SI Text

S1. Receiver Circuit Design and Characterization

Engineering of the microbial consensus consortium (MCC) began with the construction and characterization of two “receiver” circuits, designed to express a target gene in response to a specific acyl-HSL. The MCC requires that p(rhl) responds specifically to RhlR/C4HSL and that p(las) responds specifically to LasR/3OC12HSL. Therefore, we constructed the receivers shown in SI Fig. 6*a* and characterized the dosage responses of each to both C4HSL and 3OC12HSL (SI Fig. 6*b*).

To study circuit dosage responses in liquid phase, starter cultures of *Escherichia coli* JM2.300 cells [F^- *lacI*22 λ^- $e14^-$ *rpsL*135(StrR) *thi*-1] harboring the different receiver plasmids were grown to an OD of <0.3 in M9 medium (2 mM $MgSO_4$/0.2% casamino acids/0.5% glycerol/300 μM thiamine) with 50 μg ml^{-1} kanamycin at 37°C in a shaking incubator. The cultures were diluted to an effective OD of 0.0006 in wells of a 24-well plate containing 1 ml of M9 medium with 50 μg ml^{-1} kanamycin and acyl-homoserine lactone (acyl-HSL) as denoted [C4HSL from Sigma (St. Louis, MO) and 3OC12HSL from Cayman Chemical (Ann Arbor, MI)], and the plate was shaken at 37°C. When cultures reached an OD of ≈0.3, fluorescence measurements were taken on a Beckman Coulter (Fullerton, CA) Altra flow cytometer equipped with a 488-nm argon excitation laser and a 515- to 545-nm emission filter. Median fluorescence values were converted to equivalent fluorescein molecule counts by using SPHERO Rainbow Calibration Particles (Spherotech RCP-30-5A; Spherotech, Lake Forest, IL) that were measured during each session.

S2. RATE-EQUATION BASED MODEL

S2.A. Model development and specifications

2. Rate-Equation-Based Model

2a. Model development and specifications. Ordinary differential equations were used to model the MCC. In most well characterized quorum sensing systems from Gram-negative bacteria, acyl-HSL forms a complex with the R protein and binds and activates the quorum sensing controlled (qsc) promoter (1, 2). We assume that in the MCC, the R protein concentration is high and effectively constant and that the equilibrium dependence of promoters bound by the acyl-HSL/R–protein complex can be approximated as shown in Eq. **1**. In this equation, s_A is the cognate signal molecule concentration, D denotes the concentration of unbound promoter, and $D_{[rsA]2}$ is the concentration of bound promoter. The form of Eq. **1** was based on previous experiments that show a first-order dependence of $D_{[rsA]2}/D$ on the R protein concentration when acyl-HSL is in excess (1). This first-order relationship is assumed to hold when R protein is instead in excess. Eq. **2** assumes that the noncognate (i.e., crosstalk) signal s_B can similarly form a complex with the R protein and bind the promoter. Eq. **3** is based on conservation of mass, as the total number of promoters in the system D_{total} is assumed to be constant.

$$D_{[rs_A]_2} = k_1 s_A D \quad \textbf{[1]}$$

$$D_{[rs_B]_2} = k_2 s_B D \quad \textbf{[2]}$$

$$D_{total} = D + D_{[rs_A]_2} + D_{[rs_B]_2} \quad \textbf{[3]}$$

Promoters in each state (D, $D_{[rsA]2}$, and $D_{[rsB]2}$) express target genes at different rates, eventually giving rise to production of protein *i* as shown in Reactions 4–6 below. We assume that the binding reactions captured by Eqs. **1**-**3** are effectively in equilibrium with respect to the slower protein production reactions.

