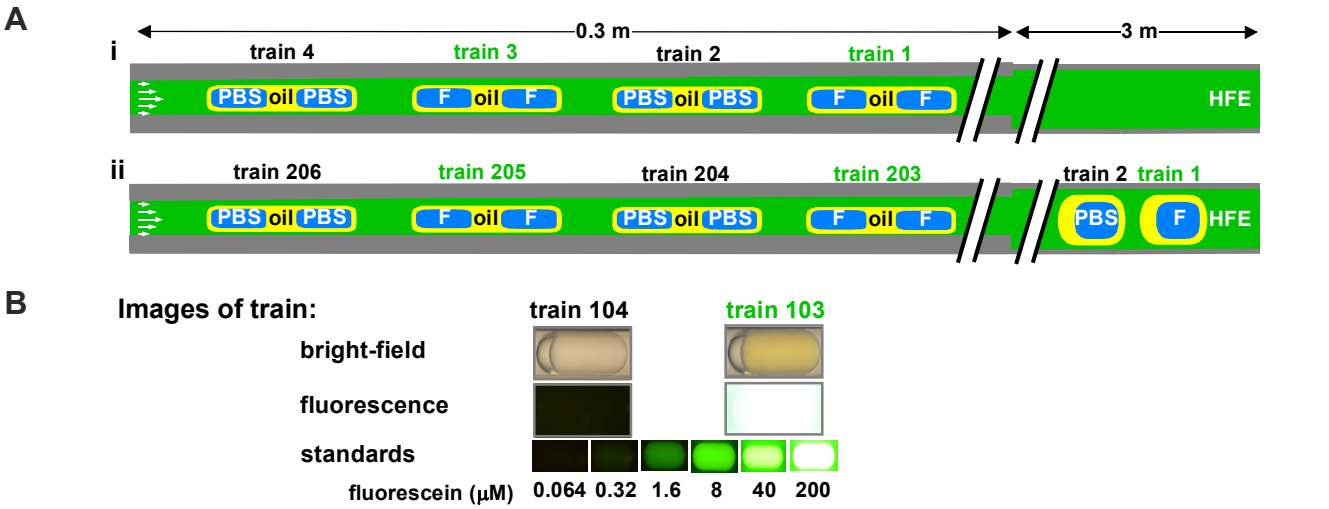

Figure S3. Negligible cross-contamination occurs during high-throughput merging of 206 pairs of drops (fluids – 50% FC40 + 50% HFE7500, PBS ± 200 μM carboxy-fluorescein, tetradecane + 1% Span80). A bipartite tube was made by joining one tube (0.3 m) with a 250-μm bore to another with a 400-μm bore (3 m). Almost all aqueous drops in a train are merged in the thinner segment, whilst merged drops "stored" in the wider segment. The wider end was attached to a syringe pump (flow rate of 0.1 ml/h for carrier fluid, 0.08 ml/h for aqueous drops, and 0.06 ml/h for separating fluid; total run-time ~2 h). The narrow end of the tube was dipped successively into the appropriate wells in 96-well plates to load 206 trains. Each train consisted of one oil drop engulfing two 50-nl drops carrying the same cargo (i.e., PBS or PBS + 200 μM carboxy-fluorescein); successive trains carried different cargoes (i.e., ± fluorescein). Results obtained with a second identical tube (with different contents) attached to a second syringe driven by the same pump at the same time are presented in Figure S2.

(A) Fluidic architecture of the first 4 trains in the bipartite tube. (i) The first and third trains each contained two PBS drops with carboxy-fluorescein, and the second and fourth trains two PBS drops without. Subsequent trains in each tube continued this alternating pattern.

(B) Bright-field and fluorescence images of trains 103 and 104 containing merged drops ± carboxy-fluorescein from the second tube (images collected 10 h after stopping flow). Fluorescence images of drops containing a dilution series of carboxy-fluorescein are included for reference. Trains containing only PBS have no detectable fluorescence. Selected trains were re-checked for any detectable fluorescence transfer after 4 days, but none was detected. Therefore, if cross-contamination occurs between trains, it does so at a level of less than one part in 625. This confirms that cross-contamination is negligible from train-to-train in the tube, and from well-to-well during loading (when the tube end is dipped hundreds of times between PBS ± fluorescein during loading). Note also that here the tube end was not "washed" in FC40 between dipping into different fluids, but such washing dips could be included if necessary.

