Encapsulating Bacteria in Agarose Microparticles Using Microfluidics for High-Throughput Cell Analysis and Isolation

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S *Supporting Information*

ABSTRACT: The high-throughput analysis and isolation of bacterial cells encapsulated in agarose microparticles using fluorescence-activated cell sorting (FACS) is described. Flow-focusing microfluidic systems were used to create monodisperse microparticles that were ~30 μm in diameter. The dimensions of these particles made them compatible with flow cytometry and FACS, and the sensitivity of these techniques reduced the incubation time for cell replication before analyses were carried out. The small volume of the microparticles (~1−50 pL) minimized the quantity of reagents needed for bacterial studies. This platform made it possible to screen and isolate bacteria and apply a combination of techniques to rapidly determine the target of biologically active small molecules. As a pilot study, *Escherichia coli* cells were encapsulated in agarose microparticles, incubated in the presence of varying concentrations of rifampicin, and analyzed using FACS. The minimum inhibitory concentration of rifampicin was determined, and spontaneous mutants that had developed resistance to the antibiotic were isolated *via* FACS and characterized by DNA sequencing. The β-subunit of RNA polymerase, RpoB, was confirmed as the target of rifampicin, and Q513L was the mutation most frequently observed. Using this approach, the time and quantity of antibiotics required for the isolation of mutants was reduced by 8- and 150-fold, respectively, compared to conventional microbiological techniques using nutrient agar plates. We envision that this technique will have an important impact on research in chemical biology, natural products chemistry, and the discovery and characterization of biologically active secondary metabolites.
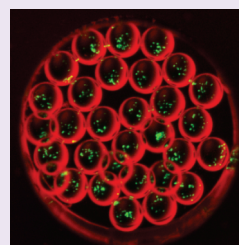
This paper describes a microfluidic technique for encapsulating, growing, and analyzing bacteria in agarose microparticles to rapidly screen and isolate cells that display phenotypes of interest. As a proof-of-principle, we used the bacterial RNA polymerase inhibitor rifampicin and identified spontaneous mutations in *Escherichia coli* that conveyed resistance to this antibiotic. We created agarose microparticles using microfluidic flow-focusing devices fabricated in poly(dimethylsiloxane) (PDMS). The microparticles are mechanically stable and have a user-defined diameter that ranges from 20 to 150 μm; these characteristics enable us to use flow cytometry (FC) and fluorescence-activated cell sorting (FACS) for the high-throughput analysis, selection, and isolation of encapsulated cells based on phenotypes.

Microbiologists frequently culture bacterial cells on agar plates to screen, select, and isolate monoclonal populations of cells. "Agar plates, toothpicks, and logic"[1] played a central role in microbiology over the past century; however, approaches based on the growth of cells on agar nutrient media have significant drawbacks for culturing organisms that grow slowly or require environmental cues.[2] The most common techniques for identifying targets of biologically active small molecules in bacteria involve large-scale screens to isolate mutants that have spontaneous resistance to the compounds, isolation and amplification of their genomic DNA, and sequencing to identify genetic changes responsible for the acquired resistance. The isolation

of mutants by growing bacteria on agar typically requires large quantities of the chemical(s) of interest and multiple iterations of screening, which can make these assays expensive and slow. These requirements can be particularly problematic if the reagents are difficult to obtain and are available only in small quantities (*e.g.*, structurally complex secondary metabolites and natural products that are not yet available through total syntheses) or when the exposure of users to hazardous compounds should be minimized. Furthermore, the time required for each cycle is constrained by the growth of an individual cell into a colony that is large enough to visualize and pick. This characteristic limits the throughput of assays for the rapid screening of biological and chemical targets.

