

**ODELAM: Rapid sequence-independent detection of drug resistance in isolates of
Mycobacterium tuberculosis.**

Thurston Herricks^{#1}, Magdalena Donczew^{#2}, David R. Sherman², John D. Aitchison^{*,1,3}

*For correspondence: email John.Aitchison@seattlechildrens.org

#Contributed equally to this work

¹Center for Global Infectious Disease Research, Seattle Children's Research Institute, Seattle, WA, 98109;

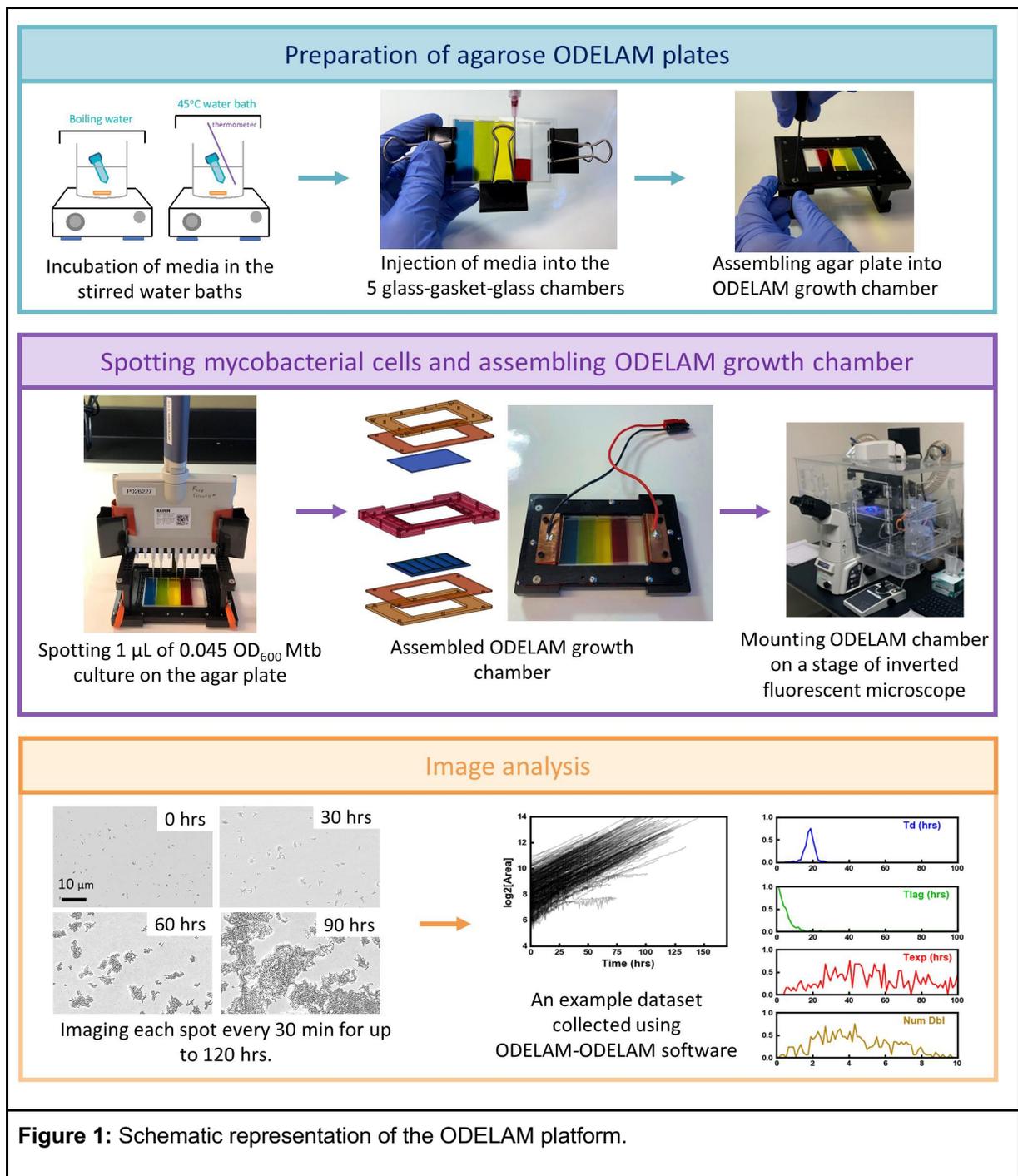
²Department of Microbiology, University of Washington, Seattle, WA 98195;

³Department of Pediatrics, University of Washington, Seattle, WA 98195.

Abstract:

Antimicrobial-resistant *Mycobacterium tuberculosis* (Mtb) causes over 200,000 deaths each year. Current assays of antimicrobial resistance require knowledge of mutations that confer drug resistance, or long periods of culture time to test growth under drug pressure. We present ODELAM (One-cell Doubling Evaluation of Living Arrays of Mycobacterium), a time-lapse microscopy-based method that observes individual cells growing into microcolonies. This protocol describes sample preparation, media preparation and instructions for assembling the ODELAM sample chamber. The ODELAM sample chamber is designed to provide a controlled environment to safely observe the growth of Mtb by time-lapse microscopy on an inverted wide field microscope. A brief description of ODELAM software is also provided. ODELAM tracks up to 1500 colony forming units per region of interest and can observe up to 96 regions for up to 7 days in a single experiment. This technique allows quantification of population heterogeneity. ODELAM enables rapid quantitative measures of growth kinetics in as little as 30 hours under a wide variety of environmental conditions.

Graphic abstract:



Keywords: Mycobacterium, tuberculosis, drug resistance, growth phenotypes, microbiology, microscopy, live imaging.

Background:

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), which is responsible for 1.4 million deaths each year (W.H.O. 2020). Despite decades of research, TB treatment is still a challenge and current chemotherapies require the combination of four different drugs (Pai, M., et al. 2016). It is becoming increasingly recognized that one of the reasons why Mtb remains a remarkably successful pathogen is its ability to produce heterogeneous phenotypes that exhibit variable response to a given drug (Dhar, N., et al. 2016; Manina, G., et al. 2015; Rego, E. H., et al. 2017; Logsdon, M. M., et al. 2018). Thus, characterizing the growth phenotypes of populations of individual Mtb cells is critical for understanding how mycobacteria sense and respond to stress conditions such as prolonged drug exposure (Santi, I. et al. 2013; Logsdon, M. M. et al. 2018).

To date, little is known about the sources and consequences of cell-to-cell differences, mostly due to limitations in the tools and techniques that allow for precise measurement of growth phenotypes of populations of individual bacterial cells. Mtb is especially challenging to study due to its very long doubling time of about 24 hrs. Recent platforms that study single bacterial cells are limited either by the number of cells that can be observed or by the extensive time required (few weeks) to monitor growth of mycobacterial colonies (Manina, G., et al. 2015; Aldridge, B. B., et al. 2012; Golchin, S. A., et al. 2012; Wakamoto, Y., et al. 2013; Choi, J., et al. 2014; Barr, D. A., et al. 2016).

