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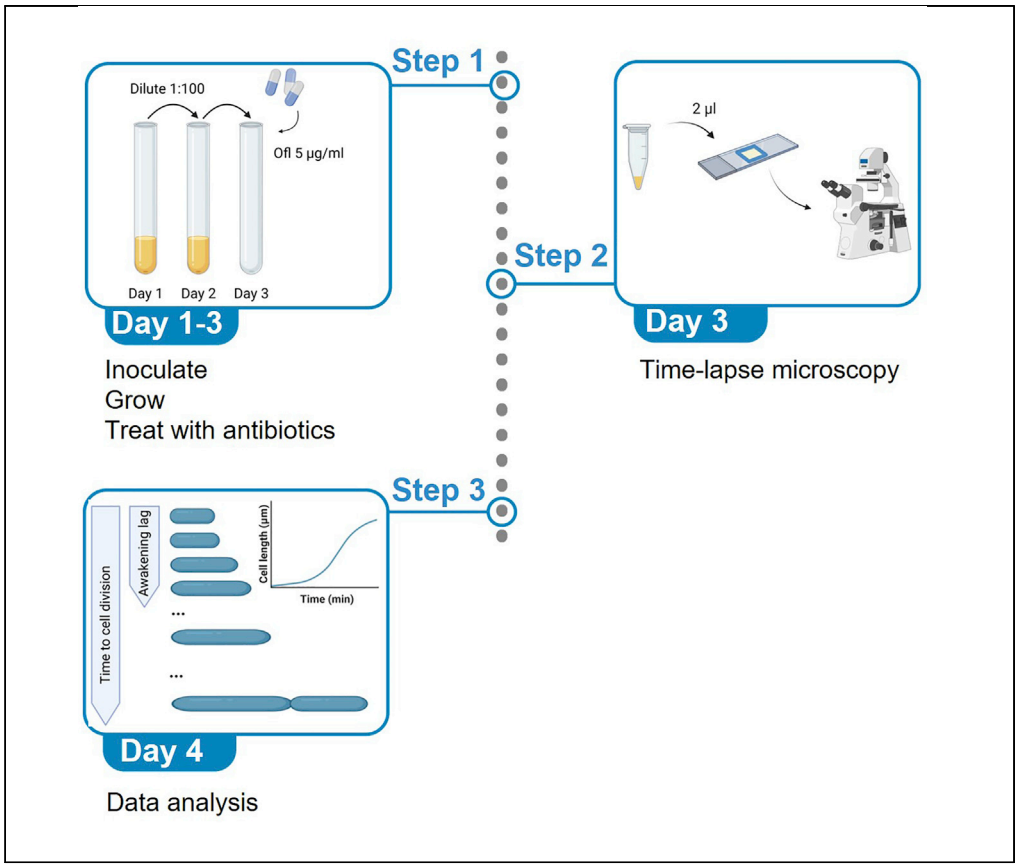
Assessing persister awakening dynamics following antibiotic treatment in *E. coli*



Given the low fraction of antibiotic-tolerant persisters and the transient nature of the persister phenotype, identifying molecular mechanisms underlying persister state exit, also called "awakening," is challenging. Here, we describe how persister awakening kinetics can be quantified at the single-cell level, enabling the identification of genes that are important for persister survival following antibiotic treatment. We report step-by-step sample preparation, dynamic recording, and data analysis. Although the setup is flexible, time-lapse microscopy requires a minimal number of persisters being present.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for assessing persister awakening dynamics via time-lapse microscopy

Experimental procedures and explanation on how to identify persisters

Guidelines on analyses and interpretation of awakening kinetics

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Assessing persister awakening dynamics following antibiotic treatment in *E. coli*

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SUMMARY

Given the low fraction of antibiotic-tolerant persisters and the transient nature of the persister phenotype, identifying molecular mechanisms underlying persister state exit, also called "awakening," is challenging. Here, we describe how persister awakening kinetics can be quantified at the single-cell level, enabling the identification of genes that are important for persister survival following antibiotic treatment. We report step-by-step sample preparation, dynamic recording, and data analysis. Although the setup is flexible, time-lapse microscopy requires a minimal number of persisters being present.

For complete details on the use and execution of this protocol, please refer to Wilmaerts et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the specific steps for *E. coli* BW25113 stationary phase cells, treated with ofloxacin and recovery on LB-agarose pads. However, we have also used this protocol for other antibiotics (ciprofloxacin, moxifloxacin, tobramycin), with other strains (UTI89, LF82 and *Pseudomonas aeruginosa*), and on a different agarose pad (M9 minimal medium supplemented with glucose).

Before any awakening kinetics are assessed, it is important to ensure that antibiotics are applied well above the minimal inhibitory concentration (MIC) and that treatment duration is sufficiently long to kill sensitive cells and reach the so-called 'persister plateau', which is determined by performing time-kill experiments (Balaban et al., 2019). Therefore, both the MIC value and biphasic killing curve need to be determined for each combination of strain and antibiotic that will be employed to assess awakening kinetics. Importantly, the MIC assay, the biphasic killing curve and the sample preparation for the time lapse microscopy should be performed in the same medium as the persister fraction can vary in different conditions.

