Chapter 12

Quantification of *Mycobacterium tuberculosis* Growth in Cell-Based Infection Assays by Time-Lapse Fluorescence Microscopy

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Abstract

Quantification of *Mycobacterium tuberculosis* (*Mtb*) growth dynamics in cell-based in vitro infection models is traditionally carried out by measurement of colony forming units (CFU). However, *Mtb* being an extremely slow growing organism (16–24 h doubling time), this approach requires at least 3 weeks of incubation to obtain measurable readouts. In this chapter, we describe an alternative approach based on time-lapse microscopy and quantitative image analysis that allows faster quantification of *Mtb* growth dynamics in host cells. In addition, this approach provides the capability to capture other readouts from the same experimental setup, such as host cell viability, bacterial localization as well as the dynamics of propagation of infection between the host cells.

Key words *Mycobacterium tuberculosis*, Macrophages, Live imaging, Time-lapse microscopy, Image analysis, Infection, Host-pathogen interaction

1 Introduction

Mycobacterium tuberculosis (*Mtb*) is a slow growing bacterial pathogen that divides once every 16–24 h under optimal in vitro conditions [1]. Consequently, plating-based assays for quantifying bacterial loads require incubation periods longer than 3 weeks [2]. Despite this, liquid culture and enumeration of colony forming units (CFU) are standard approaches used to analyze and compare growth dynamics of different *Mtb* strains or assess bacterial viability upon exposure to different stresses or drug treatments [3–5]. CFU enumeration is also routinely used to quantify intracellular bacterial burden over time in cell lysates from in vitro cell-based infection models [6–8]. In addition to the lengthy incubation periods needed to obtain the experimental readout, another limitation of the CFU enumeration approach in in vitro infection models is that these experiments often require large numbers of cells. This can

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represent a limiting factor when differentiated nondividing cells such as primary cells are used for the infection studies.

An alternative approach that is nondisruptive and amenable to high-throughput screening involves infecting cells with fluorescent or luminescent reporter strains of bacteria. Measurement of total bacterial fluorescence or luminescence is then carried out repeatedly over time to estimate the bacterial burden [3, 9–12]. Unfortunately, this approach does not distinguish between intra- and extracellular bacteria. Antibiotics, which cannot cross the plasma membrane layer of the host cell, are often included in the cell medium to prevent the replication of extracellular bacteria [13]. However, often these antibiotics have other nonspecific effects on the host cellular responses to the infection and could also potentially interfere with other small molecules being tested in cell-based in vitro infection models [14, 15]. Another limitation of this approach is that it provides limited information about the viability of the infected and uninfected cells in the assayed sample over the course of the experiment.

Time-lapse microscopy imaging is a powerful technique that overcomes most of these technical challenges and has the capability to provide multiple readouts in a single experimental setup. With time-lapse microscopy imaging, the time required to get readouts from an experiment does not depend on the doubling time of the infecting pathogen or the time it takes for the pathogen to form countable colonies on solid medium. In addition, this approach is nondisruptive and requires low number of host cells that can be continuously analyzed over time. Also, additional readouts, such as host cell viability, fraction of infected cells, bacterial load per cell, and bacterial location (intracellular vs. extracellular), can be obtained from the same dataset by just modifying the image analysis pipeline. Finally, this approach allows the investigator to capture and quantify host cell and bacterial heterogeneity at the single-cell level.

Approaches based on single-cell imaging have been implemented successfully over the last decade to compare intracellular and extracellular *Mtb* growth [16–18], quantify heterogeneity in intracellular *Mtb* growth in individual host cells [19], investigate the effect of cytokine stimulation on the control of intracellular *Mtb* [17, 19], measure intracellular *Mtb* responses to drug treatment [20], confront intracellular growth dynamics of different *Mtb* mutant strains [17, 21], characterize death of host cells infected with *Mtb* [16–18, 22, 23], and study dynamics in intracellular *Mtb* localization and host cell responses to the infection [23–28]. These achievements have been made possible due to the development of bacterial fluorescent reporters that allow the characterization of the metabolic state [20, 29], the response to stresses [30–34], and the expression of selected genes [7, 35, 36] in individual bacteria. In parallel, genetically encoded fluorescent reporters or dyes

compatible with live imaging allow labeling of specific intracellular compartments, and characterization of intracellular processes and responses to the infection in the infected host cells [23, 24, 27, 37, 38].

Here, we provide a step-by-step protocol describing how to prepare, infect, image, and analyze host cells infected with fluorescent *Mtb*. To highlight the strength of this approach, we infect murine bone marrow derived macrophages (BMDMs) with wild-type *Mtb* or with a mutant *Mtb* strain deficient in secretion of several ESX-1 secreted proteins including EsxA, EsxB, EspA, EspC, and EspB [18]. Proteins secreted by the *Mtb* ESX-1 type VII secretion system have been shown to be involved in several processes during infection including phagosomal membrane damage, bacterial translocation into the cytosol, intracellular growth, and induction of host-cell death [6, 23, 39–43]. Here, we describe how to set up an experiment to simultaneously obtain information on intracellular and extracellular *Mtb* growth dynamics, on host-cell viability, and on bacterial propagation in host cells for the two *Mtb* strains tested.

Finally, in the Subheading 4, we provide suggestions and advice on key steps of the protocol that could be modified or optimized depending on the design and the objective of the experiment.

2 Materials

2.1 Host Cells

Different types of adherent primary cells or cell lines can be used to perform this protocol (*see* **Note 1**). Here, we use murine bone marrow derived macrophages (BMDMs) that were previously collected, cryopreserved, and differentiated as described elsewhere [18, 44].

1. A culture of differentiated BMDMs. ~2–3 × 10^4 cells per condition should be used.
2. Cell scraper.
3. BMDM imaging medium: DMEM without phenol red (*see* **Note 2**), 5% heat-inactivated FBS, 1% sodium-pyruvate, 1% GlutaMax, and 5% L929-cell-conditioned medium. Store at 4 °C.
4. Trypan blue.
5. Hemocytometer and optic microscope (or automated cell counter).
6. 4-compartment 35 mm μ-dish or any other device compatible with microscopy imaging (*see* **Note 3**).

