

# 1 A robust method for generating, quantifying and testing large 2 amounts of *Escherichia coli* persisters 3

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6 Antibiotic tolerance, flow cytometry, nutrient shift, tolerant cells, persister cells, *Escherichia coli*

## 7 Running head

8 Nutrient-shift to generate antibiotic tolerant cells

9

## 10 Abstract

11 Bacteria can exhibit phenotypes, which makes them tolerant against antibiotics. However, often only  
12 a few cells of a bacterial population show such so-called persister phenotype, which makes it difficult  
13 to study this health-threatening phenotype. We recently found that certain abrupt nutrient-shifts  
14 generate *E. coli* populations that consist of almost only antibiotic tolerant persister cells. Such nearly  
15 homogeneous persister cell populations enable assessment with population-averaging experimental  
16 methods, such as high-throughput methods. In this paper, we provide a detailed protocol of how to  
17 generate such large fraction of tolerant cells using the nutrient-switch approach. Furthermore, we  
18 describe how to determine the fraction of cells that enter the tolerant state upon a sudden nutrient shift  
19 and describe a new way to assess antibiotic tolerance with flow cytometry. We envision that these  
20 methods facilitate research into the important and exciting phenotype of bacterial cells.

## 21 1. Introduction

22 Bacterial persistence is defined as the occurrence of cells within a population that are tolerant against  
23 antibiotics without carrying a genetic resistance (1). Such antibiotic tolerant cells were suggested to be

24 responsible for recurrent infections (2). Tolerant cells can be formed stochastically in exponentially  
25 growing cultures (3). Activation of toxin and anti-toxin (TAS) modules (2) and deletion of certain  
26 metabolic genes (4) has also shown to increase the number of cells entering this phenotype. Certain  
27 environmental perturbations can also induce the fraction of tolerant cells in a population, such as the  
28 entry into stationary phase (5) or certain sudden nutrient shifts, where almost all the cells in a  
29 population can enter the tolerant state (6, 7).

30 The fact that certain nutrient shifts, in *E. coli*, for instance, the one from glucose to fumarate (8), can  
31 force almost all the cells in a population into the tolerant state offers new research opportunities to  
32 investigate the molecular basis of tolerance and persistence. While the investigation of the few  
33 stochastically occurring persisters in exponentially growing cultures and the very heterogeneous  
34 stationary phase cultures require single-cell analyses or cell-sorting approaches, a population that  
35 consists of almost only tolerant cells allows the use of population-level high-throughput analyses. For  
36 instance, exploiting sudden nutrient shifts to generate almost homogeneous populations of tolerant  
37 cells, it was found that tolerant cells have increased ppGpp levels, are metabolically active with a  
38 metabolism geared towards energy generation and catabolism, and exhibit a proteome characterized  
39 by  $\sigma^S$ -mediated stress response (8).

40 Despite the now enabled use of omics techniques for the study of bacterial persistence, experiments to  
41 assess how many cells are tolerant at which level of antibiotic exposure are still necessary. Here, in  
42 most cases, classical plating assays, using different dilutions, to determine the colony-forming units  
43 are performed (e.g. (5, 6)). However, this technique is very laborious and it has a high experiment-to-  
44 experiment variability requiring many plates to obtain statistically sound results (9). Moreover, with  
45 plating fewer cells are recovered from their dormancy compared to recovery in liquid media (10). To  
46 this end, we recently introduced an alternative method to assess antibiotic tolerance with flow  
47 cytometry (8). This method can be applied to populations solely consisting of dormant cells or to  
48 heterogeneous populations with dormant and growing cells. In this method, single cell's regrowth is  
49 assessed via temporal dilution of a fluorescent signal, which can originate from a stained membrane  
50 (11) or GFP expression (12).

51 Here, we provide a detailed nutrient-shift-based protocol to generate large fractions of tolerant cells,  
52 which enables the study of tolerant cells on the population level. Further, we illustrate a method to  
53 fluorescently stain the membrane of cells, which, together with flow cytometry and a Matlab script,  
54 allows us to determine the fraction of cells that enter the tolerant state upon a sudden nutrient shift.  
55 Finally, we describe how the membrane staining procedure together with flow cytometry can be used  
56 to assess tolerance.

## 57 2. Materials

58 Prepare all media using demi water.

## 59 **2.1. The generation of large fractions of tolerant cells**

- 60 1. LB agar plates: prepare LB medium by adding tryptone (to a final concentration of 1% w/v),  
61 yeast extract (0.5% w/v) and sodium chloride (1% w/v) to a bottle with demi water. Add agar  
62 to a final concentration of 1.5% (w/v) and mix well. Autoclave for 15 minutes at 121°C and  
63 let cool down to ~55°C. Add antibiotics, if needed, and pour ~20 mL LB-agar per 10 cm petri  
64 dish (*See Note 1*). Store plates sealed with parafilm at 4°C with the agar side up. LB-plates  
65 containing antibiotics can be stored for up to one month.
- 66 2. M9 minimal medium: Prepare a base salt solution (211 mM Na<sub>2</sub>HPO<sub>4</sub>, 110 mM KH<sub>2</sub>PO<sub>4</sub>, 42.8  
67 mM NaCl, 56.7 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), a solution with trace elements (0.63 mM ZnSO<sub>4</sub>, 0.7 mM  
68 CuCl<sub>2</sub>, 0.71 mM MnSO<sub>4</sub>, 0.76 mM CoCl<sub>2</sub>), a solution with 0.1M CaCl<sub>2</sub>, one with 1 M  
69 MgSO<sub>4</sub>, one with 1.4 mM thiamine-HCl, and one with 0.1 M FeCl<sub>3</sub>. Autoclave the solutions  
70 with base salts, CaCl<sub>2</sub>, MgSO<sub>4</sub>, and trace elements, and store them at room temperature.  
71 Sterile filter the thiamine and FeCl<sub>3</sub> solutions using a 0.22 µM PES filter (*See Note 2*) and  
72 store them at 4°C. Stock solutions are sterilized so they can be stored for up to half a year. For  
73 1 L M9 minimal medium, add 200 mL base salts, 700 mL water, 10 mL trace elements, 1 mL  
74 CaCl<sub>2</sub> solution, 1 mL MgSO<sub>4</sub> solution, 0.6 mL FeCl<sub>3</sub> solution and 2 mL thiamine solution.  
75 Fill up to 1 L with water and filter sterilize the resulting solution using a PES bottle top filter  
76 into an autoclaved 1 L bottle. Store at 4°C for up to one month. The required carbon source is  
77 added fresh before the medium is used for cultivations.
- 78 3. 250 g/L glucose stock solution: For 100 mL, weigh 27.5 g D-glucose monohydrate and  
79 dissolve in 90 mL water. Adjust pH to 7 using NaOH and fill up to 100 mL with water. Filter  
80 sterilize the resulting solution using a PES bottle top filter into an autoclaved 100 mL bottle.  
81 Store at room temperature for up to one month.
- 82 4. 100 g/L fumarate stock solution: For 100 mL, weigh 14 g sodium fumarate and dissolve in 90  
83 mL water. Adjust pH to 7 using HCl and fill up to 100 mL with water. Filter sterilize the  
84 resulting solution using a PES bottle top filter into an autoclaved 100 mL bottle. Store at room  
85 temperature for up to one month.
- 86 5. M9 minimal medium supplemented with glucose to a final concentration of 5 g/L for  
87 immediate use for cultivation: Add 1 mL glucose stock solution (250 g/L) to 49 mL minimal  
88 medium in a 500 mL Erlenmeyer flask. Preheat and pre-aerate at 37°C with shaking at 300  
89 rpm before inoculation.
- 90 6. M9 minimal medium supplemented with fumarate to a final concentration on 2 g/L for  
91 immediate use for cultivation: Add 1 mL fumarate stock solution (100 g/L) to 49 mL minimal  
92 medium in a 500 mL Erlenmeyer flask. Preheat and pre-aerate at 37°C with shaking at 300  
93 rpm before inoculation.