Phenotype your cultures: MIC assay

Timing: 3 days

1. Inoculate your preculture in a glass tube containing 5 mL LB medium. Incubate at 37°C at 200 rpm for 20–24 h.
2. Prepare a stock solution of your antibiotic. For ofloxacin, prepare a stock solution of 0.5 mg/mL in milli-Q water.

3. Adjust the optical density (OD) of the overnight cultures.
   a. Add 50 μL of the culture to 950 μL 10 mM $MgSO_4$ to dissolve the bacteria in an isotonic environment. Measure the OD at 595 nm.
   b. Correct the OD_{595} to 0.1 in 10 mM $MgSO_4$. Therefore, use the following formula: $V\ (\mu l) = \frac{50\ \mu L * 0.1}{OD\ measured}$
   c. Add the correct volume of the culture (V) to an Eppendorf tube and add 10 mM $MgSO_4$ up to 1 mL.
4. Dilute this suspension 200× in 5 mL of the desired growth medium (e.g., LB).

   Note: The same medium should be used for the biphasic killing curve and for the microscopy experiment.

   Note: Your inoculum should now contain $5 * 10^5$ cells/mL.

5. Mix by pipetting up and down.
6. Transfer 150 μL of the inoculum mixture to columns 1–11 of a 96-well plate. Do this in twofold for 2 technical replicates.
7. As a negative control, add LB without cells to column 12.
8. Take a new Eppendorf tube.
   a. Multiply the highest antibiotic concentration you want to assess in the MIC assay by two.
   b. Add a volume of antibiotic stock solution, corresponding to two times the highest concentration, to the Eppendorf tube. Calculate for a final volume of 1 mL.
   c. Add up to 1 mL with inoculum mixture.
9. Add 150 μL of this mixture to the first column.
10. Make a two-fold dilution series up to column 10. Transfer 150 μL of column 1 to column 2. Take new tips, pipet up and down two times in column 2 and repeat the procedure until you have reached column 10. Discard the last 150 μL.
11. Do not add antibiotics to column 11, this is your positive control.
12. Cover the plate with a breathable membrane and lid, and incubate for 20–24 h at 37°C at 200 rpm.
13. Following incubation, remove the lid and the breathable membrane and measure the OD at 595 nm.
14. Calculate 10% of the OD from your positive control (column 11). This is your cut-off value.
15. Your MIC is the lowest antibiotic concentration where your OD is below the cut-off value calculated in step 14. An example of assessing the MIC of ofloxacin for *E. coli* BW25113 is depicted below. The MIC is 0.156 μg/mL as it is below the cut-off value of 0.0976.

μg/mL	5	2.5	1.25	1.625	0.313	0.156	0.0781	0.0391	0.0195	0.000977	+	LB
OD	0.046	0.046	0.046	0.046	0.045	**0.045**	0.276	0.791	0.95	1.112	0.976	0.044

Phenotype your culture: Biphasic killing curve

Timing: 5 days

16. Inoculate your preculture in a glass tube containing 5 mL LB medium. Incubate at 37°C at 200 rpm for 20–24 h.
17. Dilute your preculture 100-fold in 5 mL LB medium. Incubate at 37°C at 200 rpm for 16–18 h.
18. Prepare a stock solution of your antibiotic.
   a. Determine the treatment concentration. Treatment will be performed with a concentration above the MIC, which was measured in the previous experiment.

   Note: You can, for example, treat your sample with 10× MIC.

b. Calculate the concentration of the stock solution.

Note: Take into account that the stock will be diluted 100-fold.

19. Take 990 μL of the culture and add it to an empty, sterile, glass tube. Add 10 μL of the antibiotic. Avoid flaming the tube in this step, as the heat might affect the antibiotic efficacy. Incubate the tube at 37°C at 200 rpm.
    a. Check the time, this is your t=0.
20. Plate out the untreated culture at t=0.
    a. Fill up column 2 to column 8 of a 96-well plate with 180 μL of 10 mM sterile $MgSO_4$.
    b. Add 150 μL of the culture to column 1.
    c. Put the untreated culture back in the incubator after taking the sample.
    d. Make a 10-fold dilution series: take 20 μL of column 1 to column 2. Take new tips, pipet up and down two times in column 2 and repeat the procedure until you have reached column 8.
    e. Plate out 100 μL of the desired dilutions. Spread the liquid over the plate using sterile beads in order to have countable plates.
       i. For E. coli BW25113, plating out 100 μL of 10^{-6} and 10^{-7} will give you countable plates.
    f. Let the plates dry. Once they are dry, remove the beads. Put the plates in the incubator at 37°C for 40–48 h.
21. For the biphasic killing curve, add 100 μL of the treated and untreated culture to an Eppendorf tube after 1, 3, 5, 8 and, optionally, 24 h of incubation, based on your t=0. Put the cultures back in the incubator after taking the sample.
22. At every time point, plate out the untreated cultures.
    a. Fill up column 2 to column 8 of a 96-well plate with 180 μL of 10 mM sterile $MgSO_4$.
    b. Add 150 μL of the culture to column 1.
    c. Put the untreated culture back in the incubator after taking the sample.
    d. Make a 10-fold dilution series: take 20 μL of column 1 to column 2. Take new tips, pipet up and down two times in column 2 and repeat the procedure until you have reached column 8.
    e. Plate out 100 μL of the desired dilutions. Spread the liquid over the plate using sterile beads in order to have countable plates.
       i. For E. coli BW25113, plating out 100 μL of 10^{-6} and 10^{-7} will give you countable plates.
    f. Let the plates dry. Once they are dry, remove the beads. Put the plates in the incubator at 37°C for 40–48 h.
23. At every time point, plate out the treated cultures.
    a. Spin the culture down for 5 min at 3,000 × *g*. Remove the supernatant by pipetting and re-suspend in 100 μL of sterile 10 mM $MgSO_4$.
    b. Repeat the washing step.