The total rate of production of *i* shown below was derived by substituting the expressions for the different promoter states in Eqs. **1–3** into the differential equation capturing Reactions 4–7. In the resulting differential equation below, $\alpha_A = k_1$, $\alpha_{AB} = k_2$, $\eta_A = k_4 D_{total}$, $\eta_{AB} = k_5 D_{total}$, and $\eta_{A0} = k_3 D_{total}$.

$$\frac{\partial i}{\partial t} = \frac{\eta_{A0} + \eta_A \alpha_A s_A + \eta_{AB} \alpha_{AB} s_B}{1 + \alpha_A s_A + \alpha_{AB} s_B} - d_i i \quad \textbf{[8]}$$

Using the above expression for protein production, the full MCC was modeled (Eqs. **9–12**). The variable i_A is the I protein concentration in Circuit A, and s_B is the concentration of the AHL signal produced by this I protein. Likewise, i_B is the other I protein produced by Circuit B, which synthesizes the acyl-HSL signal s_A. Steady-state approximations were made for protein–protein and protein–DNA interactions, and R protein levels were assumed to be effectively constant. Saturation of acyl-HSL production from the I proteins was modeled with a Hill equation. The fluorescent reporters are not explicitly modeled, because they are assumed to be scalar multiples of the corresponding I protein concentrations in steady state.

$$\frac{\partial i_A}{\partial t} = \frac{\eta_{A0} + \eta_A \alpha_A s_A + \eta_{AB} \alpha_{AB} s_B}{1 + \alpha_A s_A + \alpha_{AB} s_B} - d_{iA} i_A \quad \textbf{[9]}$$

$$\frac{\partial s_B}{\partial t} = \frac{\rho_A \varphi_A i_A}{\chi_A + i_A} - d_{sB} s_B \quad \textbf{[10]}$$

$$\frac{\partial i_B}{\partial t} = \frac{\eta_{B0} + \eta_B \alpha_B s_B + \eta_{BA} \alpha_{BA} s_A}{1 + \alpha_B s_B + \alpha_{BA} s_A} - d_{iB} i_B \quad \textbf{[11]}$$

$$\frac{\partial s_A}{\partial t} = \frac{\rho_B \varphi_B i_B}{\chi_B + i_B} - d_{sA} s_A \quad \textbf{[12]}$$

Eqs. **9** and **10** represent Circuit A, while Eqs.**11** and **12** represent Circuit B. In Circuit A, η_{A0} is the rate of basal expression of the target gene promoter, η_A is rate of expression due to activation by the cognate, or consensus, signal s_A, and η_{AB} is the rate of "crosstalk" expression due to activation by s_B. The parameter α_A is the inverse of the concentration of s_A that gives rise to promoter response $i_A = (\eta_{A0} + \eta_A)/2$ in the absence of s_B. Similarly, α_B is the inverse of the concentration of s_B that gives rise to promoter

response $i_A = (\eta_{A0} + \eta_{AB})/2$ in the absence of s_A. At a given Circuit A population ρ_A, the maximum rate of s_B synthesis is given by ϕ_A, and χ_A is the concentration of I protein (i_A) that gives rise to half-maximal s_B synthesis. Parameters are defined similarly for Circuit B, with A and B subscripts interchanged. All d parameters are decay rates.

This system was rescaled and nondimensionalized. Variables were rescaled as follows:

$$S_A = s_A \alpha_A \quad \textbf{[13]}$$
$$S_B = s_B \alpha_B \quad \textbf{[14]}$$
$$I_A = i_A \left(d_{iA}/\eta_A\right) \quad \textbf{[15]}$$
$$I_B = i_B \left(d_{iB}/\eta_B\right) \quad \textbf{[16]}$$
$$\tau = t d_{iA} \quad \textbf{[17]}$$

The following parameter substitutions (shown below) were then made. P_{max} is defined as the maximum sustainable population in the growth environment.

