Supplemental Material for

Detecting Bacteria and Determining Their Susceptibility to Antibiotics by Stochastic Confinement in Nanoliter Droplets Using Plug-based Microfluidics

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Microfluidic device design and fabrication

Microfluidic devices were fabricated by using soft lithography[1] as described previously.[2-4] Except where noted, plugs were collected in PFA or PTFE Teflon tubing (Zeus, Orangeburg, SC) with 150 μm or 200 μm inner diameter (I.D.). The tubing was cut at a 45° angle, inserted into the outlet of the microfluidic device up to the inlet junction, and sealed into the device by using PDMS prepolymer (10:1 elastomer to curing agent). To aid in imaging of the plugs, the Teflon tubing was wound in a spiral on a glass slide, and PDMS prepolymer was poured over the tubing to fix it in place. The device with attached tubing was then autoclaved at 135 °C for 10 min to sterilize. Once sterilized, the glass slide containing the tubing was transferred to a sterile Petri dish.

Flowing solutions into the microfluidic devices

All solutions were loaded into 1700 series Gastight syringes (Hamilton, Reno, NV) with removable 27 gauge needles and 30 gauge Teflon tubing (Weico, Wire & Cable, Edgewood, NY). To maintain sterility, the syringes were filled and attached to the device within a biosafety cabinet. Syringes were connected to the microfluidic devices by using 30 gauge Teflon tubing. Solutions where flowed into the microfluidic devices by using previously described methods.[4] Flow rates were controlled by using PHD 2000 infusion syringe pumps (Harvard Apparatus, Holliston, MA).

Antibiotic preparation

Antibiotic stock solutions of ampicillin (AMP), oxacillin (OXA), cefoxitin (CFX), levofloxacin (LVF), and vancomycin (VCM) were prepared by using 150 mM $NaCl_{aq}$ at a concentration of 4000 times greater than the final concentration in the plugs, filter sterilized, and then frozen at -80 ^{o}C (AMP, Fisher Bioreagents, Fair Lawn. NJ; OXA, LVF, Fluka, Buchs, Switzerland; CFX, VCM, ERT, Sigma, St. Louis, MO). For example, AMP was tested at the breakpoint concentration of 0.25 mg/L, meaning that the stock solution was prepared at a concentration of 1000 mg/L. In the case of erythromycin (ERT), a stock solution was prepared at 1000 times the final concentration in plugs. Before each experiment, vials of the antibiotics were thawed and diluted 1000x (250x for ERT) with saline containing 80 μM fluorescein carboxylate. Fluorescein carboxylate was used to aid in indexing the resultant array of plugs. The plugs in Fig. 4 contain no fluorescein carboxylate, since indexing was not required. The blank conditions consisted of 150 mM NaCl. Antibiotic solution were further diluted on chip 1:3 (v/v) during plug formation. 20 μM fluorescein carboxylate did not interfere with the viability assay, the activity of the cells, or effectiveness of antibiotic in tests performed on 96-well plates (data not shown).

Antibiotic testing on plates

Plates were made from Mueller Hinton Agar (Fluka, Switzerland). After autoclaving, the agar was cooled, antibiotics were added, and 20 mL plates were poured. For CFX and OXA testing, 50 μL of MRSA and MSSA bacterial culture at 4×10^3 CFU/mL was spread onto separate TSA plates. The plates were incubated at 30 ^{o}C. After 16.5 h and 40 h the plates were examined for colonies. MRSA colonies appeared on CFX after 16.5 h and on the OXA plates after 40 h. Even after 40 h, MSSA colonies did not appear on the CFX or OXA plates. For AMP, ERT, LVF, and VCM, 5 μL of culture at 2×10^4 CFU/mL were spread onto plates, and the plates were incubated at 37 ^{o}C for 12 h. After 12 h, growth of colonies on the plates was considered resistance to the antibiotic and no colonies on the plates was considered sensitivity to the antibiotic. For all tests, control plates with no antibiotic were inoculated to ensure that each plate tested received many CFU during inoculation.

Comparing detection times of bacteria in nanoliter plugs and 96-well plates

For Fig. 1d, 96-well plate results for Fig. 1d were acquired in a Tecan Safire II plate reader (MTX Lab Systems, Vienna, VA) with Ex/Em 560/630 nm, gain 25, and 40 μs integration time. 200 μL of cell culture suspended in LB with 10% alamarBlue was added to wells of a Costar 96-well assay plate with black sides and clear, flat bottom (Corning, Corning, NY). Each data represents triplicate measurements taken at 37 ^{o}C. Fold change in intensity from 96-well plate results were calculated by using Eq. 1, where the well with LB and alamarBlue only was the unoccupied plug condition.

Determining the minimal inhibitory concentration of a drug against a bacterial sample

For MIC determination in plugs (Fig. 3), a procedure similar to screening susceptibility of many antibiotics was used. The input array of antibiotics consisted of plugs of CFX at a range of concentrations. Bacterial samples were MRSA or MSSA in LB at cell densities near 10^6 CFU/mL. In Fig. 3b and c, fluorescence intensity of plugs was normalized as described for Fig. 2c.

