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Tanya Parish
David M. Roberts *Editors*

Mycobacteria Protocols

Third Edition

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Preface

The mycobacteria continue to pose a threat to global health, as well as being important biological systems to understand. In the years since the Second Edition of this book, research has continued into understanding these organisms and a number of new techniques have been introduced into our technical repertoire. The aim of this book is to expand upon previous edition, providing a selection of the newest methods, as well as some of the basic methods required for a mycobacterial research laboratory. There is an increased focus on translational methods, in particular those that can be applied to drug discovery, since there is a much larger, concerted effort in that direction.

The first six chapters deal with fractionation and analysis of macromolecules, from nucleic acids to proteins, complex lipids, and metabolites. Recent advances in sequencing technologies as well as the development of metabolomics approaches provide several new methods over previous editions. Chapters regarding whole-genome sequencing, transcriptomics analysis of gene expression, and transcript mapping are all beneficiaries of the new, lower cost, higher throughput sequencing techniques. Detailed and comprehensive protocols are provided for protein and lipid/glycolipid analysis using well-established methods; these are now complemented by a metabolomics chapter in which the complement of metabolites can be profiled.

Genetic manipulation underpins our ability to investigate the biology of the mycobacteria, and chapters cover the basics of electroporation, through to advanced genetic engineering to construct sophisticated recombinant strains (gene knockouts, knockdowns, and mutations) using either homologous recombination or recombineering.

Later chapters deal with model culture systems that can be widely applied to mycobacterial species and mutant strains. These include two models of hypoxia survival and newer approaches to analyze biofilm formation. In addition, methods to look at efflux and permeability as well as the powerful approach of studying single cells are described.

Several chapters deal with methods for characterizing novel antimycobacterial agents. Since bacteria can show tolerance or resistance to the same antibiotics depending on their physiological state, several alternative methods are presented to cover a variety of conditions. Chapters covering both growth inhibition of replicating organisms, killing of replicating and nonreplicating organisms and intracellular organisms are included.

Of all the mycobacteria, *Mycobacterium tuberculosis* has become the most studied, due to the large burden of global disease it causes. Since one of its characteristics is the ability to survive and replicate in immune cells, chapters cover a range of methods, from the "simple" macrophage infection model, and the neutrophil model, analysis of bead-phagosomes, through to infection of the model organism *Dictyostelium*. Finally, a sophisticated model to look at host-pathogen interactions using high content screening is presented.

We hope that this book will be a resource both to those working in the field and to newcomers. We thank all of our authors for their generous contributions, in the knowledge that sharing their expertise and wealth of experience will enable more rapid advances in the field.

Seattle, WA, USA

Tanya Parish
David M. Roberts

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Chapter 1

Whole-Genome Sequencing for Comparative Genomics and De Novo Genome Assembly

Andrej Benjak, Claudia Sala, and Ruben C. Hartkoorn

Abstract

Next-generation sequencing technologies for whole-genome sequencing of mycobacteria are rapidly becoming an attractive alternative to more traditional sequencing methods. In particular this technology is proving useful for genome-wide identification of mutations in mycobacteria (comparative genomics) as well as for de novo assembly of whole genomes. Next-generation sequencing however generates a vast quantity of data that can only be transformed into a usable and comprehensible form using bioinformatics. Here we describe the methodology one would use to prepare libraries for whole-genome sequencing, and the basic bioinformatics to identify mutations in a genome following Illumina HiSeq or MiSeq sequencing, as well as de novo genome assembly following sequencing using Pacific Biosciences (PacBio).

Key words MiSeq, HiSeq, PacBio, Sequencing, Whole genome, Assembly, SNP, Resistance, Next-generation sequencing, MIRA, Bioinformatics, Illumina

1 Introduction

The recent impressive rise of Next-Generation Sequencing (NGS) technology platforms have made whole-genome sequencing of small bacterial genomes, such as those of mycobacteria, an attractive tool in many laboratories. NGS allows for both the assembly of entire genomes (de novo genome assembly), as well as for the detection of small and rare differences between different genomes (comparative genomics). De novo whole-genome assembly is commonly used to sequence the genome of novel bacteria, whose genome has never been sequences before, allowing for full annotation of the genome and its use as a reference for further studies. Comparative genomics can only be performed when a reference genome is already available, and is a very powerful tool for the identification of single/multiple mutations among conserved genomes.

Comparative genomics has proven to be a very powerful tool for the identification of the mechanism of resistance of bacteria to antibiotics. In particular, where resistance is due to a mutation in

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the target protein of a compound, comparative genomics has helped to unravel the mechanism of action of these unknown antibiotics. As an example, the identification of single nucleotide polymorphism (SNP), has proved essential in uncovering the mechanism of antituberculosis action of bedaquiline [1], pyridomycin [2], and Q203 [3]. Comparative genomics can also be used for other approaches where relatively little sequence variation is expected such as comparing different mycobacterial genomes [4, 5] molecular epidemiology and transmission dynamics [6, 7], phylogenetics and phylogeography [8], and estimations of mutation rates [9].

While both comparative genomics and whole-genome assembly can be performed using NGS, the approach and technology needed for these two approaches is different. In broad terms, for comparative genomics, only relatively short sequence reads are needed (100–300 bases) which can then be mapped to an annotated reference genome and compared. For de novo genome assembly much longer sequence reads are needed (with an average read length over 5 kb), which are then assembled together to generate a whole-genome sequence. The longer read length is needed to span areas of low sequence diversity such as repeats. Longer sequence reads can also be useful for comparative genomics in cases where large differences are expected between a reference genome and the genome of interest (i.e., large insertions, deletions, genome duplications, or transposon activity) [10–13].

The choice of the sequencing platform will depend on the experimental setup and the goal of the project. Table 1 summarizes key characteristics of the most common NGS platforms used in bacterial genomics. In our experience, for SNP and InDel (insertions or deletions) discovery studies Illumina currently provides the cheapest technology, especially when multiplexing on the Illumina HiSeq system (more than one genome sequenced per lane). However, for a single genome, one whole lane of the Illumina HiSeq system would give a wastefully deep coverage, and an

Table 1
Next-Generation Sequencing platforms commonly used for sequencing bacterial genomes

Platform	Typical read length	Yield per run	Paired ends	Error rate (%)[a]
Illumina HiSeq 2000[b]	100 bp	40 Gb	Yes	~0.1
Illumina MiSeq	250 bp	10 Gb	Yes	~0.1
PacBio	AVG 6–8 kb	350–500 Mb	No	~13[c]
Ion Torrent PGM	200–400[d] bp	10–1,000[d] Mb	No	~1

[a]More details and references at http://www.molecularecologist.com/next-gen-fieldguide-2014/
[b]Single lane, suitable for most bacterial genomes (there are 8 lanes in each flow cell)
[c]Accuracy will significantly improve with sufficient coverage and downstream processing
[d]Depends on the Ion Torrent machine model

alternative would be the Illumina MiSeq, which is faster than running a Illumina HiSeq system (1 day compared to 1 week), and which can sequence a small number of mycobacterial genomes in a single run. Illumina MiSeq, is also capable of sequencing longer reads, making it more suitable for finding larger mutations or even de novo assembly. Ion Torrent can be useful for quick analysis of one or a small number of samples.

Long reads are crucial for de novo genome assembly. PacBio, with its latest hardware and chemistry improvements, is currently the best solution for sequencing new bacterial genomes since it provides sufficient coverage of kilobases-long reads in a single or few runs. PacBio raw reads contain a significant number of sequencing errors, and therefore, a high depth of coverage is needed (around 100×) to generate a highly accurate consensus and optimal assembly. For genome sizes similar to that of *Mycobacterium tuberculosis* (4.3 Mb) this can currently be achieved with two PacBio Cells.

Given here is a detailed description of the methodology needed to perform both comparative genomics by Illumina MiSeq or HiSeq sequencing, and de novo whole-genome assembly using PacBio sequencing. In both cases we use *M. tuberculosis* as an example bacterium, and describe genomic DNA extraction, library preparation, and the bioinformatics steps needed for both procedures.

2 Materials

2.1 Extraction of Genomic DNA from M. tuberculosis

1. *M. tuberculosis* (*see* **Note 1**).
2. Lysis solution (SET solution): 25 % sucrose, 50 mM EDTA, 50 mM Tris–HCl pH 8.
3. 20 mg/mL lysozyme.
4. 10 mg/mL RNAse A.
5. Proteinase K solution: 400 μg/mL Proteinase K, 100 mM Tris–HCl pH 8, 0.5 % w/v SDS.
6. Phenol–chloroform–isoamyl alcohol (25:24:1).
7. Chloroform–isoamyl alcohol (24:1).
8. 3 M sodium acetate pH 5.2.
9. Isopropanol.
10. 70 % ethanol.
11. Molecular biology grade water.
12. 1 % agarose gel.
13. Spectrophotometer (*see* **Note 2**).
14. Qubit (Life Technologies) (*see* **Note 3**).
15. Fragment Analyzer (Advanced Analytical).

2.2 Library Preparation for Illumina Sequencing

1. Molecular biology grade water.
2. LoBind tubes (Eppendorf).
3. Aerosol-resistant filter tips.
4. Illumina kit for library preparation (Illumina).
5. 2 % E-Gel SizeSelect Agarose Gels and iBase (Life Technologies).
6. Qiagen Gel extraction kit (or similar).
7. Agencourt AMPure beads (Beckman Coulter).
8. Magnetic Particle Concentrator (Life Technologies) or similar equipment.
9. Covaris S-series ultrasonicator (Covaris).
10. Tubes for Covaris ultrasonicator (AFA tubes—Covaris).
11. Qubit (Life Technologies) (*see* **Note 3**).
12. Fragment Analyzer (Advanced Analytical).

2.3 Library Preparation for PacBio Sequencing

1. Molecular biology grade water.
2. LoBind tubes (Eppendorf).
3. Aerosol-resistant filter tips.
4. Pacific Biosciences DNA Template Prep kit (Pacific Biosciences).
5. Pacific Biosciences DNA Polymerase Binding kit (Pacific Biosciences).
6. MagBead kit (Pacific Biosciences).
7. AMPure PB kit (Pacific Biosciences).
8. Magnetic Particle Concentrator (Life Technologies) or similar equipment.
9. Covaris S-series ultrasonicator (Covaris).
10. Tubes for Covaris ultrasonicator (AFA tubes—Covaris).
11. Qubit (Life Technologies) (*see* **Note 3**).
12. Fragment Analyzer (Advanced Analytical).

2.4 Data Analysis

1. Workstation with at least one multi-core 64 bit CPU, 8 GB of RAM and 200 GB disk space, running under a 64 bit Unix-like operating system (ideally a recent Ubuntu-based Linux distribution) (*see* **Note 4**).

3 Methods

3.1 Extraction of Genomic DNA from M. tuberculosis (See Note 5)

1. Grow 10 mL *M. tuberculosis* culture to an OD_{600} of 0.8–1.0.
2. Pellet the bacteria by centrifugation at 3,200 × *g* for 10 min, room temperature and discard the supernatant.

3. If needed at this step the bacterial pellet can be stored by freezing at −20 °C or lower—this helps to break open some of the bacteria.
4. Resuspend the bacterial pellet in 250 μL SET solution, and add 50 μL of lysozyme.
5. Incubate the mixture overnight at 37 °C.
6. Add 10 μL of RNase A and incubate at 37 °C for 30 min.
7. Add 250 μL of Proteinase K solution and incubate at 55 °C for 2 h.
8. Add an equal volume of phenol–chloroform–isoamyl alcohol to the sample, mix vigorously by hand, and let stand at room temperature for 5 min.
9. Centrifuge at 16,000 × *g* for 10 min and recover the top aqueous layer.
10. Add 0.1 volume of 3 M sodium acetate (pH 5.2), followed by 0.7 volume of isopropanol.
11. Invert the sample until well mixed.
12. Incubate at −20 °C for at least 1 h.
13. To pellet the precipitated gDNA, centrifuge the sample (16,000 × *g*, 4 °C for 30 min). Remove supernatant and wash the pellet (not always visible) once with 70 % ethanol. Centrifuge (16,000 × *g*, 4 °C for 30 min), discard the supernatant, and air-dry the pellet.
14. Resuspend DNA in molecular biology grade water and store it at 4 °C.
15. The integrity of the gDNA can be checked by agarose gel electrophoresis (*see* **Note 6**). Intact gDNA is large and therefore migrates very slowly forming a band at the top of the gel. Any bands/smears seen in the middle of the gel could be an indication of fragmented gDNA or the presence of RNA. For a protein/solvent contamination perform a second phenol–chloroform–isoamyl alcohol gDNA extraction. For RNA contamination, perform a second RNase treatment followed by a gDNA extraction.
16. Perform quality controls on the genomic DNA by running the DNA sample on a Fragment Analyzer or equivalent equipment.
17. Run fluorometric quantitation using for instance the Qubit instrument to get an accurate reading of DNA concentration.

3.2 Library Preparation for Illumina Sequencing (See Note 7)

1. Turn on the Covaris instrument and water bath and run for 30–60 min before use to allow temperature to descend to 5–6 °C.
2. Open the software and turn ON the DEGASSER.

3. Set parameters to fragment the gDNA to 300 bp according to instructions provided by the manufacturer (*see* **Note 8**).
4. Load the sample and start fragmentation.
5. Transfer 50 μL of DNA to LoBind Eppendorf tube and perform a Qubit quantification to determine the DNA concentration.
6. Confirm correct DNA shearing using a Fragment Analyzer.
7. Add 80 μL (1.6 volumes) of thoroughly mixed AMPure beads to 50 μL of sheared DNA sample.
8. Incubate at room temperature for 15 min.
9. Place the tube in a magnetic rack for 15 min to allow beads to stick to the side of the tube.
10. Remove supernatant completely.
11. Wash the beads twice with 400 μL of 80 % ethanol, followed by separation of the beads on the magnetic rack.
12. Remove the tube from the magnetic rack and dry the beads at 37 °C for 3 min.
13. Add 52.5 μL of Resuspension Buffer, incubate at room temperature for 2 min and place the tube in the magnetic rack for 5 min.
14. Transfer 50 μL of the supernatant containing the DNA into new nuclease-free tube.
15. Add 50 μL of the fragmented DNA to 10 μL of thawed Resuspension Buffer (RSB) and 40 μL of End Repair mix (ERP) (*see* **Note 9**).
16. Mix the sample well and incubate at 30 °C for 30 min.
17. Purify DNA using AMPure beads with ratio of 1:1.6, e.g., 100 μL of sample should be mixed with 160 μL of bead (*see* Subheading 3.2, **steps 10–15**).
18. Mix 2.5 μL of purified blunt ended DNA with 12.5 μL of A-Tailing Mix (ATL) (thawed on ice) (*see* **Note 10**).
19. Mix thoroughly and incubate in a thermal cycler at 37 °C for 30 min, then 70 °C for 5 min and 4 °C hold.
20. Ligate adaptors to the DNA by adding 2.5 μL of adenylated DNA with 2.5 μL of chosen adaptor and mixing thoroughly. Add 2.5 μL of Ligation Mix (LIG) and incubate at 30 °C for 10 min. Stop reaction using 5 μL of Stop Ligation Buffer (STL) (*see* **Note 11**).
21. Purify DNA using AMPure beads with ratio of 1:1.6 as in Subheading 3.2, **steps 10–15**.
22. Load 20 μL of sample on a 2 % Egel (*see* **Note 12**).
23. Run the sample on an iBase for 16 min.

24. Excise the gel slice containing the desired fragment range (*see* **Note 13**).
25. Purify the gDNA fragments from the gel using the Qiagen Gel Extraction kit (or similar) and elute the final gDNA sample in 20 μL.
26. Perform a 18 cycle PCR using specific primers that recognize the adaptors as follows: add 20 μL of the cDNA template, 5 μL of PCR Primer Cocktail (PPC), and 25 μL of PCR Master Mix (PMM) and cycle 15 times (98 °C for 10 s/60 °C for 30 s/72 °C for 30 s), with a final 5 min elongation at 72 °C and hold at 4 °C.
27. Purify DNA sample using AMPure beads with ratio of 1:1.6 as in Subheading 3.2, **steps 10–15**.
28. Verify DNA concentration by Qubit or fragment analyzer.
29. Subject the prepared sample library to the sequencing facility for Illumina Sequencing.

3.3 Library Preparation for PacBio Sequencing (See Note 14)

1. Perform quality controls on the genomic DNA by running the DNA sample on a Fragment Analyzer or equivalent equipment (*see* **Note 15**).
2. Turn on the Covaris instrument and water bath and run for 30–60 min before use to allow temperature to descend to 5–6 °C (*see* **Note 16**).
3. Open the software and turn ON the DEGASSER.
4. Set parameters to fragment the gDNA to 2 kb according to instructions provided by the manufacturer (*see* **Note 17**).
5. Load the sample and start fragmentation.
6. Transfer DNA to LoBind Eppendorf tube and perform a Qubit quantification to determine the DNA concentration.
7. Confirm correct DNA shearing using a Fragment Analyzer.
8. To repair ends of sheared DNA, mix into a total of 30 μL, 500 ng of fragmented gDNA, with 3 μL of Template Preparation Buffer, 3 μL of ATP Hi, 1.2 μL of 10 mM dNTP mix, and 1.5 μL of End repair mix. Incubate sample at 25 °C for 15 min.
9. Add 18 μL (0.6× volume) of thoroughly mixed AMPure beads to 30 μL of end-repaired sheared gDNA sample.
10. Incubate sample at room temperature for 15 min, then place the tube in a magnetic rack for 15 min to allow beads to stick to the side of the tube.
11. Remove supernatant completely and wash the beads twice with 400 μL of 80 % ethanol, followed by separation of the beads on the magnetic rack.

12. Remove the tube from the magnetic rack and dry the beads at 37 °C for 3 min.
13. Add 32.5 μL of Elution Buffer, incubate at room temperature for 2 min and place the tube in the magnetic rack for 5 min.
14. Transfer 30 μL of the supernatant containing the DNA into new nuclease-free tube, and verify sample concentration and purity by Qubit and Nanodrop respectively.
15. Ligate hair pin adaptors to the blunt ended gDNA fragments by mixing 1 μL of 20 μM blunt adaptor to the 30 μL of end repaired fragmented gDNA. Mix in 4 μL of Template Preparation Buffer and 2 μL of ATP Lo, followed by 1 μL of Ligase.
16. Make up the reaction volume to 40 μL with water and incubate overnight at 16 °C.
17. Terminate the ligation reaction by heating the sample at 65 °C for 10 min.
18. Purify DNA using AMPure beads two consecutive times with ratio of 1:0.6 (i.e., 24 μL of AMPure beads) as in Subheading 3.3, **steps 11–15**. Elute final sample in 10 μL elution buffer.
19. Verify DNA concentration by Qubit or fragment analyzer.
20. Subject the prepared sample library to the sequencing facility for PacBio Sequencing.

3.4 Data Analysis 1: Finding Mutations in M. tuberculosis Strains (See Note 18)

This guide assumes that the reader has basic working knowledge of Unix systems, knows the basic principles of sequencing (and sequence assembly) and what assemblers do. Note that all commands given below should be written in a single line. Consecutive commands will be separated by empty lines for clarity.

1. Download MIRA (*see* **Note 19**).
2. Prepare the reference genome sequence (*see* **Note 20**).
3. Prepare the Illumina data (*see* **Note 21**).
4. Prepare the manifest file.

   A manifest file is a configuration file for MIRA which tells it what type of assembly it should do and which data it should load. It is a simple text file and it can be prepared in any text editor. Below is an example of a manifest file for Illumina single end reads (*see* **Note 22**) and three different strains (comments, which can be included in the file, are preceded by the hash character "#"):

   # START MANIFEST

   project = MyProject (*see* **Note 23**)

job = genome,mapping,accurate

define the reference sequence:
readgroup
is_reference
data = /path/to/the/reference/NC_000962.gbk
strain_name = H37Rv
# now the Illumina data for each strain:
readgroup = IlluminaSE_wt (*see* **Note 23**)
data = /path/to/wt.fastq.gz (*see* **Note 24**)
technology = solexa
strain_name = WT (*see* **Note 23**)
rename_prefix = [*reads' prefix*] WT (*see* **Note 25**)
readgroup = IlluminaSE_ mutantA
data = /path/to/mutantA.fastq.gz
technology = solexa
strain_name = mutantA
rename_prefix = [*reads' prefix*] mutantA (*see* **Note 25**)
readgroup = IlluminaSE_ mutantB
data = /path/to/mutantB.fastq.gz
technology = solexa
strain_name = mutantB
rename_prefix = [*reads' prefix*] mutantB (*see* **Note 25**)
# An additional setting to turn off the merging of Solexa reads (*see* **Note 26**):
parameters = SOLEXA_SETTINGS -CO:msr = no
# END MANIFEST

5. Execute MIRA.

   /path/to/MIRA/bin/mira manifest.conf >& log_assembly.txt

6. Obtain the table of SNPs and short InDels with *miraconvert*, a program that is bundled with MIRA (*see* **Note 27**):

   /path/to/MIRA/bin/miraconvert -t asnp *****.maf output

7. Load the *output_info_featureanalysis.txt* in a spreadsheet program.
8. Filter the list of SNPs and short InDels (*see* **Note 28**).
9. Visualize the alignments in GAP5 (*see* **Note 29**):

   tg_index -C *****.caf

   gap5 *****.g5d

3.5 Data Analysis 2: De Novo Assembly Using PacBio Reads

The recentness of the PacBio platform and the abundance of the unusual read errors (mostly InDels) make most of the current NGS assemblers inadequate for the assembly of raw PacBio reads. The easiest way to process and analyze PacBio raw data is to use the SMRT Analysis, a PacBio's open source software (*see* **Note 30**).

1. In the SMRT Portal go to *DESIGN JOB→ Import and Manage→ Import SMRT Cells* and add the locations of the Cell data (*see* **Note 31**).
2. The new SMRT Cells will be available in *DESIGN JOB→ Create New*. From there choose a name for the project and an assembly protocol (refer to the *De novo assembly* tutorials available at http://www.pacb.com/devnet/ for details), save and start the job.

4 Notes

1. Manipulation of *Mycobacterium tuberculosis* cultures must be performed under Biosafety Level 3 (BSL3) containment. Adherence to local guidelines for BSL3 work is strictly required.
2. A standard spectrophotometer, or alternative like the Nanodrop suitable.
3. Alternative methods for DNA quantification can be used, such as the Quantus Fluorometer (Promega) or the Picogreen assay [14].
4. Alternatively one can run many bioinformatics programs on external servers, like Galaxy, which is a widely used and feely available platform (http://galaxyproject.org/). For the SMRT Portal, the public SMRT Analysis Amazon Machine Image is available (with hourly rates, http://www.pacb.com/devnet/).
5. The extraction procedure of genomic DNA from bacteria is identical for both Illumina and PacBio sequencing. In both cases good quality genomic DNA (minimal contamination of proteins, RNA, or solvents) is required for high-throughput sequencing (HTS) procedures. In our experience the protocol described by Pelicic and colleagues [15] yields a few micrograms of pure, intact genomic DNA suitable for the subsequent library preparation protocols using either the Illumina or the PacBio approaches.
6. At this point it is very important to check the concentration and quality of the gDNA sample. DNA concentration can be measured spectrophotometrically, or more accurately by using a Qubit instrument (Life Technologies). A spectrophotometer can also be used to determine protein contamination (for clean gDNA, 260/280 should be greater than 1.7), and

organic solvent (often phenol) contamination (for clean gDNA, 260/230 should be greater than 2.0).

7. Now that the whole intact gDNA has been extracted, it needs to be prepared into a library of templates that can be read by the appropriate NGS instrument. The general steps for library preparation are, confirming DNA quality, shearing the intact gDNA into small fragments (for Illumina typically around 300 bp), repairing the gDNA fragments, adding adaptors to the fragments and sequencing. Sequencing can be done in single-end (DNA fragments are sequenced from one side) or paired-end mode (DNA fragments are sequenced from both sides, generating two reads per fragment). For the analysis of SNPs and short InDels either mode can be equally used. For the analysis of structural variations or de novo genome assembly of short reads (not described here), PEs are more advantageous. The procedure here described is that optimized for sequencing by Illumina HiSeq and MiSeq.
8. For the fragmentation of intact gDNA to fragments of 300 bp, mechanical shearing by ultrasonication is suggested. In our experience, the Covaris S-Series provides excellent results with high reproducibility, though other similar instruments are available. Here we describe the procedure for fragmentation using the Covaris S-Series.
9. Specific kits and protocols are then available for the library preparation and the most commonly used kits are provided by Illumina (http://www.illumina.com). Here we provide a protocol for library preparation according to the Illumina TruSeq procedure, specifying the main steps. Approximately 1 μg of pure genomic DNA is required for the library preparation according to the Illumina protocols at the time this protocol was written. It is likely that the amount of material required for HTS will decrease as the technology develops and new protocols are optimized. The shearing of gDNA leads to DNA fragments with 3′ and 5′ overhangs; however, in order to add adaptors for library preparation (next step) it is essential that all DNA ends are blunt. Therefore, to blunt-end the gDNA fragments they are "repaired" using a mixture containing a polymerase and exonuclease that fill and remove 5′ and 3′ overhangs respectively.
10. To generate a 3′ overhang on the blunt ended double stranded cDNA (needed for ligation of adaptor in the next step), the 3′ ends need to be adenylated.
11. Adaptors act as "barcodes" that can be used to identify the origin of the cDNA, and therefore, different adaptors can be used for different biological samples when sequencing them in a single lane by illumina (multiplexing). Adaptors are also needed for the next step of enrichment.

12. While DNA fragmentation by Covaris was set to generate gDNA fragments of 300 bp, this will not have been absolute. Therefore, to isolate only those fragments with a desired size for sequencing, the sample can be separated and purified by gel electrophoresis. In our experience good results are obtained using 2 % E-Gel SizeSelect Agarose Gels and an iBase.
13. Fragment sizes of 100–300 bp are recommended for sequencing on the Illumina HiSeq. For Hiseq paired-end sequencing sizes around 300–400 are better, to avoid overlapping pairs. For the Illumina MiSeq fragment size of around 500 bp is recommended, especially if sequencing paired-ends.
14. We recommend close interaction with the sequencing facility where the DNA will be subjected to PacBio sequencing for any questions or doubts about the library preparation procedure.
15. Library preparation for PacBio HTS requires the same preliminary quality control on genomic DNA as for the Illumina library preparation (i.e., Fragment Analyzer and quantitation by fluorimetry). Contrary to the Illumina procedure, the PacBio library preparation does not include a PCR amplification step. The quality of the input DNA will therefore directly influence the final results. Contaminants such as RNA, proteins or organic reagents used for DNA purification can affect the overall performance of the system. Rigorous quality controls are indeed highly recommended.
16. PacBio supplies the kits and reagents required for library preparation (http://www.pacificbiosciences.com), which starts with shearing the DNA by using the Covaris instrument or similar equipment. Approximately 20 % of the input material is lost as a result of shearing and concentration processes, and therefore, a few micrograms of pure genomic DNA is needed to start with (ideally 5–10 μg). After DNA fragmentation, the library preparation protocol continues with end repair, ligation of the adapters, annealing of the sequencing primer and finally binding of the polymerase to the template to be sequenced. Quality control of the library is carried out using the Fragment Analyzer and the Qubit fluorometer.
17. 2 kb is the fragment size of choice when this chapter was written. It is likely to increase at PacBio technology improves.
18. A common goal in *M. tuberculosis* research is to find mutations that give rise to a specific phenotype (e.g., resistance to a compound). For this we need to sequence both the wild type strain and the strains of interest. Sequencing the wild type is crucial for distinguishing SNPs derived from the natural strain variability from those responsible for a drug resistance phenotype. In essence, the analysis consists of mapping the reads to a

reference sequence, searching for SNPs and selecting the SNPs of interest. While each of these steps can be done with one or more individual programs, we suggest using MIRA [16] because it handles the first two steps automatically and provides results for the final step. Alternatively it is possible to use different programs to perform the various steps of the analysis. For an overview of the methods please refer to the literature [17–19].

19. MIRA is an open source program and it can be downloaded at http://sourceforge.net/projects/mira-assembler. Precompiled binaries are available for Linux and Mac OSX. An extensive manual of MIRA is available here: http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html. MIRA is being actively developed and frequently updated. We recommend always using the latest version, including the developmental versions.
20. MIRA can take the reference sequence in various formats (FASTA, GenBank, GFF, etc.) and we recommend the GenBank or the GFF3 format because these contain the annotations and can be downloaded from NCBI.
21. Sequence files are the FASTQ files that come from the sequencing provider (usually gunzipped and often split in smaller individual files). There is no need to preprocess them since MIRA will automatically look for adapters present in the sequence and take into account the base qualities during the mapping. Some consideration should be taken at this step regarding the theoretical coverage of the genome. With the small genome size of *M. tuberculosis* and the Illumina's high throughputs, it is often the case that the samples are over-sequenced, with average coverage of several hundreds or thousands. In such cases it is recommended to down-sample the reads to a theoretical coverage of around 100× or less to avoid long computational times and introduction of non-random sequencing errors.
22. For paired ends the library has to be defined accordingly in the manifest file. The easiest way to do it is to add the *autopairing* option so that the entry for each readgroup looks like this:

    readgroup = IlluminaPE_wt

    autopairing

    data = /path/to/your/data/wt.fastq1.gz

    data = /path/to/your/data/wt.fastq2.gz

    technology = solexa

    strain = WT
23. Any name can be given. To avoid any possible downstream problems, do not use spaces and special characters.

24. FASTQ files obtained from the Illumina sequencer are by default gunzipped. There is no need to uncompress these files since MIRA can read them. Also, Illumina HiSeq reads usually come in a number of individual FASTQ files for each sample to avoid problems of handling large files. For multiple FASTQ files per sample refer to the MIRA manual for different ways to enter them in the manifest. For example, if the FASTQ files are present in separate folders for each sample they can be defined in one line using a wildcard character like this:

    data = /path/to/your/data/**.fastq.gz

    This will also work for paired ends; you do not need to separately define groups of files for each pair.

25. If more samples were multiplexed in the same run it is recommended to rename the reads in a way that these reflect the different strains being sequenced. This will help during the manual check-up and visualization of the alignments later on since reads deriving from each sample would be quickly distinguished. The current read naming scheme for Illumina is the following:

    @<instrument>:<run number>:<flowcell ID>:<lane>:<unique read number> <additional info>

    Example of a read in a FASTQ file:

    @HISEQ:61:C3P27ACXX:7:1105:1000:12850 1:N:0:CTTGTA

    ATGCATGCATGCATGCATGCATGCATGCATGCATGC

    +

    BBBBCCCC?<A?BC?7@@???????DBBA@@@@A@@

    In this case the "HISEQ:61:C3P27ACXX:7" will be the same for all the reads from this run, and it can be replaced with something else. The option *rename_prefix* will instruct MIRA to rename the reads on the fly while loading the data. For example:

    rename_prefix = HISEQ:61:C3P27ACXX:7 mutantA

26. Please refer to the manual for details about this option. In our experience, having this option turned on when using multiple strains might produce some unwanted SNP reports. If the reader needs to use this option, she/he should first test it on a smaller dataset.

27. Upon successful finishing, MIRA will create four directories populated with files. The "chkpt" and "tmp" directories can be safely removed to save disk space (they are useful for continuing prematurely terminated runs or for troubleshooting). The "info" directory contains some statistics about the run, more useful for de novo assemblies. The "results" directory contains the CAF and MAF alignment files and the FASTA files with the consensus sequence for each strain. The *miraconvert* command should be executed in the "results" directory.

28. Look for positions where a SNP was found only in the mutants, but not in the wild type, or vice versa, the SNP is present only in the wild type but not in the mutants. Such positions indicate a strong association with the phenotype observed in the mutants. To quickly find out such positions you can use a *countif* formula in Excel, using the 4th or the 5th columns as range (SNP/InDel positions). For example, in the second raw of the first empty column to the right of the table (AA:2) type:

    =COUNTIF(D:D;D2)

    Press *Enter* and double click on the bottom right corner of the cell to apply the formula to the whole column.

    Numbers will appear in the column AA, representing the number of occurrences of each SNP/InDel position. Filter for the number of interest (number of mutants or 1) and on the C column filter for the names of samples accordingly. For example if you processed three mutants and one wild type, filter for the cells containing '3' in the column AA and filter for all the mutant names in the column C. To check for cases where only the wild type has a mutation where the mutants are the same as the reference used for mapping, select only '1's in column AA and only the wild type name in column C.

    Of interest are also mutations present in genes that are found in all mutants but at different positions. Other cases can also be of interest and the tables obtained from MIRA provide useful information for each mutation: location, nucleotide change, gene information, amino acid change, etc.

29. It is good practice to manually check any interesting or unclear positions by looking directly into the alignments. Various NGS alignments visualization tools are available [17], among which GAP5 is perfectly compatible with MIRA's default outputs. GAP5 is part of the Staden package which can be downloaded here http://sourceforge.net/projects/staden/. Upon opening the alignments, right-click on the horizontal line in the "Contig Selector" window and choose "Edit contig". The alignment browser window will open. In order to visualize the various tags set by MIRA refer to the *support/README* file of the MIRA distribution.

30. SMRT Analysis is available at http://www.pacb.com/devnet/. The SMRT Analysis software, including its graphical interface SMRT Portal, is designed for installation on larger computer clusters, but it can also be installed on single node machines (workstations) suitable for the analysis of bacterial-size genomes (instructions can be found at http://www.pacb.com/devnet/). As an alternative to the manual installation which can be difficult for beginners, a virtual machine with a preinstalled SMRT Portal is available at https://github.com/PacificBiosciences/Bioinformatics-Training/

wiki/SMRT-Analysis-Virtual-Machine-Install, including the instructions for getting the virtual machine running using Vagrant and VirtualBox in few simple steps.

31. The file structure needed to import SMRT Cells in the SMRT Portal is a top-level directory that contains the metadata.xml file (make sure to obtain this file from the sequencing provider), and an *Analysis_Results* directory that contains one *bas.h5* file, and three *bax.h5* files.

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Chapter 2

Whole-Transcriptome Sequencing for High-Resolution Transcriptomic Analysis in *Mycobacterium tuberculosis*

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Abstract

RNA-seq uses next-generation sequencing technology to determine the transcription profile of an organism in a quantitative manner. With respect to microarrays, this methodology allows greater resolution, increased dynamic range, and identification of new features such as previously unannotated genes and noncoding RNAs. Here we describe how to extract RNA from mycobacterial cultures, how to prepare libraries for Illumina sequencing, and the bioinformatics analysis of the sequencing data to determine the transcription profile.

Key words Illumina, RNA-seq, Transcriptome, RNA extraction, Library preparation

1 Introduction

With the advent of next-generation sequencing (NGS) technology, RNA-seq (RNA sequencing) is now becoming a well-established approach for transcript quantification and gene expression studies. The advantage of RNA-seq compared to transcriptome analysis using microarrays is in lower background noise (reads can be unambiguously mapped to unique regions of the genome), a higher dynamic range of expression levels and the potential for discovering novel genes and other transcribed features. A broad overview and practical guidance for the RNA-seq work flow is given in ref. [1].

For the successful use of RNA-seq (as with microarrays) a basic requirement is that there exists a reliable reference genome sequence onto which reads can be mapped (Cross-reference to WGS chapter). In principle, the amount of transcription is then determined by sequencing the RNA from the test sample(s), mapping them onto the reference genome, and subsequently quantifying the number of times any particular feature (base/gene) is covered by the sequence reads from NGS technology.

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Some aspects of RNA-seq experiments deserve more attention. In particular, the number of biological replicates is crucial for obtaining meaningful results [2]. With three or more biological replicates, the power to infer differentially expressed genes in pairwise comparisons greatly increases, while the difference in results among different statistical programs that can be used for the analysis becomes less prominent [3, 4]. The decision on the number of replicates is often affected by the sequencing costs and the available budget, but it should be pointed out that experiments without replicates are virtually the most expensive ones in respect to the amount of useful information gained over money invested. Importantly, to improve the detection power of RNA-seq experiments, it has been shown that increasing the number of biological replicate samples is significantly more beneficial than increased sequencing depth [3, 5]. Furthermore, the decrease of sequencing depth and the loss of genes covered by at least one fragment is not linear, i.e., a significant reduction in sequencing depth will be detrimental to only a small number of lowly expressed genes. To allow for the detection of numerous biological samples at the same time multiplexed sequencing is worth consideration.

To plan the experiment better, it is important to know the level of sequencing depth per sample needed to meet the requirements of the project's objectives. Haas and collaborators (2012) showed that a sequencing depth of 5-10 million non-ribosomal RNA (rRNA) fragments enables profiling the vast majority of transcriptional activity in diverse bacterial species grown under diverse culture conditions [5]. This brings us to the question concerning rRNA depletion. Commonly, more than 95 % of the total RNA-seq reads are rRNA, and therefore depletion or rRNA from the total RNA will allow for more coverage of the RNA of interest. This means that following rRNA depletion; several samples can be multiplexed on a single HiSeq lane (numerous samples per lane, decreasing sequencing cost), while sequencing total RNA requires a full single Illumina HiSeq lane (one sample per lane). The disadvantages of rRNA depletion relate to longer sample preparation time and the possible introduction of bias in the RNA population. However, in the case of a large number of samples, rRNA depletion will greatly reduce the sequencing costs, in which case it might be worth first testing and comparing the various commercially available kits on a subset of samples.

An additional consideration to be made is whether a single-end (SE) library or paired-end (PE) library should be used, as well as the sequencing length to be used. For SE-libraries, each RNA fragment is sequenced from one side, while in PE-libraries each RNA fragment is sequenced from both sides, effectively doubling the number of reads per RNA. For a comparison of gene transcription between two samples, a SE-library of any size is sufficient (default size for HiSeq is currently 100 bases), as PE-libraries do not

improve sensitivity, but increase the number of reads. In addition, sequencing of PE-libraries is more expensive than sequencing SE-libraries. Finally, strand-specific libraries are recommended for RNA-seq of mycobacteria because of high gene density and the presence of overlapping genes on opposite strands. Strand-specific reads can be assigned to their corresponding genes more accurately and can reveal potential antisense transcripts.

Here, we describe a detailed methodology for a comparative transcriptome study of mycobacteria using RNA-seq. Procedures described cover the extraction of total bacterial RNA, the preparation of strand-specific single-end library for Illumina sequencing, and detailed instruction on the basic bioinformatics methods used to map the reads to a reference genome and to count the number of reads per feature. Finally, we provide details on how to infer differentially expressed genes.

2 Materials

2.1 Extraction of RNA from M. tuberculosis (See Note 1)

1. *M. tuberculosis* (*see* **Note 2**).
2. RNA-free tubes, plasticware and glassware, and DEPC-treated water.
3. TRIzol (Life Technologies).
4. Bead beater (Biospec Products) or equivalent instrument.
5. 0.1 mm Zirconia beads (Biospec Products).
6. 1.5 mL MaXtract High Density Tubes (Qiagen).
7. Chloroform.
8. DEPC-treated 3 M sodium acetate pH 5.2.
9. Isopropanol.
10. 70 % ethanol.
11. DNase.
12. PCR or quantitative PCR reagents.
13. Agarose gel.
14. Spectrophotometer or NanoDrop.
15. Qubit (Life Technologies) (*see* **Note 3**).
16. Fragment Analyzer (Advanced Analytical).

2.2 Library Preparation for Illumina Sequencing

1. Molecular biology grade water.
2. LoBind tubes (Eppendorf).
3. Illumina TruSeq Stranded mRNA Kit for library preparation (Illumina).
4. Agencourt AMPure beads (Beckman Coulter).

5. Magnetic Particle Concentrator (Life Technologies).
6. Qubit (Life Technologies) (*see* **Note 3**).
7. Fragment Analyzer (Advanced Analytical).

2.3 Data Analysis

1. PC with at least 4 GB of RAM running under a 64-bit Unix-like operating system (*see* **Note 4**).

3 Methods

3.1 Extraction of RNA from M. tuberculosis

Good quality RNA is required for successfully performing transcriptomic analysis by RNA-seq (*see* **Note 5**). It is important to not allow RNA to be broken down, as this will impact the final expression profile. Various methods can be used for RNA preparation from *M. tuberculosis* cultures, including commercially available kits which involve column purification (*see* **Note 6**). Here we provide a protocol for RNA purification based on TRIzol reagent (*see* **Note 7**).

1. Grow the *M. tuberculosis* strain of interest to an OD_{600} of 0.3–0.4.
2. Pellet 40 mL of culture by centrifugation at 3,200 × *g* for 10 min and discard the supernatant.
3. Snap-freeze the pellet in liquid nitrogen—at this point pellets can be stored at −80 °C.
4. Remove the bacterial pellet from the liquid nitrogen (or −80 °C freezer) and immediately resuspend it in 1 mL of TRIzol.
5. Transfer the bacterial suspension to a 2 mL screw-cap tubes containing 0.5 mL zirconia beads.
6. Place the 2 mL screw-cap tubes with sample into a bead-beater and bead-beat twice for 1 min with a 2-min interval on ice.
7. Incubate the sample at room temperature for 5 min, inverting periodically.
8. Centrifuge the sample for 30 s at 10,000 × *g* and recover the top TRIzol layer.
9. Prepare a MaxTract tube by centrifugation for 30 s at 2,000 × *g*.
10. Add the TRIzol layer to the gel in the MaxTract tube.
11. Add 200 µL of chloroform and shake vigorously (do not vortex) for 15 s.
12. Stand at room temperature for 10 min.
13. Centrifuge the MaxTract tube at 12,000 × *g* for 10 min at room temperature.
14. Carefully collect the top aqueous phase into a new tube.

15. Add 0.1 volume of 3 M sodium acetate pH 5.2 and 0.7 volumes of isopropanol.
16. Invert the tube several times to mix and then store at −20 °C for at least 2 h to allow the nucleic acid to precipitate (both RNA and DNA will precipitate).
17. Centrifuge the sample for 30 min at 16,000 × *g* at 4 °C and remove the supernatant (pellet sometimes visible at the bottom of the tube).
18. Wash the nucleic acid pellet twice with 200 µL of ice cold 70 % ethanol (centrifuge, each time for 30 min at 16,000 × *g* and 4 °C).
19. Dry the pellet under vacuum, or by leaving the tube open in a clean place (*see* **Note 8**).
20. Resuspend the pellet in 96 µL of DEPC-water and add 12 µL of 10× DNase buffer and 12 µL of 1 U/µL DNase.
21. Incubate the sample for 1 h at 37 °C.
22. Perform a phenol–chloroform extraction by adding an equal volume of phenol–chloroform–isoamyl alcohol to the sample, mix vigorously by hand, and let stand at room temperature for 5 min.
23. Centrifuge at 16,000 × *g* at 4 °C for 10 min and recover the top aqueous layer.
24. Add 0.1 volume of 3 M sodium acetate (pH 5.2), followed by 0.7 volume of isopropanol.
25. Invert the sample until well mixed.
26. Incubate at −20 °C for at least 1 h.
27. To pellet the precipitated gDNA, centrifuge the sample (16,000×*g*, 4 °C for 30 min). Remove supernatant and wash the pellet (not always visible) once with 70 % ethanol. Centrifuge (16,000 × *g*, 4 °C for 30 min), discard the supernatant, and air-dry the pellet.
28. Resuspend DNA in molecular biology grade water and store it at 4 °C.
29. Perform a PCR on a housekeeping gene, for example *sigA*, to confirm that no DNA is present (no amplification product). If there is still residual DNA present (this is quite common), perform a second DNase treatment.
30. Resuspend the final RNA pellet in DEPC treated water and store at −80 °C.
31. Determine the RNA concentration and purity using a spectrophotometer, NanoDrop or Qubit.
32. Check the RNA integrity on a 1 % agarose gel—the 23S, 16S rRNA should be clearly visible (a streak of RNA rather than clear bands would suggest RNA breakdown), or if a fragment analyzer is available, use this to check RNA quality.

3.2 Library Preparation for Illumina High-Throughput Sequencing Conditions (See Note 9)

1. Fragment the RNA by mixing 1 μL of RNA (100 ng/μL), with 19.5 μL Fragment, Prime, Finish Mix (FPF buffer) (*see* **Note 10**).
2. Vortex and incubate at 94 °C for 8 min.
3. Centrifuge briefly.
4. Carry out first-strand synthesis by adding 8 μL of thawed ice-cold First Strand Mastermix (FSM) to the fragmented RNA sample. Mix well and centrifuge to collect the sample at the bottom of the tube. Incubate in a PCR machine with the following program: 10 min at 25 °C/15 min at 42 °C/15 min at 70 °C/4 °C hold.
5. Carry out second-strand synthesis to form blunt-ended double-stranded cDNA. Bring the Agencourt AMPure XP magnetic beads, Resuspension Buffer (RSB buffer), and the Second strand Mastermix (SSM) to room temperature. Place the single-stranded cDNA from the first-strand synthesis into a heat thermal cycler set to 16 °C, and add 5 μL RSB, and 20 μL SSM. Mix the reaction mixture well and incubate at 16 °C for 1 h.
6. Purify double-stranded cDNA using the Agencourt AMPure XP magnetic beads as follows: mix 90 μL of Agencourt AMPure XP magnetic beads (vortex beads prior to pipetting to properly resuspend them), and 50 μL of the double-stranded cDNA mix. Mix thoroughly and incubate for 15 min at room temperature. Load the tube onto a magnetic rack for 5 min to allow the beads to separate from the solution. Remove the supernatant and wash the beads twice with 200 μL of 80 % ethanol (using the magnetic rack to separate the beads from the 80 % ethanol). For the final wash, remove all residual supernatant with a pipette and let the beads dry for 3 min at 37 °C.
7. Add 17.5 μL Resuspension Buffer (RSB) to the dried beads, mix well, incubate for 2 min at room temperature, and apply to magnetic rack.
8. Transfer cDNA containing supernatant (15 μL) to a fresh 0.2 mL tube.
9. Mix 2.5 μL of purified cDNA with 12.5 μL of A-Tailing Mix (ATL) (thawed on ice) (*see* **Note 11**).
10. Mix thoroughly and incubate in a thermal cycler at 37 °C for 30 min, then 70 °C for 5 min and 4 °C hold.
11. Ligate adaptors to the cDNA by adding 2.5 μL of adenylated with 2.5 μL of chosen adaptor and mixed thoroughly. Add 2.5 μL of Ligation Mix (LIG) and incubate at 30 °C for 10 min. Stop reaction using 5 μL of Stop Ligation Buffer (STL) (*see* **Note 12**).

12. Purify cDNA with ligated adaptors using Agencourt AMPure XP magnetic beads. Add 42 µL of Agencourt AMPure XP magnetic beads and incubate for 15 min at room temperature.
13. Load the sample onto a magnetic rack for 5 min, and discard supernatant.
14. Wash the beads twice with 200 µL 80 % ethanol, and let the beads dry for 3 min at 37 °C.
15. To elute the cDNA resuspend the beads in 52.5 µL RSB buffer, incubate for 2 min at room temperature, place in magnetic rack, and recover the 50 µL of the supernatant that contains the cDNA.
16. Perform a second purification by adding 50 µL of Agencourt AMPure XP magnetic beads to the 50 µL cDNA.
17. Resuspend the dried beads in 22.5 µL of RSB buffer and recover 20 µL of the supernatant containing the cDNA.
18. Perform a 15 cycle PCR using specific primers that recognize the adaptors as follows: add 20 µL of the cDNA template, 5 µL of PCR Primer Cocktail (PPC), and 25 µL of PCR Master Mix (PMM), and cycled 15 times (98 °C for 10 s/60 °C for 30 s/72 °C for 30 s), with a final 5 min elongation at 72 °C and hold at 4 °C.
19. Purify the cDNA from the final PCR reaction with 50 µL Agencourt AMPure XP magnetic beads (as in **steps 12–15**), with two washes with 80 % ethanol.
20. Elute the purified DNA in 32.5 µL of RSB buffer.
21. Validate the library fragment size, purity, and concentration by Fragment Analyzer.
22. Submit the library to the sequencing facility.

3.3 Data Analysis

The data analysis workflow consists of mapping the reads against the reference genome, counting the number of reads that mapped to each gene and calculating the relative gene-to-gene expression levels between samples. This guide assumes that the reader has basic working knowledge of Unix systems and knows the basic principles of sequencing. We will describe a data analysis work flow for an *M. tuberculosis* RNA-seq experiment that includes biological replicates for two conditions (*see* **Note 13**). Note that the commands given below should be written in a single line for each step.

1. Download and install *Bowtie2* [6] (http://bowtie-bio.sourceforge.net/bowtie2) (*see* **Note 14**).
2. Download the *M. tuberculosis* H37Rv reference genome from NCBI (NC_000962.3) in FASTA format (*see* **Note 15**).
3. Build a Bowtie indexed reference:

```
bowtie2-build NC_000962.3.fasta H37Rv
```

4. Map the Illumina reads, for each sample separately. Example for sample "A":

   bowtie2 -x /path/to/bowtie2_index/H37Rv -U /path/to/A_1.fastq.gz,/path/to/A_2.fastq.gz,/path/to/A_3.fastq.gz -S A_mapped-to-H37Rv.sam (*see* **Notes 16** and **17**).

Convert SAM files to coordinate-sorted BAM files as follows:

5. Download and install *samtools* [7].
6. Convert SAM to a sorted BAM:
   samtools view -Su A_mapped-to-H37Rv.sam | samtools sort - A_mapped-to-H37Rv_sorted (*see* **Note 18**).
7. Index the BAM file (*see* **Note 19**):

   samtools index A_mapped-to-H37Rv_sorted.bam

Counting the reads over genes as follows:

8. Download and install *featureCounts* [8] (*see* **Note 20**).
9. Download the gff3 file for the corresponding reference from NCBI. For H37Rv it is NC_000962.3.gff.
10. Convert the GFF file to SAF. Simplified annotation format (SAF) is a tab delimited file that contains five columns: feature identifier, reference name, start position, end position, and strand. SAF can be generated from a GFF file in a spreadsheet program (*see* **Note 21**). An example is shown below:

GeneID	Chr	Start	End	Strand
Rv0001	gi|448814763|ref|NC_000962.3|	1	1524	+
Rv0002	gi|448814763|ref|NC_000962.3|	2052	3260	+

11. Count the reads:
    featureCounts -b -F SAF -O -a /path/to/NC_000962.3.gff -o outpuname *.bam (*see* **Note 22**). At this step we have the raw expression levels for each gene (number of reads per gene). To look for differentially expressed genes, a statistical method must be applied that accounts for differences in the sequencing depths between samples, considers the variations of the expression levels among biological replicates and compares the expression levels of each gene between the two groups of samples.
12. Install *R* and the *DESeq* package (*see* **Note 23**).
13. Prepare the count table. The table generated with *featureCounts* should be edited to look like the tab delimited table below:

gene_id	A	B	C	D	E	F
Rv0001	123	111	222	321	456	789
Rv0002	10	12	30	88	99	50

14. Run *DESeq* (*see* **Note 24**). Below is an example set of commands that could be used for a dataset as given in the example count table above, where each column represents a biological replicate, samples A, B, and C are controls, and samples D, E, and F come from an experimental condition (comments are preceded by the hash character "#"):

```
# Run R
 R
# Load the DESeq package:
library("DESeq")
# Load the count data (in this case called countable.txt):
countTable <- read.table("countable.txt", header=TRUE, row.names=1 )
# Define conditions for the samples (any names can be given; here is "ctrl" for the control samples and "treated" for the condition samples. Note that the order corresponds to the order of samples in the count table:
condition = factor( c("ctrl ", " ctrl ", " ctrl ", " treated ", " treated ", " treated " ) )
# Define the CountDataSet, the central data structure in the DESeq package:
cds = newCountDataSet( countTable, condition )
# Estimate the effective library size:
cds = estimateSizeFactors( cds )
# Estimate the dispersions:
cds = estimateDispersions( cds )
# Look for differentially expressed genes between the two conditions:
res = nbinomTest( cds, " ctrl ", " treated " )
# Save the output to a file:
write.csv( res, file="DESeq.csv" )
# Recommended; plot some useful graphs that will help assessing the quality of the dataset or possible problems as well as to inspect the results visually (refer to the vignette for details).
# Load the necessary packages:
library("RColorBrewer")
library("gplots")
# Generate the heatmap of the sample-to-sample distances. First perform the variance stabilizing transformation:
cdsBlind = newCountDataSet( countTable, condition )
cdsBlind = estimateSizeFactors( cdsBlind )
cdsBlind= estimateDispersions( cds, method = "blind" )
vsd = varianceStabilizingTransformation( cdsBlind )
# Calculate the distances:
dists = dist( t( exprs(vsd) ) )
```

```
# Generate the heatmap and save it to a file:
jpeg('Heatmap.jpg')
hmcol = colorRampPalette(brewer.pal(9, "GnBu"))(100)
mat = as.matrix( dists )
rownames(mat) = colnames(mat) = with(mat)
heatmap.2(mat, trace="none", col = rev(hmcol), margin=c(13, 13))
dev.off()

# Plot the log2 fold changes against the mean normalized counts, and save to a file:
jpeg('plotMA.jpg')
plotMA(res)
dev.off()

# Plot the per-gene estimates against the mean normalized counts per gene and overlay the fitted curve, and save to a file:
plotDispEsts( cds )
jpeg('plotDispEsts.jpg')
plotDispEsts( cds )
dev.off()

# Plot the histogram of p-values, and save to a file:
jpeg('histogram_p-values.jpg')
hist(res$pval, breaks=100, col="skyblue", border="slateblue", main="")
dev.off()
```

15. Interpret the results (*see* **Note 25**).

4 Notes

1. A clean work environment is required for performing experiments involving RNA. All glassware and plasticware must be RNase-free, wearing gloves is necessary at all times, DEPC-treated and autoclaved solutions should be used.
2. Manipulation of *Mycobacterium tuberculosis* cultures must be performed under Biosafety Level 3 (BSL3) containment. Adherence to local guidelines for BSL3 work is strictly required.
3. Alternative methods for DNA quantification can be used, such as the Quantus Fluorometer manufactured by Promega or the PicoGreen assay [9].
4. Alternatively one can run many bioinformatics programs on external servers, like Galaxy, which is a widely used and freely available platform (http://galaxyproject.org/).
5. Approximately 100 ng of total RNA is required for library preparation without ribosomal RNA depletion following the Illumina methods (http://www.illumina.com/), but this

amount is likely to decrease as new protocols are optimized. We therefore suggest careful consideration of all the available options and consultation with the sequencing facility where the library will be sequenced. If depletion will be included in the procedure, a few micrograms of total RNA will be necessary (usually between 1 and 5 μg).

6. Column purification may lead to removal of short transcripts and small RNAs. The user should therefore choose the most appropriate methodology according to the aim of his experiment.
7. In our experience the TRIzol (Life Technologies)-based protocol yields pure, intact total RNA suitable for the subsequent procedures. This method retains the small RNAs and is therefore recommended when the user is interested in obtaining a comprehensive expression profile including the small transcripts.
8. The nucleic acid pellet now contains both RNA and DNA. For RNA-seq, it is important that there is no DNA contamination of the pellet.
9. In order to prepare the purified RNA for Illumina sequencing, a cDNA library must be prepared, where the RNA needs to be fragmented, reverse-transcribed, adenylated, fitted with adaptors, and purified. Here we describe the methods used to prepare total RNA for sequencing, however, it may be of interest if experiments involve a lot of samples, to deplete ribosomal RNA from the total RNA, allowing for multiplexing. Protocols for removal of ribosomal RNA based on affinity purification are not described here, but have been developed and reported by different suppliers (ScriptSeq Complete kit by Epicentre is an example).
10. As Illumina sequencing allows for the sequencing of relatively short fragments of DNA (currently up to 250 bases), RNA needs to be fragmented to similar sized pieces.
11. To generate a 3′ overhang on the blunt-ended double-stranded cDNA (needed for ligation of adaptor in the next step), the 3′ ends need to be adenylated.
12. Adaptors act as "barcodes" that can be used to identify the origin of the cDNA, and therefore different adaptors can be used for different biological samples when sequencing them in a single lane by Illumina (multiplexing). Adaptors are also needed for the next step of enrichment.
13. While the protocol described here offers more options and flexibility for advanced usage, a new tool called Rockhopper was recently published [10] that automates the work flow described here and uses similar algorithms for each process. The usage of Rockhopper merely consists in loading the fastq

files and choosing a reference sequence. The final result is a table of gene expressions. Rockhopper also has the option for visualizing the results in the IGV browser.

14. Other mapping programs can be used [11]. In RNA-seq transcription levels are inferred by counting the number of reads that correspond to each gene. To do so, reads must first be aligned onto the annotated reference genome sequence (in this case *M. tuberculosis* strain H37Rv).
15. ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Mycobacterium_tuberculosis_H37Rv_uid57777/.
16. If multiple CPU cores are available use the "-p" option (like "bowtie2 -p 4 -x…").
17. FASTQ files obtained from the Illumina sequencer are by default gunzipped. There is no need to uncompress these files since Bowtie2 can read them. Also, Illumina reads for each sample usually come in a number of individual fastq files to avoid problems of handling one large file (therefore the three fastq files in the example, separated by commas without spaces around).
18. The dash "-" in *samtools sort* defines the standard input. The vertical bar character "|", called a "pipe" is used to pass the standard output of one program to the standard input of the following one (in this case from *samtools view* to *samtools sort*). We could execute the two commands separately and have the *samtools view* write an unsorted BAM file to the disk which could be loaded to *samtools sort*. Pipelines are recommended to avoid IO bottlenecks and excessive hard disk usage. In fact, since we do not need the SAM file anymore after it is converted to BAM, we can pipe the *bowtie2*'s output (the SAM file) directly into *samtools view* without the need of writing it onto the hard disk, in a single command:

    bowtie2 -x /path/to/bowtie2_index/H37Rv -U /path/to/A_1.fastq.gz,/path/to/A_2.fastq.gz,/path/to/A_3.fastq.gz | samtools view -Su - | samtools sort - A_mapped-to-H37Rv_sorted
19. Many programs for downstream analyses or visualization of BAM files require the corresponding BAM's index file. It is good practice to index BAM files upon their generation.
20. Other feature counting programs can be used, like *htseq-count* (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html), *BEDtools* [12] etc.
21. In some cases it might be of interest to also check for transcripts deriving from intergenic regions in order to spot potential novel genes. In that case the SAF file should be modified to include the intergenic regions as additional features. It might be a good idea to omit very short intergenic regions.

22. Multiple BAM files can be input to the program, in which case the output table will contain individual counts columns for each BAM file. If all the BAM files are present in a single directory, using the wildcard character "*" will make *featureCounts* load them all. The option "-O" means that reads will be allowed to be assigned to more than one matched feature. For example, if a read spans over gene A and gene B, both genes will be counted. For bacteria this option makes sense because many genes are packed in operons. Reads spanning multiple genes derive from single transcripts.

    Note that *featureCounts* can handle strand specific reads with the "-s" option. For the library protocol provided here, the strand specific reads are in the reverse orientation. In that case the option should be "-s 2". Since strand specific library protocols often change, one can quickly check the orientation of the strand specific reads by running *featureCounts* twice using the "-s 1" and "-s 2" options, and then manually compare the resulting counts for a few genes, taking into account their strand.

23. Refer to Bioconductor for the installation instructions (http://www.bioconductor.org/install/). Other programs can be used for differential gene expression analysis [3, 4], including the recent *DESeq2* package.

24. DESeq is an advanced program that has a number of options. For proper usage and better understanding of the program please refer to its vignette and the user manual.

25. The main output of *DESeq* is the table of genes with expression values and relative expression changes between the compared groups of samples. There are no strict rules that define statistically significant or biologically significant differentially regulated genes. As a general guidance, one could consider only genes with the *padj* value smaller than 0.05 (*padj* indicates the false discover rate) to assure statistical accuracy, and genes that have at least a twofold difference in expression values for biological significance.

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Chapter 3

RNA Sequencing for Transcript 5′-End Mapping in Mycobacteria

Scarlet S. Shell, Michael R. Chase, Thomas R. Ioerger, and Sarah M. Fortune

Abstract

Next-generation sequencing technologies facilitate the analysis of multiple important properties of the transcriptome in addition to gene expression levels. Here we describe a method for mapping RNA 5′ ends in *Mycobacterium tuberculosis*, which allows the determination of transcriptional start sites (TSSs), comparative analysis of promoter usage under different conditions, and mapping of endoribonucleolytic processing sites. We describe in detail the procedures for constructing RNA sequencing libraries appropriate for RNA 5′ end mapping using an Illumina sequencing platform. We also outline the major steps of data analysis.

Key words RNA-seq, RNA sequencing, RNA processing, Transcription start site, Transcription start point, Gene expression, Illumina library, Endoribonuclease, RNase

1 Introduction

RNA sequencing (RNA-seq) is now widely used for comparative gene expression analysis, where it serves as a more flexible alternative to microarrays [1–4]. Even more excitingly, specialized RNA-seq methods allow for high-resolution mapping of transcriptomic features that were previously not accessible on genomic scales. Transcript 5′ ends are one such feature. They can be precisely mapped by RNA-seq at single-nucleotide resolution, and the 5′ ends produced by transcriptional initiation can be distinguished from the 5′ ends produced by ribonucleolytic processing. Since these two types of transcript 5′ ends represent entirely different biological phenomena, distinguishing them is critical for understanding the biological basis of observed transcriptomic features. Identification of transcriptional start sites (TSSs) allows for the direct inference of promoter locations, facilitating analysis of transcriptional regulation. Identification of RNA processing sites provides insight into RNA degradation pathways and posttranscriptional regulation of gene expression.

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This protocol describes a method for mapping transcriptional start sites (TSSs) and endoribonucleolytic processing sites genome-wide in *Mycobacterium tuberculosis* using an Illumina sequencing platform. The protocol has two major components; the first describes in detail a procedure for construction of Illumina sequencing libraries, and the second outlines bioinformatic analysis of the resulting data. This protocol is not designed for comparative gene expression analysis, although it is appropriate for comparing alternative promoter use and RNA processing patterns in different strains or conditions. These data are highly complementary to RNA-seq gene expression datasets that can be generated using published protocols [5, 6] or commercially available kits.

To capture RNA 5′ ends, adapters are ligated directly to intact RNAs. In order to facilitate subsequent discrimination between native RNA 5′ ends (which have 5′ triphosphates and correspond to TSSs) and 5′ ends produced by endonucleolytic cleavage (which have 5′ monophosphates or 5′ hydroxyls), two parallel libraries are constructed from each RNA sample. The "converted" library captures all RNA 5′ ends that natively have 5′ triphosphates or 5′ monophosphates, and the "non-converted" library captures only RNA 5′ ends that natively have 5′ monophosphates (*see* Fig. 1). The relative sequence coverage from each of the two libraries at a given RNA 5′ end can then be used to determine if it was a TSS or an endonucleolytic processing product.

The major steps of library construction consist of differential treatment of RNA 5′ ends, ligation of adapters, shearing of RNA, cDNA synthesis, PCR to add further adapter sequences, size selection by gel, and a final PCR to enrich for properly adapted library molecules. The resulting libraries consist of DNA inserts flanked by adapters equivalent to those in standard genomic DNA paired-end TruSeq© Illumina libraries, and can be sequenced on any Illumina sequencer (*see* Fig. 1).

After mapping sequencing reads to a reference genome, the major analytical steps are identification of RNA 5′ ends and discrimination between unprocessed ends and those produced by endonucleolytic cleavage.

Fig. 1 (continued) therefore contains 5′ ends from both unprocessed and processed RNAs, while the "non-converted" library contains 5′ ends from processed RNAs only. The ratio of 5′ end sequence coverage in the two libraries can then be used to distinguish TSSs from RNA processing sites. *Thick black lines* denote unprocessed RNA and cDNA derived from such RNA; *thick gray lines* denote processed RNA and cDNA derived from such RNA; *thin black* and *gray dashed lines* denote Illumina adapter sequences; *thick black* and *gray arrows* denote Illumina sequencing primers

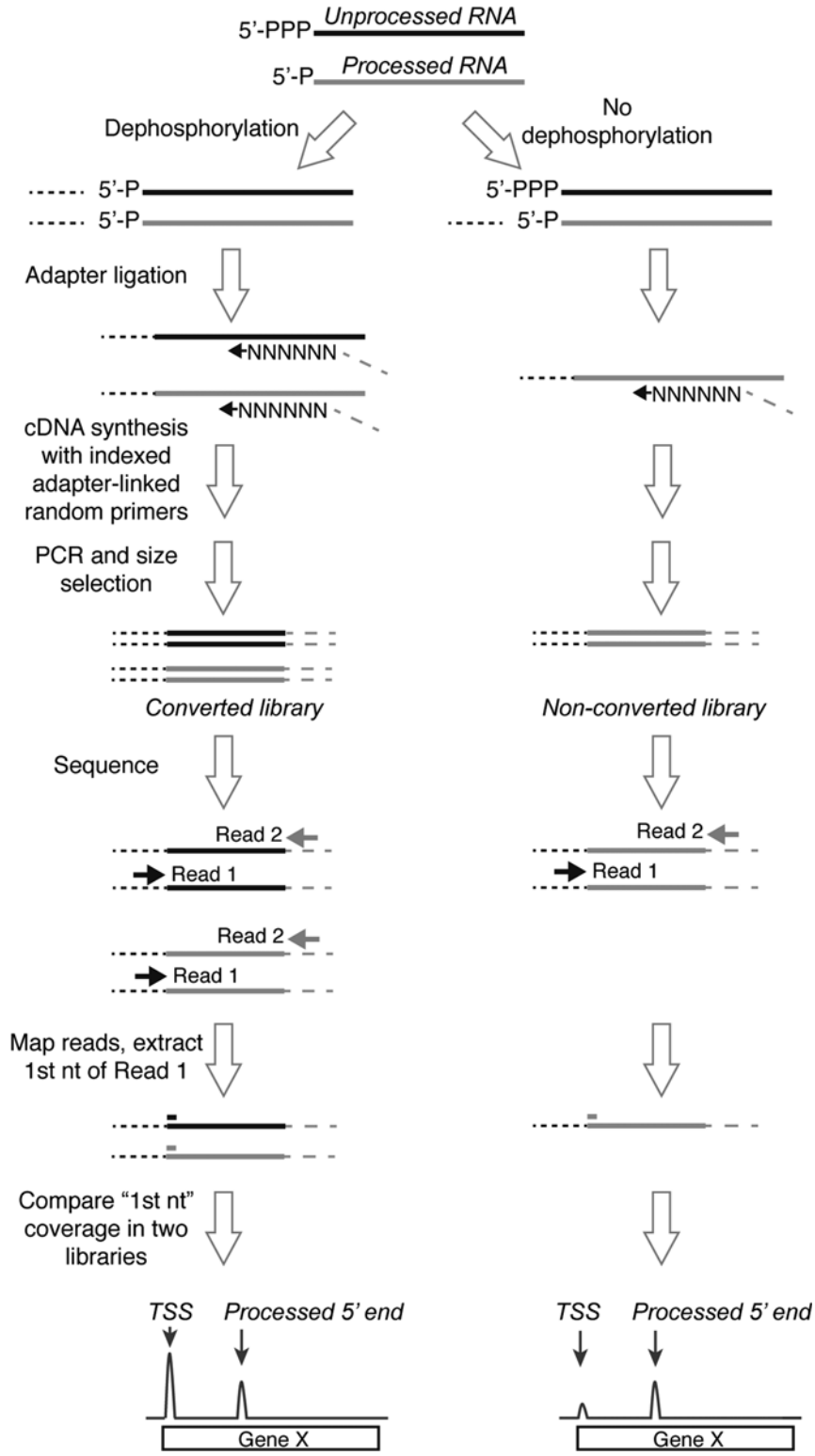

Fig. 1 Schematic of library construction for transcript 5′ end mapping. RNA samples are split into two parts, one of which is treated with 5′-polyphosphatase and one of which is left untreated. The "converted" library

2 Materials

2.1 Conversion of 5′ Triphosphates to 5′ Monophosphates in RNA

1. 0.5–2 μg rRNA-depleted, high quality RNA (*see* **Notes 1–3**).
2. 5′ Polyphosphatase and 10× buffer (Epicentre) (*see* **Note 4**).
3. RNase-free water.
4. RNeasy RNA purification kit (Qiagen) or alternative column-based RNA purification kit, e.g., RNA Clean & Concentrator (Zymo Research).
5. RNaseOUT water: RNase-free water containing 10 μL of RNaseOUT (Life Technologies) and 10 μL of 100 mM DTT per mL.
6. Tris–HCl pH 7.5: 100 mM prepared with RNase-free water.

2.2 Adapter Ligation

1. Oligo SSS392: 5′ TCC CTA CAC GAC GCT CTT CC*G AUC U* 3′, where normal font indicates deoxyribonucleotides and italic font indicates ribonucleotides, 5 μg/μL in RNase-free water (*see* **Note 5**).
2. PEG 8000: 50 % in RNase-free water.
3. RNaseOUT (Life Technologies) (or alternative ribonuclease inhibitor).
4. T4 RNA ligase I and 10× buffer.
5. ATP: 10 mM in RNase-free water.
6. DMSO.
7. RNase-free water.
8. RNeasy RNA purification kit (Qiagen) or alternative column-based RNA purification kit such as RNA Clean & Concentrator (Zymo Research).

2.3 RNA Shearing

1. Covaris sonicator (*see* **Note 6**).
2. Covaris AFA MicroTubes.
3. RNase-free water.
4. RNaseOUT (Life Technologies) (or alternative ribonuclease inhibitor).
5. DTT: 100 mM prepared with RNase-free water.
6. Tris–HCl pH 7.5: 100 mM prepared with RNase-free water.

2.4 cDNA Synthesis

1. Oligo SSS397: 5′ CTG GAG TTC AGA CGT GTG CTC TTC CGA TCT NNN NNN 3′, where "N" represents a degenerate base, 2.2 μg/μL in RNase-free water.
2. Superscript III reverse transcriptase with 5× buffer (Life Technologies).
3. dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, and 10 mM dTTP in RNase-free water.

4. RNaseOUT (Life Technologies) (or alternative ribonuclease inhibitor).
5. DTT: 100 mM prepared with RNase-free water.
6. Tris–HCl pH 7.5: 1 M prepared with RNase-free water.
7. Actinomycin D: 1 mg/mL in DMSO.
8. NaOH: 1 N.
9. EDTA: 500 mM.
10. Tris–HCl pH 7.5: 1 M.
11. MinElute DNA purification kit (Qiagen) or alternative column-based DNA purification kit.
12. Molecular-grade water.