2.2 Bacterial Culture

1. Fluorescent *Mtb* strain grown to mid exponential phase ($A_{600\ nm}$ 0.4–0.6). Here we use *Mtb* strains with a plasmid integrated in the genome (at the L5 phage attachment attB site) in which the gene encoding TdTomato was cloned downstream of a strong constitutive promoter [18]. Any *Mtb* strain expressing fluorescent proteins with excitation and emission spectra compatible with the microscope filters could be used to perform this protocol (*see* **Note 4**).
2. Centrifuge.
3. 1 mL syringe.
4. 5 μm PVDF syringe filter.
5. 1.5 mL microtubes.
6. Optional: breathable sealing film for tissue culture plates.

2.3 Optional: Tubing for Medium Replenishment/ Replacement

For experiments lasting more than 3 days, it might be useful to replace one half of the cell culture medium periodically (every 3 days) with fresh medium to ensure the viability of the infected cells. Medium replacement might also be required when the experimenter wishes to expose the infected cells to environmental stress during the experiment, such as exposure to drugs or cytokines. Having a customized tubing connected to the lid of the μ-dish facilitates the experimenter to carry out these medium changes.

1. Stainless steel pointed tweezer.
2. Bunsen burner.
3. Silicon tubing (0.8 mm inner diameter).
4. Stainless steel capillary tubes 1 mm external diameter, 0.75 mm wall thickness, 20 mm length, 2 per well.
5. Barb to female Luer lock connectors, 2 per well.
6. 0.22 μm PVDF syringe filter, 2 per well.
7. Cementit universal glue (or similar).
8. 1 mL syringes.
9. 70% ethanol.

2.4 Imaging

1. Inverted epifluorescence microscope equipped with a motorized stage, camera, and the necessary filters to image the fluorophore. Here, we use a Nikon Ti2 microscope equipped with an EMCCD camera (iXON Ultra 888, Andor) and with a mCherry (Excitation 560/40, Emission 635/60) dichroic filter.
2. Objectives with different magnifications and numerical apertures (NA) can be used, depending on the sample and on the size of the field of view of the camera. Here, we use a 40× air objective with NA = 0.95 (*see* **Note 6**).

3. A stage-top incubator connected to a gas mixer should be used to control the environmental conditions of the samples on the microscope during imaging. Here, we use a stage-top incubator (Okolab) to maintain the samples at 37 °C in a humidified environment. Air mixed to 5% CO_2 was supplied using an Okolab gas mixer. Our microscope is also enclosed within a custom-fitted plastic box to control the ambient temperature, which is kept constant at 37 °C during the experiments.

2.5 Software for Image Analysis and Statistical Analysis

1. Fiji (Image J) [45], we used version 2.9.0/1.53t.
2. Interactive Cell Lineage Tracer Fiji plugin, downloadable from: https://github.com/Biomedical-Imaging-Group/Interactive-Cell-Lineage-Tracer.
3. Microsoft Excel.
4. GraphPad Prism, we used version 9.

3 Methods

3.1 Seeding the Host Cells in the Imaging Device

1. Pre-warm the BMDM imaging medium to 37 °C.
2. Completely aspirate the culture medium from the cell culture plate or flask. Add 2 mL of BMDM imaging medium.
3. Gently detach the cells from the plate or flask using a cell scraper. Gently pipet the cells to dissolve cell aggregates and transfer the suspension to a tube.
4. Determine the cell concentration using a hemocytometer or an automated cell counter.
5. Dilute the cells to the desired concentration in BMDM imaging medium. We dilute the BMDMs to approximately 10^5 cells/mL. For other cell types, the concentration might need to be optimized (*see* **Note 5**).
6. Seed the cells in the microscopy imaging device of choice (*see* **Notes 3** and **5**). Here we use a 4-compartment 35 mm μ-dish. Add 200 μL of diluted BMDMs to each well.
7. Incubate the cells in the incubator at 37 °C, 5% CO_2 for at least 2 h or until the cells adhere to the surface of the device.
8. Add 500 μL of pre-warmed BMDM imaging medium to each well and incubate overnight (12–18 h).
9. Optional: In experiments requiring stimulation of cells with cytokines prior to infection, remove the medium from the cells, add BMDM imaging medium supplemented with the cytokine (s), and incubate for the required time before infection.

3.2 Infecting the Host Cells with Mtb

NB: When using *Mtb*, all the following procedures should be carried out in a biosafety level 3 (BSL3) facility according to the facility's guidelines and standard operating procedures.

1. Pre-warm the BMDM imaging medium to 37 °C.
2. Culture *Mtb* to exponential phase ($A_{600\ nm}$ 0.4–0.6). Collect the bacteria by centrifuging 1 mL of culture at 5000 g for 5 min at room temperature. Wash the pellet with 1 mL of BMDM imaging medium, repeat centrifugation, and resuspend in 200 μL of the same medium.
3. Filter the bacterial suspension using a 5 μm PVDF filter connected to a 1 mL syringe. This procedure removes large bacterial aggregates from the resuspended culture.
4. For each well/infection condition, mix 20 μL of filtered bacterial culture with 200 μL of BMDM imaging medium (*see* **Note 7**). Add this mix to the cells.
5. Incubate the cells and the bacteria in the incubator at 37 °C, 5% CO_2 for at least 4 h to allow the cells to internalize the bacteria.
6. Wash the cells 3 times with 500 μL of pre-warmed BMDM imaging medium to remove the extracellular bacteria.
7. Add 700 μL of BMDM imaging medium per well (*see* **Note 3**).
8. Close the lid of the μ-dish or plate (*see* Subheading 3.3 if a lid connected to tubing is required for medium replenishment/replacement during the experiment).
9. Optional: Strips of breathable sealing film for tissue culture plates can be cut and attached to the side of the μ-dish or plate to ensure safe sealing.

