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# 1 Measuring single-cell susceptibility 2 to antibiotics within monoclonal 3 bacterial populations

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12  
13 **Abstract** Given the emergence of antimicrobial drug resistance, it is critical to  
14 understand the heterogeneity of response to an antibiotic within a population of cells.  
15 Since the drug can exert a selection pressure that leads to the emergence of resistant  
16 phenotypes. To date, neither bulk nor single-cell methods are able to link the  
17 heterogeneity of single-cell susceptibility to the population-scale response to antibiotics.  
18 Here we present a platform that measures the ability of individual *E. coli* cells to form  
19 small colonies at different ciprofloxacin concentrations, by using anchored microfluidic  
20 drops and an image and data analysis pipelines. The microfluidic results are  
21 benchmarked against classical microbiology measurements of antibiotic susceptibility,  
22 showing an agreement between the pooled microfluidic chip and replated bulk  
23 measurements. Further, the experimental likelihood of a single cell to form a colony is  
24 used to provide a probabilistic antibiotic susceptibility curve. In addition to the  
25 probabilistic viewpoint, the microfluidic format enables the characterization of  
26 morphological features over time for a large number of individual cells. This pipeline  
27 can be used to compare the response of different bacterial strains to antibiotics with  
28 different action mechanisms.

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## 30 Introduction

31 Antimicrobial resistance is considered by the World Health Organization as one of the  
32 biggest threats to public health [25]. Developing new tools and methods to better under-  
33 stand bacterial resistance is becoming necessary. When addressing the question of bac-  
34 terial response to antibiotics, most microbiology studies report the Minimum Inhibitory  
35 Concentration (MIC) at which the cells stop growing, for a given initial inoculum size (usu-  
36 ally  $10^5$  cells) and after a growth time of over 24 hours. However, interpreting these MIC

37 measurements is far from trivial and could indicate many different phenomena taking  
38 place in the cultures (high variability, inoculum effect, mechanism of action of the antibi-  
39 otic) [16]. Indeed, the response of individual cells, which may then lead to colonies, can  
40 display large heterogeneity [11, 12]. For this reason the MIC is sometimes complemented  
41 with a more precise measurement, the Minimum Bactericidal Concentration (MBC). The  
42 process of determining the MBC is heavy and time-consuming and, like the MIC, only  
43 gives information about the antibiotic susceptibility on the population level. This has mo-  
44 tivated significant effort to measure the bacterial response at a more microscopic level.

45 For instance, a single-cell MIC was estimated by measuring the MIC for different inocu-  
46 lum sizes and extrapolating the value to a single cell [5]. Classical laboratory methods,  
47 however, are difficult to scale to single-cell manipulation, both in terms of the volumes  
48 of interest and also the number of experiments that are required to obtain a significant  
49 number of replicates. In contrast, the development of microfabrication methods and mi-  
50 crofluidics has allowed measurements to be made on individual cells in controlled envi-  
51 ronments [6]. In this area, two platforms which address questions of antibiotic response  
52 have emerged. First the so-called mother machine and its variations, where individual  
53 cells are trapped in thin channels and observed over a large number of generations [28].  
54 These devices rely on the tracking of the initial *mother* cells by time-lapse microscopy,  
55 while removing its daughter cells as they push out of the microchannels. By fine analysis  
56 of the images under different antibiotic treatments it is possible to learn about relations  
57 between mother and daughter cells [9], or to detect the effect of rare mutations on the  
58 fitness at the single-cell level [26].

59 In parallel, the field of droplet microfluidics has allowed studies of a different kind. By  
60 encapsulating one or a few bacterial cells within water-in-oil droplets, the development of  
61 small colonies from individual cells [15], or the signature of their metabolism [10], can be  
62 detected with optical readouts. The addition of antibiotics in solution within the droplets  
63 can then be used to determine the bacteria's susceptibility. This basic principle has, in  
64 recent years, been developed in two main directions. Either to improve the simplicity of  
65 use [13, 19] or to improve the precision of the measurements [22, 23, 27]. These droplets  
66 approaches have the potential to be transferred to clinical studies, as reviewed in recent  
67 papers [18, 30, 14].

68 Although these droplet methods constitute important milestones, they suffer from  
69 several drawbacks: First, they require specific and sophisticated equipment including pre-  
70 cise flow control systems to ensure droplet size homogeneity, as well as high-speed elec-  
71 tronics, lasers, and data acquisition, to perform the measurements on flowing droplets.  
72 Second, the latest methods do not allow the droplets to be followed in time or to relate  
73 the final state to the initial state of the droplets. Finally, the link between the single-  
74 cell measurements and the classical biological measurements has never been explicitly  
75 tested. As such, it is difficult to relate the droplet-based measurements with the vast  
76 quantity of data obtained in traditional experiments.

77 Here, we present an open-access microfluidic platform that addresses some of these  
78 issues. The platform is based on *rails and anchors* that were introduced a few years  
79 ago [1]. Those droplets are formed within microfabricated wells and remain stationary  
80 for the duration of the experiment, including for the observation of biological processes  
81 within them [2, 3]. As such, the platform only requires simple microfabrication, low-  
82 precision flow control and allows to complete time-lapse measurements. The microflu-

83 idic setup is augmented with an original and dedicated image acquisition and analysis  
84 pipeline that extracts the relevant information from the chips in an automated manner.  
85 By providing the image and data analysis as open-source code, the platform will be easy  
86 to integrate in most academic laboratories. Moreover, the current study addresses the  
87 interpretation of the biological measurements for the first time. By doing so it links the  
88 droplet-level approach, used in most droplet-based experiments, with a single-cell anal-  
89 ysis. This analysis is used to obtain unique measurements of the single-cell susceptibility  
90 to antibiotics.