2.5 PCR to Add Full-Length Illumina Adapter Sequences and Amplify Libraries

1. Oligo SSS398: 5′ AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT C 3′, 10 μM in water.
2. Reverse index primer: 5′ CAA GCA GAA GAC GGC ATA CGA GAT *XXX XXX* GTG ACT GGA GTT CAG ACG TGT GCT 3′, 10 μM in water (index indicated in *italics*; index sequences available from Illumina upon request).
3. Phusion polymerase with 5× HF buffer.
4. Betaine: 5 M.
5. dNTP mix: 25 mM dATP, 25 mM dCTP, 25 mM dGTP, and 25 mM dTTP in water.
6. Agarose.
7. TAE buffer.
8. Ethidium bromide.
9. Molecular-grade water.

2.6 Gel Purification

1. Agarose.
2. TAE buffer.
3. Ethidium bromide.
4. Gel extraction kit, e.g., Qiagen.

2.7 Solid-Phase Reversible Immobilization (SPRI) Purification

1. AMPure XP beads (Agencourt).
2. Magnetic separation stand that fits 1.5 mL microcentrifuge tubes.
3. Molecular-grade water.
4. 80 % ethanol, freshly prepared.

2.8 PCR to Enrich for Correctly Adapted Molecules

1. Oligo SSS401: 5′ AAT GAT ACG GCG ACC ACC GAG ATC 3′, 10 μM in water.
2. Oligo SSS402: 5′ CAA GCA GAA GAC GGC ATA CGA GAT 3′, 10 μM in water.

3. Phusion polymerase with 5× HF buffer.
4. Betaine: 5 M.
5. dNTP mix: 25 mM dATP, 25 mM dCTP, 25 mM dGTP, and 25 mM dTTP in water.
6. Molecular-grade water.

2.9 SPRI Purification and Gel Electrophoresis

1. AMPure XP beads (Agencourt).
2. Magnetic separation stand that fits 1.5 mL microcentrifuge tubes.
3. Molecular-grade water.
4. 80 % ethanol, freshly prepared.
5. Agarose.
6. TAE buffer.
7. Ethidium bromide.

3 Methods

3.1 Conversion of 5′ Triphosphates to 5′ Monophosphates in RNA

For each sample, perform one reaction with 5′ Polyphosphatase and one mock reaction. These will be each be carried through all subsequent steps to create the "converted" and "non-converted" libraries, respectively (*see* Fig. 1).

1. Combine:

   0.25–1 μg rRNA-depleted RNA

   2 μL 10× 5′ polyphosphatase buffer

   1 μL 5′ Polyphosphatase (or 1 μL RNase-free water for mock reaction)

   RNase-free water to a total reaction volume of 20 μL
2. Incubate at 37 °C for 1 h.
3. Add 80 μL RNase-free water to each reaction.
   Purify with RNeasy kit as follows in **steps 4–11**.
4. Add 350 μL Buffer RLT (Lysis Buffer) and mix.
5. Add 250 μL 100 % ethanol, mix well by pipetting and load sample onto RNeasy spin column.
6. Centrifuge 15 s at >10,000 × *g*, discard flow-through.
7. Apply 500 μL Buffer RPE (Wash Buffer) to the spin column, centrifuge 15 s at >10,000 × *g*, and discard flow-through.
8. Repeat **step 7** twice (three washes total), and increase centrifugation time to 2 min at the third wash.
9. After discarding the flow-through from the third wash, centrifuge column for 1 min at maximum speed.

10. Transfer column to a clean 1.5 mL tube, pipet 50 µL RNaseOUT water directly onto the membrane and centrifuge 1 min at >10,000 × *g*. Leave flow-through in tube.
11. Repeat **step 10** for a total elution volume of 100 µL per sample.
12. Add 1 µL 100 mM Tris–HCl pH 7.5.
13. Concentrate in a vacuum centrifuge to 8 µL.

3.2 Adapter Ligation

1. Combine 8 µL sample from Subheading 3.1, **step 13**, with 1 µL oligo SSS392.
2. Denature RNA at 65 °C for 10 min, then snap-cool in an ice-water bath.
3. Add the following (can be prepared as a master mix) (total volume 30 µL):

   1 µL RNaseOUT

   10 µL 50 % PEG 8000

   3 µL 10× T4 RNA ligase I buffer

   3 µL 10 mM ATP

   3 µL DMSO

   1 µL T4 RNA ligase I
4. Incubate 20 °C overnight.
5. Add 70 µL RNase-free water.
   Purify with RNeasy kit as follows in **steps 6–13**.
6. Add 350 µL Buffer RLT (Lysis Buffer) and mix.
7. Add 250 µL 100 % ethanol, mix well by pipetting and load sample onto RNeasy spin column.
8. Centrifuge 15 s at >10,000 × *g*, discard flow-through.
9. Apply 500 µL Buffer RPE (Wash Buffer) to the spin column, centrifuge 15 s at >10,000 × *g*, and discard flow-through.
10. Repeat **step 9** twice (three washes total), and increase centrifugation to 2 min at the third wash.
11. After discarding the flow-through from the third wash, centrifuge column for 1 min at maximum speed.
12. Transfer column to a clean 1.5 mL tube, pipet 63 µL RNase-free water directly onto the membrane and centrifuge 1 min at >10,000 × *g*. Leave flow-through in tube.
13. Repeat **step 12** for a total elution volume of 126 µL per sample.

3.3 RNA Shearing

Use a Covaris sonicator to shear purified, ligated RNA (*see* **Note 6**).

1. Transfer sample to a Covaris AFA MicroTube.

2. Shear with the following settings:
   Exposure time = 180 s
   Duty cycle = 10 %
   Intensity = 5
   Cycles/burst = 200
3. Transfer sample to a 1.5 mL microcentrifuge tube and add the following:
   1 μL 100 mM Tris–HCl pH 7.5
   1 μL RNaseOUT
   1 μL 100 mM DTT
4. Concentrate by vacuum centrifugation to a final volume of 11.25 μL.

3.4 cDNA Synthesis

1. Add 1 μL oligo SSS397 to concentrated sample from Subheading 3.3, **step 4**.
2. Denature RNA at 65 °C for 10 min, then snap-cool in an ice-water bath.
3. Prepare a master mix containing the following amounts per reaction:
   4 μL 5× first-strand buffer
   1 μL dNTP mix (10 mM each)
   0.5 μL RNaseOUT
   0.25 μL 1 mg/mL Actinomycin D
   1 μL 100 mM DTT
   1 μL Superscript III
4. Add 7.75 μL master mix to each cooled RNA/oligo mix on ice.
5. Incubate 42 °C overnight.
6. Add 10 μL each 1 N NaOH and 500 mM EDTA.
7. Incubate at 65 °C for 15 min.
8. Add 25 μL 1 M Tris–HCl pH 7.5.
   Purify with MinElute kit as in **steps 9–16** below.
9. Add 600 μL Buffer PB (Binding Buffer) to sample and mix well.
10. Apply to MinElute column and centrifuge for 1 min at >15,000 × *g*.
11. Pour flow-through back over column, centrifuge again for 1 min at >15,000 × *g*, and discard flow-through.
12. Apply 700 μL Buffer PE (Wash Buffer) to column, centrifuge for 1 min at >15,000 × *g*, and discard flow-through.
13. Repeat **step 12** for a total of two washes.

14. Centrifuge for 1 min at >15,000 × *g* to ensure membrane is dry, then transfer column to a clean 1.5 mL tube.
15. Pipet 30 µL molecular grade water onto membrane, centrifuge for 1 min at >15,000 × *g*, and leave flow-through in tube.
16. Repeat **step 15** for a total elution volume of 60 µL.

3.5 PCR to Add Full-Length Illumina Adapter Sequences and Amplify Libraries

Perform three reactions per sample, using SSS398 as a forward primer with an indexed reverse primer of your choice.

1. Combine the following for each reaction (can be prepared as a master mix) (50 µL final volume):

   10 µL 5× HF Phusion buffer

   2.5 µL 10 µM primer SSS398

   2.5 µL 10 µM indexed reverse primer

   20 µL 5 M betaine (*see* **Note 7**)

   0.5 µL Phusion polymerase

   0.4 µL dNTP mix (mix is 25 mM each dNTP)

   14.1 µL cDNA or molecular-grade water for no-template control
2. Perform PCR as follows, with reaction volume set to 100 µL (*see* **Note 8**):

   98 °C for 3 min

   7 cycles of:

   98 °C for 80 s

   60 °C for 30 s

   72 °C for 30 s

   1 cycle of:

   72 °C for 5 min
3. Run 5 µL of each PCR product on a 1.5 % agarose gel to assess size and abundance of products (*see* **Note 9**).

3.6 Gel Purification

1. Clean gel box, tray, and comb thoroughly with soap and rinse well with water (*see* **Note 10**).
2. Make a thick 1.5 % (w/v) agarose gel containing ethidium bromide.
3. Run all remaining PCR product from Subheading 3.5, **step 2**, dividing samples among multiple lanes as necessary (*see* **Note 9**).
4. Excise region of gel that contains majority of product but excludes primer-dimer.
5. Divide into as many microcentrifuge tubes as necessary, and add approximately 3 volumes Qiagen Buffer QG. The number of tubes and volume of Buffer QC will depend on the size of

the gel slice, which will vary depending on the fragmentation conditions as well as the width of the comb and how long the gel was run.

6. Melt gel slices at room temperature, vortexing periodically. Allow approximately 30 min for this step.
7. Plan to use one QIAquick Gel Extraction column per 1.4 mL of melted gel/Buffer QG mix; each column will be loaded twice with 700 μL.
8. Apply 700 μL melted gel in Buffer QG to QIAquick Gel Extraction column, centrifuge for 1 min at >15,000 × *g*, and discard flow-through.
9. Add up to 700 μL additional melted gel in Buffer QG to column, centrifuge for 1 min at >15,000 × *g*, and discard flow-through.
10. Apply 700 μL Buffer PE (Wash Buffer) to column, centrifuge for 1 min at >15,000 × *g*, and discard flow-through.
11. Repeat **step 10** for a total of two washes.
12. Centrifuge for 1 min at >15,000 × *g* to ensure membrane is dry, then transfer column to a clean 1.5 mL tube.
13. Pipet 40 μL molecular-grade water onto membrane, centrifuge for 1 min at >15,000 × *g*, and leave flow-through in tube.
14. Repeat **step 13** for a total elution volume of 80 μL.
15. If more than one column was used for each sample, pool eluates from the same sample.
16. Use a spectrophotometer to determine the 260/230 ratio of product. If the ratio is <1, perform the AMPure bead-based purification described in Subheading 3.7 below. If the ratio is >1, go directly to Subheading 3.8 (final PCR).

3.7 SPRI Purification

1. Concentrate sample by vacuum centrifugation to a volume of 50 μL per library.
2. Add 90 μL of AMPure XP beads and pipet up and down to mix well (1.8 volumes of beads per volume sample—*see* **Note 11**).
3. Incubate for 15 min at room temperature.
4. Place tube in the magnetic separation stand and leave for approximately 2 min or until supernatant looks mostly clear.
5. With the tube still in magnetic stand, remove the 140 μL supernatant using a pipet.
6. With the tube still in magnetic stand, add 200 μL fresh 80 % ethanol, incubate 30 s, and pipet off supernatant.
7. Repeat **step 6** for a total of two washes.
8. Use a pipet to remove any remaining supernatant and leave for 10–15 min on stand with cap open to allow evaporation of residual ethanol.

9. Remove the tube from the stand and resuspend beads in 50 μL molecular-grade water by pipetting up and down.
10. Incubate for 2 min at room temperature.
11. Return to magnetic stand, leave approximately 2 min or until supernatant looks clear, transfer supernatant to a clean tube.

3.8 PCR to Enrich for Correctly Adapted Molecules

1. Perform three reactions per library and a no-template control (*see* **Note 12**).
2. Combine the following for each reaction (can be prepared as a master mix) (50 μL final volume per reaction). If template concentrations differ, normalize all to the concentration of the most dilute library.

   10 μL 5× HF Phusion buffer

   2.5 μL 10 μM primer SSS401

   2.5 μL 10 μM primer SSS402

   20 μL 5 M betaine (*see* **Note 7**)

   0.5 μL Phusion polymerase

   0.4 μL dNTP mix (mix is 25 mM each dNTP)

   14.1 μL cDNA or molecular-grade water for no-template control.
3. Perform PCR as follows, with reaction volume set to 100 μL (*see* **Note 8**):

   98 °C for 3 min

   3 cycles of:

   98 °C for 80 s

   60 °C for 30 s

   72 °C for 30 s

   1 cycle of:

   72 °C for 5 min

3.9 SPRI Purification and Gel Electrophoresis

1. Combine the three PCR reactions per library from Subheading 3.8, **step 3**, into two aliquots in 1.5 mL microcentrifuge tubes for purification (~75 μL PCR product per tube). Purify the no-template control as well.
2. Add 1.8 volumes of AMPure XP beads per volume sample and pipet up and down to mix well (153 μL beads per library reaction and 90 μL for the no-template control; *see* **Note 11**).
3. Incubate for 15 min at room temperature.
4. Place tube in magnetic separation stand and leave for approximately 2 min or until supernatant looks mostly clear.
5. With the tube still in the magnetic stand, pipet out supernatant.

6. With the tube still in the magnetic stand, add 200 μL fresh 80 % ethanol, incubate for 30 s, and pipet off supernatant.
7. Repeat **step 6** for a total of two washes.
8. Use a pipet to remove any remaining supernatant and leave for 10–15 min on the stand with cap open to allow evaporation of residual ethanol.
9. Remove the tube from the stand and resuspend beads in 60 μL molecular-grade water (libraries) or 40 μL molecular-grade water (no-template control) by pipetting up and down.
10. Incubate for 2 min at room temperature.
11. Return the tube to the magnetic stand and leave for approximately 2 min (or until supernatant looks clear) and transfer supernatant to a clean tube.
12. Use a spectrophotometer to determine the product concentrations. If the no-template control has an apparent concentration of 10 % of the library concentration or greater, repeat the AMPure purification, using 1.8× volumes of beads and eluting in 60 μL water (40 μL for the no-template control).
13. Run 3–5 μL of each library on a 1.5 % gel to confirm that the product size is as expected, there is no primer-dimer, and the no-template control does not contain product.
14. Libraries can now be run on any Illumina sequencer (HiSeq, Genome Analyzer, or MiSeq) using a paired-end protocol and sequencing primers appropriate for TruSeq genomic DNA libraries.

3.10 Data Analysis

Here we outline the most basic steps in data analysis for RNA 5′ end mapping libraries (*see* **Note 13**).

1. Use an appropriate software package (*see* **Note 14**) to map paired-end reads to a reference genome. Read 1 represents the sequence of the 5′ end of an RNA molecule in your starting sample; Read 2 represents the reverse complement of the position that the random primer annealed to during cDNA synthesis (*see* Fig. 1).
2. Extract the genome coordinate corresponding to the first nucleotide (1st nt) of each Read 1; this represents the precise 5′ end of an RNA molecule.
3. Determine the coverage of 1st nts for each position in the genome on each strand in both the "converted" and "non-converted" libraries (*see* **Note 15**).
4. To identify RNA 5′ ends that are present at levels above background, determine the ratio of 1st nt coverage at each position in the genome to the position ten nucleotides before and ten nucleotides after on the same strand in the "converted" library.

If either of these ratios is 10 or greater, the position of the numerator may be classified as a 5′ end present above background.

5. For each 5′ end above background identified in **step 4** above, determine the ratio of 1st nt coverage in the "converted" to "non-converted" libraries. A distribution of these ratios should be bimodal. 5′ ends with ratios near 1 represent endonucleolytic processing products, while 5′ ends with ratios significantly greater than 1 represent TSSs. A rigorous cutoff could be determined by fitting the distribution to a Gaussian mixture model (in a software package like R).
6. Several additional filtering steps may be helpful. 5′ ends may be filtered by absolute 1st nt coverage level and by relative 1st nt coverage compared to a strand-specific RNA-seq expression library prepared from the same RNA sample [5]. 5′ ends mapping to adjacent positions in the genome may be "collapsed" to the position with the greatest 1st nt coverage.

4 Notes

1. This protocol was developed for *M. tuberculosis* but should be suitable for any GC-rich bacterial species.
2. *See* ref. 7 for an example of an appropriate RNA extraction protocol.
3. Ribosomal RNA can be depleted with commercially available kits such as MICROBExpress (Ambion) and Ribo-Zero (Epicentre).
4. Tobacco alkaline phosphatase (TAP) may also work adequately for this step but has not been tested.
5. This is the adapter that gets ligated to 5′ ends of target RNA molecules. Most of the oligo is composed of deoxyribonucleotides to improve stability, while five ribonucleotides are included at the 3′ end to improve ligation efficiency. Store at −80 °C.
6. Chemical fragmentation of RNA has been widely reported in the literature [5, 8, 9] and would likely be appropriate here as an alternative to sonication.
7. Betaine makes the reaction mix cloudy; this is not a cause for concern.
8. The addition of betaine, the long denaturation steps, and the setting of the reaction volume as 100 μL are all steps taken to ensure that GC rich sequences are fully represented in the final libraries. *See* ref. 10 for further explanation.

9. Add ethidium bromide to both the gel and the running buffer to ensure that small fragments can be visualized. Library reactions should give smears or bands that run higher than the primer-dimer present in the no-template control reactions. Library fragment size may vary depending on shearing or fragmentation method used.
10. Significant care must be taken to prevent cross-contamination by trace amounts of Illumina library amplicon on laboratory equipment, since all Illumina sequencing libraries contain adapter sequences that are identical in their outer portions. All gel boxes and trays should be thoroughly cleaned *before* use in preparative steps such as Subheading 3.6, and *after* all steps that involve amplified library molecules (Subheadings 3.5, 3.6 and 3.9). For best practice, designate different areas of the lab for pre-amplification and post-amplification procedures and maintain separate pipets, racks, and other equipment for each area. Change gloves after moving from the post-amplification area to any other part of the lab to minimize the spreading of amplicon.
11. The ratio of bead volume to sample volume is important for AMPure cleanups because this ratio determines the size selection properties of the cleanup [11, 12]. A ratio of 1.8 volumes AMPure beads per 1 volume of sample is appropriate for purifying away primers from PCR products. If sample volumes vary significantly from those indicated in this protocol, the volume of AMPure XP beads should be adjusted accordingly to maintain the correct ratio.
12. Primers SSS401 and SSS402 are short primers that anneal to the outer portions of the ~60 nucleotide Illumina adapter sequences. This PCR enriches for molecules that contain full-length adapters to ensure optimal attachment to the Illumina flow-cell for sequencing.
13. A complete and detailed discussion of analysis methodology is beyond the scope of this chapter. Alternative analytical strategies may be called for depending on the experimental goals.
14. Examples of freely available mapping software include Ssaha2 and Bowtie.
15. Normalization might be necessary at this step if the total number of reads differs significantly between the "converted" and "non-converted" libraries. To assess the need for normalization, compare the 1st nt coverage at the annotated 5′ ends of structural RNAs such as the 16S and 23S rRNA and tRNAs in the "converted" and "non-converted" libraries. These 5′ ends are created by endonucleolytic cleavage and endogenously have 5′ monophosphates. If they are present at equal abundance in the cognate "converted" and "non-converted" libraries, there is likely no need for normalization.

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Chapter 4

Fractionation and Analysis of Mycobacterial Proteins

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Abstract

The extraction and isolation of native bacterial proteins continue to be valuable technical pursuits in order to understand bacterial physiology, screen for virulence determinants, and describe antigens. In this chapter, methods for the manipulation of whole mycobacterial cells are described in detail. Specifically, the concentration of spent culture filtrate media is described in order to permit separation of soluble, secreted proteins; several discrete separation techniques, including precipitation of protein mixtures with ammonium sulfate and separation of proteins by hydrophobic chromatography are also provided. Similarly, the generation of whole cell lysate and facile separation of lysate into subcellular fractions to afford cell wall, cell membrane, and cytosol enriched proteins is described. Due to the hydrophobic nature of cell wall and cell membrane proteins, several extraction protocols to resolve protein subsets (such as extraction with urea and SDS) are also provided, as well as a separation technique (isoelectric focusing) that can be applied to separate hydrophobic proteins. Lastly, two commonly used analytical techniques, in-gel digestion of proteins for LC-MS and analysis of intact proteins by MALDI-ToF MS, are provided for rapid analysis of discrete proteins within subcellular or chromatographic fractions. While these methods were optimized for the manipulation of *Mycobacterium tuberculosis* cells, they have been successfully applied to extract and isolate *Mycobacterium leprae, Mycobacterium ulcerans*, and *Mycobacterium avium* proteins. In addition, a number of these methods may be applied to extract and analyze mycobacterial proteins from cell lines and host derived samples.

Key words Protein analysis, Proteome, Cellular fractionation, Culture filtrate, Antigens

1 Introduction

The proteins of pathogenic mycobacteria remain a primary focus of research targeting the development of subunit vaccines and diagnostic reagents. This is especially true in regard to *Mycobacterium tuberculosis*, where in addition to traditional vaccine and diagnostic pipelines [1], *M. tuberculosis* proteins are also included in studies exploring their potential as pathogen specific biomarkers for detection of tuberculosis cases, including latent tuberculosis infection (LTBI) and potentially during treatment response [2–5]. Traditional methods aimed at teasing out relevant

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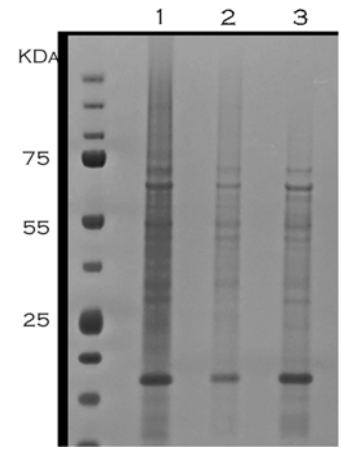

Fig. 1 SDS-PAGE of *M. tuberculosis* cell wall protein (CWP) before and after detergent extractions. From the *left*: *lane 1*, Molecular weight markers; *lane 2*, total CWP; *lane 3*, 6 M guanidine HCl extracted CWP; *lane 4*, SDS extracted CWP; *lane 5*, TX-114 extracted CWP

proteins, such as preparative methods for fractionation and discovery of antigens [6] and selective extraction of hydrophobic proteins (Fig. 1; [7]) are complemented by sensitive identification of proteins using mass spectrometry to describe proteins recognized by the host (examples provided in refs. [8–12]) and even include direct detection of *M. tuberculosis* proteins in host tissues [13–15], urine [16], and exosomes [13, 17, 18]. The generation of discrete, well-characterized native protein fractions and purified native proteins compliments these novel innovative approaches to further our understanding of tuberculosis pathogenesis.

In addition, emerging and reemerging diseases and public health concerns regarding a rise in pulmonary and cutaneous nontuberculous mycobacteria (NTM, [19, 20]) and economic impact of mycobacterial infections of livestock [15], requires the use of established biochemical and proteomics methodologies to extend studies into these understudied and important pathogens.

In this chapter, we provide methods for preparing numerous protein fractions derived from mycobacterial cells, as well as several extraction and separation methods that may be applied to these subcellular fractions or extended into clinical samples (such as tissue homogenates and cell lines). Lastly, we provide methods to identify proteins of interest using facile techniques to obtain quality proteolytic digests for shotgun proteomics (typically via liquid chromatography–tandem mass spectrometry, LC-MS/MS) or analyze intact proteins by mass spectrometry (using matrix assisted laser desorption time of flight mass spectrometry, MALDI-ToF MS).

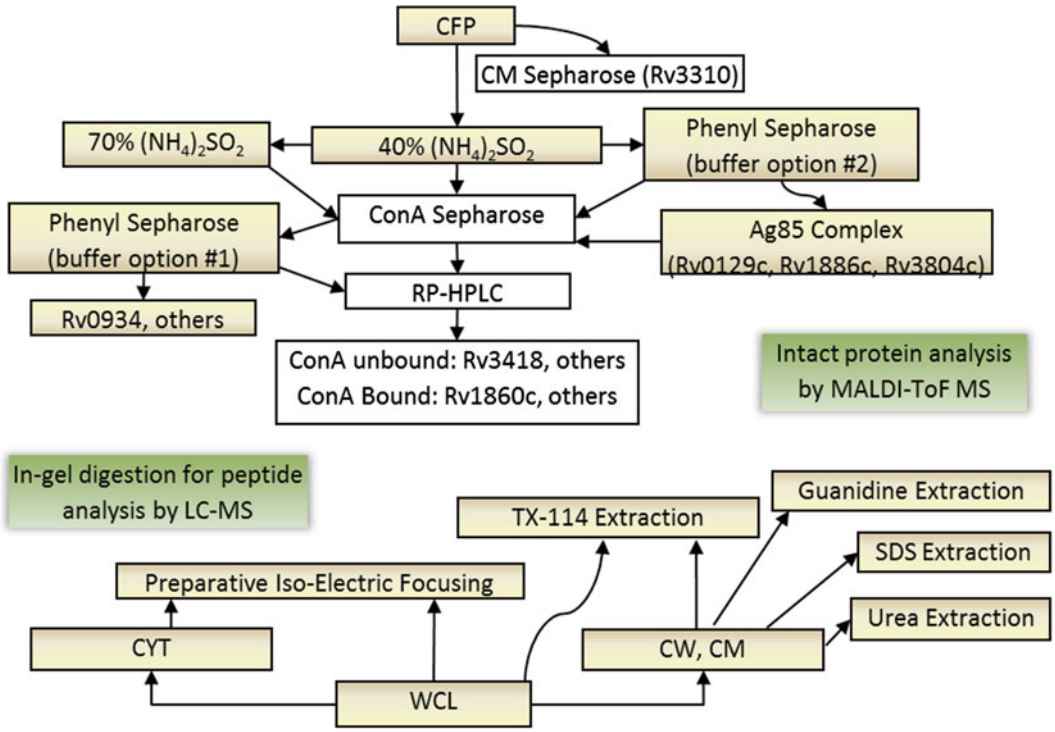

Fig. 2 Work-flow to generate *M. tuberculosis* protein fractions for subsequent purification and analysis. Highlighted methods are described in detail in this chapter and represent either separation (*beige*) or analytical (*green*) methods (Color figure online)

The methods we describe in this chapter follow the processes utilized ourselves for manipulation of *Mycobacterium* spp. cells to generate and analyze proteins. This process has been optimized to permit maximum utilization and separation of proteins from a single cell harvest (Fig. 2); methods detailed in this chapter are highlighted.

2 Materials

2.1 Concentration of Spent Culture Filtrates from Mycobacterium spp.

1. Culture Filtrate Proteins (CFP) from *Mycobacterium* spp. (*see* **Note 1**) [21].
2. 90 % v/v isopropyl alcohol.
3. 70 % v/v ethanol.
4. 10 mM NH_4HCO_3.
5. Pyrogen free, 0.2 μm filtered water.
6. Stirred Amicon ultrafiltration cell.
7. Amicon pressure vessel or reservoir.
8. Amicon tubing.

9. 5,000 MWCO Ultrafiltration membrane.
10. Compressed N_2 cylinder.
11. Nitrogen regulator.
12. 0.2 μm filter unit (*see* **Note 2**).
13. Wrench.
14. Teflon tape.
15. SDS-PAGE gel.
16. Bicinchoninic acid (BCA) protein assay kit (*see* **Note 3**).

2.2 Fracturing of Whole Mycobacterium spp. Cells by French Press

1. γ-irradiated cells (*see* **Note 4**) [22].
2. Phosphate Buffered Saline (PBS): 1.06 mM KH_2PO_4, 154 mM NaCl, 5.6 mM Na_2HPO_4, pH 7.4.
3. PBS, pH 7.4 with 1 mM EDTA.
4. Protease inhibitor, EDTA-free.
5. DNAse: 1 mg/mL stock in water (stored at −20 °C).
6. RNAse: 1 mg/mL stock in water (stored at −20 °C).
7. Glycerol.
8. French Press (SLM AMINCO).
9. French Pressure cell, 40K (*see* **Note 5**).
10. French Pressure cell stand.
11. 10 % v/v Lysol (or other broad spectrum bacterial disinfectant).
12. 70 % v/v ethanol.
13. Acid-fast staining kit.

2.3 Subcellular Fractionation of Mycobacterium spp. Whole Cell Lysate (WCL)

1. Whole cell lysate (*see* **Note 6**).
2. PBS, pH 7.4 with 1 mM EDTA.
3. Oakridge centrifuge tubes (Polypropylene or Teflon), 50 mL, sterile.
4. 40 mL ultracentrifuge tubes.
5. High-speed centrifuge and rotor.
6. Ultracentrifuge and rotor.
7. Dialysis tubing (3,500 Da MWCO).
8. 10 mM NH_4HCO_3.
9. Dialysis tank (7 L).
10. BCA Protein assay kit (*see* **Note 3**).
11. SDS-PAGE gel.

2.4 Extraction of Proteins from Subcellular Fractions Using Detergents

1. Soluble/Insoluble protein sample (such as Cell Wall or Membrane obtained in Subheading 3.3).
2. PBS, pH 7.4.
3. Oakridge centrifuge tubes (Polypropylene or Teflon), 50 mL, sterile.
4. Pyrogen free, 0.2 μm filtered water.
5. High-speed centrifuge and rotors.
6. Micro-stir bars.
7. Acetone.
8. Glacial acetic acid.
9. Triethylamine.
10. 100 % Acetone, stored at −20 °C.
11. Dialysis tubing (3,500 Da MWCO).
12. 10 mM NH_4HCO_3.
13. Dialysis tank (7 L).
14. 2 % w/v SDS in PBS (*see* **Note 7**).
15. 6 M guanidine-HCl (*see* **Note 7**).
16. 8 M urea (*see* **Note 7**): Urea should be deionized prior to use to remove reactive cyanate ions. 8 M solutions of urea may be bulk deionized using an ion exchange resin. Add AG-501-X8 (Bio-Rad, Hercules, CA) at 5 g/100 mL, stir the solution for 2 h at RT, and filter the solution to recover deionized 8 M urea.

2.5 Partitioning of Hydrophobic and Hydrophilic Proteins Using Triton X-114

1. Soluble protein sample (such as CFP obtained in Subheading 3.1 or whole cell lysate obtained in Subheading 3.2).
2. PBS, pH 7.4.
3. 32 % TX-114 Stock Solution: Combine equal parts of Triton X-114 and PBS. Stir at room temperature for several hours. Transfer to 4 °C and let stand until the mixture clears, which may take overnight (if it does not clear, it was not mixed thoroughly enough). Transfer the mixture to a 37 °C water bath or incubator until a biphase appears (*see* **Note 8**). Carefully remove and discard the top layer being sure not to disrupt the bottom Triton layer. Take note of the volume of Triton and add an equal volume of PBS. Stir at room temperature until the mixture clears, then incubate at 37 °C until a biphase appears. Remove the PBS layer and double the volume again. Repeat the stirring and incubation and remove the PBS layer. Stir the final Triton layer on a stir plate to recombine any PBS not removed. The final Triton layer is the 32 % TX-114.
4. 4 % TX-114 in PBS: Dilute 12.5 mL 32 % TX-114 stock solution to 100 mL total in PBS.

5. Polypropylene centrifuge bottles.
6. Platform mixer.
7. High-speed centrifuge and rotors.
8. 37 °C Water bath.
9. Acetone, stored at −20 °C (*see* **Note 9**).
10. Nitrogen bath.
11. Oakridge centrifuge tubes (Teflon), 50 mL, sterile.
12. PBS Saturated Phenol (*see* **Note 10**): Working in a chemical fume hood, suspend phenol crystals in PBS at 50 % w/v. Incubate in 55 °C water bath until crystals melt and a partition forms. Carefully remove the top PBS layer using a glass pipet and discard as hazardous waste. Add an equal volume of PBS back to the phenol layer. Repeat incubation and removal of top layer two more times. Keep the bottom layer as PBS saturated phenol.
13. Dialysis tubing (3,500 Da MWCO).
14. Pyrogen free, 0.2 μm filtered water.
15. SDS-PAGE gel.
16. BCA Protein assay kit (*see* **Note 3**).

2.6 Partitioning of Hydrophobic and Hydrophilic Proteins Using Ammonium Sulfate

1. Soluble protein sample (such as CFP obtained in Subheading 3.1).
2. Ammonium sulfate, $(NH_4)_2SO_4$.
3. Dialysis tubing (3,500 Da MWCO).
4. Dialysis buffer #1: 7 L of 10 mM NH_4HCO_3, 1 mM DTT.
5. Dialysis buffer #2: 7 L of 10 mM NH_4HCO_3.
6. SDS-PAGE gel.
7. BCA Protein assay kit (*see* **Note 3**).
8. Polypropylene centrifuge bottles.
9. High-speed centrifuge and rotor.
10. Freeze-dryer and flask.

2.7 Separation of Soluble Proteins by Preparative Isoelectric Focusing

1. 50 mg freeze-dried protein sample (*see* **Note 11**).
2. 8 M deionized urea (*see* Subheading 2.4, **item 14**).
3. Rotofor Buffer: 7.25 M urea, 0.4 % 3–10 Pharmalytes, 1.6 % 4–7 Pharmalytes, 1 % w/v *N*-octylthioglucoside, 2 mM DTT (*see* **Note 12**).
4. Anode Buffer: 0.1 M H_3PO_4.
5. Cathode Buffer: 0.1 M NaOH.
6. Dialysis Buffer #1: 4 M urea, 10 mM NH_4HCO_3.
7. Dialysis Buffer #2: 2 M urea, 10 mM NH_4HCO_3.

8. Dialysis Buffer #3: 10 mM NH_4HCO_3.
9. Dialysis tubing (6,000–8,000 Da MWCO).
10. SDS-PAGE gel.
11. BCA Protein assay kit (*see* **Note 3**).
12. Rotofor Cell (*see* **Note 13**).
13. Rotofor anion exchange membrane.
14. Rotofor cation exchange membrane.
15. 5 mL Culture tubes.
16. Vacuum pump.

2.8 Separation of Proteins by Hydrophobic Interaction Chromatography

1. Protein sample (such as ammonium sulfate cuts obtained in Subheading 3.6).
2. Phenyl sepharose column (*see* **Note 14**).
3. LC pump capable of buffer gradient (*see* **Note 15**).
4. Buffer System Option #1 (*see* **Note 16**):
   (a) Buffer A: 1 M $(Na_4)_2SO_4$, 50 mM NaH_2PO_4, 1 mM DTT, pH 7.4.
   (b) Buffer B: 50 mM NaH_2PO_4, 1 mM DTT, pH 7.4.

   Filter through 0.45 μm filter and store at 4 °C for no more than 2 weeks. DTT must be added fresh daily.
5. Buffer System Option #2 (*see* **Note 17**):

   Buffer A: 10 mM KH_2PO_4, 1 mM EDTA, 1 mM DTT, 0.02 % w/v NaN_3, pH 7.2.

   Buffer B: 10 mM Tris-Base, 1 mM EDTA, 1 mM DTT, 0.02 % w/v NaN_3, pH 8.9.

   Buffer C: 10 mM Tris-Base, 1 mM EDTA, 1 mM DTT, 0.02 % w/v NaN_3, pH 8.9, 50 % v/v ethylene glycol.

   Filter through 0.45 μm filter and store at 4 °C for no more than 2 weeks. DTT must be added fresh daily.
6. Pyrogen free, 0.2 μm filtered Water.
7. 20 % v/v Ethanol, 0.45 μm filtered.
8. Syringe filter or filter unit.
9. SDS-PAGE gel.
10. BCA Protein assay kit (*see* **Note 3**).
11. Centrifugal filter unit, 10 kDa NMWL.
12. 10 mM NH_4HCO_3.

2.9 Preparation of Proteins for Analysis by LC-MS

1. Coomassie blue-stained gel containing protein of interest.
2. Glass plate.
3. 70 % v/v Ethanol.

4. Pretreated 0.65 mL microcentrifuge tubes: Fill the tubes with Extraction solution (60 % v/v acetonitrile, 0.1 % v/v TFA), invert tubes to mix, incubate at room temperature for 1 h and decant the solution. Repeat this process two times for each tube. Tubes must be thoroughly dry prior to use (*see* **Note 18**).
5. 37 °C incubator.
6. Vacuum concentrator.
7. Acetonitrile, LC-MS Grade.
8. 0.2 M NH_4HCO_3.
9. Formic Acid, high purity.
10. Trifluoroacetic acid (TFA), high purity.
11. Destain solution: 60 % v/v acetonitrile, in 0.2 M NH_4HCO_3.
12. Extraction solution: 60 % v/v acetonitrile, 0.1 % v/v TFA.
13. Modified trypsin sequencing grade: Dissolve 25 μg of trypsin in 300 μL of 0.2 M NH_4HCO_3. Reconstituted trypsin should be stored at 4 °C and can be used for up to 2 weeks (*see* **Note 19**).
14. Pyrogen free, 0.2 μm filtered water (*see* **Note 20**).
15. MS Buffer: 3 % v/v acetonitrile, 0.1 % v/v formic acid.

2.10 Preparation of Intact Proteins for Analysis by MALDI-ToF MS

1. Protein sample (*see* **Note 21**).
2. 0.1 % v/v Trifluoroacetic acid in 50 % v/v acetonitrile.
3. MALDI matrix: Tare an empty 0.65 mL microcentrifuge tube on the digital balance. Weigh out Sinapinic acid into the microcentrifuge tube. Add 0.1 % TFA, 50 % acetonitrile solvent to achieve a concentration of 10 mg/mL. Bath-sonicate for 5–10 min until the matrix is fully dissolved. Matrix should be made fresh for each use.
4. Calibration standard: Protein Standard II for Mass Spectrometry (*see* **Note 22**).
5. 384-well steel MALDI target plate.

3 Methods

3.1 Concentration of Spent Culture Filtrates from Mycobacterium spp.

1. Obtain a clean Amicon reservoir; cover all openings with aluminum foil, and autoclave.
2. Obtain a clean stirred Amicon ultrafiltration cell, and tubing for connection to the Amicon reservoir. Rinse all tubing and components of the Amicon ultrafiltration unit with 70 % ethanol and allow to air-dry.
3. Equilibrate the 5,000 MWCO Amicon membrane in 90 % isopropanol for 10 min with the shiny side down, followed by an additional incubation in pyrogen free, 0.2 μm filtered water for 30 min.

4. Assemble the Amicon ultrafiltration unit with the shiny side of the Amicon membrane facing up. It is important that all O-rings are properly seated to prevent leakage.
5. Transfer stirred cell and reservoir to a 4 °C chromatography cabinet or cold room.
6. Fill Amicon reservoir with culture filtrate and close the reservoir per assembly instructions. Connect tubing from compressed nitrogen to the input port of the reservoir.
7. Connect one end of the Amicon tubing to the output port of the Amicon reservoir and the other end to the input port on the top of the Amicon ultrafiltration unit.
8. Turn on mechanism to stir the cell.
9. Place the tubing connected to the output port on the base of the Amicon ultrafiltration unit in a receptacle to collect the waste.
10. Check that all pressure release valves are closed. Turn on the nitrogen gas (*see* **Note 23**).
11. Check the Amicon ultrafiltration unit to ensure the stirrer is turning, and that there are no leaks around the lid, base, or tubing connections. Also check to see that the CFP eluate is slowly flowing from the outlet port at the base of the ultrafiltration unit (*see* **Note 24**).
12. When CFP is reduced to the desired volume (at least 20-fold concentrated), turn off both the nitrogen gas and stirring mechanism, and vent the system at both the reservoir and the stirred cell.
13. Add 10 mM NH_4HCO_3 to reservoir to begin buffer exchange (*see* **Note 25**).
14. Close all pressure release valves; turn stirring mechanism and nitrogen gas on.
15. When the CFP in the Amicon ultrafiltration unit is reduced to desired volume, turn off the nitrogen gas, turn off the stirring mechanism, and vent the system at both the reservoir and the stirred cell.
16. Disconnect all tubing and remove the lid from the ultrafiltration unit.
17. Transfer the sample into a 0.2 μm filter unit and filter-sterilize.
18. Quantitate the amount of protein by BCA. Based on the assay results, run 5 μg of each sample on an SDS-PAGE gel to visualize protein bands.
19. Optional: Western Blot may be performed to confirm the presence or absence of specific proteins (*see* **Note 26**).
20. Store the sample at −80 °C or freeze dry.

3.2 Fracturing of Whole Mycobacterium spp. Cells by French Press [23]

1. Thaw γ-irradiated *Mycobacterium* spp. cells at 4 °C to reduce proteolysis (*see* **Note 4**).
2. Make breaking buffer by adding 30 μL of DNAase and 30 μL RNAase stock and protease inhibitor (at the manufacturer's suggested ratio) to 50 mL of PBS-EDTA buffer (*see* **Note 27**).
3. Suspend cells in breaking buffer at a concentration of 2 g/mL based on wet weight of cells. Cell solution should be quite viscous.
4. Place cells on ice.
5. Use a small amount of glycerol to lubricate French Press piston and base O-ring.
6. Assemble the pressure cell base by attaching the plug, sample outlet tube, and pressure release knob, making sure knob is closed completely (*see* Fig. 3).

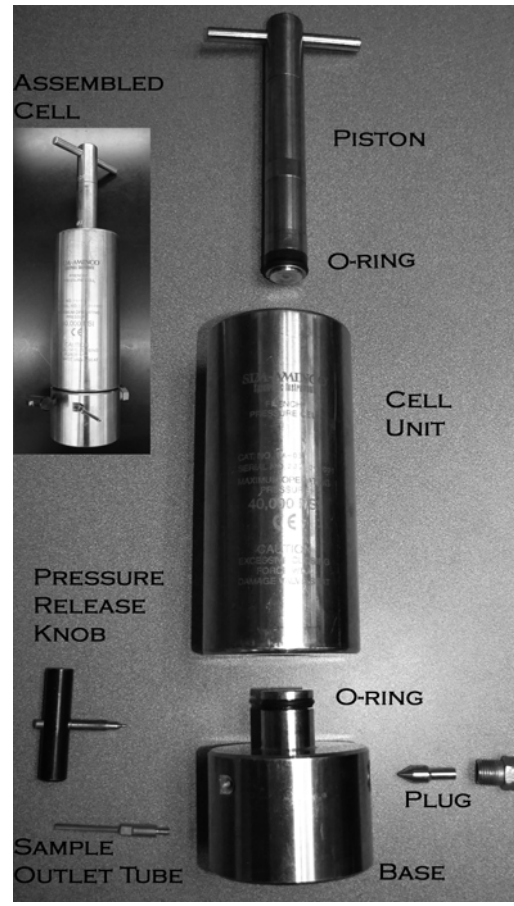

Fig. 3 Diagram of French Pressure Cell Unit with all parts. *Inset*: completely assembled unit

7. Place French Press piston into cell unit until reaching "max fill" mark.
8. Turn unit upside-down and place on French Press stand.
9. Add cell suspension to French Press cell unit, leaving enough room to attach base to the unit. Attach base to unit.
10. Keeping a tight hold of both the main cell unit and the base, turn the complete unit right-side up and place in French Press (*see* **Note 28**). Be sure that the unit is flush against the bottom pegs, and that the piston handle is perpendicular to the bracing bar.
11. Move the lever to the "High" setting and turn the French Press on.
12. Making small adjustments using the pressure release knob, keep pressure on cell between 1,000 and 1,500 while collecting eluent into a centrifuge tube (*see* **Note 29**).
13. When French Press cell is empty, turn machine lever to "Down".
14. Place eluent collection bottle on ice in ice bucket, and carefully remove cell from the French Press.
15. Repeat **steps 7–14** until the entire cell suspension has been passed through the French Press cell a total of six times.
16. Smear a small amount of cell paste on a glass slide and check breakage by acid fast staining (*see* **Note 30**).
17. When finished, thoroughly clean French Press using 10 % Lysol followed by 70 % ethanol.
18. Thoroughly clean French Press unit by completely disassembling unit, and washing each part with Lysol, distilled water, then 70 % ethanol.
19. Allow all parts to completely air-dry prior to storage.

3.3 Subcellular Fractionation of Mycobacterium spp. Whole Cell Lysate (WCL) (Fig. 4)

1. Dilute cell lysate to roughly 1 g (original weight of wet cell paste) per mL of PBS-EDTA buffer (*see* **Note 31**) and create a homogenous suspension by vortexing.
2. Centrifuge the lysate at 3,000 × *g*, 4 °C for 15 min to remove unbroken cells.
3. Decant the supernatant into clean 50 mL Oakridge centrifuge tubes and balance for centrifugation. Discard cell pellet (*see* **Note 32**).
4. Centrifuge the supernatant from **step 3** at 27,000 × *g*, 4 °C for 1 h.
5. Decant the supernatant (cytosol and membrane) into clean 50 mL Oakridge centrifuge tubes.
6. Suspend the pellet (cell wall) in breaking buffer without DNAase or RNAase (30 mL) and form a homogeneous suspension by vortexing.

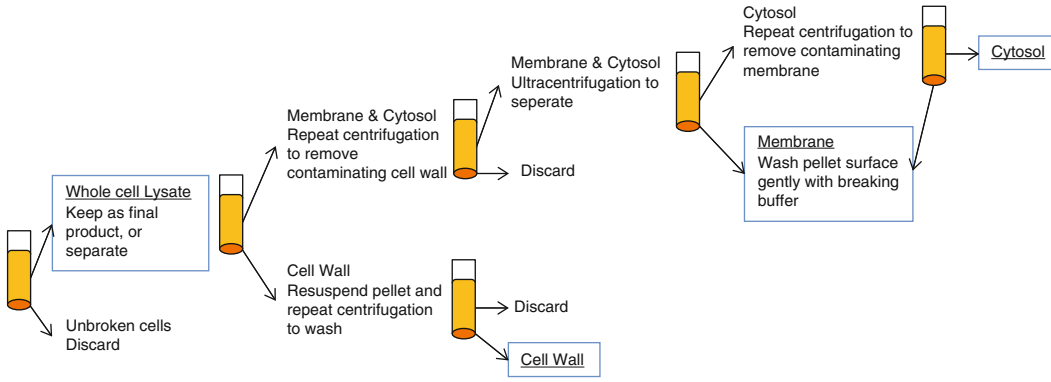

Fig. 4 Work-flow for generation of whole cell lysate, cell wall, membrane and cytosol from cells

7. Centrifuge the supernatant (cytosol and membrane) and the resuspended pellet (cell wall) at 27,000 × *g*, 4 °C for 1 h to remove residual contaminating material.
8. Decant and discard the supernatant from the pellet (cell wall) and store the pellet at 4 °C. Collect the supernatant (from the cytosol and membrane spin) in 40 mL ultracentrifuge tubes and discard the pellet.
9. Balance the ultracentrifuge tubes very precisely (*see* **Note 33**).
10. Centrifuge the supernatant (cytosol and membrane) at 100,000 × *g*, 4 °C for 4 h.
11. Collect the supernatant in clean 40 mL ultracentrifuge tubes and repeat **steps 9** and **10** (Fig. 4).
12. Gently wash the pellets from **steps 10** and **11** (membrane) in breaking buffer without DNAase or RNAase and store at 4 °C (*see* **Note 34**).
13. Collect the final 100,000 × *g* supernatant (cytosol) in 50 mL conical tubes.
14. Resuspend the reserved cell wall pellet(s) from **step 8** in 10 mM NH_4HCO_3 and pool cell wall pellets together.
15. Suspend the membrane pellets from **step 12** in 10 mM NH_4HCO_3 and pool.
16. Cut lengths of dialysis tubing long enough to fit the volumes of each product, being sure to allow room for expansion during dialysis. Boil the tubing.
17. Secure one end of boiled dialysis tube, either by tying or attaching a dialysis clip, fill with sample, and secure the other end (*see* **Note 35**).
18. Place the filled dialysis tubes in 7 L of 10 mM NH_4HCO_3 and place at 4 °C for 4–16 h.

19. Change to fresh 10 mM NH_4HCO_3 and dialyze for another 4–16 h. Repeat once more for a total of three buffer exchanges.
20. Remove the samples from their dialysis membranes and transfer to clean sample tubes.
21. Quantitate the amount of protein by BCA (*see* **Note 3**). Based on the assay results, run 3–4 μg of each product on SDS-PAGE gel to visualize protein bands.
22. Store the sample at −80 °C.

3.4 Extraction of Proteins from Subcellular Fractions Using Detergents

3.4.1 Proteins Can Be Extracted Using SDS, Guanidium, or Urea: Extract Proteins Using SDS as Follows

1. Heat 600 mL of pyrogen-free, 0.2 μm filtered water to 60 °C on a heated stir plate.
2. Transfer protein preparation to a sterile 50 mL Oakridge centrifuge tube and add 30 mL of 2 % SDS in PBS (w/v). Add micro-stir bars to samples. Cap tubes and place in heated water for 1 h, ensuring sample is stirring throughout extraction procedure [24] (*see* **Note 7**).
3. Remove Oakridge tubes from heated water bath and let cool to room temperature. Remove stir bars. Centrifuge the sample at 27,000 × *g*, 22 °C for 30 min. Collect the supernatant and reserve at 4 °C.
4. Repeat **steps 2** and **3** three additional times. SDS soluble protein is now extracted. Continue to **steps 5–9** if removal of SDS is required for downstream applications.
5. Pool the reserved supernatants into a large glass cylinder sufficient to permit dilution of sample 20-fold; this is necessary to effectively remove SDS detergent by paired ion extraction.
6. To perform a paired-ion extraction, add 85 % acetone, 5 % glacial acetic acid, 5 % triethylamine and 5 % aqueous volume of protein extract and incubate at −20 °C for 4–16 h [25].
7. Decant organic solvent (discard as hazardous waste) until a concentrated slurry can be recovered and transferred to a 50 mL Oakridge tube.
8. Centrifuge the protein slurry at 27,000 × *g*, 22 °C for 30 min.
9. Decant organic solvent (dispose as hazardous waste); add 30 mL of pyrogen free, 0.2 μm filtered water to pellet and wash gently.
10. Centrifuge the protein solution at 27,000 × *g*, 22 °C for 30 min.
11. Repeat water wash and centrifugation two additional times; remaining pellet, SDS-soluble protein fraction, may be used as needed for downstream applications.

3.4.2 Extract Proteins Using Guanidium as Follows

1. Prepare fresh 6 M Guanidine-HCl in pyrogen free, 0.2 μm filtered water just prior to protein extraction.
2. Transfer protein preparation to a sterile 50 mL Oakridge centrifuge tube and add 30 mL of 6 M Guanidine-HCl. Add micro-stir bars to sample tubes. Cap tubes and place on stir-plate.
3. Stir sample extractions at room temperature (22 °C) for 1 h.
4. Remove stir bars. Centrifuge the protein extract at 27,000 × *g*, 22 °C for 30 min. Collect the supernatant into a new tube and reserve at 4 °C.
5. Add an additional 30 mL of 6 M Guanidine-HCl solution to the remaining pellet, add stir bars and repeat extraction and centrifugation for a total of three extractions per sample.
6. Prepare dialysis tubing and exchange Guanidine-HCl extracts into 10 mM NH_4HCO_3.

3.4.3 Extract Proteins Using Urea as Follows

1. Transfer protein preparation to a sterile 50 mL Oakridge centrifuge tube and add 30 mL of 8 M urea. Add micro-stir bars to sample tubes. Cap tubes and place on stir-plate.
2. Stir sample extractions at room temperature (22 °C) for 1 h.
3. Remove stir bars. Centrifuge the protein extract at 27,000 × *g*, 22 °C for 30 min. Collect the supernatant into a new tube and reserve at 4 °C.
4. Add 30 mL of 8 M urea to the pellet, add stir bars, and repeat extraction and centrifugation for a total of three extractions per sample.
5. Prepare dialysis tubing or dialysis cassettes and exchange 8 M urea extracts first into 4 M urea at room temperature (22 °C) for 4–16 h. Repeat dialysis into 2 M urea at 4 °C for 4–16 h. Perform final dialysis into 10 mM NH_4HCO_3 (*see* **Note 36**).

3.5 Partitioning of Hydrophobic and Hydrophilic Proteins Using Triton X-114 [26]

1. Suspend protein sample in 4 % TX-114 (*see* **Note 37**). If starting from a sample that is already suspended in PBS, add 0.125 mL of 32 % TX-114 stock solution per 1 mL of PBS/protein solution to bring the total solution to 4 %. Rock at 4 °C for 8–16 h.
2. Centrifuge at 27,000 × *g*, 4 °C for 1 h. Collect the supernatant into clean centrifuge bottles and place at 4 °C for later use.
3. Resuspend the pellets in 4 % TX-114 and incubate with rocking at 4 °C for 1–2 h; repeat **step 2**.
4. Combine the two supernatants and repeat centrifugation to remove any remaining insoluble material. Transfer the supernatant to new centrifuge bottle(s) and repeat centrifugation until no visible pellet is obtained.

5. Incubate tubes containing the final clarified supernatant in a 37 °C water bath until a partition appears (1–2 h).
6. Centrifuge at 27,000 × *g*, at 25–37 °C for 1 h.
7. Using a pipette, remove the upper aqueous layer, making note of the volume being removed. Be sure to remove all of the aqueous material. To the TX-114 layer, add a volume of PBS equal to that removed.
8. Repeat **steps 5**–7 twice.
9. To the final TX-114 layer, add approximately 9× the volume of ice cold acetone and place the samples at –20 °C for 8–16 h.
10. Centrifuge the samples at 27,000 × *g*, 4 °C for 1 h. Decant the supernatant and dispose of as hazardous waste.
11. Wash the pellet with ice cold acetone, repeat centrifugation, decant and dispose supernatant.
12. Remove residual acetone by applying a gentle stream of nitrogen using a nitrogen bath, or by leaving the tubes open in a chemical fume hood until dry.
13. Suspend each acetone precipitate in 30 mL of PBS. It may be necessary gently scrape the pellet from the side of the centrifuge bottle and to slowly stir on a stir plate. The pellet will not fully go into solution.
14. Split each 30 mL sample between two 50 mL Oakridge centrifuge tubes. Add 15 mL of PBS-saturated phenol to each sample in a chemical fume hood (*see* **Note 38**). Rock at RT for 4 h.
15. Centrifuge at 27,000 × *g*, 25 °C for 1 h.
16. Remove the aqueous upper layer without disturbing the interface. Note the volume of aqueous layer removed. To the phenol layer, add a volume of PBS equal to that removed. Rock at room temperature for 4 h.
17. Repeat **step 15** and remove aqueous layer.
18. Prepare dialysis tubing and transfer the final phenol layer and interface to dialysis tubing.
19. Place in a 7 L dialysis tank and dialyze for 48–72 h with running deionized water. During dialysis, occasionally gently knead the tubing to help break up large chunks of material, being sure to wear gloves in order to prevent exposure to any residual phenol.
20. Transfer the dialysis tubing to 7 L of pyrogen free, 0.2 μm filtered water and dialyze at 4 °C for 24 h.
21. Remove the samples from their dialysis membranes and transfer to clean sample tubes. Rinse the dialysis tubing with pyrogen free, 0.2 μm filtered water to recover any particulate material.

22. Make a homogeneous suspension of the material by breaking apart large aggregates using bath sonication and/or manually breaking using a cell scraper.
23. Quantitate the amount of protein by BCA (*see* **Note 3**). Based on the assay results, run 3–4 μg of each sample on SDS-PAGE gel to visualize protein bands.

3.6 Partitioning of Hydrophobic and Hydrophilic Proteins Using Ammonium Sulfate

1. Determine the volume of your soluble protein mixture. Go to http://www.encorbio.com/protocols/AM-SO4.htm, an online calculator, and enter in the starting volume, temperature (which will be 4 °C), desired ammonium sulfate percentage (40 %), and starting ammonium sulfate percentage (0 %). The website will calculate the amount of ammonium sulfate that will need to be added (*see* **Note 39**).
2. Add a stir bar to the protein solution, place it on a stir plate, and begin stirring. Gradually add the ammonium sulfate crystals and stir until completely dissolved. Transfer sample to 4 °C (*see* **Note 40**).
3. Incubate the sample at 4 °C for 4–16 h; be sure sample is stirring at all times (*see* **Note 41**).
4. Remove the stir bar. Divide the protein solution equally among centrifuge bottles and balance. Centrifuge at 27,000 × *g*, 4 °C for 1 h.
5. Decant the supernatant into a clean container. Store the pellet at 4 °C. This pellet is the 40 % ammonium sulfate cut.
6. Determine the volume of the supernatant and repeat the online calculation as in **step 1**, entering the new volume, desired ammonium sulfate percentage (70 %), and starting ammonium sulfate percentage (40 %).
7. Repeat **steps 2–5** (*see* **Note 42**). This pellet is the 70 % ammonium sulfate cut.
8. Resuspend each pellet separately in 10 mM NH_4HCO_3.
9. Prepare dialysis tubing and add sample.
10. Place in 7 L dialysis buffer #1 and dialyze at 4 °C for 4–16 h.
11. Change to fresh dialysis buffer #1 and dialyze for another 4–16 h.
12. Change to dialysis buffer #2 (containing no DTT) and dialyze for 4–16 h.
13. Remove the samples from their dialysis membranes and transfer to clean sample tubes.
14. Quantitate the amount of protein by BCA (*see* **Note 3**). Based on the assay results, run 3–4 μg of each sample on SDS-PAGE gel to visualize protein bands.

15. Freeze the sample at –80 °C for at least 2 h. Loosen the tube lid or remove the lid and cover loosely with foil, put the sample tube in a freeze-dry flask, and place on a freeze-dryer until dry.

3.7 Separation of Soluble Proteins by Preparative Isoelectric Focusing

1. Hydrate the Rotofor membranes overnight: anion membrane in cathode buffer, cation membrane in anode buffer.
2. Resuspend the freeze-dried protein sample in 50 mL of Rotofor buffer and stir at room temperature for 8–16 h. If the protein does not fully solubilize overnight, bath sonication using a warm water bath may be used.
3. Assemble the Rotofor cell. Be sure that the membranes and anode and cathode buffers are used on the correct sides (*see* Fig. 5).
4. Fill the sample chamber with water, connect the Rotofor to a power supply, and run at 5 W for 20 min to check for proper assembly and leaks. Harvest the water out of the system and replace the tape.
5. Add the entire 50 mL of sample to the Rotofor.
6. Run at 6 W for 12 min, followed by 12 W, taking note of the volts every 15 min. The run is finished when the volts stabilize for at least 30 min (*see* Fig. 6) total run time is expected to be about 4 h. Run water (20–25 °C) through the cooling finger during the run to prevent overheating; do not use cold water as this can cause the urea to precipitate.
7. Once the voltage has stabilized, turn off the power and turn the Rotofor switch to "Harvest". This will put the Rotofor into the correct position. Use a vacuum pump to harvest the fractions into 5 mL culture tubes per instructions.

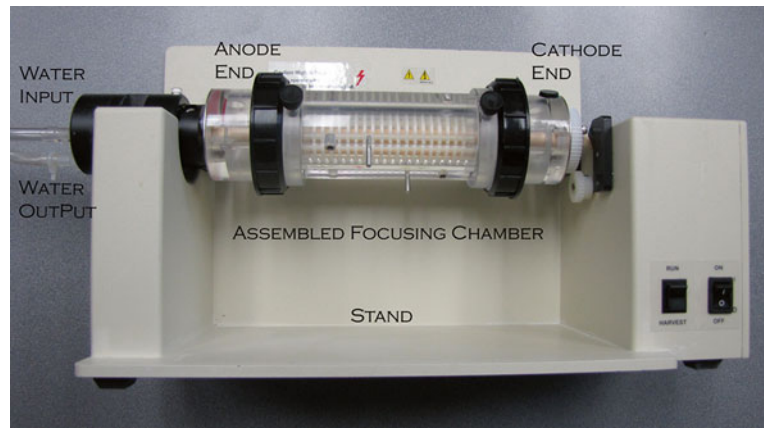

Fig. 5 Properly assembled Rotofor unit for preparative isoelectric focusing

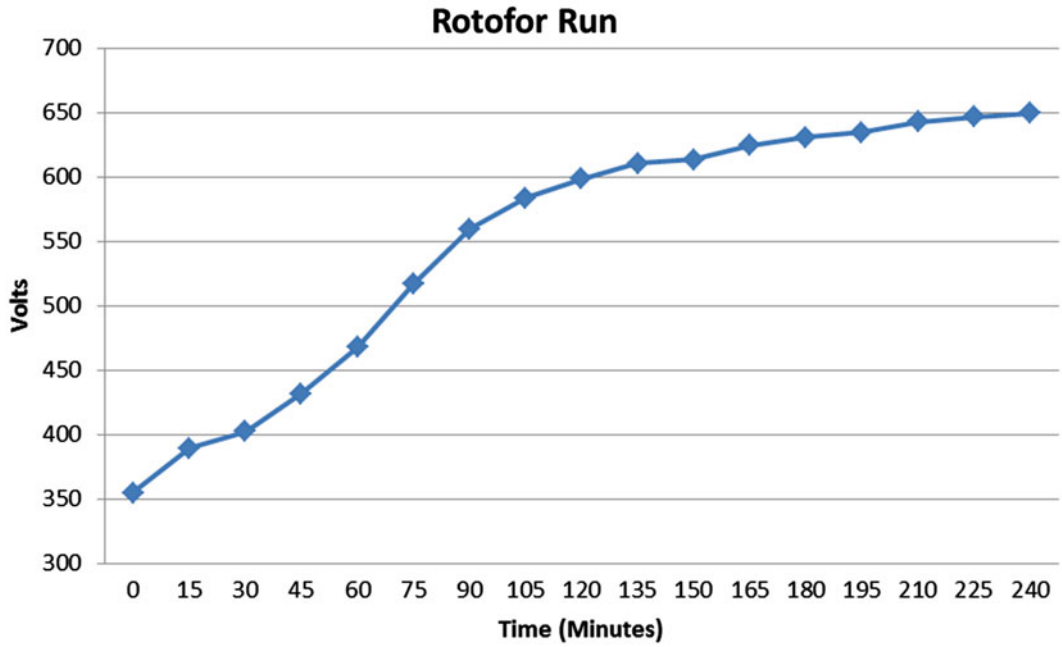

Fig. 6 Voltage recorded over time during a successful IEF run using the Rotofor apparatus

8. Run 10 µL of each fraction on SDS-PAGE gel to visualize. Once it is determined which fractions contain the protein(s) of interest, pool fractions together (*see* **Note 43**).
9. Prepare dialysis tubing and add sample.
10. Place in 7 L dialysis buffer #1 and dialyze at room temperature for 4–16 h.
11. Change to dialysis buffer #2 and dialyze at 4 °C for 4–16 h.
12. Change to dialysis buffer #3 and dialyze at 4 °C for 4–16 h.
13. Remove the sample from the dialysis membrane and transfer to a clean sample tube.
14. Quantitate the amount of protein by BCA (*see* **Note 3**). Based on the assay results, run 2 µg of sample on SDS-PAGE gel to visualize protein bands (*see* **Note 44**).

3.8 Separation of Soluble Proteins by Hydrophobic Interaction Chromatography [27]

1. Resuspend the protein sample at 1–2 mg/mL in Phenyl Sepharose Buffer A (*see* **Note 45**). Filter sample through 0.2 µm syringe filter or filter unit.
2. Prime LC pump in pyrogen free, 0.2 µm filtered water to ensure that all lines are free of air and other solutions, and then attach the phenyl sepharose column.

3. Wash the column with at least one column volume (CV) of water to remove storage buffer.
4. Prime line A in Buffer A of the selected buffer system and allow the column to equilibrate with at least one CV of Buffer A at 1 mL/min.
5. Gradient for Buffer System #1: Place line A into the prepared sample and allow it to load onto the column at 1 mL/min, being sure to collect the flow-through into a clean tube. Pass the flow-through back over the column to ensure that all of the protein has bound to the column. Program and run the gradient as follows, collecting 40 fractions during the gradient portion:

   2 CV Buffer A Wash

   20 CV Buffer A to Buffer B Gradient

   10 CV Buffer B Cleanup
6. Gradient for Buffer System #2: Place line A into the prepared sample and allow it to load onto the column at 1 mL/min, being sure to collect the flow-through into a clean tube. Program and run the gradient as follows, collecting 115 fractions starting at the A–B gradient and continuing to the end (*see* **Note 46**):

   5 CV Buffer A Wash

   1 CV Buffer A to Buffer B Gradient

   1 CV 100 % Buffer B

   1 CV 100 % Buffer B to 85 % Buffer B/15 % Buffer C Gradient

   1 CV 85 % Buffer B/15 % Buffer C

   1.5 CV 85 % Buffer B/15 % Buffer C to 40 % Buffer B/60 % Buffer C Gradient

   1 CV 40 % Buffer B/60 % Buffer C

   1 CV 40 % Buffer B/60 % Buffer C to 100 % Buffer C Gradient

   2 CV 100 % Buffer C
7. Upon completion of the LC run, clean the columns by running at least one CV of pyrogen free, 0.2 μm filtered water, followed by at least one CV of 20 % ethanol for storage.
8. Run 10 μL of each fraction on an SDS-PAGE gel to visualize protein bands (*see* **Note 47**). Store fractions at 4 °C.
9. Once it is determined which fractions contain the protein(s) of interest, pool the fractions.

10. Transfer the pool to a centrifugal filter unit with a 10 kDa MWCO (or appropriate cutoff for your protein). Centrifuge at 3,500 × *g*, 4 °C, checking approximately every 20–30 min until concentrated. If necessary, continue to add sample and concentrate until the entire sample is combined.
11. Fill the centrifugal filter unit with 10 mM NH_4HCO_3 and concentrate. Repeat two more times. Collect the final, concentrated buffer exchanged sample into a clean sample tube.
12. Quantitate the amount of protein by BCA (*see* **Note 3**). Based on the assay results, run 2 μg of each sample on SDS-PAGE gel to visualize protein bands (*see* **Note 48**).

3.9 Preparation of Proteins for Analysis by LC-MS [28, 29]

1. From a Coomassie stained 1D polyacrylamide gel, excise protein bands of interest with a razor blade on a glass plate, after cleaning both with 70 % ethanol (*see* **Note 49**).
2. Cut each spot into small pieces (~1 mm by 1 mm) and place the pieces in a pretreated microcentrifuge tube.
3. Destain by covering the gel pieces with destain solution, vortex, and incubate at 37 °C for at least 30 min or until most of the stain is removed from gel pieces.
4. Centrifuge briefly to remove condensation from the upper tube and lid and discard the destain solution. Repeat **steps 3** and **4** up to three times until the gel is completely destained.
5. Dry the gel pieces in the vacuum concentrator.
6. Add trypsin solution to the gel slices so that the gel slices are covered (generally 3–5 μL).
7. Incubate at room temperature until the trypsin solution is completely absorbed by the gel slices (~15 min).
8. Add 0.2 M NH_4HCO_3 in 10–15 μL increments to completely re-swell the gel (*see* **Note 50**).
9. Incubate the gel slices for 12–18 h at 37 °C.
10. Add 100 μL of the extraction solution to the gel slices and vortex.
11. Incubate the samples at 37 °C for 20 min, vortex, and repeat 37 °C incubation for an additional 20 min.
12. Centrifuge the samples briefly and transfer the supernatant in a new pretreated microcentrifuge tube. Avoid pipetting any gel slices into pipet tips when collecting supernatant.
13. Repeat **steps 10–12** combining the supernatant in the tube from **step 12**.

14. Place the combined supernatant extract in the vacuum concentrator to remove acetonitrile and concentrate the peptide digest (*see* **Note 51**).
15. Samples can be stored at −20 °C until further processed for LC-MS analysis.
16. Thaw samples and resuspend in MS buffer so that sample is a roughly at a concentration of 0.5–1 μg/μL.
17. Centrifuge samples at 14,000 × *g* for 5 min.
18. Gently pipet the sample out of the tube, being sure not to touch the bottom of the tube with the pipet tip (there will be a small volume left in the tube) and transfer sample to a compatible autosampler vial, being careful not to introduce any bubbles into the vial. Cap and label vials and submit for LC-MS analysis.

3.10 Preparation of Intact Proteins for Analysis by MALDI-ToF MS

1. Make a fresh MALDI matrix solution as described in Subheading 2.10.
2. Spot the calibration standard on the MALDI target plate by pipetting 1 μL of standard on plate first (otherwise, the matrix might dry before the mixing occurs). Next, pipette 1 μL of matrix on top of standard and mix well by pipetting up and down.
3. Spot sample (fraction) on the MALDI target plate by pipetting 1 μL of sample on plate first, as above. Next, pipette 1 μL of the matrix solution on top of the sample and mix well by pipetting up and down. Allow the mixture to air-dry (*see* **Note 52**).
4. Samples and standards should be arranged on the plate as shown in Table 1.
5. Submit target plate to a core facility for MALDI-ToF analysis; be sure to include plate diagram with submission. The resolution and accuracy of the instrument should provide data indicating fraction purity and separation of proteins within 1 kDa (*see* montage provided in Fig. 7).

