
Supporting Information



69451 Weinheim, Germany

**Supporting Information for**
**Microfluidic confinement of single cells of bacteria in small volumes initiates high–density behavior of quorum sensing and growth and reveals its variability**
**J. Q. Boedicker, M. E. Vincent, R. F. Ismagilov**

## Supporting Information

### *Supporting Methods*

*Device Fabrication and Setup*

To create arrays of droplets in which some droplets contained small numbers of cells, we fabricated microfluidic devices, each consisting of an array of wells (each well was 20 or 50 μm in diameter) covered by a channel (200 μm in height). The array of wells was fabricated by spinning a 10 μm layer of SU-8 2010 (MicroChem Corp., Newton, MA) onto a #1.5 glass coverslip. Each device was then baked on a hotplate by ramping the temperature at ~12°C/min from room temperature up to 95°C. The hotplate was then cooled down to 30°C using house nitrogen to assist in cooling, and each device was removed from the hotplate.

The devices were patterned by exposure to 365 nm light through a photomask containing 20 μm or 50 μm diameter holes. For the devices used at room temperature, 45 s exposure times were used to create overexposed wells resembling dimples. For devices used at 30°C (data collected for Figure 4 only), an exposure time of 17 s was used to create a deeper well with exposed glass on the bottom. Postbake procedure was identical to prebake. The devices were developed by rinsing with 1-Methoxy-2-propyl acetate (Aldrich, St. Louis, MO) for 2 minutes, and then the devices were dried with nitrogen gas. Next, the devices were then baked at 65°C overnight to evaporate any residual solvent.

A 200 μm deep microfluidic channel was made by using methods previously described.[1] The SU-8 wells and the PDMS (Sylgard, Dow Corning) channel were rinsed with ethanol, dried with nitrogen, aligned under a stereomicroscope, and placed in contact with one another. For the wells at 30°C, the SU-8 wells were first plasma cleaned for 60 seconds (SPI Plasma Prep II, Westchester, PA) prior to bringing the wells in contact with the PDMS channel. The fully assembled device, consisting of an array of SU-8 wells covered by a PDMS channel, was then sealed into a polystyrene Petri dish with a hole cut in the bottom using a UV curable epoxy (Norland Optical Adhesive 81, Norland Products, Inc., Cranbury, NJ). Next, the channel and wells were filled with FAB media and placed under a vacuum overnight.

*Forming Droplets Containing Small Numbers of Cells*

To fill the SU-8 wells with droplets containing cells, cell culture media was loaded into sterile Teflon tubing (Weico, Wire & Cable, Edgewood, NY) attached to a glass syringe (Hamilton Gastight, Reno, NV) and flowed into the inlet of the PDMS channel at 2 μL/min using a syringe pump (PHD 2000 Infusion, Harvard Apparatus, Holliston, MA). The cells were allowed to settle into the wells for 15 minutes. Then an air bubble was inserted over the wells by flowing air in through Teflon tubing attached to a glass syringe at 2 μL/min using a syringe pump. As the air swept over the SU-8 layer, it removed the cell culture on top of the wells, and a droplet formed in each well. As the air swept over the wells, it also randomly deposited cells into the droplets. More cell culture media was then flowed into the inlet to create a small air bubble covering the array of droplets; the bulk culture media filled the remainder of the channel. The tubing was cut and sealed with wax, extra media was placed in the Petri dish to maintain high humidity, and then the Petri dish was sealed with parafilm.

A single bacterium confined in a 100 fL droplet would be at a cell density of $10^{10}$ Colony Forming Units(CFU)/mL, which is above the density needed to achieve quorum sensing in bulk culture (typically $10^7$-$10^8$ CFU/mL for *Pseudomonas aeruginosa.*)[2] By controlling the cell density of the loading solution the average number of cells loaded into each droplet could be varied. For these experiments, a starting solution of ~$10^5$ CFU/mL was flowed into channels, and cells were allowed to settle. This gave loading of

between 0 and 10 cells into each droplet, with the majority of droplets containing 0, 1, or 2 cells. This method of generating droplets containing cells greatly increased the cell density in the resultant droplets.