## 91 Results

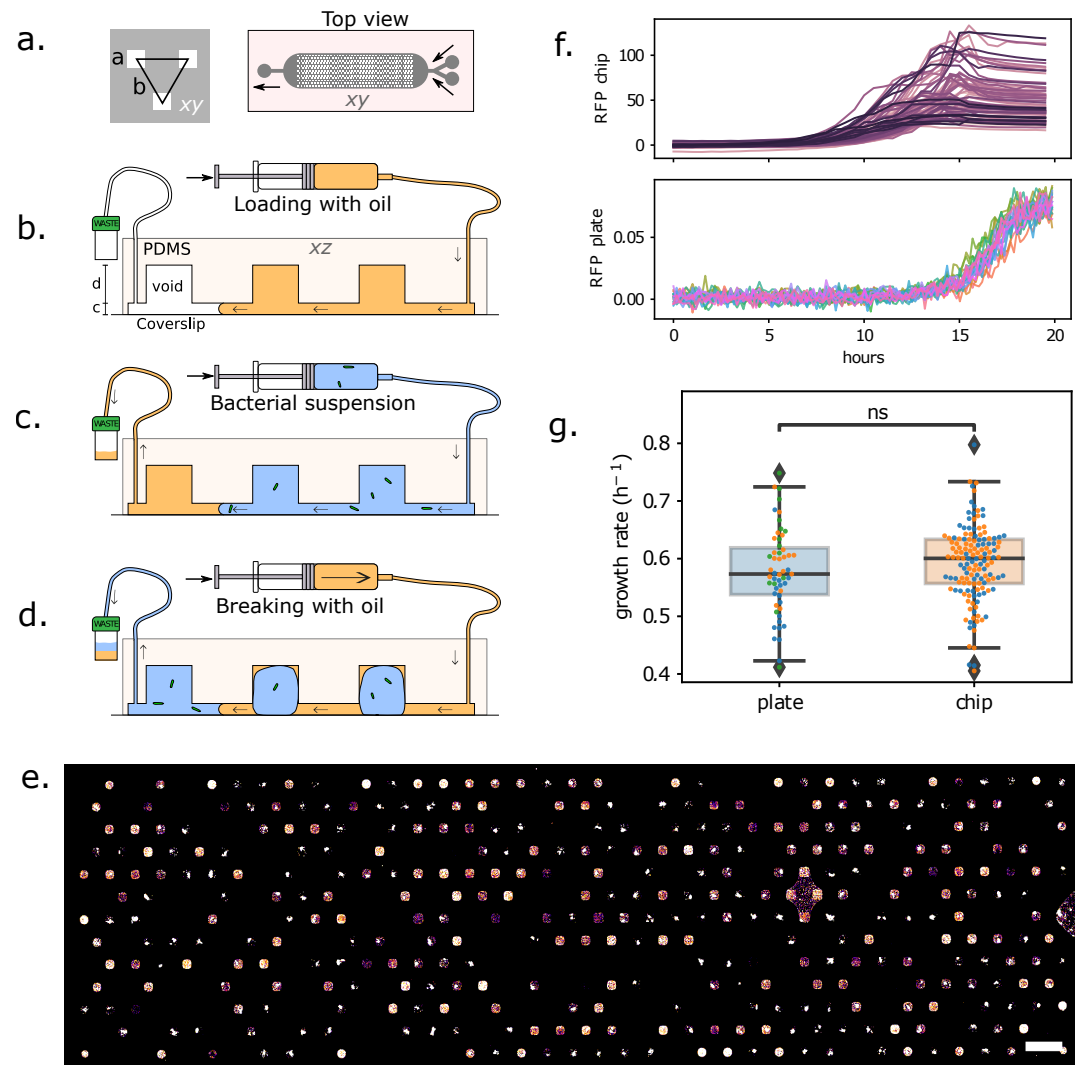
### 92 Microfluidic platform and initial observations

93 The microfluidic device used in this study is based on a geometry described previously [3,  
94 4]. It consists of a triangular array of 501 individual wells, or anchors, that can each hold a  
95 single aqueous droplet (Fig. 1a). The chip format is well suited for imaging on an inverted  
96 microscope, either using wide-field illumination or confocal mode, as discussed below.

97 Loading the chip and distributing the bacterial solution into droplets is straightfor-  
98 ward and takes about 5 min (see Method for the detailed steps, and Fig. 1 b, c, d). Briefly,  
99 the device is first entirely filled with oil (FC40, 3M) containing 0.5% surfactant (FluoSurf,  
100 Emulseo, France). Then, the aqueous suspension of bacteria is flowed through the device.  
101 In a last step, the oil is flowed again through the device, which leads to the formation and  
102 immobilization of droplets containing bacteria, directly on the anchors. The volume of  
103 each droplet is 2 nl and is primarily determined by the volume of the anchor [2]. This load-  
104 ing procedure is simple to set up and can be mastered in only a couple of trials. Indeed,  
105 the droplet formation step is robust to fluctuations in flow rate. It can be performed using  
106 syringe pumps or using hand-held syringes. Also note that the whole loading procedure  
107 is performed while the output is connected to a waste tube, and the bacterial suspen-  
108 sion is constantly encapsulated in an inert oil layer. This makes the protocol suitable in  
109 principle to handling pathogens, since the bacterial solution remains in a closed circuit.

110 *E. coli* W3110 with a fluorescent reporter (RFP) in the ptac site (ptac::RFP) allows us  
111 to detect bacterial cells and the growth of the colonies using the red fluorescent protein  
112 (RFP). A sample fluorescence image of the trapping area of the chip, acquired after 24  
113 hours of incubation, is shown in Fig. 1e. The bacteria at this stage form bright fluorescent  
114 colonies that can readily be imaged using epifluorescence or confocal microscopy. Empty  
115 droplets are also present on the device, since the cells are randomly distributed. The  
116 number of empty droplets is related to the average number of bacteria per drop, which  
117 is in turn related to the initial concentration of the bacterial suspension. Starting from  
118 different initial dilutions, therefore, allows us to tune the average number of cells per  
119 droplet and the number of positive droplets within the chip in the absence of antibiotics.

120 Before moving on to testing the effects of antibiotics in the microfluidic device, we to  
121 benchmark the bacterial fitness in the microfluidic device compared with standard multi-  
122 well plate experiment. This is done by following the growth of the fluorescent intensity in  
123 the droplets, using time-lapse epifluorescence microscopy, while performing in parallel  
124 a standard growth-curve measurement on a fluorescence plate reader from the same  
125 batch culture. The growth curves for the two cases are shown in Fig. 1f. The curves from  
126 the microfluidic device show a large variability between individual droplets, as a result



**Figure 1.** (a) Microfluidic chip design. Left: a unit cell consists of a triangular arrangement of square anchors, each of size  $a = 120 \mu\text{m}$  and distance  $b = 360 \mu\text{m}$ . Right: design of the full chip, which includes a main chamber with 501 anchors, 2 input channels on the right and 1 outlet channel on the left. (b) Side-view (not to scale), showing the channel height  $c = 30 \mu\text{m}$  and anchor depth  $d = 100 \mu\text{m}$ . The loading protocol begins by filling the chamber with oil, then (c) replacing the oil with bacterial suspension, and (d) breaking the bacterial suspension into individual droplets, anchored in their respective wells. (e) Z-projection of a confocal stack of the chip after 24 h incubation (scalebar:  $500 \mu\text{m}$ ). (f) Growth curves in 501 individual droplets on the chip (top), and in each well of a 96-well plate (bottom). (g) Measured growth rates for bacteria in the microfluidics chip (2 replicates), and in the 96-well plate (3 replicates). Growth rates were obtained by fitting an exponential function to the growth curves during the first 10 hours of growth. P-value=0.24 obtained with Welch's t-test for independent samples.

127 of distribution of the number of initial cells and of the cell-to-cell variability [7]. In con-  
128 trast, the multiwell plate experiments are insensitive to these stochastic elements and  
129 grow in a reproducible and regular manner. Fitting the individual growth curves with an  
130 exponential function for the first 10 hours of growth shows that the difference in growth  
131 rates in the microfluidic device and in the multiwell plates is statistically not significant,  
132 see Fig. 1g. Hence, the microfluidic results can be compared to standard microbiology  
133 techniques.

## 134 **Imaging and analysis pipelines**

135 Once the microfluidic device is loaded, the aim of the experiments is to identify which  
136 droplets within the array produce a population of cells after 24h and to link the final state  
137 with the initial number of cells in each droplet. These measurements are performed  
138 by first imaging the chip shortly after the loading and then after overnight incubation.  
139 Image analysis of the initial and final time points then yields a table that identifies each  
140 droplet in the array. Each droplet is then associated with measured quantities such as  
141 the initial number of cells, its final state, as well as the antibiotic concentration for a given  
142 experiment (see schematic in Fig. 2).