ODELAM (One-cell Doubling Evaluation of Living Arrays of Mycobacterium) was developed to overcome these limitations and is based on ODELAY originally developed to investigate yeast (Herrick et al. 2017; Herricks, T. et al. 2020). ODELAY is a versatile method that has been utilized to investigate the structure-function relationship of the nuclear pore complex and peroxisome biogenesis (Kim, S.J., et al. 2018; Mast, F.D., et al. 2018). ODELAM uses time-lapse microscopy to quantify growth phenotypes of populations of individual Mtb colony forming units (CFU). By direct monitoring of bacterial growth, we can assess heterogeneity of Mtb strains, arising either naturally or in response to stresses such as drug treatment. ODELAM can observe up to 1500 CFU per region of interest (ROI) and measure four main growth kinetic parameters: lag time, doubling time, exponential time (time when colonies stop growing and enter stationary phase) and number of doublings for each identified CFU. ODELAM can track up to 100,000 CFUs in one experiment, which provides statistical power to detect phenotypic variations in the population. Importantly, within each experiment ODELAM can screen between 80 to 96 samples growing in up to five media conditions. In less than 48 hours, ODELAM identifies resistant cells in a population of sensitive bacteria down to 1 per 1000 and predicts minimum inhibitory concentration (MIC) of drugs with high accuracy. Additionally, the ODELAM platform is broadly applicable as a laboratory screening tool, as it can be easily adopted to most colony forming microorganisms. ODELAM can quantify heterogeneity and detect heteroresistance in Mtb clinical isolates. This cutting-edge technology will have a meaningful impact on studies on bacterial phenotypic heterogeneity, emergence of drug resistance and help to better understand bacterial adaptation to unfavorable environmental conditions, which will facilitate development of new powerful therapies.

Materials and Reagents

A. Mycobacterium tuberculosis cell culture

1. 1.8 mL cryotubes (Nunc, catalog number: 375418)
2. 50 mL conical bottom tubes (Sarstedt, catalog number: 62.547.254)
3. 10 mL serological pipettes (VWR, catalog number: 89130-898)
4. 200 μ L tips (Genesee Scientific, catalog number: 24-412)
5. 1000 μ L tips (Genesee Scientific, catalog number: 24-430)
6. Mtb strains (storage: -80°C):
 - a. H37Rv (background strain available from BEI, catalog number: NR-123)
 - b. Other strains of interest
7. Difco Middlebrook 7H9 Broth (BD, catalog number: 271310, storage: room temperature (RT))
8. Middlebrook OADC Enrichment (BD, catalog number: 212351, storage: 4°C)
9. Glycerol (Sigma Aldrich, catalog number: G5516, storage: RT)
10. Tween 80 (Sigma-Aldrich, catalog number: P1754, storage: RT protected from light)
11. Disinfectants:
 - a. 70% ethanol (Fisher Scientific, catalog number: 04-255-92, storage: RT)
 - b. PREempt RTU Disinfectant Solution (Contec, catalog number: 21105, storage: RT)
 - c. LoPhene Concentrate Rotational Disinfectant (Decon Labs, catalog number: 8801, storage: RT)

B. ODELAM agarose gel preparation

1. 50 mL conical bottom tubes (Falcon, catalog number: 352070)
2. 15 mL conical bottom tubes (Falcon, catalog number: 352096)
3. 5 mL syringe (BD, catalog number: 309646)
4. 18 G x $1\frac{1}{2}$ needle (BD, catalog number: 305196)
5. Plain glass micro slides (VWR, catalog number: 48382-179)
6. UltraPure agarose (Invitrogen, catalog number: 16500, storage: RT)
7. Difco Middlebrook 7H9 Broth (BD, catalog number: 271310, storage: RT)
8. Middlebrook OADC Enrichment (BD, catalog number: 212351, storage: 4°C)
9. Glycerol (Sigma Aldrich, catalog number: G5516, storage: RT)

C. ODELAM spotting cell cultures

1. 96-well plate, F-bottom (Greiner bio-one, catalog number: 655180)
2. Pipette tips RT LTS 20 μ L 960A/10 (Raining, catalog number: 30389200)

Equipment

BSL1 or BSL2

1. Pipettes
 - a. eg. Rainin 2-20 μ L, 20-200 μ L, 100-1000 μ l
 - b. 0.1-10 μ l 12-multichannel pipette (Rainin Pipet-Lite Multi Pipette L12-10XLS+ (catalog number: 17013807)) *Note: ODELAM chamber is designed for this specific pipette.*
2. 2x hot plate stirrer (Corning, catalog number: PC-420D)

3. 2x 1L beakers (Sigma-Aldrich, catalog number: CLS10001L-1EA)
4. 2x stir bars (VWR, catalog number: 58948-150)
5. 2x glass giant Petri dishes to cover the beakers
6. Vortexer (Vortex-Genie 2 Scientific Instruments SI-0256)
7. Thermometer (VWR, catalog number: 89095-640)
8. Steriflip-GP Sterile Centrifuge Tube Top Filter Unit (Millipore Sigma SCGP00525)
9. Top-loading Electronic scale with 1kg capacity and 0.01 g accuracy
10. Medium (3 per one 5 glass-gasket-glass chambers) and large (2 per one single glass-gasket-glass chamber) binder clips
11. 3D printer (e.g. Ender 3D, BCN3D Sigma v19, makerbot 2x)

BSL3

1. Pipettes:
 - a. Eppendorf 2-20 μL , 20-200 μL , 100-1000 μL ,
 - b. 0.1-10 μL 12-multichannel pipette (Rainin Pipet-Lite Multi Pipette L12-10XLS+ (catalog number: 17013807)) with attached binder clips.
2. Motorized inverted microscope with heated incubator (tested on Leica DMI6000 and Nikon TiE)
3. Personal Protective Equipment (PPE):
 - a. double nitrile gloves
 - b. protective Tyvek coveralls
 - c. shoe covers
 - d. powered air purifying respirator (PAPR)
4. Absorbent pad (for working in the biosafety cabinet)

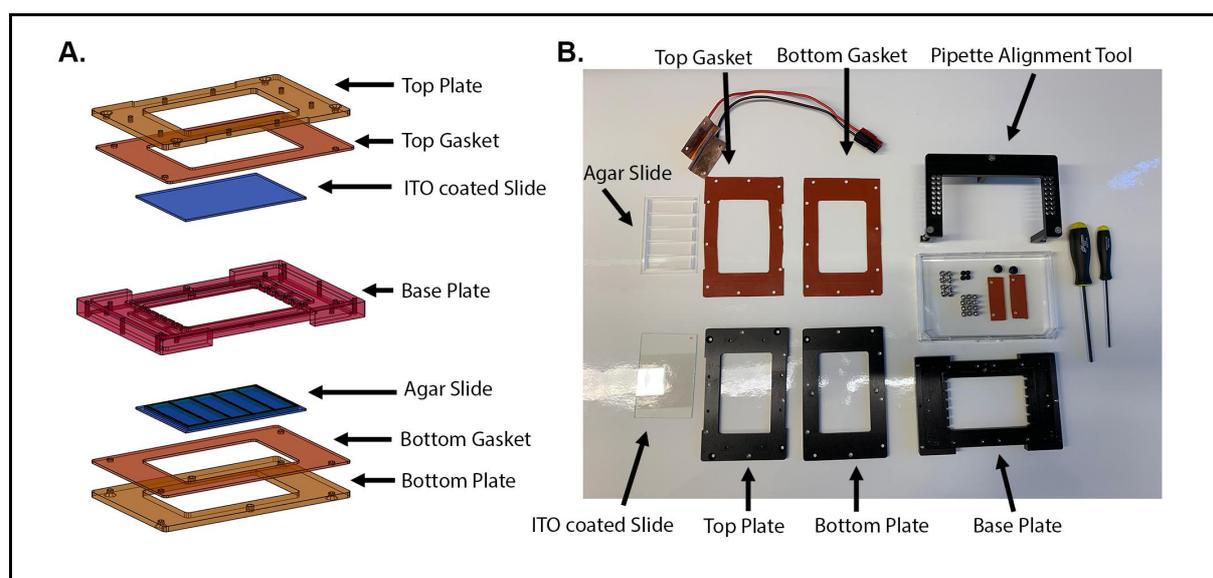


Figure 2: ODELAM sample chamber. The sample chamber for ODELAM experiments is shown in

an exploded view (A) and disassembled with all relevant parts (B).