94 **2.2. Identification and quantification of tolerant cells using membrane staining and flow**  
95 **cytometry**

- 96 1. M9 minimal medium without carbon source. Ice-cold.
- 97 2. M9 minimal medium with 1% (w/v) bovine serum albumin: dissolve 0.5 g bovine serum  
98 albumin in 50 mL M9 minimal medium and filter sterilize the resulting solution using a PES  
99 bottle top filter into an autoclaved 100 mL bottle. Store at 4°C.
- 100 3. Dye to stain cell membrane: PKH67 dye (, Sigma), keep at 4°C until use. Then dilute 50x in  
101 Diluent C, at room temperature.
- 102 4. Diluent C (Sigma, included in the PKH67 kit). Store at 4°C, let warm up to room temperature  
103 before the start of the staining.
- 104 5. M9 minimal medium without carbon source, at room temperature.
- 105 6. M9 minimal medium with fumarate added to a final concentration of 2 g/L, preheated to  
106 37°C, and pre-aerated.

107 **2.3. Assessment of antibiotic tolerance with flow cytometry**

- 108 1. Antibiotics, prepare concentrated stocks of (*See Note 3*):
  - 109 a. 100 mg/mL ampicillin: dissolve 107 mg ampicillin sodium salt in 1 mL water. Filter  
110 sterilize using a PES syringe filter and store at –20°C.
  - 111 b. 20 mg/mL tetracycline: dissolve 108 mg tetracycline hydroxide in 5 mL water. Filter  
112 sterilize using a PES syringe filter and aliquot stock in portions of 1 mL and store at –  
113 20°C. Protect against light.
  - 114 c. 50 mg/mL kanamycin: dissolve 120 mg kanamycin sulfate in 2 mL water, filter  
115 sterilize using a PES syringe filter, and store at –20°C.
  - 116 d. 35 mg/mL chloramphenicol: dissolve 35 mg chloramphenicol in 1 mL 100% EtOH,  
117 filter sterilize using a PES syringe filter and store at –20°C.
  - 118 e. 0.4 mg/mL trimethoprim: dissolve 40 mg trimethoprim in 100 mL water. Filter  
119 sterilize using a PES syringe filter and aliquot in portions of 1 mL and store at –20°C.
  - 120 f. 1 mg/mL ofloxacin: dissolve 10 mg ofloxacin in 10 mL water. Filter sterilize using a  
121 PES syringe filter and aliquot in portions of 1 mL and store at –20°C.
  - 122 g. 1.25 mg/mL rifampicin: dissolve 12.5 mg rifampicin in 10 mL water. Filter sterilize  
123 using a PES syringe filter and aliquot in portions of 1 mL and store at –20°C.
  - 124 h. 10 mg/mL Carbonyl Cyanide m-Chlorophenylhydrazine (CCCP): dissolve 20 mg  
125 CCCP in 2 mL pure methanol, filter sterilize using a PES syringe filter and store at –  
126 20°C.
- 127 2. LB medium: add tryptone (to a final concentration of 1% w/v), yeast extract (0.5% w/v) and  
128 sodium chloride (1% w/v) to a final volume of 500 mL using water. Autoclave for 15 minutes

129 at 121°C and sterilize using a 0.22 µM PES filter to remove debris, which disturbs the flow  
130 cytometric analyses. Store at room temperature.

### 131 3. Methods

#### 132 3.1. Generation of large fractions of tolerant cells

133 To investigate tolerant cells on the population level it is important to generate a cell population that  
134 consists of almost only dormant cells, despite a carbon source is available. In this section, we describe  
135 how to generate such a population, accomplished by a sudden nutrient shift. The procedure starts with  
136 generating a fully exponentially growing culture on glucose and continues with the steps to perform  
137 an abrupt switch to a different carbon source. In this section, fumarate is taken as the second carbon  
138 source because it generates the highest percentage of tolerant cells. Other gluconeogenic carbon  
139 sources can also be used for the switch (7). A schematic overview of this method is shown in Figure  
140 1A, B and 1C.

141 1. Streak out an *E.coli* strain, e.g. K12, on LB-agar and incubate overnight at 37°C. Full-grown  
142 plates can be stored up to one month at 4°C sealed with parafilm.