To address these limitations, several different methods have been proposed for encapsulating bacterial cells in aqueous droplets,[3−5] liquid plugs,[6,7] or hydrogels [8−13] for high-throughput analysis. The most widely used of these techniques encapsulate cells in agarose [2,14−17] and have been has been used to study a variety of phenomena, including (1) antibiotic susceptibility;[18,19] (2) bacterial uptake of small molecules by electroporation;[20] (3) enrichment of slow-growing bacteria;[21,22] and (4) growth of "uncultured" bacteria for phylogenetic analyses.[2]

Despite their demonstrated applications, the methods currently available for fabricating agarose particles provide limited control over the diameter of particles and produce particles with a large coefficient of variance (*e.g.*, ≥40%).[12,23−26] The large variance in the diameter of these particles can be problematic when studying cells that are present at a low frequency in a heterogeneous population of cells. Large particles with diameters that are incompatible with FC or FACS are removed by filtration prior to analysis. Strains that are underrepresented in a mixed population of cells may be encapsulated in large particles and removed before they are analyzed, which biases the composition of the sample and poses a challenge for the detection and analysis of rare cells in a mixed population.

To transcend the limitations of conventional techniques for encapsulating bacteria in agarose, we used PDMS flow-focusing microfluidic systems to form monodisperse microparticles.[27,28] The mechanism of drop formation in these microfluidic junctions is well understood and can be exploited to create monodisperse droplets and bubbles.[29−31] We isolated single cells in microparticles and demonstrated the rapid determination of the minimum inhibitory concentration (MIC) of rifampicin to *E. coli* strain MG1655 and the screening and isolation of spontaneous mutants that are resistant to the antibiotic. This approach makes it possible to isolate strains of bacteria on the basis of changes in genotype and phenotype and will be useful for evolving and engineering proteins in microbes.[4]

■ RESULTS AND DISCUSSION

The flow-focusing microfluidic devices consisted of a cross-junction with three inputs and one outlet channel through which microparticles flowed out of the device and were collected. The dispersed phase liquid admixed with cells was pumped into the junction through an inlet channel where it met the continuous phase, mineral oil, which was flowing from two orthogonally oriented channels. This geometry caused the mineral oil streams to focus the dispersed phase into a thin stream that broke into droplets as it flowed through a constriction into the outlet channel (Figure 1). The geometry of the device and the physics of fluids at the micrometer-scale ensured the stable and reproducible breakup of the dispersed phase into uniform droplets.[32]

Characterization of Microparticles. We measured the diameter of the agarose microparticles produced in the PDMS microfluidic devices at different ratios of flow rates of the mineral oil and solution of agarose. Although we fabricated microfluidic channels with a range of different dimensions, we present data from one set of devices with critical dimensions that are described in Supporting Information. As we increased the ratio of the flow rates of oil to agarose, the diameter of the microparticles decreased to ∼20 μm (Figure 1c). The coefficient of variance for the microparticle diameter at each ratio of flow rates was 10% and indicated that the microparticles were moderately monodisperse. To analyze microparticles using FACS or FC, we fabricated colloids that were ∼30 μm in diameter; we chose this diameter for the particles to accommodate a standard core size of 40 μm in most FC and FACS instruments. Microparticles with a diameter of 60−160 μm or larger can be created using other types of microfluidic devices (Supporting Information) or by fabricating channels/nozzles with dimensions that are larger than those described here.

Growth and Analysis of Encapsulated Cells. To visualize cells in microparticles *via* microscopy, FC, and FACS, we used a strain of *E. coli* MG1655 with a plasmid encoding an ampicillin
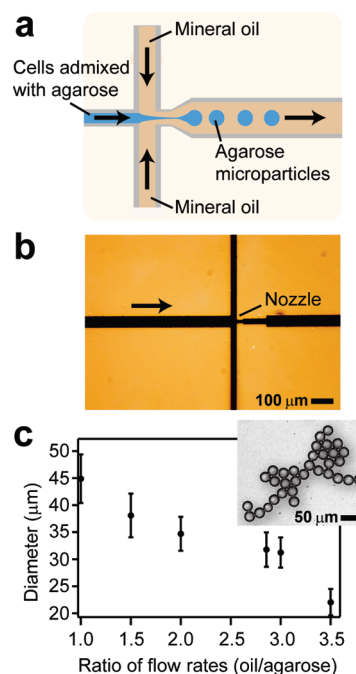
Figure 1. Production of microparticles in PDMS flow-focusing microfluidic devices. (a) A schematic diagram of the PDMS device depicting the direction of fluid flow and formation of agarose droplets suspended in mineral oil at the nozzle. A video showing droplet formation inside the device is included in the Supporting Information. (b) An image of a PDMS microfluidic device showing the nozzle region of the device; the channels were filled with black ink to increase the contrast. The arrow shows the direction of flow of the agarose solution admixed with cells. (c) A plot showing the relationship between the ratio of flow rates of mineral oil and agarose and the resulting diameter of microparticles that were formed. The error bars represent the coefficient of variance for each ratio tested. The image inset shows agarose microparticles produced using flow rates of 150 (oil) and 50 (agarose) μL h^{-1}.