5. ODELAY microscope chamber
 - a. Top Plate (machined from 6061-T6 aluminum)
6. Top PDMS Gasket (cut from McMaster-Carr Durometer 30A 9010K11)
7. Indium Tin Oxide (ITO) coated -glass slide 1.1 mm thick (SPI supplies 06404-AF)
8. Base Plate (machined from 6061-T6 aluminum)
9. Glass slide with 3D printed gasket and cast agarose media
10. Bottom PDMS gasket (cut from McMaster-Carr Durometer 30A 9010K11)
11. Bottom plate (machined from 6061-T6 aluminum)
12. Pipette spotting alignment tool (machined from 6061-T6 aluminum)
13. 1/16 hex screwdriver (McMaster-Carr 5497A23)
14. 1/16 hex screwdriver (McMaster-Carr 5497A23)
15. 12x 4-40 5/8 inch stainless steel button-head screws (McMaster-Carr 98164A433)
16. 8x 4-40 1/4 inch stainless steel flat-head screws (McMaster-Carr 90585A200)
17. 4x 4-40 1/8 inch nylon socket head screws (McMaster-Carr 95868A254)
18. Copper electrical contacts (custom made cut from copper sheet)
19. 37°C incubator (VWR 1545 Digital Incubator)
20. Culture rotator (GEL GRO Tissue culture rotator, LAB-LINE)

Software

1. Anaconda Python (<https://www.anaconda.com/>)
2. Visual Studio Code (<https://code.visualstudio.com/>)
3. ODELAY software package (AitchisonLab git repository)
4. MicroManager 1.4 or 2.0 gamma (<https://micro-manager.org/>)
5. Microsoft Office Excel or other spreadsheet compatible program (<https://www.office.com/>)
6. Cura 3D modeling (<https://ultimaker.com/>)

Procedure

A. *Mycobacterium tuberculosis* cell culture

Note: All work with Mtb should be conducted in the Biosafety Level 3 (BSL3) laboratory by trained personnel, wearing appropriate PPE and approved by the Institutional Biosafety Committee (IBC) and Environmental Health and Safety (EHS).

1. Prepare pre-culture by inoculating 10 mL of 7H9-GOT (Middlebrook 7H9 media supplemented with 0.2% glycerol, 10% OADC supplement and 0.05% (v/v) Tween-80) with 0.5 mL of the thawed Mtb glycerol stock (1:20 dilution). Grow cells in 50 mL conical tubes at 37°C for 48-72 hrs, until cultures reach OD₆₀₀=0.2-0.4 (early logarithmic phase).
2. Subculture by diluting 0.5 mL of the pre-culture in 10 mL of 7H9+GOT (1:20 dilution). Grow cells in 50 mL conical tubes at 37°C for 48-72 hours, until cultures reach OD₆₀₀=0.2-0.4 (early

logarithmic phase).

3. Dilute culture for spotting on ODELAM plate (See Part E of Procedure for further instructions)

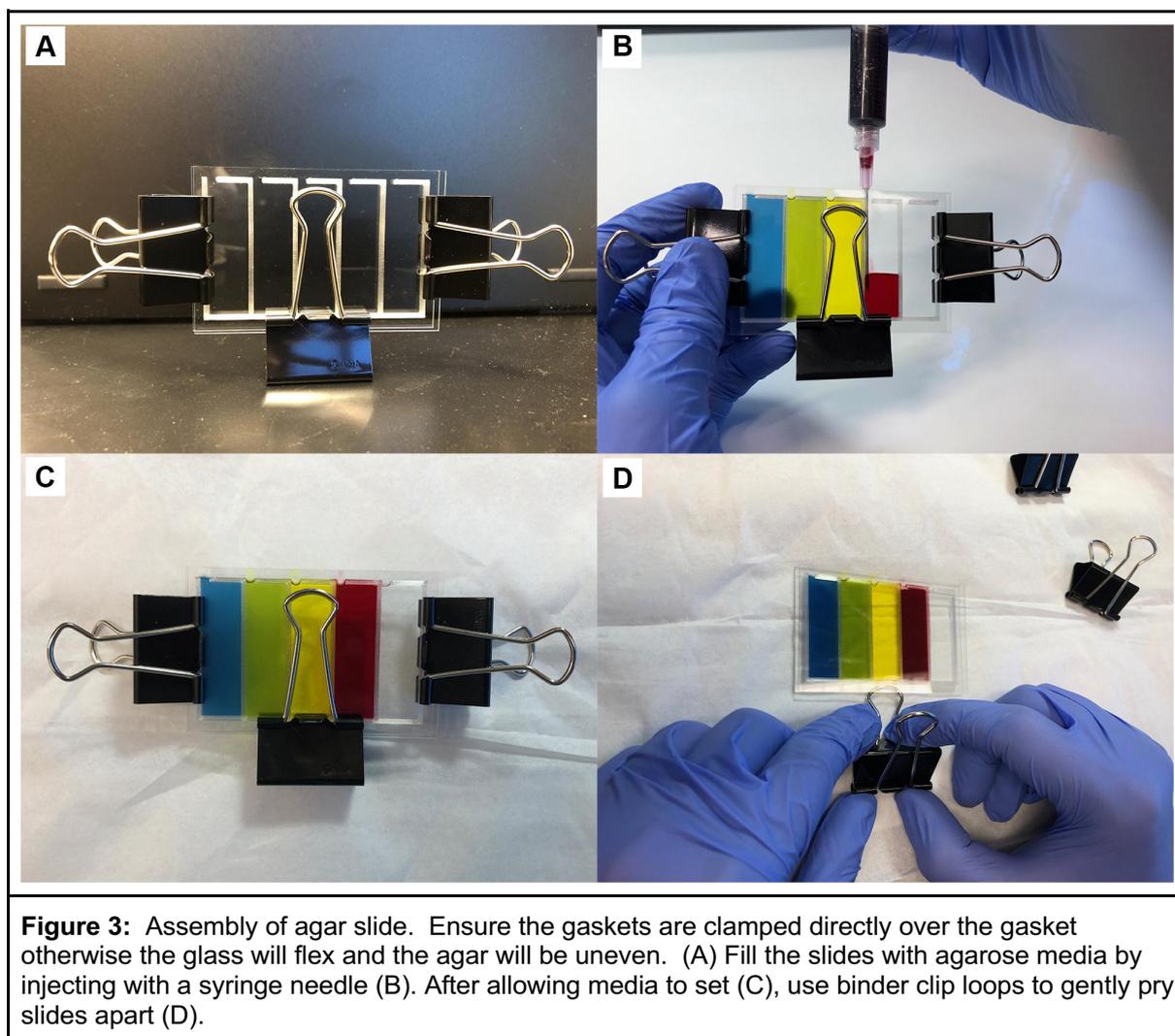
B. ODELAM agarose aliquot

1. Gather a 1 L media bottle, high purity agarose, pipettes, and falcon tubes.
2. Weigh 2 ± 0.02 g of agarose and place into 1 L media bottle.
3. Add 150 g of 18 M Ω H₂O to the media bottle.
4. Record the total weight of the bottle, agarose and water.
5. Microwave media bottle, water, and agarose until all agarose is dissolved.
6. Weigh media bottle, water and agarose again to evaluate the amount of water lost during boiling.
7. Add 18 M Ω H₂O until the current mass is equal to the previously recorded total weight.
8. Aliquot agarose by mass: 15.1 g into 50 mL Falcon conical tubes or 3.0 g into a 15 mL falcon conical tube.