143 2. Generate a culture that is fully exponentially growing on glucose, meaning that for several  
144 hours the cell number doubled at its maximal rate. Such a culture can be obtained by applying  
145 the following steps (*See Note 5*) :

146 1. (Day 1) inoculate a 100 mL flask containing 10 mL M9 minimal medium  
147 supplemented with glucose to a concentration of 5 g/L with a single colony from an  
148 agar plate at around 5 p.m. Grow overnight at 37°C and shaking at 300 rpm till  
149 around 9 a.m. the next morning.

150 2. (Day 2) towards preparing the next preculture, first, determine the cell concentration  
151 in this overnight culture. For instance, dilute an aliquot of the culture 100x, transfer  
152 200 µL to a 96-well plate (in case the used flow cytometer samples from 96-well  
153 plates) and measure the cell count (e.g. in 20 µL) with a flow cytometer (e.g.  
154 ACCURI C6, BD Biosciences) (*See Note 4*). Calculate the concentration in cells/mL,  
155  $C$ , by using the formula:

$$156 C = c \times d \times 50,$$

157 where  $c$  is the number of cells counted in 20 µL and  $d$  is the dilution factor. Then  
158 determine the volume of culture to be added to a fresh flask for having a target cell  
159 density of  $3 \cdot 10^8$  cells/mL at 5 p.m. on the same day as follows:

$$160 V_{inoc} = \frac{t_d}{2^{\frac{t}{\ln 2/\mu}}} \times C \times v,$$

161 where  $V_{inoc}$  is the volume that has to be added to the flask,  $t_d$  is the target density (in  
162 cells/mL) at the end of the day,  $\mu$  is the growth rate of the exponentially growing  
163 population (in h<sup>-1</sup>),  $t$  is time until the end of the day (in h) and  $v$  is culture volume (in

- 164 mL). Add the calculated volume to a flask containing preheated (37°C) M9 minimal  
165 medium with 5 g/L glucose, and grow the culture at 37°C and shaking at 300 rpm.
- 166 3. (Day 2) towards performing the actual nutrient-shift in the next morning (day 3) with  
167 a culture that is fully exponentially growing on glucose, perform another subculturing  
168 step in the evening before (on day 2) to have  $3 \cdot 10^8$  cells/mL at the desired time point  
169 in the morning of day 3. To calculate the volume that has to be added to a new flask,  
170 repeat step 2b. Because of the huge dilution that has to be made for the inoculation of  
171 this overnight culture, it is recommended to inoculate several parallel flasks with  
172 dilutions such that one surely obtains at least one culture at the right cell density (*See*  
173 **Note 6**). Also, to prevent lag phase behavior, which can mess up the timing, it is  
174 recommended to use preheated and per-aerated media.
- 175 3. (Day 3) to start the nutrient shift, wait until the culture has reached OD<sub>600</sub> of 0.3-0.8, first  
176 measure the cell concentration of the culture with the flow cytometer. The cell concentration  
177 should be around  $2-5 \cdot 10^8$  cells/mL. With the knowledge of the cell concentration, calculate  
178 the volume of the culture that contains  $1.5 \cdot 10^9$  cells.  
179 When staining is applied, skip the next step and go immediately to ‘Quantification of tolerant  
180 cells with membrane staining’.
- 181 4. Wash the cells to remove any residual glucose. Therefore:
- 182 1. Transfer the calculated volume to a 15 mL falcon tube.
  - 183 2. Spin down the culture for 5 minutes at 3000g, 4°C, and discard the supernatant.
  - 184 3. Wash the pellet with 5 mL ice-cold M9 minimal medium without carbon source by  
185 carefully pipetting. The ice-cold medium is used to slow down cells’ metabolism.  
186 Spin down, remove supernatant, and wash once more with the same amount of M9  
187 minimal medium.
  - 188 4. Spin down the culture for 5 minutes at 3000g, 4°C.
  - 189 5. Discard the supernatant and resuspend the pellet in 1 mL M9 minimal medium  
190 without carbon source.
- 191 5. Transfer the cells to a 500 mL flask containing 50 mL preheated M9 minimal medium with 2  
192 g/L fumarate. The cell concentration in the new flask is important because the initial cell  
193 density influences the fraction of cells that enter the tolerant state after the nutrient shift (*See*  
194 **Note 7**). With the *E. coli* wildtype strain BW25113, this procedure will result in about  
195 99.99% of cells entering the tolerant state (7, 8). With other *E. coli* strains, this fraction can be  
196 different.

197           **3.2. Identification and quantification of tolerant cells using membrane staining and flow**  
198           **cytometry**

199       To determine the fraction of cells that enter the tolerant state, e.g. after the nutrient shift, one can stain  
200       the membrane of the cells with a fluorescent dye, can follow the fluorescence of the cell population  
201       after the nutrient shift over time and can analyze the resulting single-cell fluorescence data with a  
202       computational script. The dye we propose (PKH67, Sigma) stains the membranes of the cells and  
203       equally distributes over the two new cells after cell division. Thus, the fluorescence intensity of a cell  
204       halves with every division. Knowing the initial fluorescence intensity and comparing this with the  
205       fluorescence intensity at a later time point, one can estimate back how often a cell has divided (7).  
206       Specifically, to estimate the fraction of dormant cells, a Matlab script is used that fits a mathematical  
207       model to the time course data (i.e. cell counts, fluorescence intensity distributions). The model  
208       assumes that the fluorescence intensity of a cell decreases by half with each cell division and that cells  
209       have some autofluorescence. Further, it assumes exponential growth of the growing cells from some  
210       time point after the nutrient shift onward. As the fluorescence intensities of individual cells are not  
211       identical the model fits bimodal distributions to the fluorescence intensity distributions determined at  
212       the different time points and estimates the growth rates of the two populations of cells based on the  
213       total cell counts determined and fitted bimodal distributions. The model, its rationale, and mathematics  
214       are explained in detail in our previous paper (7).

215       To determine the fraction of dormant cells that emerge after a sudden nutrient shift and to estimate the  
216       growth parameters (including among others, the growth rates of both populations), the membrane  
217       staining is applied in combination with the nutrient shift method to generate tolerant cells, meaning  
218       that the washing steps from the previous section are replaced by the following staining protocol. A  
219       schematic overview of this method is shown in Figure 1A, B, and 1C.

