Chapter 6

Observing Bacterial Persistence at Single-Cell Resolution

Emma Dawson, Emrah Şimşek, and Minsu Kim

Abstract

Within a bacterial population, there can be a subpopulation of cells with an antibiotic-tolerant persister phenotype characterized by long lag phase. Their long lag phase necessitates long (hours or days) periods of single-cell observation to capture high-quality quantitative information about persistence. We describe a method of single-cell imaging using glass bottom dishes and a nutrient agarose pad that allows for long-term single-cell microscopy observation in a stable environment. We apply this method to characterize the lag phase and persistence of individual *Escherichia coli* cells.

Keywords Persistence, Persisters, Single-cell microscopy, Lag time, Antibiotic resistance

1 Introduction

When a bacterial population is exposed to antibiotics, a subpopulation called persisters may survive while the majority of cells quickly die. Single-cell imaging has revealed that persister cells exhibit long lag times when transferred from stationary phase to fresh nutrient medium. That is, while most of the population rejuvenates and enters a reproductive state, this subpopulation remains in the non-dividing state for an extended period of time.

We designed a set of experiments to allow one to observe persistence at single-cell resolution and characterize the effect of stationary phase on lag times. The first experiment, determining the time delay in killing of growing cells (Subheading 3.1), captures the distribution of the time delay in killing growing bacteria in the presence of an antibiotic. Cells are exposed to ampicillin and the time of killing is determined using single-cell microscopy. The second experiment, single cell observation of lag time after starvation (Subheading 3.2), captures the distribution of lag times of a previously starved bacterial population. Cells are starved of nutrients (nongrowing state) and then transferred to a nutrient-rich

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environment. For each cell, the time at which it begins to grow (i.e., lag time) is determined using single-cell microscopy.

Both experiments require the observation of single cells over long periods of time (hours or days) within a stable environment. To achieve this, we make use of glass bottom microscopy dishes, which allow cells to be sandwiched in the bottom of the dish between a nutrient agarose pad and a cover glass. The nutrient agarose pad is thick and enclosed to maintain a consistent environment around the cells for the duration of the experiment, dramatically increasing the possible duration of single-cell microscopy experiments. Experiments using this method follow a basic protocol: a nutrient agarose pad is prepared for the experiment, a liquid culture is sandwiched in a glass bottom dish beneath the nutrient agarose pad, the dish is mounted on a phase-contrast microscope, single-cell images are captured for ~20 h and the resulting images are analyzed using the MicrobeJ plugin for ImageJ. The specific protocols for each experiment are outlined in Subheadings 3.1 and 3.2, with in-depth descriptions of all the shared procedures provided in Subheadings 3.3–3.6.

2 Materials

Prepare all solutions using nanopure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ·cm) and analytical grade reagents. Prepare and store all reagents at room temperature, unless otherwise indicated.

2.1 Determining Time Delay in Killing of Growing Cells

1. *E. coli* K12 NCM3772 Δ*motA* strain, stored in 25% (v/v) glucose solution at −80 °C.
2. Luria–Bertani (LB) broth: Add 600 mL water to a 1 L glass medium bottle with screw cap. Weigh 15 g of Luria–Bertani broth powder and transfer to the bottle. Close cap tightly and mix by inverting and swirling the bottle until the powder is completely dissolved. Loosen cap and autoclave at 121 °C for 30 min. Allow the solution to cool before tightening cap.
3. 100 mg/mL ampicillin solution in water, stored at 4 °C for up to a week.
4. 1 mM propidium iodide (PI) solution in water, prepare fresh.
5. Agarose powder.
6. 10 cm petri dish.
7. Glass culture tubes capable of holding 5 mL of culture and compatible caps (typical size is 20 mm).
8. 20 mL glass, screw-top scintillation vial.
9. Sterile P1000 pipette tips and compatible pipette.

10. Sterile 15 mL conical tube.
11. 35 mm glass bottom dish with a 20 mm well (*see* **Note 1**).
12. Metal scoopula (*see* **Note 2**).
13. Parafilm M laboratory film.
14. Optional: Cling film if storing pad overnight.
15. Spectrophotometer with standard cuvette.
16. Water bath, shaking at 250 rpm, 37 °C.
17. Inverted microscope fitted with an automated mechanical XY stage, 60× objective, TRITC filter, and autofocus, housed in an incubator at 37 °C.
18. ImageJ image analysis software with MicrobeJ plug-in (*see* **Note 3**).