143 As such, the ability to acquire and analyze large amounts of imaging data is funda-  
144 mental to obtain the antibiotic response curves. While the array format lends itself nat-  
145 urally to measuring droplet contents at different time points, the requirement to detect  
146 single cells at early times imposes high-resolution imaging. This runs into data han-  
147 dling limitations associated with the large file sizes and large number of experiments.  
148 These different constraints led us to develop automated imaging and analysis pipelines  
149 whose implementation was instrumental for obtaining the results below. The imaging  
150 steps are described below and the image and data analysis pipelines are provided as  
151 open-source packages at the following GitHub repository: [https://github.com/BaroudLab/  
152 anchor-droplet-chip](https://github.com/BaroudLab/anchor-droplet-chip).

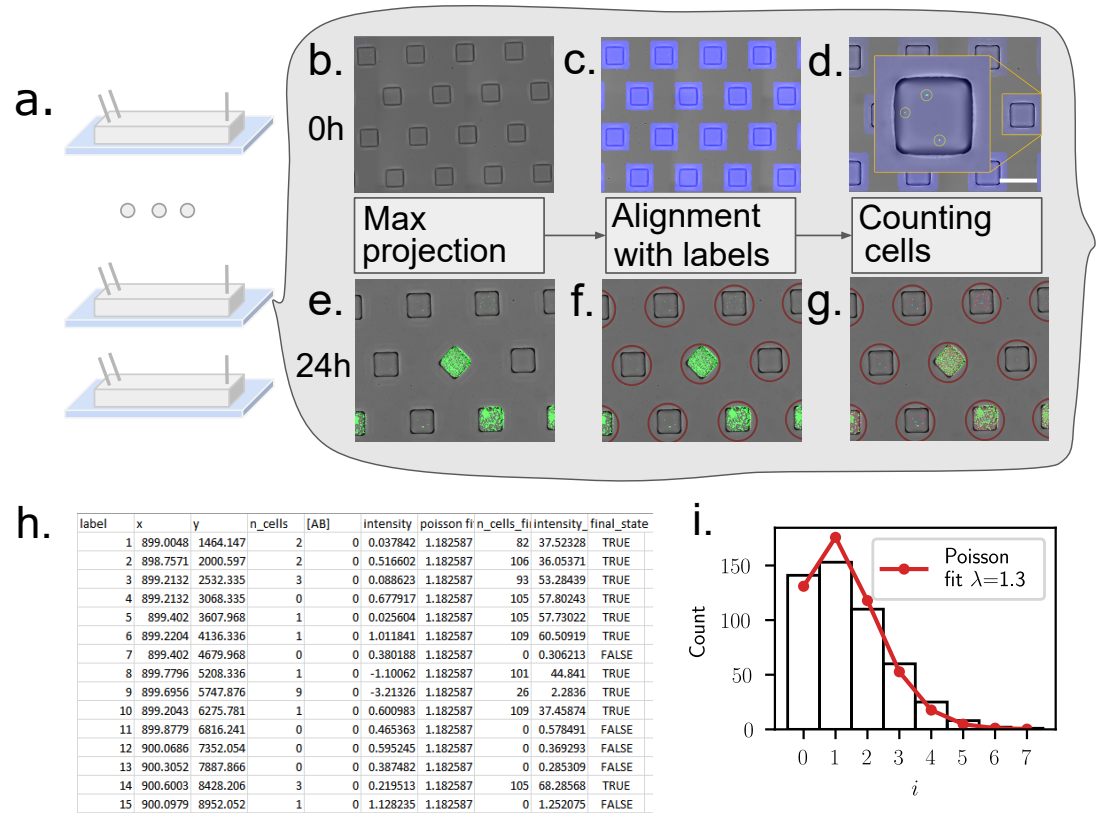
153 The aim of the pipeline is to generate a table with one line for each droplet, which  
154 includes a unique label, the antibiotic concentration, as well as the initial number of cells  
155 and final state for the droplet, as described in Fig. 2.

### 156 **Initial state.**

157 The initial number of cells per droplet is obtained by imaging the freshly loaded chip first  
158 in bright-field, and second by acquiring a confocal z-stack of the device in the RFP chan-  
159 nel. The bright-field image is used to detect the positions of the droplets, by adjusting for  
160 arbitrary shift and tilt of each acquisition (see Methods). In turn, the confocal stack is re-  
161 duced to a single image by using a maximum projection of the fluorescent intensity. The  
162 resulting fluorescent image allows us to count the number of individual bacteria within  
163 each droplet (Fig. 2c). The algorithm proved sufficiently robust to perform unsupervised  
164 automated analysis of the data from the chips.

165 Since the cells are expected to follow a Poisson distribution in the droplets [3], a quick  
166 quality control is performed at the end of the first scan, by verifying the distribution of  
167 number of cells per droplet, and checking that it indeed follows the expected shape, see  
168 Fig. 2i. This calculation also allows us to obtain a value of the Poisson parameter  $\lambda$  and  
169 to adjust the cell dilution if necessary in order to work in the desired range of  $\lambda$ .

170 Note that the loading and scanning each take about 10 minutes and the initial image



**Figure 2.** Imaging and image analysis pipeline. **(a)** The experiment begins by loading several chips with bacterial suspensions at different antibiotic concentrations. **(b)** Overlay of the bright-field image of the chip, and of the maximum projection of a fluorescent confocal z-stack. Images are acquired minutes after loading (0 h). **(c)** The bright-field image is used to identify the alignment with respect to the microscope stage and to create a unique mask around the positions of the individual anchors. **(d)** Detecting the fluorescent peaks in the maximum projection enables to count the initial number of cells per droplet. **(e)-(g)** Similar imaging and analysis operations are performed 24 h after the loading, to identify positive and negative droplets. **(h)** The data from the initial and final images are collected in a table that provides a unique label for each droplet, as well as the number of cells at 0 h and at 24 h. Each droplet is assigned a final status of positive or negative, depending on whether the final number of cells is larger than a predefined threshold of 15 cells. **(i)** A quality control step is performed using the single-cell detection at  $t = 0$  by comparing the cell number distribution with a Poisson distribution. This leads to an estimate of the Poisson parameter  $\lambda$  for each chip.

171 validation occurs within a few minutes as well. This yields a first measurement of the  
172 loading in under 30 minutes for each microfluidic chip.

### 173 Final state.

174 The contents of each droplet are later measured at the experimental endpoint, typically  
175 after an overnight incubation ( $t = 18 - 24$  h): each chip is scanned, this time using simple  
176 epi-fluorescence to optimize time and disk storage. The final image of each chip is re-  
177 aligned with the template acquired at initial times to identify each droplet, a registration  
178 step made straightforward by the fact that droplets are anchored at predefined positions.  
179 Then, cells are detected within each droplet, as shown in Fig. 2e-g. In the current exper-  
180 iments, we focus on the bottom of the microfluidic device where cells are more likely to  
181 be detected if the droplet is positive. Different quantities can be obtained from the final  
182 image as proxies for the ability of cells to grow within the drop. We count the number of  
183 cells in the final image, although mean or total fluorescence intensity can also be used.  
184 The main requirement for the measurements is to be sufficiently robust to yield a cutoff  
185 between positive and negative droplets, a classification which is also included in the data  
186 table. The final result is a csv table that contains the relevant information on the initial  
187 and final states of each droplet within each microfluidic device.