resistance and a *lac* operon for controlling the transcription of *egfp* (we refer to this strain as MG1655-p*lac*EGFP). Using the Poisson equation as a guide, we diluted a cell culture such that ∼10% of a population of agarose microparticles would contain single cells and the majority of the remaining microparticles would be empty.

We gated the data in FC experiments and analyzed the fluorescence emission of the microparticles (Figure 2). To differentiate between the autofluorescence of empty agarose microparticles (red data points in Figure 2a) and the fluorescence emitted from EGFP-expressing cells encapsulated in microparticles (black data points), we excited the sample at λ = 488 nm and compared the fluorescence emission at two wavelengths: 525 and 710 nm. The fluorescence emission of EGFP is high at λ = 525 nm and low at λ = 710 nm. Using this guideline, we gated the population of microparticles to determine the percentage containing fluorescent cells and found that approximately 11% of the microparticles contained EGFP at the initial time point (Figure 2a). This percentage of fluorescent microparticles agreed well with our calculation using the Poisson distribution, indicating that most of the fluorescent microparticles contained single cells. When we imaged the microparticles using epifluorescence microscopy, we found that some microparticles contained more than one cell, which may have arisen from four experimental variables: (1) the Poisson distribution predicts that ∼1% of the microparticles
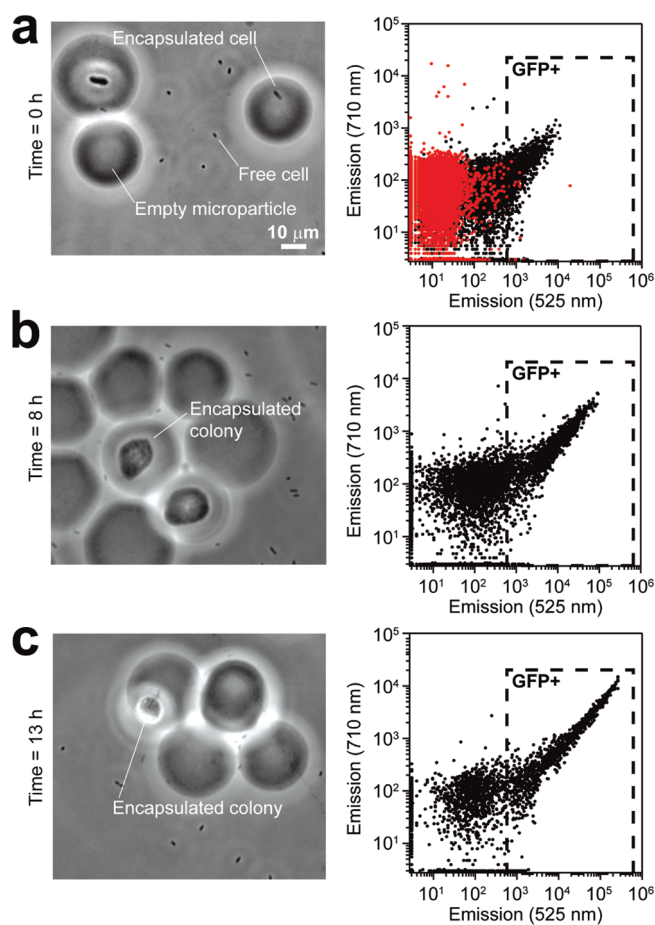
Figure 2. Growth of *E. coli* cells in agarose microparticles. Samples were collected, analyzed, and imaged at three time points: *t* = 0, 8, and 13 h. Images of microparticles are shown in the left column, corresponding to these time points. The right column contains plots of fluorescence from FC for each corresponding time point. The dotted rectangle shows the gate drawn for the GFP-positive population. Red dots in the scatter plot for the *t* = 0 h sample indicate background fluorescence emission from empty microparticles. Black dots indicate fluorescence signal from *E. coli* cells encapsulated in microparticles.