2.2 Single-Cell Observation of Lag Time After Starvation

1. *E. coli* K12 NCM3772 Δ*motA* strain, stored in 25% (v/v) glucose solution at −80 °C.
2. N^-C^- minimal medium: Dissolve 1 g K_2SO_4, 13.5 g K_2HPO_4, 4.7 g KH_2PO_4, 0.048 g $MgSO_4$, and 2.5 g NaCl in 1 L water and filter through a 0.22 μm pore MilliporeSigma filter. Store at 4 ° C (*see* **Note 4**).
3. Starvation medium: Mix 1 L N^-C^- minimal medium with 2.1 g ammonium chloride. Adjust pH to 7.
4. Glucose minimal medium: Mix 960 mL N^-C^- minimal medium with 2.1 g ammonium chloride and 40 mL of 1 M glucose solution in water. Adjust pH to 7.
5. Luria–Bertani (LB) broth: Add 600 mL water to a 1 L glass medium bottle with screw cap. Weigh 15 g of Luria–Bertani broth powder and transfer to the bottle. Close cap tightly and mix by inverting and swirling the bottle until the powder is completely dissolved. Loosen cap and autoclave at 121 °C for 30 min. Allow the solution to cool before tightening cap.
6. 100 mg/mL ampicillin solution in water, stored at 4 °C for up to a week.
7. Agarose powder.
8. 10 cm petri dish.
9. Glass culture tubes capable of holding 5 mL of culture and compatible caps (typical size is 20 mm).
10. 20 mL glass, screw-top scintillation vial.
11. Sterile P1000 pipette tips and compatible pipette.
12. Sterile 15 mL conical tube.
13. 35 mm glass bottom dish with a 20 mm well (*see* **Note 1**).
14. Metal scoopula (*see* **Note 2**).

15. Parafilm M laboratory film.
16. Optional: Cling film if storing pad overnight.
17. Spectrophotometer with standard cuvette.
18. Water bath, shaking at 250 rpm, 37 °C.
19. Inverted microscope fitted with an automated mechanical XY stage, 60× objective, and autofocus, housed in an incubator at 37 °C.
20. ImageJ image analysis software, with MicrobeJ plug-in (*see* **Note 3**).

3 Methods

3.1 Determining Time Delay in Killing of Growing Cells

1. Prepare a seed culture by taking cells from a −80 °C frozen stock and culturing in fresh LB overnight.
2. The following morning, transfer cells from the seed culture to fresh LB and culture for at least nine doublings, to an OD_{600} of 0.2–0.3 (experimental culture).
3. Following the protocol described in Subheadings 3.3 and 3.4, prepare an agarose pad of LB + 1% agarose + 100 μg/mL ampicillin + 4 μM PI (*see* **Note 5**) and use it to sandwich 4 μL of the experimental culture in a glass bottom dish. Seal the glass bottom dish with Parafilm and mount on a microscope with incubator set to 37 °C.
4. Images of the cells should be captured in a time lapse using phase contrast and a TRITC filter in 10-min time intervals, and a duration of ~20 h. *See* Subheading 3.5.
5. Time-lapse images can then be analyzed in MicrobeJ, as described in Subheading 3.6, to determine the time of death for each cell. We found PI to be a good indicator of death by ampicillin (*see* **Note 6**), however, because it stains nucleic acids, it does not identify lysed cells. Thus, we measured the time at which each cell either was stained by PI or lost its phase-contrast refractivity as the time of death (since lysed cells lose their refractivity due to the loss of intracellular materials). Example images can be found in [1].

3.2 Single-Cell Observation of Lag Time After Starvation

1. Take cells from a −80 °C frozen stock and culture in LB for 4–6 h (seed culture).
2. Transfer cells from the seed culture to glucose minimal medium at very low density (OD_{600} of ~0.0001 or below) and culture overnight (preculture) (*see* **Note 7**).
3. Dilute the preculture 20–50× into fresh glucose minimal medium and allow cells to grow exponentially for at least four doublings (experimental culture).

