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Signalling and differentiation in emulsion-based multi-compartmentalized in vitro gene circuits

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Supplementary information:

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Table of Contents

SUPPLEMENTARY METHODS	3
MATERIALS.....	3
SUPPLEMENTARY TEXT.....	8
OPTIMIZATION OF LIPID COMPOSITION	8
OSMOLARITY BALANCE.....	8
LEAKINESS OF THE LIPID BILAYERS	8
MODELS	9
GENERAL REMARKS	9
MEMBRANE-LIMITED TRANSPORT	9
COMBINED PERMEATION/DIFFUSION MODEL	11
TRANSCRIPTION	11
TRANSCRIPTION-TRANSLATION	11
PULSE MODEL	12
STATISTICS.....	14
DIFFERENTIATION MODEL	14
FITS AND SIMULATIONS	17
EFFECTIVE AREA OF PERMEATION	17
PLUX INDUCTION	18
PBAD INDUCTION	18
GUANINE PERMEABILITY	18
ARABINOSE PERMEABILITY	18
DFHBI PERMEABILITY.....	18
C6-HSL PERMEABILITY.....	18
PULSE VELOCITY	19
SUPPLEMENTARY FIGURES	20
SUPPLEMENTARY FIGURE 1	22
SUPPLEMENTARY FIGURE 2	23
SUPPLEMENTARY FIGURE 3	24
SUPPLEMENTARY FIGURE 4	25
SUPPLEMENTARY FIGURE 5	26
SUPPLEMENTARY FIGURE 6	27
SUPPLEMENTARY FIGURE 7	28
SUPPLEMENTARY FIGURE 8	29

SUPPLEMENTARY FIGURE 9	30
SUPPLEMENTARY FIGURE 10	31
SUPPLEMENTARY FIGURE 11	32
SUPPLEMENTARY FIGURE 12	33
SUPPLEMENTARY FIGURE 13	34
SUPPLEMENTARY FIGURE 14	35
<u>SUPPLEMENTARY TABLES.....</u>	<u>36</u>
SUPPLEMENTARY TABLE 1 - TRANSLOCATION EXPERIMENTAL CONDITIONS	36
SUPPLEMENTARY TABLE 2 - CHEM3D CALCULATIONS	37
SUPPLEMENTARY TABLE 3 - INDUCTION FITS	38
SUPPLEMENTARY TABLE 4 - PERMEABILITY FITS	39
SUPPLEMENTARY TABLE 5 - DNA SEQUENCES	40
<u>SUPPLEMENTARY VIDEOS</u>	<u>52</u>
<u>REFERENCES</u>	<u>53</u>

Supplementary Methods

Materials

The following chemicals were purchased from Sigma-Aldrich (Germany): hexadecane #296317, silicone oil AR 20 #10836, α -Hemolysin #H9395, MgCl_2 (1M) #63069, arabinose #A3256, rhamnose #R3875, isopropyl β -D-1-thiogalactoside (IPTG) #I6758, N-Hexanoyl-L-homoserine lactone (C6-HSL) #56395, anhydrotetracycline (aTc) #37919, Triton X-100 #X100, Na_2HPO_4 #RES20908, KH_2PO_4 #1551139, NH_4Cl #A9434, Malachite Green #38800, Atto488 #41051, 2-mercaptoethanol (β -ME) #M6250 and ammonium persulfate (APS) #A3678.

The following chemicals were purchased from Carl Roth (Germany): KCl #P017, nuclease-free water (nf H_2O) #T143, lactose #8921, Roti-Aqua-P/C/I (phenol, chloroform, isoamyl alcohol) #X985, chloroform #3313, ethanol #P076, urea #2317, TBE Buffer #3061, acrylamide #A121, TEMED #2367, agarose #2267, Tris.HCl #9090, NaCl #0962, glycerol #3783, LB Medium #X968, MgSO_4 #0682, CaCl_2 #A119, glucose #7509, casein hydrolysate (caseinh) #A157 and thiamine #T911.

The following chemicals and enzymes were purchased from New England Biolabs (NEB, USA): RNAPol Reaction Buffer #M0251, Ribonucleotide Solution Mix #N0466L, DNase I Reaction Buffer #M0303, RNase Inhibitor Murine #M0307, Pyrophosphatase Inorganic (*E. coli*) #M0361, DNase I #M0303 and T7 RNA Polymerase #M0251.

The following chemicals were purchased from Thermo Fisher Scientific (USA): guanine #AC12025, 2,4-diacetylphloroglucinol (DAPG) #AC45280, RNA Gel Loading Dye (2X) #R0641, SYBR Green II #S7586, RiboRuler Low Range RNA Ladder #SM1831, tetracycline #AAB2140822 and EDTA (0.5M, pH 8.0) #AM9260G.

The following chemicals were purchased from Avanti Polar Lipids (USA): DPhPC (4ME 16:0 PC) #850356, DOPC (18:1 (Δ 9-Cis) PC) #850375, DOPG (18:1 (Δ 9-Cis) PG) #840475 and cholesterol (20 α -hydroxycholesterol) #700156.

DFHBI #410 was purchased from Lucerna Technologies (USA).

Additionally, T7 RNA Polymerase, LacI and TetR were expressed and purified in our lab according to standard His-tag nickel purification protocols (with specific buffers for T7 RNA Polymerase¹, LacI², and TetR³).

The commercial cell-extract used in **Supplementary Fig. 5** was purchased from Arbor Biosciences (USA): “myTXTL – Sigma 70 Master Mix Kit”, #507024.

DNA was purchased from Biomers (Germany) or Eurofins (Germany) (single-stranded oligos), or from IDT (USA) (double-stranded gBlocks).

DNA template assembly. Linear DNA templates for transcription (namely 24-2 ds, 24-2-guanine ds in **Supplementary Fig. 4**, C3 ds in **Fig. 3** and MG-C3 ds in **Supplementary Fig. 10**) are assembled as follows. ssDNA template and non-template strands are mixed at 5 μM each with 1x RNAPol Reaction Buffer in nf H_2O (total volume 100 μL), in a DNA LoBind Tube (Eppendorf, Germany). The sample is heated at 95°C for 3 minutes, and then slowly cooled down at a rate of $-1^\circ\text{C}.\text{min}^{-1}$, until reaching 20°C. This allows for the two strands to fully anneal into a double-stranded (dsDNA) template.

RNA production. Purified RNA (such as Spinach in **Fig. 3**) was obtained as follows. 200 nM of the dsDNA template was mixed with 15 mM MgCl_2 , 125 mM KCl, 4 mM each rNTP, 1x RNAPol Reaction Buffer, 0.4 U/ μL RNase Inhibitor, 0.001 U/ μL Pyrophosphatase and 400 nM T7 RNA Polymerase in nf H_2O (total volume 100 μL) in a Protein LoBind Tube (Eppendorf, Germany). The sample is incubated at 37°C for at least 4 h, up to overnight. After the transcription, 1x DNaseI Buffer and 0.035 U/ μL DNaseI are added, and the sample is further incubated at 37°C for 30 min, up to 1 h, to digest the DNA template. The RNA is then extracted following a phenol-chloroform extraction protocol in a 5'-Phase

Lock Gel Heavy (VWR, USA), and precipitated with ethanol. The RNA is resuspended in *nf* H₂O and stored up to 6 months at -80°C. RNA concentration is measured by denaturing PAGE gel. The gel consists of 8 M urea, 1x TBE Buffer, 15% (v/v) Acrylamide 29:1, 0.1% (v/v) TEMED, 0.1% (w/v) APS, in double-distilled H₂O (total volume 10mL). The gel is cast in cassettes (Bolt Empty Mini, Thermo Fisher Scientific, USA), and the running buffer is 1x TBE. The gel is pre-run for 30 min at 12.5 V/cm with a running temperature of 40°C. The RNA samples are mixed with 2x RNA loading dye, denatured at 95°C for 5 min and directly put on ice to prevent the RNA from refolding. The samples are loaded onto the gel and run at 12.5 V/cm for 1 h 15 min at 40°C. The gel is stained with 1x SybR Green II, a fluorescence image of the gel is acquired, and the intensity of the RNA band is quantified against an RNA ladder (low Range Riboruler) with ImageJ.

Cloning and plasmid purification. All plasmids were cloned using standard strategies and NEB enzymes. Specifically, we alternatively used digestion/ligation, Gibson assembly⁴, Golden Gate assembly⁵ or PCR with 5'-phosphated primers followed by ligation. The plasmids were transformed into the bacterial strains DH5 α , Turbo (NEB, USA, #C2984I) or BW27783. All constructs were cloned in the plasmid backbone pSB1A3, except for pCK301 (gift from John Heap, Addgene plasmid #87767⁶), pPhIF-P2 (gift from Christopher Voigt, Addgene plasmid #74686⁷) and pBAD33-dsRedmut. The cloned bacteria were stored in glycerol stocks: an overnight culture in LB medium was mixed with glycerol to a final concentration of 25% (v/v) glycerol and stored at -80°C. To ensure that the glycerol stocks contained a monoclonal population with the correct sequence of the plasmids, an overnight culture was grown in LB medium from the glycerol stock, Mini-prepped (QIAprep Spin Miniprep Kit, Qiagen, Netherlands) and sequenced (Sanger sequencing, LightRun, GATC Biotech, Germany). Plasmids were purified prior to use in cell-extract by Miniprep followed by phenol-chloroform extraction, or by Midiprep (NucleoBond Xtra Midi, Macherey-Nagel, Germany).

Cell extract preparation. The *E. coli* cell extract was prepared according to the protocol by Sun *et al.*⁸. Shortly, a mid-log phase BL21 Rosetta2(DE3) culture was lysed by bead-beating with 0.1 mm glass beads in a Minilys device (Peqlab, Germany). The extract was incubated at 37°C for 80 min to allow the digestion of genomic DNA, and was then dialyzed for 3 h at 4°C with a cut-off of 10 kDa (Slide-A-Lyzer Dialysis Cassettes, Thermo Fisher Scientific). Protein concentration was estimated to be 30 mg/mL with a Bradford essay. In the buffer, instead of 3-phosphoglyceric acid (3-PGA), phosphoenolpyruvate (PEP) was utilized as an energy source⁹. The buffer was composed of 50 mM HEPES pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg.mL⁻¹ tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 68 μ M folinic acid, 1 mM spermidine, 30 mM PEP, 1.25 mM leucine, 1.5 mM other amino acids, 1.5 mM DTT, 3.5% PEG-8000, 80 mM K-glutamate and 6 mM Mg-glutamate. Buffer and extract were flash-frozen in liquid nitrogen, stored at -80°C and thawed on ice prior to usage. A cell-free reaction was prepared by mixing 33% (v/v) cell extract with 42% (v/v) buffer and 25% (v/v) DNA, inducers, and other additives. Reactions were conducted at 29°C.

In vitro transcription. In general, transcription was carried out with 1x RNAPol Buffer, 125 mM KCl, 15 mM MgCl₂, 4mM each rNTP, between 100 nM and 200 nM dsDNA template, and either 4 U/ μ L (NEB) or 400 nM T7 RNA Polymerase. Osmolarity between droplets was balanced with a glycerol buffer identical to the storage buffer of T7 RNA Polymerase (5 mM Tris.HCl, 100 mM NaCl, 20 mM β -ME, 1 mM EDTA, 50% (v/v) glycerol, 0.1% (v/v) Triton X-100, pH 7.9 at 25°C).

In vitro transcription-translation. Cell extract was supplemented with 5 or 10 nM plasmid DNA and 75 nM repressor protein (LacI or TetR). For C6-HSL and arabinose translocation, LuxR and AraC were expressed constitutively from the pSB1A3-pLacO-T9002-LVA and pSB1A3-AD011 plasmids.

Experiments with encapsulated bacteria. Cultures were grown overnight from glycerol stock in M9 medium (3.37 mM Na₂HPO₄, 0.2 mM KH₂PO₄, 8.55 mM NaCl, 9.35 mM NH₄Cl, 2 mM MgSO₄, 100 μM CaCl₂, 20 mM glucose, 0.20% caseinh, 0.2 mg/mL thiamine) supplemented with the appropriate antibiotics. The next day, the culture was diluted in M9 medium to a final OD 600nm ranging between 0.05 and 0.2 in receiver droplets.

Lipid preparation. The lipid-oil mixture was optimized for all experiments to be 0.25 mM cholesterol, 0.25 mM DOPG, 4 mM DOPC, 0.5 mM DPhPC in 1:1 hexadecane:AR20. However, the DFHBI screening in I1-FFL experiments (**Fig. 3e**), and the 1D/2D geometries comparison (**Fig. 3g-j**) were done in a lipid-oil mixture of 5.9 mM DPhPC in 1:1 hexadecane:AR20. The lipid or cholesterol powder was dissolved in chloroform, and the appropriate amounts of lipids and cholesterol were mixed in a glass vial. The chloroform was evaporated under a nitrogen stream, and the lipid film was dried for an additional hour under vacuum. The lipid film was then resuspended in 1:1 hexadecane:AR20 oil mix to reach the desired lipid and cholesterol concentrations, and the mix was briefly vortexed.

Experiment chambers. For the chambers, rubber O-rings (7-1 FPM 80, Herzer, Germany) were glued (UHU Plus Endfest 3000, UHU, Germany) unto glass slides (VWR, Germany). The chambers were washed before each experiment with soap, ethanol and double-distilled water, and dried at 90°C for at least 30min, to ensure that all contaminant proteins have been denatured.

Droplet production and assembly. The chamber was filled with 65 μL of the lipid:oil mix. Droplet solutions (such as sender, buffer and receiver) were prepared according to each experiment, and pipetted into heat-pulled glass capillaries with a tip diameter of around 30 μm (capillaries: 1.050x1.50x100 mm, GB150T-10, Science Products, Germany, heat-puller: DMZ-Universal Puller, Zeitz, Germany). The glass capillary was then fixed unto a house-built micromanipulator, and connected to a microinjector pump (Femtojet 4i, Eppendorf), which provided pressure pulses in the range of 20 to 100 hPa for 0.1 s. A wipe (Kimtech, USA) was lightly pressed against the tip of the capillary to take out the residual air and allow the regular flow of the solution. The pipette was brought into the lipid:oil mix and pressure injections were applied to create droplets of approximately 260 μm diameter, 10 nL volume on average. The droplets sank at the bottom of the chamber and were incubated for 15 min on average, to allow a monolayer of lipids to form around them. The glass capillary, together with the micromanipulator, was then used to move the droplets in the chamber and bring them into contact, and they spontaneously formed bilayers. The chamber was closed with a glass slide to limit evaporation and shrinking of the droplets.

Bulk experiments. To assess the functionality of the constructs, bulk experiments were conducted in three circuit environments (**Supplementary Fig. 4**). DNA/RNA circuits and bacteria constructs were assessed at 37°C, cell-extract circuits were assessed at 29°C. 15 μL (300 μL for bacteria) of the sample were pipetted in a 384-well (96-well respectively) plate (μ-Plate, IBIDI, Germany). The plate was sealed with an optically transparent film (Microseal 'B', Bio-Rad, Germany), and centrifuged for 30 s at 700 rcf. Time-fluorescence measurements were then taken in a plate reader (FLUOstar Omega or CLARIOstar, BMG Labtech, Germany).

Translocation experiments. For all translocation experiments (**Supplementary Fig. 4**), 1.5 μM α-HL was used in the sender and buffer droplets. 1 to 5 μM of a Atto655-labeled dummy DNA strand (Atto655-DNA) was used to tag the buffer droplets. **Supplementary Table 1** lists the specific conditions of each translocation experiments, and **Supplementary Table 5** displays DNA sequences.

Comparison of pore-mediated and non-specific diffusion. To compare the diffusion of C6-HSL and arabinose (**Fig. 2**, **Supplementary Fig. 7, 8** and **9**), the sender solution contained 300 mM arabinose, 1 μ M C6-HSL and 1.5 μ M α -HL, the buffer solution contained a variable amount of α -HL (**Fig. 2g**) or 1.5 μ M (**Fig. 2i**), and the receiver solution contained 5 nM of the arabinose read-out plasmid (pSB1A3-AD011) and 5 nM of the C6-HSL read-out plasmid (pSB1A3-pLacO-T9002-LVA).

Pulse experiments. For pulse experiments (**Fig. 3** and **Supplementary Fig. 10**, and **11**), 1.5 μ M of Spinach RNA was incubated with 150 nM ssDNA (C3-A) in 1x RNAPol Buffer, 125 mM KCl, 15 mM MgCl₂ at 37°C for 30 min. This allowed the RNA and the ssDNA to bind. After incubation, 4 mM each rNTP, 150 nM genelet template (C3 ds) and 1.2 μ M T7 RNA Polymerase (or as indicated in **Fig. 3**) were added to make the receiver solution. The sender solution contained 1x RNAPol Buffer, 125 mM KCl, 15 mM MgCl₂, 3 μ M DFHBI (or as indicated in **Fig. 3**), 1 μ M Atto655-DNA (as a marker), and glycerol buffer in an identical volume as T7 RNA Polymerase. For the 1D/2D geometries comparison, the concentrations were 3 μ M Spinach RNA, then 6 μ M DFHBI and 1 μ M T7 RNA Polymerase for low DFHBI, low T7 RNA Polymerase conditions, or 12 μ M DFHBI and 1.2 μ M T7 RNA Polymerase for high DFHBI, high T7 RNA Polymerase conditions.

Noise-based differentiation experiments. In the transcriptional noise experiments (**Fig. 4** and **Supplementary Fig. 12, 13** and **14**), sender solution contained 300 mM arabinose, 5 μ M Atto488 (as a marker), and 1.5 μ M α -HL for positive controls. The receiver solution contained 5 nM of the full construct (pSB1A3-AD010) or the truncated construct (pSB1A3-AD011). In the Atto488 control (**Supplementary Fig. 12a**), the sender solution contained 15 μ M Atto488, and the receiver contained 5 nM of a pBAD- α -HL plasmid (pSB1A3-AD009), 100 mM arabinose and 1.5 μ M α -HL for positive controls.