Table 1
Example of proper sample arrangement on a MALDI plate, with all unknown samples surrounding a standard for calibration purposes

Sample 1	Sample 8	Sample 7	Sample 9	Sample 16	Sample 15
Sample 2	*Standard*	Sample 6	Sample 10	*Standard*	Sample 14
Sample 3	Sample 4	Sample 5	Sample 11	Sample 12	Sample 13

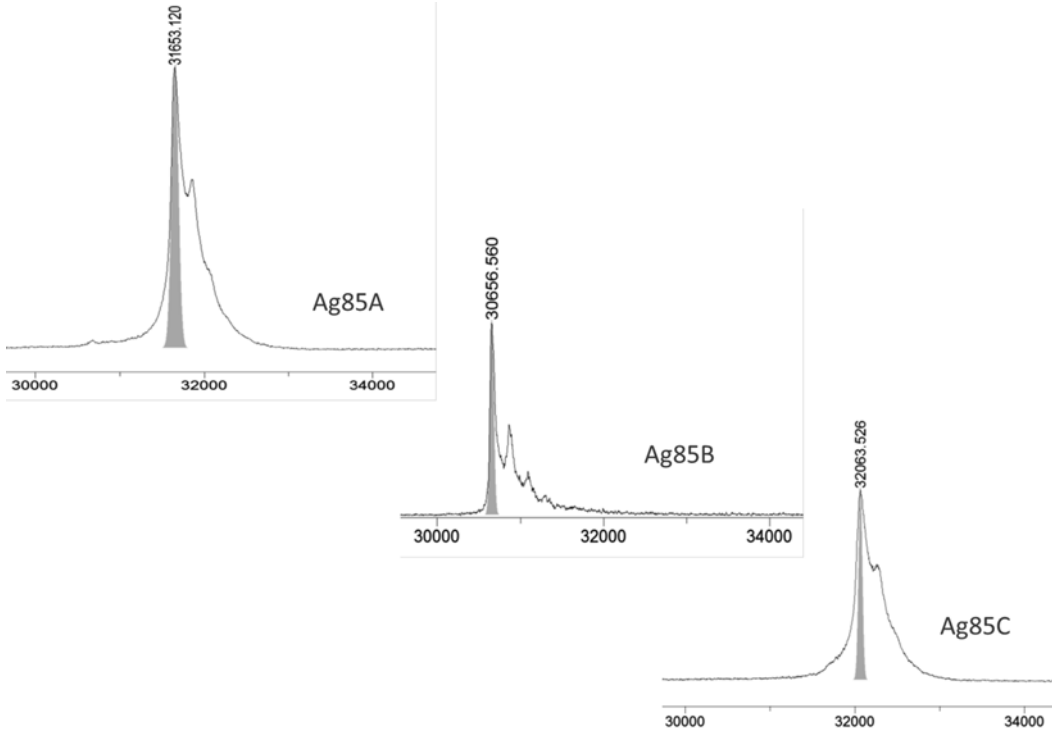

Fig. 7 Typical MALDI-ToF MS spectra demonstrating resolution of three similar proteins

4 Notes

1. If CFP is not processed immediately, 0.02 % sodium azide, or another bacteriostatic chemical should be added to prevent contamination.
2. Multiple volumes are available, so select an appropriate volume.
3. Any protein quantitation method may be used as long as it is compatible with the buffers used to concentrate the sample. Be sure to use protein standards made in the same buffer as your sample.
4. *Mycobacterium* spp. cells may be inactivated by γ-irradiation (2.4 mRad dose) or by another adequate means of destroying viable cells, such as treatment with chloroform–methanol (2:1) for 30 min or heating (80 °C/1 h).
5. French Pressure Cell (SLM-AMINCO) 40,000 PSI was used in development of this protocol. If another cell is used, scale pressures accordingly.

6. Whole cell lysate (WCL) may be generated by French Press sonication as in Subheading 3.2, or by other means of cell disruption not detailed in this chapter. WCL, and soluble fractions thereof, contain highly labile proteins and proteases; it is important to keep the samples cool at all times and perform work quickly prior to loss of activity from protease inhibitor cocktails.
7. These extraction steps were optimized for the extraction of 100 mg of freeze-dried protein sample in 50 mL Oakridge tubes. If protein sample is in aqueous solution and cannot be freeze-dried, precipitate protein out of solution by adding 4× (v/v) of 100 % ice-cold acetone and incubating at −80 °C for 1 h. Pellet protein precipitate by centrifugation and proceed with detergent extraction procedures. Adjust volumes and materials accordingly for smaller protein extracts. Recovery of extracted proteins is variable among protein sample types and detergent used for processing. Average recoveries from 100 mg of total protein sample range between 40 and 60 %, as determined by protein assay.
8. If the biphase does not appear after several h on the first incubation, or if the biphase is too small to remove, double the volume with PBS and proceed to the next stirring and incubation step. A biphase should definitely appear following the second and third incubations. If it does not, increase the incubation temperature to 55 °C.
9. Ethanol may also be used if acetone is unavailable.
10. Use caution while working with phenol. Always handle phenol in a chemical fume hood, wearing proper PPE, and only use glass pipettes.
11. Protein sample should be freeze-dried and free of salts. If the sample cannot be freeze-dried, it may also be precipitated using ice-cold acetone as described in **Note 7**. One example that uses this protocol is HspX (16 kDa, Rv2031c) [30, 31].
12. Use deionized 8 M urea to prepare this buffer. Be sure to check the percent of the purchased Pharmalyte concentrated solution to ensure that the proper final percentage is achieved in this buffer. If the buffer is prepared ahead of time, DTT should be fresh on the day of use. Store the buffer at room temperature, as the high concentration of urea will precipitate out of solution if stored at 4 °C.
13. This protocol is based on specific use of a full sized Rotofor Cell (Bio-Rad). If using a Mini Rotofor Cell or MicroRotofor Cell, please refer to the user manual and make adjustments as necessary.

14. Columns come in various sizes and applications based on the quantity of sample being separated and the type of equipment available. In general a 60 mL column can be used for large protein amounts (200–1,000 mg), a 20 mL column for protein amounts ranging between 10 and 200 mg, or a HiTrap 1 mL column for very small protein amounts (0.5–5.0 mg).
15. Higher resolution can be achieved by use of an HPLC system and increasing column flow rates to ~2 mL/min. Please refer to system manual for details on use of specific equipment.
16. This buffer system was optimized for purification of PstS1 (38 kDa, Rv0934) from a 70 % ammonium sulfate (~2.9 M) cut of culture filtrate proteins. A descending ammonium sulfate gradient is a common phenyl sepharose buffer system; however, some proteins require different initial ammonium sulfate concentrations based on their hydrophobicity in order to remain soluble and permit subsequent purification using this resin.
17. This buffer system was optimized for purification of Ag85 complex and its components (Rv3804c, Rv1886c, Rv0129c) from a 40 % ammonium sulfate cut (~1.6 M) of culture filtrate proteins. It is optimized for the separation of proteins with highly similar physiochemical properties. Sodium azide may be omitted if the fractions will be processed immediately. However, if fractions will be refrigerated for more than 48 h it is advised to keep sodium azide (0.02 %) or another bacteriostatic chemical in the samples.
18. Microcentrifuge tubes must be pretreated to remove plastic polymers prior to use in mass spectrometry.
19. This procedure can be used with proteases other than trypsin; however, the buffer for the digestions may differ for other proteases.
20. All solutions should be made using pyrogen free, 0.2 μm filtered water.
21. In the example used here, the protein samples are represented by the fractions collected during Subheading 3.8. This protocol is compatible with fractions in buffer compositions similar to those described in Subheading 2.8, option #2. Fractions containing higher concentrations of salt or other detergents should be exchanged in a compatible buffer prior to processing for mass spectrometry.
22. Protein Standard II is specifically for mass range ~20,000–70,000 Da. When ordering standard, make sure the mass range is appropriate for your protein of interest.
23. Ensure the pressure on the nitrogen regulator does not exceed limits specified by membrane *and* cell.

24. If a leak is found or the output flow is too great, shut off the flow of nitrogen and release pressure from the reservoir, then from the stirred cell. Leaks at the lid or base are generally caused by poorly seated O-rings. Leaks from tubing connections may require tightening, or the use of Teflon tape. If the output flow is too fast, this could indicate a poorly seated membrane or O-ring, or a flaw in the membrane. Once the problem is corrected, repeat startup procedures.
25. To ensure complete exchange of the buffer, 20 times the CFP volume of 10 mM NH_4HCO_3 should be added to the reservoir. For example, if there is 100 mL of CFP remaining, 2 L of 10 mM NH_4HCO_3 should be added to reservoir.
26. Suggested antibodies for *M. tuberculosis* CFP analysis by Western Blot include anti-GroES (Rv3418c), anti-SodA (Rv3846), anti-Ag85 complex (Rv0129c, Rv1886c, and Rv3804c), anti-PstS1 (Rv0934), and anti-DnaK (Rv0350) as positive controls and anti-GroEL2 (Rv0440) as a negative control. These and other anti-*Mycobacterium* antibodies are available through an NIH/NIAID biological reagent repository such as BEI resources (http://www.beiresources.org/).
27. It is important to keep the buffer cool (in a refrigerator or on ice) after the addition of the protease inhibitor tablet, DNAase, and RNAase; adjust stocks and protease inhibitor quantities accordingly depending on the volume of breaking buffer needed.
28. Care should be taken to hold onto the unit bottom, to prevent sample loss due to the bottom falling off.
29. Care should be taken to point mouth of bottle away from the face, as small air pockets inside the French Press cell may cause unpredictable, volcanic expulsion of eluent.
30. Roughly 90 % of the cells will be broken by this method. WCL will appear as a field of blue debris with few pink bacilli. If cells are not broken, pass back over the French Press until sufficient cell disruption is achieved.
31. If using lysate generated from Subheading 3.2, double the French Press lysate volume.
32. Supernatant is whole cell lysate (WCL). If WCL is one of the desired products, remove desired quantity, dialyze and store. Use remaining supernatant to continue with protocol.
33. Centrifuge tubes must be completely full and must be balanced to within 100 mg using an electronic balance.
34. Wash the pellet *before* storing. Waiting to wash the pellet will make disruption of the pellet during washing more likely. If the pellets are disrupted during the washing step, fill the centrifuge tubes with breaking buffer, balance and centrifuge as in Subheading 3.3; **steps 9** and **10**.

35. Ensure each dialysis tube is identified with its contents by labeling the clip or float so final products are not confused.
36. The step-dilution dialysis for urea helps prevent the urea from precipitating out of solution.
37. If partitioning whole cell lysate, TX-114 may be added to the breaking buffer (*see* Subheading 3.2) to a final concentration of 4 %; this procedure can be initiated at the conclusion of Subheading 3.2.
38. Be sure that the centrifuge tubes are suitable for use with phenol (the recommendation here is Teflon).
39. Any ammonium sulfate percentage may be used depending on your needs; however, many *M. tuberculosis* CFPs are differentiated for purification by beginning with a 40 % ammonium sulfate cut of CFP followed by a 70 % ammonium sulfate cut (*see* Fig. 2), hence why this example is used.
40. The solution may begin to become cloudy as protein precipitates out of solution, but it should be easy to differentiate between this and undissolved ammonium sulfate because the ammonium sulfate will appear as crystals that settle to the bottom of the container.
41. A shaker or rocker may also be used if a stir plate is unavailable for incubation.
42. There should be little to no protein left in the 70 % supernatant; the supernatant is generally discarded once it is confirmed that the protocol was successfully implemented.
43. If samples need to be stored, they can be covered and kept at 4 °C, but the urea will precipitate out of solution. Once brought back to room temperature, you can use a vortex, bath sonicator, and/or gentle heat to solubilize the urea.
44. In some cases, the protein(s) of interest may not be pure after the Rotofor. "Dirty" fractions can be freeze-dried and cleaned up by separating on the Rotofor a second time. An alternate method for proteins with minor contaminants is to use size exclusion chromatography [30].
45. Some proteins will require long incubation (12–16 h) with agitation at 4 °C in order to be fully resuspended, and may still have precipitate even after this incubation. Bath sonication may be used. If precipitate is still visible, centrifuge at 3,000 × *g*, 4 °C, for 15 min before filtration.
46. If the LC pump only has two buffer lines it may be necessary to program the gradient in two parts. This gradient is very complex and has many stopping points due to the fact that it has been specifically optimized for separation of Ag85 A, B,

and C from each other; three proteins that are very similar in all their physiochemical properties. Separation of your proteins of interest may not require such complicated gradients and should be optimized as needed empirically.

47. Each fraction containing protein may be further analyzed as necessary by western blot (if a specific protein is being purified and antibodies are available), LC-MS (*see* Subheading 3.9) or MALDI-Tof MS (*see* Subheading 3.10).
48. If additional cleanup of the protein of interest is needed, size exclusion chromatography may be useful. Alternatively, minor low molecular weight contaminants can be removed by using centrifugal filter units with a molecular weight cutoff size larger than the contaminant(s), but small enough that your protein of interest will remain in the retentate.
49. Gel can either be run to completely resolve individual bands or alternatively, as a means of removing potential contaminants (i.e., detergents, buffer components) prior to digestion of a complex sample. For the latter method, the gel is run for roughly 5 min (depending on electrophoresis system) or until sample has migrated roughly 1 cm into the gel.
50. Allow 10–15 min for swelling of the gel pieces between additions of NH_4HCO_3. The final volume should just cover the tops of the gel pieces.
51. Sample should not dry to completion in the vacuum concentrator as this may result in low peptide recovery for LC-MS due to poor solubility of some precipitated peptides in traditional LC-MS loading buffers; rather samples should be removed in ~5–20 μL of solution. It is reasonable to assume that the residual solution is acidified water and compatible with traditional LC-MS initial buffer conditions. MS buffer may be added to samples to bring all samples up to an equal volume (20 μL is the recommended volume per sample if the concentration is unknown).
52. If any of the samples contain ethylene glycol or other somewhat viscous substances that do not dry quickly, allow plate to dry overnight.

Acknowledgements

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Chapter 5

Lipid and Lipoarabinomannan Isolation and Characterization

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Abstract

Mycobacteria are microorganisms that contain a very high content of structurally diverse lipids, some of them being biologically active substances. As such the lipid composition is commonly used to characterize mycobacterial strains at the species and type-species level. This chapter describes the methods that allow the purification of the most commonly isolated biologically active lipids and those used for analyzing extractable lipids and their constituents, cell wall-linked mycolic acids and lipoarabinomannan (LAM). The latter involve simple chromatographic and analytical techniques, such as thin-layer chromatography and gas chromatography coupled to mass spectrometry.

Key words Lipids, Glycolipids, Mycolic acids, Cell wall, Lipid analysis

1 Introduction

Mycobacteria are extraordinarily rich in lipid, constituting up to 40 % of their dry weight. Compared to the lipid-rich cell wall of Gram-negative bacteria, with some 20 %, the mycobacterial cell walls contain up to 60 % lipids [1]. This high lipid content has been associated with various other singular properties of mycobacteria, such as their acid fastness, the granuloma formation, and their impermeability to antibiotics and solutes [2]. Expectedly, the sequenced mycobacterial genomes contain a high percent of genes encoding proteins putatively involved in lipid metabolism.

Mycobacterial lipids are organized to form, like Gram-negative bacteria, two membranes [3]: (1) the plasma membrane whose composition is similar to other membranes, i.e., a bilayer of phospholipids and proteins, and (2) an outer membrane (mycomembrane), whose inner leaflet consists of mycolic acyl chains (α-branched, β-hydroxylated C60–C90 fatty acyls, MA), covalently attached to the cell wall arabinogalactan, which in turn is linked to peptidoglycan [4]. The outer leaflet of the mycomembrane is thought to be composed of a diversity of exotic lipidic molecules,

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which include ubiquitous trehalose-containing glycolipids, trehalose mono- and di-mycolate (TMM and TDM, respectively), and species-specific lipids such as diesters of phthiocerol (PDIM), phenolic glycolipids (PGL), sulfoglycolipids (SGL), and glycopeptidolipids (GPL) [5–8]. Lipoarabinomannan (LAM) is distributed between the plasma membrane and the outermost layers of the mycobacterial cell envelope.

1.1 Extraction of Mycobacterial Lipids and Isolation of Their Constituents

Lipids are soluble in organic solvents, e.g., chloroform and diethyl-ether, and are easily and routinely extracted from (preferentially) wet cell pellets, provided that they are not covalently linked to the cell wall such as MA attached to the arabinogalactan-peptidoglycan complex. Due to the complexity of mycobacterial extractable "free" lipids, from the very apolar PDIM to the polar phosphatidyl inositol mannosides (PIM), fractionation of the different classes is generally realized by solvent precipitation, mild alkaline hydrolysis, and combination of various chromatographic techniques.

The MA linked to the cell wall arabinogalactan and the fatty acids (FA) commonly esterifying alcohols in extractable complex lipids are released by alkaline hydrolysis for further structural analyses. Several methods have been proposed for their isolation. Saponification is the method of choice to break the ester linkage and to obtain free fatty acids. The method of saponification should allow a good yield in FA released from all mycobacterial complex lipids containing ester linkage, not only triacylglycerol (TAG), phospholipids, trehalose esters, and glycerol monomycolate (GroMM), but also PDIM; indeed, in this last case, the presence of multibranched FA hinders the attack of the ester linkage by alkali. A caution has to be kept in mind that for the choice of the method of saponification for releasing MA it has been shown that too mild alkaline conditions cause racemization of the chiral center in carbon 2. The two diastereoisomers, namely the 2*R*, 3*R* (natural isomer) and the 2*S*, 3*R* (artifact isomer), have an opposite value of their molecular rotations (positive and negative values, respectively) and show different migrations on thin-layer chromatography (TLC) (lower and upper spot respectively), leading to a multiplication of the spots, two for each class of MA [9, 10]. Acidic conditions for releasing FA have to be avoided as they lead to the degradation of some structures, e.g., the aperture of epoxy ring, leading to a complex TLC MA methyl ester (MAME) profile [11, 12].

1.2 Structural Analyses of Mycobacterial Lipids by Mass Spectrometry

Although the structural elucidation of mycobacterial lipids is sometimes very challenging and time consuming, the recent progress in analytical techniques, mainly mass spectrometry (MS), has greatly facilitated the determination of molecules holistically by reducing sample consumption.

The pioneer work of Stenhagen and collaborators [13–15] using electron-impact (EI) MS coupled to gas chromatography

(GC) remains the reference in the field of characterization of volatile FAME by allowing the determination of the precise structure of specific multi-methyl-branched FA of *Mycobacterium tuberculosis*, resulting from the hydrolysis of PDIM, SGL, or polyphthienoyl trehalose (PPT), the so-called mycocerosic, phthioceranic, or phthienoic acids (Figs. 1 and 2) even in a mixture.

Applied to MA, the method has been very informative for fine structure characterization as the localization of functional groups on the mero-mycolate chain. Both the α-alkyl and the mero-aldehyde chains are easily identified (Fig. 1): characteristic peaks corresponding to pyrolysis fragments, due to the temperature of volatilization around 300 °C used in the method, i.e., ester C_{22}, C_{24}, or C_{26}, and mero-aldehydes are observed (Fig. 1). MAME containing one or more cyclopropane rings and a function generating an aldehyde by pyrolysis give specific cleavages of the mero-mycolic chain at the level of the rings. From these fragments, the number of methylenes m_1, m_2, and m_3 (Fig. 1) can be deduced, the intensity of

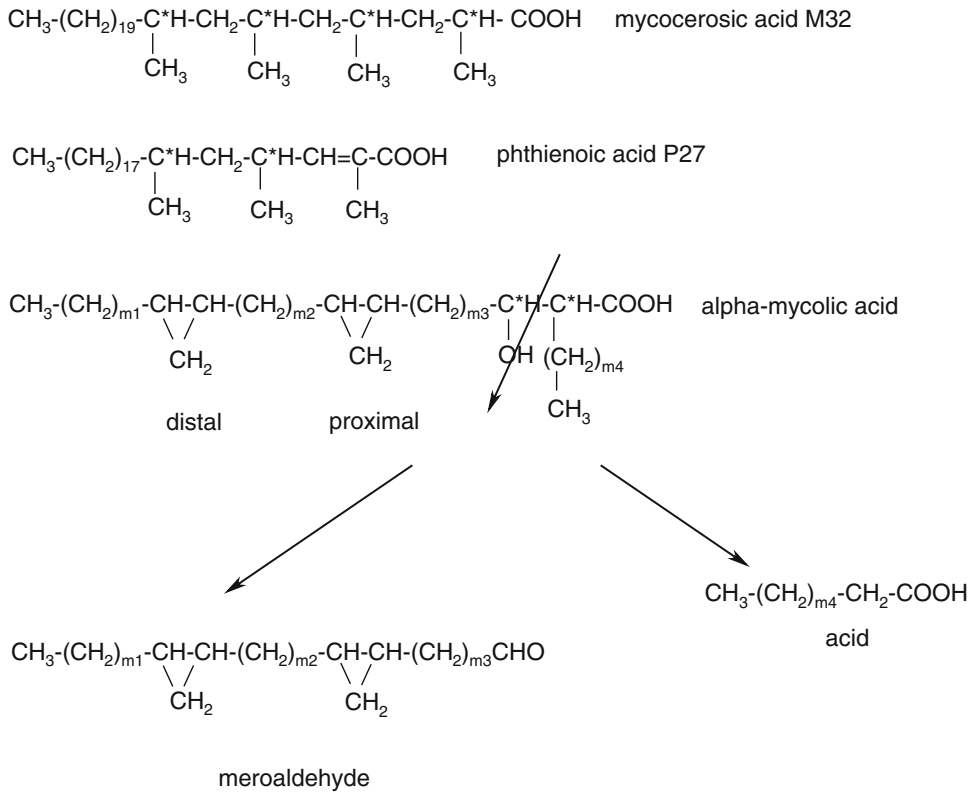
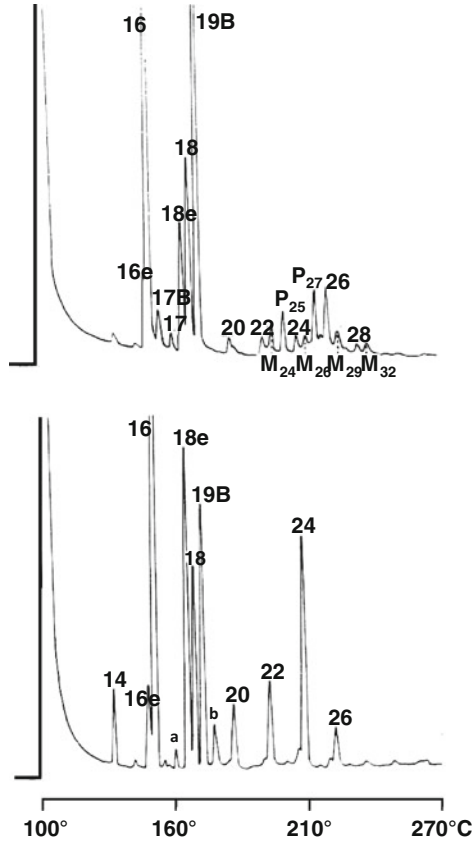


Fig. 1 Structures of representative fatty acids of *M. tuberculosis*. Mycocerosic or mycolipanolic acid (M32). Phthienoic acid (P27), also named C27 mycolipenic acid, characteristic of virulent strains. *Indicate chiral centers. P27 and M32 refer to the GC/MS profile (Fig. 2). Dicyclopropanic mycolic acid (α-mycolic acid, type I) of *M. tuberculosis* m_1, m_2, m_3, and m_4 are the methylene numbers. The *arrow* indicates the pyrolytical cleavage generating the ester and mero-aldehyde



GC	MS	Molecular weight	M detected
RT order	Characteristic peak	M methyl ester	
	m/z		
14:0	74	256	M or M-32
16:1	55	268	M
16:0	74	270	M or M-32
17B	74	284	M
(a) 2-octadecanol#	45	270	M-18
18:1	55	296	M
18:0	74	298	M and M-32
19B	74	312	M
(b) 2-eicosanol#	45	298	M-18
20:0	74	326	M or M-32
22:0	74	354	M or M-32
M24:0	88	382	M
P25:1*	127 and 169	394	M
24:0	74	382	M or M-32
M26	88	410	M
P27:1*	127 and 169	422	M
26:0	74	410	M or M-32
M29	88	452	M
M32	88	494	M

Fig. 2 GC profile of FAME and alcohols of *M. tuberculosis* (*upper*) and *M. avium* (*lower*) and identification of compounds by EI-mass spectrometry. Compounds identified by their fragmentation in MS are presented according to their retention time (RT) in GC. 16*e* and 18*e* in the GC profiles indicate ethylenic homologues, $C_{16:1}$ and $C_{18:1}$, respectively; the related methyl-branched chain acids concern tuberculostearic acid (10-methyl stearic acid) and tuberculopalmitate (10-methyl palmitic acid), 19B and 17B, respectively. (**a**) and (**b**) mean

peaks being strictly dependent upon the distance between the cyclopropane and the aldehyde [10, 16, 17]. Similarly, fragmentation of aldehydes from oxygenated MA is obtained and allows determining the position of oxygenated functions in keto- and methoxy-MA. Analysis of trimethylsilyl (TMS) derivative hydroxy-MA may also help the localization of the hydroxyl group [18].

The functional groups in the mero-mycolic chain can be localized by charge remote fragmentation in FAB-MS [19]. Careful studies on purified mero-MA of different classes (cyclopropanes, keto or methoxy) have allowed determining the exact location of the functional groups within the mero-mycolate chain [19]. However, due to the complicated fragmentation patterns obtained using the method, the determination of the molecular weight is not possible. In this context, matrix-assisted laser desorption/time-of-flight mass spectrometry (MALDI-TOF MS), by providing accurate molecular masses of the different classes of MAME, represents an attractive method by giving pseudo-molecular ion peaks, which contain sodium adducts [20]. Another alternative method for determining molecular masses of free MA consists in electrospray ionization (ESI)-MS. The requirement of free acids may pose a problem when purified MA, which result from purification steps, are analyzed because they have to be methylated for such purification [21, 22]. Finally, an interesting approach for the

Fig. 2 (continued) 2-octadecanol and 2-eicosanol, respectively. They are observed only after saponification, leading to the cleavage of the wax-ester mycolic acids; consequently, their presence is related to the occurrence of dicarboxylic mycolic acids (type VI mycolates in Fig. 3a and b) on TLC. M24 refers to mycosanoic acid: methyl 2L,4L docosanoic acid (dextrogyre). M29 and M32 correspond to mycocerosate or phthioceranate series 2, 4, 6-methyl branched with 29 and 32 carbon atoms, respectively. Both series have the same chemical structure but opposite configurations of the asymmetric carbons bearing the methyl branches, respectively, negative and positive values of the molecular rotations. According to the mycobaterial species (see text), either mycocerosates or phthioceranates are constituents of phthiocerol dimycocerosate (PDIM) and phenol glycolipid (PGL) while phthioceranates are the main acyl groups of sulfoglycolipid (SGL1). P27 and P25, the so-called phthienoic acids, a family of dextrorotatory acids considered as virulence markers encountered only in virulent strain of *M. tuberculosis* and *M. bovis.* They are engaged in polyphthienoyl trehalose (PPT) of *M. tuberculosis.* P27:1: phthienoic acid, also called mycolipenic acid, 2L,4L,6L-trimethyl tetracosen-2,3 oic acid. In addition to the peak at *m/z* 88, these acids present characteristic mass peaks* at *m/z* 127 and 169 resulting from 4,5 and 6,7 cleavages. Molecular ion (M) is not always detectable in EI MS. Instead, M-32 (loss of methanol) for FAME and M-18 (loss of water) for hydroxylated compounds are more generally observed. However, interesting fragments useful for the identification are observed (Fig. 2). The base peak (peak of higher intensity in the mass spectrum) is the marker of the structure of the acid: *m/z* 74 (Mc Lafferty rearrangement) for linear FAME. A base peak at *m/z* 88 is indicative of FAME with a methyl branch located at C2. The EI-mass spectrum of ethylenic FAME (oleic acid for example) presents a base peak at 55 and an intense molecular ion. In addition, characteristic peaks* from specific cleavage as mentioned for phthienoic acids are determinant for identification. For hydroxylated compounds, such as 2-octadecanol and 2-eicosanol, an easy confirmation of the structure is realized after silylation#: in addition to the base peak at *m/z* 73 an intense peak at *m/z* 117 corresponding to the fragment CH3-CH(OTMS) and M-15 at *m/z* 313 and 355 for octadecanol and eicosanol, respectively

analysis of lipids is the combination of HPLC with ESI-MS, which consists of separation and identification of lipid without any purification and derivatization. This method has been successfully applied to follow the MA composition in relation to changes in metabolism of the bacillus in hypoxic dormancy [21].

Mass spectrometry has also been crucial in the structural analysis of complex lipids. For instance, field-desorption EI-MS has been used to determine the molecular weight of GPL and to sequence their peptide and saccharide appendages [23]. Fast-atom bombardment (FAB) MS has also been very informative in this field [24], notably with the description of a novel sulfated GPL [25]. Structural determination of GPL has been recently realized by high-resolution multiple-stage linear ion-trap mass spectrometry ionization [26].

(MALDI-TOF) MS, using negative mode, has been applied to PIM molecular weight determination [27]; interestingly, MS/MS fragmentation from molecular ions obtained by MALDI-TOF or ESI allows to precise the nature of the fatty acids located on glycerol or on myoinositol and mannose units in the native PIM [28]. High-resolution multiple-stage linear ion trap MS with ESI has been successfully applied to determine the structure of *M. tuberculosis* SGL [29–31]. Similarly, MALDI-TOF MS has been useful for the molecular weight determination of PDIM [32] and PGL [33] as well as trehalose esters, both TMM and TDM [34–36].

1.3 The Stereochemistry of Mycobacterial Specific Acids

Mycobacteria contain genus- and species-specific lipids exhibiting chiral centers with defined stereochemistry. Among these are MA and multimethyl-branched FA found in PDIM, SGL, PGL, or PPT. In MA (Fig. 1), the stereochemistry of the centers at positions 2 and 3 is conserved in all MA characterized to date and shown to be 2*R* and 3*R*. For the simplest MA, as corynomycolic or nocardomycolic acids, the molecular rotation $[M]_D$ is +40° [37]. However, this value varies upon the occurrence of methyl branches in the mero-chain: the carbon bearing the methyl branch adjacent to the double bond or to the epoxy ring has *R* configuration, leading to a negative contribution to the molecular rotation [38–41], whereas that bearing the methyl branch adjacent to keto-, methoxy-, hydroxy-, and wax ester-MA has *S* configuration, in agreement with their biosynthetic filiation [18, 42].

In the case of multimethyl-branched FA found in PDIM, SGL, PGL, or PPT of the *M. tuberculosis* complex (Fig. 1), the stereochemistry of the carbon bearing the methyl branches at positions 2, 4, 6, or more, but always at even carbons, is different in mycocerosates (found in PDIM and PGL of *M. tuberculosis*, *M. leprae*, *M. kansasii*, *M. gastri*) and phthioceranates (found in SGL of *M. tuberculosis* and compounds structurally related to PDIM and PGL in *M. marinum* and *M. ulcerans*), reflecting

differences in enzymatic systems used for their biosynthesis [43, 44]. In the levorotatory branched fatty acids or mycocerosic (M in Fig. 2) the chiral centers have D configuration. Therefore, the C32 mycocerosic acid is the 2D, 4D, 6D, and 8D-tetramethyl octacosanoic acid esterifying the phthiocerol in PDIM and PGL (Fig. 1). The other family of branched FA esterifying trehalose in SGL has the same structure (phthioceranic acids, also labeled M in Fig. 2) but the chiral centers bearing the methyl branches have L configuration, conferring a positive value to their molecular rotation. More recently, the absolute stereochemistry of the carbon bearing the hydroxyl in the hydroxy-phthioceranic acid constitutive of diacylated sulfoglycolipids (Ac_2SGL) has been demonstrated to be *R* [45]. Then, it is worthwhile to mention here other important FA found in virulent strains of *M. tuberculosis*, the so-called phthienoic acids (P in Fig. 2); they are easily characterized by their high dextrorotation value $[\alpha]_D$ +18°. The main C_{27} homologue of this series is 2,4L,6L-trimethyl 2-*trans* tetracosanoic acid [44] (Fig. 1) esterifying trehalose and yielding PPT [46].

This chapter describes the current methods used in isolating and fractionating, purifying, and analyzing the major types of biologically active mycobacterial lipids and LAM. We describe methods used for global and rapid analyses of mycobacterial extractable lipids and their constituents, cell-wall-linked MA and LAM, which require simple chromatographic techniques such as TLC and GCMS. Then, we shall present the methods that allow the purification of the most commonly isolated biologically active lipids. The structural elucidation of mycobacterial lipids is outside the scope of this chapter.

2 Materials

2.1 Extraction of "Free" Lipids

1. A biosafety cabinet in the appropriate laboratory (*see* **Note 1**).
2. 50 mL screw-capped glass flasks.
3. Glass funnels.
4. Glass Pasteur pipettes.
5. Glass test tubes.
6. Filter paper discs 110 cm diameter.
7. HPLC-grade solvents: Methanol, chloroform, diethyl ether.
8. Rotary vacuum evaporator (*see* **Note 2**).
9. Airstream.

2.2 Characterization of Free Lipids by TLC

1. Normal-phase silica gel G60 0.25 mm plates.
2. Chromatography tank.
3. HPLC-grade solvents: Methanol, chloroform, acetone.

4. Spray flasks.
5. Dittmer and Lester reagent (*47*) commercial.
6. 0.2 % anthrone in H_2SO_4.
7. 10 % Molybdophosphoric acid in ethanol.
8. 0.2 % Ninhydrin in acetone.
9. Heater for charring the plates.

2.3 Preparation of Fatty Acid Methyl Esters (FAME) and MAME

1. Glass screw-capped tubes 18 × 1.5 cm.
2. 50 mL screw-capped glass flask.
3. Pasteur pipettes.
4. Glass test tubes.
5. KOH.
6. Nitroso-*N*-methyl urea.
7. H_2SO_4.
8. HPLC-grade solvents: Methoxy-ethanol; diethyl ether.
9. Oven at 110 °C.
10. Diazomethane: Put 3 mL of a 40 % KOH aqueous in a 50-mL flask in an icy bath under a laboratory well-ventilated hood. Add 15–20 mL diethyl ether. Add 1 g of nitroso-N-methyl urea by steps (around four additions). Shake gently after each addition. Transfer carefully the yellow supernatant diethyl ether phase with a Pasteur pipette into another flask containing dry KOH pastilles. Close the flask with a cork. Store at 4 °C before use.

2.4 Characterization of Total FAME and MAME by TLC and GC-MS

1. Silica gel G60 pre-coated plate, 20 cm × 20 cm, 0.25 mm.
2. Pasteur pipettes.
3. UV light detector for TLC plates.
4. HPLC-grade solvents: Dichloromethane; petroleum ether; diethyl ether, ethanol.
5. 10 % Molybdophosphoric acid in ethanol.
6. 0.1 % Rhodamine B in ethanol diluted to 0.01 % in 0.25 M NaH_2PO_4 solution.
7. Heater for charring the plates.
8. Gas chromatograph fitted with a TG1MS fused-silica capillary column (30 m × 0.25 mm) and connected to an ISQ™ single quadrupole mass spectrometer with electron energy of 70 eV.

2.5 Purification of Defined Biological Active "Free" Lipids

1. 0.2 M NaOH.
2. Glacial acetic acid.
3. HPLC-grade solvents: Diethyl ether, acetone, methanol, and chloroform.

4. Pasteur pipettes.
5. Glass test tubes.
6. A glass column equipped with a Teflon stopcock and a reservoir at the top to receive the solvent.
7. Round-bottom flasks.
8. A rotary vacuum evaporator (*see* **Note 2**).
9. Florisil (60–100 mesh).
10. Vortex.
11. An infrared spectrometer.
12. NaCl disks for infrared analyses.

2.6 Purification of MAME

1. Refrigerated centrifuge.
2. Glass conic centrifuge tube (Corex quality).
3. HPLC-grade solvents: Dichloromethane; petroleum ether; diethyl ether; methanol, ethanol, water.
4. Pasteur pipettes.
5. Glass test tubes.
6. Silica gel G60 pre-coated plates.
7. NO_3Ag.
8. 0.2 % dichlorofluorescein in 50 % ethanol in water.
9. Vortex.

2.7 Lipoarabinomannan (LAM)

1. Glass vessel.
2. Probe sonicator or cell disrupter.
3. Rotary vacuum evaporator (*see* **Note 2**).
4. Refrigerated centrifuge.
5. HPLC-grade ethanol, propan-1-ol, phenol, methanol, acetic acid, chloroform, cyclohexane.
6. Speedvac.
7. Peristaltic pump.
8. Fraction collector.
9. Magnetic stirrer.
10. NaCl.
11. EDTA.
12. Sodium deoxycholate.
13. $MgCl_2$.
14. Tris–HCl buffer.
15. Phosphate buffer (PBS).
16. DNase.

17. RNase.
18. α-Amylase.
19. Chymotrypsin.
20. Trypsin.
21. Dialysis MWCO 6-8000 tubing.
22. Octyl Sepharose CL-4B.
23. Bio-Gel® P-100 gel Medium or Sephacryl® S-200 HR.
24. Glass vials with Teflon seals.
25. Airstream.
26. Gas chromatograph fitted with a TG1MS fused-silica capillary column (30 m × 0.25 mm) and connected to an ISQ™ single quadrupole mass spectrometer with electron energy of 70 eV.
27. 15 % SDS-polyacrylamide gels.
28. Trichloroacetic acid (TCA).
29. Trifluoroacetic acid (TFA).
30. HCl.
31. NaOH.
32. Periodic acid.
33. $CuCl_2$.
34. Potassium carbonate.
35. Silver nitrate.
36. Formaldehyde.
37. Pyridine.
38. Hexamethyldisilazane.
39. Trimethylchlorosilane.
40. BF_3 in methanol.
41. Arabinose.
42. Mannose.
43. Mannoheptose.
44. Palmitic acid.
45. Pentadecanoic acid.

3 Methods

3.1 Extraction of "Free" Lipids"

1. Grow cells as surface pellicles (*see* **Note 1**) on either Sauton's medium [48] or Middlebrook 7H9 and variants media [41].
2. Harvest cells in the biosafety laboratory under safety hood (*see* **Note 1**) by pouring off the growth medium.
3. Add 30 mL of methanol followed by 15 mL of chloroform.

4. Shake gently the mixture to dissociate the clumps and let it stand at room temperature overnight (*see* **Note 3**).
5. Under a well-ventilated hood (*see* **Note 4**) transfer the supernatant organic extract in a round-bottom flask (250 mL) through a funnel containing a pre-weighed 110 cm diameter filter paper circles.
6. Carry out a second extraction of cells with chloroform/methanol 1:1 (v/v). Let it stand overnight and transfer as a bacterial cell suspension through the filter paper funnel.
7. Extract the bacterial pellet a third time with chloroform/methanol 2:1 (v/v). Let it stand overnight and transfer as a bacterial cell suspension through the filter paper funnel.
8. Let the delipidated bacterial cells dry at room temperature.
9. Weigh the filter paper that contains the delipidated cells.
10. Pool the organic phases in the laboratory hood.
11. Evaporate the solvents using a rotary vacuum evaporator (or under stream of air when only few mL volumes are used) (*see* **Note 2**).
12. Dissolve the pooled lipid extract in a minimum of chloroform.
13. Transfer the lipid extract in a pre-weighed glass tube.
14. Weigh the pooled lipid extracts that represent "free" lipid (*see* **Note 5**).

3.2 Characterization of "Free" Lipids by TLC

Due to the toxicity of the reagents used, conduct all steps in a well-ventilated hood, except for the migration of TLC where the hood ventilation has to be stopped to avoid turbulence in migration.

1. Dissolve the dried lipid extract in chloroform at an approximate concentration of 20 mg/mL.
2. Spot around 50 μL on the TLC plate.
3. Run the TLC with an appropriate solvent (*see* **Note 6**) and dry the plates at room temperature.

   Solvents with different polarities should be used according to the nature of the lipids to be detected.
4. Stain the TLC using specific sprays adapted to the lipid to be detected (*see* **Note 7**).
5. TLC of representative lipids are shown in Fig. 3c, d, e.

3.3 Preparation of Fatty Acid Methyl Esters (FAME) and MAME

Most of the mycobacterial fatty acids are found engaged by ester linkage and their study needs saponification, to yield the MA esterifying both complex lipids (e.g., GroMM, TDM, TMM) and arabinogalactan termini, and straight-chain and methyl-branched fatty acids from "free lipids," e.g., phospholipids, PDIM, SGL, and PPT.

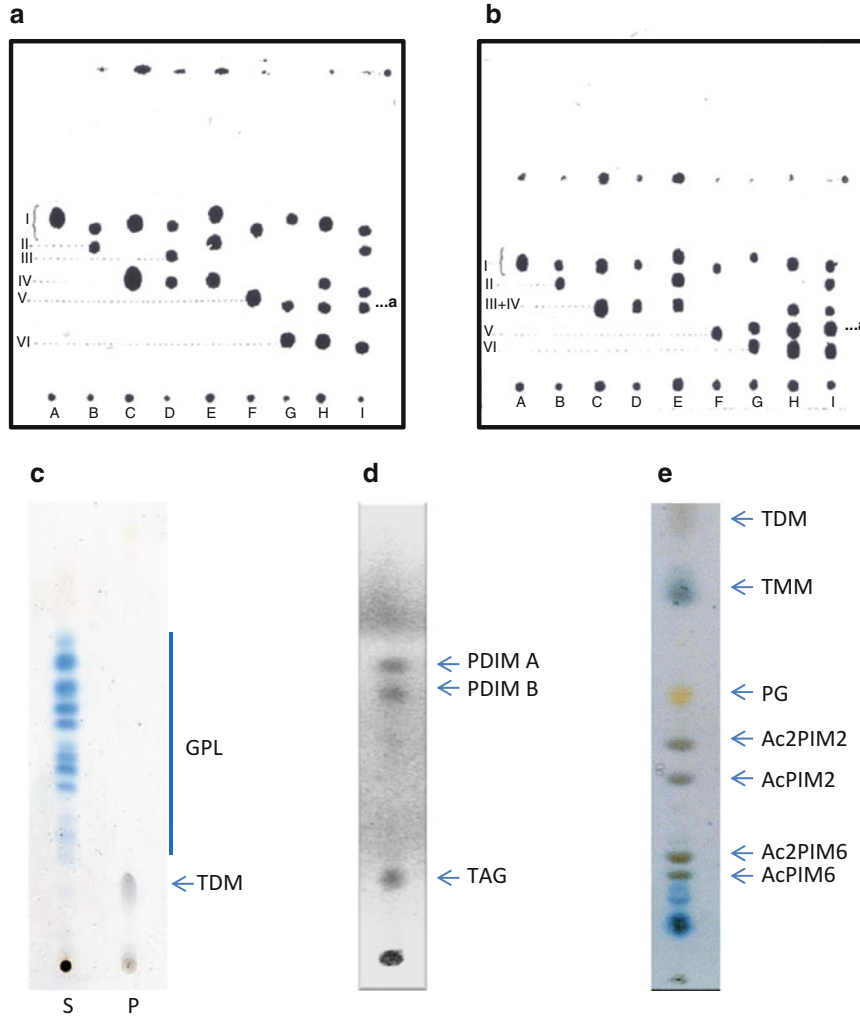


Fig. 3 TLC of mycolic acid methyl esters (MAME) from some representative strains of mycobacteria. *Roman letters* refer to mycolic acid types as in ref. 50: I α-; II α′; III methoxy-; IV keto-; V epoxy-; VI dicarboxylic-mycolic acids. Lanes (*A*) *M. fallax*; (*B*) *M. chelonae*; (*C*) *M. bovis* BCG; (*D*) *M. tuberculosis*; (*E*) *M. simiae*; (*F*) *M. fortuitum*; (*G*) *M. xenopi*; (*H*) *M. avium*; (*I*) *M. vaccae*. *a* means secondary alcohol, octadecanol, and eicosanol. Running solvents: (**a**) petroleum ether/diethyl ether 9:1 (v/v) four runs. (**b**) CH_2Cl_2. Revelation: Rhodamine B followed by charring. (**c**) TLC of "free" lipids of *M. smegmatis* after precipitation by methanol and detection with anthrone reagent. *S* soluble methanol, composed mainly of glycopeptidolipid (GPL). *P* pellet, enriched in trehalose dimycolate (TDM). Note the difference of coloration with anthrone between the *intense blue* of GPL and *grey-violet* for TDM. Running solvent: $CHCl_3/CH_3OH$ 9:1 (v/v). (**d**) TLC of "free" lipids of *M. tuberculosis* H37Rv. Phthiocerol dimycocerosate (PDIM); triacyl glycerol (TAG). Running solvent: Petroleum ether/9:1(v/v). Revelation: molybdophosphoric acid followed by charring. (**e**) TLC of "free" lipids of *M. tuberculosis* H37Rv. Trehalose dimycolate (TDM); trehalose monomycolate (TMM); phosphatidyl glycerol (PG); phosphatidyl inositol dimannoside diacylated (Ac2PIM2); phosphatidyl inositol dimannoside monoacylated (AcPIM2); phosphatidyl inositol hexamannoside diacylated (Ac2PIM6); phosphatidyl inositol hexamannoside monoacylated (AcPIM6). $CHCl_3$:CH_3OH:H_2O 60:35:8 (v/v/v). Revelation: anthrone

1. Release acids by adding 2 mL of a mixture of 40 % KOH in water and methoxy-ethanol (1:7 v/v) to whole cells or delipidated cells.
2. Heat at 110 °C for 3 h in a screw-capped tube (18 × 1.5 cm).
3. After cooling, acidify the mixture (at pH 1) with 1 mL of 20 % H_2SO_4 solution to release free fatty acids from salts.
4. Add 3 mL water and 3 mL diethyl ether and vortex.
5. Transfer the upper diethyl ether phase in a test tube (18 × 1.5 cm is recommended for an easy extraction).
6. Repeat **step 4** twice again.
7. Pool the diethyl ether phases and wash with 1 mL of distilled water, four or five times (until neutrality, i.e., pH of distilled water).
8. Dry the diethyl ether phase under airstream.
9. Add 1 mL of diazomethane to a tube containing fatty acids (up to 50 mg) until the solution becomes yellow. The methylation of acids is instantaneous (*see* **Note 8**).
10. Evaporate the reagent under airstream under a well-ventilated hood, after 5 min of contact.

3.4 Characterization of Total FAME and MAME by TLC and GC-MS

1. Dissolve FAME and MAME in diethyl ether at an approximate concentration of 10 mg/mL.
2. Spot around 50 µL on the TLC plate.

3a. Run the 1D TLC plate in dichloromethane and/or four times in 10 % diethyl ether in petroleum ether (*see* **Note 9**).

3b. Run the 2D TLC first in 5 % acetone in petroleum ether, three times, dry the plate, and run the plate in 3 % acetone in toluene (*see* **Note 9**).

4. Dry the plates at room temperature.
5. Stain the TLC plate by spraying a 10 % solution in ethanol of molybdophosphoric acid and charring or Rhodamine B and observation under UV light.
6. A 1D TLC of MA from representative mycobacteria is shown in Fig. 3.

To characterize volatile FAME (generally up to C30), GC, or more efficiently GC-MS, has to be used.

7. Dissolve FAME and MAME in diethyl ether at an approximate concentration of 10 mg/mL.
8. Inject 1 µL in the GC apparatus and run (Fig. 2).
9. Perform the identification by electron impact fragmentation (Fig. 2).

3.5 Purification of Defined Biological Active "Free" Lipids

PDIM and PGL resist mild-alkaline hydrolysis that breaks labile ester linkages (found in phospholipids and TAG) but not esters of polymethyl-branched FA and phthiocerol. The amide and osidic bonds that occur in GPL also resist this treatment [49].
To enrich "free" lipid in PDIM, PGL, and GPL

1. Solubilize the dried lipids, either the crude lipid extract ("free" lipids) or a chromatography fraction as described below.
2. Vortex.
3. Add an equal volume of 0.2 M NaOH in methanol.
4. Vortex.
5. Incubate at 37 °C for 60 min.
6. Neutralize with glacial acetic acid (some drops).
7. Concentrate under airstream.
8. Add 1 volume of $CHCl_3$ and 1 volume of water.
9. Gently vortex.
10. Dry the lower organic phase under airstream.

To enrich "free" lipids in long-chain compounds (*see* **Note 10**)

11. Dissolve up to 50 mg of lipids in a minute amount of chloroform (0.5 mL or a few drops) in a conic glass centrifuge tube.
12. Add 5 mL of methanol until a white precipitate appears.
13. Sit overnight at 4 °C.
14. Centrifuge at 4,000 × *g* for 30 min at the same temperature.
15. Transfer the methanolic supernatant with a Pasteur pipette into a weighed test tube. The pellet contains mainly MA and derivatives (TDM, TMM, GroMM, or MAME).

The procedure described for methanol precipitation above is applicable with acetone for selective enrichment of "free" lipids into phospholipids and SGL.

16. Dissolve the dry lipid extract (50–100 mg) in 0.5 mL or a few drops of chloroform in a centrifuge glass tube.
17. Add 5 mL acetone carefully until precipitate appears.
18. Vortex.
19. Sit overnight at 4 °C.
20. Centrifuge at 4,000 × *g*, at 4 °C for 30 min, to separate soluble-acetone lipids from the pellet containing insoluble phospholipids in cold acetone. Among other lipids, the soluble fraction contains mainly the SGL family [29].

When large amounts of pure specific lipids are needed, purification by column adsorption chromatography is often required. Using this method, compounds are separated according to their relative

polarity and the different types of lipids bound to the solid phase are separated by elution with solvent mixtures of increasing polarity.
Total lipid extracts are first roughly fractionated on a Florisil column.

21. Prepare the column as a slurry of Florisil in the most apolar solvent, depending on the nature of lipids, e.g., petroleum ether (apolar lipids) or chloroform (polar lipids). Use an amount of Florisil equal to around 50-fold the weight of lipids.
22. Place a small piece of cotton at the bottom of a chromatography column to retain the adsorbent.
23. Observe a good ratio between height and diameter (e.g., 2×40 cm) to ensure a good separation of lipids.
24. Pour the slurry with gentle shaking to eliminate bubbles.
25. Rinse with one volume of the solvent.
26. Allow the solvent to run slowly (by drops) through the column.
27. Dissolve the lipid extract in the minimum amount (few mL) of the most apolar solvent used.
28. With the solvent level at the top of the adsorbent, add carefully the lipid suspension on the top of the column and let it to adsorb.
29. Proceed to the elution gradient (volume 30–50 mL) (*see* **Note 11**).
30. Change the round-bottom flask after each elution.
31. Evaporate the solvent under vacuum with a rotary evaporator (*see* **Note 2**).
32. Transfer dried lipids in pre-weighed test tubes.

The fractionation of compounds is followed by TLC analysis.

33. Dissolve dried lipids in chloroform at an approximate concentration of 10 mg/mL.
34. Spot 50 μL of the fraction on plates and follow the separation by TLC with appropriate solvents (*see* **Note 6**).
35. Stain the plate with a spray adapted to the nature of compounds (*see* **Note 7**).
36. Re-chromatograph each fraction on Florisil column by adapting the solvent elution to the structure of the lipid (*see* **Note 12**).

Final purification of PDIM, TDM, and GroMM is achieved by preparative TLC.

37. Spot the sample, up to 10 mg in chloroform for 20×20 cm plate, with a Pasteur pipette or a 100 μL glass syringe as a line at the origin of the TLC plate 2. Run the plate in the TLC tank using appropriate solvents, e.g., petroleum ether/diethyl

ether 9/1 (v/v) for PDIM and TAG, and chloroform/methanol 9/1 (v/v) for GroMM and TDM.

38. After development, protect first the whole TLC surface with a glass plate except a small band on each vertical side to be visualized as a control for the detection of lipid bands by spraying.
39. Reveal the two non-covered sides of the TLC plate by carefully spraying with the appropriate reagent, anthrone (e.g., TDM), followed by heating the selected regions for visualizing glycoconjugates, or molybdophosphoric acid or Rhodamine B (e.g., PDIM, TAG).
40. Note the reagent-positive regions.
41. Scrap off the corresponding unrevealed bands with a spatula.
42. Collect the powder in a test tube.
43. Add 3 mL of the solvent used for the migration.
44. Filter the solvent extract containing the pure lipid in a preweighed test tube through a 1 cm Florisil Pasteur pipette in order to eliminate silica powder and the Rhodamine dye.
45. Dry under airstream the ether phase.

3.6 Purification of MAME

To first enrich the lipid extract in MAME, use solvent precipitation (*see* **Note 10**).

1. Dissolve up to 50 mg of MAME-containing saponified lipids in 0.5 mL or a few drops of diethyl ether in a conic glass centrifuge tube.
2. Add 5 mL of methanol until a white precipitate appears.
3. Let it sit overnight at 4 °C.
4. Centrifuge at 4,000 × *g* for 30 min at 4 °C.
5. Transfer with a Pasteur pipette the methanolic supernatant in a weighed test tube. The pellet contains mainly MAME.

To obtain fully purified MAME

6. Dissolve MAME-enriched methanol precipitate in chloroform (10 mg or 20 mg/mL).
7. Spot this suspension as a line with a glass 100 μL syringe or a Pasteur pipette on silica gel pre-coated plate, 20 cm × 20 cm, 0.25 mm. An equivalent of 7–10 mg per plate allows a good separation.
8. Run the plate once in dichloromethane or four or five times in petroleum ether/diethyl ether 9:1 (v/v) for separation of keto- and methoxy-MA [50]. When several runs are necessary, take care to stop the migration at the same level for each run.
9. Visualize the spots by spraying the plate with 0.01 % solution of Rhodamine B in sodium monophosphate 0.25 M, a reagent that does not degrade the extracted lipid.

10. Scrap off the bands visualized by UV light.
11. Extract the lipid from the silica power with around 2 × 5 mL of diethyl ether.
12. Filter the ether extract, which should contain pure MAME, in a test tube through a 1 cm Florisil Pasteur pipette, in order to eliminate silica powder and dye.
13. Evaporate the solvent under airstream.

To further separate MA according to the presence, the number, and the configuration of double bonds, argentation TLC is used (*see* **Note 13**)

14. Prepare a solution of 10 % NO_3Ag in 50 % water/50 % ethanol or 15 % $AgNO_3$ in acetonitrile.
15. Run pre-coated silica gel plates in the NO_3Ag solution. The migration is slow, around 3 h for running half of the plate.
16. Dry the impregnated TLC plate overnight at room temperature.
17. Load the sample to be purified as described for preparative TLC (**step 37** of paragraph 3.5).
18. Run the TLC plate in the dichloromethane.
19. Dry the plate at room temperature.
20. Spray a solution of 0.2 % dichlorofluorescein in alcohol, a reagent that does not degrade the extracted lipid.
21. Observe the spots that appear as orange on a yellow background under UV light.
22. Scrap off bands from the plate and treat as preparative TLC with diethyl ether (as in **steps 41–45** of paragraph 3.5).

The complete identification of pure MA is realized using sophisticated physical methods, such as polarimetry, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry [51]. For the fine structural studies of mycolic acids, refer to careful studies [19, 52].

3.7 Lipoarabinomannan

LAM is a water-soluble molecule and is not solubilized by organic solvents used to extract lipids from cells. Obtaining LAM thus requires a subsequent aqueous extraction of delipidated cells. LAM is a macroamphiphilic molecule that shows a high intrinsic molecular heterogeneity. Moreover, the mycobacterial cell envelope contains structurally related molecules such as the lipomannan (LM), the PIM, or the arabinomannan (AM) and mannan. Obtaining homogenous fractions of LAM thus requires the following critical steps: (1) elimination of protein and nucleic acid contaminants, (2) separation of lipoglycans from glycans by hydrophobic interaction chromatography, and (3) size fractionation of lipoglycans by size-exclusion chromatography [53].

1. Dry delipidated cells obtained in Subheading 3.1.
2. Resuspend them in a minimum amount of deionized water.
3. Disrupt them by probe sonication in ice or with a cell disrupter.
4. Reflux the cell lysate in a large volume of 50 % ethanol in deionized water for 4 h at 65 °C.
5. Recover the supernatant by centrifugation at 3,000 × *g*.
6. Re-extract the pellet until getting a colorless supernatant.
7. Combine the ethanol/water extracts and evaporate to dryness under vacuum with a rotary evaporator.
8. Suspend the dried ethanol/water extract in PBS at a concentration of 0.5 mg/mL.
9. Add an equal volume of phenol saturated with PBS and shake with a magnetic stirrer in a screw-cap tube or flask for 2 h at 80 °C to remove most of the proteins.
10. Allow the sample to cool down at room temperature and separate the aqueous and phenol layers by centrifugation at room temperature at 3,000 × *g*.
11. Back-extract the phenol phase with PBS as described above.
12. Combine the aqueous phases containing lipoglycans.
13. Dialyze against water (MWCO 6-8000) and evaporate to dryness under vacuum with a rotary evaporator.
14. Suspend the above dried extract in either Tris–HCl or PBS buffer, according to enzyme used, and submit to sequential enzymatic digestions by DNase, RNase (both in 20 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.5), α-amylase, chymotrypsin, and trypsin (all three in 50 M PBS, pH 7) for 8 h each enzyme using an enzyme/product ratio of 1 % (w/w).
15. Dialyze against deionized water (MWCO 6-8000) and evaporate to dryness under vacuum with a rotary evaporator or using a speedvac (depending on the volume to be evaporated).
16. Dissolve the dried sample in a minimum volume of a solution of 15 % propan-1-ol in 50 mM ammonium acetate.
17. Load the sample onto an octyl-sepharose CL-4B column previously equilibrated with the same solution (1 mL of phase is needed for 1 mg of sample) [54].
18. Elute the column at 5 mL/h with 3 column volumes of the same solution, enabling the removal of hydrophilic compounds.
19. Elute then the column with 3 column volumes of a solution of 50 % propan-1-ol in 50 mM ammonium acetate.
20. Concentrate the latter eluent to less than 1 mL and precipitate lipoglycans overnight at 4 °C with 10–20 volumes of ethanol.

21. Centrifuge at 3,000 × *g* at 4 °C.
22. Remove the ethanol and dry lipoglycans by freeze-drying or using a speedvac.
23. Resuspend lipoglycans in 0.2 M NaCl, 0.25 % sodium deoxycholate (w/v), 1 mM EDTA, and 10 mM Tris–HCl, pH 8, to a final concentration of 200 mg/mL, and incubate for 48 h at 37 °C.
24. Load onto a Bio-Gel P-100 or Sephacryl S-200 HR column (50 × 2.5 cm) previously equilibrated with the same buffer [55, 56].
25. Elute with the buffer at a flow rate of 5 mL/h.
26. Collect 1.25 mL fractions and determine the point of elution of LAM and LM by running 10 μL of each fraction by SDS-PAGE.
27. Pool separately fractions containing purified LAM and fractions containing purified LM.
28. Dialyze (MWCO 6-8000) extensively the pooled fractions for 2 days against 0.2 M NaCl, 1 mM EDTA, and 10 mM Tris–HCl, pH 8 to remove sodium deoxycholate and then against deionized water for 2 days.
29. Evaporate to dryness under vacuum with a rotary evaporator.
30. Resuspend LAM in minimum volume of water (<1 mL) and precipitate overnight at 4 °C with 10–20 volumes of ethanol.
31. Centrifuge at 3,000 × *g* at 4 °C, remove the ethanol, and lyophilize.

The homogeneity of LAM fractions can be easily and quickly determined by SDS-PAGE analysis and periodic acid-silver nitrate staining. LAM and LM migrate as broad bands around 30 kDa and 20 kDa, respectively, according to protein standards (*see* Fig. 4) [57].

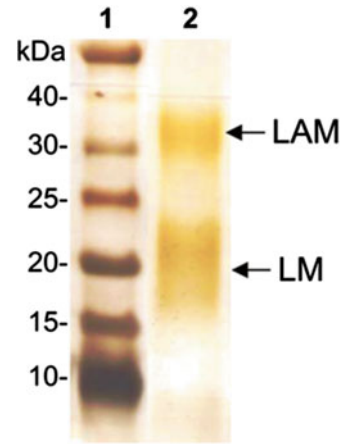


Fig. 4 SDS-PAGE analysis of *M. tuberculosis* LAM and LM. *Lane 1*: Protein MW standards. *Lane 2*: LAM and LM fraction from *M. tuberculosis* H37Rv after hydrophobic interaction chromatography

32. Submit 1 μg of LAM to classical SDS-PAGE using 15 % SDS-polyacrylamide gels.
33. Fix the gel for 20 min by gently rocking in a solution of 50 % methanol, 12 % TCA, and 2 % $CuCl_2$.
34. Remove the fixative and place the gel in a solution of 10 % ethanol and 5 % acetic acid (solution A) for 10 min.
35. Remove solution A and add a 0.7 % solution of periodic acid in 40 % ethanol and 5 % acetic acid for 10 min.
36. Wash the gel in solution A for 10 min, then in a solution of 10 % ethanol for 10 min, and finally in water for 10 min.
37. Place the gel in 0.1 % silver nitrate solution for 10 min.
38. Rinse the gel with water for 30 s, and then with a 10 % potassium carbonate solution for 1 min.
39. Develop the gel in a 2 % potassium carbonate solution containing 0.1 % formaldehyde until the gold-colored bands appear and the desired color intensity is reached; stop the reaction by placing the gel in a 10 % ethanol and 5 % acetic acid solution for 2 min. The gel can then be stored in water.

A more precise structural characterization of LAM should be performed by determining the glycosyl and fatty acyl compositions. LAM is composed of mannosyl and arabinosyl units [57–59].

40. Hydrolyze LAM (100 μg) in the presence of mannoheptose (100 nmol) as an internal standard using 200 μL of 2 M TFA at 110 °C for 2 h in glass vials with Teflon seals.
41. Allow the solution to cool down and evaporate to dryness under airstream at 40 °C or using a speedvac.
42. Add eight drops of pyridine, four drops of hexamethyldisilazane, and two drops of trimethylchlorosilane. Keep for 15 min at room temperature and evaporate to dryness under an airstream at 40 °C.
43. Resuspend the trimethylsilylated monosaccharides in 200 μL of cyclohexane and analyze by GC or GC/MS. Peak attribution can be made by a simple comparison with commercial arabinose and mannose standards submitted to the same protocol (*see* **Note 14**).

LAM contains mainly palmitic (C_{16}) and tuberculostearic (C_{19}) acids with traces of stearic acid (C_{18}) [53].

44. Deacylate LAM (200 μg) in the presence of pentadecanoic acid (8 nmol) as an internal standard by saponification using 400 μL of 1 M NaOH at 110 °C for 2 h in glass vials with Teflon seals.

45. Neutralize the reaction mixture with HCl and extract three times the liberated fatty acids with 400 μL of chloroform. Combine the chloroform extracts and evaporate to dryness under an airstream at 40 °C.
46. Add three drops of a commercial solution of BF3 in methanol. Incubate for 5 min at 60 °C and stop the reaction by the addition of 400 μL of deionized water.
47. Extract three times the fatty acid methyl esters with 400 μL of chloroform. Combine the chloroform extracts and evaporate to dryness under an airstream at 40 °C.
48. Resuspend the fatty acid methyl esters in 200 μL cyclohexane and analyze by GC or GC/MS.

4 Notes

1. All the methods herein described take into account safety conditions of work:

   Harvest of mycobacterial cells is realized under the biosafety cabinet in the appropriate laboratory NSB2 (opportunistic pathogens, e.g., *M. avium*) or NSB3 (strict pathogens, e.g., *M. tuberculosis*). If centrifugation is needed the machine has to be equipped with aerosol-free rotors and tubes. For pathogens as *M. tuberculosis*, growth as surface pellicles allows an easy collection of cells without centrifugation. The overnight contact with $CHCl_3/CH_3OH$ solvent ensures killing of cells.
2. The rotary evaporator is an indispensable tool for lipid studies but it presents risks and needs some caution: isolation of the apparatus for protecting workers by means of a screen is an indispensable precaution and, if possible, the rotary evaporator should be placed under a hood. Checking and replacement of damaged glass parts have to be regularly done. A careful examination of the round-bottom flasks before each use is indispensable. A speedvac may usefully replace the rotary evaporator in the purification of small quantities, e.g., LAM.
3. As the overnight contact with chloroform/methanol ensures killing of cells, the subsequent experiments can be conducted outside the safety laboratory.
4. As all the experiments on lipids involve solvents and chemicals, they have to be realized under a well-ventilated hood.
5. This constitutes "free" lipids. The ratio of the weight of "free" lipids *versus* that of bacterial delipidated cells on the filter plus that of "free" lipid represents the percentage of extractable "free" lipids of the cell dry weight.

6. According to the nature of the lipids to be detected, solvents with different polarities should be used:
   - For apolar lipids, i.e., triacylglycerol (TAG) and phthiocerol dimycocerosate (PDIM): Petroleum ether/diethyl ether 9:1 (v/v) [32].
   - For polyphthienoyl trehalose (PPT) present in *M. tuberculosis*: solvent, chloroform/methanol 99:1 (v/v) [46].
   - For phenolic glycolipids (PGL): chloroform/methanol 98:2 or 95/5 (v/v), depending on the nature of saccharide moiety linked to the lipid core. PGL are present in some strains of the *M. tuberculosis* complex (Beijing, canetii) but absent in H_{37} Rv strain [60].
   - For glycerol monomycolate (GroMM): chloroform/methanol 9:1 (v/v) found in all mycobacteria examined so far [61].
   - For trehalose mycolates found in all mycobacteria, i.e., trehalose dimycolate (TDM), chloroform/methanol 9:1 (v/v) or chloroform/methanol/water 30/8/1 (v/v/v), and trehalose monomycolate (TMM), chloroform/methanol/water 65/25/4 (v/v/v).
   - For glycopeptidolipids (GPL), characteristic species- or type-species glycolipids produced by some species such as *M. smegmatis*, *M. avium*: chloroform/methanol 9:1 (v/v), chloroform/methanol/water 30/8/1 (v/v/v), or chloroform/methanol/water 65/25/4 (v/v/v).
   - For sulfoglycolipids of *M. tuberculosis* (SGL), the mixture chloroform/methanol 85:15 or 80:20 (v/v) allows the separation of the three main SGL, i.e., the tetra-acylated Ac_4SGL (previously characterized by Goren as SL1), the tri-acylated (Ac_3SGL), and the diacylated (Ac_2SGL). In these solvents, Ac_4SGL co-migrates with triacyltrehalose (TAT) and the Ac_3SGL migrates with diacyltrehalose DAT [29]. Alternatively, SGL1 can be detected in a crude total lipid extract by 2D TLC using in the first-dimension chloroform/methanol/water 60:12:1 (v/v/v), and in the second direction, chloroform/methanol/water 75:11:1 (v/v/v) [62]. The plate has to dry at room temperature between the two migrations.
   - For phospholipids and amino lipids, cardiolipin (CL), phosphatidyl glycerol (PG), phosphatidylethanol amine (PE), and phosphatidylinositol mannosides (PIM) are analyzed using the reference developing solvent chloroform/methanol/water 65:25:4 (v/v/v). This solvent is also suitable for the characterization of amino lipids, e.g., the ornithine lipid (OL) characterized in *M. canetii* and *M. bovis* BCG [63].

- For the separation of the PIM family of *M. tuberculosis* and *M. bovis* BCG differing by their acylation degrees, the four acyl forms of each family of PIM (the dimannoside PIM_2 and the hexamannoside PIM_6) are separated by running the plate with chloroform/methanol/water 60:35:8 (v/v/v) [27].

7. According to the nature of the lipids to be detected, specific reagents should be used:
   - For TAG and fatty acid methyl esters (FAME) or mycolic acid methyl esters (MAME) resulting from the saponification of complex lipids: Rhodamine B (0.1 % solution in ethanol, diluted to 0.01 % in 0.25 M NaH_2PO_4 solution), a non-degradative reagent used for preparative TLC, and visualization under UV light; or molybdophosphoric acid (10 % in ethanol) and charring.
   - For phospholipids: Dittmer-Lester reagent [47].
   - For glycolipids: 0.2 % anthrone solution in sulfuric acid, followed by charring. Observe the blue spots appearing while charring; note the differences in the colors of glycolipids depending on the nature of the saccharides: intense blue for PGL or GPL, and first detected glycolipids, blue-green for PIMs, and blue-grey for trehalose esters including SGL.
   - For lipids containing free NH_2 (e.g., PE and OL): 0.2 % ninhydrin solution in acetone. After a light charring, observe the violet spots appearing. It is also possible to localize phospholipids on the same TLC by cooling the plate after revelation with ninhydrin at room temperature, and then spray with Dittmer-Lester reagent [47].
   - OL in *M. canetti* (migrates as PE): ninhydrin [63].
   - Peptidolipids from *M. fortuitum*, *M. paratuberculosis*, or *Nocardia*: cannot be detected on TLCs as they are devoid of free amino groups [8].
8. Methylation of fatty acids by diazomethane is a convenient method to convert free acids obtained by saponification into methyl esters; however, diazomethane is a toxic gas and its use needs some caution: working with small amounts (<1 g of nitroso-methylurea) with gloves under a well-ventilated hood. As diazomethane is not stable, the residual solution is easily degraded by keeping it overnight under the hood until the yellow solution is completely decolorized; the destruction of the gas can be accelerated and easily achieved by adding to the diazomethane solution a few drops of acetic acid (under the hood).
9. For 1D TLC, two plates using a solvent system are necessary: dichloromethane on the one hand and petroleum ether/diethyl ether 9:1 (v/v) using four subsequent runs on the

other hand are complementary and systematically used for the definition of the MAME composition of a given strain. Some of the MAME are not separated in dichloromethane; for example, methoxy- and keto-MA display the same migration but are well separated in petroleum ether/diethyl ether 9:1 (v/v) with four or five runs (Fig. 3a, b). When several runs are needed, stop the migration always at the same level and let the plate dry for 5 min at air between each run [50].

For 2D TLC, which is also informative in the case of complex patterns of species of mycobacteria [64] or of strains of BCG from different origins [65]: first direction petroleum ether/acetone (95:5, v/v, three runs) and second direction toluene/acetone (97:3, v/v) under a hood. Between each run, the plate is let to dry for 5 min under the hood.

10. Due to the chain length (60–100 carbon atoms), mycolic acids (MA) are insoluble in methanol. This property has been largely used as a first enrichment step of MA-containing "free lipids" such as TDM, TMM and GroMM, and MAME.
11. A typical chromatography elution for separating the main lipids would be:

    $CHCl_3$: apolar lipid, e.g., PDIM, TAG, PPT

    $CHCl_3/CH_3OH$ 99:1 (v/v): diacyl glycerol

    $CHCl_3/CH_3OH$ 98:2 (v/v): monoacyl glycerol

    $CHCl_3/CH_3OH$ 95:5 (v/v): GroMM

    $CHCl_3/CH_3OH$ 90:10 (v/v): TDM

    $CHCl_3/CH_3OH$ 85:15 (v/v): TAT

    $CHCl_3/CH_3OH$ 80:20 (v/v): peptidolipid

    $CHCl_3/CH_3OH/H_2O$ 65/25/4 (v/v/v): phospholipids (CL, PG, PE, PIM) and OL.
12. Apolar solvents for the first fractions eluted ($CHCl_3$), polar solvents ($CHCl_3/CH_3OH/H_2O$) for the last elution. For example a compound eluted in the most apolar fraction ($CHCl_3$) is fractionated by re-chromatography on a Florisil column with a gradient of less polar solvents such as diethyl ether in petroleum ether. A classical elution can be 0, 10, 20, 30, 50, and 100 % [32]. In these conditions, the PDIM fractions are isolated with 10 % diethyl ether in petroleum ether.
13. Argentation thin-layer chromatography is based on the property of double-bond π electrons to form a complex with a metal ion. Retention by silica is determined by the number and the configuration of double bonds. For instance, diethylenic MA are more retained than monoethylenic ones. A *trans* (E) double bond is less retained than the corresponding *cis* (Z) double bond. As binding force is weak, it is easily broken followed

the extraction of the compounds from the silica gel and, accordingly, is well adapted to purification by preparative TLC.

