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# Microfluidic Device for Automated Synchronization of Bacterial Cells

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## Abstract

We report the development of an automated microfluidic “baby machine” to synchronize the bacterium *Caulobacter crescentus* on-chip and to move the synchronized populations downstream for analysis. The microfluidic device is fabricated from three-layers of poly(dimethylsiloxane) and has integrated pumps and valves to control the movement of cells and media. This synchronization method decreases incubation time and media consumption and improves synchrony quality compared to the conventional plate-release technique. Synchronized populations are collected from the device at intervals as short as 10 min and at any time over four days. Flow cytometry and fluorescence cell tracking are used to determine synchrony quality, and cell populations synchronized in M2G and PYE media contain >70% and >80% swarmer cells, respectively. Our on-chip method overcomes limitations with conventional physical separation methods that consume large volumes of media, require manual manipulations, have lengthy incubation times, are limited to one collection, and lack precise temporal control of collection times.

The ability to synchronize bacterial cells has proven instrumental for the systematic study of events that occur at different times during the cell cycle (e.g., DNA replication,[1-2] protein expression,[3-4] and organelle biogenesis[3,5]). Traditional approaches to bacterial synchronization involve (1) chemical or (2) physical separation of the cells.[6] In chemical separation techniques, a mixed population of cells is exposed to a chemical that inhibits a specific phase of the cell cycle.[7] Commonly called the “arrest and release” method, these cells are collected post-arrest and released for subsequent analysis. However, the impact of this chemical approach on behavior of the cells remains uncertain. Physical separation techniques such as density gradient centrifugation and plate-release techniques are widely used to generate large populations of synchronized cells.[8-10] Centrifugation techniques typically require the addition of a density gradient medium, such as Percoll or Ludox, to the culture, but the success of this technique varies even for strains of the same species.[11] Furthermore, this approach requires relatively large culture volumes to yield visible bands suitable for recovery, and multiple rounds of centrifugation and still higher volumes to synchronize cells within a range shorter than the duration of the enriched life cycle stage. Plate-release techniques can be performed in a variety of media, but require lengthy incubation times and multiple manual operations, e.g., cell washes and centrifugation, during cell culture and collection. The baby machine[12] takes a different approach to physical separation where cells are adhered to a membrane and newborn cells are collected by

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**Supporting Information Available.** Video of cells from a mixed culture pumped into the incubation channel and video of motile cells swimming in the analysis channel under no flow. This material is available free of charge via the Internet at http://pubs.acs.org.

pushing effluent through the membrane with pressure-driven flow. This technique has been adapted for a variety of organisms and platforms[13-16] and yields synchronized populations with minimal perturbations.

Although laboratory-scale techniques are still commonly used, significant advances have been made in the development of microfluidic systems for cell biology.[17-19] The small dimensions of the microfluidic channels reduce reagent consumption and ensure laminar flow even at high flow rates. In turn, stable gradients[20] and high shear forces,[21] which are difficult to create on the macroscale, are easily generated. Microfluidic devices are also able to control and isolate small volumes of fluid precisely, facilitating multiplexed analysis[22] and the integration of sample preparation and analysis on the same device.[23] Precise fluid handling on microfluidic devices provides more control over the microenvironment of the cells and increases the growth rate of cells in microfluidic devices when compared to conventional methods.[24] Recently, mouse lymphocytic leukemia cells were synchronized on a microfluidic device that used pressure, rather than chemical attachment, to hold cells on a membrane surface.[25] This device yielded a modest number of synchronized cells (~1000 cells) during each 12-h synchronization cycle, and over three consecutive synchronization cycles (36 h of total time), 74% of these cells were determined to be in G1 phase.

Our work focuses on the development of a microfluidic baby machine for automated synchronization of the non-pathogenic freshwater bacterium *Caulobacter crescentus. C. crescentus* undergoes asymmetric binary fission that results in two genetically similar but morphologically different progeny: (1) a reproductively mature stalked cell, which is non-motile and secretes a holdfast at its pole to mediate permanent adhesion, and (2) a motile, reproductively delayed swarmer cell, which subsequently differentiates into a sessile stalk cell and thereby achieves reproductive maturity.[26] The stalked cell holdfast is a robust, natural adhesive native to the organism, which allows cells to permanently attach to our platform without the addition of an adhesive.[27] The characteristic loss of motility as the swarmer cell differentiates enables the quantification of the synchrony quality by tracking the trajectories of the cells collected from the biofilm to determine which are motile (newborn) and non-motile. Notably, the ability to synchronize *C. crescentus*, typically by selective enrichment of swarmer cells, has yielded numerous insights into the biology of the bacterial developmental cell cycle. For example, experiments with synchronized populations have demonstrated that over 500 genes are transcribed in a specific temporal pattern during the cell cycle and that timing of expression often matches the time of action of their gene products.[28]

For on-chip cell synchronization, our microfluidic device is designed to isolate the cell incubation and analysis steps and to transfer cells between those steps. To perform required fluid and cell manipulations, we integrated pneumatically actuated valves and pumps.[22,24,29-31] These pumps and valves can be operated in a normally open[32] or normally closed[33] manner, and we opted for normally closed valves to prevent biofilm formation on the valve seats, which compromises their function. The valves are individually addressable and used to isolate the analysis channels from the incubation channel until synchronized populations of newborn swarmer cells are transferred downstream to an analysis channel. Integrated pumps are used to perfuse fresh media over the biofilm as it grows and to transfer the newborn swarmer cells from the incubation channel into the analysis channels for analysis by fluorescence microscopy and collection for flow cytometry. By integrating the cell synchronization step on-chip, we have developed a closed, automated system that streamlines the steps of cell seeding, culture, synchronization, and analysis.