    Note: Since persister cells are not antibiotic-resistant, washing the treated cultures is essential as it removes the residual antibiotic.

    c. Fill up column 2 to column 6 of a 96-well plate with 180 μL of 10 mM sterile $MgSO_4$.
    d. Add 80 μL of the culture to column 1.
    e. Make a 10-fold dilution series. Transfer 20 μL of column 1 to column 2. Take new tips, pipet up and down two times in column 2 and repeat the procedure until you have reached column 8.
    f. Plate out 100 μL of the desired dilutions. Spread the liquid over the plate using sterile beads in order to have countable plates.
       i. For E. coli BW25113 treated with ofloxacin, plating out 100 μL of 10^{-4} and 10^{-5} will give you countable plates.
    g. If you plate out after 24 h of treatment, also plate out the control culture at this time point using the steps described in point 19.

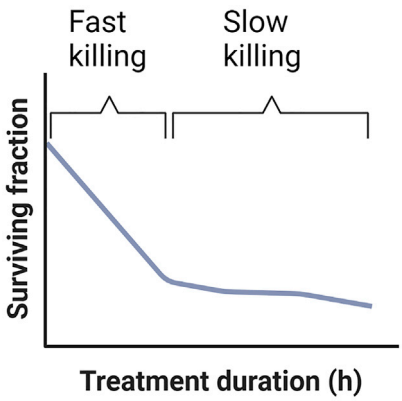

Figure 1. Illustrative example of a biphasic killing curve
In the first phase, fast killing of sensitive cells is observed. In the second phase, or the ''persister plateau'', slow to no killing of the persister cells is observed. A slow decrease in the number of surviving cells is the result of persister cells that switch back to a normal phenotype, thereby losing their tolerance (Wilmaerts et al., 2019).

h. Let the plates dry. Once they are dry, remove the beads. Put the plates in the incubator at 37°C for 40–48 h.

Optional: Plating out the untreated culture at t=1 h and t=24 h might be sufficient to determine persister fractions, if the CFU/mL does not vary significantly from t=1 h to t=8 h.

24. Count the colonies on the plates. Determine the CFU/mL of your treated and untreated cultures.
25. Determine the surviving fraction at every time point.
    a. Divide the CFU/mL of the treated cultures at the different time points over the CFU/mL of the untreated culture. This will give you a persister fraction.
    b. Take the $\log_{10}$ of these fractions and visualize the data.
    c. Ensure that you are working with persister cells by assessing whether you have a biphasic killing curve (Balaban et al., 2019).
    d. From this killing curve, you can determine the appropriate time of antibiotic treatment. This time of treatment should be a time point on the 'persister plateau', where there is no or slow killing of the cells (Figure 1) (troubleshooting 1).
        i. For ofloxacin treatment of BW25113, this is at 5 h of treatment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli Keio BW25113	Horizon	CAT#OEC5042
Chemicals, peptides, and recombinant proteins		
Ofloxacin	Sigma-Aldrich NV	CAT#O8757
Magnesium sulfate	Sigma-Aldrich NV	CAT#M2773
Tryptone	International Medical Products NV	CAT#8534595
Yeast extract	International Medical Products NV	CAT# 8534641
Agarose	Invitrogen	CAT#16500500
Agar	VWR international	CAT# 84609.0500
HCl	VWR International	CAT#1.09057.100
Sodium chloride	VWR International	CAT#1.09137.100
Deposited data		
Mendeley database: oufti test set	Mendeley	https://doi.org/10.17632/tw3ffmj4nf.1
Software and algorithms		
Nis Elements Analysis 4.3	Nikon	https://www.nikon.com/products/microscope-solutions/support/download/software/imgsfw/
Oufti		https://oufti.org/index.html

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Gene frame, 25 µL (1.0 × 1.0 cm)	Invitrogen	CAT#AB0576
Cover slips (15 × 15 mm)	VWR international	631-1566
Super premium microscope slides	VWR	CAT#631-0116
Immersion oil F2 30cc	Nikon	MXA22192
Breathable membrane 96-well plate	Greiner Bio-one	CAT#676051
Glass tubes (16 × 160 mm, 21 mL) without beaded rim	Glasatelier Saillart	CAT#112613021
96-well plates	Greiner Bio-one	CAT#655101
Microscope, able to perform time-lapse microscopy Nikon Eclipse Ti-E inverted microscope, equipped with an incubation chamber and a CFI Plan Apochromat 100× objective with a numerical aperture of 1.45	Nikon	NA
Shaking incubator (Innova 43)	Eppendorf	CAT# M1320-0012
Non-shaking incubator (Memmert Ine 700)	ProfControl	CAT#3126
Microwave	Zanussi	NA
Target2™ PTFE syringe filters	Thermo Fisher Scientific	CAT#F2500-4

MATERIALS AND EQUIPMENT

LB medium	
Components	Amount
Yeast extract	2.5 g
Tryptone	5 g
NaCl	5 g
dH_2O	500 mL
Total	500 mL

Autoclaved LB medium can be stored for several weeks at room temperature (20°C–24°C). Toss away when you see turbidity.