14. An alternative method for the analysis of glycosyl composition is through the use of capillary electrophoresis monitored by laser-induced fluorescence (LIF), which is more sensitive than GC [59]. It is based on the tagging of monosaccharides by reductive amination using 1-aminopyrene-3,6,8-trisulfonate (APTS) that can be detected with high sensitivity by LIF. Hydrolyze LAM (1 μg) in the presence of mannoheptose (1 nmol) as an internal standard using 200 μL of 2 M TFA at 110 °C for 2 h. Allow the solution to cool down and evaporate to dryness. Incubate with 0.4 μL of 0.2 M APTS in 15 % acetic acid and 0.4 μL of a 1 M sodium cyanoborohydride solution dissolved in tetrahydrofuran for 90 min at 55 °C. Stop the reaction by adding 20 μL of water. Dissolve 1–5 μL of the APTS derivative solution in 20 μL water and inject the solution in capillary electrophoresis in the reverse mode using acetic acid 1 % (w/v) and triethylamine 30 mM in water, pH 3.5 as running electrolyte.

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Chapter 6

Metabolomics of *Mycobacterium tuberculosis*

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Abstract

Enzymes fuel the biochemical activities of all cells. Their substrates and products thus offer a potential window into the physiologic state of a cell. Metabolomics focuses on the global, or systems-level, study of small molecules in a given biological system and thus provided an experimental tool with which to study cellular physiology on a global biochemical scale. While metabolomic studies of *Mycobacterium tuberculosis* are still in their infancy, recent studies have begun to deliver unique insights into the composition, organization, activity, and regulation of *M. tuberculosis*' physiologic network. Here, we outline practical methods for the culture, collection, and analysis of metabolomic samples from *Mycobacterium tuberculosis* that emphasize minimal sample perturbation, broad and native metabolite recovery, and sensitive, biologically agnostic metabolite detection.

Key words Metabolites, Enzyme function, Metabolism, Flux analysis, Carbon tracing, Metabolomics profiling

1 Introduction

1.1 Metabolomics in Mycobacterium tuberculosis

Prior to the advent of systems-level metabolomics technologies, studies of metabolism were largely limited to the use of isolated enzyme preparations and/or genetically engineered mutant strains whose significance rested on extrapolation to the native biochemical milieu of the cell and/or the accuracy and/or completeness of their accompanying bioinformatic annotations. More recently streamlined approaches, such as the one described here, have allowed for more direct measurements of the in situ biochemical function of a given enzyme or physiologic state of a given cell [1–4]. Metabolomic studies have thus helped assign novel functions to previously mis-annotated enzymes in *M. tuberculosis* [5, 6] and resolve the level and direction of metabolic reactions within the native physiologic milieu of the bacterial cytosol [7–10]. Together, such studies have revealed previously unrecognized metabolic pathways and capabilities not readily apparent in its genomic com-

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position [5, 8, 11]. Metabolomics has also impacted anti-infective development by helping to resolve the target or mechanism of action of new and existing compounds and also enabling direct measurements of their penetration and intracellular biotransformation products as recently exemplified for *para*-aminosalicylic acid [12–14].

1.2 Experimental Design

From an analytical perspective, recent advances in chromatography and mass spectrometry techniques recently made it possible to measure thousands of metabolites with subpicomole level sensitivity and in the absence of any biological preconception of the analytes of interest [1, 15]. In this chapter, we describe experimental methodology and analytical approaches to capture and characterize the *M. tuberculosis* metabolome with minimal-sampling-induced perturbation and broad and sensitive recovery of metabolites in their native state. Additional techniques such as the use of stable isotope labeling for flux analyses and pulse chase approaches to explore the kinetics of cellular reactions in intact *M. tuberculosis* are also discussed. We finally include a detailed description of an assay termed activity-based metabolomics profiling (ABMP), used to identify enzymatic activities of genes of unknown function [5, 6]. Together, these methods and assays are intended to help promote the more widespread use of metabolomics technologies in the context of *M. tuberculosis* physiology and pathogenesis.

1.3 Qualitative vs. Quantitative Measurement

Metabolite detection by LC-MS analysis enable qualitative estimations of abundance. This allows for the facile comparison of metabolite levels across samples within a single experiment. In this setting metabolite abundances are normalized to baseline measurements such as protein content or average metabolite ion intensity to facilitate this comparison. Important limitations of this approach however include the fact that measured values only represent relative, and not absolute, measures of metabolite abundance due to differences in the intrinsic ionization potentials (and hence response factors) of different metabolites. Quantitative determinations of metabolite abundance, though more labor intensive, can be achieved but requires the rigorous use of external or internal standards and/or accompanying standard curves for molecules of interest. The use of standard curves additionally allows for the determination of analytical limits of detection.

1.4 Pool Size Analysis

Metabolite pool size determinations provide a static picture of the metabolic state of the cell. This allows for comparisons across different conditions or strains, but may not represent a steady-state image of the cell. The use of multiple time points can help overcome this limitation, but is often experimentally challenging to achieve.

1.5 Carbon Tracing and Flux Analysis

Carbon tracing, and accompanying flux analysis tools, allow for the measurement of changes in individual metabolite dynamics over time, using nonradioactive heavy isotopes such as ^{13}C or ^{15}N. This allows for more direct insight into *M. tuberculosis* response to external perturbation. While the methods described herein cannot generate continuous measurements from a single sample, measurements at rapid time intervals can similarly help to overcome this limitation.

1.6 Activity-Based Metabolomic Profiling (ABMP)

ABMP is a method that was specifically developed to identify enzymatic activities for genes of unknown function. This method leverages the analytical discriminatory power of liquid chromatography-coupled high-resolution mass spectrometry (LC-MS) to analyze the impact of a recombinant enzyme and potential cofactors on a highly concentrated small-molecule extract derived from the homologous organism. By monitoring for the matched, time- and enzyme-dependent depletion and accumulation of putative substrates and products, this assay enables the discovery of catalytic, rather than simple binding, activity. By using the cellular metabolome as the input chemical library, this assay makes use of what is arguably the most physiologic chemical library of potential substrates that can be tested, in a label- and synthesis-free manner. Moreover, candidate activities assigned by this method can be confirmed using independent biochemical (including reconstitution with purified components) and genetic (using wild-type and genetic knockout, knockdown, or overexpression strains) techniques.

2 Materials

2.1 Culture System

The short biological half-lives of small metabolites require a method that enables rapid quenching of samples without prolonged manipulation or exposure to stress. We therefore use a filter culture-based system that enables nondisruptive handling, rapid quenching, and collection of bacterial cells in the absence of intermediate steps such as pipetting or centrifugation that could induce sampling-induced changes or losses. Use of this system further supports dense growth of *M. tuberculosis* in a biofilm that preserves its recently described capsule, supports bulk growth at rates similar to those associated with planktonic liquid cultures, and enables reproducible detection of most intermediates of central metabolism (Fig. 1).

1. 7H9-ADN medium: Dissolve 4.7 g Middlebrook 7H9 broth in 900 mL deionized water and add 2 mL glycerol w/v (50 %). Mix and autoclave to sterilize. Add 0.05 % w/v sterile Tween or Tyloxapol and 100 mL of a sterile solution of 5 % w/v

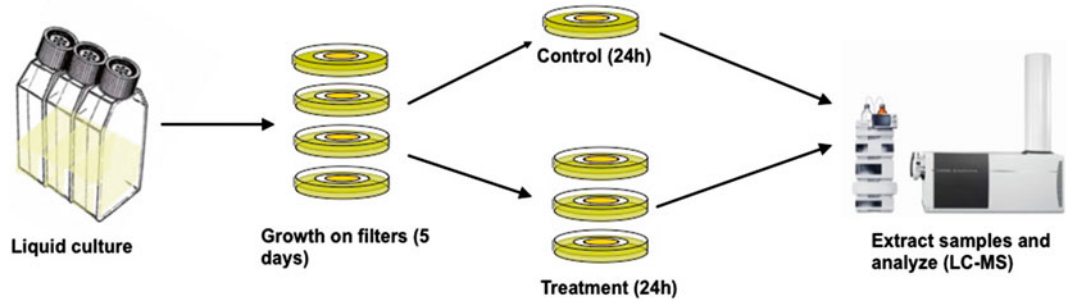

Fig. 1 Experimental design of metabolomics in *M. tuberculosis*. 1 mL of *M. tuberculosis* grown to an OD_{600} of 1 is deposited on sterile filters using vacuum filtration. These filters are transferred to 7H10 plates and incubated for 5 days to generate sufficient biomass. *M. tuberculosis*-laden filters are then transferred onto treatment plates (if required) and samples are then collected and extracted for analysis by LC-MS as described

bovine serum albumin, 2 % w/v D-glucose, 0.85 % w/v NaCl (ADN) (*see* **Note 1**). Filter sterilize before use.

2. Durapore Membrane Filters—Millipore 0.2 μm mixed cellulose ester filters (Millipore: GSWP02500) (*see* **Note 2**).
3. Swinnex Filter holder, 25 mm (Millipore).
4. Vacuum flask.
5. *M. tuberculosis* culture at a density of 5×10^8 cells/mL ($OD_{600} = 1$).
6. 7H10 agar plates: Dissolve 19 g Middlebrook 7H10 agar in 900 mL deionized water and add 5 mL glycerol. Mix and autoclave to sterilize. Add 100 mL ADN solution (*see* **Note 1**) and pour.
7. Sterile tweezers.

2.2 Sample Collection

1. Quenching buffer—freshly prepared solution of acetonitrile: methanol:water in a ratio of 2:2:1 (v/v/v). Prepare using LC-MS-grade acetonitrile and methanol with fresh Milli-Q 18 mΩ water (*see* **Note 3**).
2. Dry ice.
3. Tissue culture dish, 35 × 10 mm.
4. Bead-beating tubes filled with 0.1 mm Zirconia/silica beads (Biospec).
5. Sterile tweezers.

2.3 Sample Extraction

1. Temperature-controlled bead beater.
2. Temperature-controlled centrifuge.
3. 0.22 μm microcentrifuge filter tubes, e.g., Spin-X.

2.4 Sample Preparation

1. 0.2 % w/v formic acid in acetonitrile (LC-MS grade).
2. 0.2 % w/v formic acid solution: Prepare fresh in Milli-Q water.
3. LC-MS sampling vials with insert and rubber-seal caps, e.g., from Agilent.

2.5 LC-MS Separation Protocol for Sample Analysis

1. Cogent Diamond Hydride Type C column.
2. Agilent Accurate Mass 6220 Time of Flight (TOF) spectrometer coupled with an Agilent 1200 LC system.
3. 0.2 % w/v formic acid in acetonitrile (LC-MS grade).
4. 0.2 % formic acid solution: Prepare fresh in Milli-Q water.

2.6 Activity-Based Metabolomic Profiling

1. *Mycobacteruim bovis* (BCG) frozen stocks or culture.
2. 7H9-ADN medium (*see* Subheading 2.1, **item 1**).
3. Acidic acetonitrile: 0.2 % w/v glacial acetic acid in acetonitrile: chill on ice.
4. Purified enzyme (in a dilute chemically stable buffer, e.g., 20 mM TEA pH 7.8).
5. 20 mM Tris–HCl pH 7.2.
6. 1 M Tris–HCl pH 7.2.
7. Cell disruptor.

3 Methods

3.1 Culture System

1. Grow or concentrate *M. tuberculosis* cultures to a final density of 5×10^8 cells/ml ($OD_{600} = 1$) in 7H9-ADN medium (*see* **Note 4**).
2. Assemble filtration apparatus: Attach the filter holder to the vacuum flask using a pipette as illustrated (Fig. 2).
3. Activate vacuum and sterilize filtration apparatus using bleach followed by 70 % ethanol. Allow it to stand to dry completely.
4. Place filter on holder and slowly pipette 1 mL liquid culture onto filter. Liquid medium is drawn through filter leaving bacterial cells deposited in a uniform layer on filter surface (*see* **Note 5**).
5. Remove filter from stage with sterile tweezers, and place on solid agar medium with the bacterial layer exposed.
6. Grow at 37 °C for 5 days to accrue sufficient biomass for metabolite detection (*see* **Note 6**).
7. Once sufficient biomass is generated, filters can be moved to plates of different experimental conditions, such as antibacterial compounds of interest, as required.

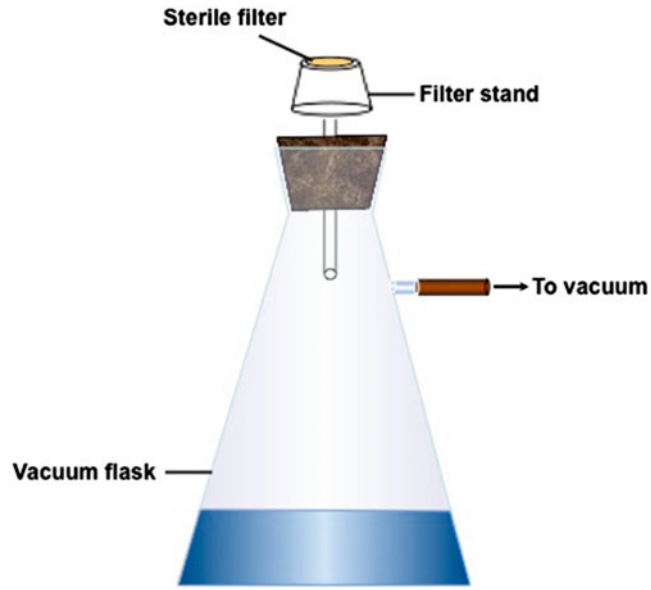

Fig. 2 Filtration apparatus for *M. tuberculosis* metabolomics. A vacuum flask is attached to a filter stand to provide the base for the inoculation of *M. tuberculosis* on sterile filters. Once the apparatus is sterilized, filters are placed on the stand, and 1 mL of *M. tuberculosis* culture is slowly pipetted on. Liquid medium is drawn through the filter and into the flask, leaving a lawn of *M. tuberculosis* deposited on the surface. *M. tuberculosis*-laden filters are then transferred onto agar plates for growth

8. Place filters under desired conditions for required incubation periods.

3.2 Sample Collection

1. Powder dry ice and place in a flat container for sample collection.
2. Place collection container (typically lid/base of a small plate) on dry ice.
3. Add 1 mL quenching buffer to plate on dry ice and allow to cool.
4. Using sterile tweezers, place *M. tuberculosis*-laden filter face down in collection buffer on dry ice (*see* **Note 7**).
5. Allow filters with *M. tuberculosis* to freeze completely.
6. Remove from dry ice, turn filters over, and allow to thaw completely.
7. Using tweezers and a pipette tip, scrape *M. tuberculosis* lawn off filter into collection buffer. Discard filter (*see* **Note 8**).
8. Pipette cell suspension into bead-beating tube (containing silica beads).

9. Scrape remaining *M. tuberculosis* biofilm into bead-beating tube using a pipette tip.
10. Seal and place tube on dry ice for processing. At this stage, samples can be stored in −80 °C freezer for a few weeks if required.

3.3 Sample Extraction

1. Thaw samples and place in cooled bead beater (0 °C or less).
2. Bead beat at 6,000 rpm, 6× times, for 30 s each (*see* **Notes 9** and **10**).
3. Spin samples at 13,000 × *g* for 10 min at 4 °C.
4. Transfer supernatant to filter tube (Spin-X tube) and centrifuge at 5,000 × *g* for 5 min at 4 °C.
5. Sample tubes can now be sterilized and transferred to the lab for LC-MS analysis.

3.4 Sample Preparation

1. Mix 75 μL of sample extract with 75 μL of the acetonitrile-0.2 % formic acid solution.
2. Vortex to mix and spin at 13,000 × *g* for 4 min.
3. Pipette 100 mL supernatant into LC vial and cap. Tap tubes to ensure that no bubbles are trapped in sample.
4. Load onto LC-MS autosampler and run separation method (Subheading 3.5).

3.5 LC-MS Separation Protocol for Sample Analysis

1. Separate samples on a Cogent Diamond Hydride Type C column linked to an Agilent Accurate Mass 6220 Time of Flight (TOF) spectrometer and coupled with an Agilent 1200 LC system (*see* **Note 11**).
2. Use a mobile phase consisting of two solvents, A = 0.2 % formic acid in water and B = 0.2 % formic acid in acetonitrile, at a flow rate of 0.4 mL/min.
3. Run a series of gradients over the course of a 24-min separation as described in Table 1.
4. After the run, equilibrate with 10 min of 85 % Solvent B prior to the beginning of a new run.
5. Using an isocratic pump, infuse a reference mass solution with the run to allow for simultaneous mass axis calibration.

3.6 Activity-Based Metabolomic Profiling

1. Inoculate 100 mL pre-warmed sterile 7H9-ADN medium with a single colony or freezer stock of *Mycobacterium bovis* BCG. BCG is used instead of *M. tuberculosis* to allow processing of samples outside of a biosafety level 3 containment laboratory (*see* **Note 12**).
2. Incubate (rolling or shaking), at 37 °C, until the OD_{600} reaches 1.0 (*see* **Note 13**).

Table 1
Solvent B (acetonitrile + 0.2 % formic acid) gradients for a 24-min separation on the LC-MS

Time (min)	Gradient (solvent B) (%)
0–2	85
3–5	80
6–7	75
8–9	70
10–11	50
11–14	20
14–24	5

3. Add the starter culture to 900 mL pre-warmed sterile complete 7H9 medium in a culture flask (*see* **Note 14**).
4. Incubate (rolling or shaking) at 37 °C until the OD_{600} reaches 1.0 (approximately 3 days).
5. Cool cells on ice for 10–15 min.
6. Transfer to appropriate containers and centrifuge at 7,000 × *g* for 15 min at 4 °C.
7. Discard supernatant and resuspend the cells in an equal volume of ice-cold sterile 7H9-ADN medium.
8. Repeat **steps 6** and **7** once (*see* **Note 15**).
9. Harvest the cells (as in **step 6**) and store on ice (*see* **Note 16**).
10. Add 1 volume cold acidic acetonitrile to the cell pellet.
11. Disrupt the cells by probe sonication (on ice).
12. Separate soluble and insoluble fractions by centrifugation at 27,000 × *g* for 1 h at 4 °C.
13. Carefully remove the soluble fraction (without disturbing the pellet) and transfer to a new container on ice. Discard the insoluble debris.
14. Flash freeze the soluble material and freeze-dry.
15. Suspend the resulting powder in 4 mL of cold 20 mM Tris–HCl pH 7.2 to generate small-molecule extracts (*see* **Note 17**).
16. Divide the solution into 100 μL aliquots in appropriate tubes and flash freeze. Store at −80 °C until ready for further processing.
17. For the enzymatic reactions, set up two tubes each containing 20 mM Tris–HCl pH 7.2 and 100 μL of small-molecule

extract. Use sterile distilled H_2O to achieve a final total volume of 980 μL (*see* **Note 18**).

18. Heat-inactivate a small aliquot (30–40 μL) of purified candidate enzyme at 95 °C for 5 min and then cool on ice for 5 min.
19. Add 20 μL of heat-inactivated purified candidate enzyme to one experimental tube and 20 μL native enzyme to the other (*see* **Notes 19** and **20**).
20. Incubate at 37 °C and remove 100 μL aliquots at 0 (prior to enzyme addition), 5, 10, 20, 30, 60, 90, and 120 min after addition of enzyme (*see* **Note 21**). Add each aliquot immediately to 300 μL cold acidic acetonitrile and store at 4 °C until ready for further processing.
21. Centrifuge the quenched aliquots at top speed (18,000 × *g*) for 10 min at 4 °C in a benchtop microfuge.
22. Carefully transfer 100 μL of the supernatant (without disturbing the pellet) to an LC-MS vial.
23. Cap and store samples at 4 °C. Samples are now ready to be analyzed by the LC-MS procedure described above (Subheading 3.5) (*see* **Note 22**).

4 Notes

1. Glucose can be replaced with other carbon sources as required.
2. The choice of filter membrane may depend on the experimental goal. Low-binding nylon/PVDF filters, e.g., Millipore: GVWP02500, can be used to prevent drugs or other chemicals from being trapped in the filter. This optimizes exposure of the bacterial lawn to chemical of interest.
3. LC-MS-grade water is not recommended due to leaching of sodium from glass bottles.
4. Higher cell densities may be used for mutants that are difficult to grow on plates.
5. For slow-growing or growth-defective strains, larger volumes of culture can be deposited on the filters, to ensure adequate biomass for LC-MS analysis at the end of the experiment.
6. Bacteria are ideally collected at mid-log-phase growth on filters. This is typically achieved after 5–7 days of filter growth on 7H10 plates with glucose and glycerol as carbon sources. The experimental design can be altered to ensure that this growth phase is achieved at sample collection when alternate media or strains/mutants are used. Shorter or longer growth intervals can also be used to observe growth phase-specific differences in metabolism.

7. The cold solution rapidly quenches bacterial metabolism while the direct use of collection buffer eliminates steps such as washing and spinning that could stress the sample.
8. Ideally the lawn should slide off the filter in one piece. This allows for ease of collection without loss of biomass.
9. If a cooling system for the beat beater is not available, samples can be cooled on ice for 30 s between runs.
10. All samples should be normalized to total protein content to account for differences in sample yield and bead-beating efficiency.
11. LC-MS equipment can be obtained from companies such as the Waters Corporation, the Bruker Corporation, and the Shimadzu Corporation.
12. 100 mL is the maximum volume permitted in the 1 L roller bottles (Nalgene) employed in our laboratory to culture *M. bovis* BCG and *M. tuberculosis* H37Rv routinely.
13. Monitor OD_{600} every 2 days to begin with. It can take from 5 to 10 days for the culture to reach the correct density.
14. We divide the culture into 10×1 L roller bottles.
15. Ideally the final harvest should result in a single-cell pellet. To achieve this, reduce the amount of wash solution used to resuspend the cells and pool samples together prior to centrifugation.
16. For long-term storage cell pellets can be flash frozen in an ethanol:dry ice bath and kept at –80 °C.
17. This volume generates a 10× extract for the conditions used in our laboratory. The buffer composition and volume used are flexible; adjust accordingly as required for the final application.
18. Add appropriate concentrations of any cofactors (e.g., pyridoxal phosphate, $NAD(P)^+/H$, Zn^{2+}) that are predicted to be required for enzyme activity to both tubes.
19. Final concentration of enzyme in the reaction mixture will vary depending on the enzyme preparation used. Final concentrations employed in our laboratory typically range from 0.5 to 5 μM.
20. If enzyme quantity is limited, heat-inactivated enzyme can be substituted with an equal volume of enzyme storage buffer.
21. If precipitation becomes visible, adjustments to the concentration of buffer, enzyme, cofactors, and small-molecule extract must be made.
22. Activity-based metabolite profiling allows for the functional characterization of orphan enzyme activities through the use of recombinant enzymes in whole-cell lysates. An unbiased metabolomics approach is used to discover catalytic activities through the identification of putative physiological products and substrates.

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Chapter 7

Electroporation of Mycobacteria

Renan Goude, David M. Roberts, and Tanya Parish

Abstract

High-efficiency transformation of DNA is integral to the study of mycobacteria, allowing genetic manipulation. Electroporation is the most widely used method for introducing DNA into mycobacterial strains. Many parameters contribute to high-efficiency transformation; these include the species per strain, the transforming DNA, the selectable marker, the growth medium additives, and the conditions of electroporation. In this chapter we provide an optimized method for the transformation of representative slow- and fast-growing species of mycobacteria—*Mycobacterium tuberculosis* and *M. smegmatis*, respectively.

Key words Transforming DNA, Selectable marker, Electrocompetent cells, Transformants

1 Introduction

Electroporation is the most widely used method for introducing DNA into mycobacterial cells, producing high efficiencies of transformation [1] and enabling the genetic manipulation of both fast- and slow-growing mycobacterial species. In this process cells are subjected to a brief, high-voltage electrical pulse which allows the entry of DNA into the cell.

Numerous species of mycobacteria have been successfully transformed with DNA by electroporation, including *Mycobacterium tuberculosis* [2–7], *M. bovis BCG* [2–4, 6, 8–15], *M. vaccae* [8, 14], *M. phlei* [11], *M. w* [14], *M. fortuitum* [11], *M. aurum* [16, 17], *M. intracellulare* [18, 15], *M. parafortuitum* [19], *M. marinum* [20], and *M. avium* [21–23]. A derivative of *M. smegmatis* ATCC607, designated mc^{2}155, is widely used yielding transformation efficiencies of 10^4–10^5 transformants/μg of DNA [24].

The reported efficiencies for electroporation vary among different mycobacterial species (Table 1). For example, some strains are notoriously difficult to transform, particularly certain clinical isolates of the *M. avium* complex [23]. However, one of the most important factors is the resistance marker used as this has a major impact on the maximum efficiency attainable.

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Table 1
Selectable markers and reported electroporation efficiencies for mycobacteria species

Species	Selection	Efficiency[a]	References
Fast growers			
M. aurum	Kanamycin	100	[16, 17]
	Streptomycin		[16, 17]
M. fortuitum	Kanamycin		[11]
M. parafortuitum	Kanamycin	300	[19]
	Streptomycin	30	[19]
M. phlei	Kanamycin		[11]
M. smegmatis ATCC607	Kanamycin	10	[10, 13]
M. smegmatis mc2 155	Kanamycin	10^5–10^6	[2, 8, 15, 24, 40]
	Hygromycin	5×10^3	[14]
	Apramycin	2×10^4	[3, 4]
	Streptomycin		[16]
	Tetracycline		[33]
	Gentamycin		[41]
	Sulfonamide		[41]
	Chloramphenicol[b]		[24]
M. vaccae	Hygromycin	10^3–10^5	[8, 14]
Slow growers			
M. avium	Kanamycin	10^2–10^4	[21–23]
	Hygromycin	104	[23]
M. avium ssp. *paratuberculosis*	Kanamycin	100	[22]
M. bovis BCG	Kanamycin	10^3–10^5	[2, 6, 8–10, 13, 15]
	Apramycin	103	[3, 4]
	Hygromycin		[12, 14]
	Chloramphenicol[c]		[11]
M. bovis Wag201 and Wag202	Kanamycin	104	[15]
M. intracellulare	Kanamycin		[15, 18]
	Gentamycin		[18]
M. marinum	Kanamycin	100	[20]
M. tuberculosis	Kanamycin	10^4–10^6	[2, 6, 42]
	Apramycin	102	[3, 4, 42]
	Hygromycin	107	[5, 7]
M. w	Hygromycin	10^3–10^5	[14]

[a]Number of transformants per μg of DNA
[b]Used for screening. Not for direct selection
[c]In conjunction with kanamycin

Most mycobacterial extrachromosomal vectors are based on the replicon pAL5000, a 4.8 kb plasmid from *M. fortuitum* [25]. Shuttle plasmids with an *E. coli* origin of replication are widely used [13] to allow for ease of DNA manipulation in an amenable host. The minimal origin has been defined and useful cloning sites introduced in several versions, e.g., pMV261 [6, 26]. The pAL5000-based vectors replicate in many mycobacterial species, including members of the *M. avium* complex which have been successfully transformed with this vector [18]. Alternative mycobacterial replicons have been derived from an *M. scrofulaceum* plasmid (pMSC262) [9], an *M. avium* plasmid (pLR7) [21], other *M. fortuitum* plasmids (pJAZ38 and pMF1) [27, 28], and a linear plasmid from *M. celatum* (pCLP). In addition, origins of replication from heterologous species which are capable of replication in both mycobacteria and *E. coli* can be used. These include plasmids pNG2 from *Corynebacterium* which was functional in both *M. bovis* BCG and *E. coli* [12], and the broad host-range gram-negative cosmid vector pJRD215 (derived from RSF1010) [16].

Integrative vectors have been developed for mycobacteria to improve plasmid stability. The most widely used integrative plasmid is derived from the mycobacteriophage L5. The minimal system only requires the phage attachment site (*attP*); the integrase (*int*) function can be provided either on the same vector or on a second vector. These vectors are stably integrated in *M. smegmatis*, *M. tuberculosis*, and *M. bovis* BCG [29]. Other integrative vectors include a derivative of mycobacteriophage Ms6 (pEA4) which is stably maintained in *M. smegmatis* [30] and plasmid pSAM2 from *Streptomyces ambofaciens*. An artificial transposon derived from the *M. avium* ssp. *paratuberculosis* insertion sequence IS*900* has also been used to integrate into the chromosome of *M. bovis* BCG and *M. smegmatis*, but the copy number per cell varied from one to five in *M. smegmatis* [8, 31].

Vectors for use in mycobacteria must encode appropriate selectable markers. The intrinsic resistance of mycobacteria to many antibiotics and the requirement to use stable drugs exhibiting a low frequency of spontaneous resistance reduce the alternatives. The first selectable marker used in mycobacteria was the *aph* gene, which confers kanamycin resistance [13]. However, since most slow growers possess only one *rrn* operon encoding ribosomal RNAs, resistance to agents such as kanamycin can arise easily via spontaneous mutations in the operon [32]. The same level of resistance is not likely to occur in fast growers since most contain two *rrn* operons. A hygromycin resistance gene (*hyg*) from *S. hygroscopicus* was reported suitable for the use in both fast and slow growers [12]. Apramycin resistance has also been used in *M. tuberculosis*; however, its use resulted in low transformation frequencies [4, 3]. Other selectable markers such as gentamycin [18], streptomycin [19], and sulfonamides [33] have been used for selection.

Tetracycline can be used in *M. smegmatis* [33]; however, this antibiotic is not suitable for slow-growing mycobacteria because tetracycline is unstable over the time required for culture (3–6 weeks). Ampicillin resistance is not suitable for use in mycobacteria because mycobacteria are intrinsically resistant to β-lactams. Chloramphenicol cannot be used for direct selection due to a high rate of spontaneous mutation of its target, although it has been used in conjunction with other antibiotic resistance genes [11, 24]. Due to their superior efficiency, kanamycin and hygromycin are used for most gene knockout experiments in mycobacteria [34].

Lastly, the choice of selection markers is dependent on the particular species of mycobacteria being used. For example, electroporation with shuttle plasmids carrying a kanamycin resistance gene as a selectable marker failed to generate transformants in *M. w* and *M. vaccae*, while efficient transformation was obtained using hygromycin selection [14].

In this chapter we describe protocols for efficient transformation of both replicating and integrating plasmids into a slow-growing species, *M. tuberculosis*, and a fast-growing species, *M. smegmatis*.

2 Materials

2.1 Electroporation of M. tuberculosis

1. Tween 80: Prepare as a 20 % v/v stock, filter sterilize through a 0.2-μm membrane, and store at 4 °C (*see* **Note 1**).
2. Complete 7H9 broth: Dissolve 4.7 Middlebrook 7H9 broth base in 900 mL deionized water, add 5 mL of 10 % w/v Tween-80, and autoclave (*see* **Note 2**).
3. Middlebrook OADC enrichment (Becton Dickinson), containing oleic acid, bovine albumin fraction V, dextrose, catalase, and NaCl: Store at 4 °C (*see* **Note 3**).
4. Roller bottles—450 cm^2.
5. 2 *M* glycine, autoclave (*see* **Note 4**).
6. 10 % w/v glycerol; sterilize by autoclaving.
7. Electroporation apparatus with pulse controller (*see* **Note 5**).
8. Electroporation cuvettes; 0.2 cm gap electrodes (*see* **Note 6**).
9. DNA in solution (*see* **Notes 6** and **14**); this should be free from salts, enzymes, and other substances. In order to clean up DNA, it can be ethanol precipitated and thoroughly washed with 70 % ethanol (this will also remove excess salts). The concentration of DNA should be about 0.2–1 mg/mL.
10. 7H10 agar; dissolve 19 g Middlebrook 7H10 agar base in 900 mL deionized water, and autoclave. Cool and add 10 % v/v OADC supplement (*see* Subheading 2.1, **item 3**). Pour plates and use within 1 week.

11. Kanamycin sulfate: 50 mg/mL stock (filter sterilize), store at –20 °C.
12. Hygromycin B: Obtained as a 50 mg/mL stock in phosphate-buffered saline; store at 4 °C in the dark.
13. Selection plates should contain 10–30 μg/mL for kanamycin resistance, and 50–100 μg/mL for hygromycin resistance (*see* Table 2 for other antibiotics).

2.2 Electroporation of M. smegmatis

1. Lemco-Tween broth: 5 g/L peptone, 5 g/L Lemco powder, 5 g/L NaCl, 5 mL/L of 10 % w/v Tween-80 (*see* Subheading 2.1, **item 1**). Autoclave (*see* **Note 2**).
2. 10 % w/v glycerol; sterilize by autoclaving.
3. Electroporation apparatus with pulse controller (*see* **Note 5**).
4. Electroporation cuvettes; 0.2 cm gap electrodes.
5. DNA in solution (*see* **Notes 6** and **14**); this should be free from salts, enzymes, and other substances. In order to clean up DNA, it can be ethanol precipitated and thoroughly washed with 70 % ethanol (this will also remove excess salts). The concentration of DNA should be about 0.2–1 mg/mL.
6. Lemco agar 5 g/L peptone, 5 g/L Lemco powder, 5 g/L NaCl, 15 g/L agar, autoclave.
7. Kanamycin sulfate: 50 mg/mL stock (filter sterilize), store at –20 °C.
8. Hygromycin B: Obtained as a 50 mg/mL stock in phosphate-buffered saline; store at 4 °C in the dark.
9. Selection plates should contain 10–30 μg/mL for kanamycin resistance, and 50–100 μg/mL for hygromycin resistance (*see* Table 2 for other antibiotics).

Table 2
Antibiotic selection for mycobacteria

Antibiotic	Stock solution	Working concentration
Chloramphenicol	34 mg/mL in ethanol	40 μg/mL
Gentamycin	50 mg/mL in water	5–20 μg/mL
Hygromycin	50 mg/mL in PBS	100 μg/mL
Kanamycin	50 mg/mL in water	10–50 μg/mL
Streptomycin	20 mg/mL in water	30 μg/mL
Apramycin	25 mg/mL in water	30–50 μg/mL

3 Methods

3.1 Electroporation of M. tuberculosis

M. tuberculosis is pathogenic to humans; therefore appropriate containment facilities should be used for all procedures (*see* **Note 7**).

1. Inoculate 10 mL of complete 7H9 broth with a loopful of mycobacteria, vortex to disperse cells, and incubate at 37 °C for 10–15 days.
2. Inoculate 100 mL complete 7H9 broth in a roller bottle with 1–10 mL of starter culture and continue incubation at 37 °C with rolling at 100 rpm for 5–7 days (*see* **Note 8**).
3. Add 0.1 volumes 2 *M* glycine (final concentration 1.5 % w/v) 16–24 h before harvesting the cells (*see* **Note 4**).
4. Wash cells three times at room temperature in 10 % glycerol. Reduce the volume each time; for example, for 100 mL, wash one, 20 mL; wash two, 10 mL; and wash three, 5 mL (*see* **Note 14**).
5. Resuspend in 1/50 to 1/100 original volume of 10 % glycerol (*see* **Note 12**).
6. Add 0.5–5 μg salt-free DNA in no more than 5 μL volume (*see* **Notes 6** and **15**) to 0.2 mL mycobacterial suspension (*see* **Note 14**).
7. Transfer to a 0.2 cm electrode gap electroporation cuvette.
8. Place the cuvette in the electroporation chamber and subject to one single pulse of 2.5 kV, 25 μF, with the pulse-controller resistance set at 1,000 Ω resistance (*see* **Notes 15–17**).
9. Recover cell suspension immediately into 10 mL of complete 7H9 broth. Wash cuvette to recover all cells (*see* **Note 18**).
10. Incubate at 37 °C for 16 h. This step allows expression of any antibiotic resistance gene carried on the DNA (*see* **Notes 15** and **18**).
11. Harvest the cells by centrifugation at 3,000 × *g* for 10 min and plate out suitable dilutions (to give 30–300 colonies/plate) on 7H10 agar plus OADC enrichment and appropriate antibiotic (*see* **Notes 19** and **20**).
12. Incubate plates at 37 °C until colonies become visible; this will take approximately 3 weeks (*see* **Note 20**) (*see* Table 3 for other species requirements).
13. Count transformants to calculate transformation efficiency (*see* **Note 23**).
14. Streak out transformants onto solid medium (7H10 plus OADC) plus selection (*see* **Notes 21** and **22**).
15. Analyze transformants as required (*see* **Note 21**).

Table 3
Growth conditions for mycobacterial transformants

Species	Growth temperature	Length of incubation
Fast growers		
M. aurum	37 °C	3–5 days
M. phlei	37 °C	3–5 days
M. smegmatis	37 °C	3–5 days
M. vaccae	30 °C	3–7 days
Slow growers		
M. avium	37 °C	2–3 weeks
M. bovis BCG	37 °C	3–4 weeks
M. intracellulare	37 °C	10–14 days
M. tuberculosis	37 °C	3–4 weeks
M. w	37 °C	10–14 days

3.2 Electroporation of M. smegmatis

1. *M. smegmatis* should be maintained in the laboratory by regular subculture on solid medium (Lemco agar) (*see* **Note 9**).
2. Inoculate 5 mL Lemco-Tween broth with a loopful of mycobacteria. Disperse the cells using a vortex (*see* **Notes 1** and **2**).
3. Incubate at 37 °C with shaking (100 rpm) overnight.
4. Inoculate large-scale culture (100–500 mL in 250–1,000 mL conical flask) with a 1/100 dilution of the overnight culture and continue incubation at 37 °C with shaking until $OD_{600} = 0.8–1.0$ (usually between 16 and 24 h; *see* **Note 10**).
5. Incubate cells on ice for 1.5 h (*see* **Note 11**).
6. Harvest cells by centrifugation at 3,000 × *g* for 10 min.
7. Wash cells three times in ice-cold 10 % glycerol. Reduce the volume each time; for example, for 100 mL, wash one, 20 mL; wash two, 10 mL; and wash three, 5 mL.
8. Resuspend in 1/10 to 1/100 original volume of ice-cold 10 % glycerol (*see* **Note 12**).
9. At this stage, cells may be frozen and stored in aliquots at −70 °C for future use. Cells frozen in this way should be thawed on ice and used as required (*see* **Note 13**).
10. Add 0.5–5 μg salt-free DNA in no more than 5 μL volume (*see* **Notes 6** and **15**) to 0.2 mL mycobacterial suspension and leave on ice for 10 min (*see* **Note 24**).
11. Transfer to a 0.2 cm electrode gap electroporation cuvette (*see* **Note 24**). The cuvette should be chilled on ice before use (*see* **Note 15**).
12. Place the cuvette in the electroporation chamber and subject to one single pulse of 2.5 kV, 25 μF, with the pulse-controller resistance set a 1,000 Ω resistance (*see* **Notes 15** and **16**).

13. Put cuvette back on ice for 10 min, transfer cell suspension to a sterile universal bottle, and 5 mL Lemco broth, and then incubate at 37 °C for 2 h (*see* **Note 18**).
14. Harvest the cells by centrifugation at 3,000 × *g* for 10 min and plate out suitable dilutions (to give 30–300 colonies/plate) on Lemco agar and appropriate antibiotic (*see* **Note 19**).
15. Incubate plates at 37 °C until colonies become visible; this will take 3–7 days.
16. Count transformants to calculate transformation efficiency (*see* **Note 23**).
17. Streak out transformants onto solid medium (Lemco) plus selection or inoculate 5 mL Lemco broth plus Tween and selection antibiotic with transformant colonies. Incubate at 37 °C with shaking (100 rpm) for 2–3 days.
18. Analyze transformants as required (*see* **Note 21**).

4 Notes

1. Growth of cells: Mycobacteria cells, particularly *M. tuberculosis*, have a tendency to clump together in culture; this is owing to the thick waxy nature of the mycobacterial coat. The addition of Tween 80, a nonionic detergent, to media reduces the amount of clumping and provides a more homogenous suspension of cells. Tween 80 can be metabolized by mycobacteria. Alternative non-metabolizable detergents such as Tyloxapol can be used instead.
2. The medium used for growth of mycobacteria for electroporation is not important and a variety of different recipes are used, the most common being Middlebrook 7H9.
3. OADC supplement is extremely heat labile and should only be added to 7H10 or 7H9 media after cooling. *M. bovis* BCG can be grown in medium supplemented with ADC (no oleic acid) rather than OADC, but growth is slower. Growth of mycobacterial cultures is enhanced by the provision of up to 10 % CO_2 in the air above the medium.
4. For slow-growing species, the addition of glycine (to a final concentration of 1.5 %) to young growing cultures improves transformation efficiencies [15, 23, 35–37]. Among Gram-positive bacteria, glycine replaces alanine during peptidoglycan synthesis. Glycine represents a poor substrate for transpeptidation resulting in decreased cell wall cross-linking [38]. In mycobacteria, glycine has been observed to increase transformation efficiency by affecting the cell wall. Ideally, glycine should be added 16–24 h prior to harvesting.

5. There are many different electroporation devices available commercially; any apparatus that can deliver high-voltage pulses can be used. There are also different makes of cuvettes available; although the gap or path length may be the same, the maximum volume of the cell suspension can vary from 50 μL to 400 μL. The volume of cell suspension used does not seem to affect the efficiency [12].
6. DNA concentration: The efficiency of electroporation depends on the choice of DNA for transformation; some vectors have been unable to transform particular mycobacterial species and the efficiency often depends on the choice of the selectable marker. The efficiency of transformation is not affected by the amount of DNA added [15]; addition of 0.5–500 ng DNA produces the same efficiency, and as much as 5 μg can be used. However, a study on *M. avium* showed that as plasmid DNA content increased from 1.5 to 3 μg there was a step increase (734-fold) in transformation frequency and doubling the DNA amount again to 6 μg yielded a further 6.4-fold increase. The volume of DNA used is critical; for small volumes of cell suspensions, the addition of a large amount of DNA in water will alter the conductivity of the suspension. Therefore, it is important that not more than 5 μL of DNA solution is added to the cell suspension.
7. Pathogenic mycobacteria represent an important biohazard; therefore, all culture and genetic manipulation must be carried out in appropriate containment facilities inside a biological safety cabinet. In most countries, genetic manipulation involving pathogenic mycobacteria or their DNA must be met with approval by the relevant authorities. In any case, risk assessment must form the first part of any experiment with pathogenic mycobacteria. A list of mycobacterial species and the type of containment required should be consulted prior to use.
8. The volume of the culture in a 450 cm^2 roller should not exceed 100 mL. It is important to pre-roll the bottles with the media 24 h before inoculation. This is to check for contamination and leaks. Cells should grow until late log phase (e.g., for *M. tuberculosis*, 7 days if inoculated 1:100 and 5 days if inoculated 4:100).
9. Mycobacteria are relatively slow-growing organisms; the fast-growing species have a generation of 2–3 h and the slow-growing species of around 20 h. This often leads to a problem with contamination of cultures because many common contaminants have a much quicker doubling time and will rapidly outgrow mycobacteria. It is extremely important to maintain good aseptic technique, especially with slow growers. It is often wise to set up duplicate cultures in case one becomes contaminated. Cultures should be checked for purity using acid-fast staining at all stages [39].

10. In general, mycobacterial cultures should be removed from the incubator when in the logarithmic phase of growth. However it has been suggested that the transformation frequency for *M. avium* is maximal at early log phase of growth [23].
11. For fast-growing species, once cultures have reached the required stage of growth, they should be removed from the incubator and incubated on ice for 1.5 h prior to harvesting. This results in a fourfold increase in transformation efficiency [1]. Longer incubations on ice result in reduced efficiency, probably owing to increased cell lysis. This may also increase the possibility of arcing during the pulse delivery.
12. For the transformation of a replicative or integrative plasmid cells can be resuspended in 1/10 of the original volume (i.e., 5 mL). However, when homologous recombination is required, it is recommended to resuspend the cells in 1/50 of the original volume (i.e., 1 mL).
13. It is recommended that competent cells that have been thawed from frozen should be harvested and resuspended in fresh 10 % glycerol prior to use. We find that the transformation efficiency generally improves for *M. smegmatis* after cells have been frozen at −80 °C and thawed.
14. For slow-growing species, cells can be kept at room temperature, but electroporation at 37 °C will increase the transformation efficiency [15, 23]. We routinely pre-warm the glycerol washes to 37 °C and centrifuge and pulse at room temperature.
15. Arcing: The use of the pulse-controller apparatus serves to reduce the probability of arcing when using high voltages applied to high-resistance media, although it may still occur. Factors that cause arcing include the presence of lysed cells in the sample or salts in the DNA solution. These factors can be minimized by ensuring that during preparation of electrocompetent cells, the preincubation on ice for fast growers is no longer than 1.5 h. Also make sure that the outside of the cuvette is dry before placing in the pulse chamber. Always ensure that the DNA for transformation is free from salts and other contaminants; ethanol precipitation and washing with 70 % ethanol can be used to clean up DNA, which should be preferably dissolved in sterile deionized, distilled water. The settings for the pulse are important as well. Increasing the parallel resistance to ∞Ω increases the possibility of arcing; therefore a setting of 1,000 Ω produces more consistent results. In some cases, arcing may be violent enough to blow the lid off the electroporation cuvette, dispersing the cell suspension over the inside of the electroporation chamber (thereby creating aerosols).

16. Pulse conditions: The use of a pulse controller in addition to the actual electroporation apparatus allows control over the parallel resistance and therefore the time constant; higher parallel resistance produces a longer time constant. Observations have shown that the optimum time constant is 15–25 ms (1,000 Ω resistance) [1]. The use of 0.2 cm electrode gap cuvettes, as opposed to the 0.4-cm gap cuvettes originally used, results in a higher field strength [1]. The electroporation medium also has an effect on the time constant. Use of diluted glycerol provides a high-resistance medium, allowing longer time constants to be achieved.
17. When working with pathogenic organisms, it is imperative that the pulse is delivered with the electroporation chamber placed inside a Class I safety cabinet and that appropriate disinfectants (e.g., freshly diluted 10 % bleach solution) are at hand.
18. The dilution of cells immediately after the pulse is important. Cells should be diluted at least tenfold and incubated for several hours prior to plating. Omission to this step leads to greatly reduced efficiencies [40]. Presumably, the dilution allows better recovery from the pulse and therefore greater survival of transformants. Slow growers should be incubated for 3–16 h before plating out.
19. The problem of clumping is also important when plating out cells after electroporation. It is important to ensure that resistant colonies have arisen from a single cell, so the cells must be thoroughly resuspended before plating. Appropriate dilutions may also help to alleviate this problem by thinning the resuspension. It is important to dilute the cells prior to plating because the cells suspensions used for electroporation are very concentrated. If the cells are not diluted, then it may be very difficult to visualize true resistant colonies against a background lawn of sensitive cells. This is owing to aggregation, which protects some cells from the effects of the antibiotic.
20. Because slow-growing organisms take up to 4 weeks to form colonies from single cells, it is important to pour plates thickly and to wrap them securely in parafilm or sealable bags to prevent drying out during the long incubation period. Cycloheximide can be added to plates (100 μg/mL) to prevent fungal contamination, which is possible during such long incubation times. The long incubation period also means that antibiotic-containing plates should be freshly poured for each experiment; this will minimize the loss of antibiotic activity.
21. Transformants may be inoculated directly into liquid medium, but we find that they grow more rapidly if they are first streaked out on plates. For most applications, three transformants should be streaked and analyzed. It is important to check the identity of extrachromosomal plasmids after transformation, since deletions and rearrangements are common.

22. Spontaneous kanamycin resistance can often be a problem with *M. tuberculosis*, owing to mutations in the *rrn* operon, of which slow-growing species possess only one. A control electroporation with no DNA to check for the frequency of such mutants can also be included. Problems of spontaneous resistance will apply to all antibiotics that act on *rrn* operon (e.g., streptomycin).
23. Mycobacteria have chemically resistant cell walls that are difficult to lyse; thus they are able to survive high voltage even when pulses have long time constants. Several factors affect the efficiency of transformation; these include the growth phase of cells when harvested, electroporation media, and the field strength and time constant of delivered pulse.
24. Pulse delivery: It is important to have an even cell suspension for electroporation because any clumping of cells will lead to arcing and reduced transformation efficiency. During the standing time on ice prior to pulse delivery, the cells may settle in the tube; it is necessary to redistribute them using a pipet or a vortex immediately prior to the high-voltage pulse. This step serves to resuspend the cells and to ensure thorough mixing of the DNA. In addition, care must be taken to ensure that no bubbles are introduced between the two electrodes of the cuvette.

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Chapter 8

Targeted Gene Knockout and Essentiality Testing by Homologous Recombination

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Abstract

This chapter provides an updated experimental protocol for generating allelic exchange mutants of mycobacteria by two-step selection using the p2NIL/pGOAL system. The types of mutants that can be generated using this approach are targeted gene knockouts marked with a drug resistance gene, unmarked deletion mutants, or strains in which a point mutation/s has been introduced into the target gene. A method for assessing the essentiality of a gene for mycobacterial growth by means of allelic exchange is also described. This method, which utilizes a merodiploid strain carrying a second copy of the gene of interest on an integration vector, allows the exploration by means of complement switching of structure–function relationships in proteins that are essential for mycobacterial growth.

Key words Allelic exchange, Complement switching, Homologous recombination, Illegitimate recombination, Phthiocerol dimycocerosate (PDIM), UV irradiation

1 Introduction

Significant advances have been made in the development of genetic tools to disrupt gene function in mycobacteria in a targeted manner [1–4]. Allelic exchange mutagenesis involves replacement of the gene of interest with a mutant version in which the function of the gene is lost either by deletion of the entire gene or a part thereof, or by insertional inactivation with a marker that confers a selectable phenotype (e.g., drug resistance), or in which the function of the gene is altered by a specific amino acid substitution/s. Allelic exchange mutagenesis relies on the process of RecA-mediated homologous recombination (HR), which involves the exchange of DNA between two molecules carrying homologous DNA sequences. Double crossover (DCO) HR between the mutant allele delivered on a vector and the wild-type allele resident on the chromosome results in allelic exchange. The methods that are commonly used for HR-mediated allelic exchange in mycobacteria differ in terms of the system used to

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deliver the mutant allele into the mycobacterial cell, with both transposon- and suicide-plasmid-mediated delivery systems having been applied widely [2, 3, 5, 6]. The methods that are based on suicide plasmid delivery also differ in terms of the markers used to identify intermediate products of single crossover (SCO) HR from which DCO recombinants are subsequently identified in two-step allelic exchange mutagenesis protocols. Various systems have been described in detail in chapters published in the previous editions of this book, and the reader is referred to these and other review articles for further information [2, 3, 7–9].

The first part of this chapter provides an updated protocol and detailed technical tips for generating allelic exchange mutants in mycobacteria by two-step selection using the p2NIL/pGOAL system [7, 8, 10]. The types of mutant strains that can be generated using this methodology are targeted gene knockouts marked with a drug resistance-encoding gene, unmarked deletion mutants, and strains in which a point mutation/s has been transferred to the target gene. Furthermore, the variations in the methodology used for these applications, as well as modifications of the protocol that have been introduced based on experience in generating targeted gene knockouts in an isolate of H37Rv that is prone towards illegitimate recombination, are described [11]. Loss of the ability of *Mycobacterium tuberculosis* H37Rv to produce the virulence lipid, phthiocerol dimycocerosate (PDIM) [12], is a relatively common occurrence during in vitro culture [13] and in colonies recovered from transformation by electroporation [14]. Inadvertent loss of PDIM production during the course of an allelic exchange experiment can complicate the downstream interpretation of the in vivo phenotype of a mutant carrying a targeted disruption in a gene associated with mycobacterial virulence; therefore, it is recommended that PDIM production is monitored using a method described herein. Finally, we describe the formal assessment by means of allelic exchange mutagenesis of the essentiality of a gene for in vitro mycobacterial growth. This method, which utilizes a merodiploid strain carrying an additional copy of the target gene, provides a convenient means of exploring by means of complement switching, the structure–function relationships in proteins that are essential for mycobacterial growth and/or survival [15–17].

1.1 Allelic Exchange Mutagenesis Using the p2NIL/pGOAL System

The first step in the allelic exchange mutagenesis procedure involves the generation of a suicide plasmid, which carries the mutant allele but lacks a mycobacterial origin of replication (*oriM*) and is therefore unable to replicate in mycobacteria (Fig. 1). The p2NIL/pGOAL system involves the use of positive selection markers encoding resistance to kanamycin (Kan) and/or hygromycin (Hyg), coupled with vector-encoded *lacZ* and *sacB* genes [10]. A SCO event results in the integration of the entire plasmid into the chromosome to generate a partial merodiploid strain that contains

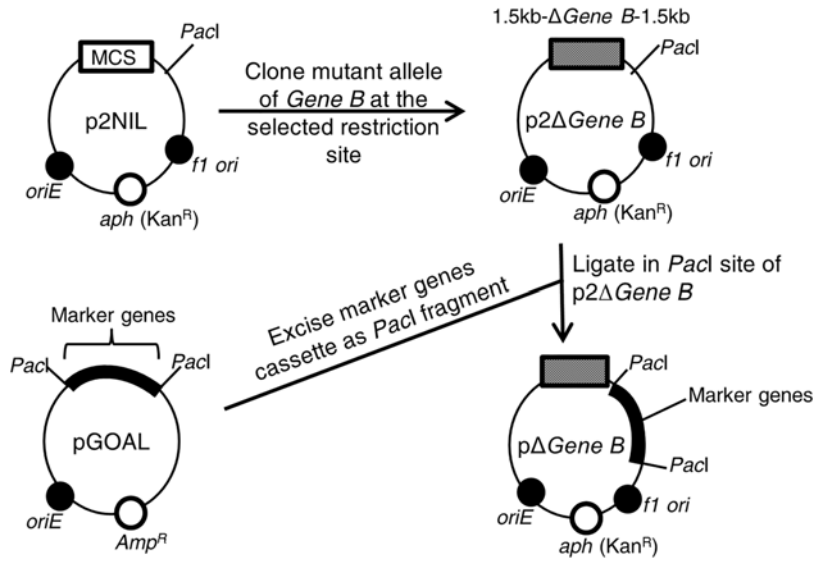

Fig. 1 Cloning strategy for generating suicide delivery vector and complementing vector. The mutant allele of the target gene (designated here as "Gene B") carrying 1.5 kb flanking regions is cloned in p2NIL. The *Pac*I marker cassette from the appropriate pGOAL vector (pGOAL17: P_{Ag85}-*lacZ*-P_{hsp60}-*sacB*; pGOAL19: *hyg*-P_{Ag85}-*lacZ*-P_{hsp60}-*sacB*) is cloned in the *Pac*I site of the resulting construct to generate the suicide delivery vector (*see* Subheading 3.1 and **Note 1**)

both wild-type and mutant alleles and is resistant to Kan and/or Hyg, appears blue when plated on solid agar medium supplemented with X-gal, and is sensitive to sucrose. A second recombination event results in expulsion of the vector from the chromosome to generate a DCO recombinant that can be selected by plating on sucrose and carries either the wild-type or mutant allele, depending on whether the recombination event had taken place on the same or opposite side of the mutation. Although marked mutants can be identified directly by plating transformed cells on media containing the corresponding drug resistance marker, the identification of unmarked or point mutants requires two-step selection using the selectable and counter-selectable markers, as described earlier [7, 8, 10].

UV irradiation of the suicide plasmid prior to electroporation has been found to increase the frequency of HR events between the plasmid and the chromosome, presumably by activating the SOS response [18, 19]. This carries the potential risk of inducing a damage tolerance system whose engagement may lead to the introduction of inadvertent, second-site mutations [19, 20]. Interestingly, however, no second-site mutations were found by whole-genome re-sequencing (WGS) of a *metH* mutant of *M. tuberculosis* that was generated by two-step allelic exchange using a mutant allele delivered on a UV-irradiated suicide vector [21]; in this case, the only mutation identified in the mutant

strain was that which had been engineered in the delivered *metH* gene [22]. Nevertheless, as WGS becomes increasingly affordable and is routinely applied in the genotypic characterization of allelic exchange mutants of *M. tuberculosis*, the magnitude of the risk of second-site mutagenesis as a result of the UV irradiation step will become clearer.

1.2 Gene Essentiality Testing by Homologous Recombination

Genes that are essential for mycobacterial growth in vitro are of particular interest as they include those that encode potential drug targets [23]. By default, targeted knockouts in essential genes are not viable, and therefore cannot be recovered through routine allelic exchange. Although the failure to obtain a targeted knockout mutant by allelic exchange is indicative of essentiality of gene function, proof of essentiality requires a formal demonstration that inactivation of the wild-type copy of the gene is possible only in the presence of a second copy, located elsewhere on the chromosome. This can be achieved by integrating a second copy of the gene into the chromosome of an SCO recombinant prior to counter-selection for DCO recombinants, as per the standard protocol. The second copy of the gene can be expressed under the control of its own promoter, or a heterologous, regulatable promoter [15, 24–26]. By producing a strain in which a complementing copy of the wild-type gene is expressed from an L5 integration site on the chromosome [27], the relationship between the gene structure and function can be explored by means of complement switching. This technique, which takes advantage of the dual integration and excision function of the L5 phage integrase that is present on the integration plasmid, allows the wild-type complementing gene, expressed from the native or a conditional promoter on an integrating vector containing a selected antibiotic marker, to be replaced by a version carrying a specific mutation/s and an alternative antibiotic marker [15–17].

2 Materials

2.1 Construction of Suicide Delivery Constructs: Mutation Marked with Drug Resistance Gene

1. Primers required for constructing the plasmid to introduce mutation/s in the target gene.
2. PCR reagents.
3. PCR product cloning vector, e.g., pGEM®-T Easy (Promega).
4. p2NIL cloning vector [10].
5. Appropriate pGOAL vector—pGOAL17 or pGOAL19 (*see* **Note 1**) [10].
6. pIJ963 plasmid [28] carrying the hygromycin resistance gene (*hyg*).
7. Competent *Escherichia coli* DH5α cells (Invitrogen).

p2NIL (Plasmid #20188), pGOAL17 (Plasmid #20189), and pGOAL19 (Plasmid #20190) can be obtained on request from Addgene (http://www.addgene.org/), a nonprofit plasmid repository.

2.2 Construction of Suicide Delivery Constructs: Unmarked Deletion Mutation

See materials in Subheading 2.1, **items 1–6**.

2.3 Construction of Suicide Delivery Constructs: Point Mutation

See materials in Subheading 2.1, **items 1–6**.

1. Mutagenic forward primer designed to generate desired mutation.
2. Forward and reverse primers (*see* Subheading 3.3 for design).
3. PCR reagents.
4. *See* materials in Subheading 2.1, **items 1–6**.

2.4 Pretreatment of DNA with UV Irradiation

1. 5 μg of suicide delivery plasmid.
2. 70% ethanol.
3. Vaccum concentrator.
4. Sterile deionized H_2O.
5. UV Stratalinker (Agilent) or similar device capable of producing 100 mJ/cm^2 ultraviolet light.

2.5 Two-Step Strategy for Allelic Exchange Mutagenesis and Gene Essentiality Testing

1. Late-logarithmic-phase culture (OD_{600} = 1.0–1.2) of *M. tuberculosis* H37RvJO, *M. tuberculosis* H37RvMA, or *M. smegmatis* mc^{2}155.
2. 30 % glycine.
3. Kanamycin sulfate: Prepare a 50 mg/mL stock solution with distilled water, filter-sterilize, and store in aliquots at –20 °C. For selection, use at a concentration of 20 μg/mL for both *M. tuberculosis* and *M. smegmatis*, and at 50 μg/mL for *E. coli*.
4. Hygromycin B (Roche): Available as a 50 mg/mL stock solution. Store at 4 °C and protect from light. For selection, use at a concentration of 50 μg/mL for both *M. tuberculosis* and *M. smegmatis* and at 150–200 μg/mL for *E. coli*.
5. Gentamicin sulfate: Available as a 50 mg/mL stock solution. Store at 4 °C. For selection, use at a concentration of 5 μg/mL for both *M. tuberculosis* and *M. smegmatis* and 20 μg/mL for *E. coli*.
6. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal): Make stock solution of 2 % in DMSO and store at –20 °C. Use 0.4 % X-gal for the selection of SCOs.
7. 10 % w/v Tween-80.

8. Oleic-acid-albumin-dextrose-catalase (OADC) supplement (BBL™, Becton Dickinson): Sterile solution, store at 4 °C.
9. Middlebrook 7H9 broth: Prepare Middlebrook 7H9 broth (Becton Dickinson) and supplement with 10 % v/v OADC, 0.05 % w/v Tween 80, and 0.2 % w/v glycerol unless otherwise specified.
10. 7H10 agar: Prepare Middlebrook 7H10 agar base (Becton Dickinson) and supplement with OADC and 0.5 % w/v glycerol, unless otherwise specified.
11. Sucrose stock solution: Dissolve 50 g of sucrose in 100 mL deionized water (50 % w/v) and filter-sterilize. Use at a final concentration of 2 % w/v.

2.6 Construction of Integrating Complementation Vector

Use one of the following vectors, depending on the required selection marker.

1. pHINT is an integrative mycobacterium-*E. coli* shuttle vector, which contains dual origin of replication, and the L5 phage-derived *attP*/integrase which mediates recombination between the core sequences of the *attP* and the chromosomal *attB* sequence. The vector also carries hygromycin and ampicillin resistance markers.
2. pGINT [29], a derivative of pHINT [30] carrying a gentamicin (Gm) resistance marker.
3. pAINT [31], a derivative of pHINT carrying a kanamycin resistance marker.

These plasmids can be obtained on request from the corresponding author.

2.7 Gene Essentiality Testing

See materials in Subheading 2.5, **items 1–11**.
See materials in Subheading 2.6, **items 1–3**.

2.8 PDIM Analysis by Thin-Layer Chromatography (TLC)

1. Middlebrook 7H9 broth—*see* Subheading 2.5, **item 9**.
2. ^{14}C-Propionate.
3. 0.3 % sodium chloride:methanol 10:1.
4. Petroleum ether.
5. Chloroform.
6. Silica gel TLC plates, 5 cm × 10 cm (L × W).
7. TLC chamber.
8. 4.5 in. Whatman paper.
9. Petroleum ether:ethyl acetate 9:2.
10. Biomax® Kodak® film or similar.

3 Methods

3.1 Construction of Suicide Delivery Constructs: Mutation Marked with a Drug Resistance Gene (See Notes 2 and 3)

1. Design primers to PCR amplify flanking regions on either side of the target gene to eliminate part or all of the coding sequence, with appropriate restriction enzyme sites constructed into their 5′- and 3′-ends. Alternatively, delete an internal segment of the gene by restriction enzyme digestion or inverse PCR.
2. Amplify and clean the PCR product and clone into appropriate cloning vector, e.g., pGEM®-T Easy.
3. Insert a Hyg-resistance-encoding cassette (*hyg*), excised as a *Bam*HI-*Bgl*II cassette from plasmid pIJ963, at the junction of the flanking regions to create a *hyg*-marked mutant allele.
4. Clone the *hyg*-marked mutant allele into the p2NIL plasmid.
5. Genotypically confirm the resulting construct by restriction enzyme mapping and/or sequencing.
6. Excise the *Pac*I-digested cassette containing the P_{Ag85}*lacZ*-P_{hsp6}*sacB* marker cassette from pGOAL17 [10], and clone into the unique *Pac*I site of the p2NIL derivative carrying the *hyg*-marked mutant allele.
7. Genotypically confirm the resulting construct by restriction mapping (*see* **Note 4**).
8. Make a large-scale preparation of the suicide delivery plasmid.

3.2 Construction of Suicide Delivery Constructs: Unmarked Deletion Mutation (See Notes 2 and 3)

1. Amplify approximately 1.5 kb of the flanking sequence up- and downstream of the target gene, eliminating all or part of the coding sequence, using primers with appropriate restriction sites to facilitate subsequent cloning in p2NIL.
2. Clean the PCR product; digest with restriction enzymes to clone into a PCR cloning vector (e.g., pGEM®-T Easy) or directly into p2NIL. Alternatively, the PCR products (devoid of any constructed restriction sites) could be cloned directly into *Pml*I-digested p2NIL (Fig. 1).
3. Genotypically confirm the resulting construct by restriction mapping and/or sequencing.
4. Excise the *hyg*-P_{Ag85}*lacZ*-P_{hsp60}*sacB* marker cassette from pGOAL19, and clone into the unique *Pac*I site of the p2NIL derivative carrying the mutant allele.
5. Confirm the construct by restriction enzyme mapping and/or sequencing.
6. Make a large-scale preparation of the suicide delivery plasmid.

3.3 Construction of Suicide Delivery Constructs: Point Mutation/s

1. The point mutation/s is introduced into the gene of interest using a PCR-based mega primer method employing three primers and two amplification steps [20, 32].

2. In the first amplification step, amplify the target gene using an internal mutagenic primer carrying the desired mutation/s and a reverse primer containing an appropriate restriction site to introduce the mutation.
3. In the second amplification step, use the resulting amplicon (approximately 200–300 bp in length) as a mega primer with a forward primer located upstream of the mutagenic site and carrying a restriction site for cloning in p2NIL [20, 32].
4. Clone the fragment carrying the mutant allele in p2NIL and prepare the corresponding suicide delivery plasmid by following **steps 3–6** of Subheading 3.2.

3.4 Pretreatment of DNA with UV Irradiation (See Notes 5 and 6)

1. Precipitate 5 μg of the suicide plasmid DNA.
2. Wash the DNA pellet twice with 70 % ethanol.
3. Dry the DNA pellet under vacuum and resuspend in 100 μL of sterile deionized water.
4. Add the DNA sample into a well of a sterile microtiter plate.
5. Remove the cover of the microtiter plate and expose to 100 mJ/cm^2 of UV irradiation.
6. Transfer the irradiated DNA into a microtube/electroporation cuvette. If necessary, the irradiated DNA can be stored at −20 °C for later use.

3.5 Two-Step Strategy for Generating Allelic Exchange Mutants (Figs. 2 and 3)

1. Use fresh log-phase pre-culture (1:100), stock culture vials (~1 mL), or a single colony from a 7H10 plate, as inoculum to start a liquid culture (150–200 mL volume) in Middlebrook 7H9 broth. Allow the cells to grow to an OD_{600} of 1.0–1.2.
2. Approximately 16–20 h prior to electroporation, add glycine (final concentration of 1.5 %) to the culture (*see* **Note 7**). Addition of glycine is not necessary for *M. smegmatis*.
3. Harvest the cells at 20 °C by low-speed centrifugation (3,901 × *g*) for 10 min in 50 mL Falcon tubes (*see* **Note 8**).
4. Decant the supernatant and resuspend the pellet initially in ~10 mL of 10 % glycerol, taking care to avoid creating bubbles and aerosols.
5. Pool the resuspended pellets from multiple tubes into a single tube (depending on the size of the original culture), wash the pellets with 10 % glycerol (volume of up to 40 mL), and centrifuge as described in **step 3**.
6. Wash the pellet twice with 10 % glycerol.
7. Resuspend the pellet in 5 mL of 10 % glycerol. The cells are now ready for use.
8. Transfer the pretreated DNA (as described in Subheading 3.4) into the electroporation cuvette (0.2 cm gap) before adding

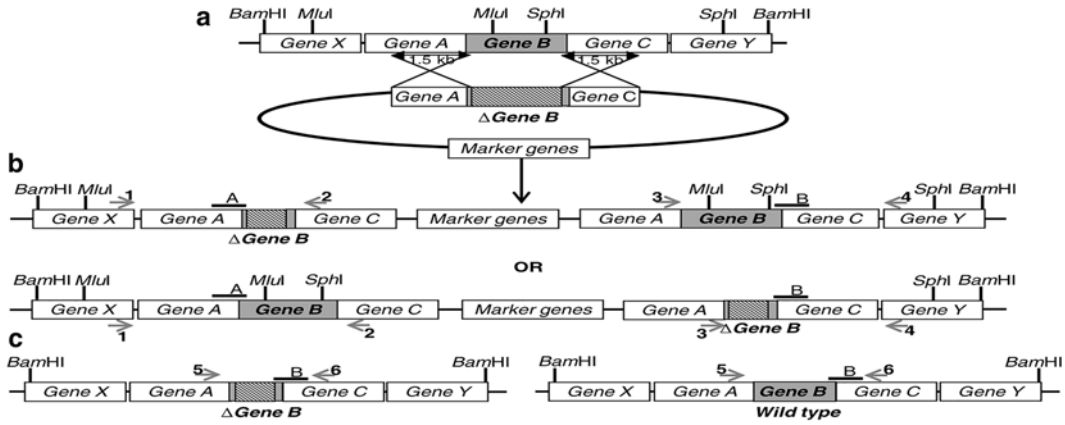

Fig. 2 Two-step strategy for allelic exchange mutagenesis with genotyping of SCO and DCO recombinants. (**a**) The strategy for targeted deletion or point mutagenesis by HR. The target gene (*Gene B*) is shown as *grey box* and the region selected for in-frame deletion or point mutation as a *hatched box*. The suicide plasmid carrying the mutant allele is electroporated into the mycobacterium enabling HR to occur. (**b**) Strategy for genotyping of recombinants. SCO recombinants contain both wild-type and mutant alleles with marker genes from the plasmid. Site-specific SCO recombination events can occur up- or downstream of the mutation, giving rise to distinct SCO genotypes which can be differentiated from one another and from illegitimate recombinants (not shown) by PCR using the primer pairs 1&2 and 3&4. SCOs can also be genotyped by Southern blot analysis probing *Mlu*I- (using probe A) or *Sph*I-digested (using probe B) chromosomal DNA. (**c**) The second HR event generates DCOs that carry either the wild-type or the mutant allele. DCOs are genotypically confirmed by PCR (using primer pair 5&6) or by Southern analysis of *Bam*HI-digested chromosomal DNA (using probe B). For point mutants, the presence of the mutation can be confirmed by restriction enzyme digestion of a PCR amplicon and/or Southern blotting for cases in which the mutation introduces a unique restriction site, or by targeted DNA sequencing

400 μL of competent *M. tuberculosis* or *M. smegmatis* cells. Include one sample without plasmid DNA as a "no plasmid" control, and a second sample with a replicating plasmid (e.g., pOLYG) as a transformation control.