contain more than one cell; (2) aggregates of cells formed in the overnight culture were not dispersed completely in the agarose solution; (3) cells aggregated in the syringe during the formation of the microparticles in the microfluidic device; and (4) single cells encapsulated in microparticles replicated during sample collection from the microfluidic outlet channel. For example, a microparticle (top left) in image for the "time zero" sample (Figure 2a) probably contained an aggregate of multiple cells.

The incubation of microparticles containing single cells led to the formation of microcolonies within the agarose and demonstrated that the nutrients in the microparticles and gas exchange through the mineral oil were sufficient for cell growth. We have found that the growth rate of *E. coli* cells in agarose microparticles suspended in mineral oil was very similar to their growth on an agarose pad. The microencapsulated cells reproduced with a doubling time of 17 ± 3 min at 37 °C, which is comparable to the doubling time of 19 ± 5 min at 37 °C on an agarose pad. We observed that the doubling time increased after 1.5 h when cells were encapsulated in 36-μm diameter microparticles that were suspended in oil. However, the maximum time of growth in

particles will depend on the medium in which they are suspended (oil *vs* nutrient broth), the size of the particles, and the number of cells encapsulated per particle.

In addition to an increase in the number of cells per particle over time, we also observed a time-dependent increase in the percentage of microparticles containing EGFP-expressing cells during the incubation of an unsorted collection of microparticles suspended in mineral oil at 37 °C without shaking (Figure 2). After 8 h of incubation in mineral oil, 24% of the microparticles were fluorescent (compared to 11% at 0 h), and after 13 h of incubation the number of fluorescent microparticles increased to 40%. We hypothesized that the increase in the number of fluorescent microparticles over time was due to the released cells from ruptured microparticles, which led to attachment of the freed cells to the surface of intact microparticles that were initially empty.

Cells formed microcolonies that were 5–20 μm in diameter within 30 μm (diameter) microparticles after 5 h of growth in mineral oil. It was difficult to determine the size of these colonies with precision using 2D optical microscopy because of the influence of the orientation of the colony/particle (Figure 2). A more reliable and sensitive approach was to use FACS to analyze the total fluorescence intensity of the encapsulated cells in the microparticles. For FACS, we transferred the colloids from mineral oil to an aqueous buffer (see Supporting Information). Some of the particles containing large colonies became mechanically unstable and released cells into the buffer during their transfer from the mineral oil. We suspected that these cells adsorbed to the surface of agarose microparticles and increased the number of fluorescent microparticles in our samples. To confirm this hypothesis we fabricated empty microparticles and admixed them with a suspension of MG1655-p*lac*EGFP cells. We immediately analyzed the sample by FC and found that ~40% of the empty microparticles were fluorescent.

The release of cells from microparticles can be an issue in some applications; however, it can be avoided by using short incubation times for cell growth and taking advantage of the sensitivity of the detectors in FC and FACS instruments. When microparticles were incubated for ≤5 h, we rarely observed cells released from microparticles. For applications that require larger microencapsulated communities, the optimization of the microparticle size, agarose concentration, and transfer of the microparticles from mineral oil to buffer will decrease colony heterogeneity that may arise from nonspecific adsorption of cells to other microparticles.

