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Detecting bacteria and determining their susceptibility to antibiotics by stochastic confinement in nanoliter droplets using plug-based microfluidics†

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This article describes plug-based microfluidic technology that enables rapid detection and drug susceptibility screening of bacteria in samples, including complex biological matrices, without pre-incubation. Unlike conventional bacterial culture and detection methods, which rely on incubation of a sample to increase the concentration of bacteria to detectable levels, this method confines individual bacteria into droplets nanoliters in volume. When single cells are confined into plugs of small volume such that the loading is less than one bacterium per plug, the detection time is proportional to plug volume. Confinement increases cell density and allows released molecules to accumulate around the cell, eliminating the pre-incubation step and reducing the time required to detect the bacteria. We refer to this approach as 'stochastic confinement'. Using the microfluidic hybrid method, this technology was used to determine the antibiogram – or chart of antibiotic sensitivity – of methicillin-resistant *Staphylococcus aureus* (MRSA) to many antibiotics in a single experiment and to measure the minimal inhibitory concentration (MIC) of the drug cefoxitin (CFX) against this strain. In addition, this technology was used to distinguish between sensitive and resistant strains of *S. aureus* in samples of human blood plasma. High-throughput microfluidic techniques combined with single-cell measurements also enable multiple tests to be performed simultaneously on a single sample containing bacteria. This technology may provide a method of rapid and effective patient-specific treatment of bacterial infections and could be extended to a variety of applications that require multiple functional tests of bacterial samples on reduced timescales.

Introduction

In this manuscript, we demonstrate a method of rapid bacterial detection and drug susceptibility screening by using plug-based microfluidics to expedite the diagnosis and treatment of bacterial infections. Bacterial infections are a major health problem, leading to more than 130 000 deaths annually just from sepsis in the United States alone.[1] These deaths are often the result of nosocomial, or hospital-acquired, infections and frequently involve drug-resistant strains of bacteria.[2,3] In addition, bacteremia, the presence of bacteria in the blood, is one of the major causes of sepsis and generally requires a minimum of a day or more to diagnose, increasing the chances of patient mortality.[4] Patient mortality rates further increase when inappropriate antimicrobial treatment is administered, which is estimated to occur in 23–30% of cases.[4]

Shortening the time necessary to detect and identify an effective antibiotic regimen to treat bacterial infections could significantly decrease the mortality rate and reduce the cost of treating patients with sepsis and other aggressive bacterial infections.[5] However, attempts to reduce the assay time of traditional diagnosis and characterization techniques are impeded by the necessity to incubate bacterial specimens for hours to days to increase the cell density in the sample to detectable levels. To overcome this challenge, new PCR-based detection methods enable diagnosis in the 1–4 hour time frame.[6,7] However, these methods only provide a genetic profile of the infecting bacterial species and lack the ability to directly test the bacteria's function, such as susceptibility to particular antibiotics. Although some types of antibiotic resistance have genetic markers, the *mecA* gene, for instance,[8] genetic markers have not been identified for all antibiotic-resistant strains of bacteria. Therefore, antibiotic susceptibility is more accurately determined by a functional assay, especially for bacterial strains with unknown resistance mechanisms.

Microfluidics is an attractive platform for rapid single-cell functional analysis.[9–17] Plugs – droplets of aqueous solution surrounded by a fluorinated carrier fluid – provide a simple platform for manipulating samples with no dispersion or losses to interfaces.[18,19] Here, we show that microfluidic plug-based assays provide the ability to reduce detection time by confining bacteria into nanoliter-sized plugs. This confinement – we refer to it as 'stochastic confinement' – decreases the detection time by confining the sample into plugs that either have a single bacterium or are empty. This approach increases the effective concentration of the bacterium and allows released molecules to accumulate in the plug. Such stochastic confinement is commonly used for single-cell analysis in microfluidic devices,[9,12,14,20–22] and similar techniques have been used for

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single-molecule and single-enzyme work.[23–28] Microfluidics also enables simultaneous execution of numerous assays of bacterial function from a single bacterial sample in the same experiment, which is especially useful for rapid antibiotic susceptibility screening. Previously, gel microdroplets have been utilized for susceptibility screening.[29,30] However, this method did not take advantage of the stochastic confinement effects in plugs or high-throughput screening methods of current microfluidic technologies. Here, we applied this technology to characterize the drug sensitivity of a drug-resistant strain of *Staphylococcus aureus* (*S. aureus*) and measure the minimal inhibitory concentration of the antibiotic cefoxitin (CFX). We also successfully distinguished between sensitive and resistant strains of *S. aureus* in infected samples of human blood plasma. This technology offers two advantages over traditional bacterial detection and drug screening methods: (1) stochastic confinement of single cells from dilute samples concentrates the bacteria, eliminates the need for pre-incubation, and reduces detection time; and (2) each assay can be performed with an individual bacterium, enabling hundreds of assays to be performed by using a single, low-density bacterial sample without pre-incubation. This technology will reduce the time needed to diagnose bacterial infections and could eventually enable patient-specific antibiotic regimens.