9. Use the following settings to perform the electroporation: 2.5 kV, 1,000 Ω, and 25 μF (*see* **Notes 9** and **10**). Immediately after each electroporation, rescue the cells by adding 700 μL of Middlebrook 7H9 broth. Transfer the rescued cells to a 1.5 mL Eppendorf tube and incubate at 37 °C overnight (for *M. tuberculosis*) or for 4 h (for *M. smegmatis*).
10. Spin the rescued cells at 9,300 × *g* for 2 min, resuspend the pellet in 100 μL of liquid medium, and plate the entire volume of cells onto Middlebrook 7H10 medium containing Hyg and Kan to select for SCO recombinants. In the case of the "no plasmid" and replicating plasmid controls, serially dilute the transformed cells. Plate 100 μL of the 10^{-1} and 10^{-2} dilutions of the "no plasmid" control onto Middlebrook 7H10 medium containing antibiotics to ascertain the frequency of spontaneous resistant mutants, and plate 100 μL of the 10^{-5}, 10^{-6}, and

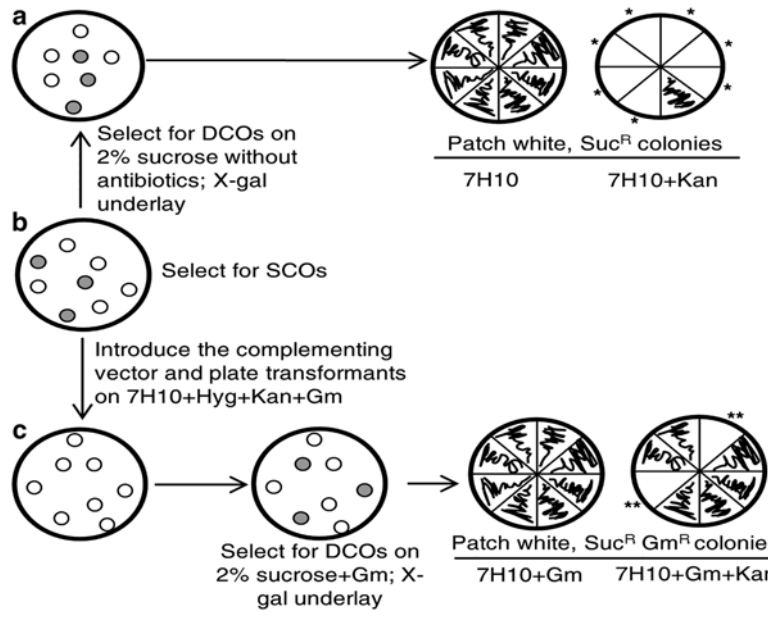

Fig. 3 Algorithm to identify DCOs from SCO recombinants. (**a**) SCOs are selected with Kan, Hyg, and X-gal. Blue colonies are denoted by *grey shading*. (**b**) Selection of DCOs in a wild-type background. White, Suc^R Kan^S clones are putative DCOs (denoted by an *asterisk*). (**c**) Selection of DCOs in a genetically complemented background. Suc^R colonies which are Kan^S and Gm^R are putative DCOs (denoted by *double asterisks*)

10^{-7} dilutions onto antibiotic-free Middlebrook 7H10 medium to enumerate the total number of viable cells. To assess the transformation efficiency, plate 100 μL of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions of the replicating plasmid control onto Middlebrook 7H10 medium containing Hyg (*see* **Note 10**). Incubate the plates at 37 °C for 3–4 weeks.

11. Perform the "X-gal underlay" as follows: carefully lift one corner of the agar medium from the bottom edge of the plate by using a filter-tipped pipette; dispense 200 μL of 0.4 % X-gal solution to cover the surface area of the plate below the agar medium; re-lay the lifted part of the agar medium; and incubate the plate at 37 °C. The colonies that appear blue after 1–2 days are SCOs (*see* **Note 11**).
12. Inoculate four individual SCOs (blue, Hyg resistant (Hyg^R) and Kan resistant (Kan^R)) into fresh Middlebrook 7H9 broth containing Kan and Hyg, and incubate at 37 °C for 1–2 weeks ($OD_{600} = 0.6$) (*see* **Notes 12** and **13**).
13. Prepare tenfold serial dilutions up to 10^{-4} and plate 100 μL from 10^0, 10^{-1}, and 10^{-2} dilutions onto Middlebrook 7H10 agar medium supplemented with 2 % sucrose and control Middlebrook 7H10 agar medium without sucrose, and incubate

at 37 °C for 4–5 weeks. Thereafter, underlay the plates with X-gal solution, as described earlier, and incubate overnight at 37 °C (*see* **Note 14**).

14. Score the number of blue and white colonies on each plate.
15. Pick at least 40 white, sucrose-resistant (Suc^R) colonies from the sucrose-containing plates and patch onto fresh Middlebrook 7H10 agar plates with and without Kan supplementation, and incubate for 3 weeks before scoring Kan sensitivity (Kan^S) (*see* **Note 14**).
16. Select at least 40 Kan^S clones and genotype by colony-boil PCR (*see* **Note 15**).
17. Select a few putative DCO clones and grow in 40 mL of Middlebrook 7H9 broth for 1–2 weeks ($OD_{600} = 0.6$) at 37 °C.
18. Extract genomic DNA from cultures and genotype after overnight restriction enzyme digestion by Southern blotting (*see* **Note 16**).

3.6 Construction of Complementation Vector (See Note 17)

1. Amplify the target gene with its native promoter using primers with appropriate restriction sites.
2. Clone the fragment, after restriction enzyme digestion, into the integrating vector, e.g., pGINT (Gm^R).
3. Genotypically confirm the complementation vector by restriction mapping and/or sequencing.
4. Make a large-scale preparation of plasmid DNA.

3.7 Gene Essentiality Testing

1. Generate a complemented SCO recombinant (merodiploid) strain (Figs. 2 and 3) by starting a liquid culture of an SCO recombinant (*obtained as described in* Subheading 3.5) in Middlebrook 7H9 broth (150 mL) supplemented with Kan and Hyg. Allow the culture to grow at 37 °C to an $OD_{600} = 1.0–1.2$.
2. Electroporate the culture with the pGINT-derived complementation vector (≤1 μg).
3. Set the settings for electroporation and rescue the cells as described in **step 9** of Subheading 3.5.
4. Briefly spin the rescued cells at 9,300 × *g* for 2 min, resuspend the pellet in 100 μL of Middlebrook 7H9 broth, and make further serial dilutions (10^{-1} to 10^{-8}) using the same. Plate 100 μL from 10^{-1} and 10^{-2} dilutions onto Middlebrook 7H10 agar plates containing Hyg, Kan, and Gm, to obtain SCO recombinants carrying the complementation vector. In addition, include the no plasmid and replicating plasmid (e.g., pOLYG) transformation controls as described in **step 10** of Subheading 3.5.
5. Incubate the plates at 37 °C for 3–4 weeks.

6. Select five Hyg^R Kan^R Gm^R transformants and streak to individual colonies.
7. Isolate DCOs from a complemented SCO recombinant (Figs. 2 and 3) by inoculating individual complemented SCO recombinant (merodiploid) strain (blue, Hyg^R, Kan^R, and Gm^R), as obtained in Subheading 3.4, **step 2**, into fresh Middlebrook 7H9 broth containing Kan, Hyg, and Gm, and incubate at 37 °C for 1–2 weeks ($OD_{600} = 0.6$).
8. Carry out *sacB*-based counter-selection as described in **steps 13–14** of Subheading 3.5 using Middlebrook 7H10 agar medium supplemented with 2 % sucrose and Gm.
9. Select ten white, Suc^R and Gm^R colonies and test for sensitivity to Kan and Hyg. Select Hyg^S Kan^S clones.
10. Genotypically analyze Suc^R Gm^R Hyg^S Kan^S clones by PCR and/or Southern blotting for the presence of introduced mutation/s in the chromosomal copy of the target gene.
11. Construct a complement switching plasmid (*see* **Notes 18** and **19**) (Fig. 4) by amplifying the target gene with its native promoter sequences using primers with chosen restriction sites.
12. Clone the fragment, after restriction enzyme digestion, into pAINT (Kan^R) at the appropriate site.
13. Genotypically confirm the construct by restriction mapping and/or sequencing.
14. Make a large-scale preparation of plasmid DNA.
15. For electroporation, start a liquid culture with 150 mL of Middlebrook 7H9 broth containing Gm, which is required to maintain integration of the complementation vector, and a fresh log-phase pre-culture of recombinant mutant strain (Gm^R), obtained as described in **steps 7–10** of Subheading 3.7. Grow to an OD_{600} of 1.0–1.2.
16. Electroporate with either pAINT (Kan^R) or a derivative carrying the intact or mutated copy of the gene under control of a promoter (native or heterologous).
17. Harvest the electroporated cells by centrifugation, resuspend the pellet in 100 μL of Middlebrook 7H9 broth, and perform tenfold serial dilutions. Plate 10^0, 10^{-1}, and 10^{-2} dilutions separately on Middlebrook 7H10 agar medium containing either Kan or Gm.
18. Incubate the plates for 3–4 weeks at 37 °C. The isolation of colonies on Kan-containing medium is indicative of a successful "complement switching" event.
19. Randomly select 10–15 Kan^R colonies and test for Gm sensitivity.
20. Select and genotypically analyze Kan^R Gm^S colonies by PCR and Southern blotting.

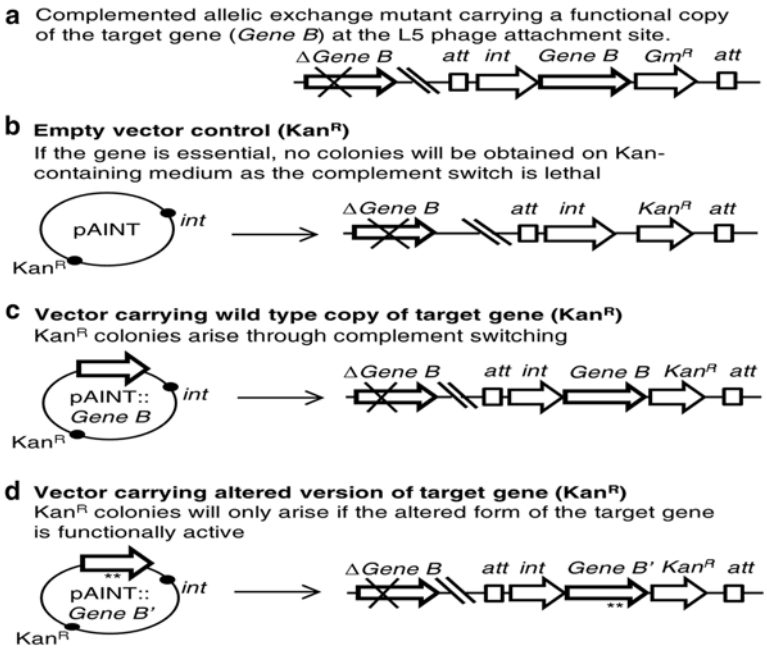

Fig. 4 Complement switching. (**a**) Genotype of a strain carrying an inactive version of an essential gene of interest (*Gene B*) at the chromosomal locus and a complementing copy of the wild-type gene integrated at the L5 phage attachment site via pGINT, constructed as described in Subheading 3.6, **step 3**. (**b**–**d**) Genotypes of the products of complement switching with an incoming pAINT-based vector carrying no insert (**b**); a wild-type copy of the target gene (**c**), or an altered version thereof (*Gene B'*) (**d**). *Asterisks* denote mutations (point mutations, deletions, insertions) in the gene

3.8 PDIM Analysis of M. tuberculosis Strains

The steps described below result in the generation of radioactive and biohazardous liquid and solid wastes, which must be treated in accordance with standard practices for safe handling and disposal.

1. Label methyl-branched lipids with ^{14}C-propionate by growing a culture of a strain of *M. tuberculosis* (e.g., an allelic exchange mutant) to early log phase ($OD_{600} = 0.5$) in Middlebrook 7H9 broth containing 0.2 % glycerol, 0.05 % Tween-80, and OADC.
2. Add 2 μL of ^{14}C-labeled propionate into a separate tissue culture flask.
3. Add 10 mL of the *M. tuberculosis* culture (from **step 1**) to the flask containing ^{14}C-propionate and incubate at 37 °C for 24–48 h.
4. To extract lipids, transfer the culture to a 15 mL conical tube.
5. Centrifuge the culture for 10 min at 3,900 × *g*. Disinfect the supernatant appropriately and dispose as liquid ^{14}C waste.

6. Add 5 mL of methanol:0.3 % sodium chloride (10:1) to the cell pellet, followed by the addition of 5 mL of petroleum ether, and vortex vigorously for 3–4 min.
7. Spin the tube at 780 × *g* for 10 min. Carefully pipette the top layer (without touching or disturbing the interface or bottom layer) into a 50 mL tube for storage.
8. Apply **steps 4** and **5** to the remaining bottom layer and re-extract.
9. At this point, the total volume of the extract should be approximately 10 mL. Add an equal volume of chloroform to the extract and leave in the chemical hood overnight to allow evaporation of chloroform and concentration of lipids for 1–2 days until approximately 1 mL remains. The lipid extracts can be kept in small glass vials for long-term storage.
10. Analyze the lipid by thin-layer chromatography (TLC) (*see* **Note 20**) by drawing a line 1 cm from the top, and another 1 cm from the bottom of a TLC plate. Mark the bottom line with points where the lipid extract will be spotted.
11. Spot approximately 1 μL aliquots onto the plate. Allow the spot to dry and repeat until the tube is empty or until 24 μL has been spotted (*see* **Note 21**).
12. Add petroleum ether:ethyl acetate (9:2 v/v) in the TLC developing chamber.
13. Place three pieces of Whatman paper in the chamber as a wick for equilibrating solvent and leave for 1–2 min.
14. Place the TLC plate in the chamber (taking care not to touch the Whatman paper) and make sure that the bottom part of the chamber is filled with solvent and that the level of solvent is well below the sample spots. Leave until the solvent border reaches the top line.
15. Air-dry the TLC plate and plastic wrap, and expose to Biomax® Kodak® film for 10 days.
16. Develop and fix the exposed X-ray film.

4 Notes

1. Both pGOAL19 and pGOAL17 carry markers for blue/white selection (*lacZ*) and sucrose sensitivity (*sacB*). The marker cassette from pGOAL19 contains an additional selectable marker (*hyg*) and is used to construct unmarked deletion mutants. The marker cassette from pGOAL17 is used to generate *hyg*-marked mutants where the *hyg* cassette is inserted into the deletion allele.
2. The regions of homology used to construct a mutant allele are PCR amplified as 1–1.5 kb fragments with restriction enzyme sites engineered at the 5′- and 3′-ends. The fragments are

sub-cloned in a PCR cloning vector (e.g., pGEM®-T Easy) prior to cloning in p2NIL.

3. The frequency of HR is dependent on the length of the homologous flanks. We generally use flanking sequences greater than 1 kb in length when constructing mutant alleles. However, the frequency at which allelic exchange mutants are obtained varies between genes/genomic loci, and between mycobacterial strains.
4. It is important to check the integrity of the suicide plasmid prior to use. In some cases, we have observed rearrangements/deletions during propagation in *E. coli*. We therefore recommend isolating the final plasmid (vector carrying mutant allele and marker cassette) from *E. coli* cells that have been grown at 30 °C or lower temperature.
5. Pretreatment of the suicide plasmid by UV irradiation requires a pure, concentrated preparation of DNA. High yields of plasmid DNA can be obtained using the standard alkali lysis protocol, followed by purification by phenol-chloroform-isoamyl alcohol extraction [7]. Alternatively, commercial plasmid purification kits may be employed, which are quicker. Importantly, UV pretreatment is only used for suicide plasmids and not for integration vectors.
6. As described above, UV pretreatment of suicide plasmids carries a risk of inadvertent introduction of second-site mutations. As an alternative, alkali pretreatment can be used to improve the transformation efficiency. In brief, the plasmid DNA (2 μg) is denatured with 0.2 M NaOH/0.2 mM EDTA, followed by incubation for 30 min at 37 °C. The denatured DNA is purified with the addition of 3 M sodium acetate (pH 4.8) and precipitated by using ethanol prior to electroporation [33].
7. Glycine treatment is thought to improve the transformation efficiency by weakening the mycobacterial cell wall. However, the addition of glycine to the culture is optional. We routinely electroporate *M. tuberculosis* H37RvMA [11] without glycine pretreatment. The glycine stock (30 %) crystallizes on storage, and can be redissolved by gentle heating in a microwave oven. If used, the glycine stock must be cooled to room temperature prior to addition to the culture.
8. It had been shown that the transformation efficiency of slow-growing mycobacteria, unlike fast growers, could be increased by washing the cells with glycerol and centrifugation at room temperature [34]. For *M. smegmatis*, the steps involve harvesting, washing, and suspending in 10 % glycerol at 4 °C or on ice.
9. Excessive salts in the DNA sample cause arcing (electrical discharge) during the electroporation procedure with a consequent drop in transformation efficiency. If possible, avoid using DNA that has been stored at −20 °C for an extended period of

time. For *M. smegmatis*, keep the electro-competent cells and cuvette on ice prior to electroporation.

10. It is important to use a replicating plasmid (e.g., pOLYG) [30] as a transformation control. A transformation efficiency of $\geq 10^5$ CFU per μg of replicating plasmid is required in order to obtain SCOs with a suicide plasmid.
11. SCO recombinants (Hyg^R Kan^R) turn blue following the X-gal underlay after 1–2 days of incubation. Colonies that appear pale blue should be re-streaked onto a fresh plate with antibiotic and X-gal for reconfirmation. Although H37RvMA and H37RvJO have comparable transformation efficiencies when transformed with episomal or integrating vectors, we have observed a significantly higher frequency of illegitimate recombination in H37RvMA than H37RvJO when transformed with suicide vectors for allelic exchange mutagenesis [11]. For this reason, we routinely select at least ten SCOs and check site specificity and orientation of recombination by PCR-based genotyping (Fig. 2). We have found that genotyping by PCR provides a rapid means of identifying putative site-specific SCOs for further confirmation by Southern blotting, and for subsequent use in DCO selection.
12. This step allows the second crossover event to occur. Avoid adding X-gal while preparing the plates. For unmarked or point mutants, no antibiotics should be included, but for *hyg*-marked mutants, include Hyg in the plates.
13. We avoid resuspending SCO cells in liquid medium by vortex mixing as it poses a risk of aerosol generation. Prior to the isolation of DCOs, we grow the SCOs in liquid medium containing appropriate antibiotics, as it is quicker than growing on solid medium.
14. Plate no more than three dilutions (10^0, 10^{-1}, 10^{-2}) on Middlebrook 7H10 agar medium with or without sucrose. Incubate the plates for 3–4 weeks (*M. tuberculosis*) or for 4–7 days (*M. smegmatis*) at 37 °C. If the mutant has a reduced growth rate, continue the incubation for up to 5 weeks. Typically, *sacB* counter-selection will result in a 4 $\log_{10}$ reduction in CFUs in 2 % sucrose-containing plates. Underlay the plates with X-gal and select white colonies to distinguish DCOs (white) from spontaneous *sacB* mutants (blue). In the case of unmarked or point mutants, DCOs carry either the mutant or the wild-type allele, depending on where the second recombination event occurred.
15. To identify DCOs, we routinely streak a minimum 40 white, Suc^R colonies for Kan sensitivity. Eight colonies can be streaked onto a single 90 mm agar plate; DCO recombinants will be Kan^S. For *hyg*-marked mutations, all Hyg^R Kan^S colonies should carry the mutant allele. For unmarked deletion or point mutations,

all Kan^S clones must be genotypically screened in order to distinguish the allelic exchange mutant from wild type, which is regenerated when the second crossover event occurs on the same side of the mutation as the first recombination event. However, the frequency at which allelic exchange mutants are recovered varies between genes/loci. Therefore, it may be necessary to screen a significantly larger number of DCOs in order to identify a mutant.

16. As the allelic exchange procedure can result in the inadvertent acquisition of second-site mutations, genetic complementation is essential in order to validate the phenotype of an allelic exchange mutant. In addition, WGS of DCO recombinants is highly recommended. In the case of unmarked and point mutants, recovery of DCOs that only carry a wild-type copy of the target gene suggests that the mutation might be lethal (i.e., the gene is essential).
17. For complementation, we routinely amplify the target gene with its native promoter. If the native promoter is unknown/obscure, the target gene can be cloned on a plasmid carrying a heterologous promoter. Occasionally, genetic complementation with an integration or episomal plasmid might not reverse the observed phenotype of the mutant fully or even partially. In such cases, complementation with the gene flanked by more adjoining sequence (approximately 5–7 kb) may be necessary. Alternatively, genetic reversion of the mutation could be carried out by "knock-in" allelic exchange mutagenesis, as reported previously [35].
18. Various vectors that carry different antibiotic resistance markers and integrate at the L5 phage attachment site are available, for example, pKP186 and pKP203 [17], pAPA3 [24], and pUC-Gm-Int [36].
19. The frequency of complement switching is reported to be very robust (10^6 transformants per μg/plasmid DNA) and co-integration of plasmids is rarely seen [17, 37]. The major consideration when applying this method is that the resident and incoming integration vectors must carry different drug resistance markers.
20. Always make sure that the TLC chamber contains enough solvent to just cover the bottom and a filter paper wick for equilibration. Never use a pen instead of a pencil to mark the line on the TLC plate. The problems of streaking and poor resolution of PDIMs after separation can be avoided by limiting the volume of sample spotted on the TLC plate. The solvent mixture of chloroform/methanol/water (100:14:0.8, by volume) should be used for the detection of sulfolipids.
21. At least four different samples can be spotted on a 5 cm × 10 cm silica TLC plate.

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Chapter 9

Construction of Conditional Knockdown Mutants in Mycobacteria

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Abstract

By definition, essential genes are fundamental to bacterial growth, yet the functions of many such genes remain unknown. Essential genes furthermore are central to the activity of most antibacterial drugs and among the most attractive targets for the development of new therapeutics. This chapter describes how synthetic genetic switches that utilize transcriptional repression, controlled proteolysis, or both to silence gene activity can be applied to construct and characterize conditional knockdown (cKD) mutants for essential genes in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*.

Key words Gene silencing, Conditional knockdown mutant, Transcriptional repression, Controlled proteolysis, Essential genes

1 Introduction

The first controlled expression system for mycobacteria was described in 1998 [1]. It utilized the transcriptional regulatory elements of the *Mycobacterium smegmatis* acetamidase gene *amiE* and acetamide served as the inducer of gene expression. Two years later, this system had been applied to study the consequences of transcriptionally inactivating an essential gene in mycobacteria for the first time. Repression of *whmD* caused filamentous growth confirming that *whmD* is required for normal cell division [2]. Since these pioneering studies, more than a dozen new regulatory systems have been described for mycobacteria and have been used to study gene functions in vitro and during infections as well as to facilitate antimycobacterial drug development [3].

The genetic toolbox for mycobacteria now includes expression systems that can be regulated with seven different chemicals: acetamide [1, 4, 5], tetracyclines ([6]:Ehrt, 2005 #23, [7–9]), arabinose [10], IPTG [11], isovaleronitrile [12], theophylline [13], and pristinamycin [8, 14]. These systems manipulate initiation of transcription, initiation of translation, and/or stability of

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the encoded protein. All of these systems have their advantages and any of them might be applied to construct conditional knockdown (cKD) mutants using the procedures described in this chapter. Their features have recently been reviewed in detail [3]. We therefore focus this chapter on the regulatory systems currently used in our laboratories.

1.1 TetON and TetOFF Systems for Mycobacteria

We defined the terms TetON and TetOFF by the effect tetracycline (tc) or anhydrotetracycline (atc) has on expression of the gene whose function is under investigation (here named *yfg* for *y*our *f*avorite *g*ene or gene product). In TetON systems, tc and atc turn expression of *yfg* on and in TetOFF systems, the addition of tc and atc turn expression of *yfg* off.

The primary regulators in all of our Tet systems were derived from the tc repressors (TetRs) present in the transposon Tn10 or the plasmid RA1. These two mobile genetic elements contain efflux-based class B and D tc resistance determinants, respectively, and the TetRs encoded by them are often referred to as TetR(B) and TetR(D) [15]. We chose these repressors because the genetic, biochemical, and structural analyses that had been performed with them resulted in an excellent understanding of their functions [16], and variants with distinct operator binding specificities [17], dimerization specificities [18, 19] and altered allosteric response to the binding of tc [20]. Their main disadvantage was that they evolved in Gram negative bacteria, which caused their codon usage to be different from genes highly expressed in mycobacteria. This was overcome by synthesizing *tetR* genes avoiding codons that are rare in the genome of *M. tuberculosis* [21].

TetR(B) and TetR(D) are homodimers that fold into N-terminal DNA-binding domains (DBDs) and a globular protein core that contains the tc binding pocket and the dimerization interface [22, 23]. Without tc, TetR binds with high affinity to an imperfectly palindromic 19 bp sequence, the tet operator (*tetO*). When *tetO* is placed close to the core elements of a promoter, TetR prevents binding of RNA polymerase and represses transcription initiation. Binding of tc or atc pushes the two DBDs into a position incompatible with binding to *tetO* [22]. As a consequence the TetR-*tetO* complex dissociates and repression is relieved. The tc derivative that binds most strongly to TetR(B) is atc [24]. Affinity of atc to TetR(B) is estimated to be 10^5 times higher than to the ribosome [16, 25], which allows it to be used as a regulator of gene expression at concentrations far below those required for its antibiotic activity.

The promoter we have most often used to generate TetON mutants is $P_{myc1tetO}$ [9]. This promoter was derived from the putative *rpsA* promoter of *M. smegmatis* by inserting one *tetO* between the −10 and −35 hexamers and a second *tetO* right upstream of the −35 hexamer. $P_{myc1tetO}$ is approximately ten times stronger than

P_{hsp60} in *M. smegmatis* but has little activity in *Escherichia coli* [9]. The repressor best suited for the construction of transcriptionally regulated TetON mutants is TetR(Bsyn1-207) which corresponds to wild-type (wt) TetR(B), but is encoded by a fully codon usage adapted gene [21]. To simplify the labeling of tubes and plates we often refer to TetR(Bsyn1-207) as TetR#10 or T10, a designation that stems from the fact that it was the tenth TetR we constructed for testing in mycobacteria.

We have used TetON systems to study several genes [26–28], but they have two disadvantages, which can complicate the characterization and generation of cKD mutants. The first is that it can be difficult to effectively remove atc in some experiments. This primarily applies to non-replicating bacteria in which atc is not diluted by cell division. It can also be difficult to remove atc from the medium, for example, when the bacteria are to be kept in low oxygen. The second disadvantage is that atc has to be present in sufficient quantities during mutant construction when the targeted gene is essential for normal growth on agar plates. Achieving this can be complicated by the degradation of atc that occurs during the weeks of incubation at 37 °C required to obtain mutant colonies.

TetOFF systems can sidestep these complications because silencing is initiated by adding atc. The first TetOFF system was constructed by using a reverse TetR (revTetR) instead of wt TetR [29]. RevTetRs are mutants that require atc to bind *tetO*. These mutants were initially found in a screen, which aimed to identify amino acids that participate in the tc-induced allosteric change that releases TetR from *tetO*. As the screen was for TetR mutants that repress gene expression in the presence of tc [30], it also allowed for the identification of mutants that only bound *tetO* when tc was present. The revTetR that performed best in mycobacteria, TetR#28 or T28 [21], was constructed using information from a later screen [20], which used a TetR(B/D) chimera originally constructed during an investigation of the dimerization specificities of TetR(B) and TetR(D) [19]. The use of T28 instead of T10 has the advantages mentioned above, but T28 is a less efficient repressor than T10 [21].

An alternative to using T28 for the construction of TetOFF mutants is to apply the Tet-Pip system developed by Boldrin et al. [8] or utilize controlled proteolysis. Controlled proteolysis requires the 3′-end of *yfg* to be modified so that it encodes a tag that can be recognized by the protease ClpXP. Degradation of tagged Yfg by ClpXP can be controlled in one of two ways: First by masking a bona fide ClpXP recognition site (e.g., the peptide encoded by the small stable RNA *ssrA*) with a second peptide that is cleaved off by the HIV-2 protease. The resulting tag is referred to as an inducible degradation (ID) tag. When expression of HIV-2 protease is controlled by a wt TetR, addition of atc will activate the HIV-2 protease gene, which clips the ID tag to unmask the ClpXP recognition site

and cause degradation of Yfg. This strategy has been implemented very successfully in *M. smegmatis* [31, 32]; but its application in *M. tuberculosis* has been hampered by toxicity of the HIV-2 protease (Eric Rubin, personal communication). The second strategy uses the DAS+4-tag instead of the ssrA-tag. The DAS+4-tag only contains a weak ClpXP recognition site and requires the accessory protein SspB, which binds to the tag and ClpXP, to initiate proteolysis [33]. In this case, regulated expression of SspB, which has no apparent toxicity in *M. smegmatis* and *M. tuberculosis*, causes proteolysis of Yfg to be inducible [34].

Controlled proteolysis and transcriptional silencing each have their advantages. Transcriptional silencing leaves the targeted protein unchanged whereas addition of a peptide tag is required to control protein stability. The degradation tag also has to be accessible and it remains to be determined if integral membrane proteins can be efficiently degraded by ClpXP. Advantages of controlled proteolysis include that one can leave the native promoter of a target unperturbed and that it actively reduces the concentration of a target protein. As a consequence of the latter, controlled proteolysis achieves faster silencing kinetics and is more efficient in non-replicating bacteria than transcriptional repression.

The extent of gene silencing achieved by transcriptional regulation and controlled proteolysis is often similar and sufficient to construct phenotypically well regulated cKD mutants for some genes but not others. For example, removal of atc from the first TetON mutant constructed to study the function of *bioA*, which encodes the 7,8-diaminopelargonic acid synthase that *M. tuberculosis* requires to synthesize biotin, reduced BioA activity 354-fold. Nevertheless, without atc this TetON mutant only showed a weak growth phenotype, which did not reproduce the strong phenotype of a *bioA* deletion mutant (Δ*bioA*). In contrast, TetON mutants that contained poor translational initiation signals to express BioA phenocopied Δ*bioA* and only grew with inducer when growth depended on biotin synthesis [26]. This strategy of minimizing the phenotypic consequences of transcriptional leakiness with weak translation initiation signals has been successful for several other targets (unpublished).

Tweaking translational initiation can improve the phenotype of cKD mutants, but it does not extend the range of a regulatory system. This is different when transcriptional repression and controlled proteolysis are combined to inactivate the same gene's activity. Implementing this for TetOFF mutants required a TetR that is inactivated by atc and controls expression of *sspB*, a revTetR to repress transcription of *yfg*-DAS+4, and two *tetO*s each of which is bound exclusively by either a wt TetR or revTetR. *TetO*-4C5G, which contains thymine to cytosine and adenine to guanine exchanges at positions 4 and 5, respectively, is not bound by wt TetR. As has been the case for wt TetR [17], the mutations E37A

and P39K were sufficient to switch the operator specificity of T28 from *tetO* to *tetO*-4C5G [35]. The respective T28 derivate was named T38. To prevent heterodimerization of T38 with T10 (which would result in a repressor with intermediate operator specificity and unknown allosteric behavior) the monomers of T10 were fused with a peptide linker [17]. This resulted in the single-chain TetR, TSC10. The dual-control (DUC) switch that was constructed with T38, TSC10, *tetO*, and *tetO*-4C5G provides a broader regulatory range than any other regulatory system available for mycobacteria and achieved rapid depletion of different target genes in growing and non-replicating conditions [35]. The primary disadvantage of the DUC switch is that it requires modifications of the promoter and the 3′-end of *yfg*, which increases the time that is required for mutant construction.

1.2 Strategies for the Construction of Conditional Knockdown Mutants

For nonessential genes (defined as dispensable for growth on standard agar plates) cKD mutants can be obtained by complementing a deletion mutant with a regulated expression plasmid. Changes in the promoter or the translational initiation site of the expression plasmid might be necessary to achieve wt behavior under inducing conditions and/or complete phenotypic inactivation of *yfg* activity under repressing conditions. But this is relatively straightforward as the same deletion mutant can be used to analyze many regulated expression plasmids.

Construction of mutants for essential genes is more complicated because *yfg* needs to be expressed throughout mutant construction. In theory, the simplest way to construct a cKD mutant for an essential gene is to transform *M. smegmatis* or *M. tuberculosis* with an expression plasmid that produces an *yfg* antisense RNA after addition of inducer. This has been successful in several cases [6, 36, 37]. However, our own attempts to silence different essential genes in *M. smegmatis* or *M. tuberculosis* with antisense RNAs of varying lengths have all failed for reasons that are not clear to us.

A strategy that has been more successful in our hands is to replace the promoter of an essential gene or modify its 3′-end to encode the ID or DAS + 4-tag. Either modification can be achieved by integrating an appropriate DNA fragment at the native locus of *yfg* (Figs. 1 and 2). These DNA fragments have been introduced into mycobacteria as temperature sensitive mycobacteriophages [38, 39], suicide plasmids [9, 29, 40], or PCR products [31, 32]. We now most frequently use PCR products which are integrated into the genome by phage-mediated recombineering [41] to achieve these and other homologous recombinations.

The advantage of in situ promoter replacement and in situ ID/DAS + 4-tagging is that cKD mutants can be constructed in a single step if the starting strain already includes the other components of the respective regulatory system (e.g., a TetR). The disadvantage of both in situ replacement strategies is that any modification

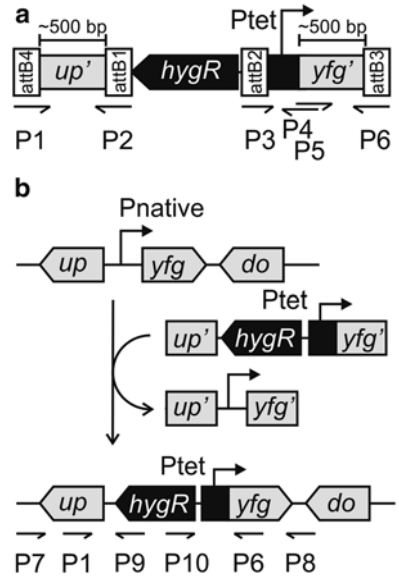

Fig. 1 Construction of cKD mutants by in situ promoter replacement. The promoter of *yfg* is replaced at its native locus. (**a**) Knockin cassette. AttB4, attB1, attB2, attB3 indicate the location of the Gateway att-sites. *Up′* and *yfg′* refer to the ~500 bp fragments required for recombineering. These sequences are identical to the region upstream of *yfg* and its 5′-end, respectively. *Ptet tetO* containing promoter, *hygR* hygromycin resistance gene, *P1 to P6* primer binding sites. (**b**) Mutant construction

that needs to be made to the regulatory system requires a new round of mutagenesis. For example, Wei and colleagues applied in situ ID-tagging to isolate *M. smegmatis* cKD mutants for alanine racemase (Alr), dihydrofolate reductase (DHFR), the beta subunit of RNA polymerase (RpoB), the A subunit of gyrase (GyrA), the enoyl reductase InhA, and the beta-ketoacyl-acyl-carrier protein synthase KasA [31]. The genes encoding all of these proteins are essential but induction of HIV-2 protease only inhibited growth of the RpoB and InhA mutants. A new round of mutagenesis would be necessary to obtain phenotypically better regulated mutants for the other targets.

An alternative to modifying *yfg* in its native chromosomal locus is to first integrate a second copy of *yfg* in the attachment site of the mycobacteriophage L5 (att-L5) and then delete the native copy of *yfg* in the merodiploid, resulting in Δ*yfg*::*yfg*-attL-5 (Fig. 3). This moves the only functional copy of *yfg* from its native locus into the att-L5 site, where it can be efficiently replaced by another plasmid that also integrates into att-L5 and can be selected for with a different antibiotic [42]. Δ*yfg*::*yfg*-att-L5 can thus be used to validate essentiality of *yfg*—by transforming with an "empty" plasmid (Fig. 3), which should not lead to colonies if the gene is indeed essential—and to analyze different regulated expression plasmids.

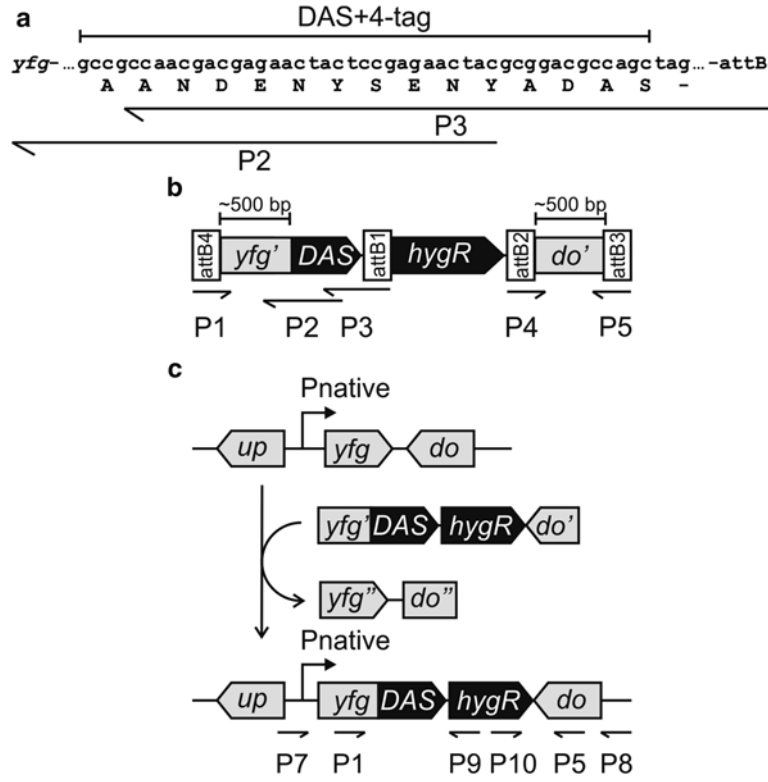

Fig. 2 Construction of cKD mutants by in situ DAS + 4 or ID-tagging. The 3′-end of *yfg* is modified at the native gene locus. (**a**) DAS + 4 tag. P2 and P3 are the primers, which add the DAS + 4-tag. Primers P2 and P3 should overlap by at least 20 bp of sequence. (**b**) Knockin cassette. AttB4, attB1, attB2, attB3 indicate the location of the Gateway att-sites. *Yfg′* and *do′* refer to the ~500 bp fragments required for recombineering. These sequences are identical to the region downstream of *yfg* and its 3′-end, respectively. *DAS* DAS + 4-tag, *hygR* hygromycin resistance gene, *P1 to P5* primer binding sites. (**c**) Mutant construction

Δ*yfg*::*yfg*-att-L5 thus provides the flexibility otherwise only available for nonessential genes, but its isolation significantly extends the time required for mutant construction. In this chapter we describe the construction and verification of in situ promoter replacement and DAS-tagged mutants, as well as mutants regulated by the DUC-switch in *M. smegmatis* and *M. tuberculosis*.

2 Materials

To obtain the plasmids mentioned below please contact either Dirk Schnappinger (dis2003@med.cornell.edu) or Sabine Ehrt (sae2004@med.cornell.edu).

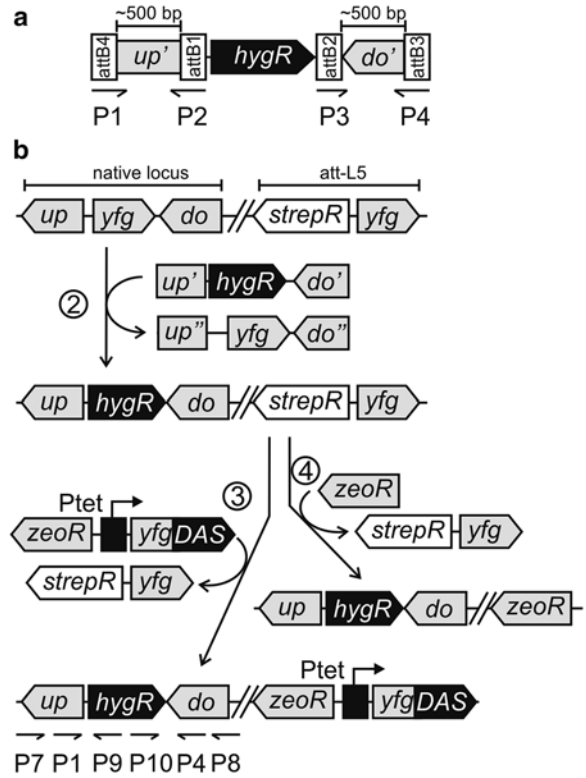

Fig. 3 Construction of DUC mutants. (**a**) Knockout cassette. AttB4, attB1, attB2, attB3 indicate the location of the gateway att-site. *Up′* and *do′* refer to the ~500 bp fragments required for recombineering. These sequences are identical to the region upstream and downstream of *yfg,* respectively. *HygR* hygromycin resistance gene, *P1 to P5* primer binding sites. (**b**) Mutant construction. In the first step (not shown) wt *M. tuberculosis* containing the recombineering plasmid is transformed with a strepR *yfg* expression plasmid that integrates into the attachment site of the phage L5 (att-L5). In step #2, the wt *yfg* is deleted, which generates Δ*yfg*::*yfg*-att-L5. Transformation with a regulated expression plasmid for DAS-tagged *yfg* is the next step (#3) for the construction of a DUC mutant. If an inducible *sspB* plasmid is not already present it can be added into the Tweety attachment site (not shown). Δ*yfg*::*yfg*-att-L5 can also be used to confirm essentiality of *yfg* by transforming with a plasmid that would delete *yfg*-attL-5 (step #4)

2.1 Plasmid Construction for Promoter-Replacement Mutants

1. pNit::ET (genbank GU459073 [31]): permits IVN-inducible expression of recombineering enzymes and counterselection with sucrose; confers kanamycin resistance (*see* **Note 1**).
2. pDE43-MCSq17: Gateway destination vector; contains *ccdB* as counterselectable marker; confers resistance to chloramphenicol and streptomycin; contains attR4 and attR3 sites for Gateway LR recombination; can integrate at the mycobacteriophage L5 *attB* site (*see* **Notes 2** and **3**).
3. pDO41A: Gateway donor vector; contains *ccdB* as counterselectable marker; confers resistance to chloramphenicol

and ampicillin; contains attP1r and attP4 sites for Gateway BP recombination.

4. pDO23A: Gateway donor vector; contains *ccdB* as counterselectable marker; confers resistance to chloramphenicol and ampicillin; contains attP2 and attP3 sites for Gateway BP recombination.
5. pEN12-HLaa: Gateway entry plasmid; contains *ccdB* as counterselectable marker; confers resistance to zeocin and hygromycin; hygromycin resistance cassette is flanked by attB1 and attB2 sites; to be used in Gateway LR recombinations.
6. pGMCSq17-10M-0X: confers resistance to streptomycin; contains TetR; integrates at the mycobacteriophage L5 *attB* site.
7. Luria Broth (LB) and LB plates.
8. Gateway Multisite Cloning BP Clonase II Plus Enzyme Mix and LR Clonase II Plus Enzyme Mix.
9. 2 μg/μL Proteinase K (Invitrogen).
10. TE buffer, pH 8.0: 10 mM Tris–HCl, 1 mM EDTA.
11. 50× TAE buffer: 1 L: 242 g Tris base, 57 mL glacial acetic acid, 100 mL 0.5 M EDTA, pH 8.0.
12. 1 % w/v agarose in 1× TAE buffer.

2.2 Plasmid Construction for In Situ DAS-Tagging

1. *See* Subheading 2.1, **items 1–5** and 7–**12**.
2. pGMCtKq28-TSC10M1-sspB: confers resistance to kanamycin; contains TetR and tetracycline inducible *sspB*; can integrate at the mycobacteriophage Tweety *attB* site.

2.3 Plasmid Construction for Dual-Control Mutants

1. *See* Subheading 2.1, **items 1–5** and 7–**12**.
2. pGMCtKq28-TSC10M1-sspB: confers resistance to kanamycin; contains TetR and tetracycline inducible *sspB*; can integrate at the mycobacteriophage Tweety *attB* site.
3. pDE43-MCZq19: Gateway destination vector; contains *ccdB* as counterselectable marker; confers resistance to zeocin and chloramphenicol; contains attR4 and attR3 sites for Gateway LR recombination; can integrate into the mycobacteriophage L5 *attB* site.
4. pDO12A: Gateway donor vector contains *ccdB* as counterselectable marker; confers resistance to chloramphenicol and ampicillin; contains attP2r and attP1 sites for Gateway BP recombination.
5. pEN41A-T02: Gateway entry plasmid; confers resistance to ampicillin; contains attB4 and attB1 sites for Gateway LR recombination; attB4 and attB1 sites are separated by the nucleotides aatt; to be used to assemble constitutive expression plasmids by LR recombination.

6. pEN41A-T38S38: Gateway entry plasmid; confers resistance to ampicillin; constitutively expresses the reverse TetR, T38, flanked by attB4 and attB1 sites; to be used to assemble regulated expression plasmids by LR recombination.
7. pEN12A-P750: Gateway entry plasmid; confers resistance to ampicillin; contains the *tetO-4C5G* promoter P750 flanked by attB1 and attB2 sites; to be used to assemble regulated expression plasmids by LR recombination.
8. pGMCZq17-0X0X: confers zeocin resistance and integrates into the mycobacteriophage L5 *attB*; to be used as control plasmid in DUC mutants; Addgene plasmid number not yet available.

2.4 Recombineering for Promoter-Replacement and In Situ DAS-Tagging

1. Liquid medium: Middlebrook 7H9 medium supplemented with 0.5 % w/v bovine serum albumin fraction V, 0.2 % w/v D-glucose, 0.085 % w/v NaCl, 0.2 % v/v glycerol, and 0.05 % v/v tyloxapol.
2. Solid medium: Middlebrook 7H10 medium supplemented with 10 % v/v OADC and 0.5 % v/v glycerol.
3. Antibiotics stock solutions: 50 mg/mL hygromycin, 40 mg/mL kanamycin, 100 mg/mL ampicillin, 25 mg/mL chloramphenicol, 25 mg/mL streptomycin, and 100 mg/mL zeocin. Add to culture medium when required at the following concentrations: 50 μg/mL hygromycin, 20 μg/mL kanamycin, 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, 25 μg/mL streptomycin, and 25 μg/mL zeocin (*see* **Note 4**).
4. 1–2 mg/mL anhydrotetracycline (atc), light sensitive.
5. 10 % v/v glycerol.
6. 1 mM isovaleronitrile (IVN) in DMSO. Store at 4 °C, stock is stable for at least 6 months.
7. 2 M glycine.
8. 60 % v/v glycerol.

2.5 Construction of Dual-Control Mutants

1. *See* Subheading 2.4, **items 1–8**.

2.6 Chromosomal DNA Preparation

1. 15 mg/mL lysozyme in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).
2. 10 % w/v sodium dodecyl sulfate (SDS).
3. 20 mg/mL Proteinase K.
4. 5 mM NaCl.
5. 10 % w/v hexadecyltrimethylammonium bromide (CTAB).
6. 3 M sodium acetate.

2.7 PCR Verification of the Recombinant Structure

1. PCR primers (*see* Subheading 3.7 and Figs. 1–3 for primer specifications).

2.8 Verification by Southern Blot

1. Hybridization buffer: 0.5 M NaCl, 5 % w/v blocking agent in ECL gold hybridization buffer (42 °C to dissolve).
2. 20× SSC: 0.3 M trisodium citrate, 3 M NaCl.
3. Primary wash buffer: 0.4 % w/v SDS, 0.5× SSC. Store for up to 3 months at 2–8 °C; stringency can be increased by lowering final SSC concentration.
4. Secondary wash buffer: 2× SSC. Store for up to 3 months at 2–8 °C.
5. Depurination solution: 250 mM HCl.
6. Denaturation solution: 1.5 M NaCl, 0.5 M NaOH.
7. Neutralizing solution: 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5.
8. Amersham ECL DIRECT Nucleic Acid Labeling and Detection System (or equivalent detection system).

2.9 Removal of pNit::ET Plasmid

1. *See* Subheading 2.4, **items 1–4** and **8**.
2. Optional: 10 % w/v sucrose.

2.10 Growth Analysis Using Discs

1. *See* Subheading 2.4, **items 1–4**.
2. Blank paper discs, 6 mm in diameter (Becton, Dickinson, and Company).

2.11 Growth Analysis in Liquid Medium

1. *See* Subheading 2.4, **items 1–4**.

3 Methods

3.1 Plasmid Construction for Promoter-Replacement Mutants

To generate a PCR product that can be used to select for promoter replacement, first clone a plasmid, pGMCS-up-hygR-P_{tet}-do, which contains (1) sequences homologous to the regions upstream and downstream of promoter insertion site, (2) *hygR* conferring hygromycin resistance for selection, and (3) a regulated promoter, e.g., a *tetO* containing promoter (P_{tet}) (*see* **Note 5**).

1. Create pEN41A-up containing homologous sequence upstream of promoter insertion site.
2. Design primers (P1 and P2) to generate a PCR product amplifying 500 bp sequence directly upstream of insertion site flanked by attB4 and attB1 sites (*see* **Note 6** and Fig. 1).
3. Execute standard PCR and gel-purify.

4. Perform a BP reaction to generate pEN41A-up with 50 fmoles of PCR product, 1 μL 150 ng/μL pDO41A, and 2 μL BP Clonase Mix II, complete to 10 μL with TE Buffer. Incubate at 25 °C for 1 h. Add 1 μL of 2 μg/μL Proteinase K and incubate for 10 min at 37 °C.
5. Transform 1 to 4 μL of the BP mix into MachI *E. coli* cells and select for transformants on LB plates containing 100 μg/mL ampicillin.
6. Streak to isolate single colonies and inoculate into LB to prepare plasmid DNA. Verify plasmid by restriction and sequencing.
7. Create pEN23A-P_{tet}-do, and sequence homologous to the 5′ end of the target gene.
8. Design primers (P3 and P4) to amplify the promoter so that P3 brings in the attB2 site and P4 includes ~20 bp of sequence homologous to the region downstream of the insertion site (*see* **Note 7**). Complementarity between P4 and P5 is important to stitch the PCR for the promoter and the PCR for the sequence downstream of the insertion site together.
9. Design primers (P5 and P6) to generate a PCR product amplifying 500 bp of sequence directly downstream of insertion site. The 3′ end of P6 brings in attB3.
10. Perform the individual PCRs for the promoter and the homologous sequence and gel-purify.
11. Using P3 and P6, perform a PCR to stitch the two PCR templates from above together and gel-purify.
12. Perform a BP using pDO23A as the donor vector.
13. Transform 1 μL into MachI *E. coli* cells and select for transformants on LB plates containing 100 μg/mL ampicillin.
14. Streak to isolate single colonies and inoculate into LB to prepare plasmid DNA. Verify plasmid by restriction and sequencing.
15. To assemble pGMCSq17-up-hygR-P_{tet}-do, perform a LR reaction with 20 fmoles of pDE43-MCSq17, 10 fmoles each of pEN41A-up, pEN12-HLaa, and pEN23A-P_{tet}-do, and 2 μL LR Clonase Mix II, complete to 10 μL with TE buffer. Incubate at 25 °C for 16 h. End LR reaction with 1 μL Proteinase K and select for transformants on LB plates containing 25 μg/mL streptomycin. Streak to isolate single colonies and inoculate into LB to prepare plasmid DNA. Verify plasmid by restriction.
16. Generate recombineering PCR product for promoter replacement using primers P1 and P6 for amplification of the regions of homology, *hygR*, and the new promoter. Gel-purify (*see* **Note 8**).

3.2 Plasmid Construction for In Situ DAS-Tagging

Generate recombineering PCR product for in-situ DAS-tagging using pGMCSq17-up-DAS-hygR-do, a plasmid that contains the 3′ region of the gene of interest fused in frame to the DAS-tag, the hygromycin cassette, and sequence homologous to the region directly downstream of the insertion site. *See* Subheading 3.1 for general cloning protocol.

1. Create pEN41A-up-DAS.
2. Design primers (P1 and P2) to amplify the last 500 bp of yfg without the stop codon. The 5′ end of P1 should bring in the attB4 site. Design P2 so that it does not include the gene's native stop codon and brings in ≥ 20 bp encoding the DAS-tag. Additionally design a third primer (P3) such that it overlaps by ≥ 20 bp with the 3′ end of P2, encodes the DAS-tag ending with a stop codon, and brings in the attB1 site (Fig. 2a).
3. Amplify this sequence in a single PCR reaction using standard concentrations of P1 and P3, but tenfold less of P2. Alternatively, the PCR can be performed in two steps if unsuccessful in one step. Gel-purify.
4. Perform the BP reaction as in Subheading 3.1.
5. Clone pEN23A-do, containing sequence homologous to that directly downstream of the insertion site.
6. Design primers (P4 and P5) to amplify ~500 bp downstream of the insertion site so that P4 brings in the attB2 site and the 3′ end of P5 includes the attB3 site.
7. Perform PCR and use the fragment in a BP as in Subheading 3.1 using pDO23A.
8. Combine pDE43-MCSq17, pEN41A-up-DAS, pEN12-HLaa, and pEN23A-do in an LR reaction to create pGMCS-up-DAS-hygR-do.
9. Generate PCR product for recombineering using primers P1 and P5.

3.3 Plasmid Construction for Dual-Control Mutants

See Subheading 3.1 for general cloning protocol.

1. Generate recombineering PCR product to replace *yfg* at its native locus with a hygromycin cassette using pGMCS-KO-*yfg*, a plasmid containing a hygromycin cassette in between the sequences required for recombination.
2. Create pEN41A-KO-*yfg*, containing 500 bp of sequence at the 5′ end of the target gene. Design primers (P1 and P2) to amplify 500 bp of the 5′ end of *yfg*. The 5′ end of P1 should bring in the attB4 site and the 3′ end of P2 should be complementary to attB1. Perform the PCR and BP reaction as in Subheading 3.1.

3. Create pEN23A-KO-*yfg*, containing 500 bp of sequence at the 3′ end of the target gene. Design primers (P3 and P4) to amplify ~500 bp at the 3′ end of *yfg* so that P3 brings in the attB2 site and the 3′ end of P4 includes the attB3 site. Perform PCR and the BP using pDO23A as the donor vector.
4. Clone pGMCSq17-KO-*yfg* by LR with pDE43-MCSq17, pEN41A-KO-*yfg*, pEN12-HLaa, and pEN23A-KO-*yfg*.
5. Generate PCR product using primers P1 and P4 and gel-purify.
6. Clone pGMCSq17-P*yfg* and pGMCZ-P*yfg* for merodiploid construction and gene essentiality check.
7. Create pEN12A-Pnative by amplifying the putative native promoter region for *yfg* flanked by attB1 and attB2 sites. Perform a BP as in Subheading 3.1 (*see* **Note 9**).
8. Create pEN23A-*yfg* by designing primers to amplify the target gene and add attB2 and attB3 sites at the 5′ and 3′ ends of the gene, respectively.
9. Perform a LR with pDE43-MCSq17, pEN41A-T02, pEN12A-Pnative, and pEN23A-*yfg* to create pGMCSq17-Pyfg. Do the same with pDE43-MCZq19 in place of pDE43-MCSq17 to create pGMCZq19-P*yfg*.
10. Create pGMCZq19-T38S38-750-*yfg*-DAS for conditional knockdown.
11. Amplify the entire target gene and add the DAS-tag and flanking attB2 and attB3 sites as in Subheading 3.2 to create pEN23A-*yfg*-DAS.
12. Combine pDE43-MCZq19, pEN41A-T38S38, pEN12A-P750, and pEN23A-*yfg*-DAS and perform a LR to create pGMCZq19-T38S38-750-*yfg*-DAS.

3.4 Recombineering for Promoter-Replacement and In Situ DAS-Tagging

1. Grow 30 mL *M. tuberculosis* to OD_{600} ~ 1.0 in liquid medium in 75 cm^2 flasks.
2. Transfer cells to 50 mL screw cap tubes and spin down for 10 min at 3,399 × *g* (room temperature).
3. Wash 1× with an equivalent volume of 10 % glycerol and wash 2× with 0.5 volume 10 % glycerol. Resuspend in 500 μL 10 % glycerol.
4. Transform 100 μL competent cells with 300 ng pNit-recET-sacB-kan by electroporation (2.5 kV, 700 Ω, 25 μF). After a 16 h outgrowth at 37 °C in 1 mL liquid medium, plate 100 μL on a 7H10 plate containing 20 μg/mL kanamycin, spin down and plate remainder on another 7H10 plate.
5. Incubate for 2–3 weeks and inoculate single colonies in 1 mL liquid medium + 20 μg/mL kanamycin in 24-well plates.

When dense, passage to larger flasks, grow to log phase, and make 1 mL stocks with 15 % final volume glycerol.

6. Grow *M. tuberculosis* carrying pNit::ET in liquid medium + 20 μg/mL kanamycin. When OD_{600} reaches 0.8–1.0, subculture 10 mL into 100 mL of liquid medium + 20 μg/mL kanamycin and incubate at 37 °C with rolling, 1.75 rpm.
7. Check OD after 2–3 days. When OD_{600} reaches 0.8–1.0, induce culture with 100 μL 1 mM IVN and continue rolling at 37 °C.
8. After ~8 h, add 10 mL of 2 M glycine and continue rolling at 37 °C.
9. After overnight incubation, prepare competent cells as in **steps 2** and **3** above and resuspend cells in 10 mL of 10 % glycerol (*see* **Note 10**).
10. For transformation, mix 500 ng of the recombinant PCR product with 400 μL cells and electroporate (as in **step 4**).
11. Dilute cells in 1 mL liquid medium and incubate overnight at 37 °C. Plate entire transformation on two 7H10 plates with 50 μg/mL hygromycin (*see* **Note 11**). Expect ~3–50 colonies per transformation, ~50 % of which are homologous recombinants (*see* **Note 12**).
12. Inoculate six to ten Hyg^R colonies into 24-well plates containing 1 mL liquid medium + 50 μg/mL hygromycin per well. When dense, prepare glycerol stocks and expand remaining culture for mutant verification by PCR and Southern blot.

3.5 Construction of Dual-Control Mutants

1. Transform *M. tuberculosis* containing pNit::ET with pGMCSq17-P*yfg* to create a merodiploid strain that is streptomycin and kanamycin resistant (*see* **Note 13**). Create glycerol stocks.
2. Use recombineering to replace the native copy of the gene with a hygromycin cassette in the merodipoloid strain containing pNit::ET (*see* Subheading 3.4, **steps 6–12**). After mutant confirmation and removal of the *recET* plasmid, create multiple glycerol stocks. This "att-site mutant" (Δ*yfg*-hygR::pGMCS-Pyfg) is the hygromycin and streptomycin resistant parent strain from which different conditional knockdowns, such as the dual-control mutant can be created.
3. Confirm essentiality of the gene by replacement transformation. Transform the att-site mutant with pGMCZq19-P*yfg* and pGMCZq17-0X0X. These two plasmids can replace pGMCSq17-P*yfg* at the L5 *attB* site. If the gene is essential, transformants should be recovered on 7H10 + 25 μg/mL zeocin plates with pGMCZq19-P*yfg*, but not pGMCZq17-0X0X (*see* **Notes 14** and **15**).

4. Replace pGMCSq17-P*yfg* in the att-site mutant with pGMCZq19-T38S38-750-*yfg*-DAS. This will result in a mutant regulated transcriptionally in the presence of atc (Δ*yfg*-hygR::pGMCZq19-T38S38-750-*yfg*-DAS, *see* **Notes 16** and **17**).
5. Transform Δ*yfg*-hygR::pGMCZq19-T38S38-750-*yfg*-DAS with pGMCtKq28-TSC10M1-sspB to create the DUC strain (Δ*yfg*-hygR::pGMCZq19-T38S38-750-*yfg*-DAS::pGMCtKq28-TSC10M1-sspB). This strain is hygromycin, zeocin, and kanamycin resistant (*see* **Note 18**).

3.6 Chromosomal DNA Preparation

This protocol is adapted from the procedure originally described by van Soolingen and colleagues [43].

1. Grow 20 mL of liquid medium until $OD_{600} \sim 0.8–1.0$. Pellet in a 15 mL tube at 3,571 × *g*, 10 min (*see* **Note 19**).
2. Resuspend pellet in 500 μL lysozyme solution, vortex, and incubate at 37 °C overnight (*see* **Note 20**). Place 10 % CTAB at 37 °C for next day.
3. Add 70–100 μL 10 % SDS, vortex, add 5 μL proteinase K, and vortex. Incubate at 65 °C on a heat block for 30 min (incubation can be extended to 3 h) (*see* **Note 21**).
4. Add 80–100 μL 5 mM NaCl, vortex, and add 80–100 μL of 10 % CTAB. Do not vortex after CTAB addition. Incubate at 65 °C for 30–60 min.
5. Transfer supernatant to 2 mL snap tubes by decanting. Add equivolume chloroform and invert. Spin at 15,588 × *g*, 5 min.
6. Transfer upper phase to new 2 mL tube.
7. Add equivolume phenol–chloroform–isoamyl alcohol, invert, and spin at 15,588 × *g*, 5 min.
8. Transfer aqueous upper phase to a new 2 mL tube, add equivolume chloroform–isoamyl alcohol, and invert. Spin at 15,588 × *g*, 5 min.
9. Transfer aqueous upper phase to a 1.5 mL tube, add 0.6 volume isopropanol and mix. DNA may be visible at this point.
10. Optional: Add 10 μL 3 M sodium acetate and put at 4 °C for 30 min.
11. Spin at 15,588 × *g* for 30 min at 4 °C.
12. Remove liquid and wash with 500 μL 75 % ethanol. Remove most of the liquid using a 200 μL pipette and let air-dry for 5 min at 37 °C.
13. Resuspend pellet in 30–50 μL TE buffer.

3.7 PCR Verification of the Recombinant Structure

All PCR verifications of recombinant DNA should be performed alongside wt *M. tuberculosis* for fragment size comparison.

1. 5′-terminal junction: Perform a PCR using primers P7 and P9. Design P7 so that it binds ~150 bp upstream of P1. This PCR will verify insertion and correct orientation within the genomic DNA.
2. 3′-terminal junction: Perform a PCR using primers P8 and P10. Design P8 so that it binds ~150 bp downstream of P6 (P6 for promoter-replacement, P5 for in situ DAS-tagging, and P4 for dual-control mutant). This PCR will verify insertion and correct orientation within the genomic DNA.
3. PCR over entire target gene: Perform a PCR using primers P7 and P8. This PCR will confirm that the entire recombinant PCR product was successfully inserted within the insertion site.
4. For the DUC mutants, an additional PCR should be performed with a primer internal to *yfg* and P7 or P8. Lack of band demonstrates that the gene no longer exists at its native locus (*see* **Note 22**).

3.8 Verification by Southern Blot

1. Design probe and find suitable restriction site for an endonuclease. Probe should be approximately 1 kb in length and bind to a fragment either upstream or downstream of the promoter insertion. The selected enzyme should cut so that the probed fragment includes the inserted promoter and hygromycin cassette in order to compare different sized fragments for the recombinant and wt strain.
2. Digest 5–6 μg chromosomal DNA overnight in a final volume of 40 μL.
3. Run 5–6 μg digested DNA on 0.8 % agarose gel at 50 V for 3 h. Photograph gel with a ruler (*see* **Note 23**).
4. Cover gel with depurination solution for 10–12 min. After bromophenol blue dye has turned yellow, stop the treatment and rinse the gel with distilled water.
5. Add denaturation solution to make single stranded DNA and agitate for about 25 min until bromophenol blue dye becomes blue again. Rinse the gel with distilled water.
6. Cover the gel with neutralizing solution for 30 min.
7. Rinse positively charged nylon membrane for a few seconds in distilled water and (optional) for 5 min in 20× SSC buffer.
8. Assemble blotting stack using 20× SSC as blotting buffer in a DNA gel box. Create Whatman bridge by placing a piece of Whatman paper flat on the gel box and extending the ends of the paper so that they touch the bottom of both legs of the gel box. Place the agarose gel on top, followed by the positively charged membrane. Layer 2 gel-sized Whatman papers on top of the membrane, and stack paper towels on the Whatman papers so that they do not touch the SSC or the Whatman bridge.

On top, place a weight, such as a heat block, and tape down securely so that it does not fall off or lean to one side, causing uneven transfer. Fill the legs of the gel box almost all of the way up with 20× SSC so that the buffer can wick up the Whatman bridge and transfer the DNA from the gel to the membrane. Blot overnight.

9. Remove paper towels and Whatman paper. Mark location of agarose gel wells on the membrane with a pencil.
10. Place the membrane in petri dish, with side that was in contact with the gel facing up. Fix DNA to the membrane using UV (auto-cross-link setting two times on Stratalink).
11. Prepare hybridization buffer and pre-warm at 42 °C. Coat sides of hybridization tube with blocking agent to avoid clumping and promote dissolution.
12. Add the membrane to hybridization buffer in tube and incubate for at least 15 min at 42 °C while rotating.
13. Label PCR probe: Boil 100 ng DNA in 10 μL final volume for 5 min. Immediately cool on ice for 5 min, add 20 μL labeling reagent, mixing gently, and add 20 μL glutaraldehyde. Incubate at 37 °C for 10 min.
14. Add labeled probe to hybridization solution, avoiding direct contact with the membrane.
15. Incubate the membrane with probe overnight at 42 °C while rotating.
16. Pre-warm primary wash buffer at 42 °C.
17. Discard hybridization buffer and add 50–100 mL of 5× SSC. Wash at 42 °C for 5 min.
18. Replace 5× SSC buffer with primary wash buffer to about 1/3 volume of the tube. Wash at 42 °C for 20 min.
19. Wash the membrane twice with primary buffer at 42 °C for 10 min.
20. Remove the membrane from tube using tweezers and place in plastic container. Wash twice with an excess of secondary buffer and incubate with agitation at room temperature for 5 min each.
21. Add 15–20 mL detection reagents (1:1) to the membrane. Detect light generated by horseradish peroxidase-catalyzed breakdown of luminol on autoradiography film, including a marker for band sizes using an X-ray film cassette in a dark room. Expose the first film for 30 s and develop. Expose a second film for a longer period of time if needed.

3.9 Removal of pNit::ET Plasmid

1. Thaw stocks for four candidates that have been positively confirmed as mutants in 5 mL liquid medium + 50 μg/mL hygromycin and grow until log phase.

2. Plate 200 μL of the mutant strains on 7H10 plates + 50 μg/mL hygromycin or 20 μg/mL kanamycin. Additionally plate dilutions on hygromycin Y-plates to isolate single colonies (*see* **Note 24**). Include additional antibiotics as required to retain plasmids containing *sspB* or *tetR*.
3. Create new glycerol stocks for these strains, recording the passage number.
4. Dilute 200 μL of the log phase cultures in 5 mL liquid medium and grow until log phase again.
5. Repeat **steps 2–4** until kanamycin plates no longer yield growth. Successful loss of *recET* plasmid can occur in as few as four passages.
6. Inoculate single colonies from Y-plate into liquid medium to create new glycerol stocks. These stocks will be the only stock used in the future, lacking the *recET* plasmid.
7. Reconfirm mutant by PCR with primers P7 and P8.

3.10 Growth Analysis Using Discs

1. For promoter-replacement mutants, transform strain with the TetR expression plasmid pGMCSq17-T10M-0X. Plate on 7H10 plates + 50 μg/mL hygromycin, 25 μg/mL streptomycin, 500 ng/mL atc to induce expression of the gene of interest (*see* **Note 25**).
2. For in situ DAS-tagged and DUC mutants, transform with pGMCtKq28-TSC10M1-sspB. Plate on 7H10 plates + 50 μg/mL hygromycin and 20 μg/mL kanamycin (*see* **Note 26**).
3. Grow cultures to log phase (OD_{600} 0.6–0.8) in liquid medium + 25 μg/mL streptomycin or 20 μg/mL kanamycin (add 500 ng/mL atc for promoter-replacement mutants). Wash 2× with equivolume of liquid medium to remove atc, if grown with atc (*see* **Note 27**).
4. Plate 100 μL on 7H10 plates + 50 μg/mL hygromycin, 25 μg/mL streptomycin or 20 μg/mL kanamycin.
5. Place a sterile 6 mm diameter paper disc in the center of each plate using tweezers.
6. Add 10 μL of sterile water or atc for final amounts of 5 ng, 50 ng, and 500 ng.
7. Incubate plates at 37 °C for 10 days. If knockdown of the target gene has a growth phenotype on 7H10 plates, growth should be dependent on atc-containing discs for promoter-replacement mutants dependent on atc for gene expression and be inhibited on atc-containing discs for in situ DAS-tagged and DUC mutants.

3.11 Growth Analysis in Liquid Medium

1. Transform mutant strains with the appropriate regulatory plasmids (*see* Subheading 3.10, **steps 1** and **2**).

2. Grow wt and mutant strains to log phase in liquid medium + 50 μg/mL hygromycin and 25 μg/mL streptomycin or 20 μg/mL kanamycin. Grow with atc if needed and wash 2× with equivolume of liquid medium to remove atc.
3. Passage bacteria into fresh liquid medium + 25 μg/mL streptomycin or 20 μg/mL kanamycin, choosing a starting OD_{600} between 0.005 and 0.01. Split wt and mutant strains into two different flasks ±500 ng/mL atc at a final volume of 10 mL (*see* **Note 28**). Follow growth of bacteria until stationary phase (*see* **Notes 29** and **30**).

4 Notes

1. Alternatively, the plasmid developed by van Kessel and Hatful can be used for recombineering [41].
2. Destination vectors and donor vectors contain *ccdB* and *Cm(R)* which are replaced when recombination between a destination vector and entry plasmids or donor vector and *attB* PCR product occurs. *ccdB* interferes with *E. coli* DNA gyrase in most strains (Mach1 and Top10) and provides a mode of negative selection against non-recombinants as cells that uptake the original vector will not survive. One Shot *ccdB* Survival 2 $T1^R$ cells are required for propagation of destination and donor vectors.
3. Resistance of the destination vector can be zeocin, kanamycin, or nourseothricin as well.
4. For *E. coli,* supplement media with 200 μg/mL hygromycin, 40 μg/mL kanamycin, and 50 μg/mL zeocin (LB plates only for zeocin).
5. Alternatively regulated promoters may be used, such as promoters induced by pristinamycin, acetamide, or isopropyl β-D-1-thiogalactopyranoside.
6. Primers should be homologous to ~40–50 bp of the template sequence.
7. It is recommended to include a terminator between *hygR* and P1 to prevent promoter activity from the *hygR* promoter from affecting expression of *yfg*. This terminator can be included at the 5′ end of the promoter between attB2 and the promoter.
8. In order to ensure high concentration PCR yields for recombination, it is often necessary to perform multiple PCR reactions and gel-purify from multiple lanes, melting the gel fragments in a 15 mL tube and running the product over the purification column through a series of spins.
9. We often use one or more heterologous promoters if the native promoter has not been identified.