Image Acquisition. The fluorescence of the networks was recorded with an inverted fluorescence microscope. Most experiments were recorded with an IX-71 microscope (Olympus, Japan), with LEDs, filters and dichroic mirrors from Thorlabs (USA), camera LucaEM from Andor (Northern Ireland, UK), objectives from Olympus (Japan), heating plate from Tokai Hit (Japan), and acquisition software MicroManager 1.4.16. Images for **Fig. 3b, c** were acquired with an Eclipse Ti2 (Nikon, Japan), with white light for excitation SOLA light engine (Lumencor, USA), filters and dichroic mirrors from Thorlabs (USA), camera Neo5.5 from Andor (Northern Ireland, UK), objectives from Nikon (Japan), incubation chamber from Okolab (Italy), and acquisition software NIS-Elements AR from Nikon (Japan). The images were further analyzed with ImageJ and MATLAB_R2015b.

Equipment and settings. Images of **Fig. 1c, d** were acquired with the IX-71 microscope, at 37°C, with a x4 objective and a bin of 2. **Fig. 1c** was acquired with an exposure time of 50ms, with LED 627nm, excitation filter 605nm-648nm, dichroic mirror 667nm and emission filter 672nm-712nm. **Fig. 1d** was acquired with an exposure time of 3s, with LED 470nm, excitation filter 450nm-490nm, dichroic mirror 510nm and emission filter 518nm-545nm.

Images from **Fig. 3b** were acquired with the Eclipse Ti2 microscope, at 37°C, with a x4 objective and a bin of 2. The red channel was acquired with an exposure time of 50ms, with excitation filter 608nm-648nm, dichroic mirror 660nm and emission filter 672nm-712nm. The green channel was acquired with an exposure time of 200ms, with excitation filter 457nm-487nm, dichroic mirror 458nm and emission filter 467nm-515nm. Images from **Fig. 3g** were acquired with the IX-71 microscope, at 37°C, with a x4 objective and a bin of 2. The red channel was acquired with an exposure time of 100ms, with LED 627nm, excitation filter 605nm-648nm, dichroic mirror 667nm and emission filter 672nm-712nm. The green channel was acquired with an exposure time of 3s (low DFHBI, low T7 RNA Polymerase conditions) or 2s (high DFHBI, high T7 RNA Polymerase conditions), with LED 470nm, excitation filter 450nm-490nm, dichroic mirror 510nm and emission filter 518nm-545nm.

Images from **Fig. 4b** were acquired with the IX-71 microscope, at 29°C, with a x1 objective and a bin of 2. The exposure time was 3s, with LED 530nm, excitation filter 530nm-550nm, dichroic mirror 576nm and for emission, a long pass filter above 590nm.

Data analysis. Droplets were tracked either with the droplet tracker software from Dr. Kapsner¹⁰ in MATLAB_R2015b, or with the plugin Time Series Analyzer in Fiji/ImageJ. The images were background subtracted. The total fluorescence intensity and radius of each droplet was extracted, and the mean intensity was calculated as $intensity\ sum/radius^2$, assuming that the microscope excited and recorded the emission from a circular slice at the center of the droplet. For the pulse, the mean intensities were filtered with a fft filter. Models were simulated with an ode23s solver, and fits were made with a "lsqnonlin" function, using the Scharfit program from Dr. Kapsner¹¹.

Chem3D. Molecular structures of the chemicals were constructed using the program ChemDraw Professional (version 16.0.1.4(77)), and loaded unto the molecular modelling program Chem3D (version 16.0.1.14). The lowest energy 3D conformations were determined using the energy minimization function of the program. The following molecular parameters were calculated for each molecule: logP (the logarithm of the octanol/water partition coefficient), hydrogen bond acceptors and donors. The molecular weight was taken from the respective company providing the chemical. To follow the Rule of Five and its recent adjustments^{12,13}, we considered the following limits for the parameters: logP from -0.4 to 5.6, molecular weight from 160 to 480 g/mol, hydrogen bond donors under 5, hydrogen bond acceptors under 10.

Supplementary Text

Optimization of lipid composition

When optimizing the composition of the lipid mixture for droplet formation, we found that 5.9 mM DPhPC was sufficient to rapidly and stably encapsulate DNA/RNA circuits and bacteria, meaning that the droplets did not fuse upon contact or in the course of the experiments (i.e., over one day), but instead formed stable bilayers. However, we encountered difficulties when encapsulating cell-extract. When the droplets were brought rapidly into contact, they tended to fuse, and when they were incubated for longer periods of time, the surface of the droplets became visibly irregular and they would not form any bilayers when brought in contact. Lipids-in strategies¹⁴, in which extruded liposomes were added to the encapsulated solution, screening of the DPhPC concentration, of the oil composition or of the incubation time did not resolve this issue. We hypothesize that proteins from the cell-extract quickly denature at the water-oil interface and make it impossible for a proper lipid monolayer to form. We then investigated mixtures of lipids with faster monolayer assembly (such as DOPC), and with stabilizing effects such as negatively charged lipids and cholesterol. Our optimized lipid mix that allowed for a fast formation of a monolayer and long-term stable bilayers was: 4 mM DOPC, 0.5 mM DPhPC, 0.25 mM DOPG and 0.25 mM cholesterol. The lipid-oil mix itself was stable at room temperature for typically 2 months, and was discarded when bilayers were no longer stable.

Osmolarity balance

Supporting a balanced osmolarity across the networks is crucial to ensure a constant volume of the compartments and to avoid fusion. For DNA/RNA circuits, high osmolarity was caused by enzymes (such as T7 RNA Polymerase), and specifically by the glycerol buffer they are stored in. We therefore used an equal amount of a self-made glycerol buffer in the droplets that did not contain enzymes. For cell-extract circuits, all droplets contained 75% (v/v) of the extract/buffer mix described above, whether or not they contained DNA circuit parts. For experiments with bacteria, an equal amount of M9 medium, found to primarily determine osmolarity, was added to all compartments of the network.

Leakiness of the lipid bilayers

When assessing the translocation dynamics of our chemical signals summarized in **Fig. 2** and **Supplementary Fig. 4**, we found that the formation of a bilayer between a sender and a receiver droplet led to a transient leak of molecules, which we attributed to the reorganization of lipids accompanying the bilayer formation. To account for this issue, we created networks that contained a buffer droplet between the sender and receiver. The buffer droplet did not contain the diffusing signal molecules and was first connected to the receiver so that any leakiness would not lead to a flux of the chemical signal. Only then was the sender droplet connected to the buffer droplet.

The composition of the droplets was as follows. For the + α -HL +chemical experiments, senders contained the chemical signal and pores, buffers contained only pores. For the - α -HL +chemical experiments, senders contained the chemical signal and pores, buffers contained no pores, so that the interface to the receiver was free of pores. For the + α -HL -chemical experiments, senders contained pores but no chemical, buffers contained pores. In all cases, receivers contained the read-out circuit.

In experiments without buffer droplets such as in the differentiation experiments shown in **Fig. 4**, leakage between senders and receivers could not be avoided, and was therefore quantified with the truncated construct pBAD-RFP. When estimating the permeability of arabinose (**Supplementary Fig. 6**), no buffer droplet was used as this led to a very low and noisy induction of the pBAD promoter.

Models

General remarks

Signal transport within our multi-compartmentalized system occurs by diffusion through the aqueous compartments and permeation of the membrane interfaces between them.

The diffusive flux j_D generated by a concentration gradient ∇n is given by Fick's law:

$$j_D = -D\nabla n \quad (1)$$

Here D is the relevant diffusion coefficient and n is the number density of the molecules in units $1/\text{m}^3$, which is related to their molar concentration c (with units $\text{mol/L} = \text{M}$) via $c = n/(1000 \times N_A)$.

For permeation of a membrane of thickness L this expression is replaced by:

$$j_M = -P\Delta n \quad (2)$$

where P is the permeability of the membrane (units: m/s) and Δn is the concentration drop across the membrane. P may be related to a diffusion coefficient for transport through the membrane via $D_M = P \cdot L$.

Depending on whether diffusion through the aqueous compartments or permeation of the membrane is limiting, we may describe the overall process with different approximations. If permeation of the signals through the interfaces is fast compared to aqueous diffusion, the overall process may be described as a free diffusion process from sender to receiver compartments. In the opposite case, transport may be described as a series of mass transfer processes, in which molecules “hop” from one compartment to the next (see below).

Diffusion through a linear array of compartments of length l connected via membranous interfaces of thickness $L \ll l$ can be described with an effective diffusion coefficient D_{eff} that fulfills¹⁵:

$$\frac{1}{D_{eff}} = \frac{1}{D} + \frac{1}{Pl} \quad (3)$$

This expression can be used to estimate which of the transport processes dominates. The diffusion coefficient of small molecules in water (as is the case for all our signaling molecules) was estimated using the Stokes-Einstein equation and is typically on the order of $D = 1000 \mu\text{m}^2/\text{s}$ (**Supplementary Table 4**). When $P \ll D/l$, then permeation of the bilayer is limiting and we have $D_{eff} \approx Pl$, whereas for $P \gg D/l$ we have $D_{eff} \approx D$.

For a typical droplet diameter of $l \approx 250 \mu\text{m}$, we get $\frac{D}{l} = 4 \times 10^{-6} \frac{\text{m}}{\text{s}}$. This corresponds well with our experimental finding that with a bilayer permeability higher than 10^{-6} m/s (as for DFHBI and C6-HSL) we observe transport limited by diffusion through the aqueous compartments, whereas for molecules with a bilayer permeability lower than 10^{-7} m/s (as for arabinose and guanine) permeation of the bilayer is limiting (**Supplementary Table 4**).

Membrane-limited transport

We first consider two droplets which contain signal molecules with homogeneous number densities n_1 and n_2 respectively, separated by a membrane of thickness L and effective area A . The permeation flux from droplet 1 to droplet 2 is given by:

$$j_{1 \rightarrow 2} = -P(n_1 - n_2)$$

Hence, the density in droplet 1 changes as follows:

$$\frac{dn_1}{dt} = \frac{1}{V_1} \frac{dN_1}{dt} = \frac{1}{V_1} j_{1 \rightarrow 2} A = -\frac{PA}{V_1} (n_1 - n_2) \quad (5)$$

In the case of non-specific permeation, A is simply the cross-section of the bilayer over which diffusion can occur. In the case of purely pore-mediated transport, A is determined by the number of incorporated pores M and their cross-section S , i.e., $A = M \times S$.

In a general network of droplets where diffusion only occurs between direct neighbors, we have:

$$\frac{dn_i}{dt} = \sum_{j \in \{\text{neighbors of } i\}} -\alpha_{ij}(n_i - n_j) \quad (6)$$

where $\alpha_{ij} = -PA_{ij}/V_i$ describes the coupling between compartments i and j , A_{ij} is the effective area over which transport occurs.

Notes:

1) For a linear array of N compartments with constant coupling α having a sender compartment 1 at one of its ends with initial concentration $n_1(t=0) = n_0$ (all other compartments $n_i(t) = 0$), this equation can be solved analytically:

$$n_{j(t)} = \frac{n_0}{N} \left[1 + 2 \sum_{k=2}^N \exp \left\{ \left(2 \cos \frac{(k-1)\pi}{N} - 2 \right) \alpha t \right\} \cdot \cos \frac{(k-1)\pi}{2N} \cdot \cos \frac{(k-1)(2j-1)\pi}{2N} \right] \quad (7)$$

2) Putting the droplets on a grid and setting all α_{ij} equal, Eq. (6) corresponds to a discretized version of the diffusion equation. Accordingly, we can also approach the problem by solving the diffusion equation within a finite domain and extracting the concentrations only at discrete points (corresponding to the centers of the compartments).

According to Crank¹⁶ the 1D diffusion profile of a substance initially confined to the interval $[0, x_0]$ into an interval of length $[0; L]$ (with initial concentration zero in $[x_0; L]$) is given by:

$$n(x, t) = \frac{n_0}{2} \sum_{k=-\infty}^{+\infty} \operatorname{erf} \frac{x_0 + 2kL - x}{\sqrt{Dt}} + \operatorname{erf} \frac{x_0 - 2kL + x}{\sqrt{Dt}} \quad (8)$$

For our parameters, this sum converges well and can be evaluated only for finite k (we chose $k=10$). As can be seen in **Supplementary Fig. 3e** and **f**, the discrete and continuous description of diffusion show remarkable agreement.

3) The agreement between the discrete and continuous diffusion model gives us confidence that in certain cases the two descriptions can be used interchangeably (by converting a permeability into an effective diffusion coefficient, or a diffusivity into a hopping rate). In general, however, none of the processes will clearly dominate. As the reaction-diffusion systems studied here could not be treated analytically, we generally resorted to numerical modeling (either using a discrete or a continuous model, as detailed below).

Combined permeation/diffusion model

In cases where transport is not clearly limited by membrane permeation, we can describe diffusion in a network using the effective diffusion coefficient D_{eff} (equation (3)). We then have:

$$\frac{dn_i}{dt} = \sum_{j \in \{\text{neighbors of } i\}} -\frac{1}{V_i} \frac{D \cdot P \cdot A_{ij}}{D + Pl} (n_i - n_j) \quad (9)$$

where the A_{ij} are the “effective” cross-sections for transport. In the case of non-specific diffusion this corresponds roughly to the area of the bilayer membrane interface, in the case of pore-mediated diffusion it is given approximately by the total cross-section of the pores in the membrane. We use this model to fit the permeability of the membranes to different chemicals, and to conclude on the appropriate approximations (**Supplementary Fig. 3 and 6**).

Transcription

We model transcription and aptamer/ligand binding with the following reactions. R is the RNA, Lig is the ligand.



RNA degradation is neglected here because it is not significant on the timescale of the experiments. We assume that binding of T7 RNAP to DNA is fast and estimate RNA production from the equilibrium concentration of the DNA-RNAP complex $[DNA \cdot Pol]_{eq}$, which is given by

$$[DNA \cdot Pol]_{eq} = \frac{1}{2} \{ [DNA]_{tot} + [Pol]_{tot} + K_d - \sqrt{([DNA]_{tot} + [Pol]_{tot} + K_d)^2 - 4[DNA]_{tot}[Pol]_{tot}} \} \quad (12)$$

with $[D]_{tot}$: total concentration of DNA template, $[P]_{tot}$: total concentration of T7 RNA polymerase and K_d : their dissociation constant. In eq. (10), k_{prod} is simply proportional to $[D \cdot P]_{eq}$ and can be estimated from bulk experiments.

Transcription-translation

We model transcription-translation when induced by a chemical I , which binds a regulatory protein to activate transcription from the DNA template D . The nascent fluorescent protein is denoted by G and the matured protein G_{mat} ¹⁷.

$$\frac{d[I]}{dt} = -\frac{[I]}{K + [I]} \quad (13)$$

$$\frac{d[D_{activated}]}{dt} = \frac{[I]}{K + [I]} \quad (14)$$

$$\frac{d[G]}{dt} = \alpha_{max} (1 - e^{-t/t_2}) \frac{[D_{activated}]^n}{K_d^n + [D_{activated}]^n} - \frac{[G]}{t_{mat}} \quad (15)$$

$$\frac{d[G_{mat}]}{dt} = \frac{[G]}{t_{mat}} \quad (16)$$

Eq. (13) and (14) describe binding of the inducer to the repressor or activator protein with a dissociation constant K , which leads to a proportional activation of the promoter. As the inducer is usually the diffusing chemical, eq. (13) allows to account for the absence of diffusion of the chemical once it is bound to its corresponding protein. In eq. (15), α_{max} is a factor to account for the efficiency of the cell extract, which can vary from batch to batch. However, in our experiments it was found to always converge to ≈ 0.0033 when fitted. t_2 is the lifetime of the RNA, which is typically 15 min in the cell extract used¹⁸. Protein expression is modeled via a Hill function involving the concentration of the activated promoter, with dissociation constant K_d and cooperativity n . t_{mat} is the protein maturation time.

It should be noted that this model does not account for resource depletion, and is therefore only valid for the beginning of the reaction (ranging from 1 hour to 3 hours, depending on the rate of expression and the maturation time of the protein). The model also does not account for leaky expression in the absence of inducer.

Pulse model

We have demonstrated above that the discrete and continuous model of signal diffusion gives us very similar results for the temporal change of the signal in the array. As transport of DFHBI through the droplet assemblies was found to be dominated by aqueous diffusion, we also treated the FFL pulse using a simple continuous reaction-diffusion model, which allowed us to explore aspects such as the influence of dimensionality of the system more easily.

In the following, we set $u := [\text{DFHBI}]$, v is the concentration of active genelet, w is the concentration of fluorescent aptamer (aptamer bound to DFHBI). We further introduce z as the concentration of the antisense RNA generated. In the experiment, aptamers for DFHBI are present in excess over the transcriptional activators. We assume that initially there is a concentration of a_b activators bound to fluorescent aptamers, while there are a_f free aptamers (cf. scheme in **Supplementary Fig. 11a**).

Transcriptional activators will be released by the DFHBI and activate the gene with rate α_b . w will be generated by this process with the same rate and also by direct binding of DFHBI to free aptamer with rate α_f . DFHBI can unbind from the aptamer with rate α_m . Active genelets generate RNA species z with a rate β , which in turn deactivate the fluorescent aptamers via hybridization at rate γ .