C. ODELAM agarose media preparation

Note: This section can be performed outside of a biological safety cabinet, as long as the lab is relatively dust free.

1. Clean previously printed gasket slide (See 3D printing gaskets of Notes section) using lab labeling tape.
2. Heat two 1 L beakers with about 750 mL of DiH₂O, 1 with boiling water and the second heat the water to 40°C.
3. Add appropriate amounts of 10x media and additional reagents needed to the prepared agarose aliquots. *Note: Don't add reagents that are temperature sensitive.*
4. Clean a 50 mm x 75 mm x 1 mm glass slide using 70 % ethanol. When dry, wipe with lens paper to remove any residual particles and fibers.



5. Place the clean glass slide on top of the printed gasket slide and secure slides with binder clips (Figure 3A).
6. Assemble the number of syringes and 20 gauge needles for each media condition.
7. Place the tube with media formulation into boiling water. The 20 mL media tubes should be in the boiling water for 18 minutes and 4 mL media tubes for 8 minutes. *Note: Make sure the cap does not go below the water surface as this will cause water to leak into the tube. Also make sure the beaker is covered otherwise agarose will not completely melt.*
8. After the appropriate amount of time and the agarose is melted, vortex the agarose media and place the media into the 40°C water bath.
9. Wait 3-5 minutes for the agarose to equilibrate at 40°C and then add the temperature-sensitive components (e.g. drug, OADC additive, etc.).
10. Vortex the tubes thoroughly to mix the media and additives.
11. Quickly draw the media into the syringe and then push the plunger down a bit and back up to remove air bubbles in the syringe.
12. Fill the glass slide by injecting the media into the gasket slide assembly (e.g. Figure 3B). *Note*

which media is injected into each chamber.

13. Wait approximately 20-30 minutes for the agarose to solidify (Figure 3C).
14. Remove the binder clips.
15. Using a binder clip loop (or other convenient leverage), slowly and carefully pry the glass slides apart (Figure 3D). Try to keep the agarose from sticking to the blank slide. *Note: This step takes some practice.*
16. Store the agarose slide in a clean plastic container (e.g. an empty tip box) for transport into the BSL-3 environment. Agarose slides can be stored for 3-4 hours at RT. Use only agarose slides prepared on the day of the ODELAM experiment.

D. Preparation for ODELAM experiment

1. Select cultures and media conditions of interest
2. Prepare directories and data storage space. Create file directories and ensure there is sufficient storage capacity for the experiment.
3. Create-Spot layout files. Double check file to ensure the strain layout is correct. Common mistakes are not correcting for flipping the array, or not sorting the array correctly.

E. ODELAM Spotting Cell Cultures

1. Clean and sterilize all ODELAM chamber components (except the agarose slide and electrical contacts) with 70% ethanol and dry with Kim wipes.
2. Use a clean plastic container to transfer the ODELAM chamber components to the Biological Safety Cabinet.
3. Measure OD600 of all Mtb culture
4. Dilute cultures to an OD600 of 0.04-0.05. The goal is to have 500-1000 cells per region of interest. The ratio of OD600 to CFU varies from strain to strain so OD600 value may need to be adjusted slightly for different organisms and strains.
5. Array 150 μ L of the diluted cultures into a 96 well plate. The locations in the 96 well plate should be determined by the spot layout pattern (See examples in Figures 6 and 7).

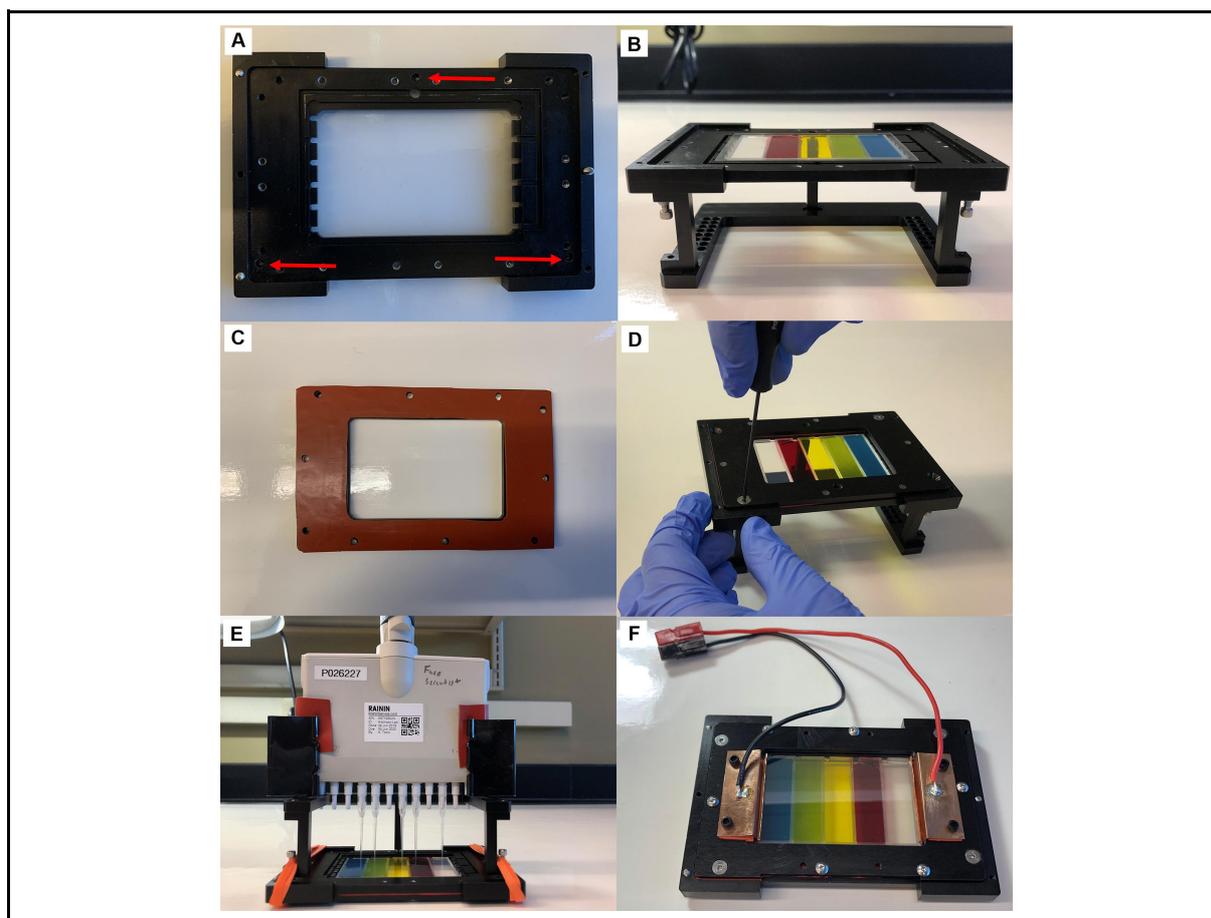


Figure 4: Assembly of ODELAM chamber. The base plate has recessed holes for mounting the pipette alignment tool as indicated by red arrows (A). Place the agar slide in the recession (B). Place the clean silicon gasket on the bottom plate (C) and secure with the appropriate button head and countersunk screws (D). Spot the cultures onto the agar pads using the correct tip sequence (E). Complete assembly of the chamber by adding the ITO slide, top plate, top gasket and electrical contacts (F). **Note: These steps should be performed in a Biological Safety Cabinet over an absorbent pad soaked in tuberculocidal agent.**