188 Note that the protocols described above can be modified for particular situations. For  
189 instance time-lapse microscopy can be performed on the chips to obtain time-resolved  
190 measurements. Similarly, confocal imaging can be used at later times to obtain a more  
191 precise cell count or fluorescent intensity, or to identify the morphology of the cells. Al-  
192 though these cases would require small modification in the pipeline, the main bricks of  
193 the analysis discussed above can still be used in a modular fashion.

### 194 **Microfluidic vs. microplate antibiogram**

195 The microfluidics and imaging protocols described above can be combined to obtain an  
196 antibiotic susceptibility curve, by loading several chips in parallel, using known concen-  
197 trations of antibiotics and bacteria in each chip. Performing these measurements is sim-  
198 plified by the standardized microfluidic format and analysis codes, making it possible to  
199 run six to twelve chips in parallel, each with a different concentration. Confronting the  
200 microfluidic measurements against standard microbiological techniques proves crucial  
201 to understand how to interpret the microfluidics data.

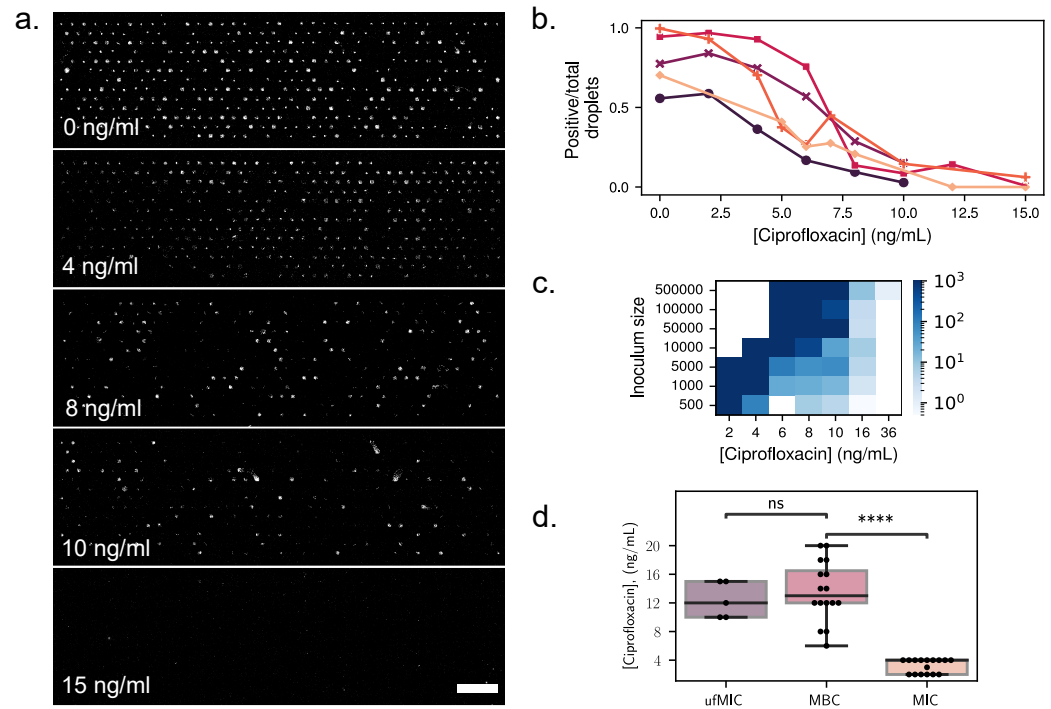
202 In each experimental run, one chip is loaded with the same bacterial concentration  
203 as the others but without any antibiotic. This control chip allows us to estimate the value  
204 of the average number of cells per droplet at initial times,  $\lambda$ , for the given run:

$$\lambda \simeq -\ln(\hat{p}_-), \quad (1)$$

205 where  $\hat{p}_-$  is the fraction of empty to total droplets on the test chip. This estimate comes  
206 from the assumption that the initial number of cells per droplet follows a Poisson distri-  
207 bution [7]. A higher value of  $\lambda$  means that drops contain a larger average number of cells  
208 initially.

209 A typical set of images from the experimental endpoint is shown in Fig. 3a. In these  
210 images the bright spots correspond to droplets where bacteria grew, while dark spots  
211 within the regular matrix correspond to droplets that do not contain a sufficient popula-  
212 tion of cells. These dark positions correspond either to droplets that did not contain any





**Figure 3. (a)** Antibiofilm chips with different concentrations of ciprofloxacin, and an initial average number of cells per droplet  $\lambda = 1.3$ . Bright spots correspond to droplets where bacterial growth occurred. Scale bar: 1 mm. Fraction of positive droplets at the end of the experiment, normalized by the total number of droplets containing at least one bacterium at the beginning of the experiment, for different concentrations of ciprofloxacin. Each color corresponds to a different run, at a different date and with a different value of  $\lambda$ . **(c)** Result of MBC experiments. Bacteria were grown in 96-well plates under different antibiotic concentrations, and starting with different inoculum sizes. The wells were scanned after 24 hours, and the contents of negative wells were replated on antibiotic-free petri dishes for 24 additional hours. The number of colonies on the petri dishes after incubation directly represents the number of surviving bacteria in each well. The color corresponds to the number of colonies on the petri dishes. **(d)** Values of the MBC (petri dishes), MIC (96-well plates) and  $\mu$ f-MIC on the chip, where the total number of cells per chip is used as inoculum size. P-values  $6e-8$  for MBC vs. MIC and  $0.9$  for ufMIC vs. MBC obtained with Welch's t-test for independent samples.

213 cells initially or to droplets where the cells did not form colonies e.g. due to the antibiotic  
214 stress. This antibiogram, allows to determine a measure of the antibiotic susceptibility  
215 of the bacteria, which we denote  $\mu\text{f-MIC}$ . The  $\mu\text{f-MIC}$  corresponds to the lowest antibiotic  
216 concentration that will inhibits the growth in 95% of the droplets.

217 As expected, the fraction of positive droplets decreases as the concentration of an-  
218 tibiotics is increased. This decrease is quantified in Fig. 3b, where the number of pos-  
219 itive droplets is shown to decrease towards zero as the concentration of the antibiotic  
220 ciprofloxacin increases, independently of the value of  $\lambda$ .

221 The interpretation of this “digital” measurement and its relation with classical micro-  
222 biology measurements is not obvious. To understand its significance, measurements  
223 from the microfluidic format were confronted with measurements in a standard mul-  
224 tiwell plate, using the same samples. Two classical microbiology measurements were  
225 performed on the samples. First, the minimum inhibitory concentration (MIC) was deter-  
226 mined with a standard microtitre broth dilution method (see Methods). The MIC corre-  
227 sponds to the lowest antibiotic concentration that will inhibits visible growth. Then, the  
228 minimum bactericidal concentration (MBC) was obtained by replating the contents of the  
229 negative wells on antibiotic-free petri dishes. The number of colonies on the petri dishes  
230 after 24 hours of incubation directly represents the number of surviving bacteria in each  
231 well. The MBC thus provides a more precise measure of the antibiotic concentration  
232 that is lethal to the bacteria, compared to the MIC. We find that the number of colonies  
233 growing from negative wells is very large below a critical value of the ciprofloxacin con-  
234 centration of 10 ng/mL, after which it drops dramatically to below  $\approx 100$  colonies, and  
235 eventually asymptotes to zero with increasing antibiotic concentration, see Fig. 3c.