The total aptamer concentration is conserved: $a_{tot} = a_f(0) + a_b(0) = a_f + a_b + w$. We also observe that $a_b(0) = a_b + v$. Hence, $a_b = a_b(0) - v$ and $a_f = a_f(0) + v - w$. This leads us to the following model equations for u, v, w, z , in which we consider v, w, z to be non-diffusing.

$$\frac{\partial u}{\partial t} = D \frac{\partial^2 u}{\partial x^2} - [\alpha_b(a_b(0) - v) + \alpha_f(a_f(0) + v - w)]u + \alpha_m w + \gamma w z \quad (17)$$

$$\frac{\partial v}{\partial t} = \alpha_b(a_b(0) - v)u \quad (18)$$

$$\frac{\partial w}{\partial t} = [\alpha_b(a_b(0) - v) + \alpha_f(a_f(0) + v - w)]u - \alpha_m w - \gamma w z \quad (19)$$

$$\frac{\partial z}{\partial t} = \beta v \quad (20)$$

Rather than attempting to fit the parameters of the model, we made the following assumptions based on previous findings:

- Diffusion: Only the DFHBI is allowed to diffuse with diffusion coefficient D , which should be in the range $100 \mu\text{m}^2/\text{s}$ - $1000 \mu\text{m}^2/\text{s}$. We set D to an intermediate value of $D = 500 \mu\text{m}^2/\text{s}$.
- Transcription: β is a transcription rate, which for genelets with split promoter regions has been found¹⁹ to be on the order of 0.01 s^{-1} .
- Hybridization: γ is a hybridization rate, which should be in the range 10^5 - $10^6 \text{ M}^{-1}.\text{s}^{-1}$. We set $\gamma = 10^6 \text{ M}^{-1}.\text{s}^{-1}$. In the model simulations, an increase in γ was found to be compensated by a decrease in β , and vice versa.
- Aptamer-ligand interactions: The on-rate for binding of DFHBI to the Spinach aptamer has been previously determined to be $\alpha_f = 8.1 \times 10^4 \text{ M}^{-1}.\text{s}^{-1}$, the corresponding off-rate was found to be $\approx 0.1 \text{ s}^{-1}$ ²⁰.

The only parameter that is completely unknown a priori is the rate α_b . We found that $\alpha_b = 0.1 \times \alpha_f$ generates satisfactory simulation results. For the model, we assumed an initial concentration of 0 for v , w , z everywhere, whereas $a_b(x) = 0.15 \mu\text{M}$ and $a_f(x) = 1.35 \mu\text{M}$ as in the experiment. Initially, u is confined to the first droplet, *i.e.*, $u(x,0) = 3 \mu\text{M}$ for $x \leq 300 \mu\text{m}$, and zero otherwise. The equations were simulated with Matlab using the pdepe function.

As can be seen in **Supplementary Fig. 11**, our simple reaction diffusion model captures the experimentally observed dynamics quite well. For comparison, we simulated the RD equations in 1D as well as in polar (2D) and spherical (3D) coordinates. It is remarkable that some of the experimental features like the overall shape of the curves (decrease in amplitude and broadening) seem to be captured better by the 2D or 3D models than the linear model (see **Supplementary Fig. 11h** and **i** for example). Obviously, treating a line of spherical compartments as a strictly one-dimensional system is a considerable simplification, and we assume that the experimental systems indeed display mixed features.

From the models and their comparison with the experiments we can make the following observations:

- the initial signal rise in the droplets closest to the sender droplet is due to diffusion into and out of the droplets, which also determines the position of the first peaks. The attenuation at long times is caused by the feedforward loop that leads to the production of the complementary RNA binding to the aptamer. Accordingly, a change in reaction rates does not affect the position of the concentration maxima in the first droplets, but only the behavior at longer times.
- a change in transcription and/or hybridization rate changes the velocity of the pulse – with higher rates leading to higher speeds, as in the experiment.
- interestingly, estimating the pulse speed only from the peaks in the first droplets, the velocity is slightly higher for the 2D and 3D case than for the 1D array. This is in agreement with the observation made for the experiment with the 2D droplet assembly. However, higher dimensionality also leads to a faster decay and broadening of the pulse.
- a typical wave velocity for nonlinear reaction-diffusion phenomena is given by $v \approx (k D)^{1/2}$, where k is a reaction rate. The pulse investigated here does not belong to this class of phenomena, as there is no positive feedback loop regenerating the propagating signal. Nevertheless, the pulse also reflects an interplay of diffusion of molecules and associated chemical dynamics (as demonstrated in **Fig. 3e** and **f**). For our case we can give a hand-waving argument for the observed pulse velocity: with the concentrations used, the DFHBI signal will initially generate $1.35 \mu\text{M}$ of fluorescent aptamer complexes. A corresponding concentration of antisense RNA has to be generated, which in our model takes $t = 1.35 \mu\text{M} / (0.15 \mu\text{M} \times 0.01 \text{ s}^{-1}) = 900 \text{ s}$. In this time, the signal will diffuse over a distance of $x \approx \sqrt{D t}$, corresponding to a velocity of $v \approx \sqrt{D/t}$, which gives $\approx 40 \mu\text{m}/\text{min}$ for $D = 500 \mu\text{m}^2/\text{s}$. This

corresponds very well with the range of pulse velocities observed in the model and the experiments (cf. **Fig. 3e, f and j**).

Statistics

For all calculations of the mean μ and the standard deviation σ for a set of N data points x_i , we used the following formula, applying Bessel's correction:

$$\mu = \frac{1}{N} \sum_{i=1}^N x_i \quad (21)$$

$$\sigma = \sqrt{\frac{1}{N-1} \sum_{i=1}^N |x_i - \mu|^2} \quad (22)$$

In **Fig. 4b**, μ and σ were calculated with the fluorescence of the four receivers of a given network. In **Fig. 4d**, all receivers of all networks under one experimental condition j were taken to calculate μ and σ . This can artificially increase the standard deviation, because external conditions may affect the overall expression efficiency in each network. The coefficient of variation η was therefore calculated for each network k :

$$\eta_k = \frac{\sigma_k}{\mu_k} \quad (23)$$

and plotted as single data points under each experimental condition in the inset of **Fig. 4d**. The mean μ and standard deviation σ of the coefficient of variation in each experimental condition j with N_j networks was then calculated:

$$\mu_j = \frac{1}{N_j} \sum_{k \in j} \eta_k, \quad \sigma_k = \sqrt{\frac{1}{N_j - 1} \sum_{k \in j} |\eta_k - \mu_j|^2} \quad (24)$$

and plotted as a bar in the inset of **Fig. 4d**. Finally, in **Fig. 4e**, we plotted the histogram of the coefficients of variation η for each experimental condition j , with a bin size of 5%.

Differentiation model

Two-droplet model

In the following, we attempt to rationalize the observations made in the positive feedback 'differentiation' system described in **Fig. 4** of the main text. We first investigate a simple model for a two-compartment sender-receiver system. The sender droplet contains a signal molecule (arabinose in the experiment), which diffuses through the membrane between sender and receiver. The receiver droplet produces membrane pores in response to the signal, which increases the permeability of the membrane for the signal. We denote the signal normalized by its induction threshold K_S by $x := [S]/K_S$, whereas the concentration of the membrane proteins A is normalized by their corresponding dissociation constant $y := [A]/K_A$. The total signal concentration in sender and receiver is $x_{tot} = x_{sender} + x = const$, and we therefore only have to consider the dynamics of x and y in the receiver droplet (see schematic model in **Supplementary Fig. 13f**). For our simplified model we assume that in the receiver:

$$\begin{aligned}\frac{dx}{dt} &= \left(\beta_0 + \frac{\beta_1 y^m}{1 + y^m} \right) \cdot (x_{tot} - 2x) \\ \frac{dy}{dt} &= \alpha_0 + \frac{\alpha_1 x^n}{1 + x^n} - \delta y\end{aligned}\tag{26}$$

In these equations, β_0 represents the leak of signal through the membrane without pores, whereas β_1 is the maximum pore-mediated transport rate. β has units of s^{-1} and is related to the permeability P via $\beta = P \times A_M/V$, where A_M is the effective membrane area and V is the volume of the compartment. α_0 considers leaky protein expression, whereas $\alpha_0 + \alpha_1$ is the expression at full induction. In order to limit the growth of y , we also include a degradation term here. In the experiment, the protein concentration also saturates due to the exhaustion of the cell extract, which is discussed below.

This dynamical system only has one stable fixed point $(\bar{x}, \bar{y})$, for which the signal concentration is simply equilibrated between the droplets (i.e., $\bar{x} = x_{tot}/2$ and, accordingly, $\bar{y} = \delta^{-1}(\alpha_0 + \alpha_1 \bar{x}^n/(1 + \bar{x}^n))$). In the absence of leaks ($\alpha_0 = 0 = \beta_0$) there would be another, unstable fixed point at $(x, y) = (0, 0)$. Overall this results in rather simple dynamics, in which both the signal and protein concentrations steadily rise in the receiver droplet until a maximum is reached.

We have to consider that rather than having protein degradation or dilution in our system, the dynamics are governed by the degradation of cell extract over time. We can incorporate this effect via a degradation function $f(t)$, which results in time-dependent expression rates $\alpha_0(t) = \alpha_0 \cdot f(t)$ and $\alpha_1(t) = \alpha_1 \cdot f(t)$. We considered a smooth exponential degradation of protein expression according to $f(t) = e^{-t/\tau_{XTL}}$, which we found to describe the experimentally observed expression dynamics qualitatively very well, and which is in line with previous findings by Karzbrun *et al.*¹⁸

Sources of variability

The species with the lowest concentrations in the experiments are the plasmids, which were present at 5 nM. Receiver droplets had a volume in the range $1 - 4 \times 10^6 \mu m^3$ (typically $2 \times 10^6 \mu m^3$, i.e., 2 nL). In this volume 5 nM corresponds to $n = c \times V = 10^{-18} \text{ mol}$, or $N = N_A \times c \times V = 6 \times 10^5$ plasmids. At such high concentrations, we actually do not expect any low molecule number effects such as stochastic gene expression. Nevertheless, there is a considerable variability in the production and assembly of the compartments, which could be regarded as ‘extrinsic noise’ sources in our experiments. Different volumes, bilayer areas and experimental variations in cell extract activities may lead to considerable variations in the expression strength and the bilayer permeability, i.e., in the parameters α_0, α_1 and β_0, β_1 in our model.

In order to evaluate the sensitivity of our system to these parameters, we first performed simulations with the simple two-droplet system, in which we only varied the α and β parameters. For both parameters, we assumed a log-normal distribution $P(x) = \frac{1}{\sqrt{2\pi}\sigma x} \exp\left(-\frac{(\ln x - \mu)^2}{2\sigma^2}\right)$. The parameters of the distribution are related to the mean and variance of x via $\mu = \ln\left(\frac{\langle x \rangle^2}{\sqrt{\text{var}(x) + \langle x \rangle^2}}\right)$ and $\sigma^2 = \ln\left(\frac{\text{var}(x)}{\langle x \rangle^2} + 1\right)$.

For the simulations, we assumed an arabinose permeability of $P = 5 \times 10^{-8} \text{ m} \cdot \text{s}^{-1}$. Given a typical bilayer area of $A_M = 1 \times 10^4 \mu m^2$ and a droplet size of $2 \times 10^6 \mu m^3$, this results in a transfer rate of $P \cdot A_M/V = 3 \times 10^{-4} \text{ s}^{-1}$. We further set $K_S = 0.036 \text{ M}$, $K_A = 12 \text{ nM}$, $m = 7$, $n = 1$. A typical maximum protein expression rate is given by 1 nM/min per nM gene template, i.e., 5 nM in our case. For the exhaustion of the cell extract, we assumed $\tau_{XTL} = 2 \text{ h}$. We further estimated a leaky gene expression rate of $\approx 3\%$ that of the fully induced rate, β_0 was set to zero in all experiments (negligible signal leak).

The results of the simulations are shown in **Supplementary Fig. 13a-c**. Apparently, the system is more sensitive to variations in gene expression strength than to variations in membrane permeability. However, both positive feedback systems (starting with zero and non-zero pore concentrations) display nearly identical, enhanced parameter sensitivities compared to the negative control.

This indicates that the enhanced variability observed for the positive feedback circuit may be caused by another source, which we assume to be the process of membrane pore formation itself. Formation of a α HL channel proceeds in several steps - adsorption of monomers to the membrane, formation of pre-pores, and membrane insertion - and the waiting time for channel incorporation can vary considerably. Furthermore, very different threshold concentrations for membrane penetration have been reported^{21, 22}.

Contrary to the positive control experiments with channels already present at the beginning, in the experiment in the absence of initial α HL, the channels have to be formed 'on the run'. This is in line with the observation that the time-point at which the fluorescence signal rises shows considerable variability only for this experimental condition (cf. **Supplementary Fig. 13e**).

We therefore attempted to incorporate the variability of channel insertion into the model. In the positive feedback system, production of α HL is driven by the leaky expression of the gene construct, which occurs with rate α_0 . In order to reach the threshold concentration K_A , we thus have to wait for a time $t_{wait} = K_A/\alpha_0$. We found that an exponentially distributed waiting time with $P(t_{wait}) = \tau^{-1}e^{-t_{wait}/\tau}$ does not yield the observed variability. However, a Gamma-distributed waiting time

$$P(t_{wait}) = \frac{1}{b^a \Gamma(a)} t_{wait}^{a-1} e^{-t_{wait}/b} \quad (27)$$

with slightly higher variance can qualitatively account for the observations. For the Gamma distribution, the mean and variance are given by ab and ab^2 , and thus the coefficient of variation $CV = 1/\sqrt{a}$. In the model we account for the variable waiting time by introducing a variable threshold concentration $K_{thr} = \alpha_0 t_{wait}$ instead of K_A . In **Supplementary Fig. 13d** and **e**, we compare the resulting traces for the fixed and the variable threshold models.

One sender and four receivers

We next applied the sender-receiver model with variable threshold to the case of one sender and four receivers ('1S-4R') as in the experiment. In **Supplementary Fig. 14a** we set the arabinose concentration in the sender droplet to 0.3 M, and otherwise used the same parameters as for **Supplementary Fig. 13e**. We next asked, whether there actually is a difference between the 1S-4R system, and a simple 1S-1R system measured repeatedly. In order to be able to compare the dynamics of these systems, we created a model that contains four identical copies of one receiver system - this ensures that the two systems start with the same initial sender concentration and end up with the same equilibrium concentration (which, trivially, is starting concentration divided by the total number of droplets). However, in the 1S-1R system with 4 receiver replicas, there is no coupling of the droplets via transient concentration imbalances. For our experimental settings, the variability observed for the 1S-4R and 1S-1R system turns out to be almost indistinguishable (**Supplementary Fig. 14e**).

It is instructive to look at the CV of the protein expression level as a function of time, and compare the 1S-4R and 1S-1R system in this respect (**Supplementary Fig. 14 c, d and g, h**). In general, for high signal concentrations the variability attains a maximum during the transition phase, in which the channels incorporate into the membrane and the system switches from leaky expression to arabinose-induced expression. Notably, the CV for the control experiments is considerably smaller in all cases. This demonstrates that the positive feedback circuit indeed makes the system highly sensitive to the various noise sources considered. However, the behavior of the 1S-4R and 1S-1R systems is found to

be very similar (compare **Supplementary Fig. 14c** and **g**) - in fact, the maximum CV for the 1S-1R is slightly higher.

We therefore next investigated whether we could create a clear difference between the 1S-4R and 1S-1R system by slightly altering the model of the system. One initial motivation for the 1S-4R system was to create a system, in which a receiver droplet stochastically creates channels and drains the sender droplet from signal before the others gain access to it. With our arabinose system (having negligible cooperativity $n \approx 1$ in gene induction), such behavior apparently cannot be achieved. A cooperative gene induction would create a sharper threshold above which gene expression is switched on, and therefore potentially increase the asymmetry in gene expression in interacting droplets. We therefore set the Hill coefficient of the model to a higher value ($n \approx 5$) and repeated the simulations of the CV (**Supplementary Fig. 14 d** and **h**). We found that in this case, indeed the CV for the 1S-4R system is increased with respect to the 1S-1R for certain parameter settings. As expected the differences are greatest for initial sender concentrations that lie in the range between a few and $\approx 10x$ of the induction threshold. In these cases, the *final* inducer concentrations in the droplets are on the order of the threshold. As an example, we show a simulation result for $n \approx 5$ and initial signal concentration at $10x$ of the threshold (compare **Supplementary Fig. 14 b** and **f**).

Overall, however, the differences between the 1S-4R and the 1S-1R case are not very strong. In order to achieve genuine ‘differentiation’ of the droplets (*i.e.*, clear symmetry breaking and/or a ‘winner-takes-all’ behavior), more complex circuitry would be required, in which the receiver droplet that is activated first actively suppresses activation of the other droplets.

Fits and simulations

Supplementary Tables 3 and 4 summarize the fixed and fitted parameters for all fits. Fitted parameters are underlined. The read-out molecule concentration (c) was transformed into fluorescence intensity (FI) using parameters α and β : $FI = \alpha c + \beta$. β was taken as FI at $t=0$ in most cases. For Hill function fits, β represented the leaky expression of the promoter with 0 M inducer.