- 220           1. Generate exponentially growing cells on glucose according to steps 1 and 2 of the previous  
221           section.
- 222           2. Before the shift to fumarate, cells will be stained with a fluorescent dye. Before the staining,  
223           take Diluent C from the PKH67 kit from the fridge and let it warm up to room temperature.  
224           The dye should stay in the fridge.
- 225           3. Determine the volume of the glucose culture that contains  $1.5 \times 10^9$  cells. Transfer the  
226           calculated volume to a 15 mL falcon tube. Spin down the culture for 5 minutes at 3000g 4°C  
227           (Eppendorf centrifuge). Discard the supernatant very carefully by pipetting. Do not lose any  
228           cells, because the dye-to-cell number ratio is very important. Losing cells will increase the  
229           dye-to-cell ratio, through which cells could be stained too intensely, which in turn can lead to  
230           cell death. A sub-optimal dye-to-cell number ratio can also affect the viability of the cells  
231           resulting in fewer cells being able to recover after antibiotic treatment.



- 232 4. During the centrifugation as mentioned in the previous step, prepare a mix of 500  $\mu\text{L}$  of  
233 room-temperature Diluent C with 10  $\mu\text{L}$  of the dye solution (*See Note 8*).
- 234 5. **When performing steps 5 to 9 act fast and respect the timings** (*See Note 9*). Resuspend the  
235 cell pellet in 500  $\mu\text{L}$  of solely Diluent C (room temperature) by carefully pipetting up and  
236 down. Make sure that the pellet is fully dissolved and that there are no droplets on the sides of  
237 the tube.
- 238 6. Add the prepared dye solution (10  $\mu\text{L}$  of dye in 500  $\mu\text{L}$  of Diluent C at room temperature) to  
239 the cell solution and mix by brief vortexing. Incubate the cells for exactly 3 minutes at room  
240 temperature (*See Note 10*).
- 241 7. Immediately after 3 minutes add 4 mL of ice-cold M9 medium with 1% (w/v) BSA and mix  
242 by brief vortexing (*See Note 11*).
- 243 8. Centrifuge the cells for 5 minutes at 3000 g and 4°C. Discard supernatant by pipetting, and  
244 resuspend the cells in 5 mL of ice-cold M9 without carbon source and centrifuge the cells  
245 again (5 minutes, 3400 rpm, 4°C).
- 246 9. Wash the cells once more in the same manner. Resuspend the cells in 1 mL room-temperature  
247 M9 medium without carbon source and transfer them to the preheated M9 minimal medium  
248 with 2 g/L fumarate (*See Note 12*) to yield an  $\text{OD}_{600}$  of approximately 0.6, which corresponds  
249 to a cell concentration of  $\sim 5 \times 10^8$  cells/mL. Check the quality of the staining using flow  
250 cytometry by measuring a proper dilution of the culture. Well-executed staining should have  
251 only one peak on the FL-1 (533 nm) signal, and the cells should be 100-fold brighter than  
252 unstained cells.

### 253 **3.3. Guide on how to use the Matlab script**

254 The Matlab script and exemplary input files are provided on GitHub ([https://github.com/molecular-](https://github.com/molecular-systems-biology)  
255 [systems-biology](https://github.com/molecular-systems-biology)). The following steps describe the data acquisition and data format requirements.

- 256 1. To assess tolerant cells, it is important to generate tolerant cells as described in section 3.1:  
257 “Generation of large fractions of tolerant cells” and to stain them as described in section 3.2:  
258 “Identification and quantification of tolerant cells using membrane staining and flow  
259 cytometry”. The staining needs to be of an appropriate quality for the script to work optimally  
260 (*See Note 13*).
- 261 2. To obtain data that can be efficiently and easily used with the Matlab script, the  
262 measurements must be done at specific times. First, the first time point must be gathered as  
263 soon as possible after the switch.
- 264 3. The next data timepoint, which is the first data timepoint used to fit the model should be one  
265 in which the growing population is becoming visible in the data. This time point varies  
266 depending on the conditions and carbon sources used (*See Note 14*).



- 267 4. After taking these first time points, it is usually advised to make measurements every 30-60  
268 minutes, depending on the growth rate of the growing population, until the growing  
269 population count is at least equal, or higher than the non-growing population count.
- 270 5. The data needs to be gathered over the growth of the culture (*See Note 15*). Certain factors  
271 need to be considered when gathering the data, for it to be usable with the script. The input  
272 data needs to be provided as a csv file containing the flow cytometry measurements for each  
273 cell at each time point, without a header (File 1). In the Matlab script, the following input  
274 needs to be provided:
- 275 a. The number identifying the column of the CSV file containing the relevant  
276 fluorescence intensity (FI) data (in case of our data, the number is 3 – variable SC).
  - 277 b. The scaling factor. Different flow cytometers have different sensitivity and numerical  
278 output values. This factor is used to accommodate for these differences and make  
279 sure that the data from different machines can be used within the script capabilities.  
280 (variable `maxval_new_FC`).
  - 281 c. The times when the samples were taken (variable `tt`)
  - 282 d. The absolute cell concentration in the culture at the respective time points (variable  
283 `cc`)
  - 284 e. The number of cells (or rows) in each CSV data file (variable `g`)
  - 285 f. A specification of which of the data files should be used for the fitting. Depending on  
286 the nutrient switch, sometimes it takes hours before the growing population is  
287 numerous enough to be detected. The first timepoint used for the fit should have this  
288 population visible. Some data points can be excluded, for example, if the  
289 measurement has failed or has been inaccurate for any scientifically justified reason  
290 (variables `indx` and `cc_indx`).

291

292 In the Matlab script, one needs to define the allowed ranges of the parameters to be estimated as well  
293 as initial parameter guesses. These values should be close to the true values. Table 1 provides an  
294 overview of the parameters that need to be set and instructions on how to estimate these values, e.g.  
295 by visual inspection of the data or by using previous knowledge. It is advisable to run the script once  
296 on the data before setting the parameters. From the then generated “Fluorescence Data” figure (Figure  
297 1), one can identify initial guesses (in matrix variable `IG`) and ranges for some of the parameters (in  
298 matrix variable `bounds`).