Experimental

Bacterial cell culture

Cells were obtained from ATCC [*S. aureus* ATCC# 25923 (MSSA) and *S. aureus* ATCC# 43300 (MRSA)]. Stock solutions of the cells were made by using Luria–Bertani media Miller formulation (LB) (BD, Sparks, MD) containing 30% (v/v) glycerol and stored at −80 °C. For each experiment, a vial of frozen stock was brought to room temperature, streaked onto a Modified Trypticase Soy Agar (TSA II, BD, Sparks, MD) plate, and incubated overnight at 30 °C. Colonies from the plates were transferred to LB and cultured at 37 °C and 140 rpm for 3 h at which point OD_{600} was 1.5–2.0. Cell densities were then adjusted by diluting in LB. To maintain sterility, all procedures were performed in a biosafety cabinet and all tubing, devices, syringes, and solutions used were either autoclaved, sterilized by EtOH, packaged sterile, or filtered through a 0.45 μm PES or PTFE filter.

Comparing detection times of bacteria in nanoliter plugs and milliliter-scale culture

Plugs were formed by using the general methods described previously.[31,32] Plugs were formed in a three-inlet PDMS device with 100 μm wide channels by flowing *S. aureus* culture in LB at 2×10^5 CFU mL^{-1} at 1 μL min^{-1}, a 20% alamarBlue solution in saline at 1 μL min^{-1}, and fluorinated carrier fluid at 5 μL min^{-1}. Twenty-five plugs were collected in the channel. Inlets and the outlet were sealed with silicon grease, and the device was placed in a Petri dish containing LB for incubation. The same aqueous solutions were mixed 1 : 1 (total volume 0.6 mL) in a 14 mL polypropylene round-bottomed tube (BD Falcon, Franklin Lakes, NJ). After 2.8 h, plugs were made from the milliliter-scale culture by using the same method, flowing the cell culture containing alamarBlue into both aqueous inlets. Both sets of plugs were immediately imaged by using an epi-fluorescence microscope (IRE2, Leica) with a Cy3 (Chroma 41007, Cy3) filter and a 10 × 0.3 NA objective for a 5 ms exposure time with binning set to 4 and gain set to 200. Fluorescence images of plugs were processed by subtracting the average background intensity from all images. Linescans [Fig. 1(b)] with a width of 25 pixels were taken along the long axis of each plug.

Experiment to compare plug volume to detection time

PDMS devices with channel widths ranging from 200 to 800 μm were prepared. Teflon tubing with a diameter similar to that of the channel was cut at a 45° angle, inserted into the device up to the inlet junction, and sealed in place by using PDMS. For Fig. 1(c) and 1(d), plugs were formed as described previously, with the exception that the 1500 nL plugs were made *via* aspiration by using a manual aspirator. In addition, 1 nL plugs were formed in PTFE tubing with an outer diameter (OD) of 200 μm and an inner diameter (ID) of 90 μm, 690 nL plugs were formed in PTFE tubing with an OD of 700 μm and an ID of 600 μm, 100 and 120 nL plugs were formed in PTFE tubing with an OD of 800 μm and an ID of 400 μm, 12.6 nL plugs were formed in PTFE tubing with an OD of 260 μm and an ID of 200 μm, and 1500 nL plugs were formed in PTFE tubing with an OD of 1100 μm and an ID of 1000 μm. Plugs were collected in the Teflon tubing, the tubing was sealed with wax, and the tubing was placed in a Petri dish containing LB for incubation and imaging. Incubation and imaging were performed in a microscope incubator (Pecon GmbH, Erbach, Germany). Time zero is defined as the time at which the sample entered the incubator, which was less than 20 min after sample preparation. Fluorescence measurements were taken with 5 ms exposure times with a 5 × 0.15 NA objective by using a 1× camera coupler for 1, 12.6, 100, and 690 nL plugs, and a 0.63× camera coupler was used for 1500 nL plugs. Plugs 140 nL in volume were imaged with 10 ms exposure times with a 5 × 0.15 NA objective by using a 0.63× camera coupler.

Plugs were analyzed by first separating them from the background by thresholding to exclude intensity below 250. The average intensity of the thresholded plugs was measured. Over time, the intensity of the plugs diverged into two groups: occupied plugs and unoccupied plugs. All occupied plugs had a change in intensity more than two-fold greater than the change in intensity of unoccupied plugs. Detection time is defined as the time at which the fold change in intensity of the occupied plugs compared to the change in intensity of unoccupied plugs reaches a maximum. The fold change in intensity is defined as the change in intensity of an occupied plug divided by the average change in intensity of an unoccupied plug [eqn (1)].

$$\text{Fold change}_{(t=ti)} = \text{Occupied plug } (I_{t=ti} - I_{t=1}) / \text{Unoccupied plug } (I_{t=ti} - I_{t=1}) \quad (1)$$

In eqn (1), $I_{t=ti}$ is intensity at time point i. The intensity of the empty plugs is the average of all empty plugs in each experiment.