Effective area of permeation

Unless otherwise specified, the volume of the droplet is taken to be 10 nL, and the radius is calculated consequently (approximately 133 μm). The thickness of the bilayer is 5 nm. The effective area of permeation for non-specific permeation is $2 \times 10^{-8} \text{ m}^2$, and the effective area of pore-mediated permeation is $1 \times 10^{-8} \text{ m}^2$. This was estimated as follows:

We found that the bilayer between two droplets on average had a diameter of approximately 160 μm , and an area of approximately $2 \times 10^4 \mu\text{m}^2$, available for non-specific permeation. The α -HL pore has a maximum outside diameter²³ of 10 nm, and we approximate its area on the bilayer to a square, as we expect steric hindrance to keep the pores from having a compact alignment. A fully covered bilayer would therefore have $\pi r_{bil}^2 / d_{pore}^2 = 2 \times 10^8$ pores. (Considering a circular pore area, the bilayer would be covered at maximum with 2.6×10^8 pores.) We assume that this number overestimates the real bilayer occupancy, as movement of the pores on the bilayer and other effects reduce the available area, and therefore took 1×10^8 pores as the maximum occupancy. In most cases, our droplets contain 1.5 μM of pores, and we can calculate the maximum number of pores in the droplet with $N = \frac{C_m V N_A}{M}$, with C_m the mass concentration, V the volume, N_A Avogadro’s number, and M the molecular weight (here 33 $\text{kg}\cdot\text{mol}^{-1}$). With $N = 9 \times 10^9$ pore monomers in the droplet, we assume that the bilayer is covered to its maximum occupancy.

pLux induction

To fit the induction of the pLux promoter by C6-HSL, we screened C6-HSL concentration and fitted the final GFP levels with a Hill fit (**Supplementary Fig. 9a**), from which we obtained K_d and n . To then fit the kinetics of translation from the induced pLux promoter (**Supplementary Fig. 9b**), we used the transcription-translation model described above. We set the initial value for K to 0.1 μM according to Urbanowski *et al.*²⁴, and fitted it to our data. $t_{mat}=7$ min for sfGFP was taken from Iizuka *et al.*²⁵. Six curves with different C6-HSL concentrations were simultaneously fitted.

pBAD induction

Similarly, for the pBAD promoter, the final RFP levels of an arabinose screening were fitted with a Hill curve (**Supplementary Fig. 9c**). For the kinetics of translation (**Supplementary Fig. 9d**), we initially set K to 0.4 mM according to Schleif²⁶, and fitted it to our experiments. $t_{mat}=102$ min for mScarlet1 was deduced from literature and fitted to our experiments^{27,28}. Nine curves with different arabinose concentrations were simultaneously fitted.

Guanine permeability

To fit the permeability of guanine, we used the general diffusion model, together with the transcription-ligand binding model. k_{prod} was fitted from separate bulk experiments and adjusted for the DNA template concentration. k_p and k_m were calculated from Paige *et al.*²⁹, with $K_d = k_m/k_p = 1.5 \mu\text{M}$. An initial delay of 20 min was estimated between start of the reaction and the beginning of data acquisition. Six curves were separately fitted and the fitted values were averaged. (**Supplementary Table 4**)

Arabinose permeability

We fitted the permeability of arabinose (**Supplementary Table 4**) with the general diffusion model together with the transcription-translation model. α was fitted for the first curve and fixed at this value for the others. An initial delay of 15 min was estimated between initiation of the reaction and the beginning of data acquisition. Six curves were separately fitted and the fitted values were averaged. K , n , K_d , α_{max} , t_2 were fixed as described in pBAD induction and **Supplementary Table 3**, and are not listed in **Supplementary Table 4**.

DFHBI permeability

General diffusion and transcription-ligand binding models were used here, as well as for guanine. k_{prod} was fitted from separate experiments and adjusted for the DNA template concentration. K_d was obtained from Paige *et al.*³⁰, k_p from Wang *et al.*²⁰; k_m was calculated from those values. An initial delay of 20 min was estimated before acquisition started. Six curves were separately fitted and the fitted values were averaged. (**Supplementary Table 4**)

C6-HSL permeability

The permeability of C6-HSL was fitted similarly as that of arabinose (**Supplementary Table 4**). An initial delay of 5 min was estimated between initiation of the reaction and the start of data acquisition. Six curves were separately fitted and the fitted values were averaged. K , n , K_d , α_{max} , t_2 were fixed as described in pLux induction and **Supplementary Table 3**, and are not listed in **Supplementary Table 4**.

For the permeability fits of both DFHBI and C6-HSL, we found that after fitting, lowering the permeability P value did not affect the fit significantly, as exemplified in **Supplementary Fig. 6**, down to $P = 9 \times 10^{-6}$ m/s for DFHBI and $P = 2 \times 10^{-6}$ m/s for C6-HSL. As transport in these cases is limited by diffusion through the aqueous compartments, the experiments only allow us to estimate a lower bound for P .

Pulse velocity

To determine the velocity of the pulse (cf. **Fig. 3** of the main text), we plotted the distance of a droplet from the sender against the time of the peak in that droplet and fitted this with a linear regression (**Supplementary Fig. 10d**). It should be noted that this is an approximation, since the pulse velocity is not constant but decreasing with distance from sender. The velocity of the pulse in at least 3 networks was thus fitted and then averaged for each DFHBI or T7 RNA Polymerase concentration, and for different dimensionalities.

Supplementary Figures

Supplementary Figure 1 shows the droplet networks assembly set-up and the chambers in which the networks are assembled.

Supplementary Figures 2 and 3 relate to **Fig. 1**. **Supplementary Fig. 2** shows the kinetics in the network displayed in **Fig. 1d** and in **Supplementary Video 1**. **Supplementary Fig. 3** describes the limit cases of the diffusion model. In these simulations, we show how the diffusion dynamics are affected in an example network of 5 droplets. We compare our discrete diffusion model to a classic 1D continuous diffusion model, in the case where the aqueous diffusion is limiting.

Supplementary Figure 4 describes the toolbox of available environments and signaling molecules for the droplet networks, and relates to **Fig. 2b and c**. The synthetic gene circuits environments that can be encapsulated in the droplets are described, the geometric and chemical restrictions of the pore are displayed, and an example is given of how the diffusion of signals is assessed with guanine. The diffusion behavior, either pore-mediated or non-specific, of guanine and 7 additional chemicals is described. Interestingly, two chemicals (lactose and tetracycline) show no diffusion at all although they are chemically and functionally close analogs of diffusing chemicals, respectively IPTG and anhydrotetracycline. The different diffusion behaviors are closely related to the hydrophobicity and size of the chemicals, as the calculated logP and molecular weight values indicate in **Supplementary Fig. 4e**: only the more hydrophobic chemicals diffuse through the bilayer. The hydrophilic chemicals, if small enough, diffuse through the pore, whereas larger chemicals do not diffuse at all (as is the case with lactose and tetracycline). It should be noted that when circuits in living *E. coli* cells are used to read out the diffusion of chemicals, the chemicals must diffuse first through the artificial lipid bilayer, and then through the cell membrane. However, all the chemicals screened are known to permeate *E. coli* cell membranes (either passively or actively) in similar experimental conditions, therefore diffusion through the lipid bilayer will be the limiting step.

Supplementary Figure 5 demonstrates the quality of our self-made cell-extract compared to a commercial equivalent.

Supplementary Figure 6 shows how the permeability of four chemicals was fitted: guanine, arabinose, DFHBI and C6-HSL. The graphs display an example experimental data set (out of the 3 or more experimental data sets used to fit the permeability), the simulated dynamics with the fitted permeability, and the simulated dynamics for other orders of magnitude of the permeability. This demonstrates how the fitted values of permeability are in the appropriate order of magnitude to reproduce the dynamics of the experiments.

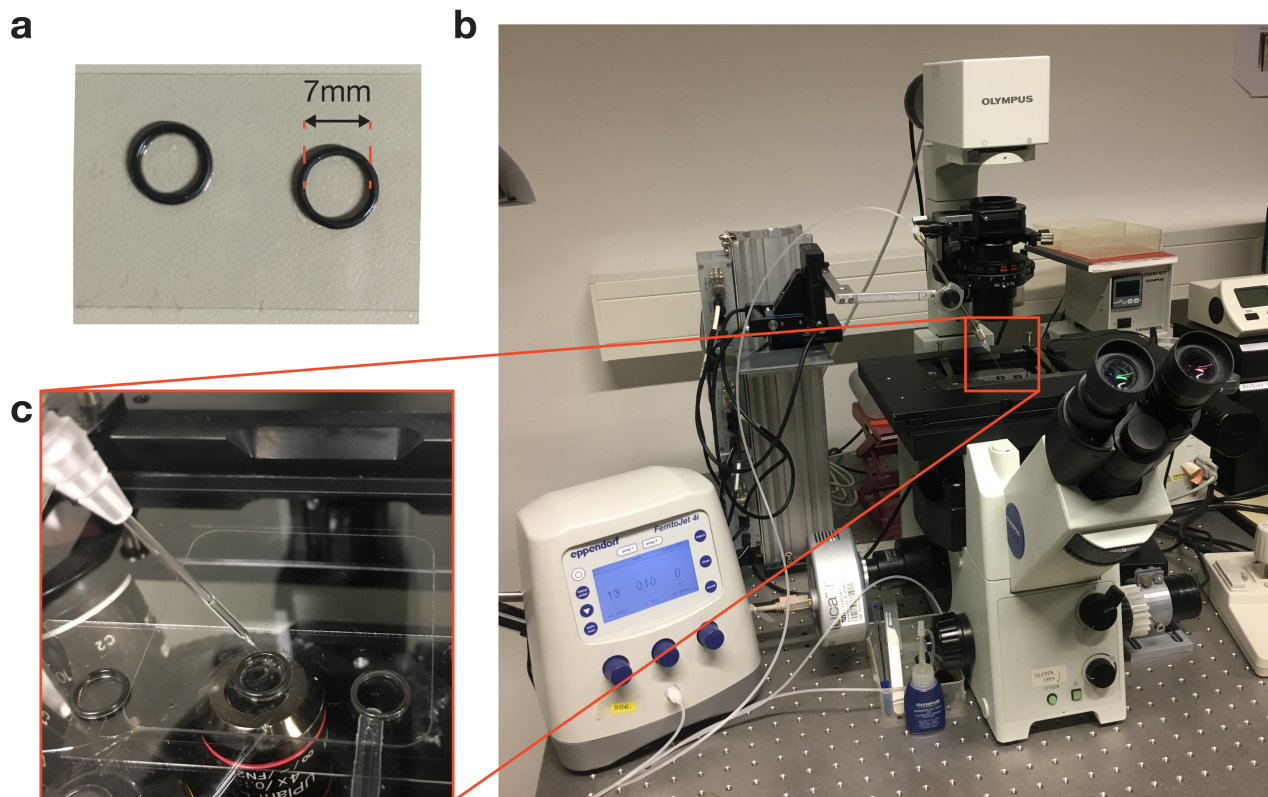
Supplementary Figures 7, 8 and 9 relate to **Figure 2**. **Supplementary Fig. 7** shows the simulated protein expression rate for the conditions tested in **Fig. 2f**, and how the RFP expression is significantly slower in droplets due to the diffusion barrier to arabinose than in bulk. **Supplementary Fig. 8** shows the simulated time traces of protein expression in the conditions of **Fig. 2i**. We see in the time traces (**Supplementary Fig. 8**) as well as in the final levels (**Fig. 2i**) that GFP expression is unaffected and RFP expression is significantly affected by the number of buffer droplets. **Supplementary Fig. 9** shows how the parameters of protein expression were determined for the pLux-GFP construct and the pBAD-RFP construct. The parameters that were fitted here and then used for the simulations of protein expression are K_d , n , K , α and β (see Models). K_d and n were fitted with a Hill function from the final protein levels in an inducer titration experiment, and K , α and β were fitted from the time traces of that same experiment.

Supplementary Figures 10 and 11 relate to **Figure 3**. In **Supplementary Fig. 10**, we show control experiments on the pulse and the method used to determine the pulse velocity. The controls include: the behavior of the circuit with and without the repressor template G in bulk and in droplets, and the DFHBI-induced release of the ssDNA trigger, measured with an orthogonal genelet template coding for

the Malachite Green fluorescent aptamer. In **Supplementary Fig. 11**, we compare our model to the experimental data. This is discussed in more details in the model section above.

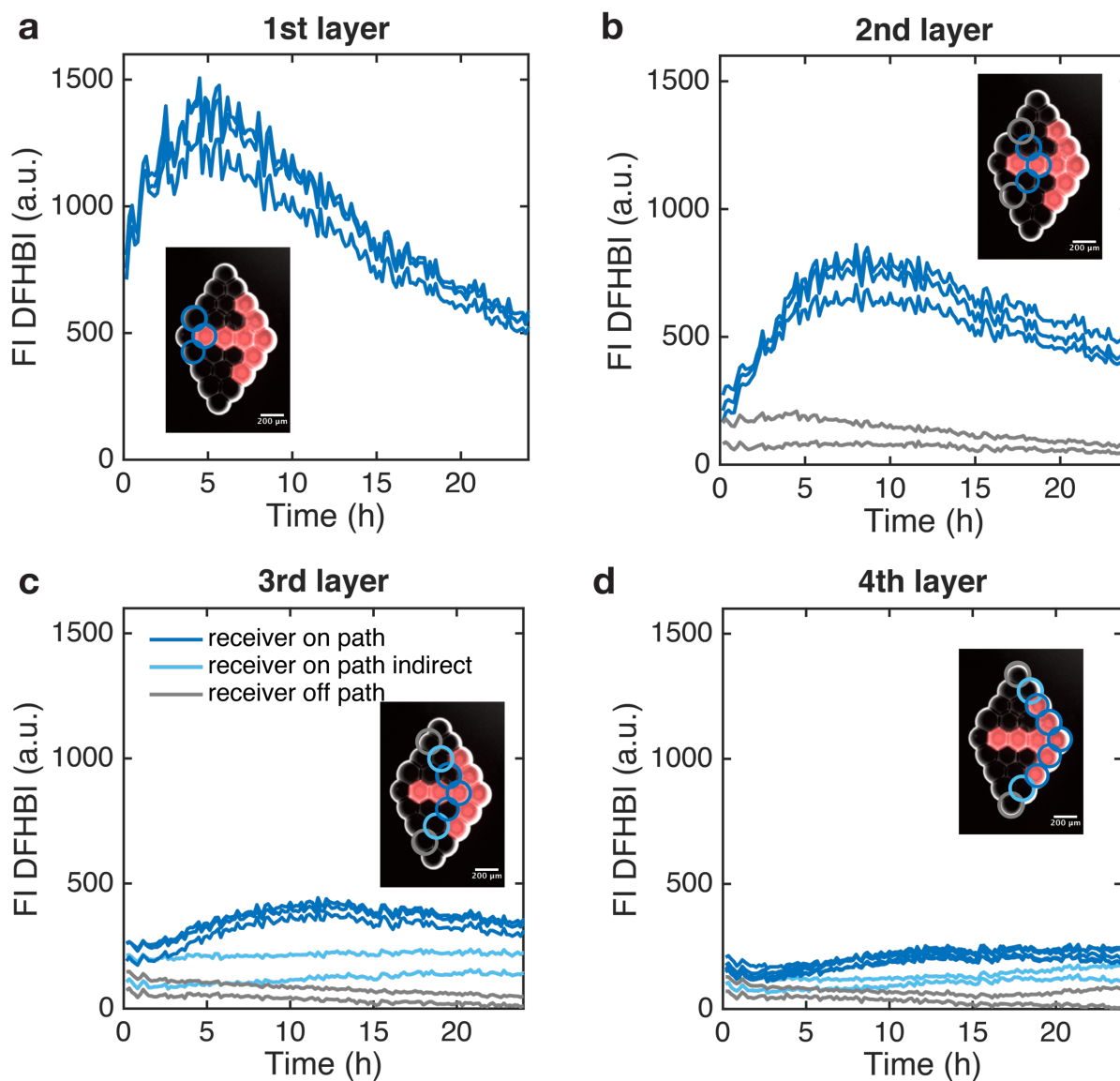
Supplementary Figures 12, 13 and 14 relate to **Figure 4**. In **Supplementary Fig. 12**, we investigate the difference in leakiness of the full construct and the reporter-only construct. We measure and fit the induction curves for those two constructs: three independent titration experiments, their fits, and the mean and standard deviation of the mean are shown for each construct. A truncated construct pBAD- α -HL is encapsulated within receivers, and we monitor the diffusion of a pore-mediated fluorophore, Atto488, in presence and absence of arabinose, to demonstrate the formation of pores. We plot the time at which each droplet crosses a given protein expression threshold (given as the final mean fluorescence of the negative control after 4 hours) to demonstrate the variation in protein expression induction in the differentiation experiment. All droplets of the positive control cross the threshold at a similar time point, consistent with fully induced protein expression. The droplets of the leak control cross the threshold at slightly distributed time points, consistent with the variability in leaky arabinose diffusion. The droplets of the differentiation experiment cross the threshold at largely distributed time points, confirming that the protein expression of each receiver is induced differently. **Supplementary Fig. 13** and **14** display simulations from the differentiation model.