3.3 Optional: Tubing Connection for Medium Replenishment/Replacement

1. Using heat-resistant gloves to hold the tweezers, heat the pointed ends of a stainless steel tweezer on the flame of a Bunsen burner.
2. Using the pointy ends of the hot tweezer, make holes on the plastic lid of a μ-dish. When using a single well 35 mm μ-dish, two holes should be made on the lid, preferably on the side of the plate where the sample is not imaged. Similarly, when using a 4-compartment 35 mm μ-dish, two holes per well should be made.
3. Insert one stainless steel capillary tube in each hole on the lid.
4. Connect a piece of silicon tubing (approx. 10 cm long) to the extremities of each capillary tube on the outer side of the μ-dish lid.
5. Seal the holes on the lid around the capillary tubes with a drop of Cementit universal glue. This step prevents leakage from the dish and stabilizes the capillary tubes in upright position. Make

sure that the inner side of the capillary tubes reaches the bottom of the plate. Let the glue dry for 30 min.

6. Connect the Barb to female Luer lock connector to the other extremity of each piece of silicon tubing. Connect a 0.22 μm PVDF syringe filter to each Luer lock connector.
7. We recommend testing the lid and the connections on the tubing before positioning the lid on the μ-dish with the cells. Place the lid on a spare μ-dish. Fill a syringe with 70% ethanol and connect it to one of the filters (inlet). Flow the ethanol in the tubing to fill the well in the μ-dish. Connect an empty syringe to the filter connected to the second tubing of the same well (outlet) and aspirate the ethanol from the well. Make sure that the connections do not leak and that the position of the capillary tube allows optimal suction of the liquid contained in the well. Repeat for all the wells.
8. Under the biosafety cabinet of the BSL3 facility, position the lid and tubing inside the stage-top incubator. Wash again the tubing and the lid with 70% ethanol as described in the previous step to ensure sterility. Before positioning the lid and tubing on the μ-dish containing the cell sample, wash the tubing with BMDM imaging medium to eliminate any residual ethanol.
9. At this point, the lid can be positioned on the μ-dish containing the infected cells.
10. Keep the empty syringes connected to the filters to avoid contamination of the outer side of the filters.

3.4 Time-Lapse Microscopy Imaging

1. Place the sealed μ-dish inside the stage-top incubator and position it securely on the microscope stage. Optional: If the μ-dish lid is connected to tubing and syringes, make sure to secure the syringes on the stage of the microscope outside the stage-top incubator.
2. Connect the gas mixer to the stage-top incubator and set the temperature of the incubator at 37 °C, with 95% humidity and 5% CO_2.
3. Select the best magnification to use (*see* **Note 6**). Here, we use a 40× air objective. This objective, in combination with the resolution of our camera, allows us to image fields of view of approximately 300 × 300 μm.
4. Manually select the positions to image. Select at least 10 XY fields per condition. Each field of view should ideally include both infected and uninfected cells.
5. Start the automated time-lapse microscopy acquisition using appropriate imaging conditions (*see* **Note 6**). Here, we imaged

each *XY* field every 2 h for 166 h. In addition, 3 z planes spaced 1.5 μm apart were acquired on each *XY* field.

6. Optional: For experiments requiring medium replenishment, connect a new 1 mL syringe prefilled with pre-warmed fresh medium to the inlet filter. Aspirate 300 μL of spent medium from the well using the empty syringe connected to the outlet filter. Inject 300 μL of fresh medium into the well using the syringe connected to the inlet. We recommend injecting the medium slowly to avoid formation of bubbles that may disturb the infected cells and affect the imaging. Once full, the syringe connected to the outlet filter can be safely removed and replaced by a new empty syringe. This step should be performed every 72 h to provide nutrients to the cells. For experiments requiring medium replacement *see* **Note 8**.
7. When the experiment is finished, stop the microscope, and save the acquired image series. Transfer the imaging device into the biosafety cabinet, disassemble, inactivate, and dispose it according to the BSL3 facility guidelines.

3.5 Image Analysis

The microscopy image series can be processed and analyzed using the Fiji image processing package.

1. Open a file with all the timepoints, channels, and stacks taken for a single *XY* field with Fiji and select the "split channels" option in the "Bio-Formats Import Options" window.
2. Select the fluorescence channel corresponding to the bacteria and perform a maximum intensity projection to project all the z-stacks into one image per timepoint. This is done using the "Z Project" function from the "Image/Stacks" menu and selecting "Max Intensity" projection type.
3. Save the fluorescence projection file.
4. Select the brightfield channel corresponding to the host cells and split the image series into individual z-stacks using the "Deinterlive" function from the "Image/Stacks/Tools" menu and entering the number of z-stacks acquired. One image series for each z-stack is generated. Choose the one with the best focus and save it as a reference channel for the host cells. Close the other image series.
5. Merge the host cells channel with the fluorescence channel corresponding to the bacteria (*see* Fig. 1) using the "Merge Channels" function from the "Image/Colors" menu. Select the merged image series obtained and combine the two channels into one by choosing the "Stack to RGB" command from the "Image/Colors" menu. Save the resulting image series.

   These files can be used for different types of analysis.