- 299 1. Two figures generated by the script are crucial at determining the quality of the fit, i.e. the  
300 figure “Cellcount curve fit check” and the multi-panel figure “Biggaussian fit for each time  
301 point”. In the Supplementary Figures, we show two examples of bad fits (files: `badfit1`,  
302 `badfit2`) and we show one example of a good fit (file: `fit3`). The files for these fits are

303 provided with the Matlab script. The two figures show the cell count and fluorescence  
304 intensity data along with the plotted model predictions for both the growing and non-growing  
305 populations, and the sum of these populations. The way the model prediction fits the data can  
306 be used to assess whether the parameters are estimated correctly.

307 2. In the first bad fit (Supplementary Figure 1), the parameter bounds are set wrong. The FI  
308 means for both populations are overestimated, and the bounds are outside of the correct  
309 values. Moreover, the nongrowing population cell concentration is underestimated.

310 3. From the plots (Figure 2A), certain problems can be deduced:

311 a. The red line on the left panel that describes the modeled cell counts does not appear  
312 to fit with the cell count points from measurements. This is caused by the  
313 underestimation of the nongrowing population cell concentration.

314 b. The green line and the red line on the right panel do not fit the bimodal distribution.  
315 This is caused, on top of cause from problem 1, the overestimation of the FI means.

316 4. Moreover, we can see from the text output of the script that some of the parameters – the  
317 growth rate of the growing population in this case – reaches the lower limit of the bounds set,  
318 and thus it is not estimated correctly. However, we know what the growth rate of the used  
319 strain in given conditions should be and the fact that the estimate is equal to one of the  
320 boundaries is just a symptom of another issue, and not the issue itself.

321 5. If we fix the first problem and correct the nongrowing population cell concentration to a value  
322 that is close to the measured value, we obtain graphs shown in Supplementary Figure 2.  
323 While the graph on the left looks like it is a good fit, the graph on the right has the similar  
324 problems as before, stemming from the overestimation of the FI means. Fixing these values as  
325 described above, we can obtain a good fit.

326 6. It is clear from the plots shown in Figure 3B and 3C that the model closely fits the data and  
327 the data output can be trusted.

328 7. The parameters obtained from the fitting are output as text. The Matlab script used as an  
329 example generates the following data:

```
330 alpha      = 0.00047173
331 mu_growin  = 0.43374
332 mu_nongrw  = 0.0026477
333 sigma_grow = 53.0783
334 sigma_nong = 55.9828
335 I_0        = 3419.6016
336 EI         = 20.0569
337 dye_degr   = 0.015
338 BG_cutoff  = 1
339 BG_magnit  = 1
340 BG_width   = 1
341 CC_weight  = 1
342 Bigaus pt = 16.0833    16.8333    17.5833    18.3333    19.0833    19.8333    20.5833
343 21.3333    22.0833    22.8333    23.5833    24.3333    25.0833    25.8333
344 CC pts    = 16.0833    16.8333    17.5833    18.3333    19.0833    19.8333    20.5833
345 21.3333    22.0833    22.8333    23.5833    24.3333    25.0833    25.8333
```

346 8. The most important obtained values are alpha (fraction of cells that entered dormancy after  
347 the nutrient shift), mu\_growin (growth rate of the growing population), mu\_nongrw (growth  
348 rate of the non-growing population). All these and other parameters, except alpha, should be  
349 checked against the lower and upper bounds set in the script and if they are equal to them, the  
350 bounds should be relaxed until they do not limit the results anymore.

### 351 **3.4. Assessment of antibiotic tolerance with flow cytometry**

352 The ability to regrow after antibiotic treatment is an indication that a cell has been in the tolerant state  
353 during treatment. While typically, such regrow experiments are done with plating assays (i.e.  
354 determination of colony-forming units, CFUs), here, we proposed a method to perform such regrow  
355 assessments with flow cytometry. These assays require the above-mentioned membrane staining.  
356 Despite the staining procedure that needs to be carried out, this technique is less laborious and has less  
357 variability than plating assays (9). Also, it was found that more cells were able to wake up after  
358 dormancy when liquid media is used compared to regrowth on plates (10). A schematic overview of  
359 this method is shown in Figure 1D.

- 360 1. To assess the antibiotic tolerance of cells, it is important to generate tolerant cells as described  
361 in section 3.1: “Generation of large fractions of tolerant cells” and to stain them as described  
362 in section 3.2: “Identification and quantification of tolerant cells using membrane staining and  
363 flow cytometry”.
- 364 2. After the transfer to the fumarate medium, it takes a certain while until the non-adapting cells  
365 to develop full tolerance against antibiotics. It is recommended to let the cells adapt for at  
366 least 2 hours after staining (*See Note 16*) before treating them with antibiotics.
- 367 3. After >2 hours, add the antibiotics. We tested ampicillin (100 µg/ml), tetracycline (20  
368 µg/mL), kanamycin (100 µg/ml), chloramphenicol (140 µg/ml), trimethoprim (5 µg/mL),  
369 rifampicin (100 µg/ml), ofloxacin (5 µg/ml) and CCCP (50 µg/ml) (8). Concentrations were  
370 obtained from literature and from survival assays on cells growing on glucose (*See Note 17*).  
371 Because some cells undergo a reductive division after the switch to fumarate, the number of  
372 cells after 2 hours is slightly higher than at time point zero. Therefore, it is required to  
373 measure a dilution of the culture direct after staining and right before antibiotics are added.
- 374 4. Incubate the cultures that now contain the antibiotics for 2 hours whilst shaking at 300 rpm at  
375 37°C. After the incubation time, measure again a dilution of the culture with the flow  
376 cytometer to determine the cell count. The cell count should not have increased after the  
377 addition of antibiotics. In some cases, the cell count can even be decreased, e.g. when a  
378 bacteriolytic antibiotic is used (*See Note 18*).
- 379 5. To assess the fraction of cells being able to recover antibiotic treatment the antibiotics are  
380 diluted out. Pipette 500 µL of the culture into 50 mL preheated and filtered LB medium, mix  
381 well and transfer a sample of the non-diluted culture in the 96 wells plate (from where the

382 flow cytometer will take the sample) and measure it straight away with the flow cytometer.  
383 The sample should not be diluted because there are now 100-fold less cells in the culture than  
384 in the flask where the cells were incubated with the antibiotics. LB contains, when only  
385 autoclaved, a lot of components that lead to a strong background signal in the flow cytometer.  
386 This will interfere with your sample because the debris will appear around the same size as  
387 your cells on the FSC-SSC dot plot and therefore it is required to use filtered LB medium to  
388 lower the background signal. The first sample taken from the LB culture is very important  
389 because it reflects all cells in their dormant state and it is used to calculate the fraction of cells  
390 that became dormant after the nutrient-switch (*See Note 19*).