10. Alternatively, for *M. smegmatis*, incubation with IVN can be shortened to 1.5 h at 37 °C and the outgrowth after electroporation only needs to be 4–5 h. Addition of glycine seems to be unnecessary in *M. smegmatis* as we have successfully obtained recombinants when it has been omitted. We have not tried omitting it with *M. tuberculosis*.
11. It is not necessary to select for pNit::ET as future steps will include passaging to encourage loss of the plasmid.
12. If there is difficulty recovering colonies via in situ DAS-tagging, the C-terminal end of the protein may not tolerate the DAS-tag and promoter replacement should be pursued instead. Additionally, some genes may be sensitive to expression levels, which may make promoter-replacement difficult as well. Repeat recombination to be sure.
13. It can be advantageous to try promoters of differing strengths in order to ensure similar expression levels to wt, especially if the gene is expected to be essential. Use of the putative native promoter and a strong and weak promoter is a good start.
14. Essentiality can be dependent on the medium used. If desired and possible, a knockout strain can be created at this step by replacing pGMCSq17-P*yfg* with pGMCZq17-0X0X.
15. Some co-integrants can be recovered from the pGMCZq17-0X0X transformation. These should be checked by streaking candidates on 7H10 + 25 μg/mL zeocin and 7H10 + 25 μg/mL streptomycin plates. Growth on both zeocin and streptomycin plates indicates that the replacement transformation was unsuccessful and instead the two plasmids are co-integrated at the L5 *attB* site. In *M. tuberculosis*, the incidence of co-integration appears to be low and only four candidates need to be analyzed, but for some genes in *M. smegmatis*, the rate of co-integration can be high.
16. This replacement transformation can be performed at the same time as confirmation of essentiality. As with above, it must be confirmed that the candidates are not co-integrants.
17. Some proteins may not tolerate the addition of the DAS-tag at the C-terminus. It is recommended to attempt the replacement transformation with pGMCZq19-T38S38-750-*yfg*-DAS twice with pGMCZq19-P*yfg* as a positive control for the transformation, and pGMCZq19-T38S38-750-*yfg* as a control for promoter strength. If no colonies are recovered with the plasmid encoding the DAS-tag at the 3′ end of the gene, but colonies are recovered for the non-DAS-tagged version and constitutively expressed *yfg*, the protein does not tolerate the DAS-tag and the DUC strain cannot be created.
18. In order to save time, in some instances the *sspB* plasmid can be co-transformed with the transcriptionally regulated plasmid.

Lack of colonies recovered from this co-transformation is not necessarily indicative of a problem with strain construction, but may instead be due to low transformation efficiency.

19. A 15 mL tube can be used with a heat block in future steps.
20. One can alternatively freeze down the pellet before addition of lysozyme for future prep.
21. 70 μL of 10 % SDS is sufficient, but the use of a repeater pipette can increase efficiency when dealing with many samples.
22. Ensure that P7 and P8 do not anneal to the plasmid used to create the merodiploid as the PCR will give a false negative for mutant confirmation.
23. It is important that the gel be as thick as the mold allows and DNA should be run until the bromophenol blue band reaches the end of the gel.
24. Alternatively, one can utilize the presence of *sacB* on the pNit::ET plasmid for counterselection. *sacB* confers sensitivity to sucrose in mycobacteria when grown on plates with 10 % sucrose. Loss of sucrose sensitivity implies loss of the pNit::ET plasmid. In our experience, not all sucrose resistant clones have become kanamycin sensitive indicating that *sacB* may have mutated through passaging. Thus, it is important to confirm that sucrose resistant candidates are also kanamycin sensitive.
25. It is important to construct wt strains with the same resistance as the mutant strain as the use of different antibiotics can affect the growth rate independently of the overall strain background. In this case, at a minimum the wt strain should be streptomycin resistant as the mutant strain must be grown with streptomycin to prevent loss of *tetR*.
26. If no colonies are recovered after two transformations, there may be enough leaky expression of *sspB* to induce degradation of the target protein, affecting cell survival in the absence of atc. In this case, *sspB* plasmids with different Shine–Dalgarno sequences can be used to alter SspB translation and counteract the effects of leaky expression.
27. When using this assay with conditional knockdowns such as DUC strains, that do not require atc for gene expression, it is not necessary to wash the culture with liquid medium to remove atc before plating in order to prevent atc carryover. Additionally, there will be an area of growth inhibition surrounding the atc-containing disc for these mutants.
28. Lower atc concentrations may be sufficient for a phenotype. A titration of different atc concentrations can be performed to determine the minimum atc concentration. Generally, 500 ng/mL is sufficient for a phenotype, but sometimes increased concentrations are required. Atc concentrations up to 1.5 μg/mL

are tolerated in wt *M. tuberculosis*. In order to ensure continued high concentrations of atc within the cell, atc should be refreshed at half of the desired concentration every 7 days, i.e., 250 ng/mL if the starting concentration is 500 ng/mL.

29. Do not create glycerol stocks or start new experiments from bacteria that have had gene activity silenced as a consequence of the presence of absence of atc as it is likely that there are suppressors. If knockdown of the target gene results in a growth defect, the strain will eventually accumulate suppressing mutations due to the presence of absence of atc depending on the type of mutant. Suppressors can be identified by plating on 7H10 plates ± atc and comparing the number of colonies that are able to grow on no atc plates compared to the number of colonies on plus atc plates.
30. Growth phenotypes can be compared between the DUC strain and transcriptionally regulated or proteolytically regulated strains if desired.

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Chapter 10

Mycobacterial Recombineering

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Abstract

The precise knockout or modification of *Mycobacterium tuberculosis* genes has been critical for the identification of functions important for the growth and pathogenicity of this important bacterium. Schemes have been previously described, using both non-replicating vectors and transducing particles, for the introduction of gene knockout substrates into *M. tuberculosis*, where the endogenous recombination systems of the host (both homologous and illegitimate) compete for transfer of the modified allele to the chromosome. Recombineering technologies, first introduced in laboratory and pathogenic strains of *Escherichia coli* over the last 16 years, have been developed for use in *M. tuberculosis*. Described in this chapter is the use of the mycobacterial Che9c phage RecET recombination system, which has been used to make gene knockouts, reporter fusions, promoter replacements, and single base pair modifications within the *M. tuberculosis* and *M. smegmatis* chromosomes at very high frequency. Higher success rates, in a shorter period of time, are routinely observed when recombineering is compared to previously described *M. tuberculosis* gene knockout protocols.

Key words Recombination, Mutation, Gene replacement, Single nucleotide polymorphisms, Mycobacteriophage

1 Introduction

In order to understand the roles of *Mycobacterium tuberculosis* genes in the physiology of the bacterium or the pathogenesis in its host, it is of utmost importance to be able to modify, tag, or knock out genes of interest within the chromosome easily. Early schemes to generate gene disruptions in *M. tuberculosis* relied on the classical approach of transformation with suicide plasmids that contain an inactivated copy of the target gene [1–3]. Relying on endogenous *M. tuberculosis* recombination pathways, rare recombinants are found by plating on appropriate media and selecting for drug resistance encoded by the plasmid. Such transformants consist of plasmid–chromosome cointegrates that contain a duplication within the chromosome, which when spontaneously resolved result in exchange of the inactivated gene with the targeted locus in a

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fraction of clones. The use of *sacB* within the non-replicating plasmid as a counterselection marker (sensitivity to sucrose) helps select for this second rare event, and has been used to make gene knockouts in *M. smegmatis*, *M. bovis* BCG and in *M. tuberculosis* [4–8]. While these schemes allowed investigators for the first time to make both marked and unmarked knockouts of *M. tuberculosis* genes, the procedures are plagued by inefficient uptake of DNA by electroporation, a low rate of plasmid–chromosome co-integrate formation and illegitimate pathways of recombination in *M. tuberculosis* (a notorious problem for mycobacterial gene replacement schemes). The illegitimate recombination events interfering with gene targeting events in mycobacteria have, however, been useful for protocols involving random mutagenesis of mycobacterial genomes [9, 10].

An improved gene knockout technology scheme for *M. tuberculosis* was described by Bardarov et al. [11] that uses phage transduction to deliver recombination substrates to the bacterium at high efficiency, solving the problem of low electroporation-mediated transformation rates for *M. tuberculosis*. The gene knockout substrate (flanked by γδ-res sites) is cloned into an *E. coli*–mycobacterial shuttle vector that encodes the essential components of a temperature sensitive *M. tuberculosis* TM4 phage packaging system and a temperature-sensitive origin of replication. After transformation into *M. smegmatis* at the permissive temperature, transducing particles are generated. The particles are used to infect *M. tuberculosis* at the nonpermissive temperature, where abortive transduction results in delivery of the recombination substrate at high efficiency. Frequencies of gene replacement are on the order of 10^{-6}, but because of the high efficiency of transduction, many colonies are readily generated. Transformation with plasmid expressing the γδ-resolvase can then be used to excise the drug resistance marker to create an unmarked gene knockout. The procedure, however, demands a number of technical steps that are time-consuming and include: (1) the construction of a plasmid containing the knockout substrate, (2) cloning this construct into an *E. coli*–mycobacterial shuttle vector containing the *ts*TM4 *M. tuberculosis* phage, (3) in vitro packaging of the shuttle vector construct into lambda phage heads, (4) infection of *E. coli* with the packaged lambda phage, (5) isolating the shuttle vector construct, (6) transforming *M. smegmatis* with the shuttle vector construct at the permissive temperature (30 °C), and finally, (7) transduction of *M. tuberculosis* with TM4 phage containing the knockout substrate at the nonpermissive temperature (37 °C).

A relatively new and simpler system for generating gene modifications in *M. tuberculosis* has developed over the last 5 years, and draws on the benefits of using a mycobacterial phage recombination system to efficiently transform linear DNA constructs electroporated into the *M. tuberculosis* genome. The system is based on the same paradigm used in *E. coli* (and other pathogens) that have utilized the phage lambda Red system for linear DNA

transformation. The procedure is known as "recombineering" (for *recombi*nogenic engi*neering*) and has vastly improved the accuracy and efficiency of engineering the *E. coli* chromosome, including foreign DNA cloned into bacterial artificial chromosomes (BACs) [12–15]. van Kessel and Hatfull [16, 17] discovered the λ Red-like RecET recombination system in mycobacterial phage Che9c, and have described its use for genetic manipulation of mycobacteria. We have further developed this technology and have employed it in numerous ways to make gene knockouts, promoter replacements, and tagged constructs in both *M. smegmatis* and *M. tuberculosis* [18–20].

For mycobacteria, DNA knockout recombineering substrates are typically generated by restriction digestion of plasmids, or by using plasmids as templates for PCRs. The linear recombineering substrates are drug cassettes (mostly *loxP-hyg-cat-loxP*) flanked by ~500 bp of target homology. The addition of *loxP* sites to the cassette allows removal of the drug resistance marker by the Cre recombinase following gene replacement. Even though the use of PCR fragments containing small regions of flanking homology (50–60 bp) work efficiently in *E. coli*, it is not recommended for use in mycobacteria. While drug-resistant transformants are easily found with these 50-bp flanking substrates, they are almost universally generated by illegitimate recombination and thus do not reflect accurate gene targeting events. In the protocol described here, linear DNA substrates containing ~500 bp flanks are electroporated into *M. tuberculosis* cells that carry a plasmid that allows the inducible expression of the phage Che9c RecET recombination system, and constitutive expression of the *sacB* gene. Following electroporation, the cells are plated on media containing the appropriate antibiotic which selects for the gene knockout, and drug-resistant recombinants are expanded and verified by PCR analysis. The RecET-expressing plasmid is lost spontaneously (or selected against by plating on sucrose). The procedure is simple and easily performed, with success rates that are equal to or greater than the previously described *M. tuberculosis* gene-modification schemes. An outline of this protocol is illustrated in Fig. 1a.

A second protocol described here is the methodology of using the Che9c RecT function in transferring single base pair mutations, additions or deletions to the *M. tuberculosis* chromosome at high frequency. This RecT-promoted oligo-mediated recombineering technology was first described by van Kessel and Hatfull in *M. smegmatis* and *M. tuberculosis* [17]. We have used this recombineering technology to verify SNPs found in *M. tuberculosis* that had putatively been identified as mutations responsible for drug-resistance [18]. Following SNP identification by whole genome sequencing of the resistant mutant, oligos containing the SNP are electroporated into *M. tuberculosis* expressing the RecT annealase. Following the same paradigm as λ Red-promoted recombineering in *E. coli*

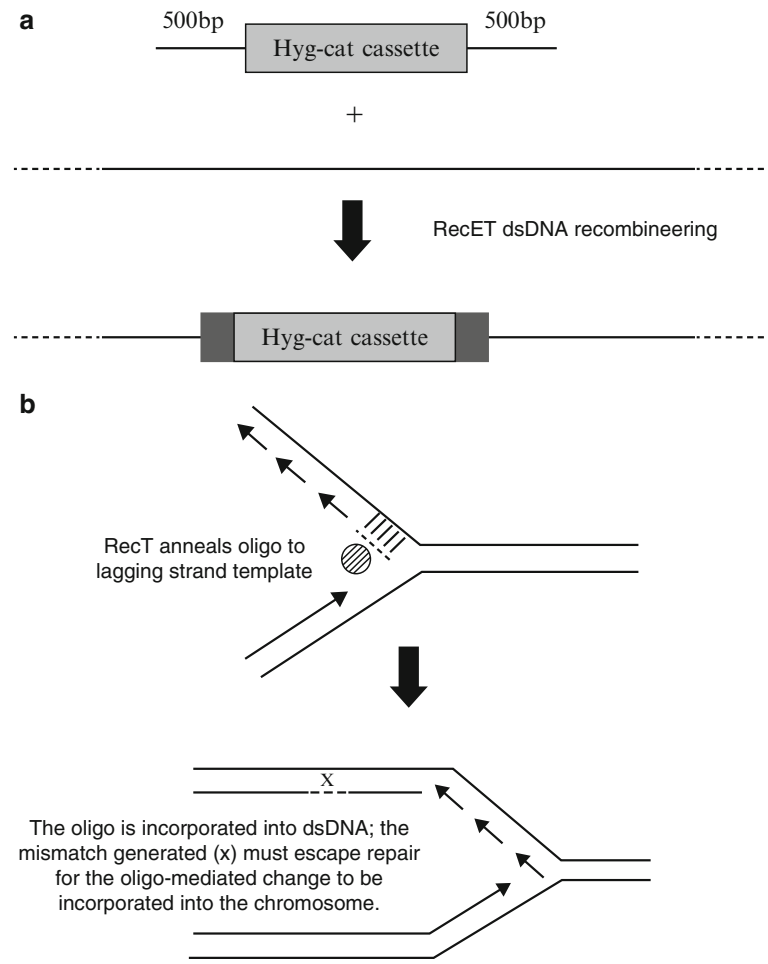

Fig. 1 Two recombineering protocols discussed in this chapter are illustrated. (**a**) Linear DNA substrates generated by plasmid digestion (or by PCR) are electroporated into *M. tuberculosis* (or *M. smegmatis*) expressing the bacteriophage Che9c RecET functions. The linear DNA contains a drug cassette (*hyg-cat*) flanked by 500 bp of homology to the regions flanking the target gene. The RecET recombineering functions promote replacement of the endogenous sequence with the cassette. While the mechanism may involve an ssDNA intermediate as demonstrated for *E. coli* [24], the same may not be true for mycobacteria, as the reaction shown here is dependent on host RecA function (unpublished observations). (**b**) A proposed mechanism of RecT-promoted oligo-mediated recombineering is shown. The Che9c RecT function promotes annealing of the oligo (*dotted line*) to ssDNA regions in the lagging strand template of the replication fork. The mismatch generated by the annealing event (denoted by the "x") must escape repair processes in order to be maintained. Following subsequent replication of the region (not shown), a recombinant chromosome is formed containing the insertion, deletion or modification designed into the oligo

[21, 22], the oligo is annealed to ssDNA regions of the *M. tuberculosis* replication fork by RecT, where it is incorporated at high frequency into the elongating lagging strand. SNPs transferred to the *M. tuberculosis* in this fashion allow us to test if the SNP alone, in an otherwise parental background, is responsible for the increase in the MIC displayed by the original drug-resistant mutant. A mechanism proposed for RecT-promoted oligo-mediated recombineering is shown in Fig. 1b.

2 Materials

2.1 Preparation of Recombineering Substrates

1. BP Clonase II and LR Clonase II Plus (Life Technologies). The following plasmids are all available from K. Papavinasa sundaram in the corresponding author's laboratory at UMass Medical School, Worcester, MA. Maintain plasmids pDO41A, pDO23A, and pKP555 in a *ccdB*-resistant *E. coli* strain and grow in LB containing chloramphenicol (20 μg/mL) to make plasmid preparations.
2. pDO41A: Donor vector for cloning upstream sequences of the target gene. Contains the sequences for *att*P4-*ccdB-cat-attP1R*. Cam^R and Amp^R.
3. pDO23A: Donor vector for cloning downstream sequences of the target gene. Contains the sequences for *att*P2R-*cat-ccdB-attP3*. Cam^R and Amp^R.
4. pKP628 (pEN-hyg-cat): Contains *loxP-hyg-cat-loxP* cassette flanked by *att*B1 and *att*B2, for use in Gateway LR reactions; Hyg^R, Cam^R and Amp^R.
5. pKP555 (pDE43-kan): Destination vector for Gateway LR reactions. Contains the *cat* and *ccdB* genes flanked by *att*R4 and *att*R3 sites; Cam^R, Kan^R.

2.2 Preparation of Recombinogenic/ Electrocompetent M. tuberculosis Cells

1. pNitET-SacB-kan: Expresses the Che9c RecET genes from the P_{nit} promoter. *See* **Note 1**. This plasmid is available upon request from the Sassetti Lab at UMass Medical School, Worceser, MA; please contact K. Papavinasasundaram (kadamba.papavinasasundaram@umassmed.edu).
2. 20 % v/v Tween 80: Mix 20 mL Tween 80 with 80 mL of distilled water, stir the solution at 50 degrees C to dissolve the Tween 80, cool to RT and filter sterilize through 0.2 μM membrane filter. Store at 4 °C for ≤2 months
3. 7H9-OADC-Tween 80: Dissolve 4.7 g of Middlebrook 7H9 powder and 2 mL glycerol in 900 mL deionized water and autoclave. Add 100 mL of Middlebrook OADC enrichment supplement and 2.5 mL of 20 % Tween 80 to 900 mL of 7H9 broth, filter-sterilize through 0.2 μM membrane filter. Store at 4 °C for ≤2 months.

4. 7H10 plates: Add 19 g of Middlebrook 7H10 powder and 5 mL glycerol to 900 mL of deionized water. Autoclave, cool to 50 °C, and add 100 mL of OADC. Mix and pour (30 mL per plate).
5. Nalgene inkwell square bottles (30 mL and 125 mL capacities).
6. Isovaleronitrile (IVN): Prepare 1,000× stock by diluting pure IVN 10,000-fold in DMSO. Store at 4 °C. Stock is stable for at least 6 months. *See* **Note 2**.
7. 2 M glycine: Dissolve 150.14 g in 1 L distilled water; filter-sterilize through 0.2 μM membrane filter. Store at room temperature for ≤6 months.
8. PBS-Tween 80: Add 2.5 mL of 20 % Tween 80 (w/v) to 1 L PBS. Filter-sterilize.
9. 10 % (v/v) glycerol: Mix 100 mL glycerol with 900 mL distilled water and autoclave.

2.3 Electroporation of Recombineering Substrates

1. Electroporation cuvettes (0.2 cm gap).
2. 7H9-OADC-Tween 80 media and 7H10 plates.
3. Small sterile disposable transfer pipettes (1 mL) individually packaged consisting of a small bulb and extended fine tip.
4. Electroporation apparatus: capable of delivering a pulse of 2.5 kV, 1,000 Ω, 25 μF setting.
5. Nalgene inkwell bottles (30 mL).
6. Aluminum foil.

2.4 Outgrowth and Verification of Allelic Replacement Mutants

1. Nalgene inkwell bottles (30 mL).
2. 7H9-OADC-Tween 80 and 7H10 plates: *see* Subheading 2.2, **items 3** and **4**.
3. Kanamycin: Dissolve in deionized water to a concentration of 20 mg/mL and filter-sterilize. Store at −20 °C for ≤6 months.
4. Hygromycin B (Roche). Supplied as a 50 mg/mL solution in PBS; store at 4 °C in the dark.
5. Hot metal bead bath set to 85 °C.
6. Taq DNA polymerase.
7. Cassette and target specific primers (*see* Fig. 4).

2.5 Generation of Unmarked Modifications by Cre-loxP

1. pCreSacB-zeo: Constitutively expresses Cre recombinase and the SacB levansucrase (Zeo^R).
2. 7H9-OADC-Tween 80 and 7H10 plates: *see* Subheading 2.2, **items 3** and **4**.

3. Taq DNA polymerase.
4. Cassette and target region specific primers (*see* Fig. 4).
5. Sterile loops.

2.6 Oligo-Mediated Recombineering in M. tuberculosis

1. Oligo (70 mer) containing mutation: Dissolve oligo from manufacturer to a final concentration of 1 μg/μL in 10 mM Tris–HCl, pH 8.0 (or water); dilute stock as needed.
2. pKM402: Expresses Che9c RecT from the inducible Ptet promoter; Kan^R.
3. 7H9-OADC-Tween 80 and 7H10 plates: *see* Subheading 2.2, **items 3** and **4**.
4. Anhydrotetracycline (ATc): Prepare 1,000× stock solution by dissolving ATc in methanol at a concentration of 500 μg/mL. Store at −20 °C in the dark.
5. Electroporation cuvettes (0.2 cm gap).
6. Small sterile disposable transfer pipettes (1 mL) individually packaged consisting of a small bulb and extended fine tip.
7. Electroporation apparatus: capable of delivering a pulse of 2.5 kV, 1,000 Ω, 25 μF setting.

2.7 Preparation of Recombinogenic/Electrocompetent M. smegmatis Cells

1. AD enrichment: For 10× stock, add 25 g of BSA, 10 g of glucose and 4.25 g NaCl to 500 mL of deionized water. When dissolved, filter-sterilize through 0.2 μM membrane filter.
2. 7H9-AD-Tween 80: To 900 mL of 7H9 broth add 100 mL of 10× AD enrichment and 2.5 mL of 20 % (v/v) Tween 80, filter-sterilize through 0.2 μM membrane filter. Store at 4 °C; stable for 4 months.
3. 7H10-AD plates: Add 19 g of Middlebrook 7H10 powder and 5 mL glycerol to 900 mL of deionized water. Autoclave, cool to 50 °C and add 100 mL of AD enrichment. Mix and pour (30 mL per plate).
4. ε-Caprolactam: Prepare 100× stock (20 %) by dissolving 200 g in 1 L of deionized water and filter-sterilize. Store at room temperature. ε-Caprolactam can be used to induce expression from the pNIT promoter in *M. smegmatis*.
5. LB plates: Add 40 g of Difco LB-Agar powder to 1 L of distilled water, mix and autoclave the suspension, and pour 30 mL per plate after cooling the solution to 50 °C.
6. 10 % glycerol.
7. Electroporation cuvettes (0.2 cm gap).

8. Small sterile disposable transfer pipettes (1 mL) individually packaged consisting of a small bulb and extended fine tip.
9. Electroporation apparatus: capable of delivering a pulse of 2.5 kV, 1,000 Ω, 25 μF setting.

3 Methods

3.1 Preparation of Recombineering Substrates

1. Clone the upstream and downstream regions of the target gene using BP clonase II as follows: Amplify two PCR products that are flanked by different *att*B sites: one amplicon should contain ~500 bp sequence upstream of the target gene, and the other amplicon should contain ~500 bp sequence downstream of the target gene (*see* **Note 3**). Incorporate the *att*B sites into the 5′ end of the primers, and include *att*B4 and *att*B1 for the upstream sequence, and *att*B2 and *att*B3 for the downstream sequence. The sequences of the *att*B sites to include in the primers are shown in Fig. 2, and the PCRs are diagrammed at the top of Fig. 3. Unique restriction sites

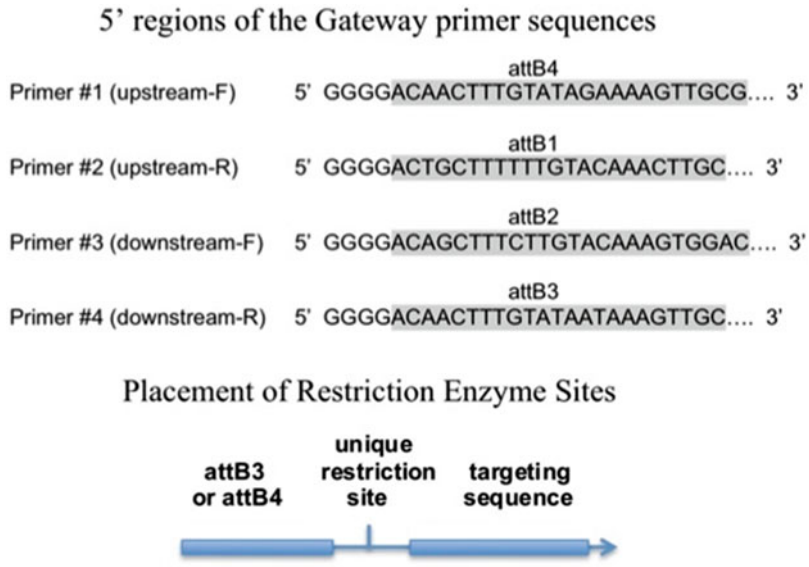

Fig. 2 Gateway *att*B sequences. *Top*: The *att*B site sequences to be used in primers #1–4 (*see* diagram in Fig. 3 for positions of primers) are shown above. Attach gene-specific primers to the 3′ end of the sequences given above in 5′ to 3′ direction (*att*B sites are in *grey*). These *att*B sites contain 1–2 extra base pairs relative to canonical *att*B sites, so as to allow in-frame readthrough should the *loxP-hyg-cat-loxP* drug marker be excised by Cre to create an in-frame unmarked gene deletion (if so desired). *Bottom*: Illustration of primers #1 and #4 (containing *att*B4 or *att*B3, respectively) and the position of unique restriction sites that should be included in these two primer sequences. These restriction sites allow for digestion of the final plasmid construct in order to liberate the linear recombineering fragment; these restriction sites should not appear in either the flanking sequences or the *hyg-cat* cassette

(ones not present in either the flanking regions or the *hyg-cat* cassette) should be designed into primers #1 and #4 (containing *att*B4 and *att*B3, respectively) and placed between the *att*B sites and the targeting sequences (*see* Fig. 2).

2. Perform two BP recombination reactions. In the first reaction, clone the PCR product containing the upstream region of the target gene (flanked by *att*B4 and *att*B1 sites) into donor vector pDO41A, generating the entry plasmid pEN41-up. In the second reaction, clone the PCR product containing the downstream region of the target gene (flanked by *att*B2 and *att*B3 sites) into donor vector pDO23A, generating the entry plasmid pEN23-down (*see* Fig. 3, step 2). Set up reactions as follows: mix 20–50 fmoles of the *att*B4 + *att*B1-containing PCR product generated in step 1 with 150 ng of pDO41A in a total volume of 8 μL. Also, mix 20–50 fmoles of the *att*B2 + *att*B3-containing PCR product generated in section step 1 with 150 ng of pDO23A in a total volume of 8 μL. Add 2 μL of the BP Clonase II enzyme solution (Life Technologies) to each mixture. Mix briefly and incubate at 25 °C for 1 h. Transform competent *E. coli* and select for Amp^R clones.
3. Perform a MultiSite Gateway LR recombination reaction that includes four vectors: the two entry vectors containing the upstream and downstream regions of the target gene (as constructed in step 2), the entry vector pKP628 containing the *hyg-cat* cassette, and the destination vector pKP555 (pDE43-kan) (Fig. 3, step 3). Set up reactions as follows: mix 25 fmoles of the two entry clones generated in step 2, 25 fmoles of the entry vector pKP628, and 60 ng of destination vector pKP555 (pDE43-kan) in a 16 μL reaction volume. Add 4 μL of LR clonase Plus enzyme solution. Mix and incubate at 25 °C for 16 h. Transform chemically competent *E. coli* cells and select for Kan^R clones (*see* **Note 4**). The product of this reaction is a plasmid that contains the *loxP-hyg-cat-loxP* cassette flanked by ~500 bp of homology upstream and downstream of the target gene of interest.
4. Generate the linear dsDNA recombineering fragment from the knockout substrate as follows: cut the plasmid generated in step 3 with the unique restriction sites engineered into the construct by primers #1 and #4 (Fig. 3). Purify the restriction enzyme digest using Qiagen PCR Purification kit, or similar kits (*see* **Note 5**); include an additional washing step to the manufacturer's instructions (*see* **Note 6**). As an alternative to plasmid digestion, generate the recombineering fragment by PCR using the plasmid as a template (*see* **Note 7**).

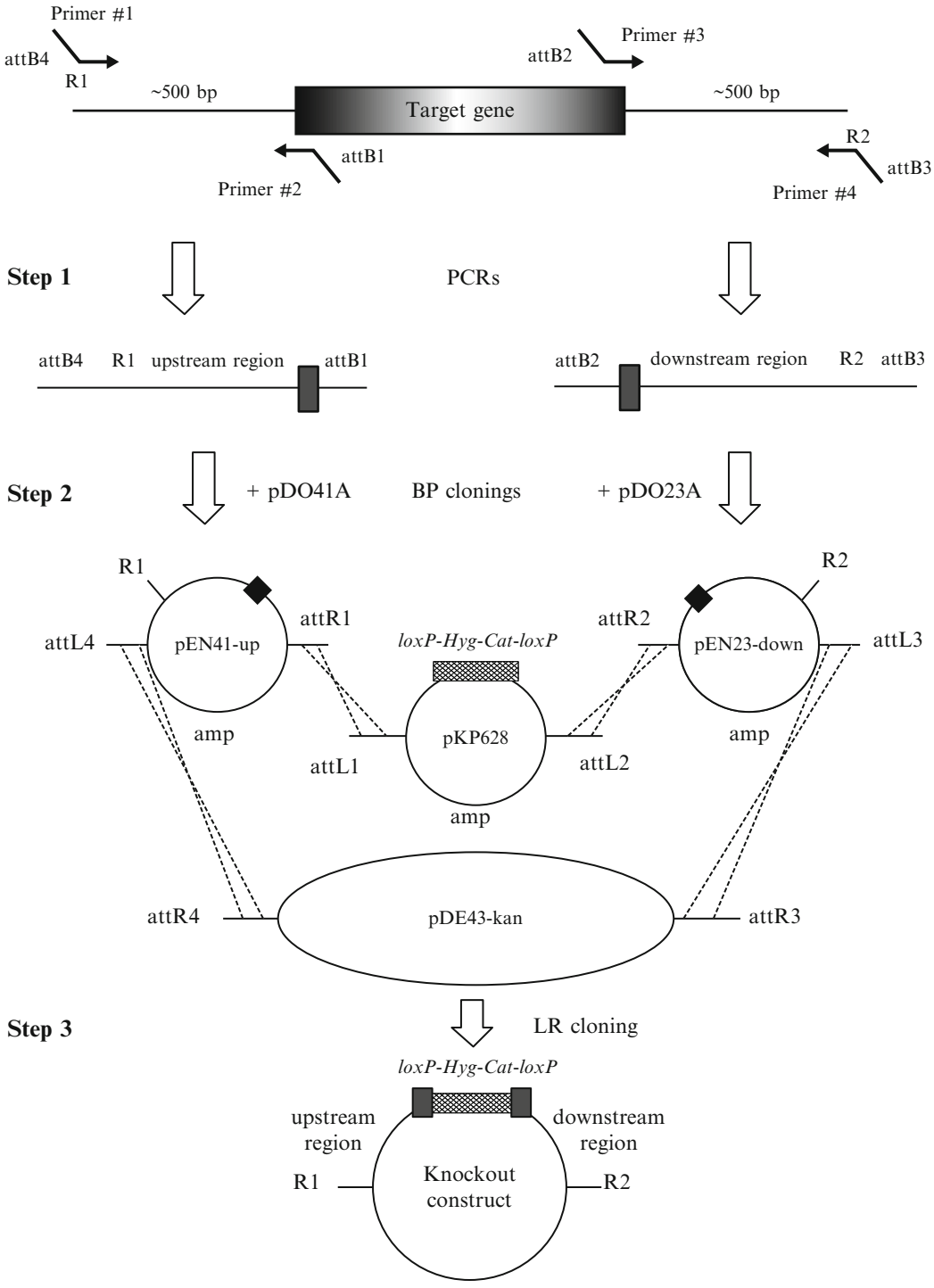

Fig. 3 Knockout construction using Gateway technology. *Step 1*: PCRs are used to generate the upstream and downstream DNA fragments of the target gene (~500 bp). Primers #1–4 contain *att*B sites at the 5′ ends (from Fig. 2) and gene specific sequences at the 3′ ends (~24 bases). Unique restriction sites (R1 and R2) should be incorporated into primers #1 and #4 between the *att*B sites and the gene-specific sequences, so as to allow

Alternatives to Gateway cloning for the generation of recombineering knockout constructs can also be performed (*see* **Note 8**).

3.2 Preparation of Recombinogenic/ Electrocompetent M. tuberculosis Cells

1. Add 0.5 mL of a glycerol stock of H37Rv carrying the pNitET-SacB-kan plasmid to 10 mL 7H9-OADC-Tween 80, plus 20 μg/mL kanamycin, in a 30 mL inkwell bottle.
2. Grow the culture with shaking at 37 °C until it reaches saturation (OD_{600} ~ 1.0).
3. Transfer 1.5 mL of the saturated culture to 30 mL of 7H9-OADC-Tween 80 medium containing 20 μg/mL kanamycin in a 125 mL inkwell bottle (starting OD_{600} of ~0.05–0.1). Grow the culture with shaking at 37 °C. The 30 mL culture contains enough cells for ~7 electroporations.
4. When the culture reaches an OD_{600} of 0.8 (4–5 days), induce the expression of the RecET proteins by adding 30 μL of isovaleronitrile stock solution.
5. Incubate with shaking at 37 °C for ~8 h.
6. Add 3 mL of 2 M glycine to the culture and continue shaking at 37 °C for 16–20 h.
7. Collect the cells by centrifugation at 3,500 × *g* for 10 min at 20 °C in a 50 mL tube.
8. At RT, add 1 mL of 10 % glycerol to the pellet and gently resuspend the cells by pipetting back and forth with a 1 mL pipet tip.
9. Bring volume of the culture up to 30 mL with 10 % glycerol. Cap the tube securely and invert it a few times to promote mixing.
10. Repeat the centrifugation and washing procedures.
11. Collect the cells by centrifugation and resuspend the pellet in 3 mL of 10 % glycerol. The cells are now electrocompetent and recombinogenic (*see* **Note 9**).

Fig. 3 (continued) liberation of the linear dsDNA recombineering fragment from the final knockout construct by restriction enzyme digestion. *Step 2*: The BP (*att*B × *att*P) cloning reactions are performed between the linear *att*B-containing PCR products from step 1 and the *att*P-containing donor vectors, pDO41A and pDO23A. The products of these reactions contain two entry vectors, which contain 500 bp of sequence upstream of the target gene (pEN41-up) and 500 bp of sequence downstream of the target gene (pEN23-down). Each of these vectors will contain the appropriate *att*R or *att*L site for use in the Multisite Gateway Three-Fragment vector construction scheme. *Step 3*. The two entry vectors from step 2 are combined with entry vector pEN12-hyg-cat (pKP628) and destination vector pDE43-kan (pKP555) in an LR cloning reaction to generate the final knockout construct. The linear recombineering fragment (~3 kb) can be generated by digestion with restriction sites R1 and R2, or the construct can be used as a template for PCR to generate ~3 kb linear dsDNA species. Both substrates contain the *hyg-cat* marker flanked by 500 bp of sequences upstream and downstream of the target gene

3.3 Electroporation of Recombineering Substrates

1. Add 400 μL of the competent *M. tuberculosis* cells to a 0.2 cm cuvette.
2. Add 0.5–1.0 μg of the recombineering substrate to the cells and mix the sample with a 200 μL pipet by gently pipetting back and forth 3–4 times. Include one electroporation as a no DNA control.
3. Place cuvette into the electroporation chamber and apply a pulse at 2.5 kV, 1,000 Ω, 25 μF setting (*see* **Note 10**).
4. Remove the cuvette from the chamber, recover the cells with a sterile 1 mL disposable transfer pipet, and transfer the cells to a 30 mL inkwell bottle containing 5 mL of 7H9-OADC-Tween 80.
5. Shake the cultures at 37 °C for 1–2 days, collect the cells by centrifugation at 3,500 × *g* for 10 min, and resuspend in ~200–400 μL of 7H9-OADC-Tween 80.
6. Plate the entire suspension on a 7H10 plate containing the appropriate drug, usually hygromycin. As a control, also plate the culture where no DNA was added to the cells.
7. Allow the plates to dry, then wrap them with two sheets of aluminum foil (*see* **Note 11**) and incubate the plates at 37 °C for 21–24 days.
8. Examine the plates for colonies indicative of gene replacement (*see* **Note 12**). The no DNA control plate should have no (or just a few) colonies.

3.4 Outgrowth and Verification of Allelic Replacement Mutants

1. Pick six to ten colonies with a sterile plastic loop and inoculate each into 30 mL inkwell bottles containing 5 mL of 7H9-OADC-Tween 80 and 50 μg/mL hygromycin.
2. After the OD_{600} reaches ~1.5, transfer 0.3 mL of culture to a screw-capped tube and heat-inactivate the cells by total immersion of the tube in hot metal bead bath (85 °C) for 1 h (*see* **Note 13**). The remaining cultures can be stored at 37 °C but need not be shaken until the heat-killed cells are verified by PCR screening.
3. Verify the structure of the recombinant by PCR analysis using Taq DNA polymerase (Fig. 4). Use 2 μL of the heat-killed bacterial suspension in a 30–50 μL PCR with the following program: step 1 (95 °C—5 min); step 2 (95 °C—30 s); step 3 (58–61 °C—30 s); step 4 (72 °C—1 min); cycle through steps 2–4 (30×); step 5 (72 °C—5 min).
4. Verify the junctions of the *M. tuberculosis* gene replacement by using primers targeting the upstream and downstream regions of the chromosome that are not contained within the flanking sequences of the recombineering substrate (*see* Fig. 4). For the upstream gene replacement junction, use target-specific

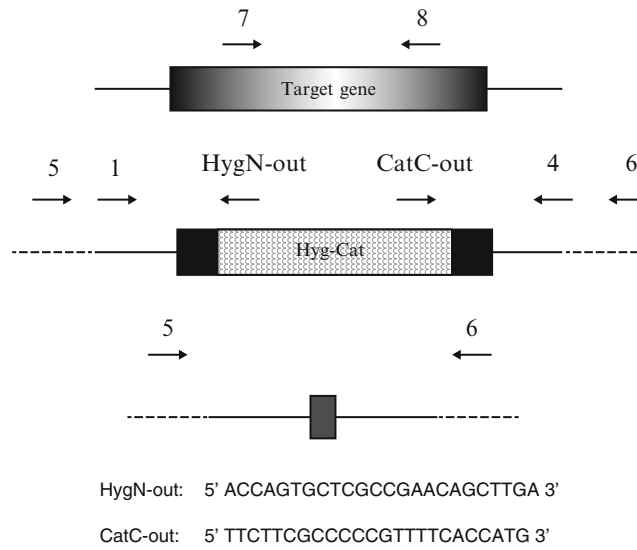

Fig. 4 Position of primers used in verification of the *M. tuberculosis* allelic replacement strain. *Top*, unmodified chromosome: Primers #7 and # 8 should be used to verify the absence of the target gene in the gene replacement strain, and the presence of the target in the parental strain. *Middle*, gene replacement: Primers #5 and Hyg-out (sequence below) should be used to verify the 5′ terminal junction, while primer #6 and CatC-out (sequence below) should be used to verify the 3′ terminal junction. Primers #5 and #6 are located in the chromosomal regions upstream and downstream of primers #1 and #4, respectively. *Bottom*, unmarked deletion (following Cre-mediated eviction of *loxP-hyg-cat-loxP*): Primers #5 and #6 should be used to verify the absence of the *hyg-cat* cassette. In all cases, the size of the PCR fragment expected for an unmarked recombinant using these primers will be ~1.2 kb (which includes both 500 bp flanking regions)

primers #5 and HygN-out; for the downstream gene replacement junction, use target-specific primer #6 and CatC-out (*see* Fig. 4) (*see* **Note 14**).

5. Verify the loss of the target gene in the knockout strain. For this purpose, we use primers that generate an internal fragment of the *M. tuberculosis* target gene in the parent, but not in the knockout strain (*see* primer positions 7 and 8 in Fig. 4) (*see* **Note 15**).
6. Screen knockout strain candidates for kanamycin sensitivity by streaking on 7H10 plates containing 20 μg/mL kanamycin and then on to drug-free 7H10 plates. Often, the RecET-producing plasmid pNitET-SacB-kan is lost spontaneously during selection for the recombinant. If this is not the case, subject cultures of the knockout strain to counterselection on 7H10 plates containing 3 % sucrose to select for cells that have lost pNitET-SacB-kan. Plate 50 μL of three serial tenfold

dilutions on to 7H10-OADC-Tween 80 plates containing 3 % sucrose. Streak out for single colonies on each plate. Incubate plates at 37 °C for 21–24 days. Restreak well-isolated colonies on 7H10-OADC-Tween 80 plates with and without 20 μg/mL kanamycin. Verify loss of pNitET-SacB-kan by screening for kanamycin sensitivity, and confirm loss of the plasmid by PCR with RecET specific primers.

3.5 Generation of Unmarked Modifications by Cre-loxP

1. Following replacement of the target gene with the *hyg-cat* cassette, transform the modified strain with 200–500 ng of pCreSacB-zeo (follow electroporation protocols described in Subheading 3.3).
2. Spread electroporation mixture on two 7H10 plates containing 25 μg/mL zeocin. Plate 100 μL and 500 μL of the mixture (and 10^{-1} and 10^{-2} dilutions) on 7H10-Zeo plates and streak out the cells with a sterile loop to isolate single colonies (*see* **Note 16**).
3. Incubate the plates at 37 °C for 21–24 days and examine the plates for single colonies. Screen for colonies that have lost the *hyg-cat* marker by streaking 20 well-isolated colonies on 7H10 plates containing 50 μg/mL hygromycin, and then onto drug-free 7H10 plates.
4. Inoculate two hygromycin-sensitive isolates into 10 mL 7H9-OADC-Tween 80 (no antibiotics) in 30 mL inkwell bottles and shake the cultures at 37 °C until the OD_{600} ~ 0.8.
5. Plate 50 μL of 10^{-1} to 10^{-3} dilutions of the cultures on 7H10 plates containing 3 % sucrose to obtain well-isolated single colonies.
6. Incubate the plates for 3 weeks until single colonies arise.
7. Streak ~20 colonies onto 7H10-OADC-Tween 80-zeocin plates and antibiotic-free plates.
8. Incubate the plates for 21–24 days and identify colonies that are sensitive to zeocin (*see* **Note 17**).
9. Transfer one Zeo^S colony with a sterile loop into an inkwell containing 10 mL 7H9-OADC-Tween 80 and grow the strain with shaking at 37 °C for 7 days. Perform whole cell Taq PCR using heat-killed cells as described in Subheading 3.4 (**step 3**) to verify absence of the chromosomal *hyg* marker. A PCR using target-specific primers #5 and #6 (*see* Fig. 4) should generate ~1.2 kb band only in the unmarked strain.
10. Sequence the PCR product to verify Cre-mediated recombination between the flanking *loxP* sites within the cassette.

3.6 Oligo-Mediated Recombineering in M. tuberculosis

1. Design a 70-mer oligo that corresponds to the lagging strand of the replication fork as it passes through the target gene (*see* **Note 18**). Position the mutation desired (single base

insertion, deletion, or missense mutation) in the middle of the 70-mer sequence.

2. Start a fresh culture of *M. tuberculosis* carrying pKM402 (Ptet-RecT-expressing plasmid) in 10 mL 7H9-OADC-Tween 80-kanamycin. Grow at 37 °C to an OD_{600} ~ 1.0.
3. Transfer 1.5 mL of the saturated culture to 30 mL of 7H9-OADC-Tween 80 medium containing 20 μg/mL kanamycin in a 125 mL inkwell bottle (starting OD_{600} of ~0.05–0.1).
4. Grow the culture with shaking at 37 °C. When the OD_{600} reaches ~0.8, induce RecT expression by the addition of 30 μL of ATc stock solution. The culture volume of 30 mL is good enough for ~7 electroporations; scale up by using multiple flasks.
5. After ~8 h, add 3 mL of 2 M glycine and continue rolling at 37 °C for an additional 16 h.
6. Collect the cells by centrifugation at 3,500 × *g* for 10 min at 20 °C in a 50 mL tube. At RT, add 1 mL of 10 % glycerol to the pellet and gently resuspend the cells by pipetting back and forth with a 1 mL pipet tip. Bring volume of the culture up to 30 mL with 10 % glycerol. Cap the tube securely and invert it a few times to promote mixing.
7. Repeat the centrifugation and washing step.
8. Collect the cells by centrifugation and resuspend the pellet in 3 mL of 10 % glycerol. The cells are now electrocompetent and recombinogenic.
9. Add 400 μL of electrocompetent cells to a 0.2 cm electroporation cuvette, add 0.1–0.5 μg of oligo, and mix the sample by gently pipetting back and forth 3–4 times with a 200 μL pipet; keep the volume of the oligo between 1 and 5 μL.
10. Place the cuvette into the electroporation chamber and apply a pulse at 2.5 kV, 1,000 Ω, 25 μF.
11. Remove the cuvette from the chamber, recover the cells with a sterile 1 mL disposable transfer pipet, and transfer the cells to a 30 mL inkwell bottle containing 10 mL of 7H9-OADC-Tween 80.
12. Shake the culture at 37 °C for 3–4 days. *See* **Note 19**.
13. Plate the cells on selective medium using 7H10 plates.
14. If desired mutant is non-selectable, plate for single colonies, pool candidates and use PCRs with mutation-specific primers to specifically identify pools of colonies that contain the modified chromosomes (*see* **Note 20**). One can increase the frequency of oligo-mediated recombineering by co-transformation with an oligo that creates a selectable phenotype (*see* **Note 21**).

3.7 Recombineering in M. smegmatis

For genetic modification of *M. smegmatis*, the protocols described above should be followed, with the following modifications:

1. Generate recombineering constructs as described above in Subheading 3.1 for *M. tuberculosis* using the Gateway technology (Life Technologies). Alternatively, we have had success recombineering in *M. smegmatis* with PCR substrates containing ~125 bp of flanking sequences (*see* **Note 22**).
2. Grow *M. smegmatis* containing pNitET-SacB-kan in 7H9-AD-Tween 80 containing 20 μg/mL kanamycin and induce RecET expression with either 0.2 % ε-caprolactam (18 mM) or 1 μM IVN (*see* **Note 23**).
3. Induce the culture at an OD_{600} of 0.5 and allow the cells to grow to an OD_{600} of 1.0 (2.5–3 h) (*see* **Note 24**). Collect the cells by centrifugation (3,500 × *g* for 10 min) and make them electrocompetent as described in Subheading 3.2 (**steps 8–11**), with the exception that the centrifugation and washing steps are carried out at 4 °C.
4. Keep cells and electroporation cuvettes at 4 °C or chilled on ice during the procedure.
5. Following electroporation, transfer the cells to a culture tube containing 2 mL of 7H9-AD-Tween 80 and allow the cells to recover for 3–4 h by rolling at 37 °C.
6. For oligo-mediated recombineering, use *M. smegmatis* carrying pKM402 and induce the culture at an OD_{600} of 0.5 with 300 ng/mL anhydrotetracycline (ATc). Allow the cells to grow to an OD_{600} of 1.0 (between 2.5 and 3 h following the addition of ATc). Following electroporation, transfer the cells to a culture tube containing 2 mL 7H9-AD-Tween 80 and allow the cells to recover (and to enable chromosome segregation) by rolling overnight at 37 °C. Plate the cultures as indicated in Subheading 3.6 (**steps 13** and **14**) using either LB or 7H10 plates containing appropriate antibiotic.

4 Notes

1. The expression of the *recET* genes is induced with IVN in *M. tuberculosis* and ε-Caprolactam in *M. smegmatis*; the plasmid is Kan^R.
2. For induction, add 30 μL of this stock to 30 mL culture to give a final concentration of 1 μM. Isovaleronitrile is toxic. Concentrated solutions should be handled with gloves in a chemical hood.
3. The region located between the outward-directed primers (primers #2 and #3; Fig. 3) will be removed in the knockout

strain. It is often advisable to design these primers such that ~15 codons of the N-terminal and C-terminal encoding regions are retained. This is especially important if in-frame deletions are desired and in cases where important regulatory signals reside in these regions for expression of neighboring genes.

4. This reaction can be scaled down by a factor of two with equivalent results. If transformation rates are low, the use of commercially available chemically competent cells is suggested.
5. The linear recombineering fragment generated by restriction digestion does not have to be gel purified, as the linearized vector fragment will not interfere with the recombineering event, and the restriction site at the ends of the digested DNA will not be incorporated into the recombinant. Also, since the plasmid does not contain an origin of replication for mycobacteria, any uncut plasmid present in the restriction digest will not generate drug-resistant transformants (false positives). The process of purifying DNA fragments on agarose gels often yields lower yields of substrate, which can decrease the number of recombinants obtained. Ideally, 0.5–1 μg of dsDNA recombineering substrates should be used.
6. This step greatly helps to remove residual salts from the DNA sample that can interfere with the efficiency of electroporation. A time constant of >20 ms should be expected for salt-free samples using 0.2 cm cuvettes. A time constant of <18 ms is indicative of salt contamination, which will result in lower efficiencies of DNA transfer across the membrane.
7. Alternatively, generate the recombineering substrate by PCR using a proofreading enzyme such as PfuUltra II Fusion HS DNA polymerase (Agilent Technologies) and the plasmid constructed in Subheading 3.1 (**step 3**) as a template to amplify a ~3 kb linear dsDNA substrate containing the drug-resistance marker flanked by 500 bp of target homology (i.e., the knockout fragment). The PCR product should be purified using the Qiagen PCR purification kit (or similar kit), and washed twice with wash buffer. PCR substrates with contaminating smaller fragments (<1 kb) can often be used without further purification. If PCR artifacts of a size similar to (or greater than) the *hyg* gene are present (≥ 1 kb) agarose gel purification should be performed.
8. Three alternative options for the preparation of recombineering substrates are available: (1) Ligation of PCR-generated fragments into vectors containing pre-existing drug-resistant cassettes, e.g., pKM342, which contains *loxP-hyg-loxP* (Hyg^R and Amp^R), or pKM343 which contains *loxP-hyg-cat-loxP*

(Hyg^R, Cam^R, Amp^R); or pKM343, respectively (contains *loxP-hyg-cat-loxP* cassette); HygR, CamR, AmpR; (2) *E. coli* recombineering of cosmid DNA containing *M. tuberculosis* sequences, or (3) direct DNA synthesis. In the first case, the upstream and downstream regions of a target gene can be generated by PCR. Unique restriction sites are incorporated into the primers that allow cutting and ligation into a *loxP-hyg-cat-loxP* containing plasmid (pKM343), or *loxP-hyg-loxP* containing plasmid (pKM342). These starting plasmids are available upon request.

In the second case, a cosmid in *E. coli* containing the gene of interest (and many kilobases of the upstream and downstream regions) can be modified by the λ Red recombination system using established protocols [13]. The *E. coli* recombineering substrate is a PCR product that contains the *loxP-hyg-cat-loxP* cassette flanked by 50 bp of homology to the target gene. The modified cosmid, which contains a replacement of the target gene with the *loxP-hyg-cat-loxP* cassette, is then used as a template for a second PCR. The primers for this PCR are selected to generate the *loxP-hyg-cat-loxP* cassette that contains 500 bp of flanking homology, which can be used directly for recombineering in mycobacteria. The advantage of this method is that no cloning is required. The disadvantage is that a cosmid clone of the target gene (and surrounding regions) must be available.

In the third case, the entire recombineering fragment can be synthesized. For the 1,264 bp *loxP-hyg-loxP* cassette flanked by 500 bp of target DNA on both sides, the cost would be a few hundred dollars, though this price is expected to drop with the development of more efficient DNA synthesis technologies. The construct is usually cloned into a plasmid and designed such that cutting of the plasmid by a restriction enzyme liberates the recombineering fragment as a linear DNA (a requirement for recombineering). Alternatively, the uncloned synthesized construct could be used as a template for a PCR that generates the recombineering fragment.

9. Even though frozen cells are adequate for transformation of replicating/integrating plasmids, freshly prepared electrocompetent cells are required for generating mutants by recombineering.

10. When working with *M. tuberculosis*, it is critical to carry out electroporations within a Class I biosafety cabinet in a BSL3 laboratory and/or in accordance with local safety regulations. If the samples contain salt or lysed cells, arcing can occur, which can cause the cover of the cuvette to fly off. One should use an electroporation device that contains a pulse controller and a cover for the cuvette holder, make sure the sample is free

of salt (*see* **Note 6**), and have disinfectant nearby in case arcing occurs. We keep the cuvette-holder inside the biosafety cabinet, and cover the cuvette holder with a towelette soaked in Vesphene as a precaution against any splashing from an arcing event.

11. The plates are wrapped in aluminum foil to keep them from drying out during the 3.5 weeks of incubation at 37 °C. Alternatively, the plates can be kept in sealed plastic boxes.
12. In our experience, this procedure should produce ~5–50 colonies/transformation with ~30–70 % of these representing true gene replacement events.
13. Follow the legal and local regulations for removal of *M. tuberculosis* samples from the laboratory. We totally immerse the vials containing *M. tuberculosis* cells in a metal bead bath set at 85 °C for 1 h. It is important to not heat the samples at much higher temperatures (or longer periods) because the cells start to lyse, and thus will not perform well as templates for whole cell PCR analysis.
14. Our primer #5 is typically ~100 bp upstream of primer #1, and primer #6 is typically ~100 bp downstream of primer #4 (*see* Fig. 4).
15. For verification of a gene replacement, it is important to not only verify the correct sequence at the junctions of the cassette with the chromosome, but also to verify the absence of the target gene. Duplications can occur (in a minority of cases). If so, one could find that a replacement takes place in one copy of the gene, with the second copy of the gene remaining intact.
16. Since the pCre-SacB-zeo plasmid carries a mycobacterial origin of replication, it will transform at high efficiency. Therefore, plate transformants to obtain well-isolated single colonies by spreading out the mixtures (or dilutions thereof) on one half of the plate, then using sterile loops to streak out for single colonies on the other half of the plate.
17. Following gene replacement, the pNitET-SacB-kan plasmid is often lost spontaneously. If this is not the case, this procedure to construct an unmarked knockout can also be used to cure the strain of pNitET-SacB-kan. Following transformation of the gene replacement strain with pCreSacB-Zeo and selection for zeocin resistance, the cells become kanamycin sensitive as well, since both plasmids use the same origin of replication and are incompatible with each other. As such, at this stage of verifying zeocin sensitivity, it is advisable to check for kanamycin sensitivity as well, and to confirm hygromycin sensitivity.
18. Rules for selection of the lagging strand of the target gene, reading the dsDNA sequence of the gene from left to right, are listed in Table 1. Another way to determine which is the lag-

Table 1
Selection for the lagging strand in *M. tuberculosis* sequence

Gene position	Gene orientation	Lagging strand[a]
0–2.2 Mb	Clockwise	Bottom strand
0–2.2 Mb	Counterclockwise	Top strand
2.3–4.4 Mb	Clockwise	Top strand
2.3–4.4 Mb	Counterclockwise	Bottom strand

[a]Reading the dsDNA sequence file of the gene from *left* to *right*

ging strand is as follows: if the gene is oriented toward the termination region (in either the right or left replicore), use the bottom strand. If the gene is pointing toward the origin (toward *dnaA*), use the top strand. Close to the *dif* site, both strands usually work equally well. Assume replication termination in mycobacteria occurs close to the *dif* site (~2.2 Mb for *M. tuberculosis*; ~3.4 Mb for *M. smegmatis*).

19. The reason for this outgrowth period is as follows: if there is more than one copy of the chromosome per cell at the time of electroporation, and only one of them is modified, the culture is grown out to segregate the modified chromosome.
20. By using mutation specific primers, one can identify which pools of colonies contain mutants by PCR. Better known as Mismatch Amplification Mutation Assay (or MAMA-PCR) [23], and described for use in *M. smegmatis* by van Kessel and Hatfull [17], primers are designed so that the 3′ terminal base of one of the primers matches the single base pair mutation inserted by the oligo. However, the penultimate base is designed to create a mismatch. Though weakened a bit for annealing to its target, it still serves to generate a PCR signal for the mutant DNA sequence. The same primer, however, cannot recognize the wild type sequence as a result of a two base mismatch at its 3′ end (i.e., due to the bp change engineered at the penultimate position and the desired oligo-mediated bp change at the 3′ end).
21. The frequency of transformation of a 70 base oligo with a single base pair mismatch, insertion or deletion is about 1:1,000 (transformants per total number of cells). To increase this frequency, the oligo can be co-transformed with a selectable oligo (for example, an oligo that confers resistance to streptomycin). For this purpose, electroporate *M. tuberculosis* with 1 μg of the oligo and 0.1 μg of an oligo that creates a streptomycin resistant mutation in the *M. tuberculosis rpsL* gene (*see* below). Proceed as described in Subheading 3.6. Following 4 days of outgrowth, dilute culture 1:25 fold into

fresh 7H9-OADC-Tween 80 containing 20 μg/mL streptomycin (5 mL total volume), and grow the culture to an OD_{600} of 1.0 (~4 days). All cells will now be strep^R and the "unselected" oligo will be incorporated into about 5–10 % of the total cell population. (The *rpsL* oligo selects for cells that pick up DNA.) The "no DNA control" from the first outgrowth should not grow when diluted in 7H9-OADC-Tween 80 with 20 μg/mL streptomycin (a good control for electroporation and recombineering competence).

Oligos conferring resistance to streptomycin (target is the *rpsL* gene):

M. tuberculosis
5′ GCGGGCAACCTTCCGAAGCGCCGAGTTCGGCTTC CTCGGAGTGGTGGTGTACACGCGGGTGCA TACACC 3′

M. smegmatis
5′ CGCGCGCGACCTTCCGGAGCGCCGAGTTCGGCTT CCTCGGAGTGGTGGTGTAAACGCGCGTGC ACACGCC 3′

22. For this purpose, an initial PCR is performed using pKM343 as a template. The primers for the PCR should have the following targeting sequences to pKM343 at their 3′ ends: Hyg-For, CGCTCTAGAACTAGTGGATCC; Cat-Rev, TCGACGGTA TCGATAAGCGT. Attach the For and Rev primers listed above to the 3′ ends of gene-specific 60 base flanking sequences. Amplify the *loxP-hyg-cat-loxP* present in pKM343 using a proofreading polymerase. Cycling condition for the first PCR: 1 cycle of 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min, and 1 cycle at 72 °C for 10 min. The product of this PCR will be the *hyg-cat* cassette flanked by 60 bp of homology upstream and downstream of the target gene. Purify the PCR product using a PCR purification kit and use it as a template with a second set of *M. smegmatis* gene-specific primers to extend the flanking homology to a total of 125–130 bp. Therefore, the second set of primers should overlap with the first set by about 20–25 bases. Cycling condition for the second PCR: 1 cycle of 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 2 min, and 1 cycle at 72 °C for 10 min. Product of the second PCR which carries ~125 bp homology to the targeting gene is then purified and electroporated into *M. smegmatis* that had been induced for the expression of the RecET functions.
23. We have also routinely used the plasmid pJV53 [16]. With this plasmid, the RecET functions are induced with 0.2 % acetamide.
24. We do not add glycine to *M. smegmatis* cultures.

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Chapter 11

In Vitro Models That Utilize Hypoxia to Induce Non-replicating Persistence in Mycobacteria

Charles D. Sohaskey and Martin I. Voskuil

Abstract

The Wayne model and Rapid Anaerobic Dormancy model are widely used methods to analyze the response of *Mycobacterium tuberculosis* to hypoxia and anaerobiosis. In these models tubercle bacilli are grown in sealed tubes in which bacilli aerobic respiration produces a temporal oxygen gradient. The gradual depletion of oxygen results in a non-replicating persistent culture capable of extended microaerobic and anaerobic survival. Here we describe both models used to induce hypoxic non-replicating persistence in *M. tuberculosis*. Additional techniques such as the isolation of RNA, the detection of nitrate reductase activity and ATP levels, and the determination of the NAD^+/NADH ratio are described.

Key words *Mycobacterium tuberculosis*, Tuberculosis, Latent infection, Hypoxia, Persistence, Dormancy

1 Introduction

1.1 Background and History

Mycobacterium tuberculosis is classified as an obligate aerobe, and thus requires oxygen to replicate. Under conditions of low oxygen it enters a non-replicating persistent (NRP) state that has been proposed to correlate to the physiological state of *M. tuberculosis* confined in necrotic granulomas or during latent infection [1, 2]. Many models have been developed to study the response of *M. tuberculosis* to decreased oxygen tension. The most widely used, because of its ease of use and reproducibility, is called the Wayne model after its main developer Lawrence Wayne [3].

The Wayne model consists of growing *M. tuberculosis* in sealed tubes so that oxygen is slowly depleted creating a temporal gradient. In the initial aerobic stages of replication the cells utilize oxygen dissolved in the medium. This growth continues until the oxygen level in the medium decreases to approximately 1 % relative saturation (using air saturated medium as the reference) or 2 mmHg [3, 4].

As oxygen levels decrease there is a sudden reduction in the growth rate of *M. tuberculosis*. When measured by optical density

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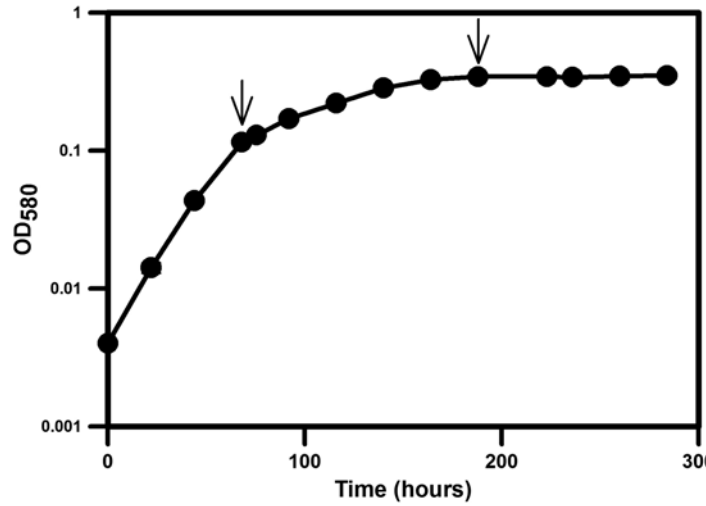

Fig. 1 Optical density growth curve of *M. tuberculosis* in the Wayne model. The shiftdown into microaerobic NRP-1 and anaerobic NRP-2 are indicated by *arrows*

cultures show a distinct shiftdown indicated by a deflection of the growth curve (Fig. 1). This microaerobic stage is termed non-replicating persistent stage 1 (NRP-1). NRP-1 is marked by induction of the DosR regulon (which also responds to other conditions that inhibit aerobic respiration), which is required for hypoxic survival [5]. Respiratory nitrate reductase activity increases due to increased expression of the nitrate transporter NarK2 [6, 7]. There is little change in ATP level or viability [3], but as cells attempt to maintain the redox balance there is an increase in NADH and decrease in NAD [4, 8, 9].

Oxygen levels continue to decrease due to cell respiration until at approximately 200 h they drop below 0.06 % saturation (0.28 mmHg) initiating non-replicating persistent stage 2 (NRP-2) [3, 4]. All replication has ceased by this time and cultures are in a dormant or non-replicating persistent state. There is an increased tolerance to many antibiotics due to decreased metabolism and replication, but increased sensitivity to metronidazole.

All that is required for growth and recovery after shiftdown to a NRP state is the introduction of air to the culture, indicating there is no limitation of nutrients or buildup of toxic products. When growth is initiated the cells will show synchronized replication for several generations [10].

A modification of the Wayne model called the Rapid Anaerobic Dormancy (RAD) model is also commonly used [8]. In this model, larger stir bars and an increased stir rate are employed resulting in a more rapid onset of NRP-1 without measurable bacterial death until after 1 month in NRP-2. An advantage of the larger stir bars and rapid stirring speed is the ability to maintain reproducible stir bar behavior that translates into consistent culture dynamics.

When comparing mutants or culture conditions that have different aerobic and hypoxic growth rates larger inoculums and less head space are also used in modified RAD models to shorten the aerobic and hypoxic growth phases. The shorter aerobic phase limits differences in cell number during models; however, the shorter NRP-1 phase may decrease long-term viability of the bacilli.

This chapter describes Wayne and RAD model methods. Techniques that are often used or those that must be modified for use with these models are also included.

2 Materials

2.1 Growth of Bacilli in the Wayne Model

1. Dubos Medium Albumin stock: dissolve 25 g of bovine serum albumin fraction V in 1 L of water. Add 37.5 g dextrose and 8.5 g of NaCl. Filter-sterilize through a 0.22 μm filter. Store at 4 °C until use.
2. Dubos Tween Albumin broth (DTA) (*see* **Note 1**). Prepare DT base medium in 180 mL batches and autoclave. Add 20 mL of Dubos Medium Albumin before use. The medium should be stored at 4 °C.
3. Screw-capped cultures tubes: 20 mm diameter by 125 mm tall.
4. Caps (*see* **Note 2**).
5. Conical screw-capped half liter flasks with 20 mm side-arms and flat (non-fluted) bases with an actual capacity of 600 mL.
6. 10 mg/mL methylene blue solution.
7. Teflon coated magnetic stir bars—8 × 1.5 mm for tubes; 8 × 50 mm for flasks.
8. Parafilm.
9. Magnetic stirrer plate (*see* **Note 3**).
10. Spectrophotometer (*see* **Note 4**).

2.2 Growth of Bacilli in the Rapid Anaerobic Dormancy (RAD) Model

1. Dubos Tween Albumin broth (DTA). *See* Subheading 2.1, **item 2**.
2. 20 mm by 125 mm glass tubes.
3. Phenolic PTFE-lined screw caps.
4. Stopcock grease.
5. Teflon coated magnetic stir bars, 12 m × 4.5 mm.
6. Magnetic tumbler stirrer.

2.3 Shift-Up from NRP State

1. Dubos Tween Albumin broth (DTA). *See* Subheading 2.1, **item 2**.
2. 20 mm by 125 mm glass tubes.

2.4 Determination of Colony Forming Units (CFU)

1. DTA base medium.
2. DTA agar: add 3.2 g of agar to 180 mL of DT base medium, autoclave, cool, add Dubos Medium Albumin and pour plates (*see* **Note 5**).
3. Teflon coated magnetic stir bars, 8 × 1.5 mm.
4. Magnetic stir plate.
5. Low-power dissecting microscope.

2.5 Determination of the Most Probable Number (MPN)

1. Dubos Tween Albumin broth (DTA). *See* Subheading 2.1, **item 2**.
2. 24-well plates.
3. Parafilm.
4. Low-power dissecting microscope.

2.6 Disruption of Cells for the Production of Cell Extracts

1. PBS–Tween: 0.05 % Tween 80 in PBS.
2. Bead beater.
3. Bead beater tubes.
4. 0.1 mm diameter zirconia/silica beads.
5. 22 μm pore-sized, low protein-binding membrane filters.

2.7 RNA Isolation

1. Guanidinium thiocyanate-phenol solution such as TRIzol or TRI Reagent.
2. 0.1 mm diameter zirconia/silica beads.
3. Bead beater.
4. Bead beater tubes.
5. Heavy Phase Lock Gel.
6. Chloroform.
7. Isopropanol.
8. DNAase.
9. RNA purification kit.

2.8 Measurement of Nitrate Reduction and Nitrite Production

1. 0.1 M HCl.
2. 0.2 % w/v sulfanilamide stock.
3. 0.1 % w/v *N*-(1 naphthyl)-ethylene diamine dihydrochloride.

2.9 Determination of Adenosine Triphosphate (ATP) Levels

1. HEPES buffer pH 7.75: 25 mM *N*-(2-hydroxyethyl) piperazine-*N*′(2-ethanesulfonic acid).
2. 25 mM HEPES buffer with 0.02 % w/v Tween 80 pH 7.75.
3. Chloroform.
4. Luciferin–luciferase reagent.
5. 13 × 100 mm glass culture tubes.

6. Heating block with holes for culture tubes.
7. Luminometer.

2.10 Determination of the NADH and NAD Ratio

1. 0.2 M HCl.
2. 0.2 M NaOH.
3. 0.2 M HCl with 20 mM 2-(Bis(2-hydroxyethyl)amino)acetic acid (Bicine).
4. 0.2 M NaOH with 20 mM 2-(Bis(2-hydroxyethyl)amino)acetic acid (Bicine).
5. Reaction buffer: 167 mM Bicine pH 8.0, 17 % w/v ethanol, 7 mM EDTA, 0.7 mM thiazolyl blue tetrazolium bromide, and 5.6 mM phenazine ethosulfate.
6. Alcohol dehydrogenase: 5 units/mL in 0.1 M Bicine buffer pH 8.0.
7. Microplate reader.

3 Methods

3.1 Growth of Bacilli in the Wayne Model

The Wayne model utilizes a head space ratio (HSR) of medium to head space air of 0.5 [3]. It can be used with either tube cultures for small volumes, or larger flask cultures (*see* **Note 6**). Because NRP cultures stop replicating at approximately 6.6×10^7 CFU/mL it is difficult to culture large volumes without a large number of tubes. In this case flask cultures can be used. *M. tuberculosis* (*see* **Note 7**) growth is monitored by measuring optical density in a spectrophotometer (*see* **Note 4**).