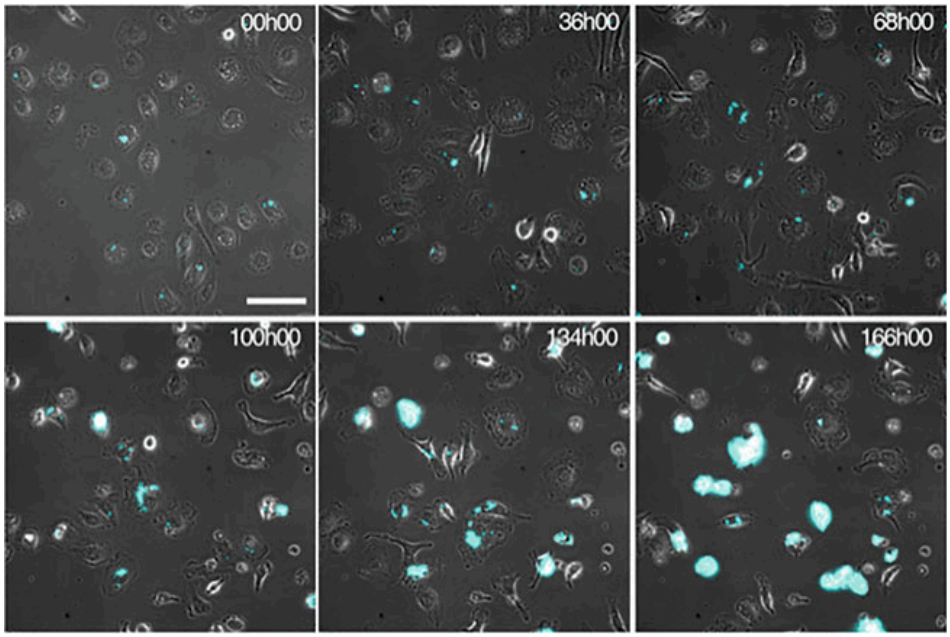

Fig. 1 Frames from a representative time-lapse microscopy image series of murine BMDMs infected with fluorescent *Mycobacterium tuberculosis*. Murine BMDMs infected with fluorescent *Mycobacterium tuberculosis* (in cyan) and imaged by time-lapse microscopy at 2 h intervals for 166 h. A subset of representative timepoints is shown. Scalebar, 50 μm

3.6 Analysis of the Total Bacterial Load

1. Use Fiji to open the file with the maximum intensity projection of the bacterial fluorescence channel.
2. Open the Interactive Cell Lineage Tracer plugin from the "Plugins" menu. In the "Lineage" tab, select "create" to create a new region of interest (ROI) with the assigned name. Draw a ROI corresponding to the whole field of view (*see* Fig. 2a) on the first frame of the image series in the image window by double-clicking and then drawing with the mouse. Select the ROI on the image window or from the "Outlines" tab of the plugin window and click on "propagate" on the plugin window to create the same ROI in all the frames of the image series. All existing ROIs can be seen upon moving the time cursor on the bottom of the image window or on the list in the "Outlines" tab of the plugin window.
3. Manually set a threshold selecting the "Threshold" command from the "Image/Adjust" menu in Fiji. The threshold used should be the same for all the image series analyzed from the same experiment and should be chosen making sure that all the bacteria in the field of view are above the threshold and that background fluorescence is not (*see* Fig. 2b).
4. Go to the "Measure" tab on the plugin and open the "Set Measurements" menu. Select "Limit to threshold" to make sure that only the areas above the threshold in the image are

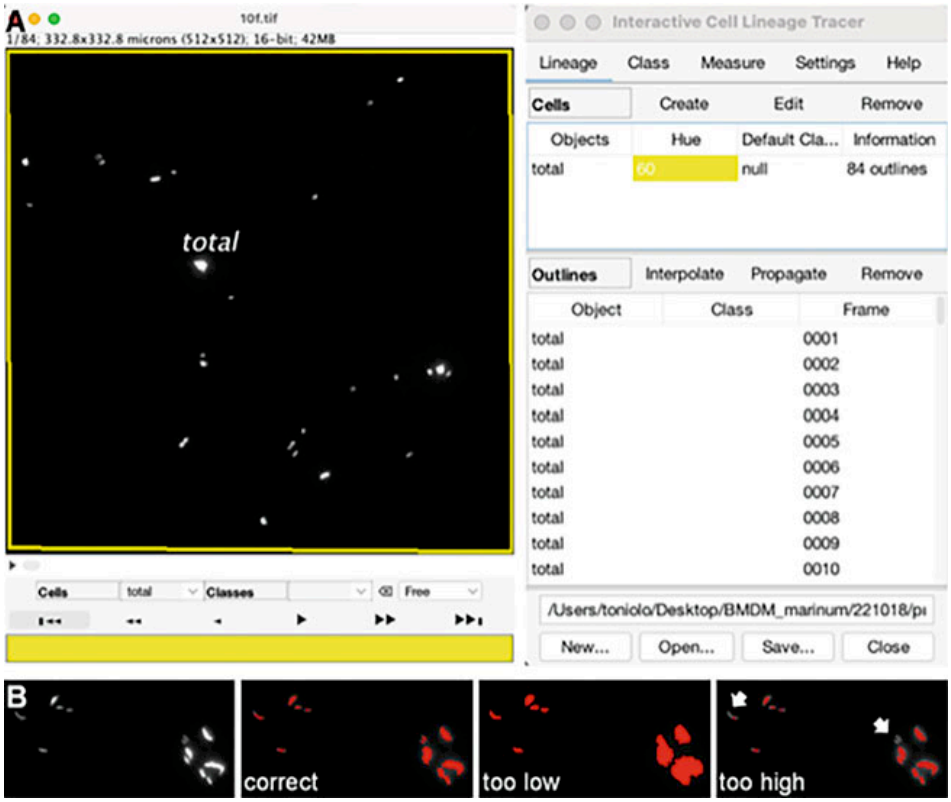

Fig. 2 ROI design for analysis of total bacterial load and setting threshold parameters. (**a**) Interactive Cell Lineage Tracer image window (left) and plugin window (right). An ROI including the whole field of view (yellow square, called "total") is drawn on the first frame of the bacterial fluorescence image series file. The same ROI is then extended to all the frames using the "Interpolate" command on the plugin window. In this window, the list of all the ROIs drawn is shown. (**b**) Different thresholds are set on the same reference image (first panel). A correct threshold segments all the bacteria in the image (second panel). If the threshold is too low, undesired background pixels such as those corresponding to the fluorescence halo around the bacterial microcolonies are included in the segmentation (third panel). If the threshold is too high, a fraction of the bacteria (indicated by white arrows) is lost in the analysis (fourth panel)

measured. Also make sure that "Area" is selected. Then click on "Measure" to measure all the ROIs in the timeseries.