Droplet volumes remained in the sub-picoliter range throughout the experiment, and the change in droplet height over 10 h was approximately 18% (Figure S7). To test the influence of media evaporation on our reporter for lasB expression, cells were grown in wells of a 96 well plate containing FAB media made at various concentrations, with relative concentrations from 0.2X to 5X, based on the concentration of media components from the initial reference.[3] Cells were imaged with an epi-fluorescence microscope (DMI 6000 B, Leica, Bannockburn, IL) and average fluorescent intensity was quantified using Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA). These experiments indicated that changing the water content of the media by up to two fold did not significantly interfere with the ability of cells to grow or report on lasB expression (Figure S8). In all other experiments, FAB media was used at 0.5X, as described below.

*Cell Culture Methods*

*Pseudomonas aeruginosa* with the plasmid pMHLB, which contains the lasB reporter P*lasB-gfp*(ASV) was generously provided by Professor Soren Molin.[4] Stocks of cells were stored at -80°C. Before each experiment, stocks were streaked onto LB agar plates (Difco LB Broth, Miller containing 2% (wt/vol) Alfa Aesar agar powder) containing 100 mg/L ampicillin and 50 mg/L gentamycin. Plates were incubated overnight at 37°C. Colonies were then diluted, transferred into tubes containing 3 mL of LB with antibiotics, and subcultured at 37°C, 160 rpm for ~5 hours, or until cell densities reached $10^5$-$10^6$ CFU/mL. Samples were taken to confirm that cells from the subculture were not expressing GFP.

The cultures were then exchanged into a modified FAB.[3] Based on data from Figure S8, 0.5 X media was used, which has a final concentration (after diluting) of 0.05 mM $CaCl_2$, 0.005 mM $FeSO_4$, 0.075 mM $(NH_4)_2SO_4$, 0.165 mM $Na_2HPO_4$, 0.1 mM $KH_2PO_4$, 0.25 mM NaCl, 0.5 mM $MgCl_2$, 5 mM sodium citrate, and 0.25% (wt/vol) casamino acids.

For experiments in Figure 4, cells were exchanged into a QS-dependent growth media, M9 with adenosine, instead of into FAB. M9 with adenosine consisted of: 24 mM $Na_2HPO_4$, 11 mM $KH_2PO_4$, 43 mM NaCl, 93 mM $NH_4Cl$, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% (vol/vol) trace mineral supplement (ATCC, Cat. No. MD-TMS), and 0.01% (wt/vol) adenosine.

All media and materials either arrived sterile, were sterilized by autoclaving, were filter sterilized, or were sterilized by rinsing in 100% ethanol.

*Image Acquisition and Processing*

Cells were imaged using an epi-fluorescence microscope (DM IRE2, Leica) equipped with either a 63× (1.40 NA, oil) or a 100× (1.35 NA, oil) objective and an L5 (Leica) filter, which were coupled with a cooled CCD camera (12-bit, 1344×1024 resolution; Hamamatsu Photonics) with a 1.0× coupler. IR (Edmund Industrial Optics, Barrington, NJ) and neutral density filters (Leica) were placed in the transmitted light path for brightfield imaging in order to reduce heating of the sample. For experiments in Figure 4, the epi-fluorescence microscope (DMI6000 B, Leica) was placed inside of a microscope incubator (Pecon GmbH, Erbach, Germany) set to 30°C. The exposure time of fluorescent and bright field images was 100 ms. Images were processed using Metamorph Imaging Software. Within each set of fluorescent images, the scale bars were adjusted to the same levels. The faint halo of autofluorescence observed around each well is due to the curvature of the side walls of the well and is not influenced by the presence of a droplet, the presence of cells (see Figure SI-6), or QS activity within the well.

For Figure SI-7, droplets containing 100 nM dextran-AF 594 (Invitrogen, Carlsbad, CA) in FAB were imaged using a confocal microscope which consisted of a Visitech Infinity 2-D array scanner confocal system (Visitech, UK) with an array of 50 μm pinholes mounted to a Leica DMI6000 inverted microscope. Images were acquired using a back-thinned electron multiplier CCD camera (16 bit, 512 x 512 pixels, Hamamatsu Photonics) and a 63× (1.40 NA, oil) objective. Fluorescent images were obtained using a 568 nm diode laser with a 0.03 s exposure time and gain set to 80. At each time point, autofocusing was performed, followed by imaging 12 planes above and below the focal plane, 0.4 μm

apart. Images were acquired using Simple PCI software and 3D reconstruction of the stacks of images were made using Metamorph software.

Movies were collected after 7.5 hours when bacteria had divided and initiated QS. Images for the movies were collected every 1 second and each frame of the movie plays 2/3 of a second.