Supplementary Figure 1



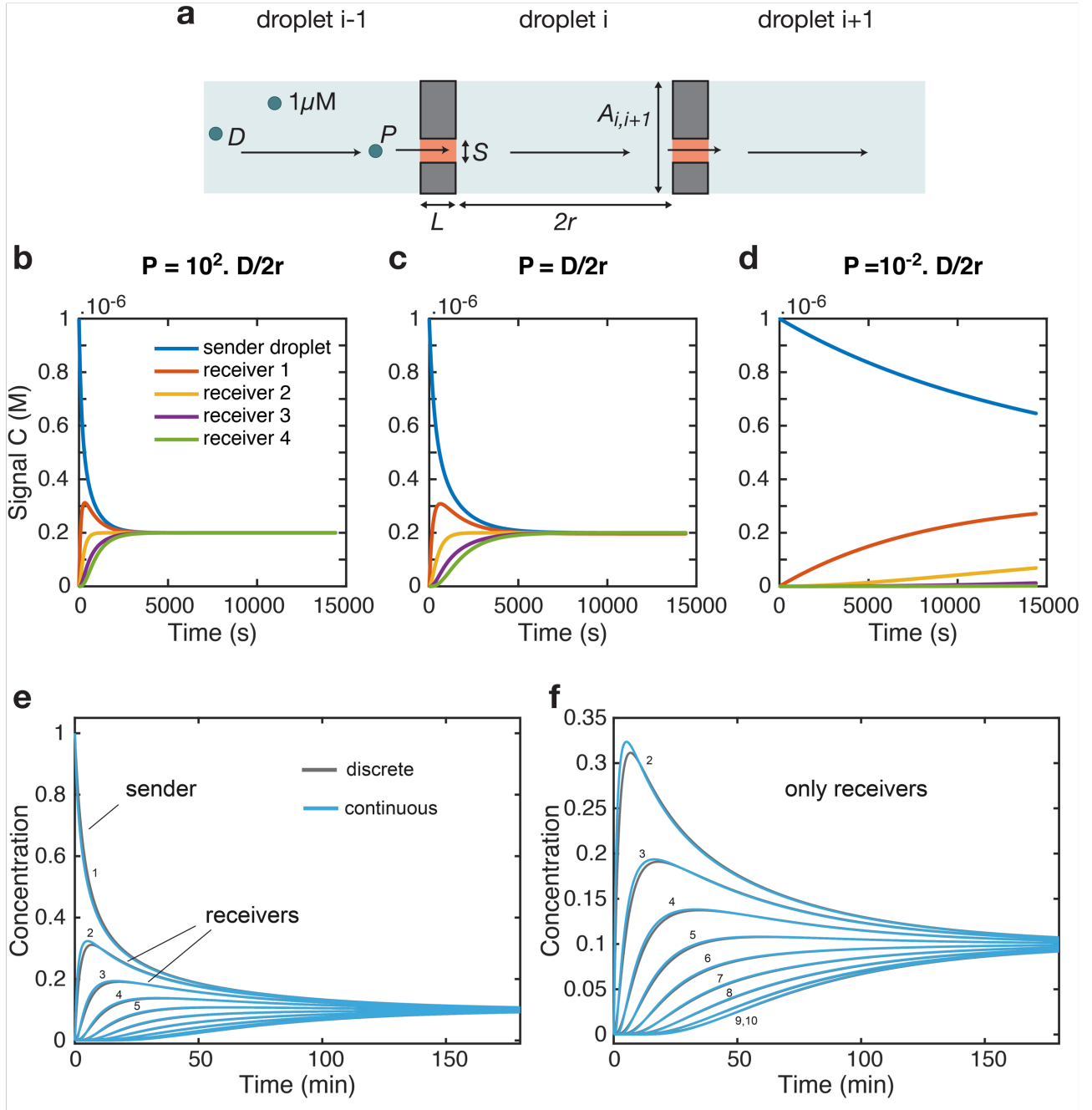
Supplementary Fig. 1 – Overview of the set-up for the assembly of droplet networks. **a**, the chambers are around 7mm inner diameter, and are tight to oil leaking once glued on a glass slide. After assembly of the networks, the chamber is closed by dropping a glass slide over it, to avoid evaporation of the droplets. **b**, Overall set-up, with chambers on the heating plate of the microscope, glass pipette mounted on the micromanipulator above the microscope, and pressure pump in the lower left. **c**, Close-up of the glass pipette.

Supplementary Figure 2



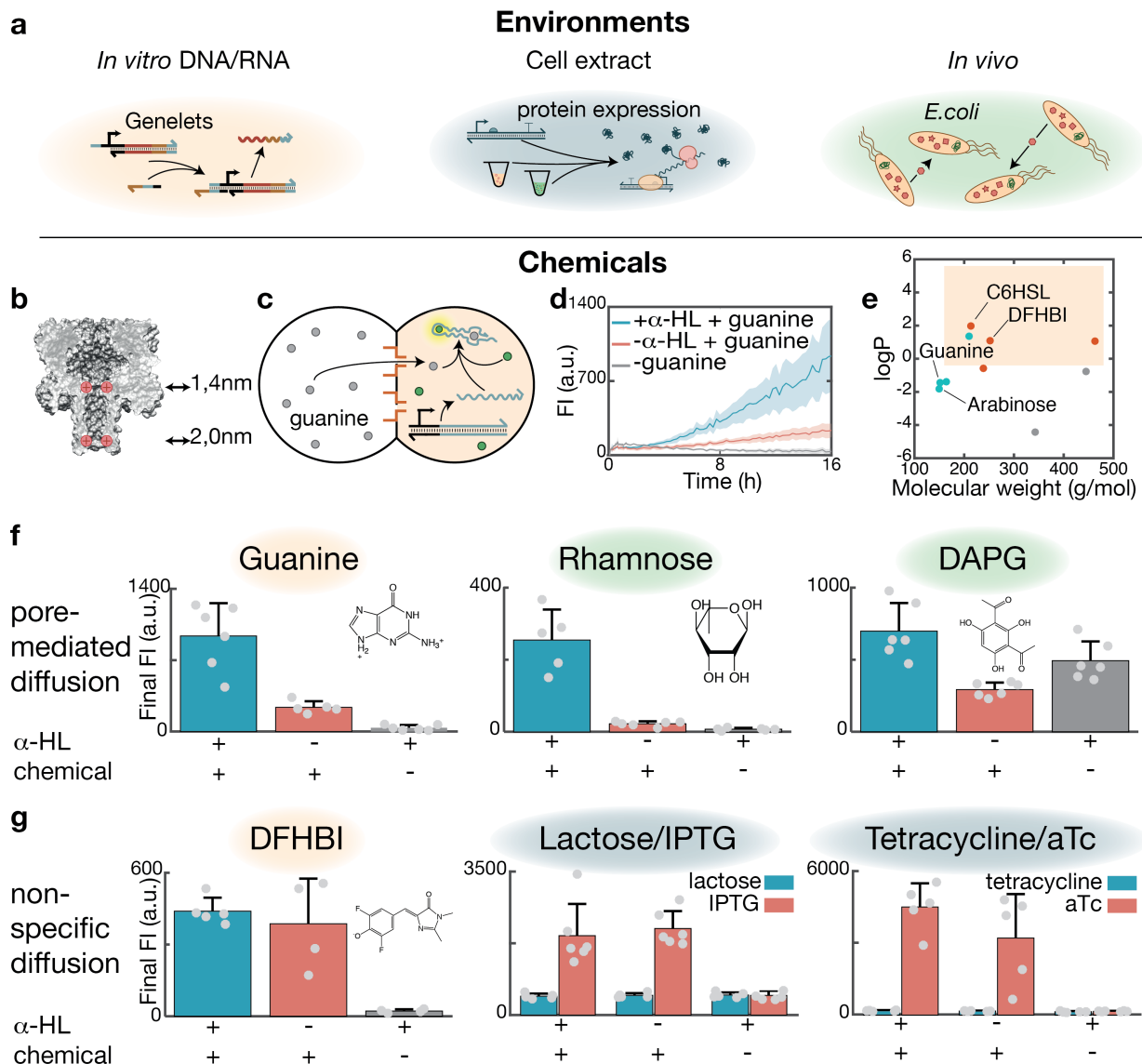
Supplementary Fig. 2 - Diffusion of guanine in a two-dimensional network. Guanine is diffusing along a path of pores (red in inset images) and in the direct neighbors. The fluorescence intensity is tracked in the droplets on the path (dark blue), *i.e.* the droplets containing pores or their neighbors; in the droplets on the path distant from the initial source (light blue), *i.e.* the droplets that although they are close to the source, are on a distant path from the source concerning the pore-mediated diffusion; and the droplets off the path (grey). Inset indicates the droplets tracked on layers starting from the sender: **a**, first layer, **b**, second layer, **c**, third layer, **d**, fourth layer.

Supplementary Figure 3



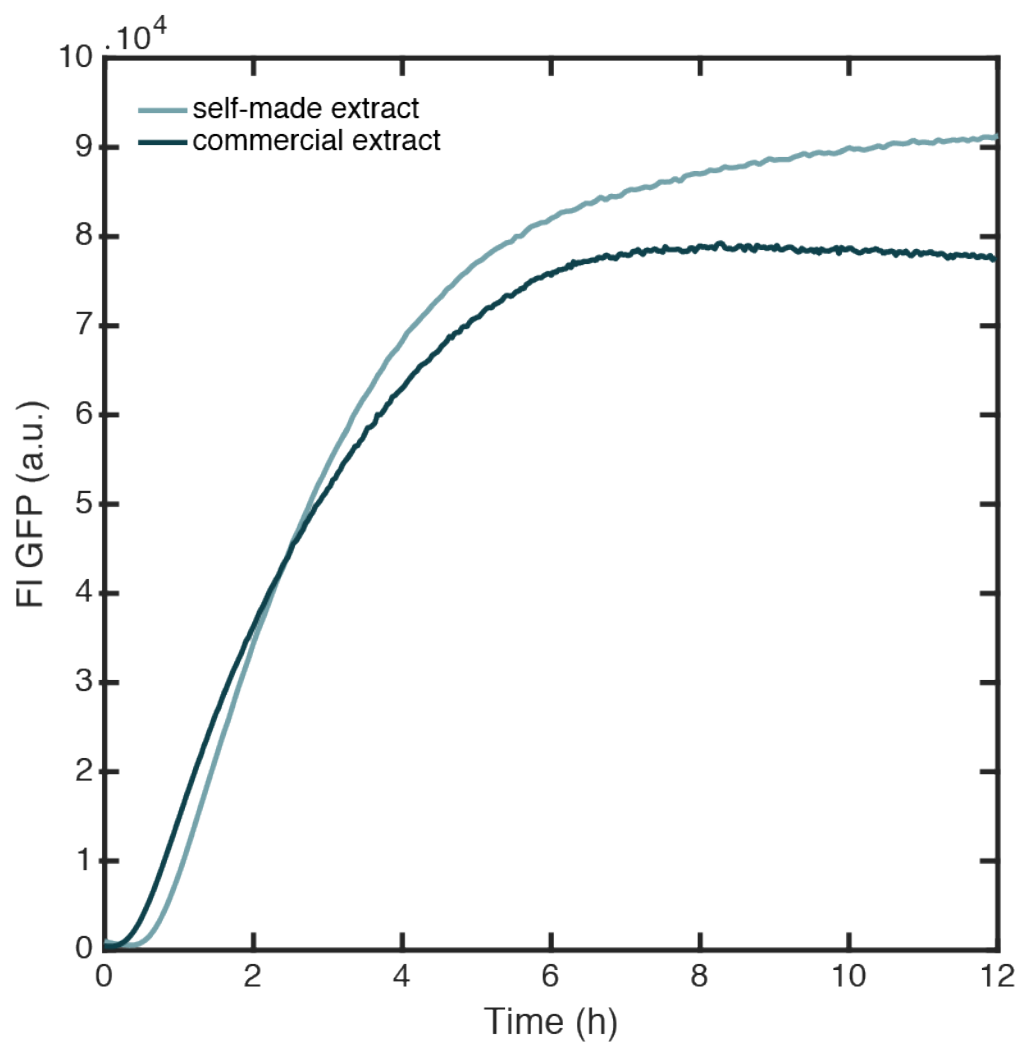
Supplementary Fig. 3 - Diffusion in an array, according to our diffusion model. **a**, Scheme of the diffusion model, with geometrical parameters, diffusivity D for aqueous diffusion and bilayer permeability P . **b-d**, Simulation of the signal concentration in a network of five droplets, for which the first (sender) droplet contains $1 \mu\text{M}$ signal at $t=0$. Diffusion coefficient for diffusion through the droplet: $D=1 \times 10^3 \mu\text{m}^2 \cdot \text{s}^{-1}$. The permeability value of the bilayer is as indicated in the figure. **b**, $P = 10^2 \times D/2r$ – diffusion through the droplet is limiting, $D_{\text{eff}} \approx D$. **c**, $P=D/2r$, both diffusion processes are of similar speed, neither can be neglected. **d**, $P = 10^{-2} \times D/2r$, diffusion through the bilayer becomes the limiting step, $D_{\text{eff}} \approx P \cdot 2r$. **e**, **f**, Comparison of a discrete diffusion model with limiting aqueous diffusion (as in **b**), and a continuous 1-dimensional diffusion model. All droplets of a 10 droplets-array are plotted in **e**, and only receivers are plotted in **f**.

Supplementary Figure 4



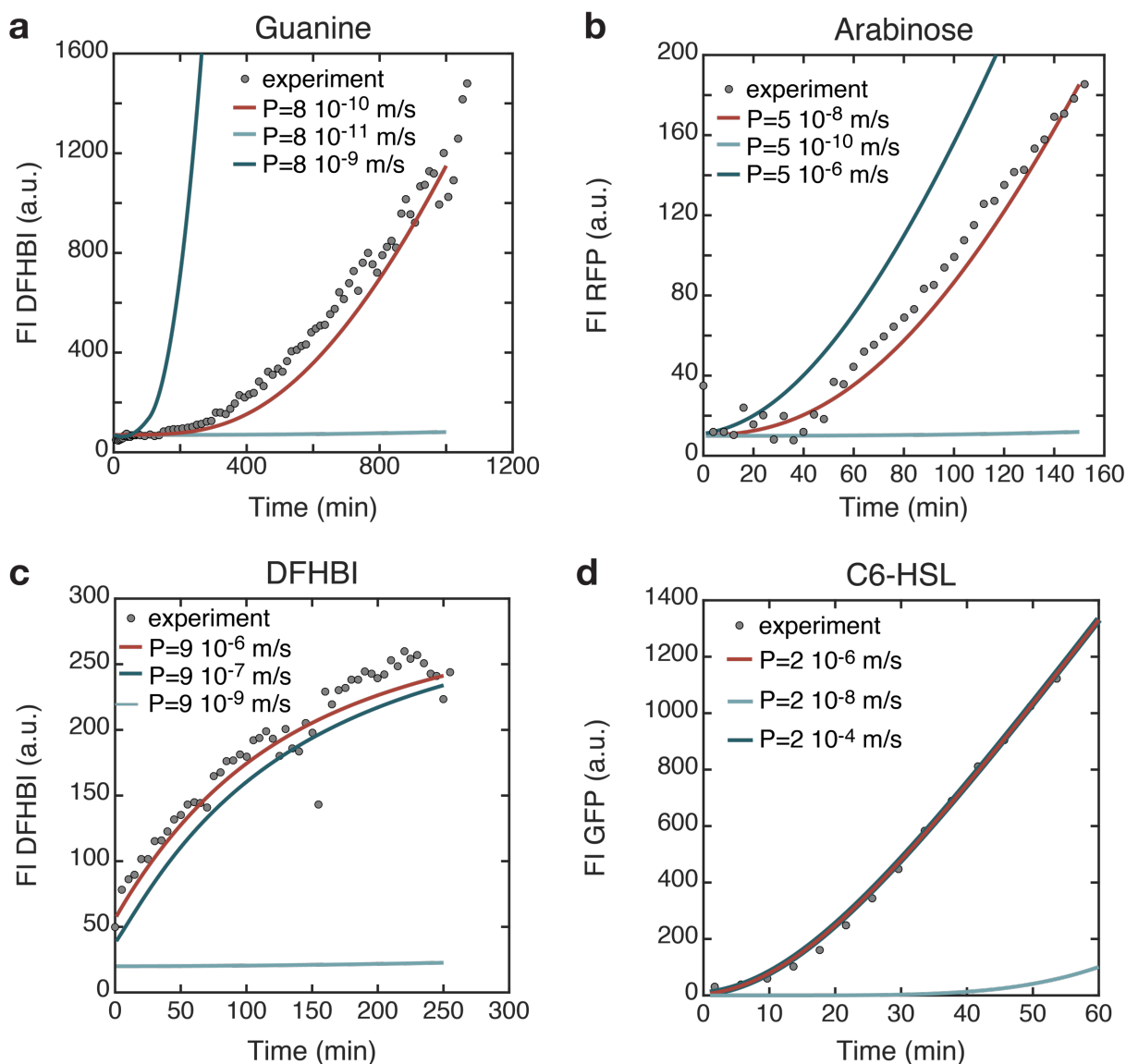
Supplementary Fig. 4 – Molecular toolbox for the encapsulation of synthetic circuits. **a**, Circuits are implemented in three environments: *in vitro*, cell extract, and *in vivo*. **b**, The α -HL pore has a constriction of 1.4 nm diameter with positively charged amino acids at both ends of the barrel. **c**, Diffusion assessing networks consist of a sender droplet with the diffusing chemical (here guanine) and a receiver droplet with a read-out circuit (here transcription of the 24-2-guanine aptamer, which increases DFHBI fluorescence upon guanine binding). **d**, The fluorescence of the receiver is compared in absence of the chemical (grey), in presence of the chemical and absence of pores (red), or in presence of the chemical and pores (blue) to determine the diffusion characteristics of the chemical. Lines and shaded areas represent the mean and standard deviation of at least three separate networks. **e**, For all assessed chemicals, their partitioning coefficient into octanol logP is plotted against their molecular weight. Pore-mediated, non-specific, and absent diffusion are indicated by blue, red and grey dots respectively. The shaded orange box represents the fulfillments of the Rule of Five criteria, *i.e.* non-specific diffusion. **f,g**, Final fluorescence intensities for read-outs of chemical diffusion, for pore-mediated diffusing chemicals (**f**) or non-specifically diffusing chemicals (**g**). Each box shows: the chemical, its structure, the environment of the read-out (shaded area), and the final fluorescence levels (mean, standard deviation, and single data points) for the three conditions listed in **d**, following the same color code.