Statistical Analysis of Antibiotic Screening Results

Unpaired t-tests were performed to compare antibiotic screening results to positive and negative controls. For Figure 2c: VCM and LVF are statistically different than positive controls and AMP, CFX, OXA, ERT, and blank conditions were all statistically different than the negative control. For Figure 3b: 8 and 24 mg/L CFX were statistically different than positive controls and 0, 0.2, 1, 2, and 4 mg/L were statistically different than the negative control. For Figure 3c: 4, 8, and 24 mg/L CFX were statistically different than positive controls and 0, 0.2, 1, and 2 mg/L were statistically different than the negative control. P values are two-tailed.

Statistical analysis of data in Figure 2c:

		average intensity	standard error	n
Blank1	above 3x	44.02	4.07	5
Blank2	above 3x	38.98	4.04	5
VCM	below 3x	8.65	0.35	49
LVF	below 3x	8.61	0.37	72
AMP	above 3x	42.49	2.17	5
CFX	above 3x	45.96	3.38	7
OXA	above 3x	46.95	3.42	11
ERT	above 3x	66.59	8.50	7

Compared to negative control, VCM below 3X

	t	p
Blank1	21.83	< 0.0001
Blank2	18.77	< 0.0001
LVF	0.09	0.9277
AMP	26.52	< 0.0001
CFX	24.45	< 0.0001
OXA	22.01	< 0.0001
ERT	18.27	< 0.0001

Compared to positive control, Blank1 above 3X

	t	p
Blank2	0.88	0.4051
VCM	21.83	< 0.0001
LVF	20.65	< 0.0001
AMP	0.33	0.7486
CFX	0.37	0.7206
OXA	0.51	0.6212
ERT	2.10	0.062

Compared to positive control, Blank2 above 3X

	t	p
Blank1	0.88	0.4051
VCM	18.77	< 0.0001
LVF	17.92	< 0.0001
AMP	0.77	0.4654
CFX	1.33	0.2136
OXA	1.38	0.1901
ERT	2.57	0.0278

Statistical analysis of data in Figure 3b:

		average intensity	standard error	n
blank1	above 3x	60.57	2.85	10
blank2	above 3x	76.01	4.87	4
0.2 mg/L	above 3x	65.71	7.06	7
1 mg/L	above 3x	80.49	5.89	11
2 mg/L	above 3x	63.36	3.79	22
4 mg/L	above 3x	53.32	4.11	13
8 mg/L	below 3x	9.90	0.97	43
24 mg/L	below 3x	11.94	0.71	75

Compared to negative control, 24 mg/L below 3x

	t	p
blank1	22.06	< 0.0001
blank2	19.63	< 0.0001
0.2 mg/L	17.34	< 0.0001
1 mg/L	23.86	< 0.0001
2 mg/L	21.25	< 0.0001
4 mg/L	17.26	< 0.0001
8 mg/L	1.72	0.0888

Compared to positive control, blank1 above 3x

	t	p
blank2	2.84	0.0149
0.2 mg/L	0.76	0.4589
1 mg/L	2.95	0.0083
2 mg/L	0.47	0.6432
4 mg/L	1.36	0.1879
8 mg/L	20.99	< 0.0001
24 mg/L	22.06	< 0.0001

Compared to positive control, blank2 above 3x

	t	p
blank1	2.84	0.0149
0.2 mg/L	1.01	0.3386
1 mg/L	0.43	0.6733
2 mg/L	1.37	0.1832
4 mg/L	2.84	0.0124
8 mg/L	19.12	< 0.0001
24 mg/L	19.63	< 0.0001

Statistical analysis of data in Figure 3c:

		average intensity	standard error	n
Blank1	above 3x	57.22	3.47	16
Blank2	above 3x	46.44	1.81	3
0.2 mg/L	above 3x	48.44	2.94	9
1 mg/L	above 3x	55.90	9.31	4
2 mg/L	above 3x	46.75	2.35	3
4 mg/L	below 3x	8.81	0.79	49
8 mg/L	below 3x	11.52	1.05	36
24 mg/L	below 3x	12.78	0.66	45

Compared to negative control, 24 mg/L below 3x

	t	p
Blank1	19.13	< 0.0001
Blank2	12.90	< 0.0001
0.2 mg/L	18.33	< 0.0001
1 mg/L	13.00	< 0.0001
2 mg/L	12.92	< 0.0001
4 mg/L	3.81	0.0002
8 mg/L	1.06	0.2902

Compared to positive control, Blank1 above 3X

	t	p
Blank2	1.31	0.2081
0.2 mg/L	1.70	0.102
1 mg/L	0.16	0.8753
2 mg/L	1.27	0.2222
4 mg/L	20.16	< 0.0001
8 mg/L	16.44	< 0.0001
24 mg/L	19.13	< 0.0001

Compared to positive control, Blank2 above 3X

	t	p
Blank1	1.31	0.2081
0.2 mg/L	0.37	0.7159
1 mg/L	0.85	0.4334
2 mg/L	0.11	0.9197
4 mg/L	11.54	< 0.0001
8 mg/L	9.44	< 0.0001
24 mg/L	12.90	< 0.0001

References

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