Determination of the Minimum Inhibitory Concentration. We used agarose microparticles to determine the MIC of rifampicin and compared the result to measurements made using techniques based on the macrodilution of batch cultures. In batch culture techniques, the MIC is the lowest concentration of antibiotic that inhibits visual growth of bacteria. These culture techniques require incubating cells for long periods of time (typically 12–18 h for *E. coli* and >24 h for slower-growing species) and consume significant amounts of the antibiotic (*e.g.*, >10 mg) as the cultures are typically grown in a dilution series and require several replicates at each concentration of compound. To decrease the amount of compound required for assay, the MIC can be determined using 96-well microtiter plates. However, performing assays in microtiter plates does not reduce the experimental time. In contrast, the microencapsulation technique we describe requires very small amounts of compounds (<1 mg) and short incubation times (≤3 h), as FC makes it possible to quantitatively measure growth by measuring small changes in the fluorescence of microparticles. We anticipated
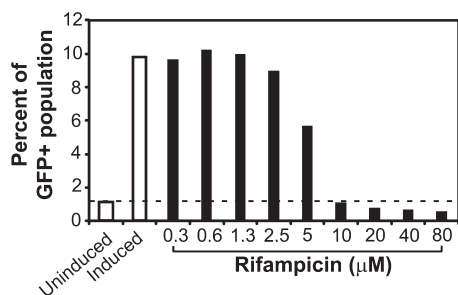
Figure 3. Determination of the minimum inhibitory concentration by encapsulating cells in agarose microparticles. The plot shows the percentage of a microparticle population that fell into the GFP-positive gate (GFP+) at various concentrations of the antibiotic (filled bars). Control samples (open bars) without antibiotic were either induced or uninduced to determine the lowest and highest percentages of cells in the GFP-positive gate. A dashed line was drawn to indicate the percent of GFP+ population from the uninduced control.

that the determination of MICs using microfluidic encapsulation would provide a useful alternative to batch culture methods.

To measure a MIC of rifampicin against *E. coli* MG1655, we constructed a strain that contained a plasmid encoding an ampicillin resistance cassette and a *tet* operon for controlling the transcription of *egfp* (MG1655-p*tet*EGFP). We encapsulated single cells of MG1655-p*tet*EGFP in agarose microparticles and analyzed fluorescence using FC. To analyze FC data from microparticles, we included two controls without rifampicin: (1) uninduced and (2) induced with anhydrotetracycline. Using these controls, we created a gate for the GFP-positive population of microparticles and used a percent population of the uninduced control that fell into the GFP-positive gate as a threshold to determine the concentration of rifampicin that completely inhibited the growth of bacteria. As we increased concentration of antibiotic the number of microparticles in the GFP-positive gate decreased (Figure 3). At 10 μM rifampicin, the percentage of the GFP-positive population approached the uninduced controls (1.1−1.2%), indicating that at this concentration growth was inhibited. The MIC we determined using microparticles (10 μM) closely matches the value determined using a dilution technique (10−12.5 μM).[33,34] The agar dilution method required 24−48 h of incubation after inoculating several plates of agar with cells and would have consumed at least 600 mL of solid media and 10 mg of antibiotic. By contrast, the microfluidic technique for determining the MIC of rifampicin reduced the total amount of reagents used by 60-fold and the experimental time by 16-fold.

Screening and Isolation of Spontaneous Mutants. The encapsulation of bacterial cells in microparticles combined with FACS may be a powerful tool for rapidly isolating strains of bacteria *via* changes in genotype and phenotype. To explore this application, we used this technique to isolate and characterize spontaneous mutants that are resistant to rifampicin. Although rifampicin is commercially available in large quantities, we used it as a surrogate of structurally complex natural products that are not readily available and for which screening and isolation of resistant mutants using conventional microbiological techniques may be challenging. The frequency of spontaneous mutants for resistance against small molecules in the rifamycin family is between 10^{-8} and 10^{-9} per bacterium per cell division.[35,36] Traditionally these mutants are selected on agar plates that contain growth media and an antibiotic at a concentration above its MIC. The isolation of spontaneous mutants using conventional approaches typically requires liters of

solid media containing a high concentration of the target antibiotic (typically tens of milligrams per liter) and takes 2−4 days of incubation to obtain visible colonies on plates from which cells are isolated. A high-throughput approach using agarose microparticles with a picoliter volume combined with FACS should make it possible to reduce the time and amount of antibiotic for the isolation of mutants.