Screening susceptibility of bacteria to antibiotics

For antibiotic screening experiments (Fig. 2), an array of 50 nL antibiotic plugs was aspirated into Teflon tubing (200 μm ID) by using a manual aspirator. Air spacers were included between

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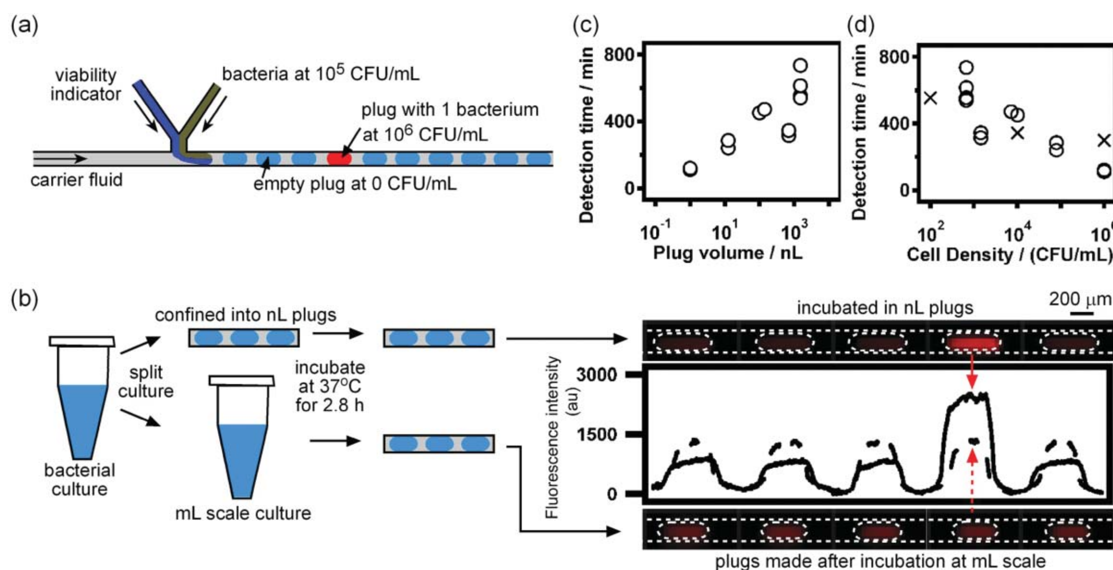


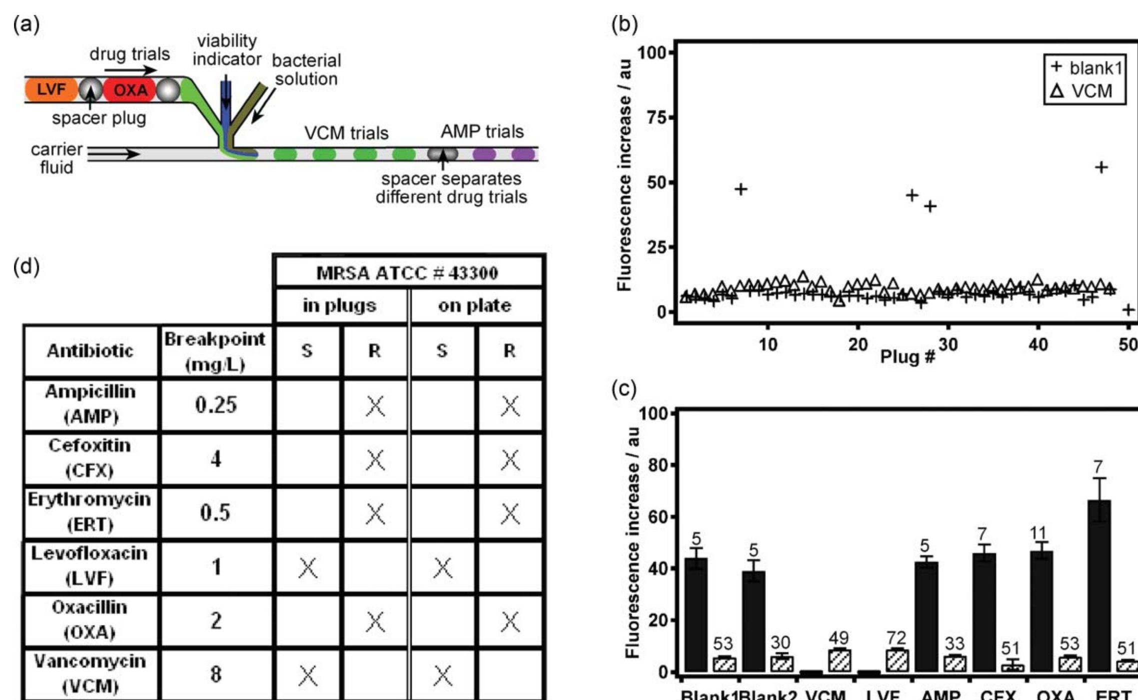
Fig. 1 Stochastic confinement of bacteria into plugs reduces detection time. (a) Schematic drawing illustrates the increase in cell density resulting from the stochastic confinement of an individual bacterium in a nanoliter-sized plug. While most plugs are empty, a few are occupied by a single bacterium at an effective concentration greater than the initial concentration. (b) Schematic drawing illustrates the experimental procedure to compare the detection of bacteria incubated in nanoliter-sized plugs and bacteria incubated in a milliliter-scale culture. See text for details. Linescans indicate that confining the bacteria at the beginning of incubation ($t = 0$) led to a few occupied plugs with a high fluorescence intensity and many empty plugs with low fluorescence intensity (solid line). All plugs made from the milliliter-scale culture had an intermediate fluorescence intensity (dotted line). (c) When confining single bacteria into plugs, the detection time decreased with the log of the plug volume. (d) The detection times measured for bacteria incubated in plugs (○) were similar to detection times measured for bacteria incubated in 96-well plates (×) with similar initial cell densities.