6. Organize and layout tips in tip-boxes for spotting. (See Notes for suggestions)
7. Place the base plate on the pipette alignment tool. Make sure the screws that level the alignment tool fit into the three recessed holes in the base plate (Figure 4A).
8. Place the previously prepared agar slide into the recessed section of the ODELAM base plate (Figure 4B).
9. Place the bottom silicon gasket on the ODELAM bottom plate. Smooth out the gasket and ensure the screw holes are correctly aligned and the gasket has a minimal overhang around the edges of the bottom plate (Figure 4C).
10. Place the bottom plate onto the base plate and secure the two with the appropriate screws (Figure 4D).
11. Flip the assembly over and place the pipette alignment tool onto the base plate. Make sure the screws protruding from each leg are set into the three corresponding holes in the base plate.
Note: Be careful the forward screw does not fall into the vent hole as this sometimes happens

and will cause the pipette tips to mark the agar.

12. Check the agar surface to see if the pipette tips mark the agar (Figure 4E). If they do, adjust the height of the alignment tool using the screws on the legs until the tips are within 1 mm of the surface. They should be really close but not mark the media.
13. Use diagrams to guide spotting patterns for transferring from 96 well plate to ODELAM (See Notes)
14. Spot 0.8-1.1 μL of culture. If needed, increase or decrease the volume of culture spotted to slightly adjust the diameter of the spotted culture.
15. Place the ITO glass slide into the recession in the base plate. Make sure the ITO conductive side of the slide is facing up. *Note: Mark the conductive side with an alcohol-resistant marker to be able to identify the conductive side.*
16. Place the upper silicon gasket onto the upper plate (similar to step 6 Figure 4C). Ensure the gasket does not overhang the center hole in the plate as this could interfere with the electrical contacts to the ITO slide.
17. Secure the top plate to the base plate with screws.
18. Screw in the vent plugs on the bottom plate.
19. Disinfect the ODELAM chamber by wiping with a disinfectant soaked towel but avoid touching or getting disinfectant on the ITO slide as this will cause condensation that will interfere with imaging.
20. Attach the electrodes and silicon gasket electrode spacers with the plastic screws to the top plate and flip the chamber over (Figure 4F).
21. Keeping track of the plate orientation, place the assembled chamber onto the microscope stage.
22. Connect electrodes to the power supply, turn on the heater power supply and ensure that current is flowing through the ITO slide. If the electrodes are in contact with aluminum, then the power supply may indicate a short or have a high current. Look for a current of 0.15 A to 0.2 A at 10 V.
23. Proceed to the next section on ODELAM microscope control.

F. ODELAM microscope control

1. Open Visual Studio Code.
2. Create a python terminal and activate the odelay virtual environment
3. Run the python file ODELAY microscopecontrol.py (eg python ODELAYmicroscopecontrol.py).
4. Select the appropriate directories, files and ODELAY experiment type for the experiment.
5. Once the new graphical user interface has loaded, check to make sure the region of interest (ROI) layout on the microscope control panel matches that of the desired experiment.
6. Start the camera by pressing the focus button.
7. Move the stage to a spot in the upper left corner of the array and where cells are present.
8. Focus the image and then move the upper left most ROI on the array. (Spot E06 is the origin)
9. Press the set origin button.

10. Move to a ROI close to a leveling screw and that has cells to focus on and use the leveling screw to focus the image.
11. Repeat this step for all three leveling screws until the image is in focus at all 3 ROI next to the leveling screws.
12. Evaluate other ROI and use the Autofocus 1 button to evaluate if those ROI can be brought into focus by the default Autofocus parameters. Increase or decrease the Autofocus as needed to ensure all ROI can be found by the Autofocus settings. (See Notes for additional Instructions).

G. ODELAM Chamber Sterilization:

1. Fill 2 plastic containers (eg. tip box lids) and a one bigger plastic container (of a size of the ODELAM chamber) with LoPhene. Prepare the biological safety cabinet with LoPhen soaked absorbent pads as a working surface.
2. After the experiment, remove the chamber from the microscope. Check for leaks. If necessary, apply disinfectant.
3. Remove the copper contacts using the 3/32 hex driver and place the contact and screws in an appropriate storage container.
4. Move the chamber to the biological safety cabinet.
5. Using the 1/16 inch driver remove the 10 flat head and button head screws from the bottom plate.
6. After removing each screw drop them into one of the two tip box lids with LoPhene.
7. Gently but firmly pry the bottom plate and gasket from the base plate. Do not use a tool for this as it could damage the gasket or either plate. Take your time as the silicon will stick to two plates together but will slowly release with firm but constant pressure.
8. Gently pry the agarose slide from the gasket and place the slide in the second tip box filled with LoPhene. Make sure LoPhene completely covers the agar. Usually some condensation forms on the edges of the slide at this point. Avoid touching it and clean gloves thoroughly as gloves can become contaminated with Mtb at this point.
9. Separate the bottom gasket completely from the bottom plate and place both in the plastic container filled with LoPhene. Make sure each part is covered.
10. Repeat these steps 5-7 for the top plate. Gently, but with constant and firm pressure, pry the top plate and top gasket from the base plate.
11. Remove the ITO coated slide and place in the tip box lid with the screws. Make sure the ITO slide is immersed in LoPhene.
12. Separate the top gasket from the top plate and the top plate, top gasket and base plate into the plastic container with LoPhene. Add additional LoPhene if needed to completely cover all the parts.
13. After about 15-20 minutes remove the agarose slide from the LoPhene. Place the agarose into a solid waste disposal within the hood and glass slide in the sharp container.
14. Dump the LoPhene that the agarose slide was in into a liquid waste container.
15. Spray LoPhene on the other containers and remove from biological safety cabinet.

16. Wash all parts and screws with generous amounts of deionized water to remove the LoPhene.
17. Finally spray all parts with 70% ethanol and dry with a paper towel.
18. Store the chamber disassembled for future use.

Data analysis

Note: This section assumes that all data analysis is performed by ODELAYTools python package executed in the command line and utilizing High Performance Compute cluster as described in the ODELAY ReadMe.md file. Please follow the instructions there to install the software and package commands. Additional features to perform analysis on standalone desktops are in development.