1. Add 17 mL of DTA medium to a 20 mm screw-capped culture tube with an 8 mm stir bar. This results in a ratio of 17 mL of medium to 8.5 mL of air (HSR 0.5). For flask cultures add 400 mL of DTA medium to a side arm flask with a 50 mm stir bar. The total volume of the flask is 600 mL including the side arm.
2. Inoculate each tube or flask with a mid-log phase culture of *M. tuberculosis* to an initial cell density of 1.1×10^6 CFU/mL (OD_{580} of 0.004) (*see* **Note 4**). Alternatively large cultures can be set up in, and then dispensed as 17 mL per tube.
3. To monitor oxygen levels methylene blue at 10 μg/mL can be added to one tube (*see* **Note 8**).
4. Seal tubes tightly and wrap with Parafilm (*see* **Note 2**).
5. Place tubes in a test tube rack on the magnetic stirrer at 120 rpm and set in a 37 °C incubator (*see* **Note 9**). For flasks 70 rpm are used.

6. Monitor growth daily by reading the optical density. To monitor growth in flasks, the culture is very gently tipped to fill the side arm. It is important to minimize the disruption of the surface (*see* **Note 10**).
7. Monitor the optical density daily to ensure a good transition into NRP-1.
8. After approximately 67 h of incubation there should be a clear deflection seen in the growth curve as the culture enters NRP-1 (Fig. 1). This shiftdown should be abrupt rather than the slow entry into stationary phase that is seen in high density cultures. If a clear transition to NRP-1 is not seen it is best to dispose of the culture and start over.
9. The transition to NRP-2 is less obvious and occurs after approximately 200 h.

3.2 Growth of Bacilli in the Rapid Anaerobic Dormancy (RAD) Model

1. Add 17 mL of DTA medium to 20 mm by 125 mm glass tubes with a 12 mm by 4.5 mm stir bar. Alternatively, add 10 mL of DTA to 16 mm by 125 mm glass Hungate tubes containing 12 mm by 4.5 mm stir bars and sealed with caps and butyl rubber septa. To shorten aerobic/hypoxic phases, tubes can be filled to 1 mL head space in modified RAD models [11].
2. Inoculate each tube with a mid-log phase culture of *M. tuberculosis* to an initial OD_{580} of 0.004.
3. Seal with phenolic PTFE-lined screw caps and stopcock grease applied to tube threads to provide an oxygen impermeable seal.
4. Place tubes in a test tube rack in a 37 °C incubator on a magnetic stirrer set at 200 rpm.

3.3 Shift-Up from NRP State (See Note 11)

1. Gently open NRP-2 tubes being very careful to avoid creating an aerosol when removing rubber sleeves.
2. From one NRP tube transfer 5 mL of culture to 3–20 mm × 125 mm glass tubes each containing 5 mL of fresh DTA. Vortex to disperse small clumps of cells and to aerate the medium.
3. Grow with shaking at 37 °C.
4. To detect synchronous replication CFUs should be determined at approximately 12 h intervals.

3.4 Determination of Colony Forming Units (CFU)

1. Aseptically dispense 900 μL of DT base into 2 mL short form screw-capped vials with an 8 × 1.5 mm stir bar (*see* **Note 12**).
2. Vortex the culture to be used to disperse the cells. Carefully open the tube.
3. Dilute 100 μL of culture into the first tube of DT base. Place on a magnetic stir plate and mix vigorously (*see* **Note 12**).

4. Pipette serial tenfold dilutions.
5. Spot 5 μL of six different dilutions along four rows (a grid under the Petri dish helps with placement).
6. Incubate at 37 °C and count colonies twice weekly using a low-power dissecting microscope until the number of colonies stabilizes (*see* **Note 5**). Spots with 5–30 colonies should be used for calculations.

3.5 Determination of the Most Probable Number (MPN) (See Note 13)

1. Dilute 100 μL of NRP-1 or -2 cultures into 900 μL fresh DTA medium in 24-well plates.
2. Perform a tenfold serial dilution out to the 10^{-10} dilution in the 24-well plates.
3. Seal plates with Parafilm and place in the incubator at 37 °C (*see* **Note 2**).
4. Check plates twice weekly for growth using a low-power dissecting microscope until the number of wells with growth stabilizes.
5. The most probable number (MPN) is determined by multiplying the lowest dilution possessing visible growth by the dilution factor.

3.6 Disruption of Cells for the Production of Cell Extracts

Cells can be disrupted to isolate proteins for enzyme assays [12]. It is important to wash the cells to remove the high level of albumin present in the medium since this protein will interfere with determining the actual cellular protein levels.

1. Place cultures on ice to chill quickly.
2. Centrifuge cultures at 4 °C at 1,200 × *g* for 20 min.
3. Carefully pour off the supernatant without disturbing the cell pellet. Centrifuge for an additional 5 min to pool residual fluid to the bottom of the tube. Carefully remove with a pipette.
4. Resuspend the bacilli in 10 mL of ice-cold PBS–Tween and vortex.
5. Harvest cells (as in **steps 2/3**) to remove all of the supernatant.
6. Resuspend cells in 750 μL of PBS–Tween and transfer to a bead beating tube.
7. Wash the NRP tube with an additional 750 μL of buffer and pool with the original sample.
8. Harvest cells at maximum speed in a microcentrifuge to remove all traces of buffer. The cells can be frozen at −70 °C until ready for use.
9. Thaw cells and add 0.1 mm zirconia/silica beads to fill approximately 1/3 of each tube. Fill the tube to the top with ice-cold PBS (*see* **Note 14**).

10. Place each tube in a beat beater and beat four times for 30 s each. In between cycles place the tubes on ice to prevent overheating.
11. Spin at 10,000 × *g* for 5 min to pellet cell debris. Carefully transfer the supernatant to a new tube. Filter-sterilize through a 22 μm pore-sized low protein-binding membrane filter. The protein concentration can be determined.

3.7 RNA Isolation (See Note 15)

1. Pellet cells by centrifugation at room temperature for 4 min at maximum speed (*see* **Note 16**).
2. Pour off supernatant and immediately freeze on crushed dry ice. Store cell pellets at –80 °C for up to 1 month.
3. Add 1 mL TRIzol to each frozen cell pellet and suspend by vortexing while pellet thaws.
4. Add suspension to 0.4 mL of 0.1 mm glass beads in an RNase-free 2 mL screw-cap tube.
5. Beat in a bead beater 3× for 1 min. Place on ice for at least 1 min between each beating cycle.
6. Centrifuge in a microcentrifuge for 45 s at maximum speed and remove TRIzol solution to a new 2 mL screw-cap tube containing 200 μL Heavy Phase Lock Gel and 300 μL chloroform.
7. Invert rapidly for 15 s, and continue inverting periodically for 2 min.
8. Centrifuge for 5 min, remove aqueous layer (540 μL) and transfer to a 1.5 mL tube containing 540 μL isopropanol. Mix by inverting several times.
9. Precipitate for a minimum of 20 min at 4 °C. Pellet in a microcentrifuge at maximum speed for 10 min at 4 °C and remove supernatant. Add 1 mL 75 % ethanol, invert, and centrifuge in a microcentrifuge for 5 min.
10. Remove ethanol by aspiration and dry under vacuum for 2 min. Do not overdry.
11. Resuspend RNA in 90 μL RNase free water.
12. Add 10 μL 10× DNaseI buffer to RNA (use no more than 80 μg total RNA) and add 4 μL DNaseI. Incubate for 30 min at 37 °C.
13. Follow kit instructions for RNA cleanup and elute RNA with 40 μL RNase free water (*see* **Note 17**).

3.8 Measurement of Nitrate Reduction and Nitrite Production

1. Dispense 3.6–3.8 mL of 0.1 HCl into 13 × 100 mm screw-cap test tubes and add 100 μL of 0.2 % sulfanilamide stock.
2. Add 10–200 μL of bacterial suspension (*see* **Note 18**). As a guide, 200 μL of culture is used for aerobic cultures, and 20 μL for NRP-1 and NRP-2. The total volume should be 3.9 mL.

3. Read and record the absorbance at 530 nm to establish a correction for samples from dense cultures.
4. Add 100 μL of 0.1 % naphthylethylenediamine dihydrochloride. Vortex and incubate at room temperature for 15 min.
5. Read and record A_{530}. Subtract the initial reading.
6. Prepare a standard curve for comparison to determine the nitrite concentration. Calculate the concentration from this curve (*see* **Note 19**).

3.9 Determination of Adenosine Triphosphate (ATP) Levels

This bioluminescent assay uses the ATP-dependent reaction of firefly luciferase and its substrate luciferin to measure ATP levels.

1. Transfer 1 mL of culture to a 1.5 mL microfuge tube and pellet by centrifuging at 10,000 × *g* for 5 min. Remove the supernatant and spin again to remove trace amounts.
2. Resuspend cells in 1 mL of HEPES buffer with Tween 80.
3. Dispense aliquots of up to 100 μL to 13 × 100 mm glass tubes and add HEPES buffer to bring the final volume to 100 μL.
4. Add 30 μL of chloroform directly to the cell suspension.
5. Place uncapped tube in an 80 °C heating block located in a vented fume hood. The air should flow across the tube. Incubate for 20 min.
6. Cool to room temperature, add 4.9 mL of HEPES buffer and vortex.
7. Mix 100 μL of this sample and 50 μL of HEPES buffer in a luminometer tube and place in the luminometer.
8. Add 50 μL of luciferin–luciferase reagent and record the light unit readings with a luminometer.
9. Prepare a standard curve to determine actual ATP levels.

3.10 Determination of the NADH and NAD Ratio

NAD and NADH levels can change very rapidly so harvesting of cells must be done rapidly.

The NAD and NADH levels are measured separately in a nucleotide cycling assay.

1. Rapidly harvest cells by centrifuging 2 mL of culture for 2 min at 10,000 × *g*.
2. Resuspend in 200 μL of 0.2 M HCl (for NAD) or 0.2 M NaOH (for NADH).
3. Heat at 80 °C for 20 min.
4. Cool and carefully neutralize with bicine-buffered 0.2 M HCl or 0.2 M NaOH (*see* **Note 20**).
5. Spin for 5 min at 10,000 × *g* to pellet cell debris and transfer the solution to a new tube.

6. Add 100 μL of reaction buffer to the wells of a 96-well plate.
7. Add 90 μL of either NAD or NADH sample to each well.
8. Start the reaction by adding 10 μL of alcohol dehydrogenase (5 units total).
9. Read the absorbance at 550 nm at 30 s intervals for 10 min.
10. The ratio of NAD to NADH can be calculated from this data. However, to determine actual concentration a standard curve must be made.

4 Notes

1. The Wayne model was originally developed with DTA although other media have been used with excellent results. Glycerol should be avoided because it greatly accelerates the depletion of oxygen which may alter results.
2. For cultures that will be incubated only into NRP-1 (up to 200 h) the standard phenolic caps with paper or PTFE liners together with stopcock grease are adequate. For longer incubation periods, or if reagents are to be injected into the tubes, tightly fitting rubber sleeve stoppers are used. With screw caps, even those sealed with Paraplast, the growth curves are not as reproducible with extended incubations. The reason for this is unknown but may be due to air leakage. Natural rubber is semipermeable to oxygen so if complete anaerobiosis is needed Hungate tubes with butyl rubber septa can be used. It can be difficult to safely insert the rubber stoppers into the tubes with cultures of *M. tuberculosis* and must be done carefully. We place each stopper on a small cap or rubber cork, before autoclaving them. The stopper can then be manipulated by holding onto the cap or cork without contaminating the culture or gloves.
3. Many versions are available and acceptable. They should be able to stir at 120 rpm without generating heat.
4. Spectrophotometers can vary greatly, and therefore, the cell density to optical density relationship must be determined for each brand. The Biochrom Libra S12 is a good spectrophotometer for growth analysis. The range of wavelengths used by different labs is 580–600 nm. With the Libra S12 an OD_{580} of 0.100 corresponds to an approximate cell count of 6.6×10^7 CFU/mL for *M. tuberculosis*.
5. DTA agar contains Tween 80 which produces smaller, softer colonies than on medium lacking this detergent. Because of the extended period of time that mycobacterial cultures are incubated plates are usually poured thicker than for other bacteria. It is also important to use an incubator with high

humidity so that plates do not dry out. Alternatively, plates can be incubated in sealed ziplock plastic bags.

6. Increasing the HSR by decreasing the volume of medium results in a less clear separation between the NRP stages. Decreasing the HSR by increasing the volume of medium produces a shorter NRP-1, and a more rapid transition to anaerobiosis resulting in decreased persistence. The amount of oxygen rather than the volume of air is important. At higher altitude adjustments can be made. For example in Denver due to decreased oxygen levels the HSR is increased to 0.65 [8].
7. The best characterized strain of *M. tuberculosis* is H37Rv, and it was with this strain that the Wayne model was developed [3]. However, the model works equally well with other strains [5]. It has also been used with other species of mycobacteria such as *Mycobacterium bovis*, both virulent and the vaccine strains of BCG [13–15], and *Mycobacterium smegmatis* [16].
8. The oxygen level in Wayne models decreases in a predictable and reproducible way [3]. Methylene blue is a commonly used redox indicator that is deep blue in the presence of oxygen but colorless during anaerobiosis. As a redox-responsive reagent methylene blue responds to the overall redox status of the culture and does not directly measure oxygen. However, it has been shown to closely mirror oxygen levels in NRP models with total loss of color upon essentially anaerobic conditions [8]. The blue color will interfere with optical density measurements taken to monitor cell growth, although the drop in absorbance can be used to determine the relative rate of oxygen consumption, as the contribution of methylene blue to the rate of absorbance change is much greater than the contribution from growing cells especially when the cultures approach anaerobiosis. As a redox active reagent methylene blue may affect the metabolism of *M. tuberculosis* although it does not appear to be generally toxic to mycobacteria as it is for many other bacteria.
9. The stir rate is set at a high enough speed to maintain the dispersion of the cells and to prevent settling, but low enough that there is no agitation of the medium surface. This results in the slow diffusion of oxygen into the medium.
10. Because of the larger air-liquid interface in flasks as compared to tubes a slower rotation speed must be used to avoid agitating the culture surface. Flasks are not sealed as tightly as tubes, and since disruption must occur when measuring growth the cultures tend to increase the optical density for a longer period of time. The shiftdown to NRP-1 is clear but the optical density may continue to increase and lengthen the NRP-1 phase.

11. After the introduction of oxygen cultures will begin to grow. If the cells are in NRP-1 they will begin to replicate with only a short or non-existent lag phase. However, if they have been in NRP-2 for extended time there will be a delay in the resumption of growth and synchronous replication will occur [10].
12. DT base is used because it contains Tween which prevents *M. tuberculosis* from clumping but does not have albumin, which reduces the expense. Phosphate buffer with Tween 80 may also be used. A low energy sonicating cleaning bath can be used to break up clumps. We have found that mixing rather than vortexing produces more accurate and reproducible colony counts possibly because cells tend to stick to glass surfaces rather than stay in suspension.
13. Liquid recovery can be used for better enumeration of viable but difficult to culture bacilli especially from NRP-2 [8]. The lower oxygen concentration in aqueous solutions compared to air likely allows for better recovery, especially of mutants, after extended anaerobiosis during NRP-2.
14. This can be PBS, PBS with DTT, or some other buffer. For enzyme assays a protease inhibitor cocktail can be used.
15. RNA levels can fluctuate rapidly and can change significantly during harvest which must be avoided. Pelleting cells packs them close together which reduces oxygen availability and can induce hypoxia-responsive genes. Chilling cells beforehand as is done with enzyme preparations will induce cold shock genes.
16. An alternative method is to combine 2–3 tubes in a 50 mL tube and pellet rapidly by centrifugation for 4 min. Use a large stir bar on outside of glass tubes to prevent stir bars from falling into centrifuge tube. RNA preservation solutions can be used to prevent any mRNA changes during centrifugation or tubes can be combined in an anaerobic glove box.
17. The RNA purification may not be necessary for all applications although it provides more consistent results and is less time consuming. The RNA column will remove small RNAs and degraded RNA. DNaseI should be inactivated at 65–70 °C for 15 min and the RNA ethanol precipitated and 75 % ethanol washed. If upon agarose gel analysis DNA contamination is observed, use a second TRIzol or RNeasy extraction.
18. In NRP cultures the tube must be opened and then discarded although samples can be taken through rubber sleeves. Aerobic cultures have weak activity while NRP ones have strong activity [6, 7]. The presence of nitrate does not alter the growth and shiftdown curve.
19. This assay measures the production of nitrite ion from nitrate ion present in culture. DETA/NO (Diethylenetriamine/nitric oxide

adduct [2,2′-(Hydroxynitrosohydrazono)bis-ethanimine]) often used as a source of nitric oxide in experiments is converted to nitrite by the acidic conditions of the assay. NADPH, azide, dithiothreitol, β-mercaptoethanol and phosphate at concentrations above 50 mM can interfere with his assay.

20. It is important to make sure the pH does not go beyond 7.0 during the neutralization step since preparation is based on the selective destruction of either NAD or NADH by pH [17].

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Chapter 12

Genetic Dissection of Mycobacterial Biofilms

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Abstract

Our understanding of the biological principles of mycobacterial tolerance to antibiotics is crucial for developing shorter anti-tuberculosis regimens. Various in vitro approaches have been developed to identify the conditions that promote mycobacterial persistence against antibiotics. In our laboratories, we have developed a detergent-free in vitro growth model, in which mycobacteria spontaneously grow at the air–medium interface as self-organized multicellular structures, called biofilms. Mycobacterial biofilms harbor a subpopulation of drug tolerant persisters at a greater frequency than their planktonic counterpart. Importantly, development of these structures is genetically programmed, and defective biofilms of isogenic mutants harbor fewer persisters. Thus, genetic analysis of mycobacterial biofilms in vitro could potentially be a powerful tool to unravel the biology of drug tolerance in mycobacteria. In this chapter we describe a method for screening biofilm-defective mutants of mycobacteria in a 96-well format, which readily yields a clonally pure mutant for further studies.

Key words Biofilm, Genetic mutants, Mycobacteria, Drug tolerance

1 Introduction

Many, if not most, microbes grow as biofilms—self-organized multicellular structures encapsulated within a matrix of extracellular polymeric substance. There are numerous diverse forms, ranging from microcolonies on submerged substrata to pellicles at air–medium interfaces [1–4]. Biofilms develop through genetically programmed pathways and harbor phenotypically heterogeneous but genetically clonal populations of constituent cells [5–16]. Microbial biofilms display a variety of behaviors that are not associated with dispersed planktonic growth, including tolerance to environmental and immunological stresses and antibiotic resistance. Biofilms represent an important persistence strategy of pathogenic microbes in chronic infections [17–19].

Mycobacterium tuberculosis is the causative agent of human tuberculosis, although most (>90–95 %) infections of immunocompetent individuals do not lead to active disease. Instead, a latent infection is established which can subsequently activate into

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disease when the immune system is compromised [20–22]. Treatment of active tuberculosis requires at least 6 months with multiple antibiotics [23], and drug resistance is an emerging problem in controlling the disease. Together, these characteristics of *M. tuberculosis* infections underscore the extraordinary ability of the pathogen to persist in the face of chemical and immunological challenges. Although it is unclear as to how and where in the host the persistent *M. tuberculosis* bacilli survive against the host-derived stresses and antibiotics, it is plausible that the mechanisms underlying in vivo persistence overlap with the intrinsic stress tolerance displayed by the bacilli when grown in vitro.

In detergent-free liquid cultures in vitro, most mycobacterial species including *M. tuberculosis* form macroscopic structures leading to the development of pellicles at the air–medium interface [5–7, 16, 24]. Over the last several years it has become apparent that mycobacterial pellicles develop through distinct stages with specific genetic requirements, and that these pellicles harbor bacilli that are phenotypically tolerant to high concentrations of antibiotics [5, 7, 16, 24, 25]. Thus, these mycobacterial pellicles represent a genetically programmed developmental process, in common with the many other microbes that form similar biofilms. Further genetic studies of these developmental processes will likely contribute towards identifying novel targets against recalcitrant infections, and facilitate an improved understanding of host–pathogen interactions. These information can subsequently be exploited for drug discovery and vaccine design against TB.

In this chapter we describe detailed methods for growing and investigating mycobacterial biofilms. Broadly, the methods involve four steps; (1) Establishing a biofilm assay in 96-well format for high-throughput screening, (2) making a high-density transposon library of mycobacteria, (3) screening the library using the 96-well format of the biofilm assay, and (4) mapping the sites of transposon insertion in the biofilm-defective mutants. While biofilm assays and construction of transposon libraries of *M. smegmatis* and *M. tuberculosis* mutants have been independently published elsewhere [7, 16, 24, 26, 27], we integrate these methods in this chapter to provide a composite workflow for studying the genetics of mycobacterial biofilms.

2 Materials

2.1 Biofilm Assay in a 96-Well Format

1. A mycobacterial strain—either *Mycobacterium smegmatis* mc^{2}155, ATCC 700084; *Mycobacterium tuberculosis* H37Rv, ATCC # 25618; or mc^{2}7000 (*see* **Note 1**).
2. *10 % w/v* D*-pantothenate*: Dissolve 1 g of D-pantothenic acid hemicalcium salt in 9 mL of deionized water. Filter-sterilize with 0.22 μm filter, and store at room temperature (*see* **Note 2**).

3. *7H9ADCTw*: Dissolve 4.7 g of Middlebrook 7H9 base in 890 mL of deionized water. Add 5 mL of 100 % glycerol. Adjust the volume to 900 mL. Dispense 90 mL aliquots in bottles, autoclave, and cool to room temperature. To each 90 mL of aliquot, add 10 mL of ADC enrichment (albumen/dextrose/catalase supplement—Becton Dickinson), and 250 μL of 20 % v/v Tween 80 (final concentration of 0.05 % v/v) (*see* **Note 2**).
4. *7H10ADC*: Dissolve 19 g of Middlebrook 7H10 agar base in 890 mL of deionized water. Add 5 mL of 100 % glycerol, and adjust the volume to 900 mL with deionized water. Heat with stirring to dissolve the agar. Autoclave and cool to 50 °C. Add 100 mL of either 10× ADC enrichment, and pour 25 mL for each 85 mm petri dish. For 7H10ADCTw, add 2.5 mL of 20 % Tween 80 per liter of the media (*see* **Note 2**).
5. *Complete Sauton's medium:* Dissolve 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 4 g L-asparagine monohydrate, 2 g citric acid, 0.05 g ferric ammonium citrate to 900 mL deionized water. Add 60 mL glycerol. Adjust pH to 7.0 with 1 M NaOH. Autoclave and store at room temperature. Just before inoculating the cells, add 0.1 mL of sterile 1 % $ZnSO_4$ per liter of medium. If culturing planktonic cells, add 2.5 mL of 20 % Tween 80 per liter of medium (*see* **Note 2**).
6. *Complete biofilm medium*: Dissolve 13.6 g of KH_2PO_4 in 900 mL of water. Add 2 g of $(NH_4)_2SO_4$. Adjust pH to 7.2 with 10 M NaOH. Add 0.5 mg of $FeSO_4{\cdot}7H_2O$ and 5 g of casamino acids. Bring up the volume to 1,000 mL with water. Autoclave and store at room temperature. Just before inoculating the cells, aseptically add 5 mL of 40 % glucose, 1.0 mL of 0.1 M $CaCl_2$, and 0.1 mL of 1 M $MgSO_4$ to 94 mL of the sterile biofilm base medium.

2.2 Construction and Screening of Transposon Library

1. A mycobacterial strain (*see* Subheading 2.1, **item 1**).
2. A shuttle phasmid, phAE781, carrying the Himar-1 transposon packaged in the genome of a temperature-sensitive derivative of mycobacteriophage TM4 [28].
3. Growth media as described in Subheading 2.1, **items 2–6**.
4. *MBTA*: Add 4.7 g 7H9 base and 7 g Bacto agar to 900 mL of deionized water. Heat to dissolve the agar. Dispense 100 mL in bottles, autoclave and store at room temperature.

2.3 Mapping Transposon Insertion Sites in Biofilm-Defective Mutants

1. Taq DNA polymerase.
2. 10× buffer for Taq DNA polymerase (with $MgCl_2$).
3. 10 mM dNTP mix.
4. Dimethyl sulfoxide (DMSO).
5. Primer 1: GGCCAGCGAGCTAACGAGACNNNNGTTGC.

6. Primer 2: CGCTTCCTCGTGCTTTACGGTATCG.
7. Primer 3: GGCCAGCGAGCTAACGAGAC.
8. Primer 4: GGCCAGCGAGCTAACGAGAC.

3 Methods

3.1 Biofilm Assay in a 96-Well Format

1. Inoculate a frozen stock of desired mycobacterial strain in 10 mL of 7H9ADCTw (*see* **Notes 2–4**).
2. Incubate the cultures at 37 °C till the OD_{600} reaches ~1.0 for *M. smegmatis* (*see* **Note 5**).
3. Inoculate 20 mL of biofilm media with 20 μL of *M. smegmatis* culture obtained from **step 2** (*see* **Note 6**).
4. Dispense 200 μL of the bacterial suspension from **step 3** in each well of a 96-well plate, and incubate the plates in humidified conditions at 30 °C for *M. smegmatis* (*see* **Note 7**).
5. A film of bacteria is visible for *M. smegmatis* cultures after 3 days of incubation that matures to robust and textured pellicles in 4–5 days (*see* **Note 8**).

3.2 Construction and Screening of Transposon Library

1. Inoculate a frozen stock of *M. smegmatis*, mc^2155, in 100 mL 7H9ADCTw.
2. Incubate the cells at 37 °C on a shaker incubator till the OD_{600} is about 0.8–1.0.
3. Mix 0.3 mL of cells, 100–1,000 plaque forming units (PFU) of phAE781 and 3 mL of MBTA pre-warmed to 42 °C. Vortex the mixture briefly and pour it evenly on an 85 mm petri dish containing 7H10ADC agar. Repeat this for at least 15–20 plates (*see* **Notes 9** and **10**).
4. Incubate the plates at 30 °C for 2–3 days, until a confluence of plaques is seen (*see* **Note 11**).
5. Overlay each of the plates with 4 mL of phage buffer and store the plates at 4 °C for a minimum of 4 h, or a maximum of overnight.
6. Pool the liquid with phages (phAE781) from the plates. Remove bacterial contaminants by filtering the lysate through 0.22 μm membrane filter. The phage stock can be kept at 4 °C up to 1 month without significant loss of viability.
7. Titer the phage stock by spotting tenfold serial dilutions on a lawn of *M. smegmatis* on 7H10ADC agar at 30 °C. A good stock will have greater than 10^{10} PFU/mL.
8. Ensure the thermal sensitivity of the phages by spotting tenfold serial dilutions on a lawn of *M. smegmatis* and incubating the plate at 37 °C for 1–2 days. The number of plaques should be reduced by several orders of magnitude.

9. Inoculate a frozen stock of *M. smegmatis* into 100 mL of 7H9ADCTw.
10. Incubate at 37 °C till the OD_{600} reaches ~1.0 (~6×10^8 cfu/mL).
11. Centrifuge the cells (3,000 × *g* at room temperature for 10 min), and resuspend the pellet in 100 mL 7H9 base (no Tween 80).
12. Incubate at 37 °C for 3 h to wash off residual Tween 80 from the bacterial surface (*see* **Note 12**).
13. Centrifuge the cells (3,000 × *g* at room temperature for 10 min) and resuspend the pellet in 0.1 volume (10 mL) of 7H9 base (no Tween 80) pre-warmed at 37 °C.
14. Add phAE781 at a multiplicity of infection (MOI) of 10. For example, 1 mL of ~10^{11} PFU for ~10^{10} cfu in a suspension is optimum.
15. Incubate the mixture at 37 °C for 30 min (*see* **Note 13**).
16. Transfer the phage-bacteria mixture into a 250 mL sterile bottle containing 90 mL of pre-warmed (37 °C) 7H9ADCTw. Incubate the content at 37 °C for 3 h (*see* **Note 14**).
17. Centrifuge the cells (3,000 × *g* at room temperature) for 10 min, resuspend the pellet in 1 mL of 7H9Tw and plate the transductants on 7H10ADCTw with 40 μg/mL of kanamycin.
18. Incubate the plates till colonies appear (*see* **Note 15**).
19. Determine the quality of the library by identifying transposon insertion sites in 10–12 random colonies by method described in Subheading 3.3. About 30–50 % of the colonies with unique junction sequences are desirable in a well-represented library. If library is satisfactory, proceed with the screening steps described below (*see* **Note 16**).
20. Stack two sets of thirty 96-well plates marked numerically, such as stack $A_{1\text{-}30}$ and stack $B_{1\text{-}30}$. Using a multichannel pipette, dispense 200 μL of 7H9ADCTw with kanamycin in each well of the plates in stack A. Keep stack B plates aside for later use.
21. Inoculate single isolated colonies individually from *M. smegmatis* library (**step 15**) in each well of the plates in stack A.
22. Wrap the plates with Parafilm thoroughly and incubate them without shaking in humidified conditions at 37 °C for 2 days to obtain primary cultures of each clone. Dispersed growth should be observed after incubation. As controls, inoculate a wild-type strain and a known biofilm mutant in separate 96-well plates to obtain their primary cultures (*see* **Note 17**).
23. In plates $B_{1\text{-}30}$, dispense 200 μL of biofilm medium using a multichannel pipette (*see* **Note 6**).
24. Using multichannel pipette, inoculate about 5 μL of bacteria from the stack A plates into the exact same positions in stack

B plates. Keep the first stack secured at either 4 °C. As controls, inoculate 5 μL of wild-type or known biofilm-negative strains from primary cultures.

25. Wrap the stack B plates and place them at 37 °C incubator under humidified conditions (*see* **Note 18**).
26. Observe the plates after 2 days. A thin film of bacteria should be visible for wild-type *M. smegmatis* cultures (Figs. 1a and 2a). The negative controls (biofilm-defective mutants) should show little to no growth at the air–medium surface (Figs. 1b and 2b). The biofilms of transposon mutants can possibly range from normal to severe deficiencies. However, each mutant with altered phenotype must be tested in a secondary screen (**steps 25–27**) to rule out false positives (*see* **Notes 8**, **19** and **20**).
27. Identify mutant clones with altered biofilms from the primary screen in stack A plates, and streak out the clones on 7H10ADC

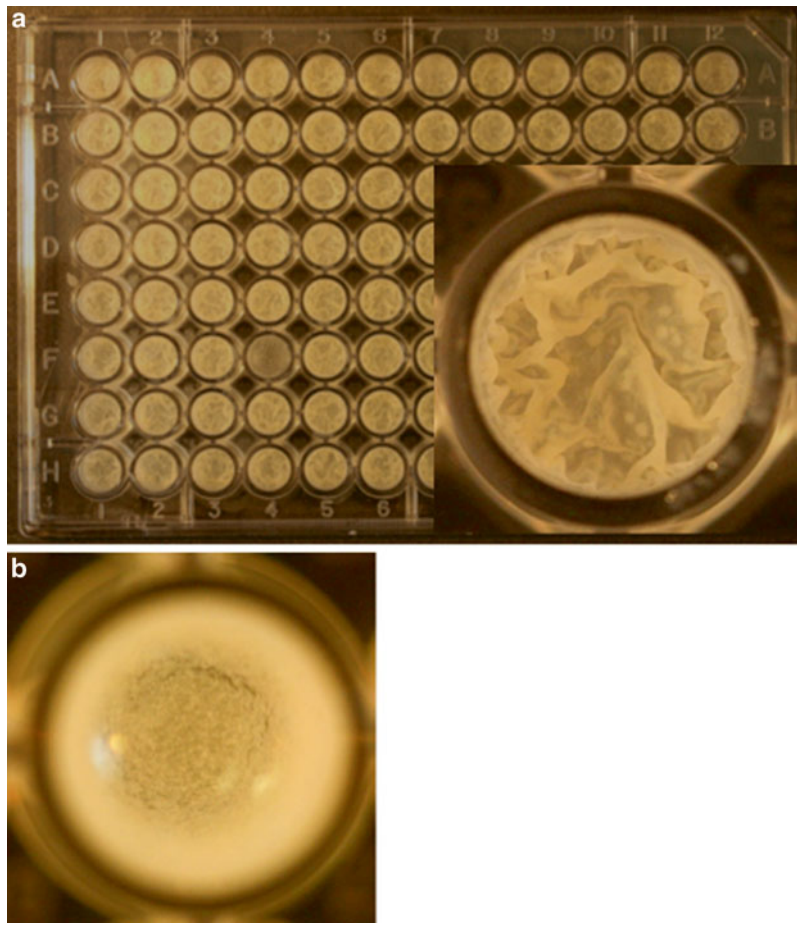

Fig. 1 (**a**) Growth of *M. smegmatis mc*^{2} 155 biofilms in a 96-well format after 5-days of Incubation at 30 °C. A top-down view of one of the typical wells is magnified in the *inset*, which shows the robust pellicles on the air–media interface. (**b**) A top-down view of mc^{2}155: *Δlsr-2* biofilms in a well of a 96-well plate

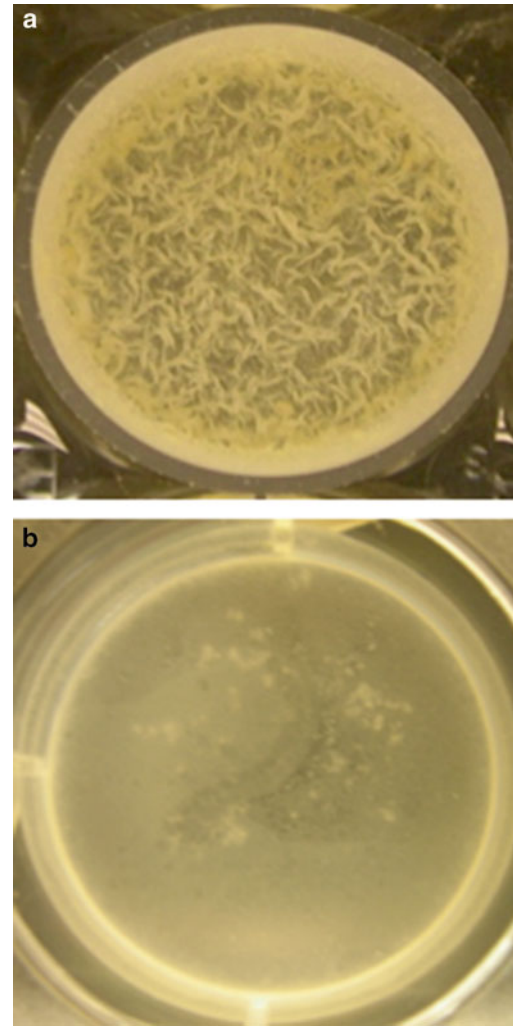

Fig. 2 A top-down view of biofilms of *M. tuberculosis* mc^{2}7000 (**a**), and mc^{2}7000: *ΔhelY* (**b**) on air–media interface in a well of a 96-well plate after 3-week incubation at 37 °C incubation

plates with 40 μg/mL of kanamycin. Incubate at 37 °C till colonies appear.

28. Inoculate a single colony of a mutant from the plates in a 50 mL polystyrene conical tube containing 5 mL 7H9ADCTw medium with 40 μg/mL of kanamycin, and culture the cells at 37 °C till saturation. As controls, inoculate wild-type strain and a known biofilm-deficient strain for the respective species.
29. For secondary screen of *M. smegmatis* mutants, inoculate 10 μL of cells in 10 mL of biofilm media in a 65 mm petri dish. Incubate at 37 °C and compare the biofilm development of the mutants with controls after 2 days (*see* **Notes 18** and **21**).

3.3 Mapping Transposon Insertion Sites in the Biofilm-Defective Mutants

1. For mutants with confirmed biofilm deficiency (Subheading 3.2), resuspend a loopful of cells from the streak (Subheading 3.2) in a microfuge tube containing 200 μL of sterile water.
2. Vortex the suspensions briefly (~30 s) and place in 95 °C heating block for 15 min (with occasional flicking every 5 min). Place them on ice.
3. Use 5 μL of the content from each tube to set up a two-step degenerate nested PCR reactions as following:

   First PCR

   Reaction contents:

   5 μL of 10× PCR buffer (with $MgCl_2$)

   5 μL of DNA

   1 μL of 10 mM dNTPs

   2.5 μL of DMSO

   1 unit of Taq DNA polymerase

   0.5 μL of 100 μM primer 1

   0.5 μL of 100 μM primer 2

   34.5 μL H_2O_2

   Reaction conditions: 95 °C for 5 min, 5 Cycles of (95 °C for 30 s, 30 °C for 30 s, 72 °C for 30 s), 25 cycles of (95 °C for 30 s, 38 °C for 30 s, 72 °C for 30 s), 72 °C for 7 min.

   Second PCR

   Reaction contents:

   5 μL of 10× PCR buffer (with $MgCl_2$)

   2 μL of the content from the first PCR

   1 μL of 10 mM dNTPs

   2.5 μL of DMSO

   1 unit of Taq DNA polymerase

   0.5 μL of 100 μM primer 3

   0.5 μL of 100 μM primer 4

   39.5 μL of H_2O_2

   Reaction conditions: 95 °C for 5 min, 30 Cycles of (95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s), 72 °C for 7 min.
4. Purify the PCR products from the second reaction and sequence using Primer 4.
5. A typical sequencing read will represent the transposon insertion site with a sequence reading, $_{\text{(transposon DNA)}}$CAGCCAACC TGTT/$A_{\text{(mycobacterial DNA)}}$

4 Notes

1. Experiments with *M. tuberculosis* H37Rv require a Biosafety Level 3 laboratory (BSL-3). Because BSL-3 facilities are highly restrictive and may not be readily accessible to investigators, an attenuated derivative of H37Rv, mc^{2}7000, was constructed. mc^{2}7000 contains deletions in the region of difference 1 (RD1) locus and *panCD* operons, which render the strain incapable of growth in many mammalian hosts [7]. As a result, the strain can be safely used in a Biosafety Level 3 (BSL-2) laboratory.
2. ADC enrichment containing oleic acid (OADC) is used for *M. tuberculosis* strains. For culturing mc^{2}7000, add 1 mL of 10 % D-pantothenate stock in 1 L of medium (final concentration of 100 μg/mL).
3. The protocol described here was developed for a high efficiency transformable strain of *M. smegmatis*, mc^{2}155 [29]. Some of the strains in the ATCC collection may be naturally deficient in biofilm formation and must be verified before initiating the screen.
4. The protocol for *M. tuberculosis* biofilms was developed using an attenuated strain, mc^{2}7000 [7]. Because *panCD* mutation confers pantothenate auxotrophy in mc^{2}7000, the strain requires pantothenate supplementation (100 μg/mL) in the medium. However, the protocol described in this chapter can be adopted for any strain of *M. tuberculosis*. All virulent strains of *M. tuberculosis* must be cultured under BSL-3 containment.
5. For *M. tuberculosis* use culture with $OD_{600} \sim 0.2–0.4$. It is important to use an early log phase culture of *M. tuberculosis* to achieve reproducibility in these biofilm assays. A late log phase or early stationary phase culture often has inconsistent growth patterns of biofilms.
6. For *M. tuberculosis*, inoculate 20 mL of Sauton's media with 0.2 mL of primary culture. While biofilms of *M. smegmatis* are cultured in a modified M63 media (called biofilm media), *M. tuberculosis* biofilms are best obtained in Sauton's media.
7. Incubate *M. tuberculosis* biofilms at 37 °C. Moreover, incubation under humidified conditions is particularly critical for growing *M. tuberculosis* biofilms in 96-well plates to avoid risk of liquid evaporation during the extended incubation at 37 °C. Keeping the plates undisturbed during incubation is important to avoid well-to-well contamination and to allow attachment of bacteria to the substrata.
8. For *M. tuberculosis*, it takes about 3 weeks to form biofilms, and an additional 2 weeks for full maturation (Figs. 1a and 2a).

A longer incubation period (4–5 weeks) is necessary for complete maturation of *M. tuberculosis* biofilms. However, in the 96-well format excessive bacterial outgrowth associated with mature biofilms increases the risk of well-to-well contamination. Therefore, an early (2–3 weeks) stage when a thin film is formed at the interface is best for mutant screening.

9. phAE781 is a recombinant TM4^{ts} carrying shuttle phasmid carrying Himar-1 transposon, packaged into a TM4^{ts} phage (phAE159). At permissive temperature (30 °C), the recombinant phage carrying transposable elements can replicate in *M. smegmatis*, while at restrictive temperature (37 °C) it delivers the transposon in mycobacteria, but does not replicate.
10. MBTA should be kept at around 42 °C to avoid loss of bacterial or phage viability, while keeping the top agar in liquid form.
11. For a high titer stock, a confluence of plaques with clearly distinct boundaries on a bacterial lawn is optimum. A totally clear plate due to excessive phage input produces low titer stocks.
12. Incubation time to wash off Tween 80 from *M. tuberculosis* is 16 h.
13. Incubation time for phage infection in *M. tuberculosis* is 3 h.
14. Outgrowth incubation time for *M. tuberculosis* is 16–18 h.
15. Often it is necessary to calibrate the plating volume for the transductants to achieve well-separated 200–300 colonies on an 85 mm petri dish. In a typical scenario, 10, 50, 100, and 200 μL of transductants are diluted with 7H9Tw to a final volume of 500 μL, and plated on separate plates. It takes about 2–3 days for *M. smegmatis*, and 3–4 weeks for *M. tuberculosis* transductants form colonies.
16. We have described methods for screening ~3,000 independent mutants, although the method can be scaled up.
17. Incubate *M. tuberculosis* at 37 °C for about 3 weeks.
18. *M. smegmatis* produces greater biomass when forming biofilms at 30 °C, and this temperature is usually preferred for studying *M. smegmatis* biofilms. However, 37 °C is preferred for screening transposon mutants of *M. smegmatis*, because residual transducing phages (phAE781) in the primary colonies can inhibit bacterial growth at 30 °C.
19. It is important to be mindful of the distinction between a growth-deficient mutant and a biofilm-deficient mutant. While the former grows slowly under all conditions, the latter will show deficiency only when grown under biofilm-specific conditions, without apparent growth defects in planktonic form. The slow-growing mutants are likely to appear as false positives

in the primary screen and therefore must be resolved in the secondary screen.

20. Occurrence of false positives in the 96-well assay is usually more frequent in *M. tuberculosis* than *M. smegmatis*. *M. tuberculosis* is quite fastidious and even slight variations in growth conditions across the wells, particularly with respect to headspace air, can significantly impact in the timing of biofilm formation for otherwise normal cells.
21. For secondary screening of *M. tuberculosis* mutants, inoculate 250 µL of cells in a 250 mL bottle containing 25 mL of Sauton's medium. Tighten the lid firmly and incubate undisturbed at 37 °C for 3 weeks, after which loosen the lids and follow the growth of the mutant biofilms for the next 2 weeks. Compare the biofilms with a wild-type control in a secondary screen.

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Chapter 13

Measuring Efflux and Permeability in Mycobacteria

Liliana Rodrigues, Miguel Viveiros, and José A. Aínsa

Abstract

The intrinsic resistance of mycobacteria to most antimicrobial agents is mainly attributed to the synergy between their relatively impermeable cell wall and efflux systems. The mycobacterial cell wall is rich in lipids and polysaccharides making a compact envelope that limits drug uptake. Changes in cell wall composition or structure lead to variations in susceptibility to drugs. Bacterial efflux pumps are membrane proteins that are capable of actively transporting a broad range of substrates, including drugs, from the cytoplasm to the extracellular environment. Increased expression of efflux pump genes confers a low level resistance phenotype, and under these conditions, bacteria may have greater chances of acquiring chromosomal mutation(s) conferring higher levels of drug resistance. In order to develop effective antimycobacterial therapeutic strategies, the contributions to drug resistance made by the limited permeability of the cell wall and the increased expression of efflux pumps must be understood. In this chapter, we describe a method that allows: (1) the quantification of general efflux activity of mycobacterial strains (clinical isolates, mutants impaired in efflux or permeability) by the study of the transport (influx and efflux) of fluorescent compounds, such as ethidium bromide; and (2) the screening of compounds in search of inhibitors of efflux pumps, which could restore the effectiveness of antimicrobials that are subject to efflux.

Key words *Mycobacterium*, Efflux pumps, Efflux inhibitors, Ethidium bromide, Fluorometry, Accumulation assay, Efflux assay

1 Introduction

Over the years, several methods have been used to measure efflux and permeability using radiolabeled, metal-labeled or fluorescent substrates in bacterial cells [1–5]. In particular, fluorometry allows the detection of the transport of fluorescent substrates through the bacterial cell wall, which can be correlated with altered efflux activity and/or permeability of the bacteria [6–8]. Mycobacteria, similarly to other bacterial genera, have a large number of active efflux pumps that have a role in low level resistance to drugs such as tetracycline, aminoglycosides, fluoroquinolones [9], tolerance to drugs such as isoniazid and rifampicin [10], and other physiological processes such as redox potential and bacterial and colony morphology [8, 11]. Therefore, efflux pumps must be taken into

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account in drug discovery programs [12–14] and their inhibition could contribute to new therapeutic strategies for tuberculosis and drug-resistant tuberculosis [15].

We have developed a fluorometric method for the assessment of efflux activity in mycobacteria that uses the common efflux substrate ethidium bromide (EtBr) [4, 6, 16, 17]. EtBr has been shown to be a particularly suitable probe for these studies since it emits weak fluorescence in aqueous solution (extracellular medium) and becomes strongly fluorescent as it accumulates in nonpolar and hydrophobic environments, such as the periplasmic space of gram-negative bacteria or cytoplasm of gram-positive bacteria [2]. This methodology allows easy and accurate detection and quantification of the transport of EtBr through the bacterial cell wall. Kinetics of EtBr accumulation and efflux can be assayed separately, hence dissecting the contribution of both processes to EtBr transport [4, 6, 7, 16].

This method allows the evaluation of accumulation or efflux activity in real-time of a large number of samples simultaneously, with the possibility of testing different experimental conditions with the same set of bacterial suspensions, hence maximizing reproducibility and significance. Moreover, it also allows the screening of efflux inhibitors that may be used in the future as adjuvants of antimycobacterial therapy [15].

The original method was developed with the use of a real-time PCR instrument (Rotor-Gene™ 3000, Corbett Research), and subsequently has been adapted for 96-well plate fluorometers. Both methods allow accurate real-time measurements of accumulation and efflux of EtBr [4, 6, 7, 16, 17].

The EtBr accumulation assay assesses the ability of the strain to confront increasing concentrations of EtBr. The concept behind this assay is that when the ability of the efflux systems to extrude EtBr is exceeded, EtBr will accumulate over a period of time, ultimately reaching a quasi steady-state. Figure 1 shows an example of this type of assay with *M. smegmatis mc²155* strain; accumulation of EtBr is relatively stable (no increase over time) at the concentration of 0.5 μg/mL, over a period of 60 min. In the presence of an efflux inhibitor, such as thioridazine (TZ), chlorpromazine (CPZ), or verapamil (VP), accumulation of EtBr increases due to the inhibition of efflux (Fig. 2).

In an efflux assay, (Fig. 3) bacterial cells are first de-energized and loaded with EtBr and then placed in EtBr-free solution. In the presence of a carbon source, such as glucose, efflux pumps are able to transport EtBr out and so fluorescence decreases quickly with time; in the absence of glucose, fluorescence decreases more slowly; in the presence of efflux inhibitors fluorescence decreases at an even lower rate.

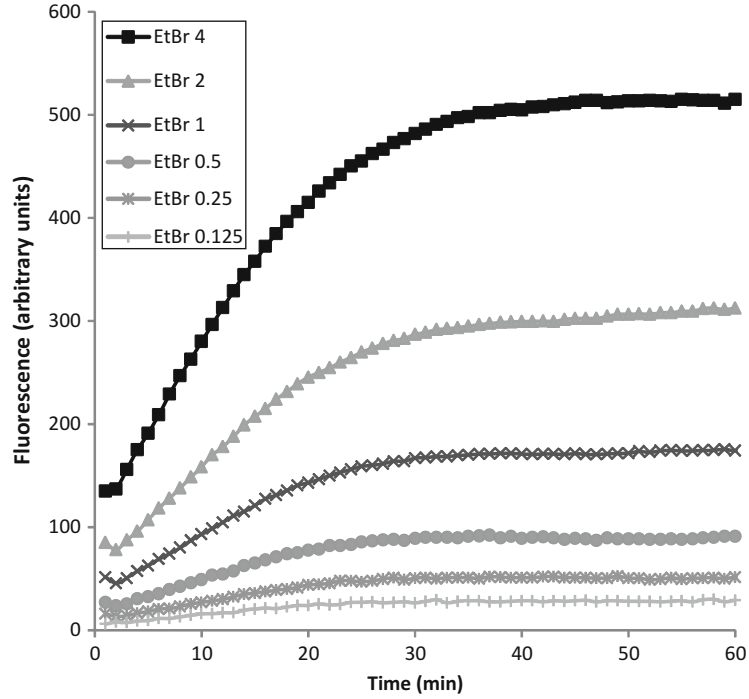

Fig. 1 Accumulation of increasing concentrations of EtBr (0.125–4 μg/mL) by *M. smegmatis* mc²155

Accumulation and efflux assays can be used to quantify and compare EtBr transport activity between mycobacterial strains (e.g., clinical isolates or mutants impaired in efflux or permeability). For example, Fig. 4 (adapted from Fig. 2 from ref. 17) presents accumulation of EtBr at 1 μg/mL in three *M. tuberculosis* strains: *M. tuberculosis* H37Rv (wild-type), H37RvMmrKO (a mutant of H37Rv with the *mmr* efflux pump gene inactivated), and H37Rv::pCVZ2 (an H37Rv derivative containing a plasmid overexpressing *mmr*). The H37RvMmrKO mutant showed an increased accumulation of EtBr relative to the wild-type strain, whereas the strain overexpressing *mmr* showed a decreased accumulation of EtBr. These results indicate that the Mmr efflux pump is associated with the efflux of EtBr in *M. tuberculosis* [17].

In summary, this methodology can be employed to evaluate influx and efflux of fluorescent substrates by mycobacteria (conditions should be optimized for each particular mycobacterial species and fluorescent substrate), making use of any fluorometric tube or plate reader and this information is of great value to predict the contribution of the efflux activity to the drug resistance phenotype.

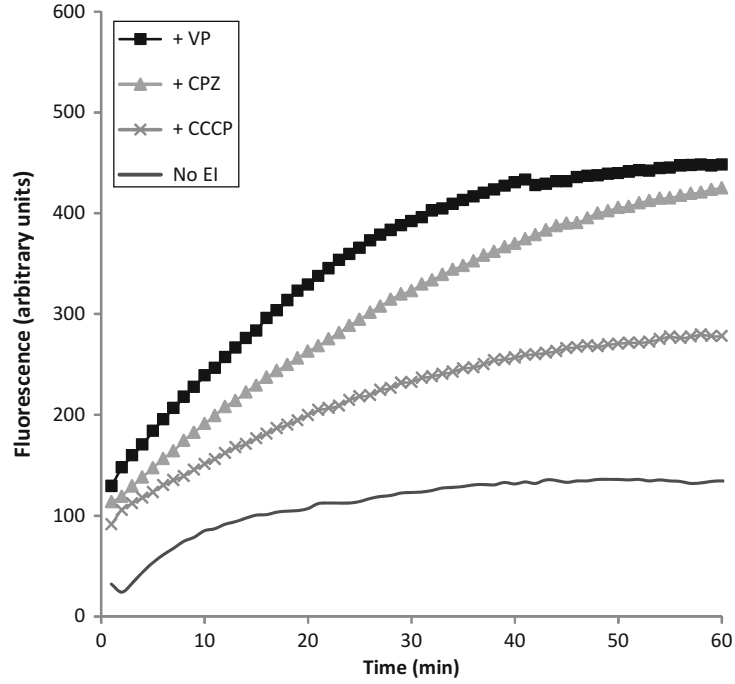

Fig. 2 Effect of efflux inhibitors on the accumulation of EtBr by *M. smegmatis* mc^2155. EtBr was used at of 0.5 μg/mL and efflux inhibitors at one-half the MIC to not compromise cellular viability. *CCCP* carbonyl cyanide m-chlorophenylhydrazone, *CPZ* chlorpromazine, *EI* efflux inhibitor, *VP* verapamil

2 Materials

2.1 Preliminary Assays: Determination of Minimum Inhibitory Concentrations (MICs)

1. Mycobacterial strains. Commonly, laboratory reference strains such as *M. tuberculosis* H37Rv can be used for these protocols. Other strains such as mutants deleted in genes encoding efflux pumps, or other mycobacterial species can be used as well (*see* **Note 1**).
2. 7H9 medium plus Tween: dissolve 4.7 g Middlebrook 7H9 broth medium in 900 mL water, add Tween 80 to 0.05 % w/v. Autoclave and add 100 mL Albumin–dextrose–catalase supplement (ADC) (Becton Dickinson) (*see* **Note 2**).
3. 7H9 medium plus glycerol: dissolve 4.7 g Middlebrook 7H9 broth medium in 900 mL water, add glycerol to 0.5 % w/v. Autoclave and add 100 mL Albumin–dextrose–catalase supplement (ADC) (Becton Dickinson).

2.2 Preparation of Cultures and Bacterial Suspensions for Accumulation and Efflux Assays

1. 7H9 medium. *See* Subheading 2.1, **items 2** and **3**.
2. Resazurin: stock solution at 0.1 mg/mL in deionized, sterile water. Store at 4 °C and protect from light.
3. PBS-Tw: PBS (*See* subheading 2.3 item 2) plus 0.05 % w/v Tween 80.

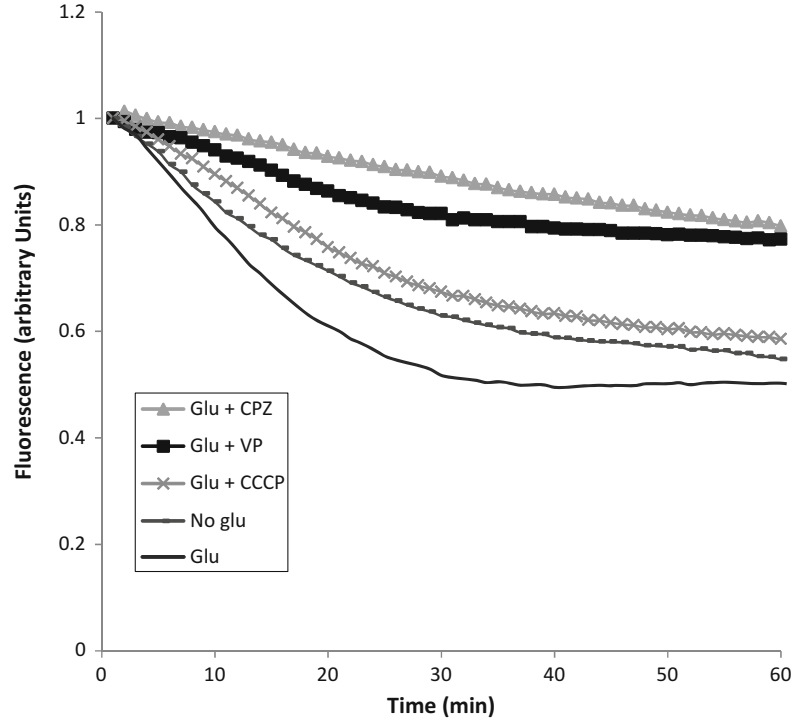

Fig. 3 Efflux of EtBr by *M. smegmatis* mc^{2}155. Efflux takes place at 37 °C in the presence of glucose and is inhibited by the efflux inhibitors. Efflux inhibitors were used at one-half the MIC to not compromise cellular viability. *CCCP* carbonyl cyanide m-chlorophenylhydrazone, *CPZ* chlorpromazine, *EI* efflux inhibitor, *VP* verapamil, *Glu* glucose

2.3 Determination of the Steady-State EtBr Concentration

1. 7H9 medium. *See* Subheading 2.1, **items 2** and **3**.
2. Phosphate buffered saline (PBS): dissolve 8 g NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of deionized water. Adjust pH to 7.4 with HCl. Adjust volume to 1 L with additional deionized H_2O. Sterilize by autoclaving for 15 min at 121 °C (*see* **Note 3**).

2.4 Demonstration of the Effect of Efflux Inhibitors on EtBr Accumulation

1. 7H9 medium. *See* Subheading 2.1, **items 2** and **3**.
2. PBS. *See* Subheading 2.3, **item 2**.
3. Glucose: 20 % (w/v) stock solution in deionized, sterile water. Store in aliquots at 4 °C (*see* **Note 4**).
4. EtBr (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide): stock solution of 1 mg/mL in deionized, sterile water. Store at 4 °C and protect from light (*see* **Note 5**).
5. Fluorometer (e.g., Synergy HT detection microplate reader) (*see* **Note 6**).
6. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP): 1 mg/mL in dimethyl sulfoxide (DMSO). Store at −20 °C and protect from light.

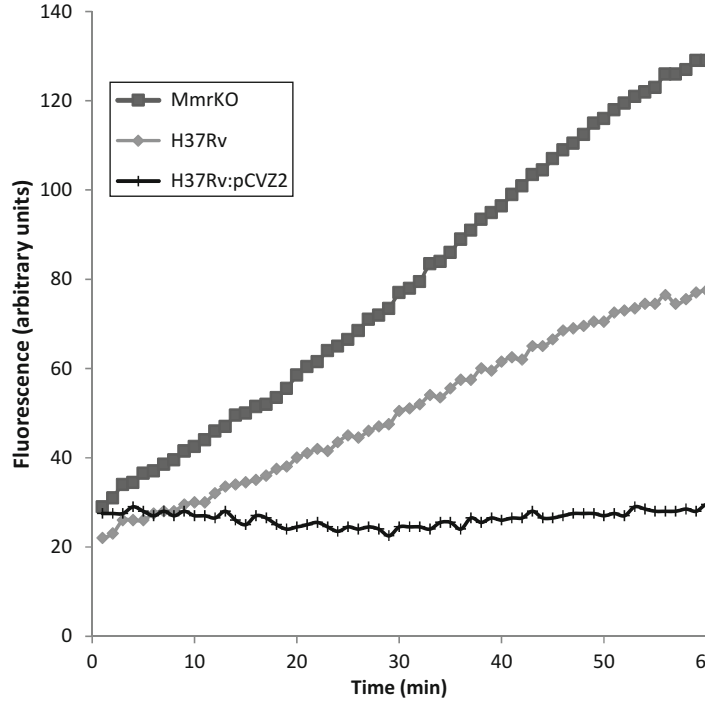

Fig. 4 Accumulation of EtBr at 1 μg/mL by *M. tuberculosis* H37Rv, H37RvMmrKO (H37Rv Δ*mmr*), and H37Rv::pCVZ2 (H37Rv containing pCVZ2) (adapted from ref. 17)

7. Chlorpromazine (CPZ): 1 mg/mL in deionized, sterile water. Store at −20 °C and protect from light.
8. Thioridazine (TZ): 1 mg/mL in deionized, sterile water. Store at −20 °C and protect from light.
9. Verapamil (VP): 10 mg/mL in deionized, sterile water. Store at −20 °C and protect from light.

2.5 Ethidium Bromide Efflux Assay

1. *See* Subheading 2.4, **items 1–9**.

3 Methods

3.1 Preliminary Assays: Determination of Minimum Inhibitory Concentrations (MICs)

Before starting any EtBr accumulation or efflux assay, the minimum inhibitory concentration (MIC) of EtBr and efflux inhibitors to be used must be determined

1. Grow mycobacterial strains at 37 °C in 7H9 medium plus Tween until an OD_{600} of 0.6–0.8.
2. Dilute the mycobacterial cultures in 7H9 medium plus glycerol in order to obtain a final concentration of 10^5 CFU/mL.

3. Transfer 100 μL to the wells of a 96-well plate that contain 100 μL of each compound at concentrations prepared from twofold serial dilutions in 7H9 medium plus glycerol.
4. Incubate the plates for 6 days at 37 °C.
5. Add 30 μL of resazurin and incubate at 37 °C for 2 days.
6. A change from blue to pink indicates reduction of resazurin and, therefore, bacterial growth. The MIC is defined as the lowest concentration of compound that prevents this color change.

3.2 Preparation of Cultures and Bacterial Suspensions for Accumulation and Efflux Assays

1. Grow mycobacterial strains (*see* **Note 1**) at 37 °C in 50 mL (*see* **Note 7**) of 7H9 medium plus Tween 80 until they reach OD_{600} of 0.6–0.8.
2. Centrifuge cultures at 3,000–4,000 × *g* for 10 min and discard the supernatant.
3. Wash the pellet in PBS-Tw (*see* **Note 8**) and adjust the OD_{600} to 0.8 with PBS-Tw (*see* **Note 9**).

3.3 Determination of the Steady-State EtBr Concentration

1. Add glucose to yield a final concentration of 0.4 % to the bacterial suspension (*see* **Note 4**).
2. In a 96-well plate (*see* **Note 10**), prepare serial dilutions of EtBr from 0.125 to 8 μg/mL in PBS-Tw plus 0.4 % glucose in a volume of 100 μL per well (Fig. 5).
3. Aliquot 100 μL of bacterial suspension into the wells of a 96-well plate containing the serial dilutions of EtBr (*see* **Note 11**). A final volume of 200 μL should be obtained in each well.
4. Include the following controls:

   Control 1: add 100 μL of PBS to 100 μL of EtBr at the selected concentrations; no bacterial cells must be added.

   Control 2: add 100 μL of PBS and 100 μL of the bacterial suspension with no EtBr.
5. Place the 96-well plate in the fluorometer and program the instrument with the following settings: a temperature of 37 °C; a unit of time for each measurement (cycle) of 60 s; the number of cycles necessary to obtain the total period of time (i.e., 60 cycles to obtain a 60 min assay); and excitation and detection wavelengths of 530 nm and the 590 nm, respectively (*see* **Note 6**).
6. Select the highest EtBr concentration that allows a readable steady-state equilibrium without compromising bacterial viability, for use in demonstration of the effect of efflux inhibitors on EtBr accumulation (*see* **Note 6**).

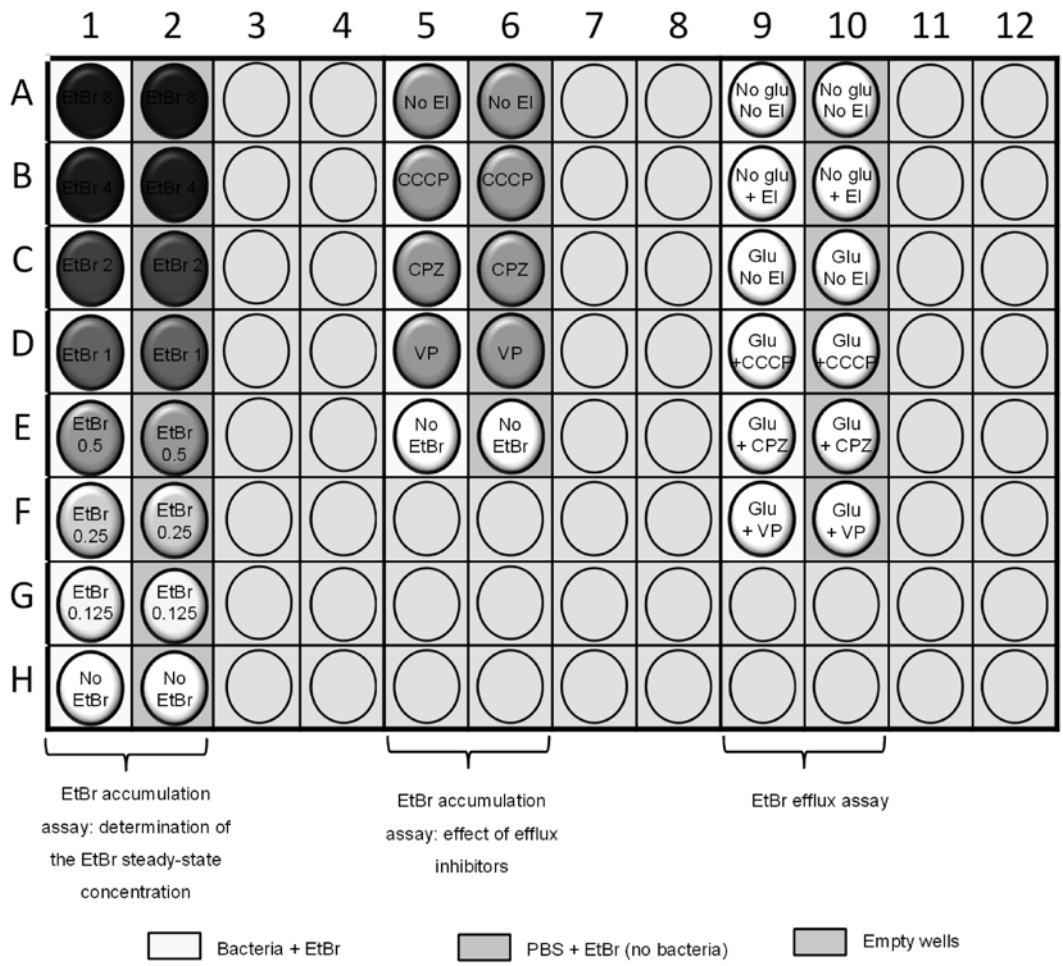

Fig. 5 Schematic representation of EtBr accumulation and efflux assays prepared in a 96-well plate. *CCCP* carbonyl cyanide m-chlorophenylhydrazone, *CPZ* chlorpromazine, *EI* efflux inhibitor, *EtBr* ethidium bromide, *Glu* glucose, *PBS* phosphate buffered saline, *VP* verapamil

3.4 Demonstration of the Effect of Efflux Inhibitors on EtBr Accumulation

1. Prepare a dilution of EtBr in PBS with 0.05 % Tween and 0.4 % glucose to reach two times the highest EtBr concentration that allows a readable steady-state equilibrium without compromising bacterial viability, selected through the protocol above. Transfer 100 μL of this EtBr dilution to the wells of the 96-well plate.
2. Add 10 μL of one efflux inhibitor (CCCP, CPZ, TZ, VP or any other, *see* **Note 12**) (Fig. 5).
3. Add 100 μL of bacterial suspension.
4. Include the following controls:

   Control 1: add 100 μL of PBS to 100 μL of EtBr at the selected concentration; no bacterial cells must be added.

Control 2: add 100 μL of PBS and 100 μL of the bacterial suspension with no EtBr.

Control 3: mix 190 μL of PBS and 10 μL of efflux inhibitor.

Control 4: mix 90 μL of PBS, 10 μL of efflux inhibitor and 100 μL of EtBr dilution.

5. Place the 96-well plate in the fluorometer and program the instrument with the following settings: a temperature of 37 °C; a unit of time for each measurement (cycle) of 60 s; the number of cycles necessary to obtain the total period of time (i.e., 60 cycles to obtain a 60 min assay); and excitation and detection wavelengths of 530 nm and the 590 nm, respectively (*see* **Note 5**).
6. Select the efflux inhibitor resulting in the highest accumulation of EtBr by bacterial cells for being used in EtBr efflux assays.

3.5 Ethidium Bromide Efflux Assay

1. To 10 mL of bacterial suspension at 0.8 OD_{600}, add 10 μL of EtBr stock solution (1 mg/mL) to obtain a final concentration of 1 μg/mL and add the efflux inhibitor selected through the protocol above (*see* **Note 12**). Mix and incubate at 25 °C for 60 min (*see* **Note 13**).
2. Harvest the EtBr-loaded cells at 3,000–4,000 × *g* for 5 min at 4 °C, discard the supernatant and resuspend to an OD_{600} of 0.8 in EtBr-free cold PBS-Tw.
3. Keep cells on ice until use in order to minimize EtBr efflux; otherwise, EtBr efflux would start immediately (*see* **Note 14**). Proceed to the next step immediately.
4. Aliquot 100 μL of cells into the wells of a 96-well plate (*see* **Note 15**).
5. Add glucose to yield a final concentration of 0.4 % and add the efflux inhibitors at one-half the MIC in order to obtain the following conditions: (a) Minimal efflux control = bacteria without glucose + efflux inhibitor; (b) Baseline efflux control = bacteria without glucose; (c) Maximal efflux control = bacteria with glucose; and (d) Efflux inhibitor control = bacteria with glucose + efflux inhibitor (Fig. 5).
6. Place the 96-well plate in the fluorometer and program the instrument with the following settings: a temperature of 37 °C; a unit of time for each measurement (cycle) of 60 s; the number of cycles necessary to obtain the total period of time (i.e., 60 cycles to obtain a 60 min assay); and excitation and detection wavelengths of 530 nm and the 590 nm, respectively (*see* **Note 16**).

3.6 Normalization of Data and Presentation of Results

The final results of the accumulation assay are calculated as follows:

1. Subtract the EtBr baseline fluorescence obtained in Control 1 (PBS + EtBr, with no bacterial cells) from the one corresponding to the bacteria + EtBr.

2. Make sure that fluorescence measurements in Control 2 should be very low, below those in Control 1, with no increases over time.
3. If the latter is not the case, conditions should be checked.

The results of the accumulation assay in the presence of efflux inhibitors are calculated as follows:

1. Subtract the EtBr baseline fluorescence obtained in Control 4 (PBS + efflux inhibitor + EtBr, with no bacterial cells) from the one corresponding to the bacteria + efflux inhibitor + EtBr.
2. Make sure that fluorescence measurements in Control 3 should be very low, below those in Control 4, with no increases over time.
3. If the latter is not the case, conditions should be checked.

In any of these experiments, fluorescence can be expressed in terms of relative final fluorescence (RFF) at the last time point (minute 60) of the assay in comparison with reference conditions by using the formula (RFassay – RFref)/RFref, where RFassay is the relative fluorescence at the last time point of the ethidium bromide accumulation assay and RFref is the relative fluorescence at the last time point of the ethidium bromide accumulation assay under the reference conditions [17, 18] (*see* **Note 17**).

The results of efflux experiments are presented in terms of relative fluorescence, calculated by comparing the data obtained for the bacterial population under conditions that allow maximum efflux (bacteria in the presence of glucose and absence of efflux inhibitor) against the data obtained from the control that contains the EtBr-loaded cells under conditions that restrict efflux (presence of efflux inhibitor and no glucose). The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relative to the EtBr-loaded cells.

