Appendix A

Supplementary information for Chapter 2:
Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium
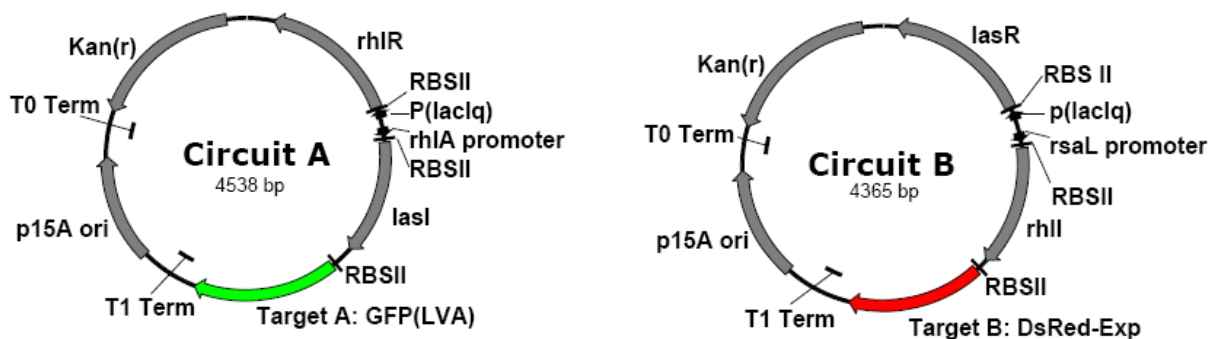
A.1 Plasmids encoding Circuits A and B

Figure A1 Plasmids encoding the MCC. In Circuit A, LasI and Target A [GFP(lva)] are under control of p(rhlA) and RBSII, while in Circuit B, RhlI and Target B (DsRed-exp) are under control of p(rsaL)-RBSII.

A.2 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 the materials and methods section of the paper, 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 over 50-fold greater than the responses of the circuits in isolation (Figure 2.3 and A2).
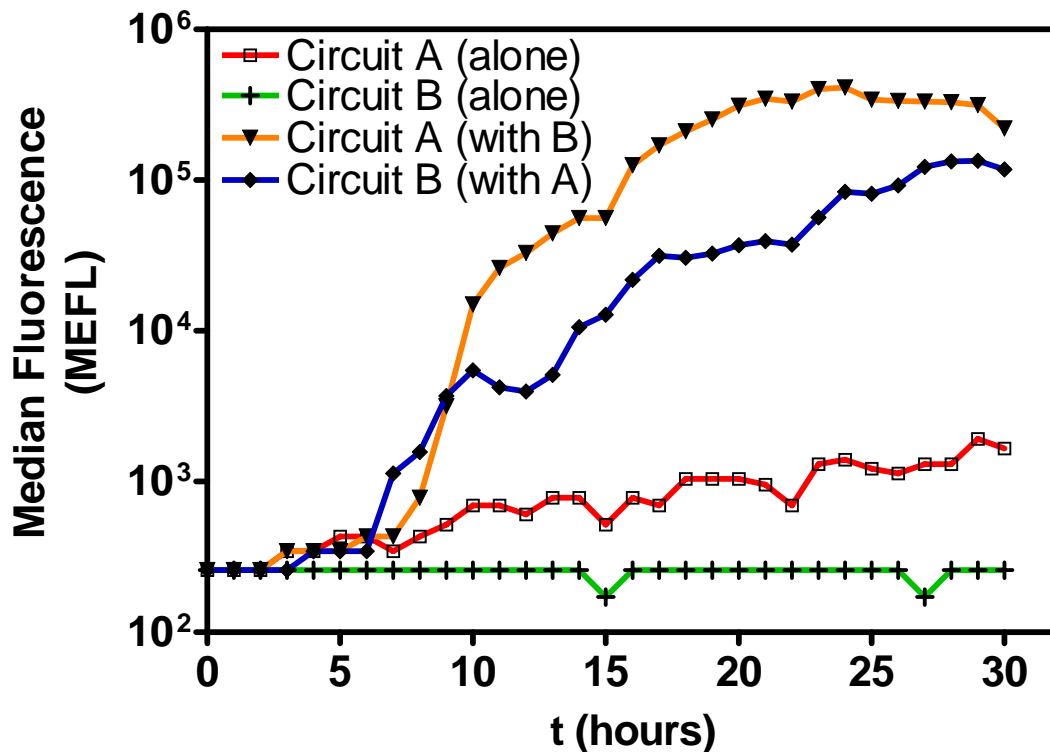

Figure A2 Fluorescence of cells containing Circuits A and B rises with time when the two are grown in communication with one another. Neither cell population fluoresces significantly in the absence of the other population.

A.3   Solid phase imaging equipment and settings

Axiovision 4.5 software was used to capture mosaic images every 30 minutes on a Zeiss Axiovert 200M microscope equipped with an AxioCam MR CCD camera. Images were captured using a 2.5x 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 μm by 9.8039 μm. The number of tiles per mosaic and pixels per tile for the experiment in Figures 2.4A, B were as follows: 8x5 tile mosaic with 282x188 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 hours). 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.