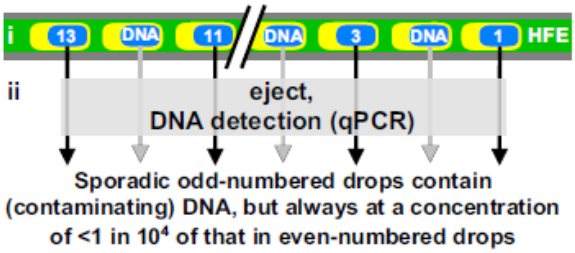

Figure S4. Assessing cross-contamination using PCR (150-µm tube; fluids – 50% HFE7500 + 50% FC40, water ± DNA, tetradecane + 1% Span80). (i) A set of 13 trains. Each train contains one 30-nl oil super-drop engulfing a 30-nl water drop (with 200 nl HFE7500 between oil super-drops); even-numbered water drops contain 3 ng DNA (~5 x10^8 molecules) whilst odd-numbered drops contain no DNA. Once loaded, flow was first stopped for 5 min to give DNA time to exchange between drops and/or bind to the tube wall (if it were able to do so), and then reversed so that individual aqueous drops could be ejected into different micro-centrifuge tubes. The tip of the 150-µm tube was "washed" by dipping for 1-2 s in FC40 after loading each train, and after ejecting each water drop. (ii) The amount of DNA in each ejected aqueous drop was assessed using qRT-PCR. All even-numbered drops contained the expected high concentration of DNA, whilst most odd-numbered drops contained no DNA detectable after 40 cycles. However, sporadic odd-numbered drops did contain DNA, but always at a concentration that was <1 in 10^4 of that in even-numbered drops. Analysis of similar sets of trains on different days gave substantially similar results, indicating that sporadic contamination at a low level does occur (as in any laboratory conducting molecular biology). Levels can, of course, be reduced by including additional "washes" in FC40 between individual samples during loading and ejection. Therefore, we suggest that contamination levels should be assessed prior to the use of any new application.

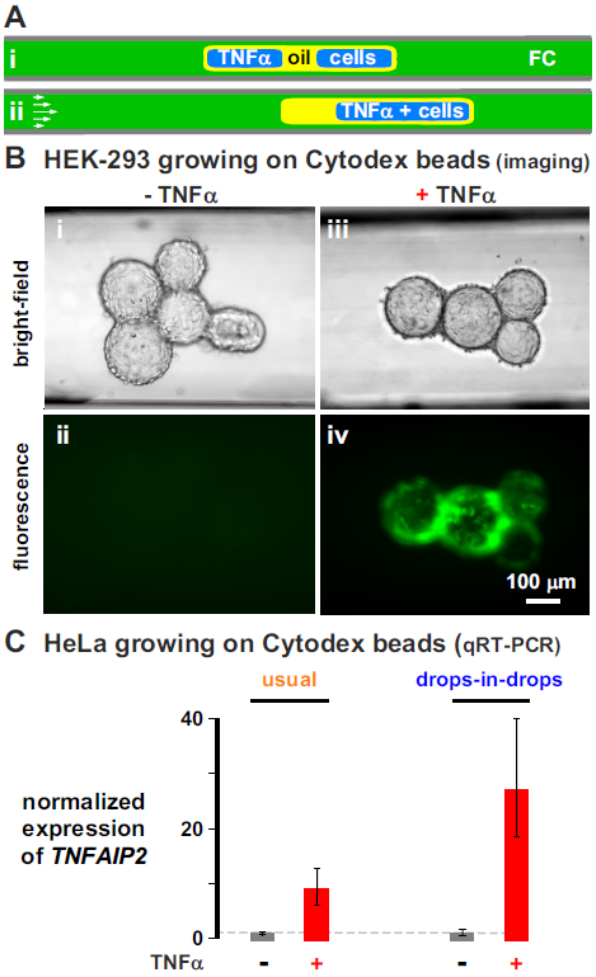

Figure S5. The effects of TNFα on gene expression in different cells growing on the surface of Cytodex beads (560-µm tubes). FC is fluorocarbon. Fluids: HFE7500 for (B) and 50% FC40 + 50% HFE7500 for (C), growth media, 5-cSt silicone oil + 0.25% AbilEM180.

(A) Approach. (i) Initially, one oil drop engulfs 2 aqueous drops; the first contains cells on beads, and the second growth media ± TNFα. (ii) Flow then induces the two aqueous drops to merge and mix. Tubes are now incubated at 37°C.

(B) HEK-293 cells encoding an NF-κB-responsive *GFP*-reporter gene. Tubes containing cells on Cytodex beads were grown ± TNFα for 20 h, before bright-field and fluorescence images of the tubes were collected. (i, ii) In the absence of TNFα, only background levels of fluorescence are seen associated with the 5 beads in the field. (iii,iv) TNFα induces bright (GFP) fluorescence in the cells growing on the 4 cells in the field.

(C) Comparison of HeLa cells grown conventionally on the surface of plates ("usual") with those grown on beads in tubes ("drops-in-drops"). *TNFAIP2* is an endogenous gene that responds to TNFα. After incubation (2.5 h), drops were ejected from tubes, and levels of *TNFAIP2* mRNA assessed using quantitative RT-PCR (qRT-PCR; values are averages ± maximum and minimum values from 5 drops or plates normalized first relative to the unchanging levels of 5S RNA, and then relative to the control without TNFα). TNFα increases *TNFAIP2* expression in cells grown in both ways, despite the very different environments.