## Experimental Section

### Materials

We purchased SYTOX green nucleic acid stain from Life Technologies, glass slides (50 mm × 75 mm × 1.5 mm) from Corning, Inc.; poly(dimethylsiloxane) (PDMS, Sylgard 184) from Dow Corning Corp.; Shipley 1813 photoresist, SU-8 2010, and Nano PG Developer from MicroChem Corp.; and all other chemicals from Sigma Aldrich. Complex peptone yeast extract (PYE) medium[26] and minimal growth medium with 0.2% glucose (M2G)[34] were prepared as previously reported.

### Device Fabrication

Microfluidic devices were fabricated from three layers (fluid, control, and membrane) of PDMS. The fluid and control layers were cast from SU-8 masters created by standard photolithography. Glass sides were cleaned with methanol and spin-coated with 2% of titanium diisopropoxide bis(acetylacetonate) in propanol and a 15-μm thick layer of SU-8. This initial layer of SU-8 created a robust film on top of which the fluid and control channels were created. This layer was pre-baked on a programmable hotplate (732P, TMC Industries) at 65 °C for 3 min and 95 °C for 1 min, exposed to 270 mJ/cm$^2$ of UV light (205S, Optical Associates, Inc.), and post-baked similar to the pre-bake. For the fluid-layer master, a single 20-μm thick layer was spin-coated onto the substrate, and the SU-8 layer was exposed to 270 mJ/cm$^2$ of UV light through a first photomask (Output City). The fluid layer design is shown in Figure 1. For the control-layer master, two 20-μm thick layers of SU-8 (total thickness of 40 μm) were spin-coated onto the substrate and exposed to 350 mJ/cm$^2$ of UV light through a second photomask. The masters were developed for 2 min in Nano PG Developer, rinsed with 2-propanol, and dried with nitrogen.

PDMS replicas of the control and fluid layers were prepared by creating a boundary around the masters with tape before spreading 9 mL and 6 mL of uncured PDMS, respectively. The PDMS was degassed under vacuum for 30 min, then cured in the oven at 65 °C for 2 h. The pressure and vacuum access holes were punched through the control layer with a leather punch. To create the PDMS membrane layer, a glass slide was coated with Shipley 1813 photoresist and cured at 115 °C for 1 min, and uncured PDMS was spread on top of the photoresist-covered slide. The photoresist prevented adhesion of the cured PDMS to the glass slide. After the PDMS was degassed for 30 min, the glass substrate was spun at 1000 rpm for 35 s to create a layer of PDMS that was approximately 100-μm thick. The PDMS was cured at 65 °C for 15 min. The control and membrane layers were plasma cleaned (PDC-32G, Harrick Plasma), brought into direct contact with each other, and cured at 65 °C for 1 h to insure proper bonding between the membrane and control layers. The bonded control and membrane layers were slowly peeled off the glass substrate, and fluid access holes were punched through the PDMS. The fluid and control layers were aligned and reversibly sealed together before use. These devices were then autoclaved for 30 min at 121 °C to sterilize the device before use with bacterial cultures. Four devices fabricated similarly were used for the experiments reported herein and are designated devices 1, 2, 3, and 4.

### Device Operation

After sterilization, the fluidic device was coupled to an AssayMark system (Microfluidic Innovations, LLC). The AssayMark system interfaces with the microfluidic device through a stainless steel manifold that contains pressure and vacuum connections, which align with the pressure and vacuum holes in the control layer of the device. Each pressure and vacuum connection of the manifold is connected to a 3/2 solenoid valve. The state of the solenoid valve is controlled through software by application of either pressure (5 psi, on state) or vacuum (−10 psi, off state). The control software operates in either a manual or programmed

mode (Microfluidic Innovations, LLC). The manual mode contains toggles for each valve and pump whereas the programmed mode accepts a script that specifies the state of each valve and pump for a given time period. The programmed mode automates the actuation of pumps and valves over multiple days with no user input. To describe device operation, valves are referred to by the name of the closest reservoir, pump, or location relative to the incubation channel in Figure 1.

## Cell Culture

*C. crescentus* cells (CB15::Tn*7gfp3*, YB4789) expressing cytoplasmic green fluorescent protein were used in this study. To grow a mixed culture for device seeding, 3 mL of PYE medium was placed in a test tube, inoculated with a single colony, and shaken in a reciprocal water bath (R76, New Brunswick Scientific) for 5-6 h at 30 °C until the culture reached an $OD_{600nm} \approx 0.1$.