A.4 Biofilm experimental setup, imaging, and image processing

A.4.1 Equipment specifications

An image of the biofilm flow apparatus can be found in Figure A3. 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 μgml^{-1} kanamycin and 20 μgml^{-1} tetracycline) was placed in the sterile reservoirs every 24 hours. Medium was pumped from the reservoirs by a Watson-Marlowe peristaltic pump (205U) 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 (Stovall Life Sciences, Inc., ACCFL0001) and finally exited the flow apparatus into sterile effluent reservoirs. The flow chambers and tubing and medium approaching them were maintained at 30ºC in a small custom-built incubated chamber (not shown).

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

Figure A3 Photograph of the flow apparatus. The entire interior was kept sterile, and flow chambers were maintained at 30ºC in an incubator (not shown).

A.4.2 Monoculture dosage experiments

Circuit A monoculture dosage experiments were initiated as described above. To enable

identification of all bacteria in the biofilm, an eCFP expression plasmid, pMP4641 [68],

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 hours with medium containing acyl-HSL prior to 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 timepoint and with a constant flowrate of 125 μlmin^{-1} (speed setting 1.5 on the Watson-Marlowe 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 eCFP 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 hours prior to 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 hours prior to imaging. Hence, after 42 total hours of growth, dosage response imaging took place for Circuit B biofilms. At this timepoint and with a constant flowrate of 125 μlmin^{-1} (speed setting 1.5 on the Watson-Marlowe peristaltic pump), Circuit B formed conformal monolayer biofilms which coated the substrate to provide reproducible imaging data.

A.4.3 MCC experiments

In MCC biofilms used for quantitative imaging (Figure 2.5), 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

prior to inoculation of the MCC biofilms. Biofilms were incubated after inoculation,

coverslip-down without flow, for one hour. Flow was then resumed at a flow rate of 20

μlmin^{-1}, (speed setting 0.5 on the Watson-Marlowe peristaltic pump), and flow chambers

were left coverslip-down for a total of 24 hours. 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 hours past inoculation.

For the four-color images of the MCC biofilm (Figure 2.6), cells containing the Circuit A

plasmid also contained plasmid pMP4658, which is identical to plasmid pMP4641 but

which constitutively expresses eYFP in place of eCFP [68]. Cells containing the Circuit

B plasmid also contained pMP4641. The images generated by these biofilms were not

used for quantitative analysis because (1) the two different fluorophores may interact

differently with the cellular environments, and (2) 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.

A.5 Biofilm imaging equipment and settings

A.5.1 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.

A.5.2 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

A.5.3 Settings specific to dosage experiments

Channel 1: green   Pinhole setting: 250 (2.39 Airy)

Excitation: 488 nm Argon laser, 11%   Gain: 800

Dichroic: 488/543   Amplifier Offset: -0.048

Secondary Beamsplitter: NFT 545 nm   Amplifier Gain: 1

Emission filter: BP 500–530 nm

Channel 2: cyan   Pinhole setting: 250 (2.46 Airy)

Excitation: 458 nm Argon laser, 76%   Gain: 1000

Dichroic: 458/514   Amplifier Offset: 0.1

Emission filter: BP 480–520 nm   Amplifier Gain: 1

A.5.4 Settings specific to MCC experiments

Channel 1: green   Pinhole setting: 250 (2.39 Airy)

Excitation: 488 nm Argon laser, 11%   Gain: 875

Dichroic: 488/543 nm   Amplifier Offset: -0.043

Secondary Beamsplitter: NFT 545 nm   Amplifier Gain: 1

Emission filter: BP 500–530 nm

Channel 2: cyan   Pinhole setting: 250 (2.46 Airy)

Excitation: 458 nm Argon laser, 80%   Gain: 1000

Dichroic: 458/514 nm   Amplifier Offset: -0.04

Emission filter: BP 480–520 nm   Amplifier Gain: 1

Channel 3: red

Excitation: 543 nm HeNe laser, 80%   Pinhole setting: 250 (2.12 Airy)

Dichroic: 488/543 nm   Gain: 1000

Secondary Beamsplitter: NFT 545 nm   Amplifier Offset: 0.1

Emission filter: BP 480–520 nm   Amplifier Gain: 1

A.5.5 Settings specific to 4-color MCC imaging

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

Pinhole setting: 117 (0.99 Airy)

Gain: 1000

Amplifier Offset: -0.0

Secondary Beamsplitter: NFT 545 nm

Emission Filter: LP 650 nm

Amplifier Gain: 1

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: 1000

Amplifier Offset: -0.05

Amplifier Gain: 1

A.6 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, prior to moving to a new depth. 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.

A.7 Image processing

Step 1—The input

The input is always a set of 512x512 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 hours 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, Figure 2.5) or for each day (MCC experiments, Figure 2.6). 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 to 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 Figure 2.5.

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 Figure 2.5, insets (dosages), and Figure 2.6B (MCC experiments).