236 A comparison of the measurements obtained from the three techniques ( $\mu\text{f-MIC}$ , MIC,  
237 and MBC) is shown in Fig. 3d, as a function of inoculum size. The inoculum size used for  
238 the  $\mu\text{f-MIC}$  corresponds to the total number of cells per chip. The classical MIC measure-  
239 ment (at  $t = 24$  h) is shown to be the least sensitive of the three measurements, since it  
240 finds a critical value of the concentration that is well below the value that is necessary  
241 to kill all of the cells. In contrast, the values of the MBC and of the  $\mu\text{f-MIC}$  are in the  
242 same range. They both show similar trends with the inoculum size, namely a slow but  
243 detectable increase with the initial number of bacterial cells.

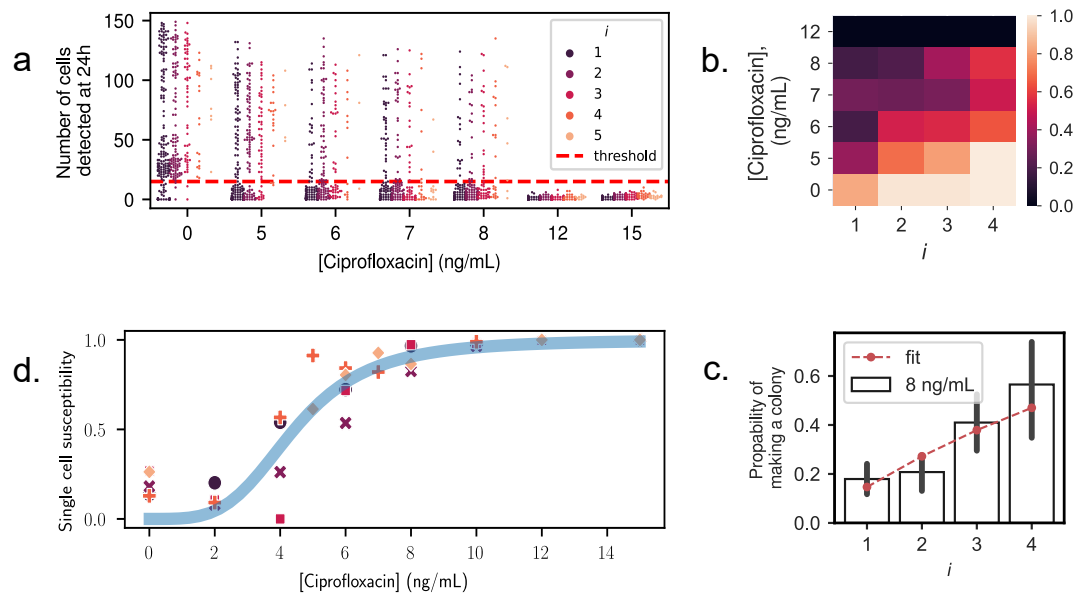
244 This close correspondence between the MBC and the  $\mu\text{f-MIC}$ , obtained by pooling  
245 together the total number of cells in the chip, indicates that the results in the microfluidic  
246 chip can be treated as a population-level measurement: all the bacteria from a single chip  
247 form a small population, whose ability to survive to a given concentration of antibiotics  
248 depends on its initial size. This result indicates that cells are behaving in an independent  
249 manner.

## 250 **Computing single-cell antibiotic susceptibility**

251 Beyond population level measurements, the objective is to provide insights about the an-  
252 tibiotic susceptibility of individual cells within a monoclonal population. This is achieved  
253 by taking a probabilistic viewpoint on the ability for a single cell to produce a colony at a  
254 given antibiotic concentration. Experimentally, we count the number of bacteria in each  
255 droplet at the beginning and at the end of an experiment for six antibiotic concentrations,  
256 see Fig. 4a. At the end of an experiment, the image analysis algorithm counts the number  
257 of bacteria at the bottom of the droplets. While this number does not reflect the exact

258 total number of cells in the droplet at late times, it is an acceptable proxy to differentiate  
 259 positive from negative droplets .

260 A threshold value to distinguish positive from negative droplets is chosen at  $n_{\text{final}} =$   
 261 15 cells to separate best the two populations (colony or no colony). The number of pos-  
 262 itive and negative droplets for each condition and each initial number of cells is then  
 263 determined. As a result, the probability of a droplet to contain a colony at the end-point  
 264 can be plotted as a function of its initial number of bacteria  $i$ , and of the antibiotic con-  
 265 centration  $C_{\text{AB}}$ . We call this probability  $p(i, C_{\text{AB}})$ : it represents the probability to produce  
 266 a colony starting from  $i$  cells, under a concentration of antibiotics  $C_{\text{AB}}$ . The evolution of  
 267  $p$  is shown as a heat-map in Fig. 4b. We observe that  $p(i, C_{\text{AB}})$  decreases as the antibiotic  
 268 concentration increases, but that it increases with the initial number of cells in a droplet;  
 269 when more cells are in a droplet initially, more antibiotics are needed to prevent the  
 270 growth of a colony after an overnight incubation.



**Figure 4.** Single-cell antibiotic susceptibility. **(a)** Final number of cells, counted at the bottom of each droplet at  $t = 24 h$ , as a function of antibiotic concentration. Colors indicate the initial number of cells  $i$  in the droplet at  $t = 0$ . The horizontal dashed line is the threshold, fixed at 15 cells, chosen to define the final droplet state as positive or negative. **(b)** Survival probability, computed using Eq. (3), as a function of the initial cell number  $i$  and antibiotic concentration. **(c)** The probability to produce a colony as a function of initial number of cells, for  $C_{\text{AB}} = 8 \text{ ng/mL}$ . Bars indicate experimental measurements from counting positive cells. Dashed line shows fit according to Eq. (2), assuming independent outcomes for each cell and fitting for  $q$ . For additional fits, see Supp. Fig. S1 **(d)** The single-cell antibiotic susceptibility is plotted for all antibiotic concentrations and all experimental replicates (points). The data is well-fitted with a two-parameter Hill function (line):  $h(C_{\text{AB}}) = C_{\text{AB}}^n / (K + C_{\text{AB}}^n)$ , with best fit values  $n = 3.9$  and  $K = 4.4$ .