$K_{AB} = \frac{\alpha_{AB}}{\alpha_B}$	$K_{BA} = \frac{\alpha_{BA}}{\alpha_A}$	$\gamma_{A0} = \frac{\eta_{A0}}{\eta_A}$	$\gamma_{B0} = \frac{\eta_{B0}}{\eta_B}$	$\gamma_{BA} = \frac{\eta_{BA}}{\eta_B}$
$\gamma_{AB} = \frac{\eta_{AB}}{\eta_A}$	$\theta_A = \frac{\varphi_A \alpha_B P_{max}}{d_{iA}}$	$\theta_B = \frac{\varphi_B \alpha_A P_{max}}{d_{iA}}$	$\lambda_{SA} = \frac{d_{sA}}{d_{iA}}$	$\lambda_{SB} = \frac{d_{sB}}{d_{iA}}$
$\lambda_{IB} = \frac{d_{iB}}{d_{iA}}$	$p_A = \frac{\rho_A}{P_{max}}$	$p_B = \frac{\rho_B}{P_{max}}$	$R_A = \frac{d_{iA}\chi_A}{\eta_A}$	$R_B = \frac{d_{iB}\chi_B}{\eta_B}$

This results in the following nondimensionalized system:

$$\frac{\partial I_A}{\partial \tau} = \frac{\gamma_{A0} + S_A + \gamma_{AB} K_{AB} S_B}{1 + S_A + K_{AB} S_B} - I_A \quad \textbf{[18]}$$

$$\frac{\partial S_B}{\partial \tau} = \frac{\theta_A p_A I_A}{R_A + I_A} - \lambda_{SB} S_B \quad \textbf{[19]}$$

$$\frac{\partial I_B}{\partial \tau} = \lambda_{IB}\left(\frac{\gamma_{B0} + S_B + \gamma_{BA} K_{BA} S_A}{1 + S_B + K_{BA} S_A} - I_B\right) \quad \textbf{[20]}$$

$$\frac{\partial S_A}{\partial \tau} = \frac{\theta_B p_B I_B}{R_B + I_B} - \lambda_{SA} S_A \quad \textbf{[21]}$$

We first analyzed the steady-state behavior of circuits grown in isolation. Each circuit can potentially activate in isolation due to crosstalk. The steady-state behavior of a single circuit in isolation is represented by Eqs. **22** and **23**.

$$\frac{\partial I_A}{\partial \tau} = \frac{\gamma_{A0} + \gamma_{AB} K_{AB} S_B}{1 + K_{AB} S_B} - I_A = 0 \quad \textbf{[22]}$$

$$\frac{\partial S_B}{\partial \tau} = \frac{\theta_A p_A I_A}{R_A + I_A} - \lambda_{SB} S_B = 0 \quad \textbf{[23]}$$

Steady state solutions are given in Eqs. **24–27**:

$$\left\{ I_A = \frac{\sqrt{\xi^2 + z} + \xi}{2(p_A \theta_A K_{AB} + \lambda_{SB})}, \quad S_B = \frac{p_A \theta_A \left(\xi + \sqrt{\xi^2 + z}\right)}{\lambda_{SB}\left(2R_A (p_A \theta_A K_{AB} + \lambda_{SB}) + \xi + \sqrt{\xi^2 + z}\right)} \right\} \quad \textbf{[24]}$$

$$\left\{ I_A = \frac{-\sqrt{\xi^2 + z} + \xi}{2(p_A \theta_A K_{AB} + \lambda_{SB})}, \quad S_B = \frac{p_A \theta_A \left(\xi - \sqrt{\xi^2 + z}\right)}{\lambda_{SB}\left(2R_A (p_A \theta_A K_{AB} + \lambda_{SB}) + \xi - \sqrt{\xi^2 + z}\right)} \right\} \quad \textbf{[25]}$$

$$\xi = p_A \theta_A \gamma_{AB} K_{AB} - \lambda_{SB} R_A + \lambda_{SB} \gamma_{A0} \quad \textbf{[26]}$$

$$z = 4\gamma_{A0} \lambda_{SB} R_A (\lambda_{SB} + K_{AB} \theta_A p_A) \quad \textbf{[27]}$$