***Supporting Movies***

**Movie SI-1.** A movie in bright field showing an array of wells with bacteria moving around in droplets after being confined for 7.5 hours.

**Movie SI-2**. A movie showing bacteria in an array of wells. Some of the bacteria had initiated QS after 7.5 hours of being confined.

***Supporting Figures***

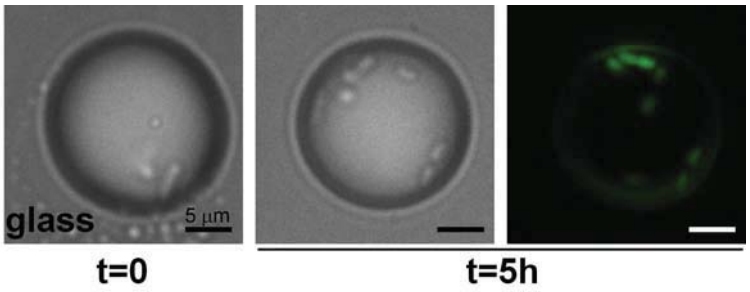


**Figure SI-1**. Initiation of QS by small groups of cells on a glass coverslip. Small aqueous droplets covered by air were formed by flowing cell culture media, followed by air, through a PDMS channel placed over a glass coverslip. Bacteria in droplets retain their motility and growth capabilities. Small numbers of bacteria in these aqueous droplets initiated QS, a pattern similar to that of droplets formed inside of SU-8 wells.

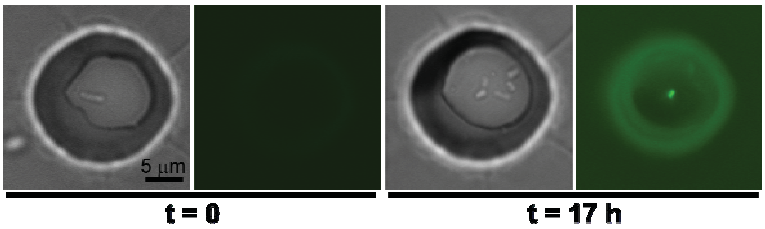


**Figure SI-2.** When starting from a single cell at t=0, not all of the daughter cells in the same droplet initiated QS after 17 h. At t=0, the droplet contained a single cell (brightfield image, far left), and QS had not been initiated (fluorescent image, center left). After 17h, the droplet contained 5 cells (brightfield image, center right), and QS had been initiated in one cell but not in the others (fluorescent image, far right). These results demonstrate heterogeneity in the ability of cells from a clonal population within the same droplet to initiate QS.

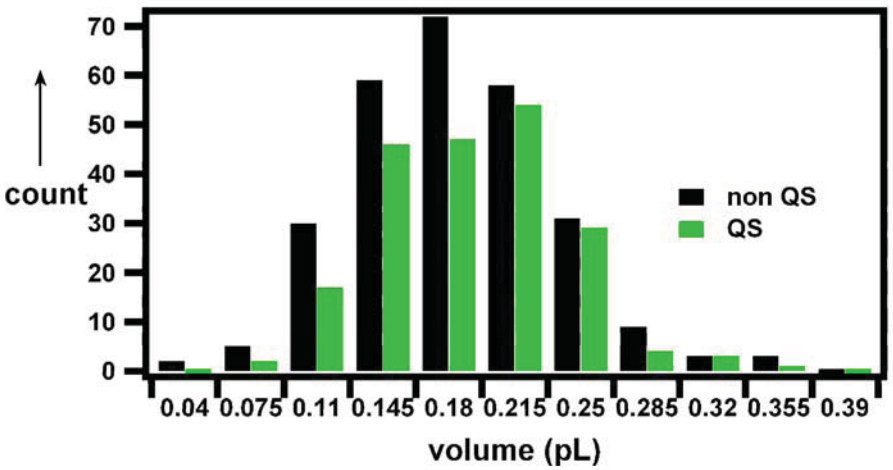


**Figure SI-3**. The volume of the droplet does not correlate with whether or not QS is initiated by the cells in that droplet. Volumes of droplets were estimated for the data presented in Figure 2E by measuring the diameter of the droplets and assuming a height of 2.5 μm based on confocal measurements. The droplets contained 1 to 14 cells at t=0. Droplets containing cells which activated QS after 10 h were categorized as QS (green bars) and droplets containing cells which did not activate QS after 10 h were categorized as non QS (black bars). Chi-squared p = 0.8098.