1. Activate the ODELAY python environment. (eg >conda activate "name of odelay environment")
2. Create a data directory where the processed data for the experiment will be written.
3. Set the image directory where the microscope images were written using the command "odelay set-image-dir", and enter the path to the image directory at the command prompt.
4. Set the data directory using the command "odelay set-data-dir".
5. Initialize the data processing with "odelay initialize". Wait for the response that the experiment is initialized.
6. Finally enter the command prompt >odelay process all.
7. Make sure that a *Spot-Layout.xlsx file is correctly filled out and in the data directory.
8. After the data has finished processing enter the command >odelay summarize-experiment. This command will reduce the dataset to a single hdf5 file.
9. Plot a summary histograms of all regions of interest using the command >odelay plot-summary Mtb.
10. Plot growth curves for with histograms with the command >odelay plot-gc all Mtb. This will plot all regions of interest successfully processed in the experiment.

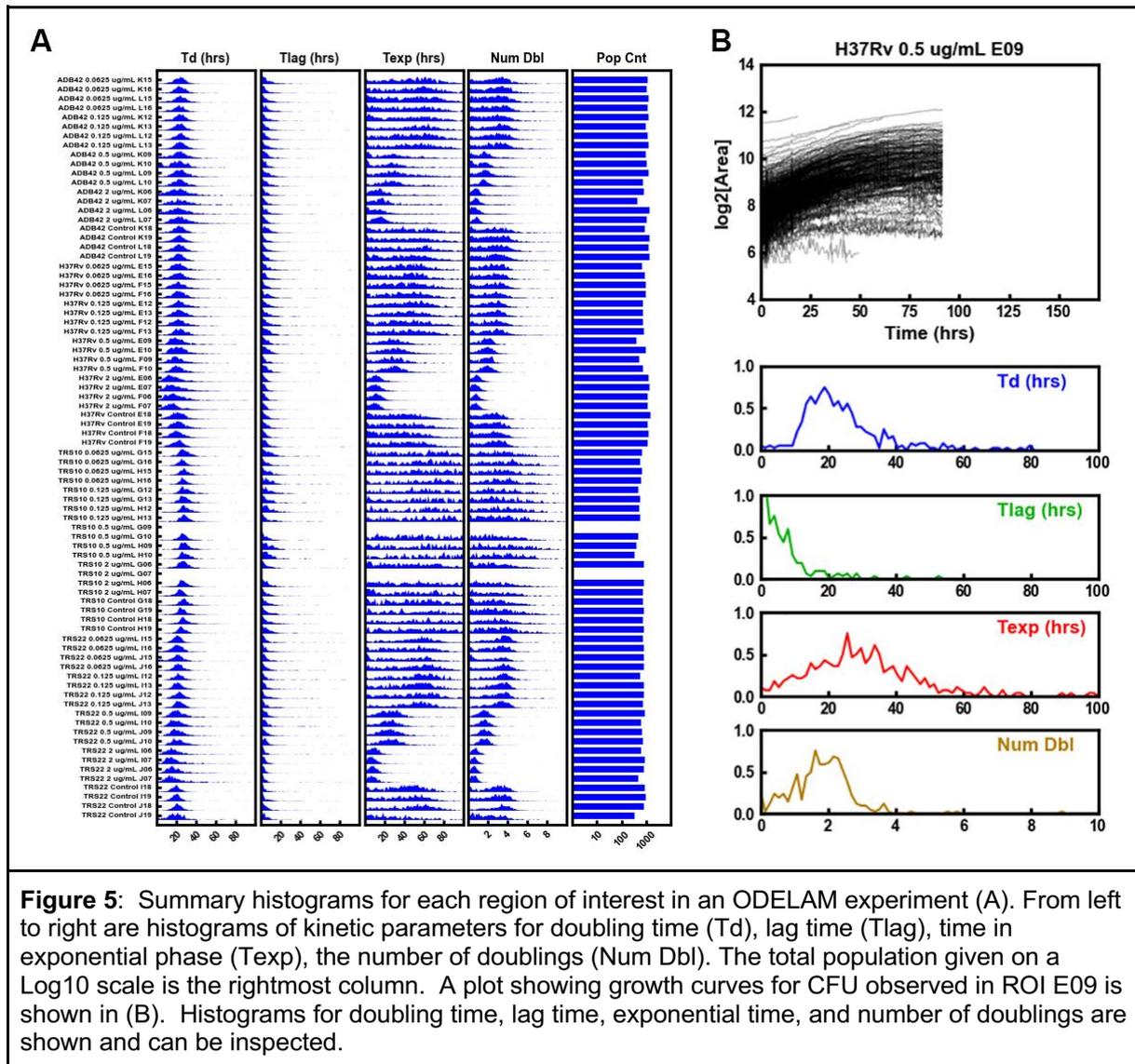


Figure 5: Summary histograms for each region of interest in an ODELAM experiment (A). From left to right are histograms of kinetic parameters for doubling time (Td), lag time (Tlag), time in exponential phase (Texp), the number of doublings (Num Dbl). The total population given on a Log10 scale is the rightmost column. A plot showing growth curves for CFU observed in ROI E09 is shown in (B). Histograms for doubling time, lag time, exponential time, and number of doublings are shown and can be inspected.

Notes

96 Well Layout Patterns

ODELAM spotting requires practice and organization to transfer samples from a 96 well plate to the 384 well pitch of the ODELAM plate. The following examples describe a couple of strategies for arranging samples in a 96 well source plate. The first example is how to spot a 96 well plate.

1. Arrange the 96 well plate according to how many samples are required. Usually multiples of 4 are best.
2. Arrange the tips in the tip boxes according to the number of conditions required for the configuration of the agar pads (Figure 5A).
3. Alternate from left to right every row when spotting to generate a checkerboard pattern on the agarose slide (Figure 5B).
4. Repeat step 3 for the rows that were skipped in the previous step (Figure 5C).