Because $z \geq 0$, only the first solution has a positive value for I_A. The maximum gene expression levels in Circuits A and B due to isolation activation are γ_{AB} and γ_{BA}, respectively. When Circuit A or Circuit B is grown in isolation, target gene expression will be halfway between the minimum and maximum (the midpoint between γ_{A0} and γ_{AB} or γ_{B0} and γ_{BA}) at a population density of Ω_A or Ω_B, respectively. The population of Circuit A that gives rise to the expression level $I_A = (\gamma_{A0} + \gamma_{AB})/2$ is:

$$\Omega_A = \frac{\lambda_{SB}(\gamma_{AB} + \gamma_{A0} + 2R_A)}{K_{AB} \theta_A (\gamma_{AB} + \gamma_{A0})} \quad \textbf{[28]}$$

In examining the full system when Circuits A and B are grown together, it is useful to understand when both circuits produce target gene responses that are distinguishable from the maximum possible expression of the circuits in isolation. Thus, we determined the populations Ψ_A and Ψ_B representative of a situation in which both Circuits A and B are halfway between maximum possible expression in isolation ($I_A = \gamma_{AB}$ or $I_B = \gamma_{BA}$) and maximum possible expression overall ($I_A = 1$ and $I_B = 1$), defined as $I_A = (\gamma_{AB} + 1)/2$ and $I_B = (\gamma_{BA} + 1)/2$ at steady state. Eqs. **19** and **21** can be rewritten to give expressions for S_B and S_A in terms of p_A and p_B, respectively. Substituting these expressions for S_A and S_B

into Eqs. **18** and **20** and plugging in $I_A = (\gamma_{AB} + 1)/2$ and $I_B = (\gamma_{BA} + 1)/2$ results in a nonhomogeneous set of equations that are linear with respect to p. The solution for this set of populations (Eqs. **29–32**) exists and is unique, and the populations are positive provided $\gamma_{AB} < 1$, $\gamma_{BA} < 1$, $K_{AB}K_{BA} < 1$, $\gamma_{A0} < (\gamma_{AB} + 1)/2$, and $\gamma_{B0} < (\gamma_{BA} + 1)/2$. Note that although the values may correspond to populations that are too high to be sustained in practice, we would still like to know how theoretically "close" we are to getting proper consensus response. For this reason, we do not constrain the populations.

$$\Psi_A = \frac{\lambda_{SB}(2R_A + 1 + \gamma_{AB})(FK_{BA} + G)}{\theta_A(1+\gamma_{AB})(1-\gamma_{AB})(1-\gamma_{BA})(1-K_{AB}K_{BA})} \quad \textbf{[29]}$$

$$\Psi_B = \frac{\lambda_{SA}(2R_B + 1 + \gamma_{BA})(GK_{AB} + F)}{\theta_B(1+\gamma_{BA})(1-\gamma_{AB})(1-\gamma_{BA})(1-K_{AB}K_{BA})} \quad \textbf{[30]}$$

$$F = (1-\gamma_{BA})(1+\gamma_{AB}-2\gamma_{A0}) \quad \textbf{[31]}$$

$$G = (1-\gamma_{AB})(1+\gamma_{BA}-2\gamma_{B0}) \quad \textbf{[32]}$$

Note that the populations Ψ_A and Ψ_B do not always define a true threshold that p_A and p_B must cross to ensure expression levels $I_A \geq (1 + \gamma_{AB})/2$ and $I_B \geq (1 + \gamma_{BA})/2$. For example, for some parameter sets it may be possible to raise p_A but lower p_B below Ψ_B and still attain expression levels such that I_A is at least $(1 + \gamma_{AB})/2$ and I_B is at least $(1 + \gamma_{BA})/2$. In such a case, Ψ_B would not define a true threshold that p_B must cross to reach these levels of I_A and I_B. To gain an understanding of when Ψ_A and Ψ_B are thresholds, we used Matlab's ode15s to numerically find the steady state values of I_A and I_B for random parameter sets over a range of different p_A and p_B values in the vicinity of (Ψ_A,Ψ_B). Based on the results (SI Fig. 7), the most important parameters in determining whether Ψ_A and Ψ_B are thresholds are R_A and R_B, respectively. Provided R_A and R_B are not too large, Ψ_A and Ψ_B are true thresholds. However, even for scenarios in which Ψ_A and Ψ_B do not define true thresholds, (Ψ_A, Ψ_B) still defines a unique, representative point in (p_A, p_B) space for which consensus behavior occurs.