We encapsulated cells of strain MG1655-p*tet*EGFP in agarose microparticles and dosed them with rifampicin. As expected, most of the cells in microparticles did not express EGFP (99.8% of microparticles), indicating their sensitivity to rifampicin.[35] A small subpopulation of microparticles (0.2%; approximately 8 × 10^3 out of the 4 × 10^6 microparticles that we analyzed) was GFP-positive and was sorted into a tube containing liquid nutrient media. From these microparticles we isolated mutants, sequenced them, and identified an A1538T base-pair mutation in the *rpoB* ORF (Figure 4). This mutation results in a Q513L substitution, which has been described previously as conferring resistance to rifampicin.[33,34] Overall, the screen used a total of 65 μg of rifampicin and took 6 h to complete, compared to 15 mg of compound that would have been required for a 48 h screen using a traditional approach with agar plates.[34]

The microencapsulation technique has several advantages over conventional, Petri dish based approaches for isolating bacteria on the basis of phenotypes: (1) The volume of the microparticles (*e.g.*, several picoliters) minimizes the experimental time and consumption of reagents. Encapsulating cells in agarose microparticles makes it possible to grow the cells through only a few cycles of division and detect them by FACS. In contrast, a typical colony on an agar plate contains ~10^9 bacterial cells;[37] a visible colony requires ~20 h of growth from a single cell that has a doubling time of 40 min. (2) FACS is a sensitive technique that can detect low levels of fluorescence in a colloidal sample. This characteristic can be used to detect changes in fluorescence in individual encapsulated cells.[38,39] Using FACS for the analysis of cells expands the number of genotypes and phenotypes that can be simultaneously quantified in a high-throughput screen, as each can be coupled to a fluorescent reporter. Many FACS instruments can simultaneously measure up to 16 different fluorophores in a sample, which makes it possible to multiplex analyses. (3) The encapsulation and growth of cells in microparticles that are suspended in oil can protect the user from toxic reagents that are either incorporated into the colloids or are produced by cells. These compounds only diffuse out of the colloids if they are soluble in mineral oil. Replacing the mineral oil as the continuous phase with fluorous liquids, such as perfluorodecalin, will improve the insulation of the user from these compounds by reducing their solubility in the continuous phase fluid.[8] This characteristic may be particularly useful in research with biosafety level two and three organisms and select chemicals. (4) The use of agarose as the hydrogel has several advantages, including biocompatibility, a gelling temperature that is close to the optimal growth temperatures for bacteria, relatively low cost, and availability in most biological laboratories. (5) PDMS devices are straightforward to fabricate and operate and users can easily manipulate the layout and dimensions of the systems using soft lithographic techniques.[28] The devices described in this manuscript produce agarose microparticles at a frequency of >500 Hz. Droplet formation at frequencies of 10^3−10^4 Hz have been reported in the literature for similar microfluidic systems,[29,31] which will reduce the time required to encapsulate a large population of cells for analysis and selection.