LB-agar plates	
Components	Amount
Yeast extract	2.5 g
Tryptone	5 g
NaCl	5 g
Agar	7.5 g
dH_2O	500 mL
Total	500 mL

Autoclaved LB-agar can be stored at 50°C for a couple of days. Poured plates can be stored at 4°C for several weeks at 4°C.

LB-agarose pads	
Components	Amount
Yeast extract	0.5 g
Tryptone	1 g
NaCl	1 g
Agarose	2 g
dH_2O	100 mL
Total	100 mL

LB-agarose can be kept at 50°C for a day.

Note: Microwave the solution until it starts to boil. Gently stir after 1 min of microwaving.

⚠ CRITICAL: Do not reheat old agarose. Always prepare a fresh bottle.

Ofloxacin 100× stock solution (0.5 mg/mL)		
Components: Step 1	Final concentration	Amount
Ofloxacin		25 mg
Milli-Q water		5 mL
Total	5 mg/mL	5 mL
Components: step 2		
Ofloxacin 5 mg/mL		50 µL
Milli-Q water		450 µL
Total		**500 µL**
Ofloxacin stocks should be stored at −20°C in the dark. Stocks can be stored for 4 months.		

Note: Ofloxacin stocks should be filter sterilized using a filter pore size of 0.2 µm.

Note: Ofloxacin dissolves poorly in H_2O. Add two drops of HCl (1 M) and shake the solution. Add additional drops if necessary.

10 mM $MgSO_4$		
Reagent	Final concentration	Amount
$MgSO_4.7H_2O$	10 mM	1.23 g
dH_2O	n/a	500 mL
Total	**10 mM**	**500 mL**
Autoclaved $MgSO_4$ solution can be stored for several months at room temperature (20°C–24°C).		

STEP-BY-STEP METHOD DETAILS

Prepare the sample for microscopy

Timing: 4 days

Using the appropriate antibiotic concentration (see MIC assay), and following the correct treatment time (see biphasic killing curve), persister awakening kinetics can be assessed. Cells are treated with antibiotics and, following several washing steps, are spotted on the LB-agarose pad.

1. Day 1: Inoculate your preculture in a glass tube containing 5 mL LB medium. Incubate at 37°C at 200 rpm for 20–24 h.
2. Day 2: Dilute your preculture 100-fold in 5 mL LB medium. Incubate at 37°C at 200 rpm for 16–18 h.
3. Day 3: Take 990 µL of the culture and add it to an empty, sterile, glass tube. Add 10 µL of the ofloxacin stock solution. This will result in an ofloxacin concentration of 5 µg/mL.

Optional: Use another antibiotic of your choice, in a concentration calculated from the MIC assay and a treatment time determined from the biphasic killing curve. The duration of treatment depends on when the persister plateau is reached in the biphasic killing curve and should be assessed for every combination of antibiotic and bacterial strain.

⚠ CRITICAL: Avoid flaming the tube in this step, as the heat might affect the antibiotic efficacy. Work in a sterile environment to avoid contamination.

a. Incubate the tube at 37°C at 200 rpm for 5 h.

4. In the meantime, prepare the LB agarose.
5. Prepare the microscopy slide.
   a. Remove the protection layer from the gene frame and stick it to a glass microscopy slide (Figure 2A).
   b. Take approximately 200 μL of the heated LB-agarose and slowly pipet it in the middle of the gene frame until the frame is completely filled (Figure 2B). This is easier if you use a 1 mL pipet, as the tip opening is larger.
   c. Add heated LB-agarose until your gene frame is filled.

   Note: The gene frames used here have a capacity of 25 μL.

   d. Take a glass cover slip and gently press it on the gene frame (without removing the protective layer of the gene frame), thereby flattening the LB-agarose (Figure 2C).
   e. Let it dry for 2–5 h at room temperature.

   Note: If the LB-agarose pad has not dried completely, it will shrink during your time-lapse experiment which can cause XY drift or losing of focus in the Z direction.

6. Make sure the microscope incubation chamber is at its correct temperature (37°C).
7. Following 5 h treatment with ofloxacin, pipet 100 μL of your sample in an Eppendorf tube.
   a. Centrifuge the cells at 3,000 × *g* for 5 min.
   b. Resuspend the pellet in 100 μL of pre-heated LB.
   c. Repeat the washing step.
8. During the last washing step, prepare your LB-agarose pad.
   a. Gently remove the cover slip on top of the agarose by sliding.
   b. Take a scalpel and go over the inner border of the gene frame. This will prevent the agarose pad to be damaged when you remove the protective layer from the sticky blue border of the gene frame.
9. Add 2 μL of the washed culture in the middle of your LB-agarose pad.
   a. Gently tap the side of the slide against the bench, spreading the culture.
   b. Let it dry for a couple of minutes.

   Note: The liquid should be completely evaporated.

   c. Remove the protective layer from the sticky blue border of the gene frame.
   d. Put a glass cover slip on top of the gene frame.
   e. Gently press it.
   f. Note down the time.