Supplementary Figure 5



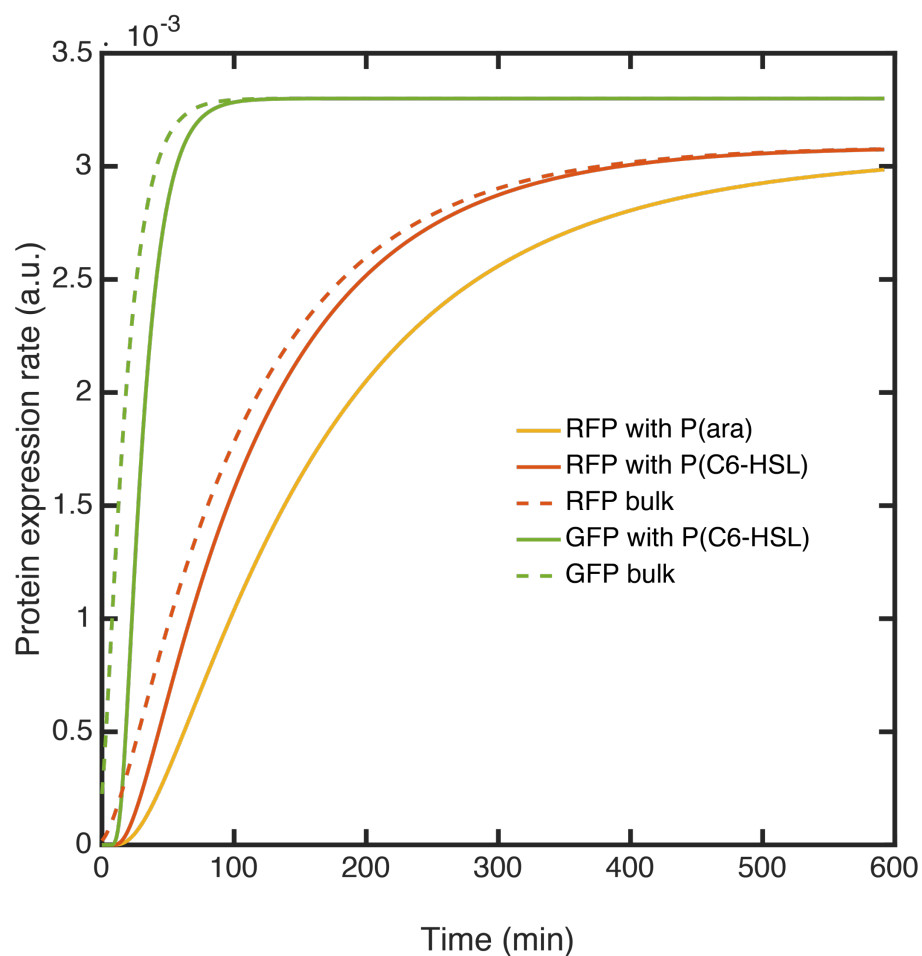
Supplementary Fig. 5 – Comparison of the quality of our self-made cell-extract with a commercial equivalent. We express GFP from 5nM of a pLux-GFP plasmid (pSB1A3-pLacO-T9002-LVA) induced with 1 μ M C6-HSL. The expression efficiency and the kinetics are roughly equivalent between commercial and self-made cell-extract.

Supplementary Figure 6



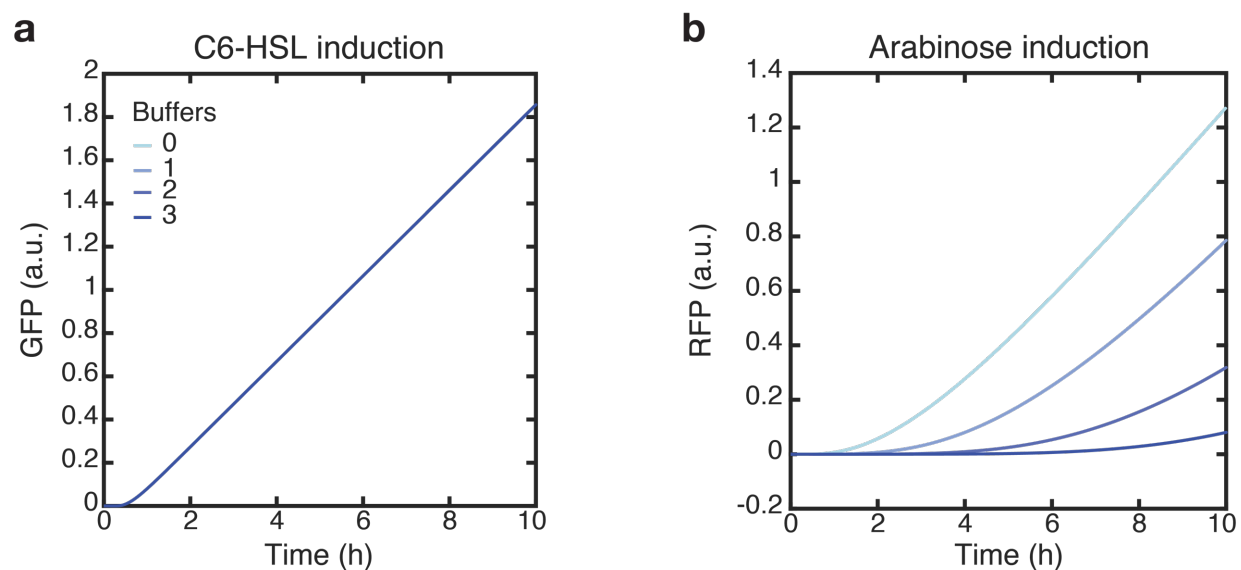
Supplementary Fig. 6 - Fitting of P from translocation experiments. D was determined for the different species via the Stokes-Einstein equation. All figures show: grey dots, one of six data sets that were fitted; red line, simulation with fitted permeability; light and dark blue lines, simulations with different permeabilities, indicating the significance of the fitted permeability. **a**, Guanine, P was fitted at 8×10^{-10} m/s (i.e., permeation through the bilayer is limiting). **b**, Arabinose, P was fitted at 5×10^{-8} m/s (permeation through the bilayer is limiting). **c**, DFHBI, P was fitted at 9×10^{-6} m/s or above (i.e., diffusion through the droplet is limiting). **d**, C6-HSL, P was fitted at 2×10^{-6} m/s or above (diffusion through the droplet is limiting).

Supplementary Figure 7



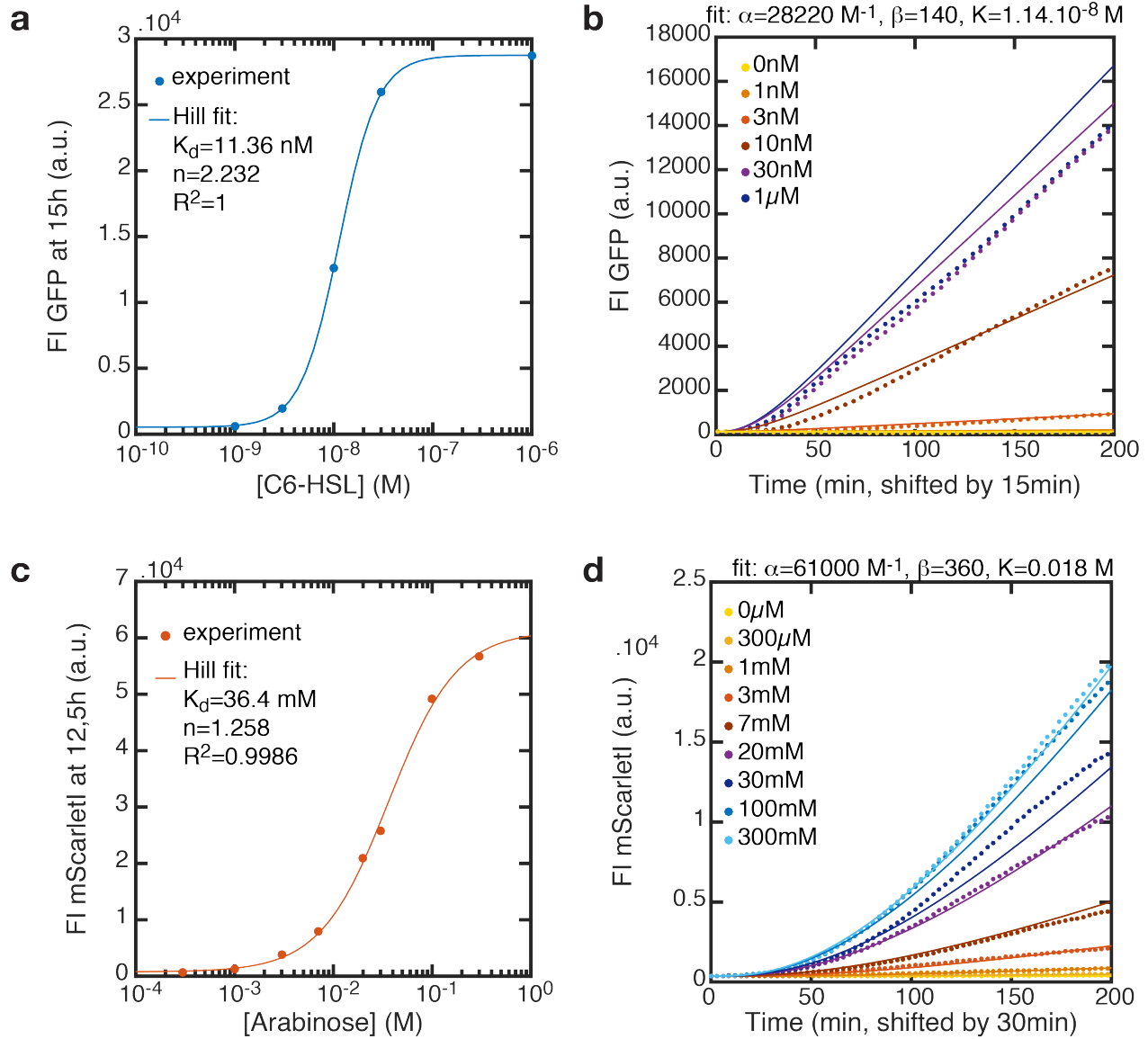
Supplementary Fig. 7 - Comparison of simulated protein expression rates when the inducer is freely available (bulk), or when it is retained through a permeability barrier (lipid bilayer, in droplet networks). The expression rate of GFP is compared in bulk (dotted green) and in droplet when the permeability of the bilayer to the inducer (C6-HSL here) is its fitted permeability (continuous green). The expression rate of RFP is compared in bulk (dotted red), in droplet when the permeability of the bilayer to the inducer (arabinose here) is its fitted permeability (continuous yellow), or when the permeability is taken to be that of C6-HSL (continuous red). This demonstrates that the RFP expression when in droplets is significantly slower due to the slower diffusion of arabinose compared to C6-HSL.

Supplementary Figure 8



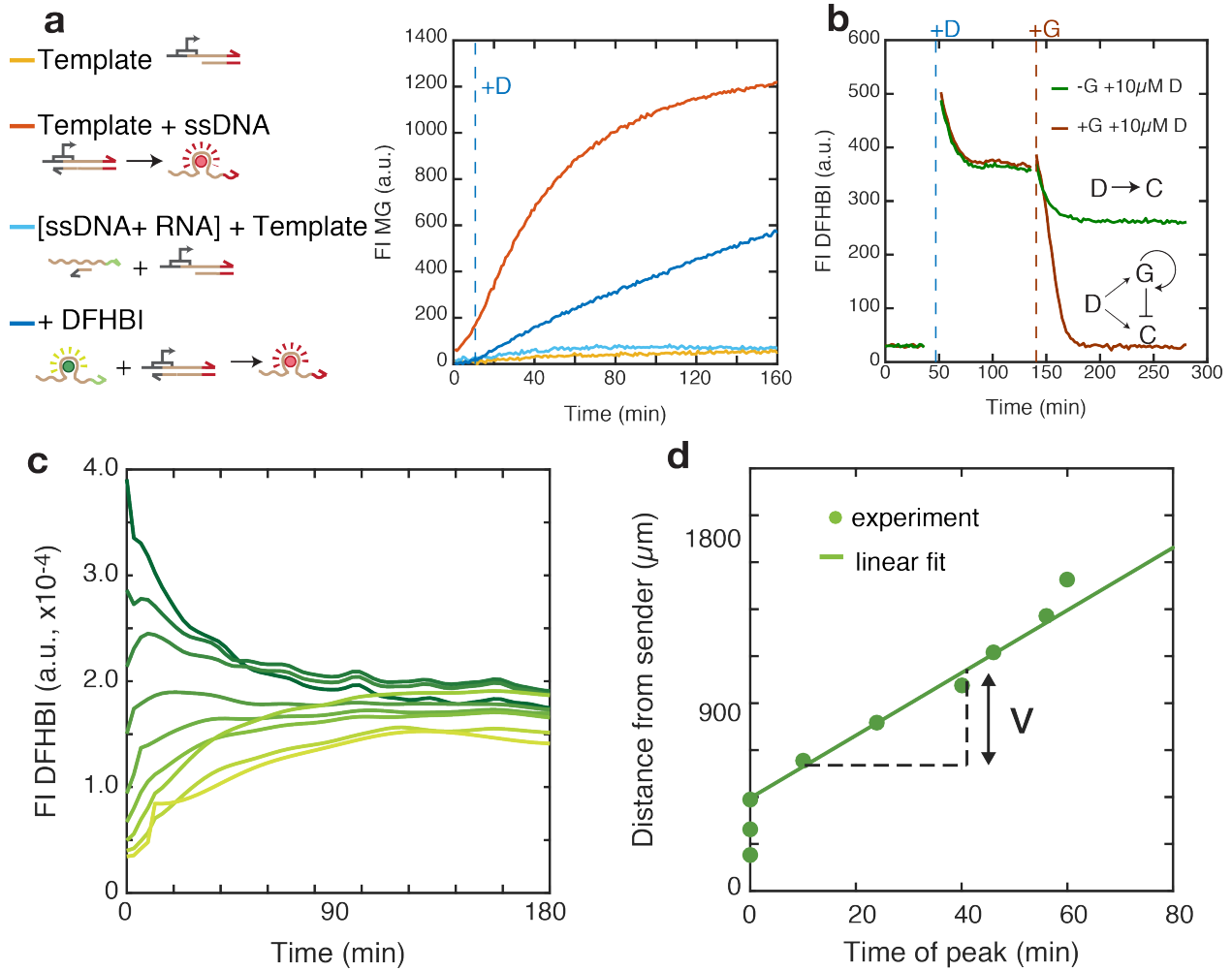
Supplementary Fig. 8 - Simulation of protein expression induced by C6-HSL or arabinose, with 0, 1, 2 or 3 buffer droplets between sender and receiver. **b**, In all cases, C6-HSL reaches concentrations in the receiver well above K_d and gene expression is fully induced, consistent with experiments. **c**, In the case of arabinose, the number of buffer droplets strongly affects the arabinose concentration that reaches the receiver in the time span considered. Accordingly, the concentrations in the receiver stay below K_d , which reduces protein expression with increasing number of droplets, consistent with the experiments.

Supplementary Figure 9



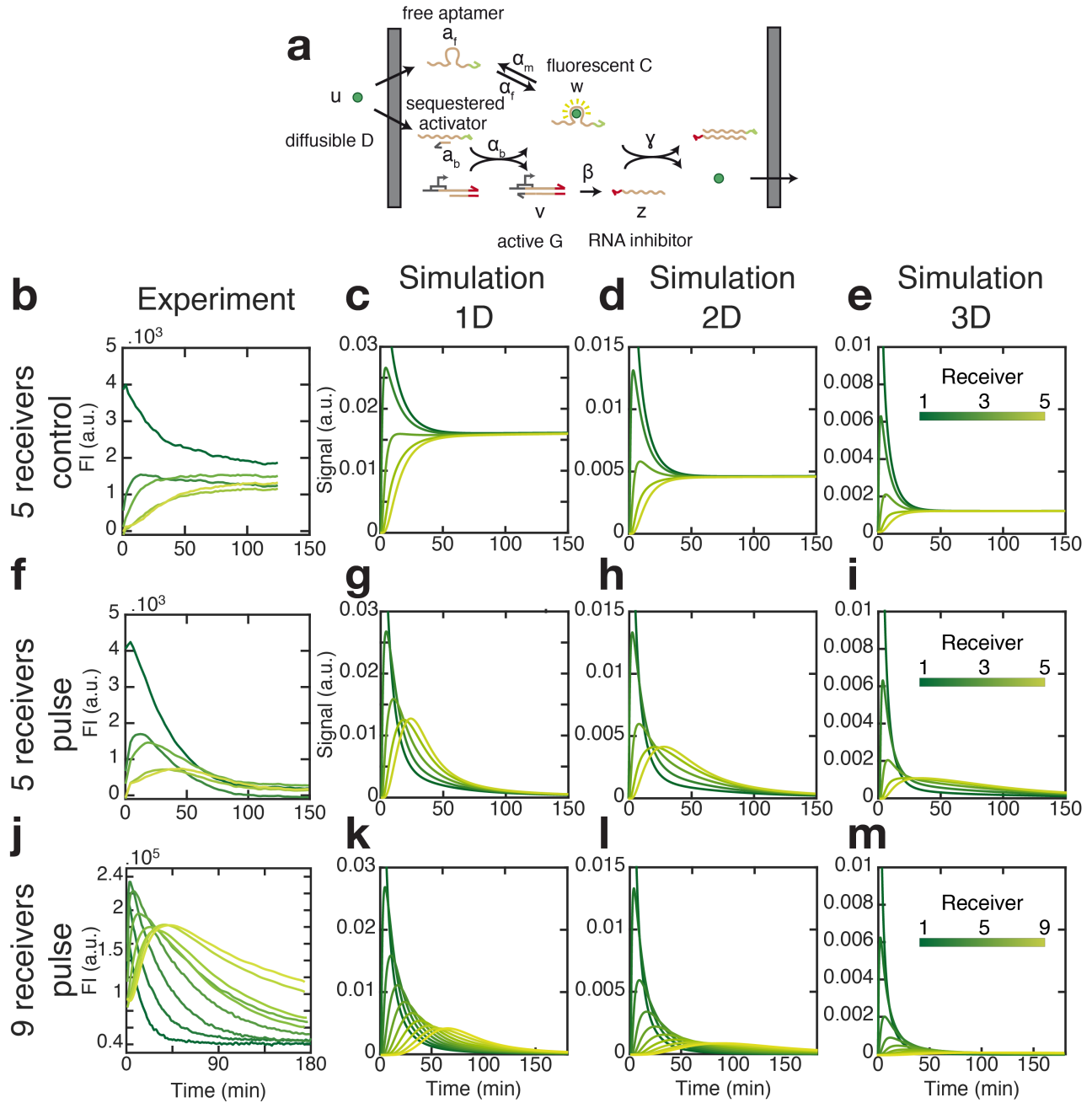
Supplementary Fig. 9 – Comparison of the experimentally determined induction characteristics of the pLux and pBAD promoter. **a,c**, Final fluorescence intensities obtained from expression of fluorescent proteins under the respective promoters for varying inducer concentrations. Experimental data are fitted with a Hill curve, from which the K_d and Hill exponent were obtained. **b,d**, Initial protein expression kinetics is fitted with an ODE model. Dots represent experimental data and lines represent fits. α and β are parameters relating protein concentration to fluorescence, and K is the binding affinity between the chemical (C6-HSL or arabinose) and the activator protein (LuxR or AraC, respectively), initially taken from literature and fitted to the experimental data (α has units of M $^{-1}$).