271 Counting the number of bacteria at initial times, and the fraction of positive droplets  
 272 at the end of the experiment, enables to infer the susceptibility of a single cell to a con-

273 centration of antibiotic  $C_{AB}$ . This single-cell susceptibility, which we denote  $q(C_{AB}) =$   
274  $1 - p(1, C_{AB})$ , is defined as the probability for a single cell to die (equivalently not form  
275 a colony) at concentration  $C_{AB}$ . If additionally all bacteria are assumed to behave inde-  
276 pendently, the probability for  $i$  cells to die is  $q(C_{AB})^i$ . The probability for  $i$  cells to form a  
277 colony is therefore the probability for at least one of them to form a colony, and we then  
278 have:

$$p(i, C_{AB}) = 1 - q(C_{AB})^i. \quad (2)$$

279 For each concentration of antibiotics, the probability  $p(i, C_{AB})$  was estimated by count-  
280 ing the number  $\mathcal{N}_i$  of droplets containing exactly  $i$  cells at the beginning of the experi-  
281 ment. Among these  $\mathcal{N}_i$  droplets, a number  $N^+(i, C_{AB})$  droplets were positive at the end  
282 of the experiment. We then have:

$$p(i, C_{AB}) = \frac{N^+(i, C_{AB})}{\mathcal{N}_i}. \quad (3)$$

283 The probability  $p(i, C_{AB})$  was fitted to the functional form of Eq. (2) with  $q(C_{AB})$  as a  
284 single fit parameter, as shown in Fig. 4c (see SI Fig. S1 for all data). The good agreement  
285 between the data and the theory confirms that the bacteria may indeed be considered  
286 as independent of each other (see in Fig. 4c), at least for the low number of cells present  
287 initially at the beginning of the experiments. Then if we write  $q^*(C_{AB})$  the best fit value  
288 of  $q(C_{AB})$ , this value provides the best estimate of the single-cell susceptibility to drug  
289 concentration  $C_{AB}$ , using all experimental data at hand and assuming that all cells are  
290 independent.

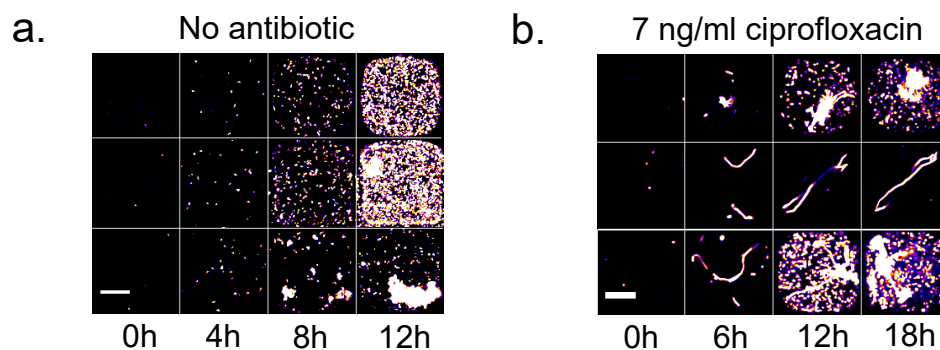
291 The single-cell susceptibility  $q^*(C_{AB})$  is expected to take a sigmoidal shape, with a  
292 value near 0 in the absence of drugs (all bacteria form colonies), and increases non-  
293 linearly with the drug concentration until reaching a plateau at  $q^*(C_{AB}) = 1$ . This is con-  
294 firmed by the experimental measurements, as shown in Fig. 4d. These data are arbitrarily  
295 fitted to a Hill function:  $h(C_{AB}) = C_{AB}^n / (K + C_{AB}^n)$ , with  $K$  and  $n$  being two fit parameters,  
296 providing a good match between experiments and the fitted function.

## 297 **Identifying morphology changes under antibiotic stress**

298 This microfluidic device and image analysis pipeline is a very powerful tool to obtain quan-  
299 titative information at the single-cell level as seen in Fig. 4. But more than quantitative  
300 information, the anchored droplet format provides a unique ability to access qualitative  
301 data and to follow the evolution of the bacterial colonies within each droplet over time.  
302 The tracking of the droplet contents can be performed by time-lapse microscopy on the  
303 chips. Since the droplet position is invariant throughout the experiment, identifying the  
304 progeny of an individual cell can be achieved. Examples of a sample droplet contents can  
305 be seen in Fig. 5 and the accompanying movies, first in the absence of antibiotics (Fig. 5a)  
306 or under a sub-MIC concentration of ciprofloxacin (Fig.5b).

307 In the absence of antibiotics, almost all droplets display bacteria in their planktonic  
308 state, swimming in the droplets and showing the typical size and shape for *E. coli* in cul-  
309 ture. A few hours into the experiments and as the populations begin to grow, cells in  
310 some droplets start to adhere to each other, eventually forming clumps. The presence  
311 of these clumps could be attributed to the presence of adhesion proteins like fimbrial  
312 adhesins that may then contribute to biofilm formation of *E. coli* [8, 21].

313 The morphological change of bacteria in the presence of antibiotics often begins with  
314 the elongation of the cells into long filaments: this corresponds to 83% of cases in the



**Figure 5.** Example of bacterial growth in three independent droplets growth **(a)** without antibiotic, and **(b)** with sub-MIC concentrations of ciprofloxacin (7 ng/mL). Scale bar: 50  $\mu$ m. Even at sub-MIC concentrations, the presence of ciprofloxacin leads to a filamentous bacterial morphology.

315 experiments at sub-MIC antibiotic concentration (7 ng/mL). The filamentation is due to  
316 the SOS response triggered by the presence of ciprofloxacin; cell-division stops but the  
317 cell metabolism continues, leading to cell volume growth. The elongated cells can then  
318 begin to produce offspring after several hours (50% of all cases) or can arrest their growth  
319 and stay in the filamentous form (33% of all cases). This is in agreement with observations  
320 on agar plates [11].

321 In some cases, no elongation is found. These droplets correspond either to cells that  
322 were in a dormant state (10% of all cases is expected) [17] or to cells that suffered too  
323 much damage and died due to the antibiotics (7% of all cases) as no fluorescence can  
324 be detected. As a result of these dynamics, nearly all of the droplets that form colonies  
325 contain cells having the filamentous form as well. Nevertheless, the ability to image the  
326 contents of the droplets provides a very precise indication of the state of the cells within  
327 them. This in turn is informative about the ability of the cells to overcome the antibiotic  
328 stress.

## 329 Discussion

330 As the emergence of antibiotic resistance is accelerating, it is crucial to understand the  
331 variability of antibiotic response on the single-cell level. This has motivated work using  
332 dilution methods to determine the MIC on petri dishes [12] or in liquid media [5], or us-  
333 ing precision microscopy on agar [11]. In parallel, microfluidic methods have been used  
334 to provide better controlled conditions and statistics over a large number of individual  
335 cells [6, 28]. In recent years a flurry of droplet-based approaches has provided informa-  
336 tion showing the heteroresistance of a bacterial population [23], digital antibiotic sus-  
337 ceptibility [27, 29], or even providing pathogen identification [14]. These droplet-based  
338 methods have shown that the encapsulation within droplets can be used to explore the  
339 progeny of individual cells as they respond to antibiotic stress. By relying on snapshots of  
340 moving droplets, these methods cannot relate the initial and final states of each droplet,  
341 nor can they identify the biological mechanisms that allow cells to overcome the anti-  
342 biotic stress at sub-MIC concentrations.