- 391 6. From now on, subject the LB cultures to flow cytometric analyses every 30 minutes for a  
392 period of 4 hours. Cells have a short doubling time on LB and thus cell counts can quickly  
393 enter a range that is beyond the linear range of the flow cytometer. Take notice of the cell  
394 count in each sample and determine the appropriate dilution for the next sample (*See Note*  
395 **20**).
- 396 7. The fraction of tolerant cells is calculated by subtracting the number of nondividing cells  
397 (cells that do not exit the bottom right part of Figure 3A) in each time point from the initial  
398 number of nondividing cells.
- 399 8. Samples measured directly after staining, after the addition of antibiotics, and right before the  
400 nutrient shift are solely used as quality controls for the experiment. The cells should show one  
401 single small peak directly after the switch to fumarate and the population should have  
402 significantly slowed down growth before antibiotics have been added. After the two hour  
403 treatment with antibiotics the growth should have completely stagnated or the cell number  
404 could even have dropped in case a bacteriolytic (e.g. ampicillin) has been used.

#### 405 **3.4.1 Data analysis**

- 406 1. For data analysis of the total fraction of tolerant cells, the cells are followed for four hours  
407 after the switch to LB, and the loss of fluorescence as well as the size of the cells is used to  
408 determine the fraction of the population being able to escape dormancy after antibiotic  
409 treatment. It is required to set up a dot plot to gate the cells (FSC-H vs SSC-H) and a dot plot  
410 using cell size and fluorescence (FL1-A vs FSC-A).
- 411 2. To determine the fraction of cells escaping dormancy the number of cells transferred to LB is  
412 taken at time point zero (T0). For each following time point, the number of nondividing cells  
413 is subtracted from the cells transferred at T0. It is not possible to use the number of escaping  
414 cells since they start dividing after the switch to LB (*See Note 21*) as shown in Figure 3A.
- 415 3. To define the non-growing population, the quadrant function. As in Figure 3A, it needs to be  
416 adjusted manually for each sample. Particularly in samples where a big part of the population  
417 just started to divide it can be hard to distinguish the non-growing population from the

418 growing population. It is advised to use the later time-points to set the quadrant and use the  
419 same settings for the early division samples (*See Note 22*). When one is only interested in the  
420 final number of tolerant cells and not in the recovery dynamics one can decide to not analyze  
421 these samples. An example of the recovery dynamics is shown in Figure 3B.

422 4. The data from 4 hours on LB is used to calculate the final fraction of tolerant cells. Take the  
423 number of nondividing cells and subtract it from the number of cells added to LB at T=0. It is  
424 optional to take the average of the last two, or the last three measurements to calculate the  
425 final fraction of tolerant cells (only if the recovered fraction reached steady state). Averages  
426 and standard deviations are calculated from three individual experiments and shown as box  
427 plots.

#### 428 4. Notes

- 429 1. If antibiotics are required for the particular *E.coli* strain it is important that the antibiotic is  
430 always added after autoclaving the LB-agar and after the LB agar has cooled down to ~55°C,  
431 to avoid degradation of the antibiotic. It is recommended to prepare 1000x concentrated  
432 stocks of the required antibiotics, to store them at -20°C and thaw them just before pouring  
433 plates.
- 434 2. When using the glucose-to-fumarate rapid nutrient shift to generate large fractions of tolerant  
435 cells, it is important to not use cellulose acetate filters because these filters could release  
436 acetate into the medium (13), which *E. coli* can use as a carbon source. As *E. coli* can have  
437 different preferences on which gluconeogenic carbon source to use first, even a small  
438 concentration of acetate could affect the results. PES filters are equally priced and do not  
439 release any compound that can act as a substrate for *E. coli*.
- 440 3. The antibiotic concentrations used in the working solutions are based on literature research  
441 and on experiments where we tested the antibiotics on *E.coli* cell growing on fumarate. The  
442 concentration of the antibiotic stocks is based on the solubility of the antibiotic. For ampicillin  
443 and tetracycline, the solubility is very high so it was decided to make a 1000x concentrated  
444 stock.
- 445 4. When measuring and counting *E. coli* cells, the flow cytometer needs to be calibrated for  
446 small cells. Typically, the default settings are not suitable for *E. coli* cells. With the BD  
447 Accuri C6, the threshold for *E. coli* should be 8000 for the forward scatter (FSC-H) and 500  
448 for the side scatter (SSC-H). Further, debris in the medium can disturb the measurement.  
449 Thus, it is required to only use filtered media when measuring samples with the flow  
450 cytometer. For reliable cell counts with the Accuri C6, the events measured by the flow  
451 cytometer should be between 10 000 and 100 000 cells in 20 µL of the measured sample. This  
452 level ensures a high enough amount of cells in comparison to machine noise, and low enough  
453 to not cause over-saturation of the instrument.