For the on-chip cell culture, biofilm seeding was executed in manual mode, and biofilm incubation was performed overnight in programmed mode. Prior to biofilm seeding, PYE medium was pumped from the media reservoir through the incubation channel to the waste reservoir with pumps 1 and 2. The mixed culture (~15 μL) was added to the cell reservoir and drawn from the cell reservoir through the incubation channel by pumps 1 and 2 to fill the incubation channel with cells. The valves that comprise pumps 1 and 2 were then opened for 10 min, which provided sufficient time for permanent attachment of the cells to the microchannel surface.[35] After cell adhesion, the biofilm in the incubation channel was flushed with fresh media from the media reservoir for 1 min every 15 min by actuation of pumps 1 and 2. During the 15 min incubation, the exit valve of the incubation channel was closed to prevent hydrostatic flow over the biofilm. The incubation reservoir holds ~250 μL of media to ensure that media is not exhausted over the course of the overnight biofilm growth. Because biofilm growth is affected by the efficiency of the initial seeding step, we monitored the fluorescence of the biofilm. When the ratio of the fluorescence intensity of the biofilm relative to the fluorescence intensity after the initial seeding step was >2, we started day 1 collections of synchronized cells.

## Cell Synchronization

To optimize synchrony collection, the media reservoir was rinsed thoroughly with fresh media to remove any debris or unadhered cells and refilled with media. Because the device was inoculated from the cell reservoir, biofilm formation due to bacterial back-growth into the media reservoir was not observed. The control program was switched to manual mode, and fresh media was pumped by pumps 1 and 2 through the incubation channel to the waste reservoir for 10 min to remove unadhered cells from the incubation region. For devices 1, 2, and 3, the PYE medium was replaced with M2G medium six hours prior to the first cell collection to allow sufficient time for cells to be conditioned to M2G medium. M2G medium has a lower background fluorescence signal in flow cytometry and, therefore, is preferred for this analysis. Device 4 used PYE medium for the entire experiment.

For devices 1 and 2, six consecutive cell collections were performed at 10-min intervals on day 1. Each 10-min synchronization cycle consisted of a 5-min release time, during which the incubation exit valve was closed and newborn swarmer cells were released into the media in the incubation channel, and a 5-min pump time, during which pumps 1 and 3 pushed the media with the newborn swarmer cells from the incubation channel into one of five analysis channels. On days 2-4, three consecutive collections were made at 10-min intervals under the same conditions. For each day, synchronized cells were pumped into a different, uncontaminated analysis channel, and synchrony was assessed both on-chip by fluorescence microscopy and off-chip by flow cytometry analysis of collected cells.

Periodically, we observed the presence of non-motile cells in the intersection after the incubation channel that were not removed during the flush steps. To prevent these cells from negatively impacting the synchrony results, cell collection was optimized for devices 3 and 4. For these devices, pumps 2 and 3 pushed media from the analysis reservoir to the waste reservoir during the 5-min release time. During the 5-min pump time, cells were pushed into the analysis channel as in devices 1 and 2.

To compare results from our device to a standard synchronization technique, we collected populations from a mixed culture and a plate-release technique[4,35-36] and analyzed the samples by flow cytometry. For the plate-release technique, a 1-mL aliquot of a mixed culture was diluted in 50 mL of PYE medium in a 150-mm diameter sterile polystyrene petri dish and was agitated overnight (16-18 h) at room temperature on a reciprocal shaker at 70 rpm. Four hours before collection, the plate was rinsed with 100 mL of sterile ultrapure water (18 MΩ·cm, Millipore, Inc.), and an additional 50 mL of PYE medium was added to promote exponential growth of the cells. To initiate synchronous cell release, the plate was rinsed with 100 mL of sterile PYE medium. After the addition of 1 mL of PYE medium, the plate was shaken for 5 min on the reciprocal shaker at 70 rpm. The 1-mL volume of the swarmer cell enriched media was collected from the plate, centrifuged at 5000 × g for 5 min at 4 °C, and concentrated to a final volume of 100 μL by removal of the supernatant. This 100-μL aliquot was added to 900 μL of M2G, mixed, centrifuged again at 5000 × g for 5 min at 4 °C, and concentrated to a final volume of 100 μL to wash the cells of most of the PYE medium for an improved signal-to-noise ratio with flow cytometry. A similar plate-release protocol was followed for M2G medium to compare synchronized populations from both media.

## Fluorescence Microscopy

An inverted optical microscope (IX71, Olympus, Inc.) configured for epifluorescence and equipped with a high-sensitivity CCD camera (C9100-13, Hamamatsu, Inc.) was used to acquire images of the seeding process, biofilm, and synchronized cells. Video data were collected with MetaMorph imaging software (Molecular Devices, LLC.). To determine the percent swarmer cell population in each synchrony, video was collected with and without flow. Six frames of the video without flow were analyzed with the ParticleTracker plug-in[37] for ImageJ to obtain trajectories for each cell in the field of view. The ParticleTracker plug-in has five parameters that are used to identify cells and trajectories: cutoff, intensity percentile, link range, displacement, and radius. At 20× and 60× magnifications, the cutoff was set to 0, the intensity percentile was varied from 0.1% to 5%, depending on the cell density present, and link range was set to 3. For video obtained at 20× magnification, the displacement was set to 12, and the radius was set to 5. For video obtained at 60× magnification, the displacement was set to 20 and the radius was set to 10.