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dx.doi.org/10.1021/cb100336p |*ACS Chem. Biol.* 2011, 6, 260–266
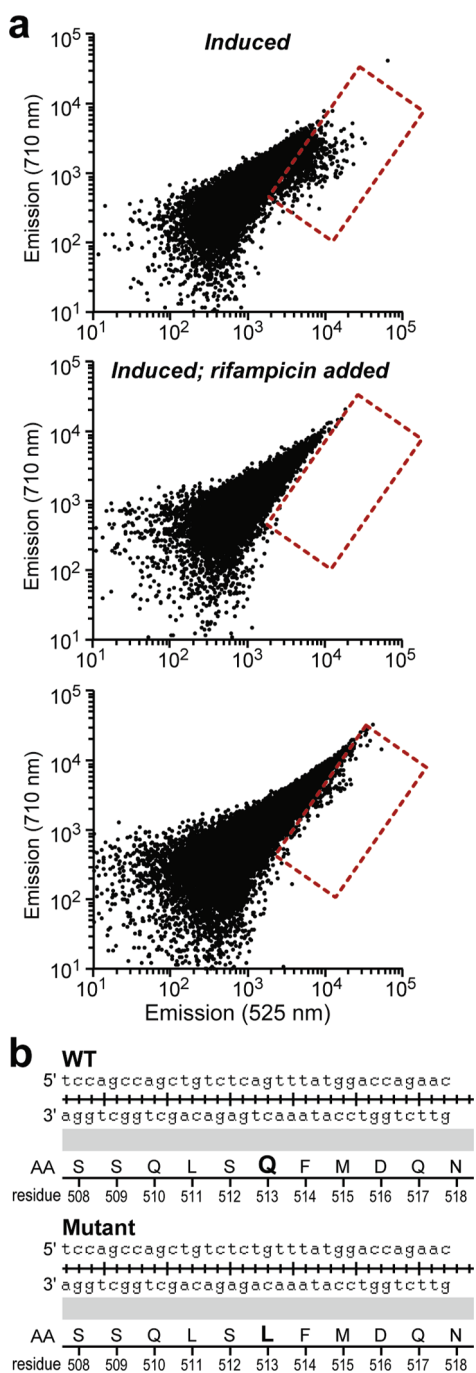
Figure 4. Spontaneous mutant screen of *E. coli* cells in agarose microparticles. (a) We analyzed the microparticles using FC. By comparing the fluorescence emission at wavelengths of 525 and 710 nm, we differentiated a subpopulation with a positive EGFP signal for the induced control (the uppermost plot). The GFP-positive gate is drawn as a dashed box. As a negative control, we analyzed microparticles containing both inducer and rifampicin (the middle plot). The negative control had negligible emission in the GFP-positive gate since a monoculture of wildtype *E. coli* cells was sensitive to the antibiotic. By contrast, a small population of microparticles that contained cells from a pool of 480 independent cultures showed fluorescence emission in the GFP-positive gate despite the presence of rifampicin (the bottom plot). This GFP-positive subpopulation were isolated *via* FACS. (b) Mutants that were isolated by FACS contained a mutation in the *rpoB* gene, resulting in a Q513L substitution. The panel shows the DNA sequence of the region, the corresponding amino acids (AA), and residue numbers. The mutated amino acid is indicated with a bold letter.

The devices in the manuscript produce monodisperse microparticles with a user-defined diameter, which makes it possible to analyze samples by FACS without first filtering out particles that are too large for the instrument. The ability to omit the filtration step avoids sample bias.

There are several characteristics of this approach that currently limit its application. Measuring the fluorescence emission of cells encapsulated in microparticles requires that cells produce fluorescent molecules or proteins. In many cases this criteria is not an issue and can be solved by genetic engineering. A fundamental limitation is that it may be difficult to rely on fluorescence detection when isolating and amplifying bacteria that have not been previously cultured or that are genetically intractable. A potential solution is to use side light scattering for measuring cell growth in microparticles. In addition, long incubation periods can lead to release of cells from microparticles, which will be eliminated from analysis or which may adsorb on the surface of other colloids and contaminate them. The optimization of growth time and conditions can transcend this limitation. Finally, the small molecule we tested is an antibiotic and provides an obvious phenotype for screening as it prevents cell replication. The biological target of other small molecules may be more difficult to discern using this approach and may require the development of more sophisticated methods of analysis.

In addition to its application in the high-throughput screen for studying target proteins of biologically active small molecules in microorganisms, we anticipate that encapsulating and analyzing single bacterial cells in agarose microparticles will have a variety of applications in environmental microbiology and for studying the human microbiome. Since encapsulation of cells makes it possible to detect and analyze slow-growing and rare species in a mixed population by using FACS, bacterial strains that are isolated and amplified in microparticles can be sequenced to catalog microbes associated with a particular ecological niche. Manipulating the liquid in which the microparticles are dispersed and incubated provides control over the diffusion of secondary metabolites in and out of the colloids, which may promote growth of species that are otherwise difficult to culture using standard media.[2,40] Finally, this technique may find applications in areas that require phenotype-based screening of bacteria, including biosensing, biological engineering, and synthetic biology.