SI Table 1 shows how different modifications affect system parameters and the ratio Ψ_A/Ω_A. For example, modifying pH to alter acyl-HSL decay will change the λ_s values. However, these λ_s values cancel out in the ratios, so modifying pH does not change the

ratio of critical populations. Similarly, the θ values cancel out in the ratio. Thus, we examined the effects of R_A and K_{AB} on Ψ_A/Ω_A. SI Fig. 8 depicts the importance of building the system from receivers with low K_{AB} values. Although K_{AB} could be lowered by reducing the R protein expression level, this would also raise K_{BA}, thus having the adverse affect of raising Ψ_B/Ω_B. However, even if K_{BA} is high, Ψ_A/Ω_A can be reduced by increasing R_A through changes in the I protein RBS or addition of a degradation tail. This constitutes an advantage of the positive feedback design over a design in which both I proteins are constitutively expressed. In the case of constitutive expression ($\chi = R = 0$), we lose the ability to mitigate the effect of potentially high K_{AB} values through modification of the I protein expression and degradation rates.

Results from Receiver experiments (*Receiver Circuit Design and Characterization*) and the rate-equation based model (this section) led to construction of MCC plasmids mapped in Fig. 1a and detailed in SI Fig. 9.

2b. Validation of model in liquid culture. We validated the model-informed design choices by testing cells containing Circuits A and B in liquid culture. Cells containing each circuit were grown both in isolation and in communication with one another. As per details provided in *Materials and Methods*, isolated circuits were unable to produce a significant response, but when cells containing the two circuits were grown to sufficient density in separate chambers that allowed passage of small molecules between the two populations, responses from both were >50-fold greater than the responses of the circuits in isolation (Fig. 2*B* and SI Fig. 10).

3. Solid-Phase Imaging Equipment and Settings

Axiovision 4.5 software was used to capture mosaic images every 30 min on a Zeiss (Thornwood, NY) Axiovert 200M microscope equipped with an AxioCam MR CCD camera. Images were captured by using a ×2.5 objective and a GFP filter with 470/40 excitation and 525/50 emission, and the exposure time for all mosaic tiles was 50 ms. Image acquisition bit depth was 12, and each mosaic tile was stored as a 16-bit grayscale

image. Each pixel represents an area of 9.8039×9.8039 μm. The number of tiles per mosaic and pixels per tile for the experiment in Fig. 3 *A* and *B* were as follows: 8×5 tile mosaic with 282×188 pixels in each tile. Tiles overlapped 10% to form the full mosaic. Control experiments were performed in which two rectangular agarose slices containing Circuit A were placed in contact with one another, and two slices containing Circuit B cells were placed in contact with one another. In both of these control experiments, no gradient formed at the interface between the adjacent agarose slices.

Matlab was used to perform background correction and image normalization. Background correction was implemented by first selecting a set of tiles within the agarose regions of the mosaic from the initial image (time = 0 h). A single, representative "background" tile was created from the median intensities of this set of tiles. This background tile was then subtracted from all tiles over all times. A 51-pixel moving average filter was used to further remove the effects of bias within each tile. A one-dimensional spatial representation for each time point was then created by taking the mean of each column of pixels.

4. Biofilm Experimental Setup

4a. Equipment specifications. An image of the biofilm flow apparatus can be found in SI Fig. 11. The interior of the biofilm flow apparatus was kept sterile during the duration of each experiment. Biofilms were grown in M9 biofilm medium which was not recycled and which was maintained at room temperature. Freshly prepared medium with appropriate antibiotics (50 μg ml^{-1} kanamycin and 20 μg ml^{-1} tetracycline) was placed in the sterile reservoirs every 24 h. Medium was pumped from the reservoirs by a Watson–Marlow peristaltic pump (205U; Watson–Marlow Bredel, Wilmington, MA) with 16-channel capacity. Oxygen-permeable Tygon tubing (ABW00002) carried medium from the reservoirs, through bubble traps which reduced pulsatile action in the flow (Biosurface Technologies, BSTFC34), and through a custom-made heat-strip which prevented bacteria from swimming upstream to the medium reservoirs. Medium then entered the flow chambers (ACCFL0001; Stovall Life Sciences, Greensboro, NC) and

finally exited the flow apparatus into sterile effluent reservoirs. The flow chambers and tubing and medium approaching them were maintained at 30°C in a small custom-built incubated chamber (not shown).