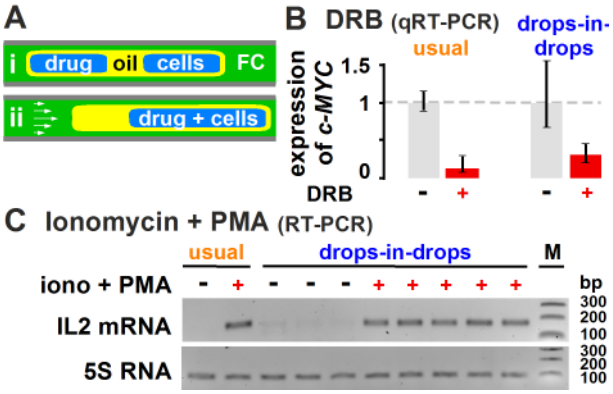

Figure S6. Effects of an inhibitor and an activator on mRNA levels in Jurkat cells growing in suspension (560-µm tubes).
(A) Approach. Fluids: fluorocarbon (FC; HFE7500; growth medium ± cells or drug; 5-cSt silicone oil + 0.25% AbilEM180). Cells grown conventionally in plates ("usual") provide controls. (i,ii) Flow delivers drug to cells. Tubes are now incubated (37°C) for 4 h before the merged drop is ejected and its contents analyzed.
(B) An inhibitor: DRB reduces levels of c-MYC mRNA (analyzed using quantitative RT-PCR; averages ± maximum and minimum values from 5 drops normalized first to the unchanging levels of 5S RNA, and then to the untreated control).
(C). An activator: ionomycin and PMA increase levels of IL2 mRNA in individual drops but not those of 5S RNA used as a control (assessed using RT-PCR and gel electrophoresis; intensities of 171- and 90-bp bands reflect IL2 mRNA or 5S RNA levels; M shows 100-, 200-, 300-bp markers).

Table S1. Sequences of oligonucleotides used for PCR.

PCR		sequence (5' to 3')
pcDNA3 fw		gtgtaggtcgttcgctccaa
pcDNA3 rev		gcgtcagaccccgtagaaaa
IL2 fw		tgtcacaaacagtgcacctact
IL2 rev		agttctgtggccttcttggg
TNFAIP2 fw		cagaattggcaggtacccca
TNFAIP2 rev		cgtgtctacagtggcgatga
c-MYC fw		ctgggaggagacattggttgaac
c-MYC rev		agaagccgctccacatacag
5srRNA fw		tacggccataccaccctgaa
5srRNA rev		gcggtctcccatccaagtac

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Movies

Movie S1

Merging and mixing of two water drops (the second contains red dye) as in Figure 1B. The stage carrying the tube is moved manually to maintain the train in the field of view.

Movie S2

Multiplexed drop merging (movie of experiment in Figure 6B). Four bipartite tubes are attached to 4 syringes driven by one pump (out of sight at top left); it operates in the "withdraw"/"stop" mode as the other ends of the 4 tubes are dipped by the robot successively into fluids contained in Eppendorf tubes on the cooling plate (not used here) in the (repeating) order: fluorocarbon, water + red dye, oil, water + red dye, fluorocarbon, water + blue dye, oil, water + blue dye, fluorocarbon. Then, series of trains form in each tube. Each train contains an oil drop engulfing two 50-nl water drops containing dye of the same color; successive trains carry drops with different-colored dyes. Flow induces the 2 drops in each train to merge by the time the train reaches the point where the tube is inserted into slots cut into the thermal block (not used here). Trains carrying red and blue dye usually alternate in a tube; however, two successive trains carrying the same dye are included for indexing purposes, and such trains carrying red dye can be seen at the right ends of the segments of tubes slotted into the thermal block.

Movie S3

Multiplexed train generation and drop merging (from an analogous experiment to the one in Movie S2). Here, the robot dips the ends of 10 bipartite tubes successively into fluids contained in Eppendorf tubes on the cooling plate (not used) in the (repeating) order: fluorocarbon, water + blue dye, oil, water + blue dye, fluorocarbon, water + red dye, oil, water + red dye, fluorocarbon, water + yellow dye, oil, water + yellow dye, fluorocarbon. A series of trains form in each tube; each train is composed of an oil drop engulfing two 50-nl aqueous drops containing dye of the same color, and successive trains carry drops with different-colored dyes. Flow induces the 2 drops within each train to merge, as in Movie S2.

Movie S4

Transfer (advection) between two water drops (the first contains red dye) demonstrating dye transfer from leading to lagging drop. The stage carrying the tube is moved manually to maintain the train in the field of view.

Movie S5

Advection between aqueous drops to create a concentration gradient. The stage carrying the tube is moved manually to maintain the train(s) in the field of view. Initially, there are 6 trains; each train is composed of an oil drop engulfing an aqueous drop, and the first aqueous drop contains red dye. During flow, the second train catches up the first, then the third catches up the now-joined 1 + 2, and so on. The result is a series of aqueous compartments connected by aqueous channels around each oil drop. Red dye is transferred from the first compartment to the last, to create a dilution series.