The trajectories determined by the ParticleTracker plug-in were used to count the number of cells, and the paths of the trajectories were used to determine which of the cells were motile and non-motile. Cells adhered to the walls of the microchannel were excluded from the population of non-motile cells. To exclude adhered cells from the data, frames of the channel under flow were analyzed to determine which of the non-motile cells were adhered to the channel walls.

## Flow Cytometry

During the 5-min pump time, media containing newborn swarmer cells were collected in the reservoirs at the end of a designated analysis channel. Aliquots (~5 μL) containing the synchronized populations were removed from the reservoirs and chemically fixed in 210 μL of ice-cold ethanol. To the fixed cell suspension, 700 μL of 1 μM SYTOX green nucleic

acid stain in 20 mM TRIS-HCl (pH 7.5) and 150 mM sodium chloride was added. Finally, 85 μL of M2G was added to bring the volume to 1 mL. Samples were run on a flow cytometer (FACSCalibur, BD, Inc.), and the data were analyzed with FlowJo (Tree Star, Inc.).

## Results and Discussion

### Device Design

Our microfluidic device is designed to maintain a cell culture over several days and to release synchronized cell populations on timescales of minutes to hours to days. The device layout (Figure 1) contains a single incubation channel and five analysis channels into which synchronized cells are released. Cells are initially seeded into the device, allowed to adhere, and cultured by infusion of fresh media at regular time intervals, e.g., 15 min. The synchronized cell populations are released and analyzed off-chip by flow cytometry and on-chip by fluorescence microscopy. The incubation channel has a serpentine geometry (73 mm in length) to increase the surface area for biofilm formation, and five parallel analysis channels permit collection of multiple data sets without contamination from adjacent experiments. Use of two input channels prevents uncontrolled bacterial growth in the media reservoirs during cell culture, and therefore, separate reservoirs are used for the seeding and incubation steps. Integrated valves isolate the channels from each other and make possible the study of cellular response to different stimuli in series or parallel. The number of input, incubation, and analysis channels can easily be increased if more complex incubation or analysis conditions are desired.

### Cell Seeding and Biofilm Growth

Each experiment began with seeding cells into the incubation channel and allowing cells to adhere to the channel surface. To ensure proper device operation, fluorescence images of the cells were taken during device seeding, incubation, and collection to monitor the growth of the biofilm. Figure 2a is a fluorescence image of the entrance to the incubation channel as an exponential mixed culture is pumped through the valves highlighted in region *a* of Figure 1. Video 1 in the Supporting Information shows the cells being delivered to the incubation channel. Biofilm seeding was monitored over 10 min to ensure irreversible adhesion of cells to the microchannel walls. After a sufficient number of cells adhered to the channel to promote biofilm growth, the programmed mode for incubation was selected and run until biofilm fluorescence intensity was >2 times the fluorescence intensity of initial seeding. The uniformity of the biofilm in Figure 2b (region *b* in Figure 1) indicates that the device maintains a homogeneous environment throughout the incubation channel and efficiently removes unadhered and loosely adhered cells as the biofilm develops. As discussed below, maintaining a uniform biofilm leads to high quality synchronies over longer periods of time, e.g., days. Figures 2c-d are, respectively, bright-field and fluorescence images of a valve seat at the end of the incubation channel (region *c* in Figure 1). Figure 2d shows that the valves are able to confine the biofilm growth to the incubation channel. The biofilm (bright region) grows up to the boundary set by the closed valve, but not under or beyond the valve.

### Comparison to Standard Techniques

We compared the performance of our on-chip synchronization device with a non-synchronous mixed culture and the plate-release technique in both PYE and M2G media (Figure 3). All cell collections were analyzed by flow cytometry. Collections obtained from the microfluidic device contained a swarmer cell enrichment that is comparable to the plate-release technique in both media, and populations obtained from days 1 and 2 from the microfluidic device contain on average 75% and 89% swarmer cells in M2G and PYE

media, respectively. The plate-release technique includes cell collections from day 1 only and yielded 70% and 87% swarmer cells in M2G and PYE media, respectively.

The structure of the biofilm formed by *C. crescentus* depends on the incubation media used.[38-39] Biofilms formed in PYE medium are robust, monolayer-type films where all cells are attached to the surface by the holdfast. This monolayer structure assists in the collection of quality synchronies with minimal release of large aggregates from the layer. Conversely, biofilms formed in M2G medium exhibit mushroom-shaped structures where cells are attached to the surface and each other. Cell aggregates composed of stalked cells are released from the more complex mushroom-shaped structures, mix with the swarmer cells, and are counted in the synchronized cell population. The difference in biofilm structure explains the lower synchrony quality in M2G medium for both the plate-release technique and our microfluidic device.