each antibiotic plug to prevent merging of adjacent antibiotic plugs and to enable indexing of plugs in the output array. Plugs of saline solution were included as the first and last plugs in the pre-formed array to serve as positive controls and are referred to as blank conditions. The Teflon tubing containing the array of antibiotic plugs was sealed into a device inlet by using wax (Hampton Research, Aliso Viejo, CA). To screen the susceptibility of MRSA (methicillin-resistant *S. aureus*) and MSSA (methicillin-sensitive *S. aureus*) to each antibiotic, bacterial samples and indicator were merged with the pre-formed array of antibiotic conditions by using the microfluidic hybrid method.[31] Bacterial samples were at a density of 4×10^5 CFU mL^{-1} in LB, and viability indicator solution was made by mixing four parts of alamarBlue solution (AbD Serotec, Oxford, UK) with six parts of 150 mM NaCl. The flow rate of the antibiotic array was 0.25 μL min^{-1}, the flow rate of the bacterial solution was 0.5 μL min^{-1}, and the flow rate of the viability indicator was 0.25 μL min^{-1}. The carrier fluid was FC40 (Acros Organics, Morris Plains, NJ) with a flow rate of 1.6 μL min^{-1}. For each antibiotic plug in the pre-formed array, approximately 50 smaller plugs (4 nL in volume) were formed, each potentially containing a single bacterium. The resulting plugs were collected in a coil of Teflon tubing (150 μm ID).

After plug formation, the tubing was disconnected from the PDMS device, and the ends were sealed with wax. The Petri dish containing the tubing was filled with 20 mL of LB solution to prevent evaporation of the plugs during incubation. The plugs were immediately transferred to a microscope incubator (Pecon GmbH, Erbach, Germany). Time zero is defined as the time when the plugs entered the incubator, which was about 20 min after plugs were formed. Fluorescence measurements for the plugs were recorded by using an inverted epi-fluorescence microscope (DMI6000, Leica, Bannockburn, IL) with a 10 × 0.3 NA objective (HCX PL Fluotar) coupled to a CCD camera ORCA ERG 1394 (12-bit, 1344 × 1024 resolution) (Hamamatsu Photonics) by using a 0.63× camera coupler. Images were taken of each plug by using Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA) every 30 min with exposure times of 5 ms. Plugs were analyzed by first separating them from the background by thresholding to exclude intensity below 250. The average intensity of the thresholded plugs was measured. The change in intensity at time point *ti* is $I_{t=ti} - I_{t=1}$. In the experiments described in Fig. 2(c), 3(b) and 3(c), the fluorescence intensity of the plugs was normalized by setting the intensity of the brightest plug to 100.

Detection and drug susceptibility screening of MRSA and MSSA in human blood plasma

For Fig. 4, cells were suspended in a 1 : 1 mixture of human blood plasma (pooled normal plasma George King Bio-Medical, Overland Park, KS) and LB containing 40% alamarBlue. Plugs were formed and collected in Teflon tubing (200 μm ID). Images were taken with a 5 × 0.15 NA objective with a 0.63× camera coupler. Texas red pictures were taken every 10 min with exposure times of 25 ms. A brightfield image was taken at the beginning and end of the experiment. Linescans of original plug images were taken at time 0 and time 7.5 h. Adobe Photoshop was used to enhance the contrast of the plugs shown in Fig. 4. Fluorescence intensities were normalized to the maximum concentration at $t = 0$ for Fig. 4(b)–(e).



Antibiotic	Breakpoint (mg/L)	MRSA ATCC # 43300 in plugs S	in plugs R	on plate S	on plate R
Ampicillin (AMP)	0.25		X		X
Cefoxitin (CFX)	4		X		X
Erythromycin (ERT)	0.5		X		X
Levofloxacin (LVF)	1	X		X	
Oxacillin (OXA)	2		X		X
Vancomycin (VCM)	8	X		X	

Fig. 2 A combination of stochastic confinement with the microfluidic hybrid method was used to screen many antibiotics against the same bacterial sample. (a) Schematic drawing illustrates the formation of plugs of bacteria, viability indicator, and antibiotic from a pre-formed array of plugs of different antibiotics. Approximately 50 plugs were formed with each antibiotic in the screen. (b) The increase in fluorescence intensity of the control plugs with no antibiotic (+, Blank1, positive control) and vancomycin (△, VCM, negative control) are shown. After incubation, four plugs contained live bacteria in the positive control sample, but no plugs contained live bacteria in the negative control sample treated with vancomycin, indicating that bacteria were not resistant to this antibiotic. (c) Bar graph shows the results of the antibiotic screen against the methicillin-resistant *S. aureus* (MRSA), indicating that this strain of MRSA was resistant to four antibiotics, but sensitive to two. The bars show the average increase in fluorescence intensity of all plugs that were above (solid) or below (striped) three times the intensity of the VCM baseline (see Experimental for details). *N*, shown above each bar, equals the number of plugs for each condition, and the error bars show the standard error. See ESI† for statistical analysis. (d) Chart shows the agreement between the susceptibility profiles (S, sensitive and R, resistant) of MRSA determined by the plug-based microfluidic screen and the control susceptibility screen using Mueller–Hinton plates. See text for details.