4. Wash the experimental culture. Begin by adding 5 mL of experimental culture to a prewarmed sterile 15 mL conical tube.
5. In a centrifuge prewarmed to 37 °C, centrifuge the experimental culture at 1900 rcf for 5 min.
6. Discard supernatant and resuspend cells in 5 mL of starvation medium.
7. Repeat **steps 5** and **6**.
8. Transfer cells to a sterile glass culture tube and culture in starvation medium (starvation culture).
9. Following the protocol described in Subheadings 3.3 and 3.4, prepare an agarose pad with glucose minimal medium + 1% agarose + 100 μg/mL ampicillin and use it to sandwich 5 μL of the starvation culture in a glass bottom dish. Seal the glass bottom dish with Parafilm and mount the dish on a microscope with incubator set to 37 °C.
10. Images of the cells should be captured in a time-lapse using phase-contrast exposure, 10-min time intervals, and a duration of ~20 h, *see* Subheading 3.5.
11. Time-lapse images can then be analyzed in MicrobeJ, as described in Subheading 3.6, to measure cell length in each frame. The lag time of each cell is then determined by the time after sandwiching at which its length begins to increase (*see* **Note 8**).

3.3 Preparing the Agarose Pad

1. Prewarm a rack of P100 pipette tips (*see* **Note 9**).
2. Add 3 mL of medium and 0.3 g agarose to a 20 mL glass, screw-top scintillation vial. Screw the cap loosely closed (to allow venting) and place the vial in a microwave. Warm the medium in short (<1 s) pulses, bringing the medium just to boiling in each pulse before stopping the microwave. Every few pulses, remove the vial and swirl gently (*see* **Note 10**), making sure that the medium does not touch the cap. Continue until all agarose crystals have dissolved.
3. Allow the medium to cool for ~1 min to 55–65 °C (warm, but not painful to the touch). Gently turn the vial to collect any condensation back into the medium, again being careful that the medium does not touch the cap.
4. Add 100 μg/mL ampicillin and use a pipette with prewarmed tip to mix by pipetting up and down gently, being careful not to introduce bubbles. For determining the time delay in killing of living cells (Subheading 3.1), also add 4 μM PI.
5. Using a pipette with prewarmed pipette tips, transfer 2.5 mL of medium to a glass bottom dish, being careful not to introduce any bubbles. Return the lid to the dish.

6. Allow the pad to cool for 4 h at room temperature.
7. The pad may be used immediately or stored for up to 24 h. For storing, wrap the edge of the dish in Parafilm and cover with cling film, store at 20 °C.

3.4 Sandwiching Cells

1. Unwrap the glass bottom dish (if stored overnight). Sterilize a scoopula with 100% ethanol and dry.
2. Gently remove the agarose pad from the glass bottom dish using the sterile scoopula. Run the scoopula along the wall of the dish to release the pad and then lift the pad out of the dish on the scoopula. Return the pad to the glass bottom dish.
3. Place the glass bottom dish into the base of a 10 cm Petri dish, crack the lid of the glass bottom dish, and place in an incubator at 37 °C to prewarm for 30 min (*see* **Note 11**). Prewarm the microscope enclosure to 37 °C.
4. Use a sterile scoopula to gently lift the prewarmed agarose pad out of the glass bottom dish, and balance as securely as possible on the scoopula. Using two pieces of tape, secure the scoopula to a test-tube rack (or similar) so that the end with the agarose pad is suspended in the air, being careful to prevent the agarose pad from touching any surfaces.
5. Dry the inside of the glass bottom dish using microfiltered forced air, if available.
6. Pipette 5 μL of liquid culture into the well of the glass bottom dish, using several droplets to spread the cells across the entire well, but not outside the well.
7. Remove tape to free the scoopula and place the agarose pad back into the glass bottom dish, running the scoopula along the edge to lower it as evenly as possible. Use the scoopula to gently press the pad down to remove any bubbles (*see* **Note 2**).
8. Wrap the edge of the dish in Parafilm (*see* **Note 12**).

3.5 Microscopy

1. Mount the glass bottom dish onto the microscope with an enclosure prewarmed to 37 °C. We use an oil-immersed 60× objective. The objective should be high enough magnification to facilitate accurate segmentation of individual cells.
2. To prepare the time-lapse, scan across the well of the dish to select positions at random for imaging. Positions should be centered around a cell, be nonoverlapping, and be spread as evenly as possible throughout the well. The time-lapse should be set to capture images of each position twice per doubling (~10 min for this experiment) with phase-contrast imaging. For determining the time of killing in living cells (Subheading 3.1), a red florescence exposure should also be used with a TRITC filter.
3. The time lapse should be set to run for 20 h (*see* **Note 13**).