Inoculation of bacteria into the flow chambers was performed with sterile 1-ml syringes directly into the Tygon tubing, ≈3 cm before the flow chambers. Flow chambers were left to incubate coverslip-down for 1 h without flow, and then were incubated coverslip-down for an additional 24 h with flow. They were then incubated coverslip-up with flow for the remainder of experiments and all imaging.

4b. Monoculture dosage experiments. Circuit A monoculture dosage experiments were initiated as described above. To enable identification of all bacteria in the biofilm, an enhanced cyan fluorescence protein (eCFP) expression plasmid, pMP4641 (3), provided a constitutive marker in all Circuit A cells. This plasmid was chosen for its demonstrated retention in *E. coli* cells, even in the absence of antibiotic pressure. Tetracycline was administered with the biofilm medium to maintain the plasmid. However, its degradation properties in this medium are uncharacterized. Thus, retention in the absence of antibiotic was an attractive feature.

After the first 48 total hours of Circuit A incubation, sterile M9 biofilm medium containing the appropriate antibiotics and concentrations of C4HSL (Sigma) was placed into the medium reservoirs. The Circuit A biofilm was then incubated for 18 h with medium containing acyl-HSL before dosage response imaging. This induction time was chosen based upon the determined time points of maximal expression in the solid-phase MCC studies. Dosage response imaging therefore took place after 66 total hours of Circuit A biofilm growth. At this time point and with a constant flowrate of 125 $\mu l/min^{-1}$ (speed setting 1.5 on the Watson–Marlow peristaltic pump), Circuit A biofilms were robust monolayers which provided reproducible imaging data.

Circuit B monoculture dosage experiments were also initiated as described above. Again, to enable identification of all bacteria in the biofilm, the eCYFP expression plasmid

pMP4641 provided a constitutive marker in all Circuit A cells. Circuit B biofilms grow and thicken more quickly than Circuit A biofilms, so Circuit B biofilms were incubated for a total of 24 h before induction with C12HSL (Sigma). The Circuit B biofilm was induced with M9 biofilm medium containing appropriate antibiotics and concentrations of C12HSL for an additional 18 h before imaging. Hence, after 42 total hours of growth, dosage-response imaging took place for Circuit B biofilms. At this time point and with a constant flow rate of 125 $\mu l/min^{-1}$ (speed setting 1.5 on the Watson–Marlow peristaltic pump), Circuit B formed conformal monolayer biofilms which coated the substrate to provide reproducible imaging data.

4c. MCC experiments. In MCC biofilms used for quantitative imaging (Fig. 5), separate cultures of Circuit A and Circuit B cells, all containing the eCFP plasmid pMP4641, were first grown to saturation and then diluted to OD 0.2. These were mixed in a 50/50 ratio immediately before inoculation of the MCC biofilms. Biofilms were incubated after inoculation, coverslip-down without flow, for 1 h. Flow was then resumed at a flow rate of 20 $\mu l/min^{-1}$, (speed setting 0.5 on the Watson–Marlowe peristaltic pump), and flow chambers were left coverslip-down for a total of 24 h. For the remainder of the experiment and for all imaging, flow chambers were left coverslip-up. Images were taken a total of 24, 48, 72, 96, and 120 h after inoculation.