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A detailed explanation of following procedures can be found in the supplemental material: microfluidic device design and fabrication, flowing solutions into the microfluidic devices, antibiotic preparation, antibiotic testing on plates, comparing detection times of bacteria in nanoliter plugs and 96-well plates, determining the minimal inhibitory concentration of a drug against a bacterial sample, and statistical analysis of screening results.

Results and discussion

Stochastic confinement of individual bacteria into plugs nanoliters in volume reduces detection time

To reduce the time required to detect bacteria in a sample, we designed a microfluidic device to confine a single bacterium into plugs nanoliters in volume. In principle, when generating plugs with a small volume from a solution with a low concentration of bacteria, much of the volume of the initial solution forms plugs that contain no bacteria. There are a few plugs occupied by a single bacterium. As a result, the concentration of bacteria in the occupied plugs is greater than the concentration of bacteria in the initial solution. For example, if plugs nanoliters in volume were made from a culture with an initial bacterial concentration of 10^5 CFU mL^{-1}, one in ten plugs would receive a single bacterium, as illustrated in Fig. 1(a). The concentration of cells in these occupied plugs would be one bacterium per nanoliter or 10^6 CFU mL^{-1}. In other words, 10^5 CFU mL^{-1} corresponds on average to 0.1 bacterium per 1 nL, and confining this solution into nanoliter plugs creates many plugs with no bacteria per nanoliter and a few plugs with one bacterium per nanoliter. CFU mL^{-1} refers to the colony forming units (CFU), a measure of live bacteria, per milliliter.

To monitor the presence of metabolically active bacteria in plugs, a fluorescent viability indicator, alamarBlue, was added to the cultures. The active ingredient of alamarBlue is the fluorescent redox indicator resazurin.[33] Resazurin is reduced by electron receptors used in cellular metabolic activity, such as NADH and FADH, to produce the fluorescent molecule resofurin. Therefore, fluorescence intensity in a plug is correlated with the presence and metabolic activity of a cell, in this case, a bacterium. Because resazurin indicates cell viability, resazurin-based assays have been used previously in antibiotic testing.[34–37] Here, we use resazurin to detect both the presence of a live bacterium and the response of bacteria to drugs, such as antibiotics. We postulated that stochastic confinement would decrease the detection time, because the bacterium is at an effectively higher concentration when confined in a plug

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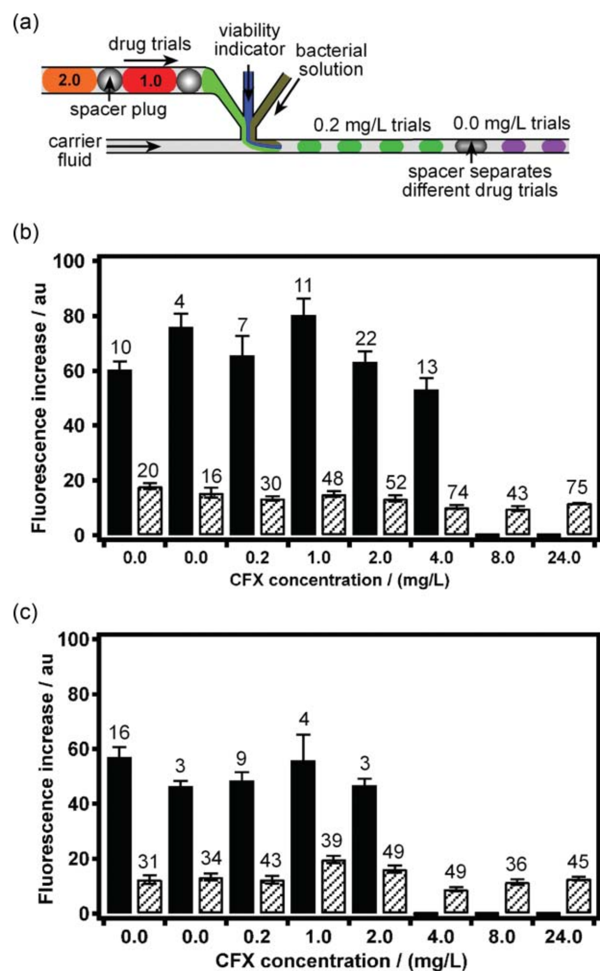

Fig. 3 A microfluidic plug-based assay was used to identify the minimal inhibitory concentration (MIC) of cefoxitin (CFX) for methicillin-sensitive *S. aureus* (MSSA) and methacillin-resistant *S. aureus* (MRSA). (a) Schematic drawing illustrates the formation of plugs of bacteria, viability indicator, and an antibiotic at varying concentrations. In the schematic, numbers inside the pre-formed plugs symbolize the final concentration of CFX in those plugs. (b and c) Using 24 mg L^{-1} CFX as the baseline, graphs show the average change in fluorescence intensity of plugs greater than (solid) and less than (striped) three times the baseline for MRSA (b) and MSSA (c). *N*, shown above each bar, equals the number of plugs for each condition, and the error bars show the standard error. These results indicated that MRSA has a higher MIC than MSSA. See ESI† for statistical analysis.