5. Copy all the measurements from the "measure" window that pops up and paste them to an Excel file.

3.7 Analysis of Growth Parameters of Individual Bacterial Microcolonies

1. Use Fiji to open the merged file including the brightfield and the fluorescence channel corresponding to the host cells and the bacteria, respectively.
2. Open the Interactive Cell Lineage Tracer plugin. In the "Lineage" tab, select "create" to create a new region of interest (ROI) with the assigned name. For this analysis, create and draw one ROI per bacterial microcolony on the first frame of the image series on the image window. ROIs should be designed around intracellular and extracellular microcolonies and should not overlap with other microcolonies (examples in

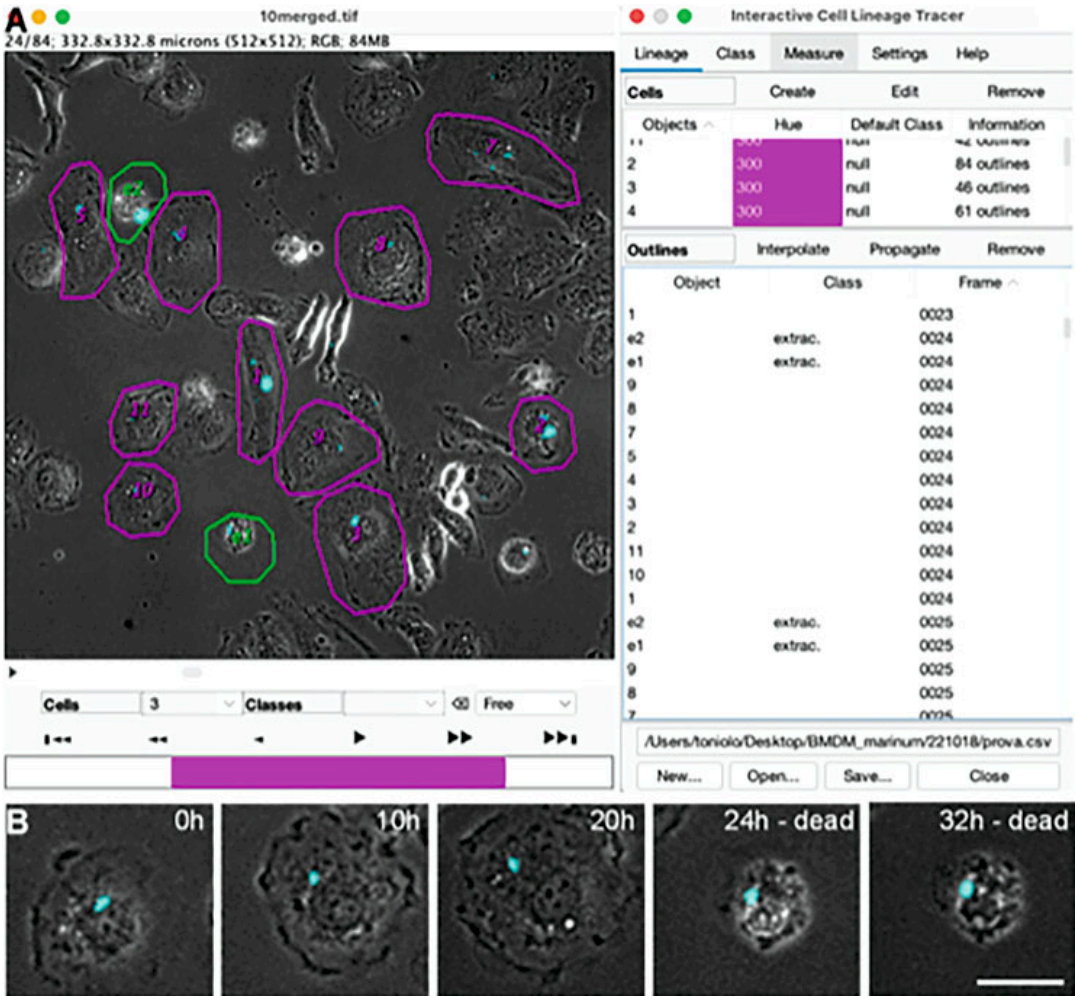

Fig. 3 ROI design for measurement of growth of intracellular and extracellular *Mtb* and for identification of host cell death. (**a**) Interactive Cell Lineage Tracer image window (left) and plugin window (right). ROIs for all the intracellular (magenta) and extracellular (green) microcolonies (in cyan) are created in the plugin window and drawn on the image window. Each ROI is drawn on all the frames where the same microcolony is visualized. In the plugin window, the list of all the microcolonies tracked (top list) and all the ROIs drawn (bottom list) are shown. (**b**) Representative image series of a dying infected macrophage. Scalebar, 20 μm. From 0 h to 24 h the *Mtb* microcolony (cyan) is considered intracellular. After 24 h the microcolony is marked as extracellular

Fig. 3a). The name associated to each ROI should define if microcolonies are intracellular and extracellular. For example, we label each intracellular microcolony sequentially with a number and each extracellular microcolony with the prefix "e."

3. Each ROI drawn on the first frame is extended to subsequent frames to track microcolonies over time. This is done by selecting an ROI, moving to the next frame, and drawing the ROI again. Repeat for all the frames and for all the ROIs. If over time the microcolony does not change drastically its XY coordinates (due to host cell movements), the "propagate" or the "interpolate" commands in the plugin window can be used to speed up manual annotation of the image series. They both

work on the specific ROI selected. The "propagate" command draws the same ROI in all the following frames. The "interpolate" command automatically draws ROIs in all the image frames between two frames where ROIs have been manually designed.

4. When selected, ROIs can be adjusted or redesigned using the mouse, or deleted (by clicking on "Remove" in the "Outlines" tab). All the ROIs designed for the same microcolony can be deleted by selecting the microcolony from the list in the "Objects" tab of the plugin and clicking on "Remove."
5. If a microcolony becomes extracellular over the course of the imaging due to lysis of the host cell (see instructions on how to identify death or lysis of the host cell in the Subheading 3.8 below), this can be noted by adding a label to the ROIs designed on the frames where bacteria are extracellular. This can be done by going to the "Class" tab and creating a new class with the assigned name using the "create" command. Once the class is created, it can be selected from the "Classes" menu in the image window and be assigned to the selected ROI. Alternatively, the newly formed extracellular microcolony can be treated as a new microcolony and a new name can be assigned to it.
6. Similarly, if extracellular microcolonies are completely or partially internalized by host cells, they should be treated as new microcolonies from the time point where the uptake event takes place.
7. Once ROIs have been designed for all the microcolonies in all the frames, save the annotation in a .csv file by clicking on "save" in the plugin window. Close the image window and the plugin.
8. Open the file with the maximum intensity projection of the bacterial fluorescence channel in Fiji and open the Interactive Cell Lineage Tracer plugin. Select "Open" on the plugin window and open the .csv file. All the ROIs previously designed will be visible on the open image series.
9. Manually set a threshold and measure the fluorescence values for all the ROIs drawn following the instructions described in the Subheading 3.6, **steps 3** and **4**.
10. Copy the measurements from the results window and paste them to an Excel file.