Start the time lapse

Timing: 20 min for setting up the microscope

Following sample preparation, cells are imaged to follow their recovery and determine their awakening kinetics. It is important that this imaging is initiated as soon as possible.

10. Visualize the sample using a 100× magnification (troubleshooting 2).
    a. Choose several different XY positions throughout the sample. Spread your positions well. Take 5–8 positions close to each other, then move further across your sample. Spreading has several advantages. E.g., if an air bubble in your immersion oil disrupts focus, it is likely that not all of your positions are affected.

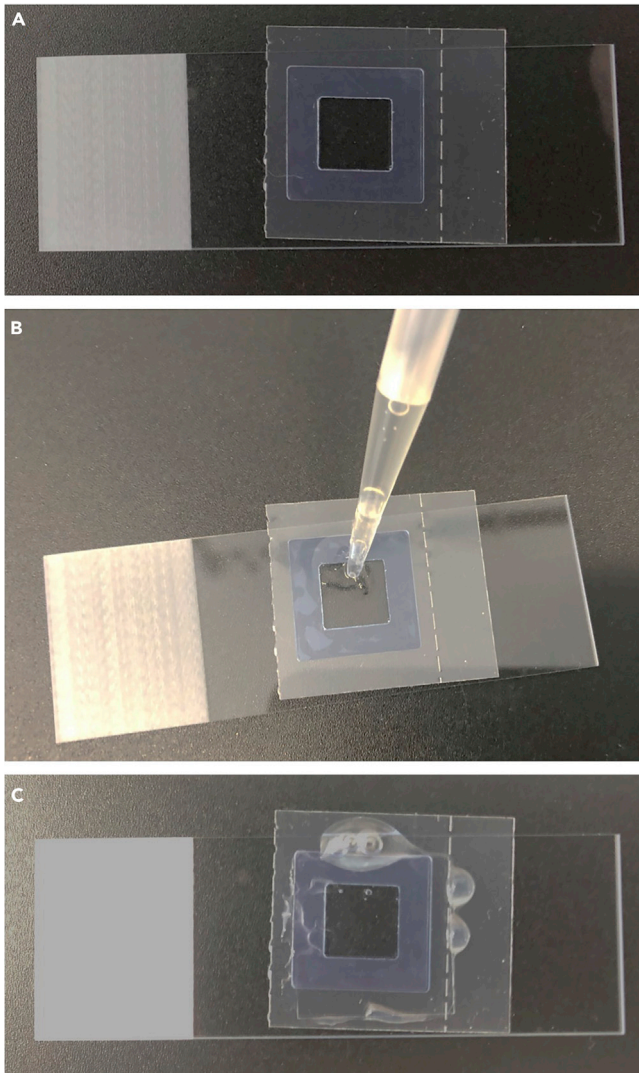

Figure 2. Preparation of the LB-agarose pad

(A) Stick the gene frame to a glass slide.

(B) Fill the gene frame with LB-agarose.

(C) Remove excess agarose by gently pressing the cover slip on the agarose.

Note: The number of cells in the frame is important. Avoid positions with too many cells (Figure 3A). It will complicate data analysis.

Note: Avoid positions that are right next to a big "wall" of cells (Figure 3B).

Note: Ideally, take a combination of frames that contain a different number of cells. Examples are depicted in Figures 3C and 3D.

11. The number of XY positions to select depends on your persister fraction, which is related the probability of observing a persister cell.
    a. For *E. coli* BW25113 in stationary phase following ofloxacin treatment, 25 XY positions usually suffice.

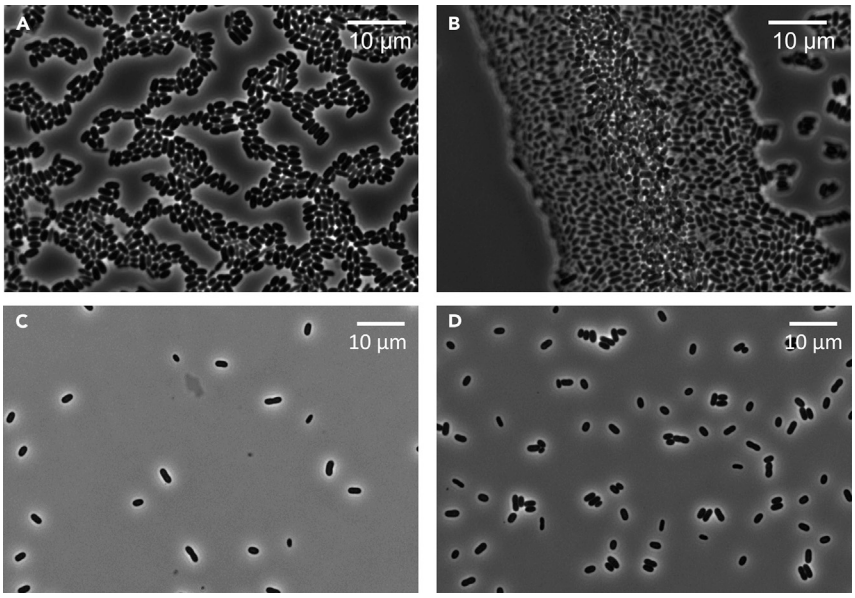

Figure 3. Choosing your XY positions
(A) This position is too crowded.
(B) Avoid imaging in the near proximity of a big "wall" of cells.
(C and D) Examples of ideal XY positions.