- 454 5. For the successful generation of almost 100% tolerant cells with a glucose-to-fumarate shift,  
455 the cells in the glucose culture must be in a fully exponentially growing state on glucose for at  
456 least 24 hours before the nutrient shift. Because cells typically have a lag phase after  
457 inoculation from the LB plate, we recommend starting culturing cells at least one day before  
458 the actual nutrient shift to ensure the maximal growth rate on glucose.
- 459 6. To ensure cells that cells keep on growing exponentially also after the re-inoculation, it is  
460 crucial to pre-heat the media before dilution of the cultures. But even then, typically short lag  
461 phases occur after diluting, and it is thus recommended to not only inoculate the calculated  
462 number of cells but also to prepare a preculture starting with 2x the number of cells required,  
463 such that at the desired time point in the morning one surely obtains a culture with the proper  
464 cell density.
- 465 7. Previous research has shown that the initial cell density after the nutrient shift influences the  
466 fraction of dormant cells after nutrient shift (14). The absolute number of adapting cells seems  
467 to be constant regardless of the initial cell density after a glucose to fumarate shift, resulting  
468 in a higher fraction of cells adapting when a flask is inoculated with a low cell density (13).  
469 Furthermore, note that with different *E. coli* wildtype strain strains different fractions of  
470 dormant cells can emerge.
- 471 8. Preparing this mixture too soon will cause the dye to clump and staining intensity will be sub-  
472 optimal. The solution can be prepared during the first centrifugation step, although the  
473 Diluent C must be brought to room temperature earlier. When multiple samples are stained in  
474 parallel, always change the pipette tip to prevent water transfer to the vial containing the dye  
475 stock. The dye-to-cell number ratio is very important for proper staining. The ratio is good,  
476 when the cells clump a bit during staining, as indicated by a slightly cloudy solution.
- 477 9. Leaving droplets on the side on the tube will create a fraction of unstained cells because  
478 droplets containing cells do not get in touch with the dye. This will be seen as a separate  
479 unstained fraction and will influence your results or even make them unusable.
- 480 10. Longer incubation will cause the cells to die, whereas shorter incubation will cause the cells  
481 to be stained sub-optimally. If you stain cells of multiple different samples in parallel, add the  
482 dye in 20-30 second intervals, such that the incubation time can be strictly adhered to.
- 483 11. BSA blocks the remaining dye molecules, thereby preventing the cells from being killed. It is  
484 recommended to use a timer and make sure you have the M9 medium with BSA in your  
485 pipette ready so that you only have to release it from the pipette into the tube containing the  
486 cells when the 3 minutes incubation time is over.
- 487 12. It is recommended to prepare the flasks with preheated and pre-aerated M9 medium with 2  
488 g/L fumarate before harvesting the cells.
- 489 13. To obtain a good fit of the data to the model and thus reliable parameter estimates, the data  
490 needs to fulfill certain criteria. First, the fluorescence intensity of stained cells should be at

491 least two orders of magnitude higher than the background fluorescence of unstained cells.  
492 This, in turn, means that the fluorescence intensity decrease can be tracked over 5-6 divisions,  
493 which is enough for most carbon source switches. Moreover, the fluorescence intensity data  
494 of stained cells should form a single peak in the histogram. The narrower the peak, the better,  
495 as the growing and non-growing populations will be better separated from each other in the  
496 obtained data. While in our experiments we had the best results using the green fluorescent  
497 dye, we have also used a red fluorescent dye (PKH26), which was giving us data of lesser  
498 quality. In principle, any product that utilizes a fluorophore linked to an aliphatic chain that  
499 intercalates in the cell membrane could be utilized to generate the data for the script, and there  
500 are many alternative products available on the market that have different excitation and  
501 emission properties that might be best suitable for the equipment used. However, due to  
502 different protocols and properties of these dyes, appropriate controls would have to be made  
503 to exclude the effect of the staining procedure on the obtained results.

504 14. The higher the fraction of adapting cells, the sooner the first usable data point can be taken. In  
505 the case of a glucose-to-fumarate switch, 15-16 hours after the switch is usually a good  
506 starting point, as the growing population starts to be large enough to be detectable, while it  
507 still has a fluorescence intensity that is above the autofluorescence. Capturing time points, in  
508 which the growing population still has some fluorescence above the cellular autofluorescence  
509 is crucial for the script to estimate the fraction of growing cells accurately.

510 15. The fluorescence intensity data from the flow cytometer needs to be in log<sub>10</sub> space.  
511 Depending on the cytometer used, a transformation needs to be done on the data (as we do in  
512 case of the Accuri C6 cytometer) or is already done in the cytometer or its software.  
513 Moreover, depending on the flow cytometer, the numerical range of values can be different  
514 and this needs to be addressed by setting the scaling factor.

515 16. After transferred to the fumarate medium, several cells will undergo a reductive cell division  
516 within about the first hour. Experiments have shown that after 2 hours on fumarate, all cells  
517 of the population become tolerant against ampicillin (8). Adding antibiotics too early might  
518 result in fewer cells surviving treatment, whereas incubating longer than 2 hours is not  
519 increasing the tolerant population.

520 17. For each antibiotic used in our experiments, we checked in the literature which concentrations  
521 were used in *E.coli* inhibition experiments. We used this information as a starting point to  
522 explore which concentration of each antibiotic kills growing cells. Before the tolerance  
523 experiments were done we carried out identical experiments with glucose grown cells to  
524 determine the concentration of antibiotics needed to kill a growing population.

525 18. When switching the cells to fumarate 0.01% of the population will adapt and start  
526 proliferating (7). Those cells will be sensitive to antibiotic treatment. Especially when the  
527 tolerant cells are kept for longer periods (~24 h after the nutrient shift) a significant



528 population of growing cells is visible which are sensitive to antibiotics. When a bacteriolytic  
529 antibiotic such as ampicillin is used, the treatment will cause these cells to lyse. Therefore, the  
530 cell count after treatment can be lower than before treatment.

531 19. To check cells' ability to survive antibiotic treatment they are transferred to LB medium. No  
532 washing steps are applied to prevent the cells from extra stress. By only transferring 500  $\mu$ L  
533 in 50 mL the antibiotics are diluted 100 fold, enough to nullify their inhibiting effect.

534 20. Bacteria grow fast on LB medium,  $\mu = 1.9 \text{ h}^{-1}$  (15). Therefore, it is essential to take regular  
535 measurements, to generate reliable data points. Dead cells do not proliferate, meaning that  
536 their number will remain the same and they will not lose their fluorescence. They will be  
537 visible as a stained cloud of small cells in the FL1-A versus forward scatter (FSC-A) plot.  
538 However, when the descendants of the small growing population keep increasing their  
539 number, they will outgrow the linear range of the flow cytometer and a bigger dilution must  
540 be made to measure the sample. This has the risk to lose the visibility of the population of  
541 dead cells because they excessive diluted and their cell count is not reliable. In particular,  
542 when only a small fraction of cells are not dividing a small dilution will introduce a big  
543 measurement error by dilution out the number of non-growing cells.