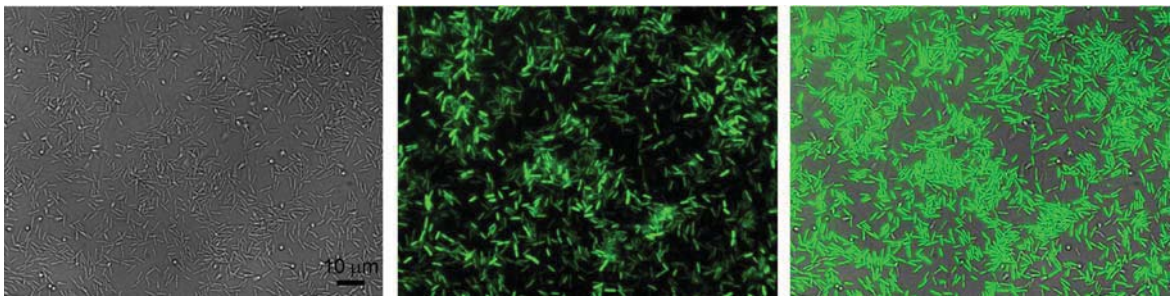


**Figure SI-4.** Brightfield (left) and fluorescent (center) pictures of the *P. aeruginosa* lasB reporter strain growing in the bulk cell culture located at the outlet of a microfluidic device demonstrate that cells are more homogeneous in bulk cultures containing large numbers of cells than in droplets containing small groups of confined cells. Cells were loaded into the device at $10^5$ CFU/mL, and the cells then divided at room temperature for 13 h. By 13 h, the cells had divided to a dense culture and had initiated QS. An overlay (right) of the brightfield and fluorescent images indicate that nearly all of the cells in the bulk culture had initiated QS. In the overlay image, a few bacteria observed did not overlap in the brightfield and fluorescent images, perhaps due to QS heterogeneity in the population or due to the bacteria moving out of position during image acquisition.

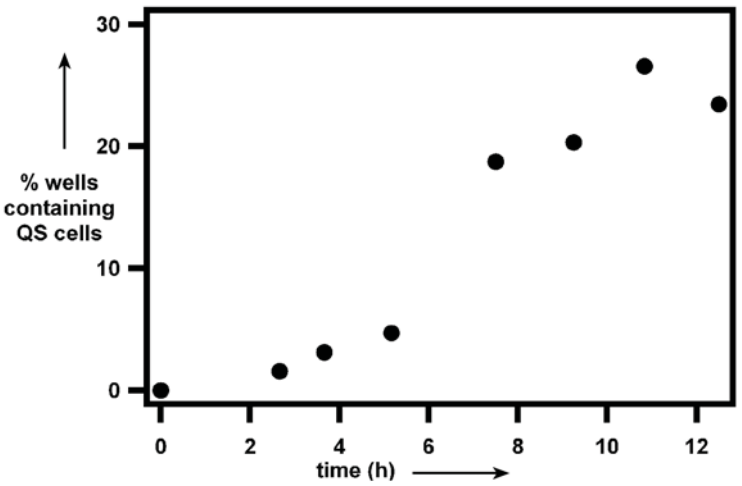


**Figure SI-5.** Plot shows the percentage of droplets containing fluorescent cells at each time point. During the 13.3 h experiment, 17 of 64 droplets contained cells which initiated QS. Most of the cells which initiated QS during the experiment did so between 6 and 8 h, suggesting that the duration of the experiment was long enough to pick up most of the QS initiation events. The dip observed after 11 h was due to a well which previously contained QS activated cells no longer remaining fluorescent, likely due to deactivation of QS and degradation of the unstable version of GFP.

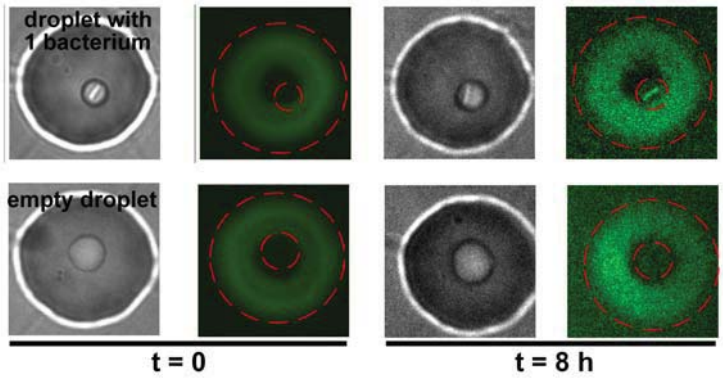


**Figure SI-6**. Another example of initiation of QS by a single bacterium confined into a droplet of small volume. The top set of panels shows a well with a droplet containing a single bacterium at t=0 and t=8 h. The bottom set of panels shows an empty droplet. The faint halo of autofluorescence observed around each well is due to the curvature of the well and is not influenced by the presence of a droplet, the presence of cells, or QS activity within the well.