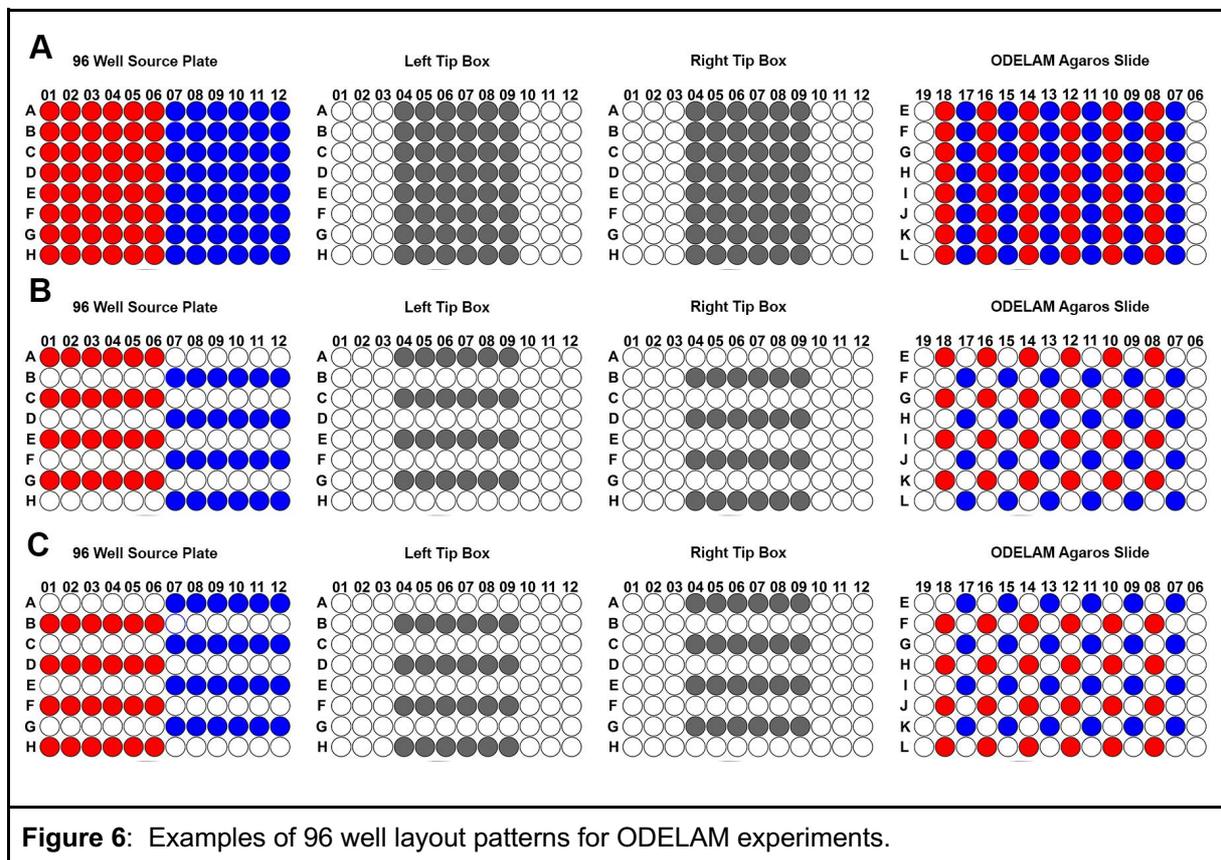
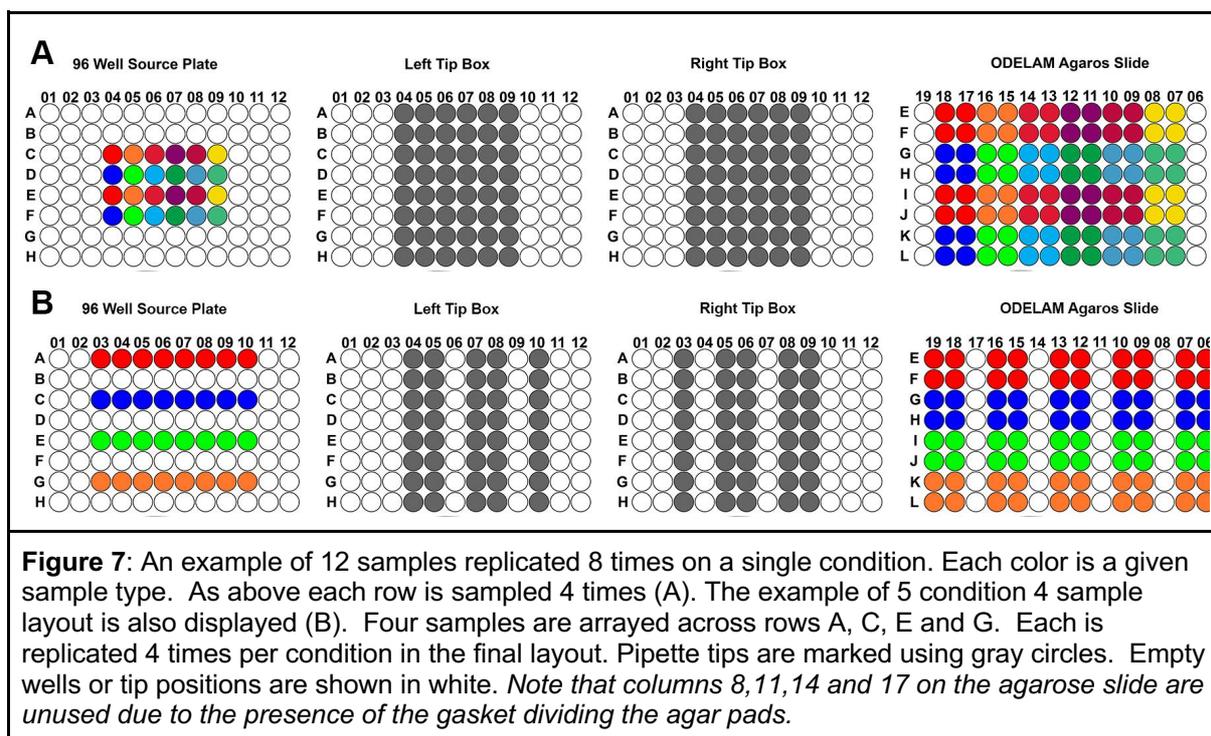


Figure 6: Examples of 96 well layout patterns for ODELAM experiments.

5. Alternate patterns can be generated for spotting fewer colonies. A 12 or 24 sample pattern can be generated (Figure 6A) or for spotting on multiple conditions (Figure 6B).



Autofocus Settings

The autofocus settings are flexible to ensure that all spots will stay in focus throughout the experiment. There are two autofocus settings, Autofocus 1 and Autofocus 2. Autofocus 1 is utilized to find focus of the first image taken of an ROI. Thereafter Autofocus 2 uses settings that increase the speed of image acquisition. When setting Autofocus 1, make sure the range is set such that all ROI are within \pm range setting. With larger ranges it is important to increase the number of images collected so that the focus algorithm will converge on a good focus. Ideally, a good print of the gasket should enable ROI focus to within 60 μ m of each other across the spotted array.

3D Printing Gaskets

Successfully printing 3D gaskets onto a microscope slide requires editing the G-code file that defines the printer movements. Most 3D printing software does not allow printing an object above the build plate which, in this case, is required to print on a 1 mm thick glass slide. 3D printing TPU gaskets can be tricky and inconsistent. The printing speed, feed rates, extrusion temperature, and print cooling parameters that work will vary depending on the filament diameter and printer utilized. In general, a print head speed of 20 mm/s, removing filament retraction, an extruding temperature of 230°C, and a build plate temperature of 65°C are good starting points. We have found that drying the filament overnight at 70°C in an oven can dramatically increase print quality. This is due to water becoming absorbed into the polymer filament and then boiling out when the filament is melted. The voids created from the water vaporizing make the extruded filament uneven causing flaws in the print.

The following gives a general description on how to generate G-code files and then how to edit the G-code so that the printer will print a gasket directly onto a glass slide. G-code files are text files that text commands that tell the printer where and how to move the printing head. Correctly following these steps helps generate a gasket that allows molding of the agarose media for reliable time lapse imaging.

Printing Slide Build Plate mount:

1. Generate a *.stl file of a rectangular frame with an inner opening of about 50.75 mm x 75.75 mm and an outer measurement of 70 mm x 95 mm and about 0.5 mm to 0.75 mm thick.
2. Center the frame on the build plate and generate the G-code file using standard PLA filament parameters. Do not use a raft or any support material.
3. Transfer the G-code file to the printer.
4. Print the frame on a glass plate and tape afterwards use Kapton tape around the outer edges of the frame to hold it down. This frame will center the glass slide for printing the gasket and can be used several times. It is recommended to have a dedicated glass plate for printing slide gaskets that can be reliably removed and relocated on the build plate.

To generate G-Code for printing a gasket:

1. Import gasket design (usually a *.stl file) into the software for the 3D printer (eg. Cura3D)
2. Center the gasket on the center of the build plate.
3. Enter the appropriate printing parameters for TPU and generate the G-code file.
4. Open the ZchangerGcode.py file and change fileName variable to the G-code file generated in the previous step.