Supplementary Figure 10



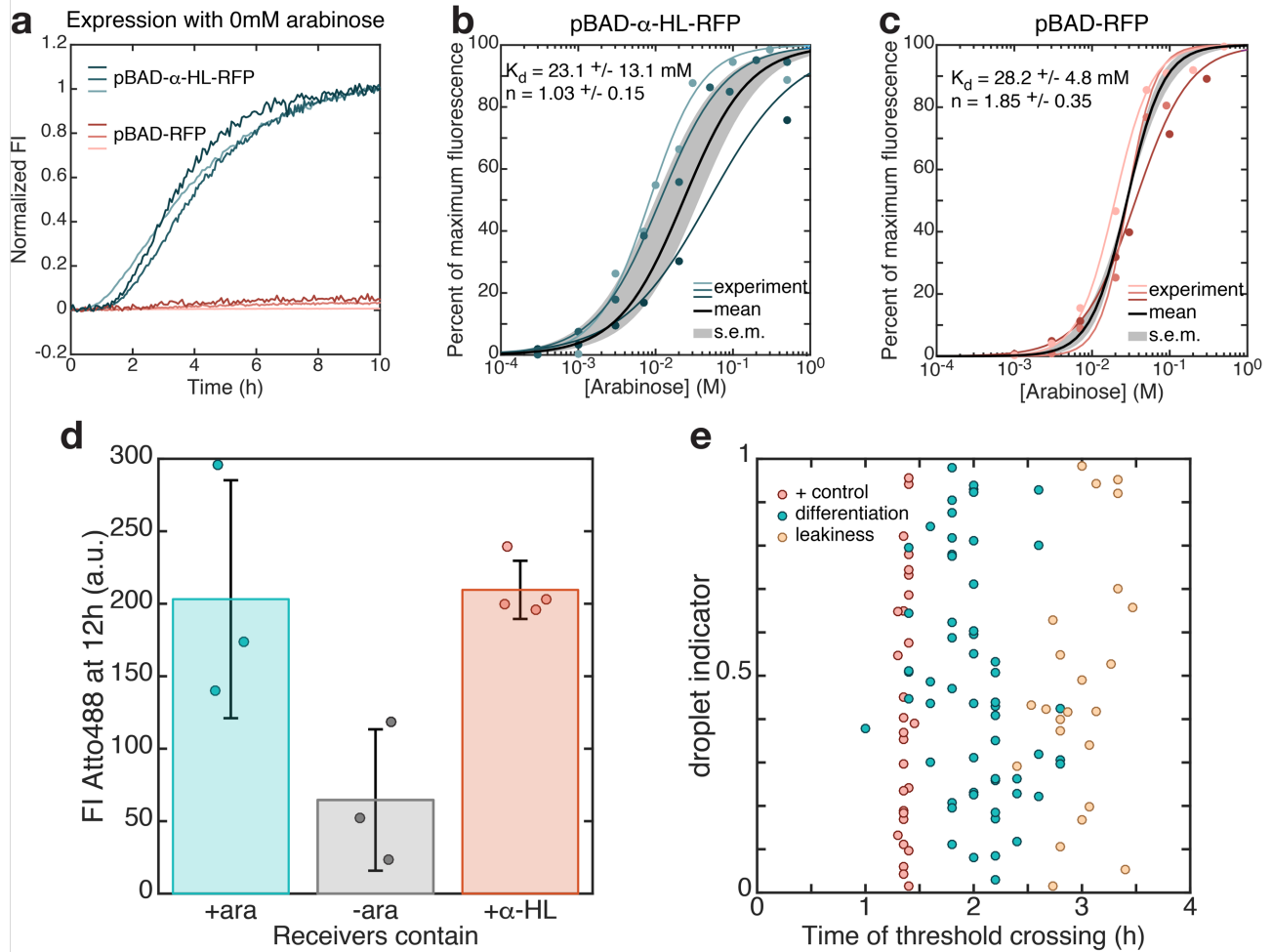
Supplementary Fig. 10 - a, The tightness of the RNA-ssDNA duplex is tested with a genelet coding for the Malachite Green fluorescent aptamer, MG-C3 ds³¹ (5 μM of the fluorophore MG were added to the samples). The MG genelet here takes the place of the repressor template G in the usual pulse experiments. Yellow: The template is incomplete, and therefore no transcription occurs. Red: The template together with the ssDNA is active, transcription occurs, Malachite Green fluorescence increases. Light blue: When the ssDNA is preincubated with the Spinach aptamer, and the template is added later, no transcription occurs. Dark blue: When DFHBI is added, the ssDNA is released from the Spinach aptamer, and transcription of MG aptamer is activated. **b**, In this experiment, the usual genelet G is used. Transcription from G creates a negative feedback that displaces DFHBI from the RNA. DFHBI (+D vertical line, at 30 min) is added to a preincubated RNA-ssDNA duplex and fluorescence levels rise due to the formation of the Spinach aptamer. When G is added (+G vertical line, at 55 min), fluorescence decreases (red curve), indicating the displacement of DFHBI by the circuit. When water is added instead of G (green line), fluorescence stays constant after an initial drop due to dilution of the aptamer. **c**, In a linear network of droplets not containing the genelet G, diffusion of DFHBI only results in an equilibration of the fluorescence level across the whole network - in contrast to the fluorescence pulse observed when G is present. **d**, To obtain the pulse velocity, the distance from the sender of each droplet was plotted against the time of the pulse maximum in this droplet. A linear regression fit is used to estimate the pulse velocity from the slope. The droplets which maximum was reached before the start of the data acquisition were not considered for the regression.

Supplementary Figure 11



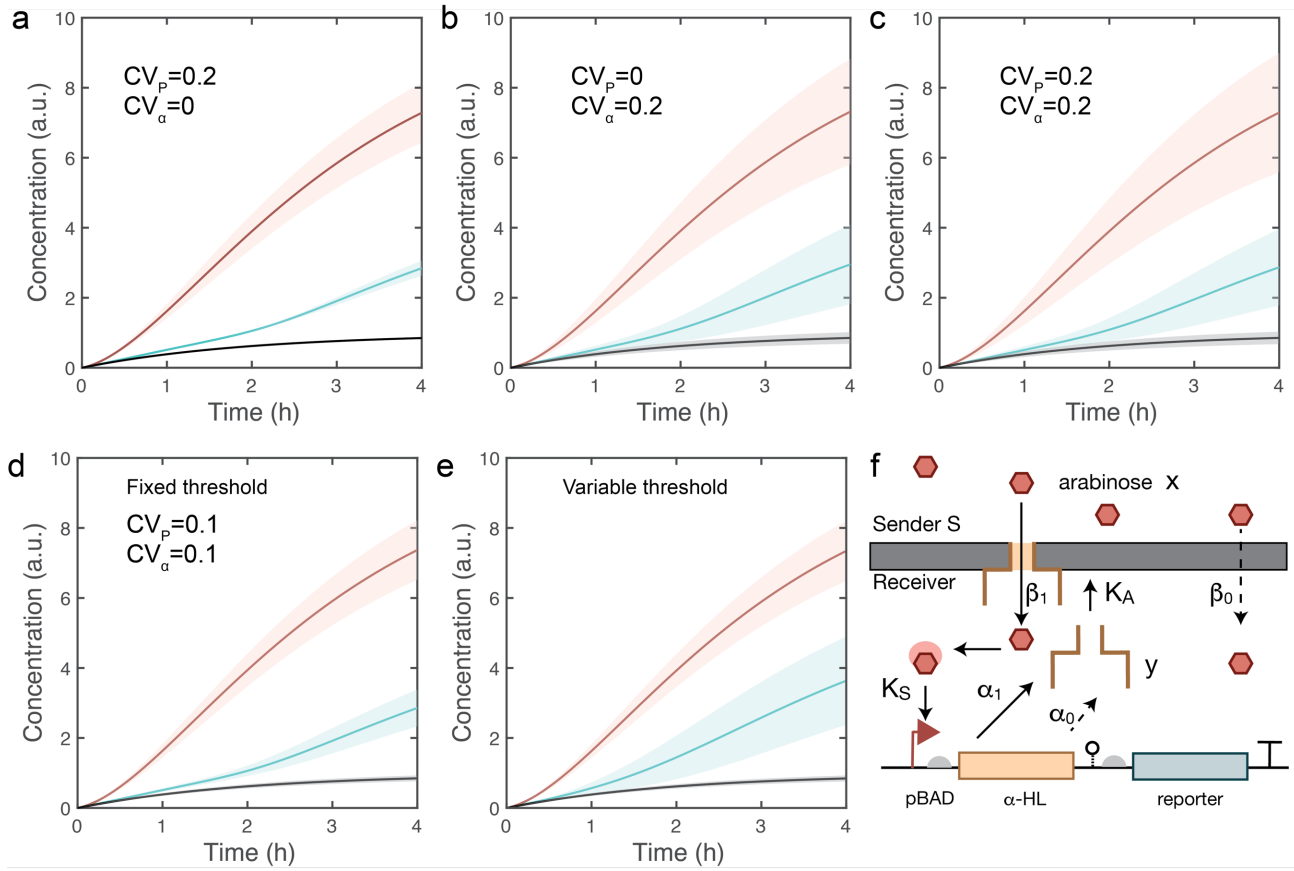
Supplementary Fig. 11 – Comparison of the model and the experimental results for linear assemblies of the pulse. **a**, Scheme of the model, with species DFHBI u , free aptamer a_f , sequestered activator a_b , active template v , fluorescent Spinach-DFHBI complex w , antisense RNA z , and rates of DFHBI binding/unbinding the free aptamer α_f and α_m , rate of DFHBI binding the bound aptamer and activating the template α_b , rate of transcription β and rate of antisense RNA and Spinach aptamer binding γ . **b-m** Experimental data (**b**, **f**, **g**) compared with simulations of the continuous model in 1D (**c**, **g**, **k**), 2D (**d**, **h**, **l**), and 3D (**e**, **i**, **m**) for a control experiment with 5 receivers (**b-e**), a pulse experiment with 5 receivers (**f-i**) and a pulse experiment with 9 receivers (**j-m**). Although the model captures the behavior of the pulse circuit (both in control and full experiments), the system studied cannot be readily approximated to a 1D or 2D geometry, and the diffusion of DFHBI in all 3 dimensions plays a role in the dynamics. All simulation graphs have been truncated for readability, as the first droplet presents a high peak.

Supplementary Figure 12



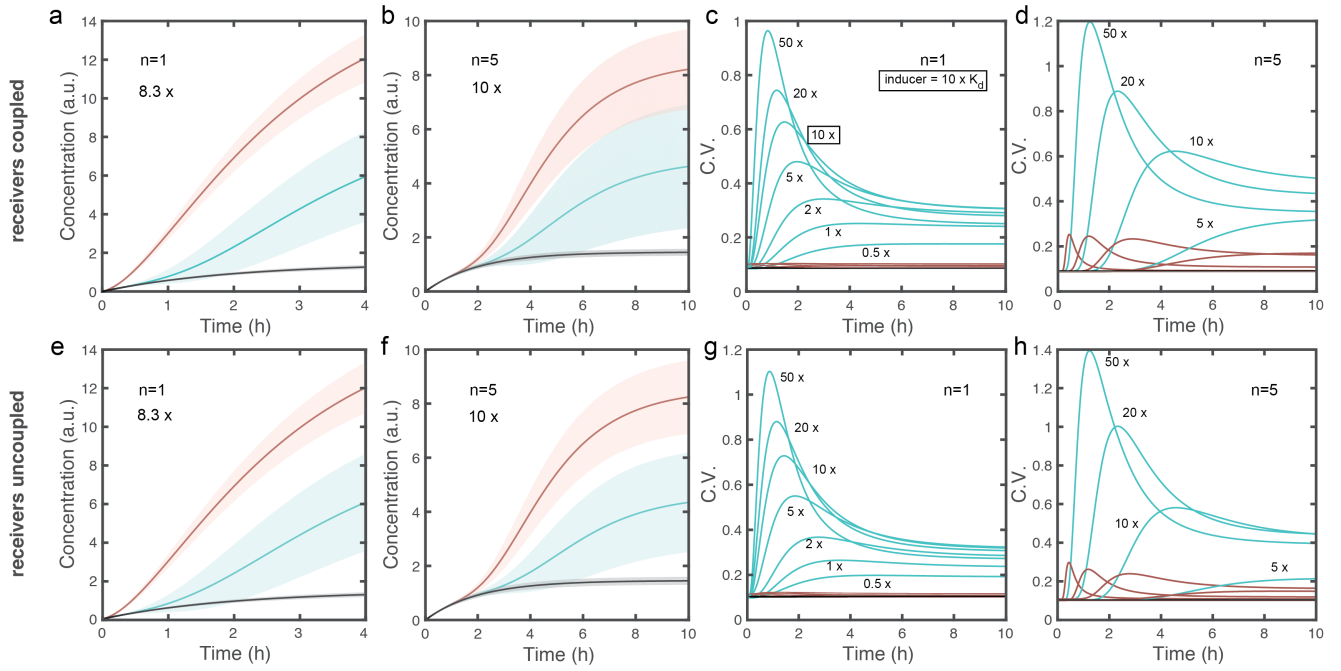
Supplementary Fig. 12 - a, The full construct pBAD- α -HL-RFP is leakier than the truncated construct pBAD-RFP: with 0mM arabinose, the full construct expresses at least 20 times more RFP than the truncated one. **b-c**, Hill function fits for the induction of pBAD- α -HL-RFP and the truncated construct pBAD-RFP. The final fluorescence intensity at 10h is taken (dots) for different arabinose concentration, and fitted with a Hill function (colored lines). Mean (black line) and s.e.m. (shaded grey area) of the dissociation constant K_d and the Hill exponent n are calculated for each construct over the three repeats. The K_d values are equal within the error range, indicating the same induction response to arabinose. The cooperativity of the induction (n) is stronger in the truncated construct, which we assume to be due to sequence-specific context, and consistent with the lower leakiness of the truncated construct in absence of arabinose (**a**). **d**, Control experiment showing the formation of α -HL pores by the construct pBAD- α -HL. Senders contain Atto488, which has a pore-mediated diffusion (data not shown). Receivers contain the construct with or without 100 mM arabinose, or with 1.5 μ M α -HL. Mean and standard deviation of the FI of at least three networks are shown at 12h of acquisition (dots show single networks). Without arabinose (grey), the final fluorescence is lower than in the other experiments, indicating less translocation of Atto488. The final fluorescence when arabinose induces the expression of pores (blue) or when purified pores are added (red) is similar, indicating translocation of Atto488. This experiment demonstrates that arabinose induces the correct formation of pores and enables influx of Atto488. **e**, To quantify the kinetics of expression per droplet, the time where each droplet crosses a given threshold of protein expression is plotted. The threshold is chosen as the final mean protein expression of the negative control (conditions 3 in **Fig. 4**). The droplets of the positive control (red, conditions 2), the differentiation experiment (blue, conditions 1) and the leakiness control (yellow, conditions 4) are plotted.

Supplementary Figure 13



Supplementary Fig. 13 – Simulations of protein expression in a system consisting of one sender and one receiver compartment. Protein expression in the receiver droplet is shown. Thick line is the mean of a simulation of 1000 traces, the shaded area indicates the standard deviation. **a**, assuming only a variation in the permeability of the membrane between sender and receiver with a $CV_p = 0.2$. **b**, assuming only a variation in the protein expression rate in the receiver ($CV_a = 0.2$). **c**, Varying both permeability and expression rate ($CV_p = CV_a = 0.2$). **d**, **e**, Varying both permeability and expression rate ($CV_p = CV_a = 0.1$) with a fixed threshold for membrane pore formation (**d**) or a variable waiting time for pore formation (**e**), where we set $CV_{wait} = 2$. **f**, Scheme summarizing the main parameters of the model: α_0 and α_1 the leak and induced expression of pores y , with an induction threshold K_S depending on arabinose x , β_0 and β_1 the leak and pore-mediated permeability of the bilayer to arabinose x , where the pores y incorporate with a dissociation constant K_A .