3.8 Analysis of Host Cell Viability and Propagation of Infection

Cell-death events are identified by visual comparison of adjacent frames in image series, captured by brightfield or phase-contrast or differential interference contrast imaging. Dying macrophages rapidly shrink, lose membrane integrity, and stop moving. We define the time of death for individual macrophages as the first image

frame in which the cell stops moving and loses their membrane integrity. Often at this timepoint, the intracellular bacteria identified on the fluorescent channel and the intracellular structures visible in the brightfield images also stop moving (*see* Fig. 3b). When infected host cells die, intracellular bacteria become extracellular and can propagate to neighboring cells.

1. Using Fiji, open the merged file that comprises the brightfield and the fluorescence channel corresponding to the host cells and the bacteria, respectively.
2. Image series are analyzed manually by visual inspection. On an Excel file, add a new entry for each cell present on the first frame, and note down the infection status (infected or bystander) and the frame of death. Cells that stably overlap with bacteria in the fluorescence channel should be annotated as infected, the other uninfected cells in the same sample should be considered "bystander."
3. If bystander cells become infected (i.e., internalize extracellular bacteria), note down the frame in the Excel file as the time of infection. This can also be done for previously infected cells that further interact with extracellular microcolonies.

3.9 Data Analysis

3.9.1 Total Bacterial Load Over Time

The total bacterial load over time is calculated from the total fluorescent area per field of view. The values obtained from this analysis can be considered comparable to values obtained from CFU enumeration at different timepoints post infection or from the total fluorescence or luminescence over time in cells infected with fluorescent or luminescent bacteria.

1. In the Excel file with the measures from the image series, the columns labeled as "Frame" and "Area" are used for this analysis. Sort the column with the total fluorescent area ("Area") by frame number and normalize the total fluorescent area of each frame by dividing these values for the total fluorescent area measured for the first frame. Repeat this procedure for all the fields of view imaged.
2. In Prism GraphPad, create a new XY data table and graph. Choose the number of replicate values to enter in Y depending on the number of fields of view per condition analyzed.
3. In the data table, add the timepoints in the X column. Name each "Group" with the name of the condition tested and copy the normalized total fluorescent area per frame previously calculated in Excel to the columns. Each field of view imaged for the same condition should be added as Y replicate in the respective group.
4. In the graph, select the individual dataset and in the "Style" tab select "mean and error" under the menu "Appearance" and

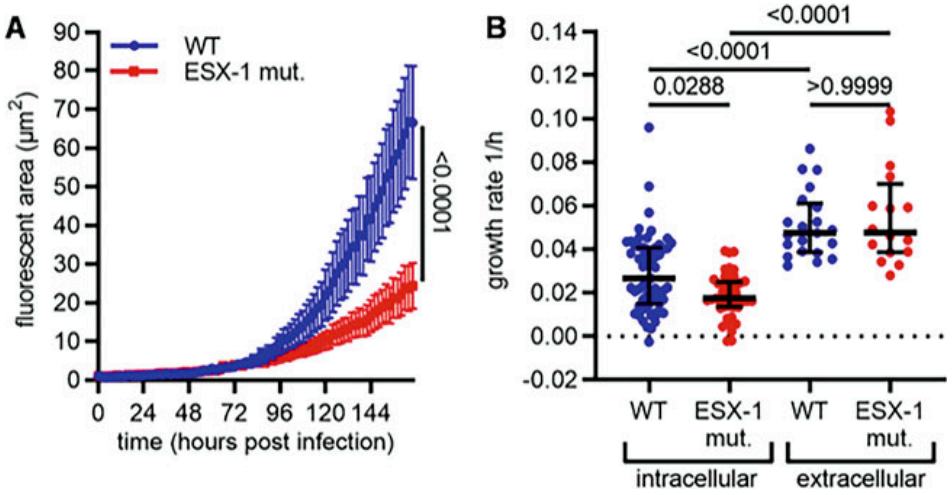

Fig. 4 Growth of *Mycobacterium tuberculosis* in the murine BMDMs infection model. (**a**) Total relative fluorescent area occupied by the indicated *M. tuberculosis* strains (bacterial load) per field of view measured over time. Values are normalized to timepoint 0. Symbols represent the average bacterial load ($n = 8$), bars represent standard errors of the mean. P-value was calculated using an unpaired t test. (**b**) Growth rate of intracellular ($n = 59$ for WT and 45 for ESX-1 mutant) and extracellular ($n = 21$ for WT and 16 for ESX-1 mutant) bacterial microcolonies. Each symbol represents a single microcolony. Black lines and error bars represent median values and interquartile range. *P*-value calculated using an unpaired Mann-Whitney test

"SEM" under the menu "Plot." Colors can be selected for the different datasets (or conditions) and a line connecting the dots of the different timepoints can be added.

5. Calculate statistical significance in differences between conditions by comparing the distributions of the normalized values measured for the last timepoints.

Upon analysis of bacterial loads in our sample dataset (*see* Fig. 4a), we can observe that the ESX-1 mutant strain grows significantly less over time than the wild-type strain in the murine BMDM infection model. The potential reasons for the slow growth include I) the strain may have a general growth defect and II) the bacteria may be attenuated for growth when taken up by BMDM. Analysis of the total bacterial load over time, which is like CFU enumeration, does not allow us to distinguish between these two possibilities. These options can however be evaluated by comparing the intracellular and extracellular growth rate of the two *Mtb* strains.

3.9.2 Intracellular Versus Extracellular Bacterial Growth Rate

The growth of the individual microcolonies can be calculated and compared to the other microcolonies cultured in the same condition or under different conditions. Because to the annotation performed during the image analysis step, it is also possible to distinguish intracellular from extracellular microcolonies.

1. In the Excel file with the measures from the image series, the columns labeled as "Cell," "Frame," and "Area" will be used

for this analysis. Reorganize the data to obtain one column per microcolony ("Cell") with all the fluorescent area values sorted in increasing order of frame number.