Note: If this fraction is approximately 1% you will need somewhere around 25 different XY positions. If it is higher (e.g., 10%), 15 frames might be sufficient. However, optimization might be necessary here.

⚠ CRITICAL: Keep in mind that the number of XY positions is limited. The limitation depends on the time interval between time points and speed of your microscope. The interval between the different time points should be 15–20 min, which cannot be reached if there are too many positions.

12. Choose an interval of 15 min for a total duration of 18 h (troubleshooting 3).

Note: The total duration of the experiment depends on the chosen strain and antibiotic. As a rule of thumb, 18 h is often sufficient for *E. coli* strains.

13. Note down the time. The time between step 13 and in step 9 should be taken into account during the data analysis.

Optional: If the persister fraction is high enough, you can increase throughput by combining two experiments on the same agarose pad. Therefore, carefully divide the agarose pad in two using a scalpel. This division line will be visible under the microscope. Make sure your cultures are well-separated and take XY positions far from the division line, to make sure you are visualizing the correct strain.

Note: The antibiotic concentration used might influence awakening kinetics. Note that this concentration should always be above the MIC.

Analyze the data

Timing: 1 day

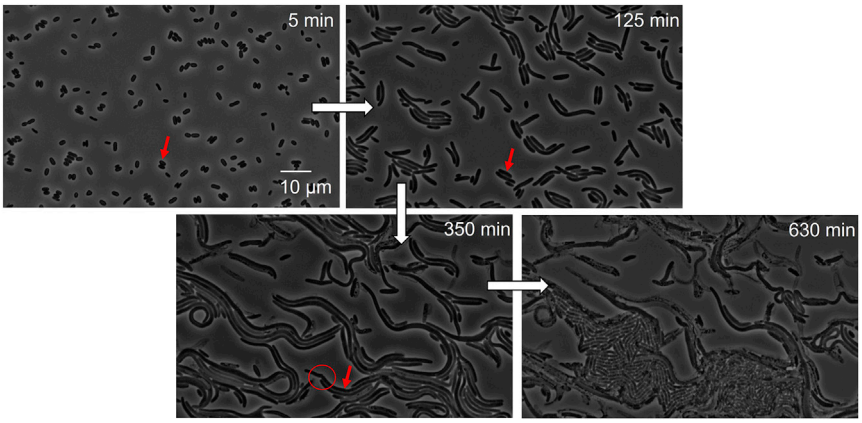

Figure 4. Representative example of recovering BW25113 following ofloxacin treatment

The red arrow indicates the persister cell, all the other cells are antibiotic-sensitive. The cell division plane is indicated with a red circle. The persister cell undergoes multiple cell divisions, leading to the formation of a micro-colony, in contrast to the sensitive cells.

Following ofloxacin treatment, strong filamentation is observed in both sensitive cells and persister cells during recovery, resulting from the induction of the SOS response due to double-strand break formation (Goormaghtigh and Van Melderen, 2019; Murawski and Brynildsen, 2021; Wilmaerts et al., 2022). Some of these filamented sensitive cells will even undergo cell division. However, a sensitive cell will not form viable offspring, whereas a persister cell will create so-called micro-colonies, as shown in Figure 4. Following the identification of the persister cells, awakening parameters can be determined.

14. Take into account the time between putting the cells on agarose (step 9) and the time the time-lapse was initiated (step 13).
15. Identify the persister cells. An example of *E. coli* BW25113 following ofloxacin treatment is shown in Figure 4 (troubleshooting 4 and 5).
    a. Persister cells are defined as cells that are able to divide multiple times (n > 4), resulting in a substantial number of offspring. Eventually, this will result in a micro-colony, as can be observed in Figure 4 at time point 630 min.
    b. Sensitive cells are cells that do not undergo multiple cell divisions, and as a result will not form a micro-colony.

Note: Antibiotic-sensitive cell elongation/division depends on the applied antibiotic. For example, we do not observe cell elongation or cell division following tobramycin treatment of *E. coli* BW25113. Looking for microcolonies is an ideal way to quickly scan for persister cells in your time-lapse microscopy data.

16. Analyze the persister awakening kinetics.
    a. Follow the length of the persister cell in time. Note down in an excel file.
    b. Determine the awakening lag from this growth curve. This is the time a cell needs to reach twice its initial size.

Optional: If other antibiotics are used (e.g., aminoglycosides), the threshold to determine the awakening lag can be set to 1.5-fold its initial size as not all the persister cells elongate to 2-fold the initial size before division during post-antibiotic recovery occurs.

    c. Determine when cell division occurs. Do this in a consistent manner: e.g., set the time of cell division when a cell division plane is visible.
    d. For every cell, calculate the time in between the awakening lag and cell division.