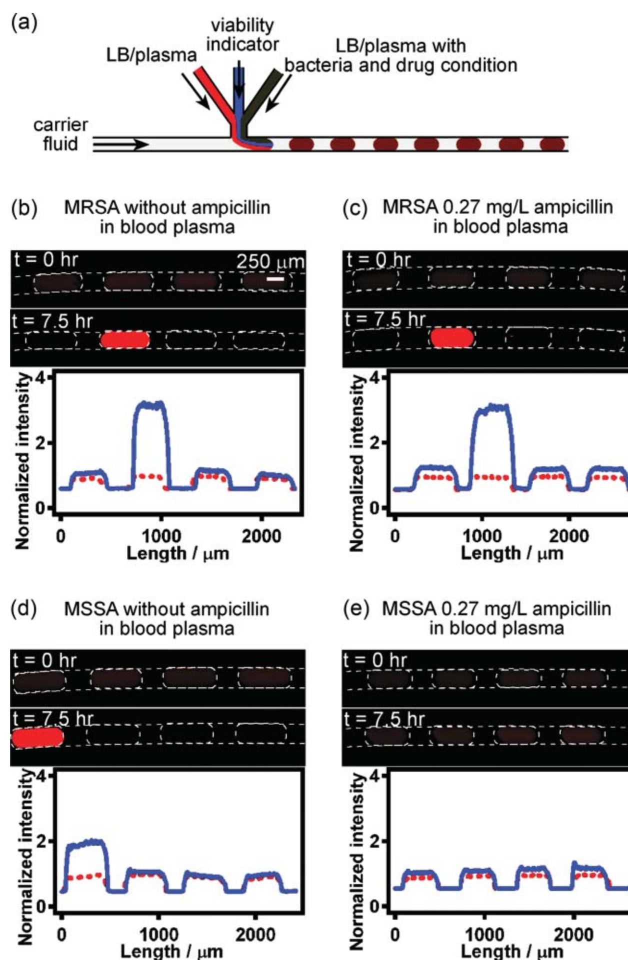

Fig. 4 A combination of stochastic confinement with the plug-based microfluidic assay was used to determine susceptibility of bacteria to an antibiotic in a natural matrix, blood plasma. (a) Schematic drawing illustrates the formation of the plugs of bacteria, viability indicator, antibiotic, and the plasma/LB mixture. (b–e) Linescans indicate that fluorescence increases after 7.5 h (blue solid line) in the cases of no antibiotic or bacteria resistant to the antibiotic. (b and c) Images and linescans of four representative plugs made from a 1 : 1 blood plasma/LB sample inoculated with MRSA without (left) and with (right) the addition of ampicillin (AMP). (d and e) Images and linescans of four representative plugs made from a 1 : 1 blood plasma/LB sample inoculated with MSSA without (left) and with (right) the addition of AMP. Linescans show a change in fluorescence intensity across width of the images for plugs at $t = 0$ (red dashed line) and $t = 7.5$ h (solid blue line). The scale bar in (b) applies to all images. All images were contrast-enhanced for clarity and should be interpreted qualitatively; linescans of raw intensities are provided to convey the information quantitatively.

than in the starting solution, and the signal-to-noise required for detection would be reached sooner as the product of the reduction of resazurin accumulates in the plug more rapidly.

To demonstrate the ability of stochastic confinement to reduce the detection time, a single sample of *S. aureus* containing the fluorescent viability indicator was split – half of the culture was used to generate plugs nanoliters in volume, and the other half remained as a milliliter-scale culture. Both the nanoliter plugs and the milliliter-scale culture were incubated for 2.8 h at 37 °C. After incubation, the milliliter-scale culture was also used to form plugs. This experimental procedure is illustrated in Fig. 1(b). Confining bacteria into plugs nanoliters in volume reduced the time required to detect a change in fluorescence intensity of the viability indicator. Bacteria confined to and incubated in nanoliter-sized plugs showed a greater change in fluorescence intensity after 2.8 h than the bacteria incubated in the 'unconfined' milliliter-scale culture. Linescans of the plugs of bacteria that were incubated in plugs showed many empty plugs with low fluorescence intensity and a few occupied plugs with high fluorescence intensity [Fig. 1(b), top row of plugs and solid line]. However, linescans of plugs of bacteria that were incubated in the milliliter-scale culture have a lower, uniform fluorescence intensity [Fig. 1(b), bottom row of plugs and dotted line]. Therefore, bacteria confined to nanoliter-sized plugs could be detected earlier than bacteria in a milliliter-scale culture.

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In plugs containing a single bacterium, the detection time was proportional to plug volume. The detection time was defined as the time at which the increase in fluorescence intensity reached a maximum. When single bacteria were confined in plugs ranging from 1 to 1500 nL in volume, the detection time increased with the log of plug volume [Fig. 1(c)], implying that bacteria were dividing exponentially inside the plugs. This result is similar to previous estimates that the detection time decreases by about 1.5 h for every order of magnitude increase in cell density.[38] The detection times measured for bacteria incubated in plugs were similar to detection times measured for bacteria incubated in a 96-well plate from cultures with similar initial cell densities [Fig. 1(d)]. This result implies that incubation in plugs had no adverse effects on the growth of bacteria and suggests that the reduction in detection time is the result of higher initial cell density due to confinement.