For the four-color images of the MCC biofilm (Fig. 5), cells containing the Circuit A plasmid also contained plasmid pMP4658, which is identical to plasmid pMP4641 but which constitutively expresses enhanced yellow fluorescence protein (eYFP) in place of eCFP (3). Cells containing the Circuit B plasmid also contained pMP4641. The images generated by these biofilms were not used for quantitative analysis because (*i*) the two different fluorophores may interact differently with the cellular environments and (*ii*) differentiation between the potentially overlapping spectra of these four fluorophores with certainty is difficult. These biofilms were otherwise prepared and analyzed by a method identical to that described above for quantitative MCC biofilms.

5. Biofilm Imaging Equipment and Settings

5a. Microscope settings. Images taken for quantitative analysis were all taken with identical settings. All images taken for a given dosage analysis were taken from the same experiment: at the same time and on the same day. In addition, all biofilms described in this paper were grown and imaged at the same time of day, and the microscope and flow apparatus were maintained in the same room with the same lighting and temperature. Controlling these variables enabled reliable, reproducible growth of the biofilms.

Settings for all imaging:

Microscope:	Zeiss 510 upright CLSM
Control software:	Carl Zeiss AIM
Objective:	Zeiss Achroplan 40x/0.8 W
Pixel resolution:	512x512
Data depth:	12 bit
Scan speed:	5–12.8 µs pixel time
Averaging:	2 for all single images, 1 for stacked images

Dosage experiments:

Channel 1: green
Excitation: 488 nm Argon laser, 11%
Dichroic: 488/543
Secondary Beamsplitter: NFT 545 nm
Emission filter: BP 500-530 nm

Pinhole setting: 250 (2.39 Airy)
Gain: 800
Amplifier Offset: -0.048
Amplifier Gain: 1

Channel 2: cyan
Excitation: 458 nm Argon laser, 76%
Dichroic: 458/514
Emission filter: BP 480-520 nm

Pinhole setting: 250 (2.46 Airy)
Gain: 1000
Amplifier Offset: 0.1
Amplifier Gain: 1

MCC experiments:

Channel 1: green
Excitation: 488 nm Argon laser, 11%
Dichroic: 488/543 nm
Secondary Beamsplitter: NFT 545 nm
Emission filter: BP 500-530 nm

Pinhole setting: 250 (2.39 Airy)
Gain: 875
Amplifier Offset: -0.043
Amplifier Gain: 1

Dichroic: 458/514 nm
Emission filter: BP 480-520 nm

Channel 2: cyan
Excitation: 458 nm Argon laser, 80%

Pinhole setting: 250 (2.46 Airy)

Gain: 1000
Amplifier Offset: -0.04
Amplifier Gain: 1

Channel 3: red
Excitation: 543 nm HeNe laser, 80%
Dichroic: 488/543 nm
Secondary Beamsplitter: NFT 545 nm
Emission filter: BP 480-520 nm
Pinhole setting: 250 (2.12 Airy)
Gain: 1000
Amplifier Offset: 0.1
Amplifier Gain: 1

4-Color MCC Stacks:

Channel 1: green
Excitation: 488 nm Argon laser, 11%
Dichroic: 488/543 nm
Secondary Beamsplitter: NFT 545 nm
Emission filter: BP 500-530 nm
Pinhole setting: 104 (0.99 Airy)
Gain: 860
Amplifier Offset: -0.038
Amplifier Gain: 1

Channel 2: cyan
Excitation: 458 nm Argon laser, 80%
Dichroic: 458/514 nm
Emission filter: BP 480-520 nm
Pinhole setting: 102 (1.00 Airy)
Gain: 1000
Amplifier Offset: -0.04
Amplifier Gain: 1

Channel 3: red
Excitation: 543 nm HeNe laser, 80%
Dichroic: 488/543 nm
Secondary Beamsplitter: NFT 545 nm
Emission Filter: LP 650 nm
Amplifier Gain: 1
Pinhole setting: 117 (0.99 Airy)
Gain: 1000
Amplifier Offset: -0.05

Channel 4: yellow
Excitation: 514 nm Argon laser, 80%
Dichroic: 458/514 nm
Emission filter: LP 530 nm
Pinhole setting: 115 (1.00 Airy)
Gain: 1,000
Amplifier Offset: -0.05
Amplifier Gain: 1