Supplementary Figure 14



Supplementary Fig. 14 – Simulation of the four-receiver system. **a**, Simulation corresponding to the experiment's conditions (Hill exponent $n=1$ and initial signal is in 8.3-fold excess over the threshold). **b**, Simulation at a higher Hill exponent of the arabinose induction $n=5$ and ten-fold excess of initial signal over the threshold (*i.e.*, $[S]_0/K_A = 10$). **c**, Coefficient of variation for $n=1$ as a function of time (the numbers indicated are $[S]_0/K_A$ in the sender). **d**, $CV(t)$ for $n=5$. **e-h**, Simulations corresponding for **a-d** for a system with four identical copies of a single receiver droplet (receiver dynamics without coupling of the droplets via the sender droplet, but with the same starting and end concentrations of the inducer molecules).

Supplementary Tables

Supplementary Table 1 - Translocation experimental conditions

Chemical	C in sender	Read-out	Environment	Final FI
Guanine	4 mM	24-2-guanine RNA (DFHBI fluorescence)	<i>in vitro</i>	16 h
Arabinose	100 mM	pBAD33-dsRedmut (RFP)	<i>in vivo</i>	13 h
Rhamnose	40 mg/mL	pCK301 (GFP)	<i>in vivo</i>	22 h
DAPG	250 μ M	pPhIF-P2 + 1 mM IPTG (YFP)	<i>in vivo</i>	20 h
DFHBI	4 μ M	24-2 Spinach RNA (DFHBI fluorescence)	<i>in vitro</i>	16 h
C6-HSL	5 μ M	pSB1A3-pLacO-T9002-LVA (GFP)	cell-extract	16 h
IPTG/lactose	4.5 mM	pSB1A3-pLacO-GFP (GFP)	cell-extract	5 h
Tc/aTc	2 μ M	pSB1A3-l13521 (RFP)	cell-extract	4 h

Supplementary Table 2 - Chem3D calculations

Chemical	Molecular weight (g.mol ⁻¹)	Connolly excluded volume (Å ³)	Radius (Å)	H-bond acceptors	H-bond donors	logP
aTc	462.9000	N.C.	N.C.	8	5	1.0686
arabinose	150.1300	118.083	3.04	5	4	-1.8245
C6-HSL	213.2330	186.1240	3.54	2	1	1.9708
DAPG	210.1850	150.8570	3.30	5	3	1.3533
DFHBI	252.2200	172.4380	3.45	5	0	1.0888
guanine	151.1300	85.4350	2.73	3	3	-1.4483
IPTG	238.2980	204.4620	3.65	5	4	-0.5786
lactose	342.3000	274.5410	4.03	11	8	-4.4127
rhamnose	164.1600	134.9840	3.18	5	4	-1.3749
Tc	444.4350	N.C.	N.C.	9	6	-0.7617

N.C.: not calculated. Chem3D calculations returned 0.

Supplementary Table 3 - Induction fits

Experiment	α (M ⁻¹)	β	K_d (M)	n	K (M)	$\alpha_{\max}$	t ₂ (min)	t _{mat} (min)	R ²
pLux induction (Supplementary Fig. 9a)	<u>28220</u>	<u>534.7</u>	<u>11.36 10⁻⁹</u>	<u>2.232</u>					1
pLux kinetics (Supplementary Fig. 9b)	28220	140	11.36 10 ⁻⁹	2.232	<u>11.4 10⁻⁹</u>	0.0033	15	7	
pBAD induction (Supplementary Fig. 9c)	<u>60530</u>	<u>756.8</u>	<u>36.4 10⁻³</u>	<u>1.258</u>					0.9986
pBAD kinetics (Supplementary Fig. 9d)	60530	360	36.4 10 ⁻³	1.258	<u>18 10⁻³</u>	0.0033	15	102	

Underlined numbers were fitted in the experiments shown in the corresponding figures.

Supplementary Table 4 - Permeability fits

Experiment	α (M ⁻¹)	β	k_{prod} (M.s ⁻¹)	k_p (M ⁻¹ .s ⁻¹)	k_m (s ⁻¹)	D (m ² .s ⁻¹)	P (m.s ⁻¹)	V (L)
guanine permeability (Supp. Fig. 6a)	<u>5.0.10⁸</u>	70	5.75.10 ⁻¹⁰	5.5	8.25.10 ⁻⁶	1.2.10 ⁻⁹	<u>8.10⁻¹⁰</u>	1.3.10 ⁻⁸
arabinose permeability (Supp. Fig. 6b)	<u>1000</u>	-10	N.A.	N.A.	N.A.	9.0.10 ⁻¹⁰	<u>5.10⁻⁸</u>	6.10 ⁻⁹
DFHBI permeability (Supp. Fig. 6c)	<u>9.4.10⁷</u>	-30	<u>9.83.10⁻¹⁰</u>	8.17.10 ⁴	0.105	9.5.10 ⁻¹⁰	<u>> 9.10⁻⁶</u>	1.0.10 ⁻⁸
C6-HSL permeability (Supp. Fig. 6d)	<u>1.9.10¹⁵</u>	0	N.A.	N.A.	N.A.	7.7.10 ⁻¹⁰	<u>> 2.10⁻⁶</u>	1.0.10 ⁻⁸

N.A.: not applicable. Underlined numbers were fitted in the experiments shown in the corresponding figures.

Supplementary Table 5 - DNA sequences

For all double-stranded sequences, the non-template, (+), coding strand is given. Underlined sequences are inverted. [pSB1A3-AD009](#), [pSB1A3-AD010](#) and [pSB1A3-AD011](#) are available on [Addgene](#).

DNA	Sequence
24-2 ds from Paige <i>et al.</i> ³⁰ promoter aptamer	GGAATGATAATACGACTCACTATAGGGGACGCGACCGAAATGGTGAAGGACGGGTCCA GTGCTTCGGCACTGTTGAGTAGAGTGTGAGCTCCGTAAGTGGTCGCGTC
24-2-guanine ds from Paige <i>et al.</i> ²⁹	GGAATGATAATACGACTCACTATAGGG GACGCGACTGAATGAAATGGTGAAGGACGGGTCCAGATAATCGCGTGGATATGGCAGC CAAGTTTCTACCGGGCACCCTAAATGTCCGACTCTTGTGAGTAGAGTGTGAGCTCCGT AACTAGTCGCGTC
C3-A	TATTACGGAGCTCACACT
C3 ds non-template complete strand	AGTGTGAGCTCCGTAAATACGACTCACTATAGGGGACTCCAGTTACGGAGCTCACACTCT ACTCAACAGTGCCGAAGCACTGGACCCGTCTTCACCATTTCCGGTCGCGTCTAT
C3 ds template incomplete strand	ATAGACGCGACCGAAATGGTGAAGGACGGGTCCAGTGCTTCGGCACTGTTGAGTAGAG TGTGAGCTCCGTAAGTGGAGTCCCCATAGTGAGTTCG
MG-C3 non-template	AGCACCGTAATACGACTCACTATAGGGATCCCGACTGGCGAGAGCCAGGTAACGAATG GATCCAGTACAT
MG-C3 template	ATGTAAGTGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCATAGTGAGTTCG
Atto655-DNA	Atto655- TTGATGTAT
pPhIF-P2 pTac ribozyme RBS PhIF terminator pPhIF YFP from Nielsen <i>et al.</i> ⁷ plasmid map: https://benchling.com/s/seq-XFd0xQ4JrTRpudYM WtVL	TGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGCTCACAATTAGCG GTCAACGCATGTGCTTTGCGTTCTGATGAGACAGTGATGTGAAACCGCCTCTACAAAT AATTTTGTAAAGGAGCTATGGACTATGTTTGAAAGGCTGAAATACTAGATGACACGTAC CCCGAGCCGTAGCAGCATTGGTAGCCTGCGTAGTCCGCATACCCATAAAGCAATTCTGA CCAGCACCATTTGAAATCCTGAAAGAATGTGGTTATAGCGGTCTGAGCATTGAAAGCGTT GCACGTCGTGCCGGTGCAAGCAAACCGACCATTTATCGTTGGTGGACCAATAAAGCAG CACTGATTGCCGAAGTGATGAAAATGAAAGCGAAGAGGTGCGTAAATTTCCGGATCTG GGTAGCTTTAAAGCCGATCTGGATTTTCTGCTGCGTAATCTGTGAAAGTTTGCGGTGAA ACCATTGTGGTGAAGCATTTGTTGTGTTATTGCAAGACACAGCTGGACCCGCAAC CCTGACCCAGCTGAAAGATCAGTTTATGGAACGTCGTCGTGAGATGCCGAAAAAACTGG TTGAAATGCCATTAGCAATGGTGAAGTCCGAAAGATACCAATCGTGAAGTCTGCTG GATATGATTTTGGTTTGTGTTGATCGCCTGCTGACCGAACAGCTGACCGTTGAACAG GATATTGAAGAATTTACCTTCCTGCTGATTAATGGTGTGTTGTCGGGTACACAGCGTTAA GGAAACACAGAAAAAGCCCGCACCTGACAGTGCGGGCTTTTTTTTTTCGACCAAAGGTA CTCGACGTACGGTGGAACTCTGATTCTGTTACCAATTGACATGATACGAAACGTACCGTATC GTAAAGGTAGCTGTCAACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACA GCCTCTACAAATAATTTTGTAAATACTAGAGAAAGAGGGGAAATACTAGATGTGAGCA AGGGCGAGGAGCTGTTACCGGGGTGGTGGCCATCCTGGTCGAGCTGGACGGCGACG TAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA AGCTGACCCTGAAGTTCATCTGCACACAGGCAAGCTGCCCGTGCCCTGGCCACCCCT CGTGACCACCTTCGGCTACGGCCTGCAATGCTTCGCCCGCTACCCCGACCATGAAG CTGCACGACTTCTCAAGTCCGCCATGCCGGAAGGCTACGTCCAGGAGCGCACCATCTT CTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGG GGCACAAGCTGGAGTACAATAACAGCCACAACGTCTATATCATGGCCGACAAGCAG AAGAACGGCATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGACGGCAGCGTGC AGCTCGCCGACCACTACCAGCAGAACACCCCAATCGGCGACGGCCCCGTGCTGCTGCC CGACAACCACTACCTTAGCTACCAAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGC GCTACATGGTCTGCTGGAGTTCTGACCGCCGCGGGATCACTCTCGGCTGAGGACG AGCTGTACAAGTAAGTCTGGTACCAAAATTCAGAAAAAGAGCCCTCCCGAAAGGGGGCC TTTTTTCGTTTTTGGTCCAATGGCGGCGGCCATCGAATGGCGCAAAACCTTTTCGCGGTA TGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATATGAAACAGTAAC GTTATACGATGTGCGAGAGTATGCCGGTGTCTTATCAGACCGTTTCCCGCGTGGTGA ACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGCGCATGGCGG

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pCK301 pRhaBAD sfGFP from Kelly <i>et al.</i>⁶ plasmid map: https://benchling.com/s/seq-4YmBvKy6NsJuUAQI uTtt	CCACAATTGAGCAAATTGTGAACATCATCACGTTTCATCTTTCCCTGGTTGCCAATGGCCC ATTTTCTGTGAGTAACGAGAAGGTCGCGTATTCAGGCGCTTTTTCAGACTGGTCGTAATG AAACGGTTTCCCTCTAGAAATAATTTGTTAACTTTTAGGAGGTAAACATATGCGTAAA GGCGAAGAGCTGTTCACTGGTGTGCTCCCTATTCTGGTGGAACTGGATGGTGATGTCAA CGGTGATAAGTTTCCGTGCGTGCGGAGGGTGAAGGTGACGCAACTAATGGTAACTGA CGCTGAAGTTCATCTGTACTACTGGTAACTGCCGGTACCTTGCCGACTCTGGTAACG ACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGA CTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCTTTAAGG ATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAAC CGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCT GGAATACAATTTTAAACAGCCACAATGTTTATCATCCGCGGATAAACAACAAAAATGGCAT TAAAGCGAATTTTAAATTTCCGCCACAACGTGGAGGATGGCAGCTGCAGCTGGCTGATC ACTACCAGCAAAACACTCCAATCGGTGATGGTCTGTTCTGCTGCCAGACAATCACTAT CTGAGCACGCAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCT GCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAAGTGTACAAAGGC TCCGGCTCCGGCTCCACCATCACCATCACCATTGATAACTCGAGCCCCAAGGGCGAC

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pSB1A3-pLacO-GFP pLacO-1 RBS GFPmut3 (BBa_E0040) terminator plasmid map: https://benchling.com/s/seq-6XyH5h3vss0N7I0ly cL	CTGATAAATGTGAGCGGATAACATTGACATTGTGAGCGGATAACAAGATACTGAGCACCT TATGGACTATGTTTTCACATACGAGGGGGATTAGATGCGTAAAGGAGAAGAACTTTTCAC TGGAGTTGTCCCAATTCTTGTGAATTAGATGGTGATGTTAATGGGCACAAATTTCTGT CAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACCTACCTTAAATTTATTGCA CTACTGGA AAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTTCGGTTATGGTGTT AATGCTTTGCGAGATACCCAGATCATATGAACAGCATGACTTTTTCAAGAGTGCCATGC CCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACATAAGACAC GTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTAAATAGAATCGAGTTAAAGGTATTG ATTTTAAAGAAGATGGAACATTCTTGGACACAAATTGGAATACAACATAACTCACACAA TGTATACATCATGGCAGACAAAACAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACA CAACATTGAAGATGGAAGCGTTCACTAGCAGACCATTATCAACAAAATACTCCAATTGG CGATGGCCCTGTCTTTTACCAGACAACCATACCTGTCCACACAATCTGCCCTTTCGAA AGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAAACAGCTGCTGGGA TTACACATGGCATGGATGAACTATACAAATAAGGAAAACACAGAAAAAGCCCGCACCTG ACAGTGCGGGCTTTTTTTTTTCGACCAAAGGGGCTTTTTTGTATTCTGCAGTCCGGCAA AAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTAAACCGAAAAGATTACTTCGCG TTATGCAGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTGGCTGCGGCGAGC GGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACCGAG GAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAGAACGGCCGCTT GCTGGCGTTTTTCCACAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAA GTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTC TCCCTTCGGGAAGCGTGCGCTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTG TAGGTCGTTCTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTACGCCCGACCGCT GCGCCTTATCCGGTAACATATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGC GCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAACA AACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTGCAAGCAGCAGATTACGCGCAGAAAAA AAGGATCTCAAGAAGATCCTTTTGATCTTTTACGCGGTCTGACGCTACGTTGAACGAA AACTACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTT TTAAATTA AAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACA GTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCTGTTTATCCA TAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGC CCCAGTGCTGCAATGATACCGCGAGACCCACGCTACCCGGCTCCAGATTTATCAGCAAT AAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCCCTCC ATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCCGCCAGTTAATAGTTTG CGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCTGTTGGTATGGC TTCATTACAGCTCCGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCA AAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTGAGAAGTAAGTTGGCCGCAATG TTACTCATGTTTATGGCAGCACTGCATAATTTCTTACTGTGATGCCATCCGTAAGA TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGA CCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTT AAAAGTGCTCATCATTGGA AACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGC TGTTGAGATCCAGTTTCGATATAACCCACTCGTGACCCCACTGATCTTCAGCATCTTTTA CTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGA ATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCTTTTCAATATTATTGAAGCA TTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTA TTATCATGACATTAACCTATAAAAAATAGGCGTATCACGAGGCAGAAATTCAGATAAAAAAA ATCCTTAGCTTTTCGCTAAGGATGATTCTGGAATTCATT
pSB1A3-I13521 pLTetO-1 (BBa_R0040) RBS mRFP (BBa_E1010) terminator	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACCTACTAG AGAAAGAGGAGAAA TACTAGATGGCTTCTCCGAAGACGTTATCAAAGAGTTTCATGCGT TTCAAAGTTTCGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGG TGAAGGTCGTCGTCAGGAAGGTACCCGACCGCTAAACTGAAAGTTACCAAAGGTGGTC CGCTGCCGTTTCGTTGGGACATCTGTCCCCGAGTTCCAGTACGGTTCCAAAGCTTAC GTAAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCTTCCCGGAAGGTTTCAA

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Supplementary Videos

- **Supplementary Video 1:** This video shows the time lapse of **Fig. 1b-c**. The first image shows the droplets containing α -HL (labeled with a red dye), and the following images show the time lapse of guanine-induced fluorescence into the network. The diffusing guanine binds a bifunctional aptamer (24-2-guanine), allowing for a binding pocket for DFHBI to form on this aptamer. The subsequent binding of DFHBI gives rise to fluorescence, scaled in blue here. Time is given in hh:mm.
- **Supplementary Video 2:** Time lapse of the diffusion of DFHBI into a network of receivers containing a Spinach RNA transcription. This exemplifies the behavior of a non-specifically diffusing chemical, in contrast to pore-mediated diffusing chemical such as guanine in **Supplementary Video 1**. The first frame indicates the sender S, and the path of α -HL pore-containing receivers, visible in black by contrast to the red labeled receivers that do not contain pores. The DFHBI diffuses through the entire network, irrespective of the presence or absence of pores in the receivers.
- **Supplementary Video 3:** This video shows 6 arrays containing the pulse propagation circuit, with different numbers of receivers. The sender droplet is labeled with a red dye, and the receivers show fluorescence in the green channel. The array shown in **Fig. 3b** is indicated by an arrow.
- **Supplementary Videos 4 and 5:** These videos show 2D networks containing the pulse generator. Sender droplet is indicated by "S". **Supplementary Video 5** shows specifically a time-lapse of **Fig. 3g**.
- **Supplementary Video 6:** This video shows a differentiating network of one sender (indicated by S) and 4 receivers containing the circuit described in **Fig. 4b**, left panel.

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