### Isolation of Synchronized Cells as Needed Over Four Days

A major drawback to many physical separation techniques is that the collection of multiple synchronies over minutes, hours, and days from the same parent population is difficult. For example, cell collections obtained from the plate-release technique contain a high percentage of swarmer cells on day 1, but by day 2 the biofilm is overgrown and cannot be used to collect a synchronized population of swarmer cells. Our device extends the lifetime of the biofilm, which permits synchronized populations to be collected over multiple days. Continuous perfusion of media across the biofilm maintains an environment that supports healthy cell growth, minimizes the formation of cellular aggregates in the microchannel, and greatly reduces the likelihood of contamination by elimination of manual manipulation of fluids for collection of the synchronized population. Figure 4 shows the percent swarmer cells obtained on day 1 at 10-min intervals for 60 min on our device and analyzed by flow cytometry. Swarmer cell enrichment remains consistent across the entire 60-min collection period for the microfluidic device. For comparison, cell collections at 10-min intervals over a 30-min period for the plate-release method are shown and are comparable.

In addition to the analysis of consecutive collections at short time intervals, e.g., 10 min, device performance was assessed over four days (Figure 5). Synchronized populations of cells were collected from four devices in both M2G and PYE media on days 1 to 4. Cell populations from devices 1, 2, and 3 were synchronized and collected in M2G, whereas populations from device 4 were synchronized and collected in PYE. A 10-min synchronization cycle (5-min release time plus 5-min pump time), during which newborn cells are released into the media in the incubation channel, was performed for all devices. For devices 1 and 2, the media in the incubation channel was pumped directly into the analysis channel to maximize the volume collected for analysis. However, unadhered and non-motile cells were periodically observed in the intersection between the analysis channels and the end of the incubation channel, which served as a source of contamination for the swarmer cell enrichment. For devices 3 and 4, we removed this plug of media with unwanted non-motile and unadhered cells by filling the analysis channel with fresh, sterile media prior to the incubation period. Media from the analysis channel was routed into the waste reservoir to remove these aggregates before the newborn cells were pumped into the analysis channel.

Three consecutive cell collections taken at 10-min intervals were averaged to determine percent swarmer cells for each day. On average, the percentage of swarmer cells collected in PYE medium was higher than the percentage of swarmer cells collected in M2G medium, because biofilms formed in M2G contain complex structures that lead to release of aggregates from the biofilm during cell collection. Synchronies from the device on days 1 and 2 are comparable to the plate-release technique (Figure 3). In the incubation channel, the

cell density (cells/mL; discussed below) on day 1 is lower than day 2, but both days yield high quality swarmer cell enrichments. Synchrony quality is lower on day 3 and continues to decline by day 4. By day 5 (data not shown), large unadhered aggregates were present in each collection that mixed with the newborn swarmer cells. Typical lifetimes of the microfluidic devices were three days (~four days from initial seeding) to obtain at least 70% swarmer cells in M2G and at least 80-swarmer cells in PYE.

If cells had been collected from the biofilm every ten minutes for all three days, our device could produce 432 synchronized cell populations. To illustrate the vast reduction of reagent consumption our platform achieves, only 3 mL of media is required for seeding of the biofilm and collection of all 432 populations. On the other hand, the plate-release technique on a 150-mm diameter petri dish requires ~200 mL of media for the growth and incubation steps and another 100 mL for each collection. If 432 collections could even be obtained from one biofilm, the plate-release technique would require over 43 L of media. Of course, the plate-release technique can be scaled down, but will never match the precise fluid handling capabilities and small volume of the microfluidic device. Moreover, the plate-release technique yields poorly synchronized populations after day 1 and requires a technician to conduct each step in the process, e.g., cell washes, centrifugation, and concentration.

## Fluorescence Microscopy

In addition to analysis by flow cytometry, we evaluated the quality of each cell synchronization by fluorescence microscopy. Determination of the synchrony quality by fluorescence microscopy permits the analysis to be performed on the microfluidic device without transfer of collected cells to, for example, a flow cytometer. Although we successfully demonstrated flow cytometric analysis of our samples, as described above, the manual transfer of the sample from the device and the preparation required for analysis by flow cytometry introduce the possibility of contamination or sample degradation before analysis. To perform the analysis of the synchronized population by microscopy, we closed the valve at the entrance to the specified analysis channel to suppress flow in the media that contained newborn swarmer cells. Video, similar to Video 2 in the Supporting Information, was taken of the cells in the analysis channel without flow for several seconds before flow was resumed. Frames from the video collected without flow were analyzed by the ParticleTracker plug-in for Image J to determine the number of cells in the field of view and the trajectories of those cells. Because intersecting cell trajectories can interfere with the ParticleTracker analysis, we used only six frames of video to minimize the number of intersections but to produce trajectories long enough to identify motile cells (Figure 6a). The trajectories tracked with the ParticleTracker plug-in were then visualized to determine which cells were motile. Because adhered cells were not considered in the flow cytometry analysis and we cannot determine if the adhered cell is a swarmer or stalked cell, video frames of the analysis channel under flow were analyzed to determine which non-motile cells were adhered to the channel walls (Figure 6b).

To evaluate our microscopy technique, the percent swarmer cells determined by flow cytometry are compared to the percent swarmer cells determined by fluorescence cell tracking. Figure 5 shows that the overall trend in synchrony quality and percent swarmer cells are the same for data analyzed by flow cytometry and fluorescence microscopy. Although flow cytometry is an efficient method to distinguish cells with one chromosome (swarmer cells) from cells with more than one chromosome (stalked and predivisional cells undergoing DNA replication), we are able to accurately differentiate cells on-chip by both movement and size with video microscopy and, consequently, precisely identify populations of motile, non-motile, and predivisional cells. Therefore, we show that data collected by

flow cytometry and fluorescence microscopy are comparable, but fluorescence microscopy allows for real-time observation of synchrony quality on-chip.