5b. Three-dimensional image rendering. The stacked images were captured with settings listed above, at 1-μm spacing. The entire field was captured for each channel at each depth before moving to a new depth. Laser scanning microscope (LSM) files from the stacks were imported directly into Imaris 4.5.2. Throughout the depth, cyan was used to mask the red channel (red pixels without cyan were set to 0) and yellow was used to mask the green channel (green pixels without yellow were set to 0). All channels were then rendered in Imaris as isoforms (lower threshold cutoffs of 100, Gaussian filter diameter of 1.584 μm). Colors were generated by a default full-range linear look up table.

5c. Image processing.
Step 1: The input: The input is always a set of 512×512 pixel RGB TIFF-chunky images exported from LSM files. For each image, RGB colors correspond to detector channels on the microscope ("R" is emission from dsRed, "G" is emission from GFPlva, "B" is emission from eCFP). All images for a given dosage experiment were taken on a single day, 18 h after induction with acyl-HSL. All images for a given day in the MCC experiments were taken at the same time on that day. Each biofilm grew in one "lane" of a flow chamber and at least two lanes were used for each acyl-HSL concentration (dosages, Fig. 4) or for each day (MCC experiments, Fig. 5). Images were directly imported into Matlab.

Step 2: Obtaining information from the eCFP image: Maxima were first extracted from the "B" layer of the RGB image (eCFP emission). The image containing only these maxima was then adjusted to fill the entire spectrum, and the regional maxima were extracted from it. The image containing only these regional maxima was essentially a digital matrix of pixels which were "1" if a "significantly cyan" pixel is present, and "0" if not. A significantly cyan pixel could be assumed to be associated with a cell in the biofilm, because all cells in the biofilm constitutively express eCFP. The total number of

cell-associated pixels in the histogram was counted and assigned to the variable TotalCyan.

Step 3: Using eCFP information to threshold the GFP image. The “G” layer of the TIFF image reports GFPlva expression, or circuit-function-related green fluorescence. From the raw histogram for the “G” image, the top TotalCyan pixels were chosen for inclusion in a new “green” histogram. When DsRed-exp was also present (MCC experiments), the “R” layer of the TIFF image reported DsRed-exp expression. The top TotalCyan pixels were chosen from it for inclusion in a new “red” histogram. Pixels were chosen from the top intensity bin first, then the next intensity bin, and so on, until TotalCyan pixels were incorporated into the new histogram. The new histograms therefore included only cell-related green or red pixels.

Step 4: Generating comparable histograms from all images. All intensity bins in the cell-related fluorescence histograms (“green” and “red”) were divided by TotalCyan such that they represented a percentage of total pixels in the image, rather than a raw total. This enabled quantitative comparison of various images, even when they did not contain the same number of cell-related pixels. We called these the percentage histograms.

Step 5: Intensity weighting. The percentage histograms were retained (only “green” histograms for dosages, and both “green” and “red” for MCC experiments), but also used to generate weighted histograms. Each element (bin) of the percentage histogram was multiplied by the intensity it represented (1–256), yielding a weighted histogram for mean calculations.

Step 6: Averaging over a single concentration or lane. Percentage histograms for images taken of lanes that are induced with the same acyl-HSL concentration (dosages) or on the same day (MCC experiments) were averaged by intensity bin. This resulted in an average intensity histogram for each acyl-HSL concentration or day. The “green” averaged percentage histograms reporting dosage experiment results were displayed in Fig. 4.

Step 7: Mean intensity calculations. The weighted histograms for images taken of lanes that were induced with the same acyl-HSL concentration (dosages) or on the same day (MCC experiments) were averaged by intensity bin. Then, for each concentration or day, the mean of this averaged-weighted histogram was taken. This calculation yielded a mean intensity for each dosage or day, for each fluorophore present. The mean intensity for each color for each day was then plotted, in Figs. 4 *Insets* (dosages) and 5*B* (MCC experiments).

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