2. In Prism GraphPad, create a new "*XY*" data table and graph. Choose the option where a single *Y* value is entered for each point.
3. In the data table, add the timepoints in the *X* column. Copy the area of fluorescence per timepoint to the *Y* columns. Use one column for each microcolony.
4. The growth rate is calculated by interpolating an exponential growth equation to the fluorescence area values. Click on the "Analyze" button, select all the dataset on the right tab of the window, and select "Nonlinear regression (curve fit)" in the "*XY* analyses" menu. Select "Exponential growth equation" from the "Exponential" menu and launch the analysis. Results of the interpolation are found in "Results."
5. The growth rate of each individual microcolony corresponds to the reciprocal of the doubling time value calculated in Prism GraphPad.
6. Compare the distributions of the growth rate values calculated for all the microcolonies of each condition by plotting the values in a new "Column" data table and graph. Here, individual values can be visualized choosing "Scatter dot plot" in the "Appearance" menu. The shape and the broadness of the distribution can potentially provide information on the heterogeneity of the single-cell host-pathogen interaction. Please note that these distributions often are not normal, it is thus recommended to compare them using a Mann-Whitney test.

In our example of microcolony growth rate analysis (*see* Fig. 4b), we can observe that the wild-type and the ESX-1 mutant microcolonies have the same growth rate when extracellular, suggesting that the mutant strain does not have a general growth defect. However, the intracellular microcolonies of the ESX-1 mutant grow significantly slower than the wild-type counterparts, confirming that this mutant strain is attenuated in BMDMs.

The two analyses presented so far focus on the bacterial growth dynamics, however it might also be useful to understand if the strains differentially affect host cell viability and how the respective strains propagate to uninfected host cells.

3.9.3 Host Cell Viability and Propagation of Infection

If we look at a snapshot of infected cells, we observe a distribution of live uninfected cells (or bystanders), live infected cells, extracellular bacterial microcolonies on dead host cell debris, and uninfected debris of dead cells. In different samples, we may observe a different distribution of these subsets (*see* Fig. 5a, b); however, single timepoint snapshots may not be sufficient to explain why

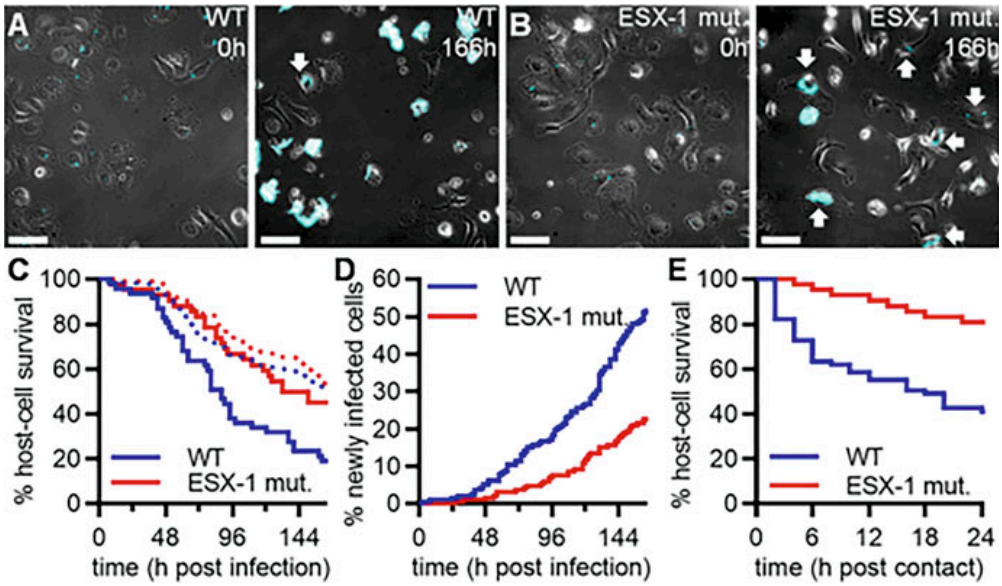

Fig. 5 Propagation of *M. tuberculosis* infection and plotting host-cell survival dynamics in the *Mtb*-BMDMs infection model. (**a**, **b**) Representative first and last frames from image series of BMDMs infected with WT (**a**) or ESX-1 mutant (**b**) *Mtb* (in cyan). White arrows indicate *Mtb* microcolonies associated with live BMDMs at the end of the experiment. Scalebars, 50 μm. (**c**) Percentage survival over time of infected (solid lines) versus uninfected bystander macrophages (dotted lines). (**d**) Percentage of uninfected bystander macrophages becoming infected over the course of the experiment. (**e**) Percentage survival over time of macrophages interacting with extracellular *Mtb* microcolonies released from dead macrophages. Timepoint zero corresponds to the time when a macrophage interacts with a microcolony

the samples are different. For example, uninfected debris might originate from a dead uninfected cell or from a dead infected cell whose bacterial cargo has subsequently been taken up by another cell. Similarly, a highly infected dead host cell might originate from a cell in which bacteria grew fast or from a cell that has internalized many slow-replicating bacteria as a single event from an extracellular microcolony. Analysis of time-lapse image series allows tracking over time each individual host cell and its interaction with bacteria providing dynamic information that may lead to better understanding of their phenotypes.

1. To quantify *viability over time* of infected and bystander cells, use the manually annotated data for the viability of the cells. Filter the data in the Excel file to select only the subset of infected cells that were infected already in the first frame and the subset of bystander cells that do not become infected over the course of the experiment. For cells that do not die over the course of the experiment, assign an arbitrary time of death equal to the number frames imaged +1.
2. In Prism GraphPad, create a new "Survival" data table and graph. Copy the column with the time of death values from the Excel file to the *X* column of the data table. Assign each subset of cells to a different *Y* column (e.g., infected with WT

Mtb to Group A, bystander of infected with WT *Mtb* to Group B, infected with mutant *Mtb* to Group C, bystander of infected with mutant *Mtb* to Group D). For each death event annotated in the *X* column, add a "1" to the *Y* column corresponding to that event. To obtain a graph like the one in Fig. 5c, plot the data as "probability of survival" in the "Graphs" tab. This plot allows comparison of viability of different subset of cells over time.