544 21. Bacteria escaping the dormant state do this by resuming their growth. Since it is impossible to  
545 determine the individual growth rate for each cell there is no way to use the growing  
546 population for calculating the fraction of cells able to escape dormancy. However, since we  
547 know the original number of cells transferred to LB and we can distinguish growing from  
548 non-dividing cells we can use the number of non-dividing cells and the number of cells at T0  
549 to calculate the fraction of the population which has survived antibiotic treatment.

550 22. When cells start waking up from their dormant state, they will first get bigger, followed by  
551 the loss of fluorescence. This can be seen in a dot plot as in Figure 3A (FL1-A vs FSC-A).  
552 However, in some cases, the cells may wake up a bit slow, and in the earlier time-points of  
553 the recovery experiment, the population of cells increasing size might overlap the nondividing  
554 population. In that case, it is advised to determine the non-dividing population at a later time-  
555 point and use those settings in the more indefinite sample.

556

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560

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598

599 **Figure captions**

600

601 **Figure 1 – Schematic overview of the procedure for generating persisters and for testing for**  
602 **their antibiotic tolerance.** (A) prepare an exponentially growing culture. Add a colony to 50 mL  
603 glucose minimal medium and grow overnight. Dilute this culture and grow during the day, dilute once  
604 more, and grow overnight. Make sure the culture is in an exponential phase when starting the staining.  
605 (B) wash or stain the cells to remove all residual glucose and optional, to stain the cells for tracking  
606 them with flow cytometry. (C) add the washed or stained cells to fumarate minimal medium and  
607 follow their growth at different time points using flow cytometric analysis. (D) to test antibiotic  
608 tolerance, add antibiotics after 2 hours in fumarate minimal medium. Incubate for 2 hours and transfer  
609 500  $\mu$ L to 50 mL LB medium. Follow regrowth for 4 hours using flow cytometry.

610

611 **Figure 2 – Finding initial parameters and example of a good fit.** (A) How to find the initial  
612 parameter guesses using the Fluorescence Data figure generated by the script. The model needs a  
613 good estimation input to make it generating proper estimations. Therefore the mean fluorescence of  
614 the growing and the non-growing population needs to be estimated. To make a good estimation pick  
615 the average of the fluorescence in between the left arrows for growing cells and the average of the  
616 peak on the right for non-growing cells. (B) The cell count curve fit check. Empty disk – cell count at  
617  $t = 0$ , red disks – cell counts used for the model fit, red line – predicted total cell count, cyan line –  
618 cell count of non-growing cells, magenta line – cell count of growing cells. (C) The model fit to the  
619 fluorescence data at each time point. Blue line – experimental data; green line – the distribution  
620 corresponding to the growing population; red line - the distribution corresponding to the non-growing  
621 population; black line – the sum of the distributions pictured by red and green line.

622

623 **Figure 3 – Example of regrowth in LB medium after antibiotic treatment.** (A) Exemplary flow  
624 cytometry graphs over time: When tolerant cells (stained with fluorescent dye) are transferred to LB  
625 they first increase in size followed by a loss in fluorescence as a consequence of their divisions. Cells  
626 in Q1 are big and have lost their fluorescence. Cells in Q2 are big and have a high fluorescent  
627 intensity. Cells in Q3 are small and have lost their fluorescence. Cells in Q4 are small and are  
628 fluorescent. (B) Left, the formula of how the fraction of tolerant cells is calculated. Right, an example  
629 graph of treatment with 2 different antibiotics. The fraction of cells for each time point is calculated  
630 by 1 minus the number of cells in Q4 divided of the number of cells in Q4 on timepoint 0. Cells that  
631 are killed by antibiotics will not regrow in LB medium and therefore will not leave section Q4 in the  
632 flow cytometer graph. After 4 hours a steady state is reached and the fraction of cells on  $T = 4$  can,  
633 therefore, be used as the ultimate fraction of viable cells.

634

635 **Supplementary figure 1 – Bad fit 1.** (A) The cell count curve fit check. Empty disk – cell count at  $t = 0$ ,  
636 red disks – cell counts used for the model fit, red line – predicted total cell count, cyan line – non-  
637 growing cell count, magenta line – growing cell count. (B) The model fit to the fluorescence data at  
638 each time point. Blue line – experimental data; green line – the distribution corresponding to the  
639 growing population; red line - the distribution corresponding to the non-growing population; black  
640 line – the sum of the distributions pictured by red and green line.

641

642 **Supplementary figure 2 – Bad fit 2.** (A)The cell count curve fit check. Empty disk – cell count at  $t =$   
643  $0$ , red disks – cell counts used for the model fit, red line – predicted total cell count, cyan line – non-  
644 growing cell count, magenta line – growing cell count. (B)The model fit to the fluorescence data at  
645 each time point. Blue line – experimental data; green line – the distribution corresponding to the

646 growing population; red line - the distribution corresponding to the non-growing population; black  
647 line – the sum of the distributions pictured by red and green line.

648

#### 649 **Table captions**

650 **Table 1 – Overview of the parameters needed to be set for Matlab script.** Left column: overview  
651 of parameters that need to be set. Right column: instructions on how to estimate these values.

652

#### 653 **Tables**

#### 654 **Table 1**

<b>Parameter</b>	<b>How to determine</b>
nongrowing population initial FI mean	From “Fluorescence Data” figure – see Figure 2A
nongrowing population FI stdev	From “Fluorescence Data” figure – see Figure 2A
nongrowing population cell concentration	The cell concentration after the nutrient switch is usually a good initial guess, except for nutrient switches in which the non-growing population is small. Upper and lower bounds can be very relaxed.
growth rate of non-growing population	From previous experiments
growing population initial FI mean	From “Fluorescence Data” figure – see Figure 2A
growing population FI stdev	From “Fluorescence Data” figure – see Figure 2A
initial growing population cell concentration	A guesstimate based on the expected number of adapting cells for a particular carbon source switch. Upper and lower bounds can be very relaxed.
growth rate of the population that starts to grow normally	From previous experiments determining growth rate on particular carbon source
unstained cell background autofluorescence	By analyzing data for unstained cells, or by checking the FI for very late samples, when cells have lost all their fluorescence.

655