343 In this context the device and analysis pipeline presented here combine the advan-  
344 tages of microscopy with those of droplet-based methods. Indeed the ability to iden-  
345 tify the initial state of each droplet allows us to work at much higher cell numbers per

346 droplet, which translates to similar statistics as previous papers [27] while using a much  
347 lower number of droplets in total. Moreover the ability to provide time-resolved measure-  
348 ments on each droplet provides unique qualitative information about the adaptation of  
349 the cells, similarly to other microscopy-based methods [11]. Finally, the streamlined exper-  
350 imental and analysis pipeline allows us to load and image the chip in under 30 minutes,  
351 followed by an overnight incubation and a second scan requiring only a few minutes. As  
352 a result the complete campaign for obtaining an antibiogram can be performed robustly  
353 in a few hours. In contrast, the classical (petri-dish) method requires an initial overnight  
354 incubation in a 96 well plate, followed by a second overnight incubation on 50 to 80 petri  
355 dishes and manual counting of the colonies thereafter.

356 Beyond the performance aspects, it is important to benchmark the microfluidic mea-  
357 surements against standard microbiology protocols. The comparison of MIC, MBC, and  
358  $\mu\text{f-MIC}$  shows that the results in the microfluidic chip can be treated as a population-level  
359 measurement, since the  $\mu\text{f-MIC}$  matches the MBC when accounting for the starting inocu-  
360 lum size. In addition to this, the detection of individual cells and the ability to perform a  
361 large number of single-cell assays in parallel allow us to develop a probabilistic treatment  
362 of the outcome within each droplet forming a colony after 24 hours. The probability for  
363 an individual cell to produce a colony at a given antibiotic concentration thus describes  
364 the heterogeneity of responses in the population.

365 This platform can now be used to address the single-cell response to antibiotics while  
366 screening different bacteria and molecules having different mechanisms of action. The  
367 expectation is that this screening will translate into both quantitative (shape of the sin-  
368 gle cell susceptibility curve) and qualitative (shape of the cells) differences among the  
369 conditions. The ability to encapsulate tens or hundreds of cells within the droplets, in  
370 a controlled manner, will then allow the exploration of collective behaviors and non-  
371 monotonic time-evolution of the response to antibiotics [24]. More complex experiments  
372 can also be envisaged, e.g. by recovering the contents of individual drops and perform-  
373 ing -omics measurements on them or by varying the antibiotic concentration in time, as  
374 described previously [4]. Taken together, the different operations that can be combined  
375 into this platform constitute a major step forward in the study of antibiotic response both  
376 for scientific questions and for medical applications.

## 377 **Methods**

### 378 **Microfluidics and microfabrication**

#### 379 **Microfabrication and chip design**

380 To produce microfluidic chips for the experiments, a custom mold was made using 2-  
381 layer-SU-8 photoresist lithography. The bottom layer contains the channels with 2 inputs  
382 and 1 output. The upper layer contains inverted microwells of squares shapes 120x120  
383  $\mu\text{m}$  and space 240  $\mu\text{m}$  apart. As shown in Fig. 1 (a), the wells are organized in 13 rows of  
384 39 and 38 wells respectively for odds and even rows. The channel height is 30  $\mu\text{m}$  and  
385 the well height is 130  $\mu\text{m}$ .

386 Next, PDMS and its curing agent (PDMS SYLGARD 184, Dow Corning) are mixed at a  
387 1:10 ratio and poured into the mold. The mold is placed in a vacuum chamber for 30  
388 minutes to eliminate air bubbles and then cured for 3 hours at 70 °C. Once the PDMS  
389 is cured, the chip is cut off from the mold and plasma bonded (CUTE Plasma, Femto

390 Science) to the coverslip.

### 391 Hydrophobic treatment

392 Prior to loading, the chips are surface treated with hydrophobic solution (NOVEC 1720  
393 surface modifier/electronic grade coating 3M). To do so, two surface treatments were  
394 done after the plasma bonding by filling the chip with the hydrophobic solution and cur-  
395 ing it for 10 minutes at 110°C. A third surface treatment is done prior to loading.

### 396 Chip loading

397 As described in Fig. 1, using a syringe pump (NEMESYS), the chip is first filled through  
398 the first input with a continuous oil phase (3M Fluorinert FC40 with non-ionic surfactant  
399 RAN fluoSurf final concentration 1 %) while purging the air bubbles. Then, through the  
400 second input, the continuous oil phase is replaced by the bacterial suspension. Finally,  
401 the continuous oil phase is injected again at a very low flow rate, breaking the droplets  
402 apart and leaving them locked in the wells.

## 403 Cell culture and preparation

### 404 Strain

405 The experiments were performed using the *E. coli* W3110 strain JEK1037 [20] labeled with  
406 red fluorescent protein (lacYZ:RFP).

### 407 Antibiotic Solution

408 Ciprofloxacin (Sigma-Aldrich) was solubilized in 0.1 N HCl (Sigma-Aldrich) at 25 mg/mL.  
409 The stock was then diluted with MiliQ water to 1  $\mu\text{g}/\text{mL}$ . The final concentration of ciprofloxacin  
410 used was between 0 and 32 ng/mL.

### 411 Cell culture

412 From the -80°C stock, the cells were streaked on LB agar plate and incubated overnight  
413 at 37°C. The next day one isolated colony is inoculated in supplemented minimal media  
414 (MOPS with glucose final concentration 0.4%) and IPTG is added at 0.05 mM to induce  
415 the expression of the RFP. The bacterial suspension is incubated overnight at 37°C with  
416 shaking.

### 417 Cell dilution

418 In order to get one to five cells per droplet, the optical density of the solution, measured  
419 at 600nm, was calibrated. This was achieved as follows: the calibration was known by  
420 using digital counting: the chip was loaded with the diluted bacterial suspension without  
421 antibiotics, incubated at 37°C overnight, and then imaged. The empty wells were counted,  
422 and assuming a well can only be empty if no cells is loaded, the initial loading parameter  
423  $\lambda$  was computed.  $\lambda$  is the Poisson parameter which corresponds to the mean number  
424 of cells per droplet and can be directly obtained by  $-\ln(N_{(-)}/N_{total})$ . Where  $N_{(-)}$  is the  
425 number of negative droplets and  $N_{total}$  is the total number of droplets.  $\lambda$  is monitored by  
426 the concentration of the bacterial suspension and directly linked to the optical density.

## 427 Growth characterization and antibiotic susceptibility

## 428 Growth curves

429 For growth characterization cells were loaded at different dilutions (500 to 500,000 cells  
430 per well) in a 96-well plate. The plate was then placed in the plate reader (Thermo Sci-  
431 entific Varioskan LUX) for 24 hours at 37°C with shaking. The optical density at 600 nm  
432 (OD) and the RFP fluorescence signal (excitation 488 nm and emission 520 nm) were mea-  
433 sured every 10 minutes.

434

435 In parallel, cells were loaded to the chip with an average of one cell per droplet. This  
436 corresponds to one cell per droplet on average. The chip was then placed under the  
437 microscope and the RFP signal was measured every 30 minutes.

## 438 MIC and MBC

439 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration  
440 (MBC) were obtained to characterize the antibiotic susceptibility at the population level.

441

442 Cells are loaded in a 96 well plate at different dilutions (500 to 500,000 cells per well)  
443 and with different ciprofloxacin concentration ranging from 2 to 36 ng/mL. The plate is  
444 then incubated for 24 hours at 37°C with shaking.