Table 1. Example of persister cell regrowth following ofloxacin treatment

Time	Length (μm)
5	2.57
20	2.65
35	2.85
50	3.35
65	3.63
80	3.95
95	4.35
110	4.7
125	**5.16**
140	5.58
155	5.97
170	6.5
185	6.87
200	7.24
215	7.69
230	7.96
245	8.69
260	11.69
275	14.31
290	17.68
305	22.37
320	27.37
335	**cell division**

17. Analyze growth kinetics of sensitive cells, in cases where they elongate.
    a. Randomly select sensitive cells from the same frames that contain persister cells.
    b. Follow the cell length in time for at least the same number of sensitive cells as there are persister cells. However, the more the better.
    c. Follow the length of sensitive cells in time for 180 min. Following ofloxacin treatment, this time frame allows all sensitive cells to elongate.

Note: This time frame might need optimization if other antibiotics/strains/media/... are used.

    d. Determine the awakening lag for the sensitive cells. This is the time a cell needs to reach twice its initial size.

EXPECTED OUTCOMES

Strong filamentation is observed following fluoroquinolone treatment, resulting from the induction of the SOS response due to double-strand break formation (Goormaghtigh and Van Melderen, 2019; Murawski and Brynildsen, 2021; Wilmaerts et al., 2022). After ofloxacin treatment (5 μg/mL) of stationary phase BW25113 cells, filamentation of both sensitive and persister cells can be expected (Figure 4).

Note: Filamentation does not occur following treatment with aminoglycosides.

In this example (Table 1 and Figure 4), the awakening lag is 125 min, and the timing of cell division is 350 min. The maximum cell length before cell division is measured from the frame before cell division (at 335 min) and is 27.37 μm. However, as can be observed from Figures 5A and 5B, these values can be very heterogeneous.

Upon treatment of BW25113 with ofloxacin, the following persister awakening kinetics are expected (Figure 5A): an awakening lag that ranges from 75 to 664.5 min, with an average of 262.4 min; a time

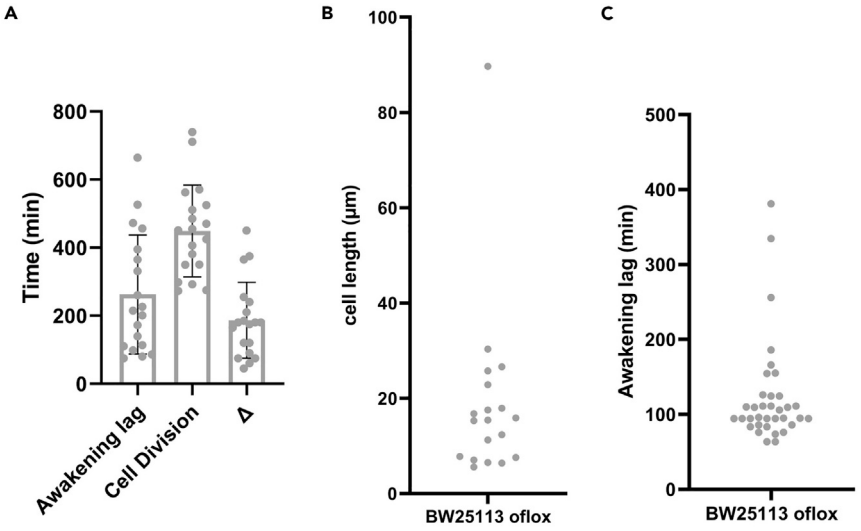

Figure 5. Persister awakening kinetics and cell elongation following ofloxacin treatment of BW25113

(A) Expected outcomes of the awakening lag, the timing of cell division and the difference between the timing of cell division and the awakening lag (here Δ) for BW25113 (WT). Data are represented as mean ± SD. Every dot represents the data of one persister cell.

(B) Following ofloxacin treatment, persister cells elongate strongly before cell division. Maximal cell lengths before cell division of persister cells are shown.

(C) The awakening lag of sensitive cells following ofloxacin treatment of BW25113.

of cell division that ranges from 273 to 739.5 min, with an average of 449 min; a difference between cell division and awakening lag (here Δ) that ranges from 45 to 450 min, with an average of 186.6 min.

Strong filamentation is observed, both for persister cells as for sensitive cells. The maximal cell length of persister cells before cell division is depicted in Figure 5B, and ranges from 5.59 μm to 89.69 μm with a median of 15.44 μm. For sensitive cells, the awakening lag ranges from 63.5 min to 381 min, with a median of 96 min.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Assess the distribution of the different datasets. If possible, normalize the data when not normally distributed, for example using Box Cox transformation. Otherwise, use non-parametric statistical tests.

   Optional: In addition to the awakening lag, the timing of cell division and the time between the awakening lag and the cell division, one might opt to examine other parameters such as maximal cell length, growth speed or cell volume.

   Note: The data distribution can give important information on the underlying mechanism. We refer to (Norman et al., 2015).

2. To assess if a gene is important for awakening or persister survival following antibiotic treatment, consider following points:
   a. The effect of deletion of a gene on the persister fraction of the cells.
      i. If the persister fraction is decreased, this gene might be either essential for persister survival following antibiotic treatment, or to exit the persister state.
   b. The effect of gene deletion on the persister awakening dynamics (awakening lag, timing of cell division and the time between the awakening lag and cell division).

c. The alterations in the awakening dynamics should be persister-specific.

Note: Following ofloxacin treatment, sensitive cells start to elongate, and measuring the awakening lag to compare with persister cells is possible. However, elongation of sensitive cells is not always observed but depends on the used antibiotic. If elongation is not observed in sensitive cells, comparing the awakening lag with persister cells is impossible. Instead, a persister-specific effect could be validated by assessing the exit from the stationary phase, without antibiotic treatment, of the mutant and compare this with a wild-type strain.