To determine the population density of synchronized cells obtained from our device, we used the fluorescence microscopy data to estimate the number of cells per unit volume. From these data, we estimate that our baby machine device produced an average swarmer cell density of $1 \times 10^8$ cells/mL on day 1 and $4 \times 10^8$ cells/mL on days 2, 3, and 4. The four-fold increase in cell density was expected due to continued growth of the biofilm. The volume of the incubation channel is 0.44 μL (20-μm deep, 300-μm wide, and 73-mm long) and, therefore, produced $2 \times 10^5$ swarmer cells per synchronization cycle on days 2-4.

### Cell Cycle Analysis

To ensure that our system does not physiologically perturb the cells, we conducted a cell cycle analysis to track the division of the collected populations. A synchronized population was collected and resuspended in 1 mL of media, and flow cytometric analysis on this population was undertaken every 30 min (Figure 7). At 30 and 60 min, the population contained 80% and 89% swarmer cells, respectively. Notably, a small but distinct peak of predivisional cells (fluorescence intensity > 220 in Figure 7) is observed at 30 min. This peak suggests that some predivisional cells escaped the biofilm during cell collection, presumably facilitated by activation of the flagellar motor in these cells. Subsequent division of these cells gives rise to both motile swarmer and non-motile stalked cells, explaining the observed increase in the “swarmer cell” density observed at 60 min by flow cytometry. Stalked cells born from predivisional cells can also explain the small fraction of non-motile cells observed in on-chip fluorescence microscopy (Figure 5). By 120 min, the DNA peak widened, indicating that cells had entered the DNA replication phase as cells moved toward the predivisional stage, and by 180 min, the percentage of swarmer cells had decreased to 45%, indicating a mixed population of swarmer cells and predivisional cells. These data confirm that cells undergo division, and therefore, our device produces viable synchronized populations.

## Conclusions

We have developed the first bacterial baby machine on a microfluidic platform. Specific advantages of our device over traditional physical separation techniques include continuous cell collection over minutes, hours, and days, high-quality swarmer cell enrichment confirmed on-chip by fluorescence cell tracking and off-chip by flow cytometry, significantly lower reagent consumption, and automated device operation with integrated pumps and valves that minimize contamination. In addition, our device is disposable, air permeable, programmable, scalable, and easy to fabricate. Future device designs can include longer or parallel incubation channels to increase the number of swarmer cells produced, integration of additional buffer channels and pumps after the incubation channel for downstream experiments, and control of the temperature and gas composition in the incubation channel.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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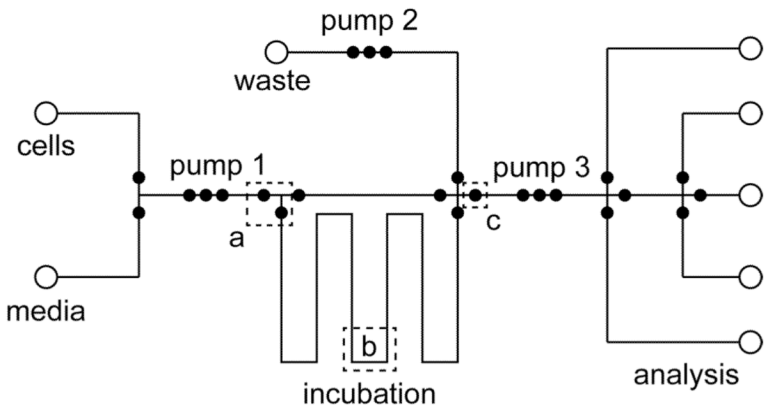


**Figure 1.**

Schematic of the microfluidic device for on-chip cell synchronization. Open circles represent reservoirs, and closed circles represent valves. Three valves in series are used as peristaltic pumps and are labeled pumps 1, 2, and 3. The biofilm is seeded and grown in the incubation channel. Synchronized cells released from the biofilm are transferred to one of five analysis channels, analyzed on-chip by fluorescence cell tracking, and collected for flow cytometry. Dashed regions *a, b*, and *c* correspond to images in Figure 2.