■ METHODS

Fabrication of Microfluidic Devices. We used photolithography and soft lithography to fabricate microfluidic devices. Details of the fabrication process can be found in the Supporting Information.

Fabrication and Characterization of Microparticles. We dissolved agarose in water or growth media at a final concentration of 2% v/v and used light mineral oil as the continuous phase for the emulsification of the agarose solution. To stabilize the pregelled agarose droplets and prevent coalescence, we added 4% (v/v) of the surfactant Span80 to the mineral oil. We filtered the agarose solutions and oil through 0.22 μm pore size syringe filters and loaded them into glass gastight syringes. We used positive displacement syringe pumps (Harvard Apparatus) to control the flow rate of fluids in the microfluidic devices. To prevent gelation of the agarose in the syringe and in the device during the production of microparticles, we heated the syringes on the pumps with heating tape to maintain a temperature of 40 °C; we heated the microfluidic device to 37 °C. Procedures for imaging microparticles and encapsulating cells are described in the Supporting Information.

Microbial Cell Culture and Growth Conditions. Details on bacterial strains and growth conditions can be found in the Supporting Information.

Using a Poisson Distribution to Encapsulate Single Cells. To guide the dilution of cells to encapsulate one cell per microparticle, we used the Poisson equation. Detailed description of the calculations can be found in the Supporting Information.

Flow Cytometry and Fluorescence-Activated Cell Sorting. We used FACS to sort the population of microparticles containing EGFP-expressing cells into the wells of 96-well microplates, culture tubes, or onto LB agar plates (Supplementary Figure S1). For these experiments, we used a SORP BD FACS Aria instrument at the Flow Cytometry Facility at the UW Carbone Cancer Center. Prior to analyzing the microparticles using FACS, we extracted the microparticles from the oil phase and transferred them into a solution of PBS or growth medium containing the appropriate antibiotics and small molecule inducers. To transfer the microparticles from oil to the buffer, we rinsed the microparticles with pure mineral oil seven times to remove the surfactant. For the first three rinse cycles, we spun the microparticles in microcentrifuge tubes at $100g$ for 3 min; we reduced the centrifugation time to 30 s for the subsequent four cycles. After rinsing with mineral oil, we carefully pipetted the microparticle−oil emulsion on top of $700\,\mu$L of growth medium or PBS in a microcentrifuge tube. The density of the microparticles was higher than the oil and the particles gradually moved into the aqueous solution. To prevent the possibility of clogging the FC or FACS, we filtered the aqueous suspension of microparticles through a Nylon filter with 60-μm diameter pores. This step was not necessary but was carried out at the request of the FACS facility. We acquired FC data by collecting >10,000 data points for each sample; for FACS, we used an 85 μm diameter nozzle tip at a liquid pressure of 45 psi.

Determination of the Minimum Inhibitory Concentration. We determined the minimum inhibitory concentration (MIC) of rifampicin that prevents the growth of strain MG1655-p*tet*EGFP in liquid cultures (*i.e.*, macrodilution method)[41] and in agarose microparticles. For the encapsulated cells in microparticles, we incubated the samples for 3 h and induced the cells with anhydrotetracycline for 45 min prior to using FC. Details on MIC determination can be found in the Supporting Information.

Screen and Isolation of Spontaneous Mutants. To increase the probability of isolating spontaneous mutants to rifampicin, we incubated 480 independent cultures of strain MG1655-p*lac*EGFP in 96-well microplates (200 μL per well). We treated encapsulated cells with the antibiotic for 3 h at 37 °C prior to FACS. We incubated sorted microparticles at 37 °C for 17 h for release of cells and subsequently plated the released cells on LB agar to obtain single colonies. Ten colonies were selected and their *rpoB* gene was amplified and sequenced. Details on this experiment can be found in the Supporting Information.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

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