The sensitivity of a bacterial strain to many antibiotics can be screened in a single experiment by using plug-based microfluidics

Next, we extended this system to screen a single bacterial sample against many antibiotics to generate an antibiogram, or chart of drug susceptibility. A pre-formed array of plugs of six antibiotics – two beta-lactams (ampicillin, AMP, and oxicillin, OXA); a cephalosporin (cefoxitin, CFX); a fluoroquinolone (levofloxicin, LVF); vancomycin, VCM; and a macrolide (erythromycin, ERT) – was generated by aspiration. Antibiotics were tested at the breakpoint concentration,[39] the accepted concentration of antibiotic at which bacterial susceptibility is determined [Fig. 2(d)]. Using the microfluidic hybrid method,[31] the plugs in the antibiotic array were merged with methicillin-resistant *S. aureus* (MRSA, ATCC# 43300) at an initial cell density of 4×10^5 CFU mL^{-1} and the viability indicator on-chip to form plugs approximately 4 nL in volume, as illustrated in Fig. 2(a). Since stochastic confinement of the bacterium into nanoliter-sized plugs generates many empty plugs, approximately 50 plugs were generated for each antibiotic condition such that it was statistically likely that each condition would contain several plugs occupied by a single bacterium. In total, 400–500 plugs were formed for each screen, which consisted of six drug conditions and two blank conditions. All 400–500 plugs were collected in the same coil of tubing. A blank condition was located at the beginning and end of the array to ensure that the position in the array did not affect the assay results. The merged plugs were collected and incubated for 7 h at 37 °C. After incubation, the fluorescence intensity of the plugs was measured.

Occupied plugs containing an antibiotic to which the bacterial strain was resistant showed increased fluorescence intensity, whereas plugs containing an antibiotic to which the bacterial strain was sensitive showed no significant increase in fluorescence intensity [Fig. 2(c)]. Plugs containing VCM were used as a negative control, because VCM inhibited this *S. aureus* strain in macro-scale experiments, in agreement with expectations[40] (data not shown). The average increase in fluorescence from all plugs containing VCM was used as a baseline to which the increase in fluorescence intensity of all other plugs was compared [Fig. 2(b): △, VCM]. Five out of 58 control plugs (9%) with no antibiotic [Fig. 2(b): +, Blank1, only the first 50 plugs shown] showed an increase in fluorescence intensity more than three times greater than the VCM baseline, indicating that they were occupied by bacteria. However, the other plugs with no antibiotic showed an increase in fluorescence intensity similar to the baseline, indicating that they were unoccupied [Fig. 2(b): +, Blank1].

By comparing the fluorescence increase in each plug with the VCM baseline, we can determine which antibiotics were toxic to the bacteria. Plugs occupied with a viable bacterium showed an increase in fluorescence intensity greater than three times the VCM baseline. Fig. 2(c) shows the average intensities of plugs that showed an increase in fluorescence intensity greater than three times the baseline (black bars) and plugs that showed an increase in fluorescence intensity less than three times the baseline (striped bars). No plugs containing VCM or LVF had a fluorescence increase greater than three times the baseline, indicating that MRSA was sensitive to these antibiotics (see ESI† for statistical analysis). Poisson statistics [eqn (2)] can be used to predict the probability of not loading a bacterium into any of plugs in the conditions LVF or VCM. In other words, eqn (2) predicts the possibility of the LVF or VCM results being false-negative.

$$f(k,\lambda) = \frac{\lambda^k e^{-\lambda}}{k!} \tag{2}$$

In eqn (2), f is the probability of having k bacteria in a plug given an average bacterial loading of λ bacteria per plug. The experimentally determined λ was 0.11, as 11% of control plugs with no antibiotics received bacteria [Fig. 2(c): Blank1 and Blank2]. For $k = 0$ and $\lambda = 0.11$, we calculated the probability of having an unoccupied plug to be 0.896. The probability of having 49 unoccupied plugs is 0.896^{49}, or 0.0046. Given that LVF and VCM had at least 49 plugs, the probability of a false-negative due to loading is less than 0.5%.

The results from the MRSA antibiotic screen were used to make the antibiogram in Fig. 2(d). The antibiotics were tested at the breakpoint concentration, and the fluorescence data were used to determine whether the bacterial strain was sensitive (S) or resistant (R) to the antibiotic. Sensitive means that no plugs containing a specific antibiotic showed an increase in fluorescence intensity greater than three times the VCM baseline. Resistant means that at least one plug containing a specific antibiotic showed increased fluorescence intensity greater than three times the VCM baseline. The susceptibility profile generated for MRSA by using the microfluidic screen [Fig. 2(d)] was identical to the profile generated by using Mueller–Hinton agar plate tests and similar to previous reports in the literature for MRSA.[40] However, antibiotic sensitivity testing is influenced by many factors, including bacterial load, culturing conditions, temperature, bacterial strain, and type of assay used to detect sensitivity. In addition, a cell population might contain sub-populations of cells with variable sensitivity to a given antibiotic. All of these factors should be considered and further characterized before formulating guidelines for implementing plug-based antibiotic sensitivity assays.