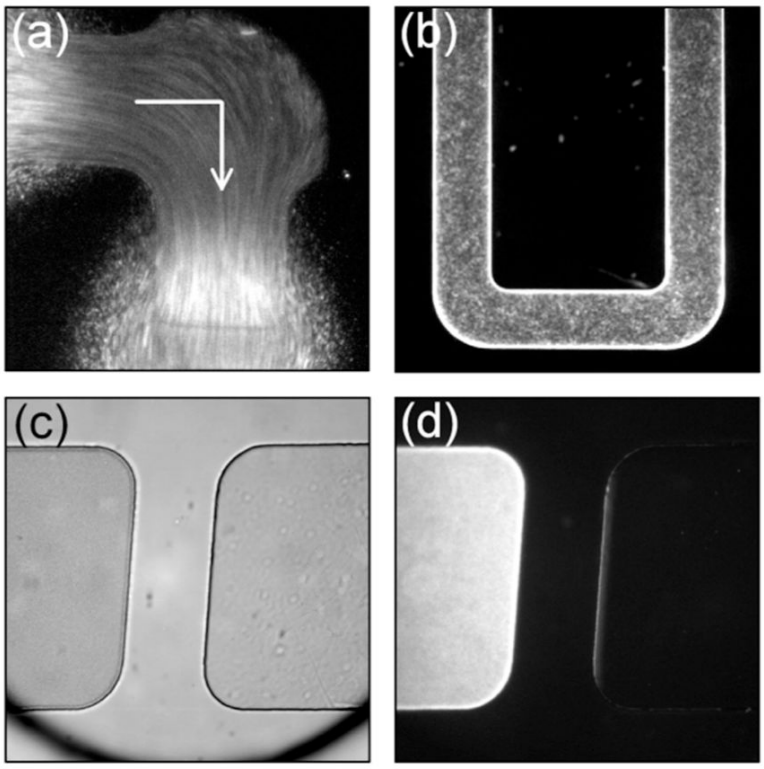


**Figure 2.**

(a) Fluorescence image of an exponential mixed culture pumped into the incubation channel for biofilm seeding (region *a* in Figure 1). Arrow indicates direction of flow. (b) Fluorescence image of a uniform biofilm formed in the incubation channel (region *b* in Figure 1). (c) Bright-field image of a valve seat at the end of the incubation channel (region *c* in Figure 1). (d) Fluorescence image of the biofilm (bright region) grown up to, but not beyond, the valve seat in panel (c).

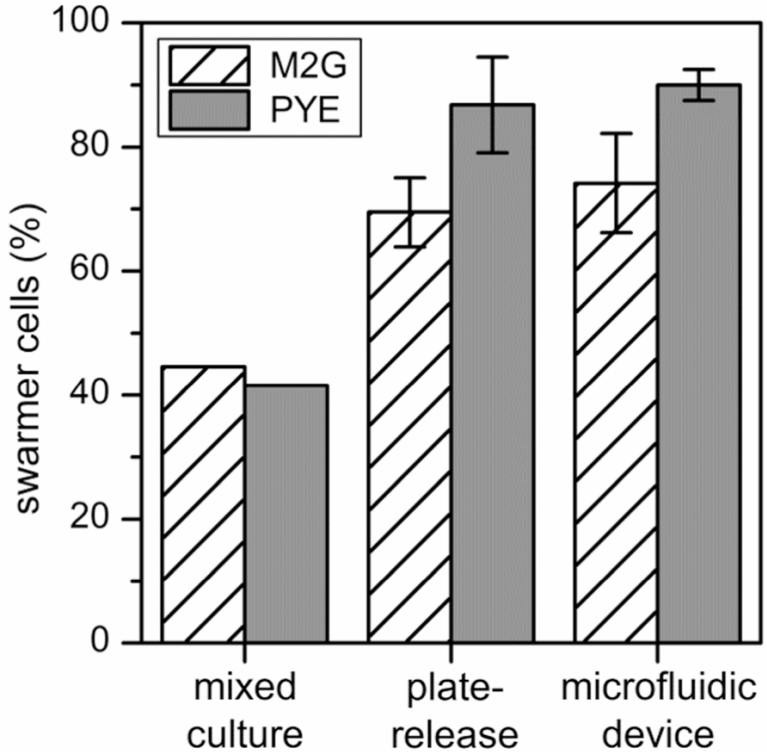


**Figure 3.**
Percentage of swarmer cells from a mixed culture, plate-release technique, and microfluidic device for M2G and PYE media. Samples were collected on day 1 and analyzed by flow cytometry.