Note: The line 19 of ZchangerGcode.py ';TYPE:WALL-OUTER' may need to be changed to a different value depending on the printer software generating the G-code. Generally, there are some notes added to the G-code. It may take some inspection of the file and trial and error to get this section correct.

5. Also change the writeFileName to a name that is easy to recognize.
6. Run the file using >python ZchangerGcode.py. This will create a file where the z-height of the gasket is offset by 1.1mm from the print bed and allow the gasket to be printed on a 1 mm thick slide.
7. Transfer the file (named from writeFileName) the printer.
8. Place a 50 x 75 x 1 mm glass slide into the rectangular frame printed previously.
9. Tape the corners of the slide down to the rectangular frame using Kapton tape.
10. Print the slide gasket.
11. Repeat steps 8-10 as necessary.

Recipes

Formulating 7H9-GOT media (1000 mL)

1. Weigh out 4.7 g 7H9 powder into 1000 mL glass media bottle
2. Add 900 mL of 18 MΩ H₂O and dissolve the 7H9 powder
3. Add 4 mL of sterile 50% glycerol

4. Autoclave at 120°C for 30 min.
5. Allow liquid to cool to 55°C
6. Add aseptically following ingredients:
 - a. 2.5 mL of sterile 20% Tween 80
 - b. 100 mL of sterile OADC enrichment

Formulating 10x 7H9-G media (41.6 mL)

1. Weigh out 1.88 g 7H9 powder into 50 mL Falcon tube.
2. Add 40 mL of 18 MΩ H₂O and dissolve the 7H9 powder by vortexing and slight heating.
3. Add 1.6 mL of 50% glycerol.
4. Filter sterilize using 50 mL steriflip filter and conical tube.

Table 1 Volume recipes for 7H9-GO Media

Final Media Volume	1.33 % w.v. agarose	10x 7H9-G media	18 MΩ H ₂ O	OADC	1000x Drug (optional)
20 mL	15.1 g	2 mL	1 mL	2 mL	20 μL
10 mL	7.5 g	1 mL	0.5 mL	1 mL	10 μL
4 mL	3.05 g	0.4 mL	0.2 mL	0.4 mL	4 μL

Acknowledgments

This project has been funded with National Institutes of Health U19 AI106761

Competing interests

The Authors declare no competing interests.

References

- Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., & Fortune, S. M. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science (New York, N.Y.)*, 335(6064), 100–104. <https://doi.org/10.1126/science.1216166> PMID: 22174129
- Barr, D. A., Kamdolozi, M., Nishihara, Y., Ndhlovu, V., Khonga, M., Davies, G. R., & Sloan, D. J. (2016). Serial image analysis of *Mycobacterium tuberculosis* colony growth reveals a persistent subpopulation in sputum during treatment of pulmonary TB. *Tuberculosis (Edinburgh, Scotland)*, 98, 110–115. <https://doi.org/10.1016/j.tube.2016.03.001> PMID: 27156626
- Choi, J., Yoo, J., Lee, M., Kim, E. G., Lee, J. S., Lee, S., Joo, S., Song, S. H., Kim, E. C., Lee, J. C., Kim, H. C., Jung, Y. G., & Kwon, S. (2014). A rapid antimicrobial susceptibility test based on single-cell morphological analysis. *Science translational medicine*, 6(267), 267ra174. <https://doi.org/10.1126/scitranslmed.3009650> PMID: 25520395
- Dhar, N., McKinney, J., & Manina, G. (2016). Phenotypic Heterogeneity in *Mycobacterium tuberculosis*. *Microbiology spectrum*, 4(6), 10.1128/microbiolspec.TB2-0021-2016. <https://doi.org/10.1128/microbiolspec.TB2-0021-2016> PMID: 27837741.
- Golchin, S. A., Stratford, J., Curry, R. J., & McFadden, J. (2012). A microfluidic system for long-term time-lapse microscopy studies of mycobacteria. *Tuberculosis (Edinburgh, Scotland)*, 92(6), 489–496. <https://doi.org/10.1016/j.tube.2012.06.006> PMID: 22954584
- Herricks, T., Donczew, M., Mast, F. D., Rustad, T., Morrison, R., Sterling, T. R., Sherman, D. R., & Aitchison, J. D. (2020). ODELAM, rapid sequence-independent detection of drug resistance in isolates of *Mycobacterium tuberculosis*. *eLife*, 9, e56613. <https://doi.org/10.7554/eLife.56613> PMID: 32401195
- Herricks, T., Dilworth, D. J., Mast, F. D., Li, S., Smith, J. J., Ratushny, A. V., & Aitchison, J. D. (2017). One-Cell Doubling Evaluation by Living Arrays of Yeast, ODELAY!. *G3 (Bethesda, Md.)*, 7(1), 279–288. <https://doi.org/10.1534/g3.116.037044>
- Kim, S. J., Fernandez-Martinez, J., Nudelman, I., Shi, Y., Zhang, W., Raveh, B., Herricks, T., Slaughter, B. D., Hogan, J. A., Upla, P., Chemmama, I. E., Pellarin, R., Echeverria, I., Shivaraju, M., Chaudhury, A. S., Wang, J., Williams, R., Unruh, J. R., Greenberg, C. H., Jacobs, E. Y., ... Rout, M. P. (2018). Integrative structure and functional anatomy of a nuclear pore complex. *Nature*, 555(7697), 475–482. <https://doi.org/10.1038/nature26003>
- Logsdon, M. M., & Aldridge, B. B. (2018). Stable Regulation of Cell Cycle Events in Mycobacteria: Insights From Inherently Heterogeneous Bacterial Populations. *Frontiers in microbiology*, 9, 514. <https://doi.org/10.3389/fmicb.2018.00514> PMID: 29619019
- Manina, G., Dhar, N., & McKinney, J. D. (2015). Stress and host immunity amplify *Mycobacterium*

tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell host & microbe*, 17(1), 32–46. <https://doi.org/10.1016/j.chom.2014.11.016> PMID: 25543231

Mast, F. D., Herricks, T., Strehler, K. M., Miller, L. R., Saleem, R. A., Rachubinski, R. A., & Aitchison, J. D. (2018). ESCRT-III is required for scissioning new peroxisomes from the endoplasmic reticulum. *The Journal of cell biology*, 217(6), 2087–2102. <https://doi.org/10.1083/jcb.201706044>

Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., Ginsberg, A., Swaminathan, S., Spigelman, M., Getahun, H., Menzies, D., & Raviglione, M. (2016). Tuberculosis. *Nature reviews. Disease primers*, 2, 16076. <https://doi.org/10.1038/nrdp.2016.76> PMID: 27784885.

Rego, E. H., Audette, R. E., & Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature*, 546(7656), 153–157. <https://doi.org/10.1038/nature22361> PMID: 28569798

Santi, I., Dhar, N., Bousbaine, D., Wakamoto, Y., & McKinney, J. D. (2013). Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. *Nature communications*, 4, 2470. <https://doi.org/10.1038/ncomms3470> PMID: 24036848

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., & McKinney, J. D. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science (New York, N.Y.)*, 339(6115), 91–95. <https://doi.org/10.1126/science.1229858> PMID: 23288538

World Health Organization, *Global Tuberculosis Report, 2020*, https://www.who.int/tb/publications/global_report/en/