Plug-based methods can also be used to determine the minimal inhibitory concentration (MIC) of an antibiotic against a bacterial sample

Next, this microfluidic approach was used to determine the MIC of the antibiotic cefoxitin (CFX) for MRSA and MSSA (Fig. 3).

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This assay was similar to the antibiotic screening assay described above, except that the pre-formed array of antibiotic plugs all contained the same antibiotic and the concentration of that antibiotic in each plug of the pre-formed array was different. Again, plugs containing saline solution (blank condition) were included at the beginning and end of the array to serve as negative controls and to ensure that the first and last plugs of the array gave similar assay results. The positive control plugs consisted of CFX at a concentration of 24 mg L^{-1}, as both strains were shown to be inhibited by CFX at this concentration. Using the microfluidic hybrid method,[31] plugs of the antibiotic array were merged with bacteria and the fluorescent viability indicator as illustrated in Fig. 3(a) and incubated at 32 °C. Plugs with MRSA were incubated for 6.75 h and plugs with MSSA were incubated for 6.5 h. It should be noted that temperature can affect the results of antibiotic sensitivity assays. Here, the difference in MIC of MRSA and MSSA was discerned by assays conducted at 32 °C.

After incubation, the fluorescence intensity of the plugs was measured. Here, the average increase in fluorescence intensity of plugs containing 24 mg L^{-1} CFX was used as the baseline to which the increase in fluorescence intensity of other plugs was compared. Because MRSA is resistant to many beta-lactam antibiotics, CFX should be less effective against the strain MRSA. As expected, the MIC of CFX was higher for MRSA (<8 mg mL^{-1}) than the MIC of CFX for MSSA (<4.0 mg mL^{-1}) [Fig. 3(b) and 3(c)]. These results validate the use of this plug-based technology for screening both the susceptibility and the minimal inhibitory concentration of many antibiotics against a single bacterial sample.

Microfluidic bacterial detection and drug screening are applicable to complex, natural matrices, including human blood plasma

To validate the applicability of this method to detecting bacteria in natural matrices, this method was used to detect bacteria in a sample of human blood plasma. Bacterial strains MSSA or MRSA were inoculated into pooled human blood plasma at a concentration of 3×10^5 CFU mL^{-1}. To test the sensitivity of the bacteria to beta-lactams, the antibiotic ampicillin (AMP) was added to the culture at the breakpoint concentration. The inoculated plasma was then combined on-chip with viability indicator as illustrated in Fig. 4(a). After 7.5 h of incubation at 37 °C, plasma samples infected with MRSA were distinguishable from samples infected with MSSA. While plugs containing MRSA and AMP showed a similar increase in fluorescence intensity to plugs containing MRSA and no AMP [Fig. 4(b) and 4(c)], plugs containing MSSA and AMP showed no increase in fluorescence intensity [Fig. 4(d) and 4(e)].

Conclusions

Stochastic confinement combined with plug-based microfluidic handling methods accelerates bacterial detection and enables rapid functional antibiotic screening. By using this method, assays can be performed on a single bacterium, potentially eliminating the need for pre-incubation. By confining and analyzing single bacterium in plugs, the detection time is now determined by plug volume. We were able to achieve detailed functional characterization of a bacterial sample in less than 7 h. We also demonstrated that a bacterium in a 1 nL plug can be detected in as little as 2 h. The detection time is limited by the formation and measurement of plugs of small volume and is less dependent on the initial concentration and growth rate of bacteria in the sample. This feature could be potentially important for accelerated detection of slowly-growing species such as *Mycobacterium tuberculosis*, a pathogen of significant importance world-wide.[41] Here, we have demonstrated a screen with 400–500 nL plugs. High-throughput screens with more conditions and increased concentration of the sample would require methods that can handle larger numbers of smaller plugs, including methods for automated sorting and analysis.[42–45] Upon incorporating such methods for handling and sorting large numbers of plugs of small volume, this technique could be used for the detection of bacteria in a sample at a cell density much lower than 10^5 CFU mL^{-1}. Since we are measuring the activity of single cells, it is conceivable that detecting the presence of even a single bacterium in a sample could be feasible.

Given that a typical 5 mL blood sample from a patient with bacteremia contains a cell density of 100 CFU mL^{-1},[46] this method is capable of performing dozens of functional tests on such a sample. Patient-specific characterization of bacterial species would not only lead to more rapid and effective treatment, but such an advance would also enable in-depth characterization of bacterial infections at the population level. Such detailed characterization may aid in tracking and identifying new resistance patterns in bacterial pathogens.[47,48] While clinical implementation of this technique would require further testing to determine appropriate guidelines for the execution and interpretation of plug-based sensitivity assays, this paper highlights the advantages of the confinement of single cells for biological assays. The principles of these methods, stochastic single-cell confinement and multiple functional assays without sample pre-incubation could also be applied to other areas, including performing functional tests on field samples, detecting contamination of food or water, separating and testing samples with mixtures of species, measuring functional heterogeneity in bacterial populations, and monitoring industrial bioprocesses.

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