445

446 The MIC is determined as the antibiotic concentration of the first negative well, i.e.  
447 the well at which the OD is the same as the OD of an empty well.

448 Then, the contents of the negative wells are plated on LB agar plates and incubated  
449 for 24 hours at 37°C. The number of colonies from each of these plates is counted. The  
450 MBC is determined as the concentration where the number of colonies decrease sharply  
451 from more than a hundred cells to less than a dozen cells.

## 452 Single-cell susceptibility

453 To characterize the single-cell susceptibility to antibiotic, at least 10 microfluidic chips are  
454 fabricated and loaded per experiment : a control chip without antibiotic, and nine more  
455 with serial concentrations of ciprofloxacin (from 2 to 36 ng/mL). The bacterial suspension  
456 is prepared with the antibiotic and immediately loaded into the chip. The chip is imaged,  
457 using fluorescence microscopy, right after in order to determine the exact number of  
458 cells in each well.

459 Then, the chip is immersed in a container filled with Milli Q water to prevent evapo-  
460 ration and incubated overnight at 37°C. After incubation, a scan of each chip, measuring  
461 the RFP signal is performed, using fluorescence microscopy.

462

## 463 Microscopy and image acquisition

464 Microscopy images are acquired using spinning disk confocal microscope (Nikon Ti2 +  
465 Yokogawa) with a 20x 0.7 NA air objective lens (Nikon Inc.) and with 2x2 pixels binning  
466 (set directly in camera properties (Hamamatsu Orca 4)). Images of the complete chip  
467 are obtained by stitching individual images with a 5% overlap. The imaging rate is opti-  
468 mized by acquiring first a bright-field image of the complete chip. The RFP signal is then  
469 obtained either in confocal mode, using 3D stack.



470 For the 3D stack, using triggered NIDAQ Piezo Z, planes are acquired with a step of  
471  $1\ \mu\text{m}$ , for a total penetration of  $120\ \mu\text{m}$ .

472 The total area of the device is about 1.4 by 0.4 mm, which translates to 40 kpix long  
473 dataset using 350 nm pixel size. However, some chips could be tilted due to manual  
474 bonding, which effectively increases the necessary scanning area, resulting in much big-  
475 ger images.

## 476 **Image analysis**

### 477 **Image registration**

478 First the 3D fluorescence stack is converted to 2D using maximum projection (see Fig. S2  
479 b,e). Both channels, 2D bright-field and 2D fluorescence images, are merged together  
480 and saved as a tif stack. Then a well-labelled template image and a well-labelled mask  
481 are made and aligned to the experimental images (see Fig. S2 c,f).

482 Note that the protocols described above can be modified for particular situations. For  
483 instance time-lapse microscopy can be performed on the chips, in order to obtain time-  
484 resolved measurements. Similarly, confocal imaging can be used at later times in order  
485 to obtain a more precise cell count or fluorescent intensity, or to identify the morphology  
486 of the cells in particular cases. Although these cases would require small modification  
487 in the pipeline, the main bricks of the analysis discussed above can still be used in a  
488 modular fashion, without major changes in the general approach.

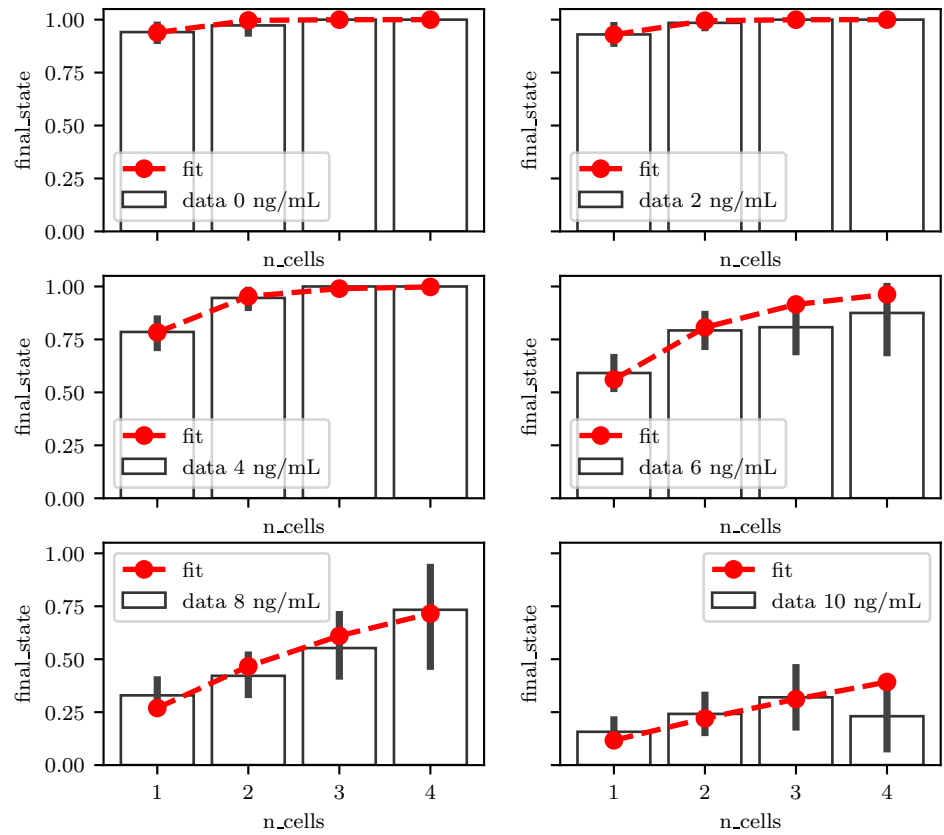
### 489 **Cell counting**

490 After image registration, every droplet is associated with the labeled area defined by the  
491 mask. Inside this mask, peak detection is performed to detect single fluorescent cells.  
492 To avoid false detection, due to noise, a preprocessing is performed as follows: First, a  
493 Gaussian filter, and subsequent peak detection using Scipy function `peak_local_max` with  
494 an absolute threshold of two. The number of peak per label is then recorded into a table  
495 for further processing.

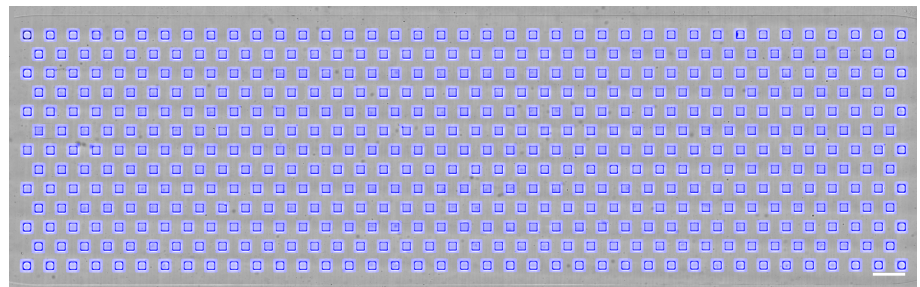
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## 500 **Supplementary information**



**Figure S1.** Fitting susceptibility to susceptibility for different concentrations of ciprofloxacin.



**Figure S2.** Template (grayscale) + mask (blue) used for alignment and segmenting the droplets. Scalebar 500  $\mu$ m

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