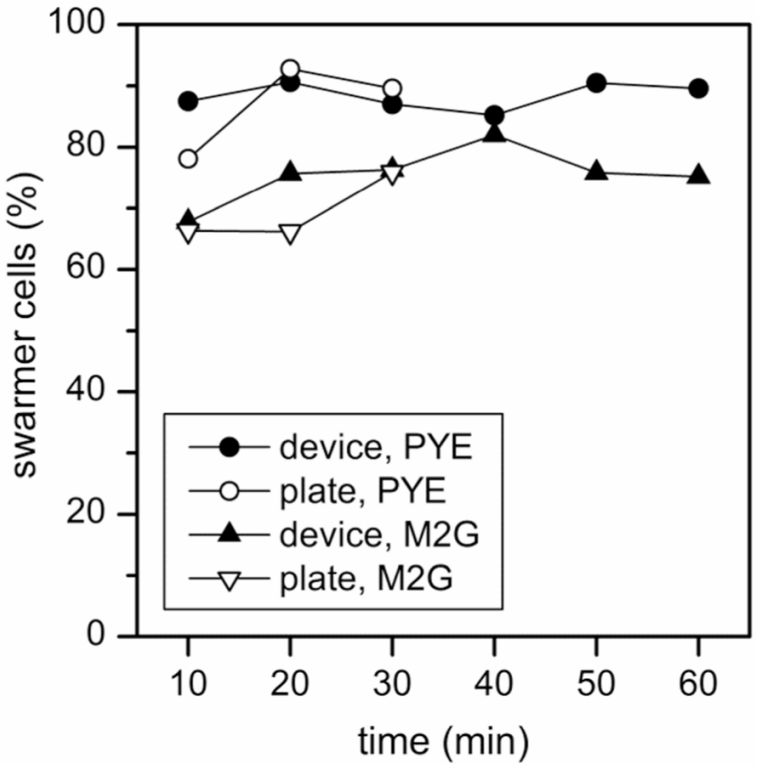


**Figure 4.**
Percentage of swarmer cells from six consecutive 10-min synchronization cycles from a microfluidic device and three consecutive 10-min synchronization cycles from the plate-release technique. Samples were collected on day 1 and analyzed by flow cytometry.

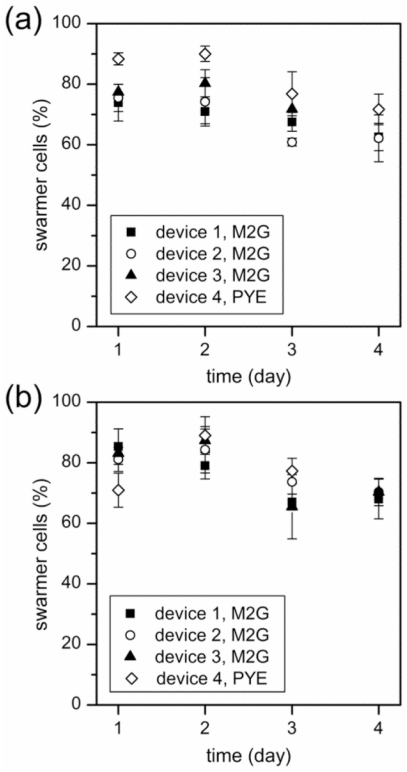


**Figure 5.**
Percentage of swarmer cells collected over four days from devices 1, 2, 3, and 4 and analyzed by (a) flow cytometry and (b) fluorescence cell tracking. For devices 1 and 2, the synchronized cells were transferred into the analysis channel immediately after a 5-min release time. For devices 3 and 4, non-motile cells present at the exit of the incubation channel were routed to the waste reservoir prior to transfer of the synchronized cells into the analysis channel. Both M2G and PYE media were evaluated.

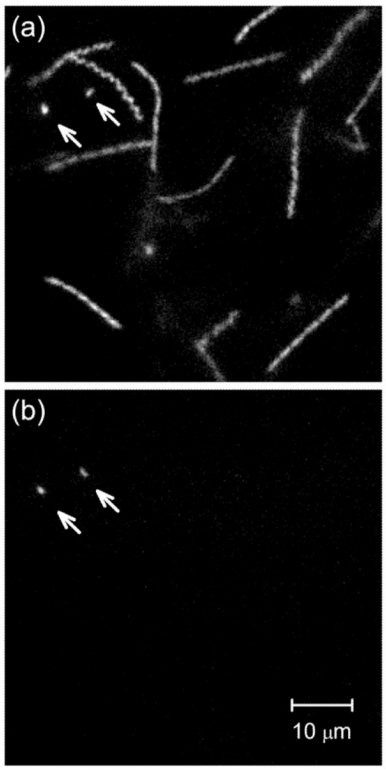


**Figure 6.**
(a) Composite fluorescence image of motile cell movement in the analysis channel under no flow over six consecutive frames. Cell trajectories are tracked with the ParticleTracker plug-in for Image J. (b) Fluorescence image of two non-motile cells adhered to the analysis channel under flow. Panels (a) and (b) are the same field of view, and the arrows highlight the position of the adhered cells in both panels.

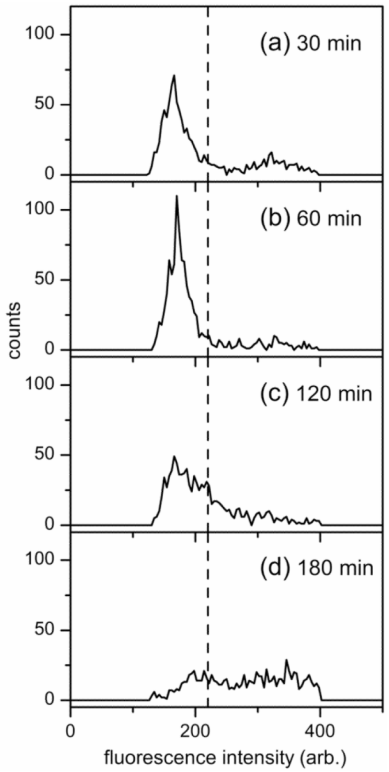


**Figure 7.**

Histograms of synchronized cells cultured for (a) 30 min, (b) 60 min, (c) 120 min, and (d) 180 min after collection from the microfluidic device. Cell samples were analyzed with flow cytometry, and fluorescence intensities below 220 are swarmer cells and above 220 are predivisional cells. At 30, 60, 120, and 180 min, the cell samples contained 80%, 89%, 85%, and 45% swarmer cells, respectively.