3.6 MicrobeJ Analysis

1. Load a phase-contrast image, along with any corresponding exposures, into ImageJ and open MicrobeJ by selecting "Plugins"→"MicrobeJ"→"MicrobeJ."
2. Under the "Bacteria" tab, select the phase-contrast image to segment the bacteria. Checking the "shape" and "intensity" options will provide information about the shape (length, area, aspect ratio, etc.) and intensity (max, min, average intensity within each cell for each channel) (*see* **Note 14**).
3. Use the pencil icon in the bottom left of the "bacteria" tab to detect the cells and open the manual editing interface. This will overlay the phase-contrast image with the outlines of cells it has detected. In the manual editing interface, you can check and correct the segmentation by outlining any cells that have been missed, removing incorrectly detected cells, and adjusting the segmentation of cells (splitting two cells that have been outlined as one, adjusting the boundary of cells that have been improperly outlined, etc.) (*see* **Note 15**).
4. Once cells have been satisfactorily segmented, view the results using the MicrobeJ results table (bar graph icon in the top bar of the experiment editor). Here you will be able to view and save information about the position, shape, intensity, and so on of the cells you have segmented.

4 Notes

1. We use CellVis 35 mm glass bottom dishes. You will need the appropriate cover glass thickness for your microscope.
2. Bending one end of the scoopula so it has a foot of ~1 cm perpendicular to the handle helps to provide a gentle way to adjust (especially, to press down) the agarose pad.
3. ImageJ can be downloaded for free at: imagej.nih.gov/ij. The MicrobeJ plug-in can be downloaded for free at: microbej.com.
4. This solution may be made at 4× concentration and stored at −20 °C for convenience.
5. This concentration was chosen because it has minimal impact on cell viability in starvation while still being detectable.
6. We confirmed that PI is a good indicator for cell death by ampicillin by incubating cells with ampicillin and PI for 80 min before spreading them on an LB agar plate (no ampicillin). Across five biological replicates a total of ~25 PI+ cells were examined, and none grew. We additionally used a fluorescent glucose analog (2-NBDG) alongside PI to compare metabolic activity (uptake of 2-NBDG) and PI staining in

cells exposed to ampicillin. We found two distinct populations of cells, those that were PI stained with no uptake of 2-NBDG and those that were not PI stained with uptake of 2-NBDG, further confirming PI as a good indicator of cell death by ampicillin. More information can be found in [1].

7. The culture should still be growing exponentially the next morning.
8. An example of the results is given in [2].
9. The temperature of prewarm is not especially important for this step. Having the pipette tips warm simply helps to keep the agarose warm while transferring the agarose to the glass bottom dish and prevents the agarose from solidifying on the walls of the pipette tip. We prewarm to 80 °C, but even a prewarm to 37 °C would be better than using room temperature tips and may be more readily available.
10. An 11 × 21 cm Kimwipe (or similar), folded twice longwise ("hotdog" style) makes a good sling to wrap around the vial, so you do not need to hold the hot vial.
11. The agarose pad should be prewarmed long enough to come to 37 °C and ideally no longer. In our lab conditions, 30 min is optimal, but you may need to adjust the prewarming time. Use a consistent prewarming time to prevent differences in the pad due to different levels of drying.
12. Parafilm is gas-permeable with low water permeability. It allows oxygen and carbon dioxide to pass while preventing drying up the agarose pad.
13. For these experiments, 20 h was sufficient, but an experiment may be run for 48 h or longer. This is a major benefit of using the agarose pad to sandwich cells in the glass bottom dish.
14. We find that the "Local Default" segmentation method works well. If MicrobeJ is having difficulty segmenting your cells correctly, you will want to try using different segmentation methods. Some segmentation methods are computationally heavy, so it is helpful to use the "Rectangle" tool of ImageJ to select a smaller section of the image for MicrobeJ to work on while you are testing methods.
15. If MicrobeJ is consistently accepting noncells as cells or vice versa, you can adjust the segmentation parameters in the "Bacteria" tab. This allows you to set minimum and/or maximum values for the shape and intensity of objects it accepts as cells (area, length, intensity, etc.).

References

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