3. When phase-contrast microscopy is employed, time-lapse microscopy images can be analyzed using Oufti (Paintdakhi et al., 2016). The parameters that can be used to analyze filamented *E. coli* cells are available in the Mendeley database, in combination with a sample data set. However, automated analysis is difficult when cells elongate strongly, and additional optimization of the parameters might be necessary.
   a. Load the frames in Oufti (test set persister).
   b. Load in the parameters (testpersister_parameters).
   c. Locate the persister cell (indicated with a red circle in persister.png).
   d. When cells are not growing or not elongating rapidly and growing into neighboring cells (from frame 1–20 in the example dataset), automated analysis of the persister cell is possible. Refine the mesh wherever necessary.
   e. When cells elongate rapidly and grow into neighboring cells (from frame 21 onwards in the example dataset), manually adding the elongated persister cell is necessary. Change the attrCoeff to 0.4 and manually add the persister cell. Refine the mesh when necessary. Keep in mind that manually added cells will be assigned a new cell number on each frame, which needs to be taken into account for downstream analysis.
   f. Cell length (and other parameters) can be exported in CSV or in Matlab format.

LIMITATIONS

Visualizing persister cells in cultures with low frequency of persisters (e.g., 0.001%) will be challenging, if not impossible using a microscope set-up. In addition, this protocol relies on a microscope that is able to perform time-lapse microscopy: a motorized XY stage and Z focus, combined with temperature control.

Furthermore, automated high-throughput analysis of time-lapse microscopy with strong filamented cells is yet impossible. Significant hands-on time to analyze the data is needed.

TROUBLESHOOTING

Problem 1

Phenotype your culture: biphasic killing curve, step 25d.

The biphasic killing curve does not show two different rates of killing, but shows a constant decline.

Potential solutions

As described in (Balaban et al., 2019), this indicates that you are working with an antibiotic-tolerant population. You can still assess awakening kinetics, but keep in mind that these cells originate from a population which is entirely antibiotic-tolerant, rather than from a subpopulation of persister cells.

Problem 2

Start the time lapse, step 10.

When visualizing the cells on the LB-agarose pad, the cells are not fixed but are moving around.

Potential solutions

Your culture was not completely dried before putting on the glass cover slip on top of the agar. Check for positions closer to the edge of the culture to see if there are positions with fixed cells.

If not, prepare a fresh LB-agarose pad. However, keep in mind that this might give issues as a fresh pad tends to shrink substantially during the imaging.

Problem 3

Start the time lapse, step 13.

Your time-lapse microscopy loses focus after a couple of hours.

Potential solutions

There are different possible reasons why the focus is lost in time.

You have used the Perfect Focus System (PFS) of Nikon, or a similar hardware solution: Do not use the PFS option of the microscope (if applicable), as the differences in Z-drift might be too large for PFS to resolve. Use autofocus instead, with a total range of 14 μm and steps of 0.4 μm. Make sure the reference Z is saved after each round of imaging. Note that using autofocus increases the time needed to visualize each frame.

The agarose pad was too fresh upon use. Make sure it is prepared at least 2 h before using.

Refresh your immersion oil. We have observed that immersion oil that has been open for a while results in more air bubbles that interfere with imaging during time-lapse microscopy.

Problem 4

Analyze the data, step 15.

You do not observe persister cells.

Potential solutions

Make sure you have performed enough washing steps. Washing twice is sufficient for ofloxacin, but this might not be the case for other antibiotics. This can be validated by assessing survival by plating out after different washing steps.

Verify the % of persister cells in the culture. If it is too low (e.g., 0.001%), observing a persister cell will be very difficult.

If your fraction of persister cells is sufficiently high, try again and take more XY positions, but make sure that all positions can be imaged within your interval time (±15 min).

It might be that your XY positions are too crowded, making it difficult to track cell divisions over time. If so, take frames with well-separated cells, as depicted in Figures 3C and 3D.

Problem 5

Analyze the data, step 15.

You have too many persister cells in the same frame, making it difficult or impossible to observe or analyze persisters with a longer lag phase because the frame is quickly overgrown.

Potential solutions

You can try to dilute the culture before spotting on the agarose pad, but even then it might be difficult to observe persisters with a longer lag phase.

Other set-ups, such as a mother machine, could be useful in this specific case (sample with very high persister fraction). Here, cells are separated in specific growth channels (Windels et al., 2019). Note that the design of the mother machine is important: if you only have 100 channels, on average you will have 1 persister cell if your persister fraction is 1%. Hence, for populations with lower persister fractions, agarose pads are the preferred solution. Of note, in our set-up with 1% of persister cells, we were able to detect a variation in the awakening lag of the persister cells from 75 (minimal) to 664 min (maximal) within one sample (Wilmaerts et al., 2022).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jan Michiels, jan.michiels@kuleuven.be.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The dataset generated during this study is available at Mendeley Data: 10.17632/tw3ffmj4nf.1.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.W. and J.M.; methodology, D.W.; validation, D.W.; investigation, D.W.; writing, D.W.; editing, D.W., S.G., and J.M.; formal analysis, D.W. and S.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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