3. To quantify *infection propagation*, use the manually annotated data for the infection status of the cells. Filter the data on the Excel file to select only the bystander cells that are not infected at the beginning of the experiment. For cells that do not get infected over the course of the experiment, assign an arbitrary time of infection equal to the number frames imaged +1.
4. In Prism GraphPad, create a new "Survival" data table and graph. Copy the column with the time of infection values from the Excel file to the *X* column of the data table. Data for each condition analyzed (in this case the two *Mtb* strains) are assigned to different *Y* columns. For each infection event annotated in the *X* column, add a "1" to the *Y* column corresponding to that event. To obtain a plot like the one in Fig. 5d, plot the data as "probability of death" in the "Graphs" tab. This plot allows comparison of infection propagation to bystander cells for the different strains.
5. To quantify *viability upon interaction with an extracellular microcolony*, use the manually annotated data for the time of infection and time of death events for all the cells that interacted with extracellular bacterial microcolonies. Calculate the time from infection to death by subtracting the time of infection from the time of death. For cells that do not die over the course of the experiment, assign an arbitrary time from infection to death equal to the number frames imaged +1.
6. In Prism GraphPad, create a new "Survival" data table and graph. Copy the column with the time from infection to death values from the Excel file to the *X* column of the Prism GraphPad data table. Data for each condition analyzed (in this case the two *Mtb* strains) are assigned to different *Y* columns. For each infection event annotated in the *X* column, add a "1" to the *Y* column corresponding to that event. To obtain a plot like the one in Fig. 5e, plot the data as "probability of survival" in the "Graphs" tab. This plot allows comparison of viability of host cells over time upon interaction with an extracellular microcolony of different *Mtb* strains.

From these analyses, we can observe that cells infected with the WT *Mtb* strain die more often than the bystander cells (*see* Fig. 5c). Cells infected with the ESX-1 mutant strain have a higher survival

over time and do not seem to die more often than bystander cells (*see* Fig. 5c). This suggests that a lower number of microcolonies of the ESX-1 mutant strain become extracellular over time, and consequently the infection propagates to a smaller number of bystander cells compared to the WT strain (*see* Fig. 5d). Interestingly, the WT *Mtb* strain also propagates better because cells that internalize WT extracellular microcolonies die faster than cells that interact with ESX-1 mutant extracellular microcolonies (*see* Fig. 5e).

4 Notes

1. The protocol described here is compatible with any adherent cell lines or primary cells. However, we recommend using differentiated cells (preferably slow- or nongrowing), especially for experiments lasting more than 48 h. Fast-proliferating cells or cell lines that double every day, could end up overpopulating the imaged area, thereby affecting the bacteria-to-cell ratio, the microenvironment and availability of nutrients and make it extremely challenging to track individual cells over time.
2. Phenol red in cell culture medium may increase background fluorescence [46]. We thus advise using phenol red-free cell culture medium while imaging.
3. Depending on the number of conditions to image in parallel, different types of devices can be used. We tested different devices including single well 35 mm μ-dishes, 4-compartment 35 mm μ-dishes, and 24-well plates (all from Ibidi). Please note that different adapters may be required to fix the devices on the stage of the microscope. When choosing the imaging device, it might also be important to consider what objective will be used to image (*see* **Note 6**). During imaging, we recommend filling the single well 35 mm μ-dishes with 1.5 mL of medium, and each well of the 4-compartment 35 mm μ-dishes or the 24-well plates with 700 μL.
4. It is recommended to use integrative vectors for expression of fluorescent cassettes. This is especially important when studying phenotypic heterogeneity and target reporter gene expression levels at the single-cell level. Expression of fluorescent genes from episomal plasmids is often misleading as the heterogeneity observed is confounded by the variability in plasmid copy number.
5. Using approximately 2–3 × 10^4 BMDMs per well, we usually obtain 40–50% cell confluency. This number of cells is optimized for BMDMs seeded in single well 35 mm μ-dishes, 4-compartment 35 mm μ-dishes, or 24-well plates. For

different imaging devices or cell types, the number of cells to seed in each well may have to be optimized. 40–50% cell confluency is optimal because cells do not overlap and are easily trackable over time during image analysis. If fast-proliferating cells are being imaged, a lower starting density may be desirable.

6. We recommend using a 20× or a 40× air objective. The magnification should be selected depending on the field of view and the resolution of the microscope camera. Ideally, each field of view should fit 15–30 host cells (depending on cell density and size) and fluorescent bacteria should be clearly visible. Oil objectives with higher magnifications (60×–100×) may be used to capture better resolution of labeled intracellular compartments or individual intracellular bacteria. Notably, oil objectives are not optimal to image and travel across large XY distances. Therefore, they should only be used to stably image small surface areas and a limited number of conditions per experiment. In our hands, we managed to successfully image up to 4 conditions in a 4-compartment 35 mm μ-dishes using a 60× or a 100× oil objective for several days, but we faced significant challenges with oil stability when we attempted to image more than four conditions in 24-well plates. Please, also note that the oils used in microscopy could potentially damage the bottom of the imaging device. Make sure to use an oil that has been shown to be compatible with the imaging device. Finally, to avoid phototoxicity, it is recommended to use the minimum intensity and exposure times that provide good signal in the fluorescence channels.
7. Using the conditions described, we normally infect the cells with a multiplicity of infection (MOI) of 1 bacterium per BMDM cell. The MOI can be estimated by counting the number of bacteria and cells per field of view. Please note that an MOI of 1 does not imply that each cell in the dish is infected with one bacterium. Typically, some cells are infected with several individual bacteria and a certain fraction of cells are not infected. We encourage the user to optimize the number of bacteria used for the infection according to the desired MOI and as per the infectivity of the specific cells used.
8. If during the experiment the medium in the well needs to be completely replaced to switch to an entirely different culture condition, it is important to remember not to tilt or move the μ-dish on the stage of the microscope. While it may not be possible to aspirate all the medium in the well using the syringe connected to the outlet tubing in a single go, carrying out multiple complete medium changes, effectively dilutes out any residual medium to negligible concentrations.

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