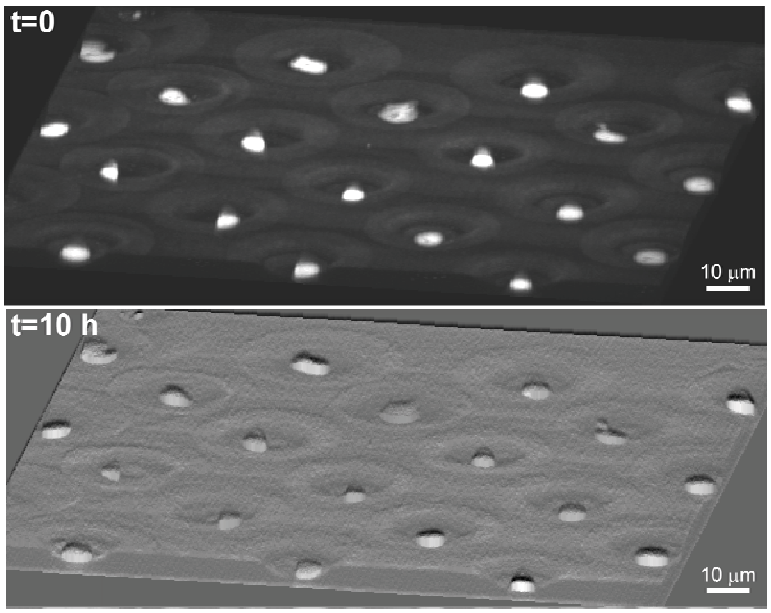


**Figure SI-7.** Wells containing droplets ~200 fL in volume do not evaporate over 10 h. Using confocal microscopy, we imaged droplets containing dextran-alexa594 over time at room temperature. At 10 h (bottom), droplet fluorescence is reduced due to photobleaching and levels were adjusted.

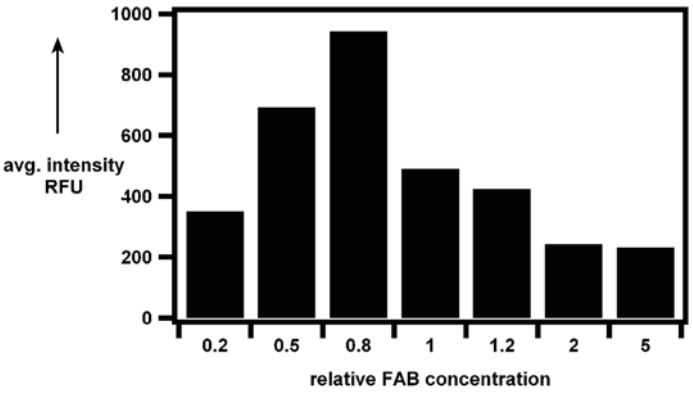


**Figure SI-8**. Changing the concentration of media components in FAB does not prevent initiation of QS, as measured by using a fluorescent reporter for lasB expression. Components of FAB media were varied from 0.2 to 5X the standard recipe (see text for details). Cells were inoculated into each media condition, and the fluorescence of the cells was measured after 6.5 h.

**Supporting References**

[1] H. Song, R. F. Ismagilov, *J. Am. Chem. Soc.* **2003**, *125*, 14613.

[2] L. C. Wu, C. Holbrook, O. Zaborina, E. Ploplys, F. Rocha, D. Pelham, E. Chang, M. Musch, J. Alverdy, *Ann. Surg.* **2003**, *238*, 754.

[3] T. R. De Kievit, R. Gillis, S. Marx, C. Brown, B. H. Iglewski, *Appl. Environ. Microbiol.* **2001**, *67*, 1865.

[4] M. Hentzer, K. Riedel, T. B. Rasmussen, A. Heydorn, J. B. Andersen, M. R. Parsek, S. A. Rice, L. Eberl, S. Molin, N. Hoiby, S. Kjelleberg, M. Givskov, *Microbiol.-Sgm* **2002**, *148*, 87.