4 Notes

1. Some mycobacterial strains are pathogenic so cultures and further manipulations must be done in biosafety level 2 or 3 laboratories according to the requirements for each specific strain.
2. According to the manufacturer, ADC must be added to Middlebrook 7H9 broth medium. However, in many laboratories, OADC (oleic acid-albumin-dextrose-catalase) is used instead of ADC. The choice between ADC and OADC may depend on many factors, such as availability, price, and the particular growth requirements of mycobacterial species or strains to be assayed.

3. Alternatively, some suppliers provide PBS tablets (Sigma Aldrich). In this case, dissolve one tablet in 200 mL of deionized sterile water, according to the manufacturer's instructions.
4. Glucose solutions do not need to be sterilized if used immediately. If they are kept at 4 °C they should be used within 3 days of preparation. Glucose is added to provide an energy source for the activity of the efflux systems.
5. EtBr is a powerful teratogen. Safety precautions must be followed when working with EtBr. Non-absorbent gloves (e.g., nitrile) should be worn when handling EBr and stock solutions should be prepared under a fume hood. Care should be taken to avoid contaminating the working area. All disposable materials used for EtBr preparation or manipulation should be identified as contaminated and disposed according to local laws and regulations, such as in appropriate container for subsequent incineration.
6. Other fluorometers may be used. In our experience, the Safire from Tecan (microplate system) and the Rotor Gene™ 3000/6000 from Corbett Lifesciences (0.2 mL microtube system) are suitable instruments to be used in these assays. Any equipment should be set up for excitation at 530 nm and detection of fluorescence of EtBr at 590 nm. Most fluorometers provide measurements of fluorescence at every time point during the course of the experiment, along with a graphical plot of the increase of fluorescence over time. This allows the real-time visualization of when the steady-state equilibrium is reached at a given EtBr concentration (such as that shown in Fig. 1 for a concentration of 0.5 μg/mL). This should be reached at no more than 10 % of the relative fluorescence that the instrument can record [7].
7. A culture of 50 mL will give approximately 35 mL of bacterial suspension at an OD_{600} of 0.8, which is a volume large enough to test several experimental conditions and to carry out the control experiments. The volume of the culture can be scaled up or down depending on the number of experiments to be done.
8. PBS is supplemented with Tween 80 at a final concentration of 0.05 % to get stable and disperse suspensions of mycobacterial cells and avoid the formation of bacterial clumps.
9. If using bacterial suspensions adjusted at 0.8 OD_{600} does not result in detection of accumulation or efflux, test other bacterial suspensions adjusted at 0.4, 0.6, or 1.0 OD_{600}. This optimization may be necessary according to the instrument used to record the fluorescence emitted by the fluorescent substrate accumulated inside the mycobacterial cells.

10. The assay can be done in 96-well plates or in microcentrifuge tubes depending on the requirements of the fluorometer to be used.
11. Other fluorescent compounds such as Hoechst 33342, Ethidium monoazide (EMA), Rhodamine 123 or orange acridine can be used instead of EtBr. If so, excitation and emission wavelengths should be modified and levels of relative fluorescence sensitivity of the assays will also vary according to the excitability threshold of the compound used for efflux substrate. Relative fluorescence will also have to be adjusted according to the compound used.
12. Other efflux inhibitors such as Phe-Arg-β-naphthylamide (PAβN) may be used. Minimal inhibitory concentrations (MICs) of the efflux inhibitors to be tested on each bacterial strain must be conducted prior to their use, to test for antibacterial activity. Each inhibitor is used at a final concentration that should not exceed one-half of the MIC in order to not compromise the cellular viability.
13. In order to measure EtBr efflux, it is necessary to promote the maximum accumulation of EtBr. This is accomplished by exposing the bacteria to EtBr under conditions that limit efflux to its minimal activity, namely: a temperature of 25 °C, absence of glucose, pH and the presence of the most active efflux inhibitor selected from accumulation experiments. All these conditions that diminish/reduce efflux must be optimized experimentally according to the instrument used to record the fluorescence emitted by the fluorescent substrate accumulated inside the mycobacterial cells [4, 6].
14. We have found that for most strains, efflux of EtBr takes place within the first 10–15 min of the assay at 37 °C, and this period of time may be shorter in the case of drug resistant strains or strains overexpressing efflux-pumps.
15. It is advisable to keep the 96-well plate on ice until all reagents are added to wells (*see* **Note 9**).
16. In the case of strains with overexpression of efflux pumps it might be necessary to adjust the measurement time, performing shorter acquisition cycles due to the increased efflux activity.
17. When assaying different strains and mutants of *M. tuberculosis*, the reference condition could be the assay of reference strain H37Rv, whereas when assaying the effect of efflux inhibitors, the reference condition is the assay in the absence of any efflux inhibitor. In both cases, high RFF values indicated that cells accumulated more ethidium bromide under the tested conditions than under the reference conditions and vice versa for negative RFF values.

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Chapter 14

Single-Cell Analysis of Mycobacteria Using Microfluidics and Time-Lapse Microscopy

Neeraj Dhar and Giulia Manina

Abstract

The crucial role of phenotypic heterogeneity in bacterial physiology and adaptive responses has required the introduction of new ways to investigate bacterial individuality. Time-lapse microscopy is a powerful technique for evaluating phenotypic diversity in bacteria at the single-cell level, whether exploring the dynamics of gene expression and protein localization or characterizing the heterogeneous phenotypic response to perturbations. Here, we present protocols to carry out time-lapse imaging of mycobacteria at the single-cell level using either agarose pads or customized microfluidic devices. The sequences of images obtained can be analyzed using programs such as ImageJ and allow the investigator not only to extract various parameters of growth and gene expression dynamics but also to unravel the physiological basis behind phenomenon such as persistence against stresses.

Key words Single-cell, Time-lapse, Microscopy, Microfluidics, Mycobacterium, Cell growth, Cell division, Fluorescence, Gene expression, Single-cell analysis

1 Introduction

Bacterial populations, even though largely clonal, are extremely heterogeneous. However, this heterogeneity is often masked by traditional microbiological assays, which are essentially population averaging methods [1, 2]. The general assumption behind such assays is that the average of the population reflects the state of each cell in that population. However, this would be a too simplistic assumption, considering the stochastic nature of biological processes and the variability each individual cell faces, which allows it to explore an almost infinite phenotypic space. This phenotypic heterogeneity is not just an unexpected result of noisiness of the biological processes but has been shown to have functional consequences on the behavior and long-term survival of bacterial populations, especially when facing unforeseen perturbations [4–9].

Heterogeneity is manifested in diverse forms: from variation in cell size, interdivision time and growth rates, to large dynamic

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ranges in gene expression and stress responses [1–11]. Therefore, it is essential to develop sensitive techniques that allow us to capture cell-to-cell variability. There are several methods by which single-cell phenotypes can be measured such as flow cytometry, single-cell sequencing, single-cell-omics, single-cell spectroscopy, and time-lapse microscopy. While some techniques such as flow cytometry allow us to capture the phenotypic diversity in a population and enable multiparameter analysis, they, however, provide just a snapshot of the dynamic range in the population [10]. In the current chapter, we focus on time-lapse microscopy technique, which in the last decade has revolutionized the way we look at individual cells and has allowed us to characterize the phenotypic heterogeneity in bacterial populations.

Time-lapse microscopy has been used extensively to study gene expression dynamics, protein localization, single-cell behavior as well as their response to fluctuations in the environment. Most of these studies have focused on model organisms such as *Escherichia coli*, *Bacillus subtilis*, and *Caulobacter crescentus* [3–8, 11–19]. However, in the last few years there have been substantial efforts to characterize mycobacterial species at the single-cell level [9, 20–28]. Time-lapse imaging of mycobacteria poses several challenges—(1) Genus *Mycobacterium* comprises members with very long generation times, ranging from ~3 h in case of "fast-growing" species such as *Mycobacterium smegmatis* to ~20 h in case of "slow-growing" species such as *Mycobacterium tuberculosis*. As a result the time-lapse experiments extend from several days to weeks, posing a challenge to the stability and robustness of the imaging setup; (2) With their unique snapping mode of division [29] and their tendency to clump and form long cords, segmentation analysis of individual mycobacteria in image sequences is very difficult; (3) Since the processes of cytokinesis and cell separation are temporally distinct events [26] it is not possible to accurately determine cell size after division or the interdivision time without specific fluorescent markers for septum formation; (4) Due to the high GC content of mycobacterial genomes, it is often difficult to optimize expression of fluorescent reporter genes (which are usually from low GC content organisms). Moreover, since most mycobacterial promoters are weak, characterizing native transcriptional signals is more challenging.

To construct transcriptional reporter strains, the fluorescent protein (FP) along with an optimized ribosome-binding site should be cloned downstream of the promoter of interest [6, 9]. This can be done either by knocking-in the FP downstream of the promoter at the native locus in the chromosome or by cloning this construct into a plasmid that can be integrated into one of the phage attachment sites in the chromosome. Integrative plasmids are recommended so as to reduce cell-to-cell variability caused by episomal plasmid copy number variations. Using similar approaches translational reporter

fusions or protein fusions can also be constructed by cloning the FP in-frame with the ORF of interest [9, 26, 27]. The choice for an N-terminal or a C-terminal FP fusion depends on the structure and the location of functional domains in the protein under investigation. It is always a good practice to add a protein linker, comprising of 6-18 amino acids (serine and glycine), between the target protein and the FP. It is important to make sure that the localization and behavior of the FP fusion accurately reflects the behavior of the endogenous protein. Therefore, necessary controls include (1) confirmation of localization of endogenous protein by other techniques such as immunolocalization or electron microscopy or by generating fusions using different FPs; (2) verifying that the FP fusion does not alter the functional activity of the native protein; (3) confirming that the FP fusion does not alter the expression level or stability of the tagged protein. While most of the reported single-cell studies employed agarose pads to carry out time-lapse microscopy, some studies have utilized customized microfluidic devices. In the following sections we describe protocols to carry out time-lapse microscopy of mycobacteria using both these techniques.

2 Materials

Prepare all media and reagents using deionized water. Filter-sterilize using 0.22 μm filters. Store all reagents at 4 °C unless specified differently.

2.1 Bacterial Culture and Preparation

1. Glycerol: 50 % (v/v).
2. Tyloxapol: 10 % (w/v). Protect from light (*see* **Note 1**).
3. Albumin–dextrose–saline (ADS): 5 % w/v Bovine Serum Albumin (Fraction V), 2 % w/v D-glucose, 0.81 % w/v NaCl in water.
4. Complete 7H9 medium: Dissolve 4.7 g Middlebrook 7H9 broth base (Difco) in 900 mL deionized water. Add 10 mL of 50 % glycerol, 2 mL of 10 % Tyloxapol, and 100 mL of ADS supplement (*see* **Note 2**).
5. 30 mL PETG bottles (Nalgene).
6. 5 μm filter unit, PVDF membrane.
7. 1 mL syringes.

2.2 Agarose Pad Microscopy

1. Low-melting point agarose.
2. 2× concentrated Complete 7H9 medium: Dissolve 4.7 g Middlebrook 7H9 broth base (Difco) in 400 mL deionized water. Add 10 mL of 50 % glycerol, 2 mL of 10 % Tyloxapol, and 100 mL of ADS supplement.

3. Sterile 50 and 15 mL polypropylene conical centrifuge tubes.
4. #1 Glass coverslips: 24 × 60 mm; 21 × 26 mm; 25 mm round.
5. 100 mm petri dishes.
6. Scalpel blade.
7. Biopsy punch, e.g., Harris Uni-Core™, different sizes.
8. μ-Dish (Ibidi).
9. Glue.
10. Immersion oil.

2.3 Preparation of Semipermeable Membranes

1. Semipermeable cellulose dialysis membrane, 25 kDa cutoff.
2. Teflon cylindrical column (diameter 25 mm), custom-made.
3. Methanol.
4. Vacuum chamber.
5. Broad flat-tip forceps.
6. Whatman™ Filter paper.
7. Microscope glass slides.
8. Binder clips.

2.4 Microfluidic Device Fabrication and Assembly (Fig. 1)

1. SU-8 Silicon wafer master (*see* **Note 3**).
2. Polydimethylsiloxane (PDMS), heat-curable silicone elastomer (Sylgard 184, Dow Corning).
3. Square petri dish, 120 × 120 × 17 mm.
4. Vacuum chamber.
5. Baking oven.
6. Surgical scalpel blade.
7. Biopsy punch, e.g., Harris Uni-Core™, different sizes.
8. Silicone tubing, 0.76–1.65 mm (inner–outer diameter), e.g., from Helix Medical.
9. Male and female Luer connectors.
10. Round coverslip, 25 mm.
11. Broad flat-tip forceps, used for handling filters, wafers, coverslips.
12. Whatman™ filter paper, Grade 4, 90 mm.
13. Petri dish, 100 mm.
14. Nylon screws, e.g., Craftech Industries.
15. Acrylic frame, custom-built (Fig. 1)
16. Syringe pump.
17. Immersion oil.
18. Isopropanol.
19. 70 % ethanol.

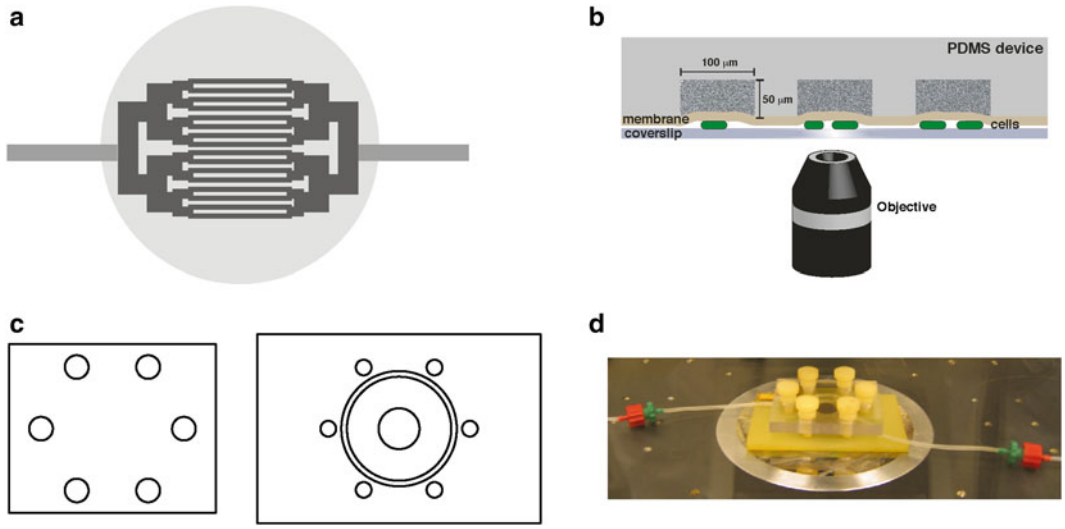

Fig. 1 Schematic view of a microfluidic device used for single-cell time-lapse microscopy (also *see* ref. [9]). (**a**) Outline of the PDMS microfluidic chip showing the layout of the channels and the inlet/outlet tubings. (**b**) Cross-sectional view of the assembled microfluidic setup. Bacteria are sandwiched between the glass coverslip and a semipermeable membrane and are fed by diffusion of medium, being pumped through the channels in the PDMS device. (**c**) Outline of the acrylic upper and lower frames that are used to assemble the microfluidic device. After assembly, the device is clamped between the two frames using six screws. The middle region in the lower frame (*right panel*) is in a slightly recessed region for placing the coverslip. The objective is in contact with the coverslip through the hole in the middle of the lower frame. (**d**) Picture of the assembled device with the tubings connected

20. Water-bath sonicator.
21. Tuberculocidal disinfectant.

2.5 Time-Lapse Microscopy (See Note 4)

1. Inverted microscope with fluorescence capability (PersonalDV, Applied Precision).
2. Environmental chamber enclosing the optical components and the stage (Weather station Precision Control, Applied Precision).
3. High-resolution and sensitive CCD camera (Photometrics CoolSnap HQ^2).
4. Motorized stage, for multipoint visiting and for autofocusing on the z axis (25 mm Flexure stage, Applied Precision).
5. Light source for epifluorescence (250 W Xenon light source or Solid state Illumination module, Applied Precision).
6. Motorized shutters for different filters (Applied Precision).
7. Olympus 100× objective, UPLFL 1.30 NA, 0.2 mm WD.
8. Image acquisition computer: with acquisition software and to control the microscope (Workstation with softWoRx® 5.0, Applied Precision).

9. Clamps, to hold the microfluidic device in position.
10. Antivibration table (Applied Precision).
11. Image analysis computer.

2.6 Image Analysis (See Note 5)

1. ImageJ 1.47q (Wayne Rasband, NIH, USA) (http://imagej.nih.gov/ij/index.html).

3 Methods

3.1 Bacterial Culture and Preparation

1. Inoculate 6 mL of complete 7H9 medium in a 30 mL PETG bottle with 100–200 µL of a frozen stock of the mycobacterial reporter strain (*see* **Note 6**).
2. Grow the culture in a 37 °C incubator with shaking at 100 rpm for 12–24 h (in case of fast growing species) or for about 5–7 days (in case of slow-growing species), till it reaches mid-exponential phase ($OD_{600} = 0.4–0.6$) (*see* **Note 7**).
3. Harvest bacteria from 2 × 1 mL of culture by centrifugation in screw-cap tubes at 2,400 × *g* for 5 min.
4. Resuspend the bacterial pellet in 200 µL of pre-warmed (37 °C) complete 7H9 medium.
5. Pass the concentrated bacteria through a 5 µm filter using a 1 mL syringe (*see* **Note 8**), to remove large bacterial clumps.
6. The filtrate contains predominantly single-cell bacteria and can be used for imaging.

3.2 Agarose Pad Microscopy

1. Prepare 1.5 % (w/v) low-melting point agarose in 10 mL of sterile deionized water in a 50 mL tube and dissolve in the microwave (*see* **Note 9**).
2. After the agarose has cooled down to around 40–50 °C, add 10 mL of pre-warmed (37 °C) 2× concentrated complete 7H9 medium (*see* **Note 2**) and mix by vortexing.
3. Gently dispense 1–2 mL onto a glass cover slide placed inside a 100 mm petri dish on a flat surface.
4. Place a glass coverslip on the top to create an agarose sandwich (*see* **Note 10**).
5. Close the petri dish and wait for 30–60 min till the agarose solidifies (*see* **Note 11**).
6. Remove the upper coverslip gently using a scalpel blade and cut out the required size of the agarose pad using a scalpel blade or a biopsy punch and place it on a glass slide.
7. Seed 2 µL of the bacterial sample on top of the agarose pad and wait for 5–10 min to allow it to be absorbed into the agarose.

8. Flip the agarose pad gently onto a coverslip and carry out snapshot imaging of the bacteria from the bottom through the coverslip (*see* **Note 12**).
9. Alternatively, for short-term time-lapse microscopy experiments, the agarose pad (or several agarose pads, each with a different strain of bacteria) can be flipped onto the bottom of a μDish so that the bacteria are trapped between the agarose pad and the coverslip bottom. Close and seal the lid of the μDish using grease to minimize drying of the pad (*see* **Note 13**).
10. Place a drop of immersion oil on the 100× objective and mount the coverslip or the μDish on the microscope stage, such that the glass side faces the objective.
11. Carry out snapshot imaging or setup a time-lapse microscopy experiment.

3.3 Preparation of Semipermeable Membranes

1. Take a few long (~20 cm) pieces of dialysis membrane and open by cutting lengthwise with a scalpel blade or scissors.
2. Soak the membranes in 500 mL of double distilled water in a 1 L beaker for 2–3 h. Stir the water occasionally and change the water a few times during this period (*see* **Note 14**).
3. Remove the membranes and cut out several round disks of membrane (similar diameter as the coverslip ~26 mm) using a cylindrical column and a scalpel blade (*see* **Note 15**).
4. Transfer the round membrane disks into another beaker with fresh double distilled water and allow them to soak for 1 h. Do not let the membranes dry between transfers.
5. Take out the membrane disks from the water and remove excess water by placing them on a filter paper.
6. Soak the membranes in 500 mL methanol overnight, in a chemical fume hood.
7. Remove membranes from methanol and remove excess methanol by placing them on a filter paper.
8. Place the membranes between sheets of rectangular filter paper and sandwich them between two glass slides, clamping the slides using a binder clip.
9. Place the assembled membranes in a vacuum chamber under vacuum for at least 2 days.

3.4 Microfluidic Device Fabrication and Assembly (See Note 16) (Fig. 1)

1. Secure the SU-8 patterned Silicon wafer to the bottom of a square petri dish using clean tape.
2. Combine 10 g of Sylgard 184 curing agent and 100 g of Sylgard 184 pre-polymer base for making the PDMS mix.
3. Using a clean plastic fork or spreader mix the reagents to a milky consistency until the curing agent is evenly distributed.

4. Pour the PDMS mix onto the Silicon wafer in the petri dish.
5. Place the petri dish in the vacuum chamber and apply vacuum. As the vacuum builds up, bubbles will appear. Vent the chamber a few times initially to burst the bubbles and then leave it for complete degassing for 15–30 min.
6. Remove the petri dish and incubate at 80 °C in an oven for 2 h to cure the PDMS.
7. Using a scalpel gently cut out the PDMS block corresponding to the patterned area and place the block in a fresh petri dish keeping the features on top. Avoid damaging the silicon wafer when separating the PDMS block from the wafer.
8. Using the biopsy punch (1.6 mm), punch out holes in the PDMS block to connect to the patterned channels, for the inlet and outlet ports.
9. Insert silicone tubings into the ports and seal the connections using a small amount of PDMS-mix and curing it for 1 h.
10. Attach female luer connectors to the inlet and outlet tubings. The device is now ready to use.
11. Carry out the handling and assembly of the microfluidic device under aseptic conditions preferably in a biosafety cabinet.
12. Clean a set of broad flat-tip forceps using 70 % ethanol.
13. Place a Whatman filter paper into a petri dish and wet it with 5 mL of pre-warmed complete 7H9 medium. Pour out the excess medium (*see* **Note 17**).
14. Take out one of the semipermeable membrane disks from the stack of membranes prepared before (from Subheading 3.3) and place it on the wet Whatman filter paper. When the dried semipermeable membrane is placed on the wet filter paper, it will instantly curl up. Wait for a few minutes and then gently uncurl and flatten the membrane using clean forceps.
15. Dispense 3–5 μL of filtered bacteria (from Subheading 3.1) onto the center of the membrane and spread the inoculum gently using the side of a pipette tip.
16. Leave the petri dish open in a biosafety cabinet under the laminar flow for a few minutes, until the medium is absorbed on the membrane. Do not let the membrane dry out.
17. Take the lower part of the acrylic frame and apply a small amount of immersion oil around the hole in the middle (Fig. 1c) (*see* **Note 18**).
18. Gently pick up a 25 mm coverslip using the flat forceps and place it on the hole in the base (the immersion oil helps the coverslip adhere to the surface).
19. Place the sterilized microfluidic device in the middle of the top frame (Fig. 1c), the channels side up. Apply moderate pressure

on the edges of the microfluidic device to make it adhere to the acrylic surface (*see* **Note 19**).

20. Gently lift the semipermeable membrane from the Whatman filter and immediately place it on the microfluidic device, with the bacteria side face up. Make sure there are no air bubbles trapped between the membrane and the channels.
21. In one movement, flip the upper frame and place it on the lower frame, aligning the microfluidic device with the coverslip on the base.
22. Hold the frame by applying pressure on the center of the device with your index finger and start inserting the screws. Do not tighten the screws fully. Once all the screws have been positioned, start tightening them, making sure that you apply equal pressure and tighten each screw to the same extent (*see* **Note 20**).
23. Fill a 60 mL syringe with pre-warmed complete 7H9 medium and connect it to the inlet tubing through a female Luer connector. Prime the tubing with medium to get rid of air bubbles.
24. Connect the inlet and outlet tubings to the tubing connectors of the microfluidic device using male Luer connectors (Fig. 1d).
25. Place the outlet tubing into a waste receptacle; apply gentle force on the syringe to perfuse the microfluidic channels with the medium and check for any leaks.
26. Transfer the assembled device to the microscope. Put a drop of immersion oil on the bottom of the coverslip as well as on the 100× objective lens and clamp the device frame to the microscope stage, aligned with the objective lens (*see* **Note 21**).
27. Attach the syringe to the syringe pump and place the outlet tubing into a waste bottle containing disinfectant. Switch on the pump and maintain the flow rate at 25 μL/min (*see* **Note 22**).
28. Carry out the time-lapse microscopy experiment.
29. At the end of the experiment, transfer the microfluidic setup into a biosafety cabinet and perfuse the tubings and device with 70 % ethanol.
30. Transfer the device and tubings into a 500 mL beaker containing 200 mL isopropanol. Cover the beaker with aluminum foil.
31. Place the beaker in an ultrasonic water bath and clean the device by sonication for 60 min (*see* **Note 23**).
32. Remove the device from isopropanol and wash the channels and tubings by flowing 70 % ethanol using a 5 mL syringe.
33. Rinse the device and tubings extensively (8–10 purges) with double distilled water, using a 5 mL syringe.

34. Remove traces of water from the tubings and device using filter paper or a jet of compressed air.
35. Place the microfluidic device in a 250 mL beaker keeping the channel-side facing up. Pack the tubings in aluminum foil and place it in the beaker. Cover the beaker with aluminum foil and autoclave it. The device is now ready for reuse.

3.5 Time-Lapse Microscopy

1. Make sure the temperature in the environmental chamber is correct and has been allowed to stabilize for 24 h (*see* **Note 24**).
2. Observe cells from the eyepiece using the phase-channel and record the location [x, y, z] of points in the device, which contain few [1–3] bacterial cells (*see* **Note 25**).
3. Set the acquisition conditions—imaging channels; exposure conditions; list of positions; imaging frequency; experiment duration; autofocus settings, in the image acquisition software. Even though these settings are variable and depend on the fluorescence signal and experimental setup, below are listed some conditions we use typically in our experiments when imaging mycobacteria constitutively expressing a fluorescent protein (Fig. 2).
4. Imaging channels: The channels to be imaged depend on the number of fluorescent reporters. Typically 3–4 channels can be imaged simultaneously. An example of the excitation/ emission filters and dichroics that can be used—DAPI (Ex:360/40; Em:457/50); FITC (Ex:490/20; Em:528/38); RD-TR-PE (Ex:555/28; Em:617/73); GFP (Ex:475/28; Em:525/50);

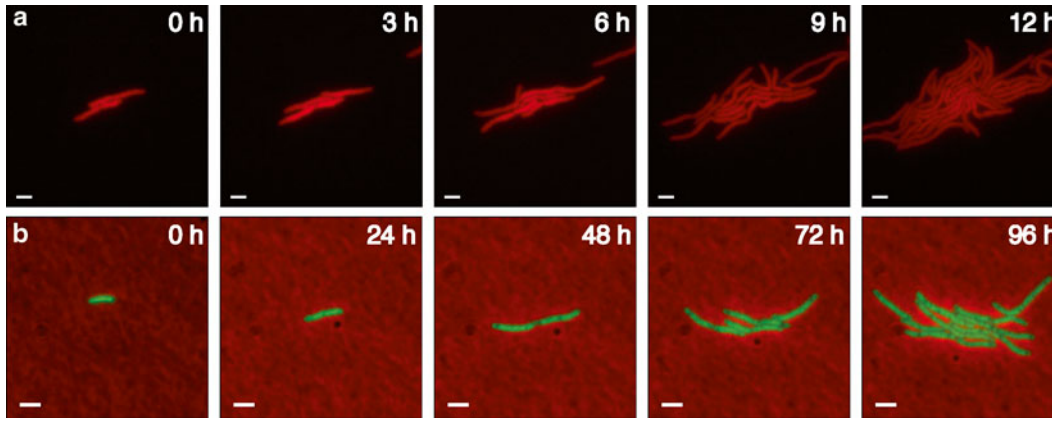

Fig. 2 Time-lapse microscopy snapshots of mycobacteria growing in the microfluidic device. (**a**) Representative image sequence of an *M. smegmatis* strain expressing dsRed2 protein constitutively from an integrative plasmid, growing in a microfluidic device. Only the fluorescent channel is shown (*red*).(**b**) Representative image sequence of an *M. tuberculosis* strain expressing GFP protein constitutively from an integrative plasmid, growing in a microfluidic device. Images shown are composite images generated by merging the phase (*red*) and fluorescence (*green*) channels. Numbers shown on *top right*, indicate the number of hours elapsed. Scale bar, 2 μm

mCherry (Ex:575/25; Em:632/60); Cy5 (Ex:640/20; Em:685/40). Polychroic beam splitter (suitable for DAPI, FITC, RD-TR-PE, Cy5); Dichroic (GFP/mCherry).

5. Imaging frequency: This again depends on the time scales of the dynamic process being studied. Typically, for experiments using *M. smegmatis*, image every 10–15 min, resulting in 18–12 images per cell cycle (~3 h). For experiments using *M. tuberculosis*, image every 1–2 h, resulting in 20-10 images per cell cycle (~20 h) (Fig. 2) (*see* **Note 26**).
6. List of positions: This is often dependent on the imaging frequency and exposure conditions. In our experiments, we typically image between 30 and 60 positions in case of *M. smegmatis* and 50–200 positions in case of *M. tuberculosis*.
7. Exposure conditions: This is one of the most crucial experimental parameters and should be empirically determined for each fluorescent reporter and imaging frequency to maximize signal intensity and to minimize phototoxicity and photobleaching. If the fluorescence signal is weak, binning can be done to increase the signal and keep the exposure time to minimum. Typical exposure settings for *M. smegmatis*: Fluorescent channel (FITC/GFP/TRITC): 50 % transmittance; 0.1 s exposure; Phase channel: 50 % transmittance; 0.1 s exposure. For *M. tuberculosis*: Fluorescent channel (FITC/GFP/TRITC): 10–32 % transmittance; 0.05 s exposure; Phase channel: 50 % transmittance; 0.1 s exposure. For time-lapse experiments, a filter for blocking out UV light from the excitation light should be included in the optical path.
8. Autofocus: Due to the long timeline of these experiments, there is bound to be drifting on the *z*-axis, which could affect the focusing of the sample. Therefore, an autofocus regime needs to be implemented, preferably on the phase-channel to avoid phototoxicity issues. For autofocusing on the phase channel when imaging mycobacteria, we typically scan 6–7 μm on the *z*-axis in 0.2 μm steps (*see* **Note 27**).
9. Once all the parameters have been set and saved in the software, image acquisition can be initiated. The images are all saved automatically, usually in the customized format of the acquisition software being used.
10. Monitor the acquisition of the initial few time points, to make sure the autofocus and other parameters are functioning correctly.

3.6 Image Analysis

While there are several software packages that have been developed and published for the automated segmentation and analysis of time-lapse microscopy images of bacteria, these are more suitable for bacteria such as *E. coli* or *B. subtilis*, where the bacterial growth,

shapes and division are more amenable to algorithm based segmentation. Automated segmentation of mycobacterial cells and identification of cell division events is tricky because the bacteria grow as long rods that tend to stick together and because the events of cytokinesis and cell separation are not simultaneous [26]. Therefore, so far we have been carrying out analysis of the time-lapse images by manual segmentation using programs such as ImageJ.

1. Import the image sequence stack into ImageJ (*see* **Note 28**).
2. Use the ImageJ function "*Adjust—Brightness/Contrast*" for optimal visualization of the cells for segmentation.
3. Choose the parameters you want to measure such as cell size (Area) and fluorescence intensity (Mean gray value) using the ImageJ function "*Set Measurements.*"
4. Carry out manual segmentation of the images by drawing polygons around individual cells. For analysis of time-lapse image sequence, the polygon has to be modified frame by frame as the cells grow over time and consecutive measurements carried out to determine dynamics of the different cellular parameters (*see* **Note 29**).
5. Use the ImageJ function "*Measure*" to acquire the measurements on the segmented polygon. The measurements can be exported as a .txt or .csv file.
6. Cell lineage information can also be documented to generate lineage trees and to plot growth rates and fluorescence as a function of genetic relatedness (*see* **Note 30**).
7. The measurements can be finally assembled in a program like Microsoft Excel for further analysis.

4 Notes

1. Tween 80 (0.05 %, final concentration) can also be used, but is not recommended due to its instability.
2. Even though we list only Middlebrook 7H9 medium here, in practice any mycobacterial media, e.g., Sauton, Dubos, M9 minimal medium, can be used. Minimal medium such as M9 are particularly attractive for fluorescence imaging due to low autofluorescence.
3. Silicon wafer master can essentially be made with any design (Fig. 1). There are several reviews describing in detail the photolithography procedures to make these master templates [30–32].
4. Different microscope vendors (Olympus, Zeiss, Nikon, Leica, etc.) provide integrated microscope setups suited for live cell microscopy. Here we list the components that we use in our lab.

5. Besides ImageJ, there are several software packages (freely available), which can be adapted for analysis of image sequences, such as Fiji [33], Icy [34], Schnitzcells [19], CellProfiler [35], MicrobeTracker [36], and CellTracer [37].
6. While designing the bacterial fluorescent reporters, it is important to choose a suitable FP, that is bright, has a fast maturation time, is photostable and excitation of which does not harm the cells. Proteins such as GFP, dsRed, and mCherry have been expressed in mycobacteria successfully [9, 20, 22, 26, 27]. Mycobacteria exhibit autofluorescence when excited at 405 nm, so this has limited the use of cyan FPs [38]. It is essential to carry out control experiments to verify that exposure to fluorescent excitation is not affecting the growth rate or behavior of mycobacteria. If monitoring multiple FPs, choose ones with minimal spectral overlap.
7. When culturing *M. tuberculosis*, put the PETG bottle in a secondary container before placing it in the shaker.
8. Before putting the concentrated bacteria in the syringe filter, the filter can be pre-wetted with 100 μL of pre-warmed medium to prevent sample loss.
9. Use freshly prepared high-purity low-melting point agarose.
10. For a quick snapshot, mycobacteria can also be imaged directly without using an agarose pad. Place 0.5–1 μL of culture between two glass coverslip slides (24 × 60 mm). In case of *M. tuberculosis*, a smaller sized coverslip can be used on top and the edges sealed with silicone grease or any other sealant. While this method is adequate to have a quick look at the bacterial strain, it should not be used for quantitative analysis, as the bacteria are usually not in a single plane and tend to move around. When working with *M. tuberculosis*, handle the coverslips with great care, as they are very fragile. Transfer the slide from biosafety cabinet to the microscope in a secondary container.
11. The agarose pad should be even and without any bubbles. Try to avoid overdrying the agarose pads, as this will cause unevenness of the surface. The agarose pads can be stored at 4 °C for a few hours.
12. Use a scalpel or forceps to transfer the agarose pads. Avoid damaging the surfaces, as this will affect the seeding as well as imaging of bacteria.
13. Use of agarose pads in μDish allows imaging of multiple strains or different conditions in parallel. These dishes can also be sealed with grease to avoid evaporation or when working with *M. tuberculosis*. When conducting short-term time-lapse experiments, medium can be dispensed inside the dish at the edge, to delay evaporation.

14. These washing steps are required to get rid of sodium azide from the membranes.
15. A cylindrical piece of plastic or Teflon (~25 mm), with smooth edges can be fabricated in a workshop for cutting out the membranes. To cut the membranes uniformly, apply gentle pressure using the Teflon cylinder and cut the membrane along the edges using a sharp scalpel. If the membrane gets wrinkled or damaged, discard it.
16. When preparing the microfluidic device, work in a dust-free environment and clean all surfaces that come in contact with the PDMS. When preparing the PDMS mold, wear a lab coat, a clean pair of gloves, hair-cover, and face-mask.
17. The filter paper should be wet but not soaking in medium, as this will cause the bacteria to float away.
18. Avoid using excess of immersion oil because it can overflow onto the top of the coverslip and come into contact with the bacteria, affecting their growth and imaging.
19. Avoid touching the channels, as the smallest of particles can compromise the imaging, block the channels or result in formation of air bubbles.
20. Do not over tighten the screws, as this might cause the coverslip to crack. Do not keep it too loose, as this might cause leakage.
21. Make sure that the device assembly is clamped firmly to the stage. Because the imaging is done using a 100× objective, even small drifts can cause loss of the chosen imaging points and focusing.
22. When switching medium or syringes check for air bubbles and purge them by gently pushing the syringe plunger manually.
23. When working with *M. tuberculosis*, the device should be autoclaved before sonication.
24. Switch on the environmental chamber at least 24 h before using the microscope and allow all the microscope components (stage, immersion oil, environmental chamber, medium) to stabilize to the correct temperature. Small differences in temperature can cause drifting of the device as well as focusing issues.
25. When choosing positions for imaging, avoid points that are close to one another, to minimize photobleaching and phototoxicity. Also choose the positions within a narrow region of the device, so as to avoid loss of immersion oil during movement of the stage.
26. The frequency of imaging is dependent on the exposure conditions. To minimize phototoxicity, decrease the exposure times when imaging multiple fluorescent channels or image the points less frequently. Mycobacteria growing on minimal medium are more sensitive to phototoxicity.

27. Hardware based autofocus regimes can be implemented instead of image-based autofocusing. Most microscope vendors now provide this option, for example—Olympus's Z-Drift Compensator, Nikon's Perfect Focus System, Zeiss's Definite Focus, Leica's Adaptive Focus Control.
28. ImageJ or Fiji has the capability of importing most file formats generated by different microscope vendors. Once imported the files can be saved as .TIF sequences. These image sequences can also be annotated in ImageJ and exported as movie files.
29. When quantifying fluorescence dynamics over time, it is important to make sure that the fluorescent lamp intensity variation is corrected for during analysis. Background subtraction should always be done. It is useful to have a different fluorescent protein expressed constitutively to serve as an internal control.
30. In case of mycobacteria, since the events of cytokinesis and cell separation are temporally distinct events, it is difficult to quantify cell division events or interdivision time using phase or cytoplasmic fluorescence imaging alone. Septation markers such as Wag31 [26] or membrane staining using fluorescent dyes should be used to better define these events.

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Chapter 15

Antimicrobial Susceptibility Testing for *Mycobacterium sp.*

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Abstract

The concept of antimicrobial susceptibility testing is an essential part of clinical microbiology. Antimicrobial testing has played a central role in the identification of new antibiotics and defining their clinical uses. Here we describe different approaches to determine the activity of compounds in medium- or high-throughput format.

Key words Antimicrobial susceptibility test, HTS whole cell assay, Minimum inhibitory concentration (MIC), Resazurin, ATP measurement and oxygen consumption

1 Introduction

Tuberculosis (TB) is one of the leading causes of death in the world. In addition, multidrug-resistant (MDR)-TB incidence is raising in many areas around the world with 5-10% being extensively drug resistant (XDR). The development of new types of antituberculous drugs is urgently needed [1]. Nowadays, most if not all of the known antitubercular have been identified through phenotypic assays, so the development of fast and reliable ways to determine the antitubercular activity of chemical entities is still a must. The methods described below have been adapted to screen the activity of thousands of compounds against *Mycobacterium tuberculosis* under replicating conditions [2].

In the dawn of microbiology, identification of substances that induced or blocked the growth of microorganisms led to the development of methods for the quantitative estimation of these growth-promoting or growth-inhibiting substances. Many references to antibiosis can be found in the works of Pasteur, Koch, and Paul Ehrlich. Their observations were supported by experimental evidence (from in vitro growth inhibition to animal protection studies), although they did not establish practical methodologies that were predictive of the clinical outcome.

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Since these original studies, the concept of antimicrobial susceptibility testing has become an essential part of clinical microbiology. Antimicrobial testing has played a central role in the identification of new antibiotics and defining their clinical uses; different institutions and researchers have collaborated in the development of standards and guidelines accepted by the vast majority of the scientific community working in the antibacterial field. In the case of first line and most second line agents, different semiautomatic methods have been developed to define the clinical cutoff based on bacterial susceptibility and the plasma concentrations obtained using current doses [3].

The main drawbacks for the development of a well-established method for antitubercular testing come from the physiology of the bacteria; on the one hand the slow rate of growth implies long incubation periods and on the other hand the complexity of the disease makes it very difficult to predict the clinical outcome based on a single in vitro assay. In the case of *M. tuberculosis*, a consensus regarding the medium pH, composition, carbon source, temperature, time of incubation strains has yet to be reached.

Most laboratories have solved the first issue by using different surrogates of bacterial growth; this has allowed a reduction in the testing time from a month to a week without affecting the reliability of the measure and also has made it possible to miniaturize the assay to be run in microtiter plates. In this search of new drugs, determining the Minimum Inhibitory Concentration (MIC) of each compound is a key tool.

In order to increase throughput, compounds can be evaluated in a single shot by defining a statically significant cutoff regarding growth inhibition. The surrogate of growth used for high-throughput screening (HTS) will have a great impact in the robustness of the assay.

The *Z*-factor is used to judge the robustness of the data obtained with a particular assay. The *Z*-factor is defined in terms of four parameters: the means and standard deviations of both the positive (p) and negative (n) controls (μ_p, σ_p, and μ_n, σ_n). Given these values, the *Z*-factor is defined as:

$$Z - \text{Factor} = \frac{1 \quad 3\left(\sigma_p + \sigma_n\right)}{\left|\mu_p - \mu_n\right|}$$

In our assays, the *Z*-factor lower limit had been established at 0.4. Plates with *Z*-factor values below this cutoff are rejected.

This chapter will describe different approaches to determine the antitubercular activity of a compound in liquid and solid medium and inside the THP1 cells. We have tested four different readouts as surrogates of growth: resazurin reduction, ATP measurement, oxygen consumption, and optical density (OD). The differences between the 4 different readouts of bacterial growth are summarized in Table 1.

Table 1
Choice of readout for monitoring mycobacterial growth

	Robustness	Interference	False +/– ves	Throughput	Price
Resazurin	√√	√√	√	√√	√√√√
ATP	√√√	√√	√	√√√√	√√
Oxigen	√√√	√√√	√√	√√√	√√
OD	√	√√√√	√√√	√	√√√√

1.1 Methods for Monitoring Growth

Resazurin is a nonfluorescent, blue dye that is reduced to the fluorescent, pink derivative (resorufin) by oxidoreductases within viable cells [4]. Although inexpensive, resazurin has some inconveniences, such a low signal–noise ratio and frequent problems associated with the reactivity of the dye and the compounds under study. Alternative methodologies with higher sensitivity amenable to be used in high-throughput format (384-well) are available. An alternative method which is also dependent on metabolic activity is measuring ATP levels using the "BacTiter-Glo™ Microbial Cell Viability Assay" [2, 5, 6].

M. tuberculosis is an obligate aerobe bacterium that uses oxygen as the terminal electron acceptor of the electron transport chain. The recent use of quenched-fluorescence oxygen sensing is an extremely simple, broadly applicable noninvasive assay that rapidly provides informative insights into oxygen consumption in cell culture models [7–11]. The intensity and lifetime of the emitted fluorescence varies inversely with oxygen concentration. Oxygen-sensing probes have been shown to be a simple, high-throughput way to evaluate oxygen consumption in isolated mitochondria in vitro [10, 11]. An oxygen sensitive fluorescent compound (*tris*-1,7-diphenyl-1,10 phenanthroline ruthenium (II) chloride) is embedded in a gas permeable and hydrophobic matrix permanently attached to the bottom of plates. Oxygen quenches fluorescence from the sensor at the bottom of the well. When oxygen is consumed, the biosensor fluoresces, providing a signal that can be correlated to cell growth. This system has been adapted to evaluate the growth of *M. tuberculosis* and it is the system most used in the clinic (BD BACTEC™ MGIT™) [12].

Finally, growth can also be determined by measuring OD of cultures. This is a very simple and cheap method. The big disadvantages of this method are the low signal–background ratio and the need of a careful mixing step before reading to avoid bubble formation.

1.2 Intracellular Activity

Human THP-1 Monocytes have been shown as a good model to study the intracellular stages of *M. tuberculosis* [13]. In this chapter we also describe an assay to determine the effect of the

compounds on H37Rv growing inside monocytes. The assay uses a luminescent strain of *M. tuberculosis* to infect the monocytes, and luciferase activity is then measured using a commercial reagent (Bright-Glo™). The reagent causes cell lysis and generates a luminescent signal which is proportional to the activity of luciferase present which in turn is proportional to the number of viable cells in the culture. The assay relies on the activity of a thermostable luciferase and in the properties of a buffer formulation for extracting luciferase from bacteria.

2 Materials

2.1 Plate Preparation

1. 96-well V-bottom plates.
2. DMSO (*see* **Note 1**).
3. 0.1 μg/mL rifampicin.
4. Antitubercular standards. Use a range of standards such as isoniazid, ethionamide, and fluoroquinolones.
5. Millex-GV syringe-driven filter units with a membrane pore size of 0.22 μm.

2.2 Preparation of Inoculum

1. *Mycobacterium* strain of interest, e.g., *M. tuberculosis H37Rv*, *Mycobacterium bovis BCG* Pasteur, *Mycobacterium smegmatis* mc^2155.
2. 7H9-ADC-Tyloxapol: dissolve 4.7 g Middlebrook 7H9 broth base in 900 mL deionized water, Add 5 mL of 10 % w/v Tyloxapol and 10 % Albumin–Dextrose–Catalase (ADC) enrichment (Becton Dickinson).
3. 25 cm^2 cell culture Flasks.

2.3 Measuring Growth Using Resazurin

1. 96-Well, flat bottom, microtiter plates.
2. Resazurin Solution: Dissolve one tablet of Resazurin Tablets in 30 mL of PBS. Sterilize by filtration (0.22 μm).
3. Fluorimeter, e.g., Spectramax M5.

2.4 Measuring Growth by ATP

1. Sterile 96-well or 384-well white plates.
2. Bactiter-GloTM Microbial Cell viability Assay reagent (Promega).
3. Luminometer, e.g., Spectramax M5.
4. Antitubercular standards. Use a range of standards such as isoniazid, ethionamide, and fluoroquinolones (Table 2).

2.5 Measuring Growth by Oxygen Consumption

1. 96/384-Well oxygen consumption plates (BD Biosciences).
2. Transparent film.
3. Fluorimeter, e.g., Spectramax M5.

Table 2
MIC_{90} of standards using either ATP or resazurin to monitor growth

		MIC (μg/mL)								
		INH	Rif	Eth	Strepto	Kan	Moxi	Oflo	Cipro	Line
ATP	*M. smegmatis mc²155*	36	8.77	0.51	0.82	2.12	ND	ND	ND	ND
	M. bovis BCG	0.23	0.0004	1.23	0.15	2.37	0.036	0.32	ND	0.39
	M. tuberculosis H37Rv	0.23	0.004	1.41	0.63	3.25	ND	ND	ND	ND
Resazurin	*M. smegmatis mc²155*	125	9	0.5	0.9	2.2	ND	ND	ND	ND
	M. bovis BCG	0.5	0.002	2.7	0.18	1.2	0.14	0.16	0.09	0.4
	M. tuberculosis H37Rv	0.25	0.002	2.5	1.25	2.5	0.06	0.6	0.5	0.6

2.6 Measuring Growth by Optical Density

1. Sterile 96-well or 384-well plates.
2. Spectrometer, e.g., Spectramax M5.

2.7 MIC on Solid Medium

1. 96-Well V-bottom plates.
2. 7H10 agar: dissolve 9.5 g Middlebrook 7H10 agar base in 450 mL water and add 10 % v/v oleic acid–albumin–dextrose–catalase (OADC) enrichment (Becton Dickinson).
3. Sterile 24-well plates, flat bottom with lid.

2.8 Measuring Intracellular MIC

1. *M. tuberculosis H37Rv* containing the *Photinus pyralis* luciferase gene (Hygromycin resistant plasmid).
2. 7H9-ADC-Tyloxapol (*see* Subheading 2.2, **item 2**).
3. 4 mm glass beads.
4. RPMI complete medium: RPMI 1640 HEPES modification, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 % fetal bovine serum.
5. RPMI plus 0.05 % Tyloxapol.
6. Monocytes (THP1) (ATCC® TIB-202™).
7. 490 cm^2 roller bottles.
8. RPMI plus PMA: add 20 nM of Phormol 12-myristate 13-acetate (PMA). Sterile 384-well white plate with tissue culture treated surface.
9. Bright-Glo™ Luciferase Assay System (Promega).
10. Luminometer.

2.9 Calculation of % Inhibition, MIC, pIC_{50}, and IC_{50}

1. Analysis software for 96-well plates, e.g., Grafit 5 (Erithacus Software Limited) or GraphPad Prism.
2. Analysis software for 384-well plates, e.g., ActivityBase (ID Business Solutions Limited).

3 Methods

3.1 Plate Preparation (Fig. 1)

1. Dispense stock solution of compound (*see* **Note 2**) in Column 1 and 13 of 384-well plates or in column 1 of 96-well plates (V bottom plates) (*see* **Note 3**). Not the dilution factor will be 1:100 for the final assay plates. Use the particular plate type specified for the readout you will be using.
2. Add DMSO to columns 2–5, 7–12, and 14–17, and 19–24 (columns 6 and 18 are reserved for controls) in 384-well plates or columns 2–10 in 96-well plates.
3. Perform 1:2 or 1:3 serial dilutions across the row from column 1–12 (missing column 6) and from column 13–24 (missing column 18) in DMSO for 384-well plates. Note that columns 6 and 18 are used for controls. For 96-well plates dilute across the row from column 1–10.
4. Add DMSO (control 1) to column 6 and add 0.1 μg/mL rifampicin in DMSO (control 2) to column 18 in 384-well plates; for 96-well plates add control 1 to column 11 and control 2 to column 12.

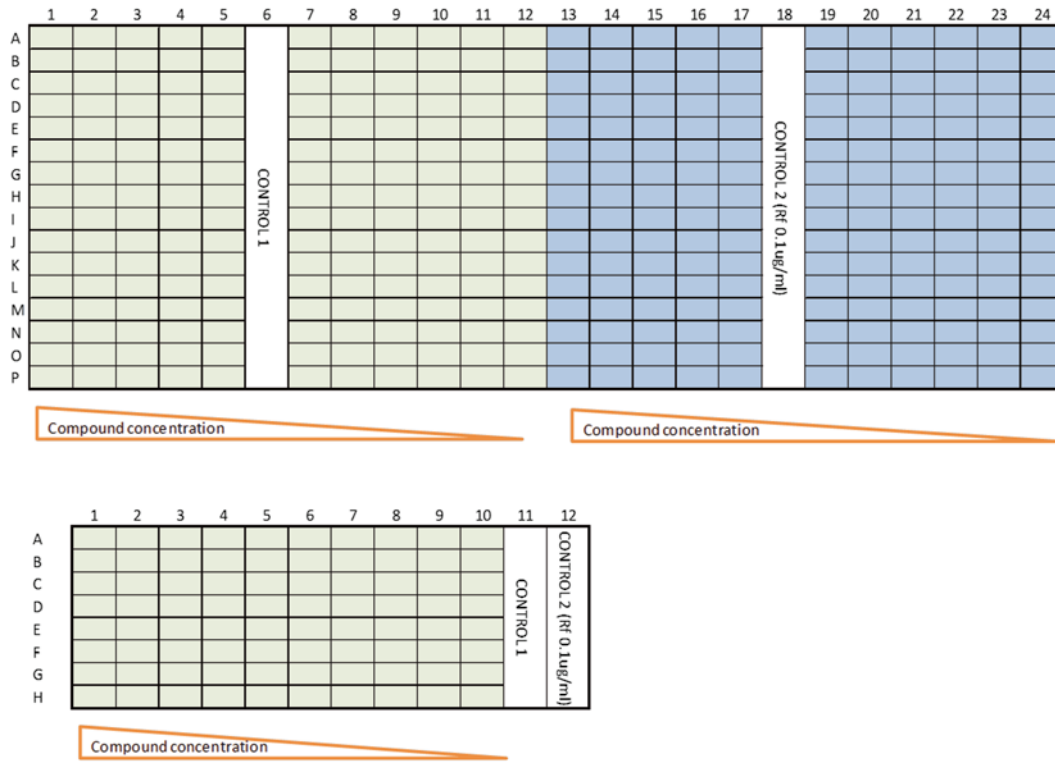

Fig. 1 Plate layouts. Plate layouts for both 384-well (**a**) and 96-well plates (**b**) are shown

5. Add isoniazid or another standard drug in one row as a control.
6. Prepare the assay plates, by transferring 250 nL of each well into the 384-well assay plates (or 2 μL for 96-well plates) (*see* **Note 4**).

3.2 Preparation of Inoculum

1. Culture *M. tuberculosis* H37Rv, *M. bovis* BCG, or *M. smegmatis* in 7H9-ADC-Tyloxapol in a 25 cm^2 flask (*see* **Note 5**).
2. Incubate at 37 °C for approximately 10 days (*M. tuberculosis* and *M. bovis BCG*) or for 3 days (*M. smegmatis*).
3. Check purity and subculture in 7H9-ADC-Tyloxapol to $OD_{600} = 0.01$ (*see* **Note 6**).
4. Incubate at 37 °C for 4–6 days (*M. tuberculosis* and *M. bovis BCG*) or 16 h (*M. smegmatis*).
5. Standardize the inoculum to approximately 1×10^7 CFU/mL by measuring the OD_{600} (*see* **Note 7**). Dilute 1:200 in 7H9-ADC-Tyloxapol so the final concentration is 5×10^4 CFU/mL.

3.3 Measuring Growth Using Resazurin

1. Dispense 200 μL of inoculum (from Subheading 3.2) to the entire 96-well compound plate (from Subheading 3.1).
2. Wrap the plates in aluminum foil and place them in a sealed box to prevent evaporation.
3. Incubate at 37 °C for 6 days for *M. tuberculosis* or BCG or 2 days for *M. smegmatis*.
4. Add 25 μL/well of resazurin solution (*see* **Note 8**).
5. Incubate at 37 °C for 2 days (*M. tuberculosis* and *M. bovis BCG*) or 1 day (*M. smegmatis*).
6. Measure fluorescence with Excitation 530 nm; Emission 590 nm (Cutoff 570 nm) (*see* **Notes 9** and **10**).
7. Analyze data as described in Subheading 3.9.

3.4 Measuring Growth by ATP

This assay measures growth by determining the amount of ATP, which is related to the number of metabolically active bacteria (*see* **Note 11**).

1. Dispense 200 μL (96-well plate) or 25 μL (384-well plate) of inoculum (from Subheading 3.2) to the entire compound plate (from Subheading 3.1).
2. Wrap the plates in aluminum foil and place them in a sealed box to prevent evaporation.
3. Incubate at 37 °C for 2 days (*M. smegmatis*) or 7 days (*M. bovis* BCG and *M. tuberculosis* H37Rv) in sealed box.
4. Remove the plates from the incubator and allow them to equilibrate at room temperature (*see* **Note 12**).

5. Add 25 μL of reconstituted BacTiter-Glo™ reagent to each well for 384-well plates and 100 μL for 96-well plates (*see* **Notes 13** and **14**).
6. Incubate at room temperature: 5 min for *M. smegmatis* mc^2155, 8 min for *M. bovis* BCG, and 30 min for *M. tuberculosis* H37Rv (*see* **Note 15**).
7. Measure luminescence; all wavelengths emitted with integration time 250 ms (endpoint).
8. Analyze data as described in Subheading 3.9.

3.5 Measuring Growth by Oxygen Consumption

1. Dispense 200 μL (96-well plate) or 25 μL (384-well plate) of inoculum (from Subheading 3.2) to the entire compound plate (from Subheading 3.1).
2. Cover plates with a transparent film and sterile lid.
3. Wrap the plates in aluminum foil to prevent evaporation and incubate at 37 °C for 7 days for *M. tuberculosis* and *M. bovis BCG* and 2 days for *M. smegmatis*.
4. Measure fluorescence using a bottom read, five readings per well at 485 nm excitation, 630 nm emission, 495 nm cutoff.

Analyze data as described in Subheading 3.9.

3.6 Measuring Growth by Optical Density

1. Dispense 200 μL (96-well plate) or 25 μL (384-well plate) of inoculum (from Subheading 3.2) to the entire compound plate (from Subheading 3.1).
2. Wrap the plates in aluminum foil and place them in a sealed box to prevent evaporation.
3. Incubate in a sealed box at 37 °C for 10 days (*M. tuberculosis* or *M. bovis BCG*) or 2 days (*M. smegmatis*).
4. Homogenize the culture by pipetting up and down; take much care not to introduce bubbles (*see* **Note 16**).
5. Read OD_{600}.
6. Analyze data as described in Subheading 3.9.

3.7 MIC on Solid Medium

1. Perform 1:2 serial dilutions of the compounds in 96-well V-bottom plates, from well 1 to well 11. Leave well 12 for a blank control.
2. Mix 10 μL of compounds from 96-well V-bottom plates with 1 mL of 7H10-0ADC (at 55 °C) (*see* **Note 17**).
3. Dispense into 24-well flat bottom plates in wells A1 to B5. Add 10 μL of DMSO in well B6 (growth control).
4. Spot 5 μL of culture at 1×10^5 CFU/mL onto the solidified media in wells A1 to B6.
5. Incubate for 2 weeks at 37 °C (*M. tuberculosis, M. bovis BCG*) or 3 days (*M. smegmatis*).

6. Count CFU.
7. Determine the MIC_{95} as the concentration of compound in the well where ≤5 CFU are present (95 % of growth inhibition).

3.8 Measuring Intracellular MIC

1. Culture *M. tuberculosis* H37Rv: *luc* in 7H9-ADC-Tyloxapol until the OD_{600} is 0.5–0.8.
2. Divide 160 mL of culture into 4 × 50 mL tubes and pellet at 4,000 × *g* for 10 min.
3. Add 10 4 mm glass beads and disperse the bacterial pellet of each tube by shaking for 60 s.
4. Add 6 mL of fresh RPMI medium and leave on the bench for 5 min (*see* **Note 18**).
5. Carefully decant 5 mL of the supernatant and discard the rest.
6. Collect all the supernatants into the same sterile tube and centrifuge at 750 × *g* for 5 min to avoid any remaining clumps; discard the pellet.
7. Measure OD_{600} of dispersed bacterial suspension using a 1:3 or 1:4 dilution into RPMI-0.05 % Tyloxapol.
8. Calculate the volume needed to have a multiplicity of infection (MOI) of 1, using the following conversion: An OD of $0.1 = 1 \times 10^7$ CFU/mL (*see* **Note 19**).
9. Prepare THP1 cells by maintaining them in complete RPMI1640 and incubate at 37 °C with 5 % CO_2.
10. Infect THP1 Monocytes (4×10^5 cell/mL) for 4 h in a roller bottle with a MOI of 1 in RPMI-PMA.
11. Remove excess bacteria by washing five times in complete RPMI (5 × 750 × *g*, 5 min).
12. Dispense 50 μL/well (10,000 cells/well) of infected THP1 cells in 384-well white plates with compound (*see* **Note 20**).
13. Incubate for 5 days at 37 °C 5 % CO_2.
14. Add 25 μL of reconstituted Bright-Glo™ to each well.
15. Incubate at room temperature for 30 min.
16. Measure luminescence (Kinetic Time: 2:00:00; Interval: 1:07; Reads: 108, Luminescence Integration: 500; Top read Emission Lm1 All Automix: Off Calibrate: Off PMT: Medium Settle Time: Off Column Priority C. Speed: Normal; Reads/Well: 1; Lag Time: 0:00; End Time: 2:00:00; RLU Min: 0 RLU Max: 500000).

3.9 Calculation of % Inhibition, MIC, pIC_{50}, and IC_{50}

The activity of a compound can be presented in different ways.

1. Calculate the % Inhibition: 100 × [(data − control1)/(control2 − control1)]; % remaining signal: 100 − % Inhibition. Control 1 is maximum activity, i.e., no inhibition of bacterial growth. Control 2 is complete inhibition of bacterial growth.

2. Calculate the MIC as the lowest concentration of the compound that inhibits at least 90 % of the growth (MIC_{90}) or 50 % of the growth (MIC_{50}).
3. Determine the pIC_{50} or IC_{50} as a nonlinear regression curve fit with program like Grafit 5 or GraphPad Prism for 96-well plates and with ActivityBase for 384-well plates.

4 Notes

1. When we have compounds of unknown solubility in the different solvents, we use DMSO to dissolve them. Heating the sample or a sonication bath can help to dissolve them.
2. The concentration of compound depends on the MIC for that compound. For example if the highest concentration that you have to test is 125 μM, you have to add 12.5 mM (dilution factor from master plate to assay plate is 1:100). The volume to add depends of the number of assay plates that you are to prepare and the volume required to perform the serial dilutions.
3. An alternative way to test more compounds is to assay at a single shot concentration. The master plate is prepared in the same way without performing the serial dilution. Add test compounds in each well of the plates. Normally this process is done when you have a lot of compounds with a low probability to be actives. Once the inactive compounds are discarded you perform the dose–response curves with the actives compounds to determine the exact MIC or pIC50.
4. The volume to use in the assay plate depends on the concentration of the master plate and the concentration that you have to test. The limitation is the concentration of the solvent that it is not toxic for the mycobacteria. The maximum concentration of DMSO that allows bacteria viability of mycobacteria is 5 %. In the case of the luminescence assay, DMSO could affect the signal depending on the luminometer that you use.
5. Stocks of bacteria are kept in 15–20 % glycerol at −80 °C. Bacterial cultures should not be passed many times to avoid selection and accumulation of spontaneous mutations in the strain.
6. A Ziehl–Neelsen stain can be useful to check the purity of the culture or plate the culture in non-mycobacterial selective agar plates, e.g., EJ, chocolate blood agar, or LB agar.
7. It is very important and useful to have the correlation between the OD and CFUs/mL. The most important factors in this ratio are the culture media, the culture conditions, and the fluorimeter. In our case this correlation is OD_{600} $0.125 = 1 \times 10^7$ CFUs/mL.

8. The signal–noise in the resazurin readout in 384-well plates is not very good so it is a readout to avoid with these kinds of plates.
9. In the resazurin method, the plates can be read visually. The MIC value is considered as the compound concentration in the first blue well (minimum concentration of compound that completely inhibits visible growth of the organism so the well where the resazurin (blue) has not been reduced to resorufin (pink).
10. Compounds may directly interfere with the readout by reducing the resazurin. In this case the MIC is calculated where the fluorescent signal reaches a plateau.
11. ATP is measured using a commercial reagent (BacTiter-Glo™). The reagent causes bacterial cell lysis and generates a luminescent signal which is proportional to the amount of ATP present. The assay relies on the activity of a thermostable luciferase and in the properties of a buffer formulation for extracting ATP from bacteria [6]. The signal for cell wall synthesis inhibitors is higher than the signal expected so the MIC for this kind of inhibitors could be underestimated. At sub-inhibitory concentrations cell wall inhibitors facilitate the bacterial break and ATP release.
12. It is essential to equilibrate the plates and the BacTiter-Glo™ reagent at room temperature to achieve better sensitivity. Insufficient equilibration leads to the decay of the luminescent signal.
13. We have observed variability in the signal depending on the batch of BacTiter-Glo™, so we advise to test each batch before starting a big HTS campaign in order to ensure the best results.
14. The amount of BacTiter-Glo™ reagent used has been optimized to develop the luminescent signal and save reagent. We use 10 μL.
15. The times of incubation established with the BacTiter-Glo™ reagent for the different mycobacteria species is crucial in order to obtain the best sensitivity. For each strain it is necessary to determine the time at which the highest level of luminescence is achieved. The first time this system is used with a new strain, perform a kinetic curve of luminescence to determine the optimal time.
16. Bubbles interfere with the readout. If bubbles have been produced, a quick centrifugation can remove them.
17. For the determination of the MIC on solid medium, compounds are added to molten medium after cooling it up to 50 °C so that the compound is homogenously distributed in the media. It is very important to be sure the temperature is correct in order not to inactivate the compound. For example, rifampicin is inactivated at high temperature, so we apply rifampicin on the top of solidified media.

18. In the single cell suspension step, after the glass bead treatment, it is important to add more volume than that needed finally. For instance, if you need 5 mL from every tube, add 6 mL and take only 5 mL. This tip will ensure that the bigger clumps are already discarded and the subsequent centrifugation will do the rest.
19. It is recommended to build standard curves of OD versus CFUs and Luminescence versus CFUs during the setup of the protocol with your conditions and luminometer and spectrophotometer. It is also possible to use pre-counted glycerol aliquots stored at –80 °C up to 3 months.
20. For THP1 cells the maximum tolerated concentration of DMSO is 0.5 %.

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Chapter 16

Determination of Compound Kill Kinetics Against *Mycobacterium tuberculosis*

Julie Early and Torey Alling

Abstract

In this chapter, we describe how to determine the kill kinetics and minimum bactericidal concentration (MBC) of a compound against *Mycobacterium tuberculosis*. Techniques are described for three conditions: actively growing aerobic bacteria, and non-replicating bacteria induced by nutrient starvation and/or low pH. Each technique involves determining the number of viable bacteria in the presence of several concentrations of compound over 3 weeks. Guidelines for how to interpret the results, to determine if growth-inhibitory compounds are bactericidal or bacteriostatic and also whether compounds exhibit time-dependent or concentration-dependent kill are provided.

Key words Minimum bactericidal concentration, Replicating, Non-replicating starvation, Time-dependent, Concentration-dependent, Low pH, *M. tuberculosis*

1 Introduction

Once a compound is known to have activity against bacteria, kill curve experiments are often the next step to determine if the compound is bacteriostatic or bactericidal, and what the kinetics of kill are. The minimum bactericidal concentration (MBC) is usually defined as the lowest concentration of compound needed to kill 3 logs of bacteria in 24 h under a given set of conditions [1]. The doubling time for most bacteria is far shorter (~20 min for *Escherichia coli*) than that of *Mycobacterium tuberculosis* (~24 h), and most antibiotics, such as rifampin and isoniazid work best on actively diving *M. tuberculosis* [2, 3]. It makes sense, therefore, that *M. tuberculosis* drugs often require longer incubation times than drugs used to treat other bacteria. For this reason, we define MBC as the lowest concentration of compound needed to kill 3 logs of *M. tuberculosis* in 21 days under the given conditions. Compounds are considered bactericidal if the MBC is no more than four times the minimum inhibitory concentration (MIC), and bacteriostatic if the MBC is more than four times the MIC [4].

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Compounds that are bactericidal may also exhibit time-dependent killing or concentration-dependent killing. For compounds that have time-dependent kill, any concentration at or above the MBC will result in a constant rate of kill of the bacteria [1, 5]. The length of time it takes to kill depends on the compound being tested, and can be as fast as a few days or a long as a few weeks in *M. tuberculosis*. In order to detect the kill at a variety of rates, this protocol monitors every 7 days over the course of 21 days. For compounds that are concentration dependent, the rate of kill will increase as the concentration of compound increases [1, 5]. The lowest concentration needed to kill the bacteria will vary for each compound. In order to detect kill with these compounds, this protocol tests at several concentrations at and above the MIC over the course of 21 days.

Understanding if a compound is time-dependent or concentration-dependent can influence downstream steps in a drug discovery program, especially relating to pharmacokinetic studies. A compound with time-dependent kill will work best if one can optimize the length of time that the serum concentration remains above the MIC [5]. In contrast, for a compound with concentration-dependent kill, area under the curve (AUC)/MIC and C_{max}/MIC ratios are the primary concepts to optimize [5].

Knowledge of the MBC is also helpful for physicians, because it can lead to a shorter course of antibiotics, and is especially useful for treatment of patients with compromised immune systems [1, 6]. The therapeutic use of bacteriostatic drugs requires an intact host immune system to clear the existing organisms. In contrast, drugs that are bactericidal and administered appropriately may be able to clear the existing organisms with very little aid from the immune system [6].

During the natural course of infection, *M. tuberculosis* is found in various states such as persistent and actively growing [7]. Some drugs are also bacteriostatic in one condition and bactericidal in another, or have different rates of kill depending on the condition [2, 8]. It is helpful, therefore, to test MBC under several conditions, especially conditions similar to the host. For that reason, MBC is sometimes determined in human sera [1, 9]. In this chapter, we describe methods to determine MBC for *M. tuberculosis* in three conditions: replicating in 7H9 broth, starvation, and low pH (*see* **Note 1**).

2 Materials

2.1 Preparation of Starting Culture

1. Complete 7H9 broth: Dissolve 4.7 g Middlebrook 7H9 powder in 900 mL distilled water, add 5 mL of 10 % w/v Tween 80 and then filter-sterilize with a 0.22 μm filter.

Aseptically add 100 mL of oleic acid–albumin–dextrose–catalase supplement (OADC) [Becton Dickinson] (*see* **Note 2**).

2. Conical tubes: 50 mL.
3. Roller bottles.
4. *M. tuberculosis* strain of interest.

2.2 Kill Kinetics Against Aerobic, Replicating Bacteria

1. Conical tubes: 50 mL.
2. Compound of interest.
3. DMSO.
4. Complete 7H9 broth (*see* Subheading 2.1, **item 1**).
5. 7H10 agar plates: Add 19 g Middlebrook 7H10 base to 900 mL distilled water. Autoclave for 15 min, then cool to 55 °C, add 100 mL OADC, swirl to mix before pouring into petri dishes (*see* **Note 3**).

2.3 Kill Kinetics Against Bacteria Under Nutrient Starvation

1. Conical tubes: 50 mL.
2. Compound of interest.
3. DMSO.
4. Phosphate-buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL of water. Adjust the pH to 7.4 with HCl and then add water to 1 L. Sterilize by autoclaving.
5. 10 % w/v Tyloxapol: warm distilled water and Tyloxapol, individually, to 55 °C. Add 10 mL of warmed Tyloxapol to 90 mL of warmed water, swirl to mix, and continue to heat until dissolved. Filter-sterilize with a 0.22 μm filter.
6. PBS–Tyloxapol: Add 5 mL 10 % Tyloxapol to 995 mL of PBS.
7. 7H10 agar plates (*see* Subheading 2.2, **item 5**).

2.4 Kill Kinetics Against Bacteria Under Acidic Conditions

1. Conical tubes: 50 mL.
2. Compound of interest.
3. DMSO.
4. Phosphate citrate buffer, pH 4.5: Combine 448 mL of 0.2 M dibasic sodium phosphate and 552 mL of 0.1 M citric acid. Adjust to pH 4.5 with 0.1 M citric Acid or 0.2 M dibasic sodium phosphate solution as needed. Add 5 mL 10 % Tyloxapol per L and check pH. Filter through 0.22 μm filter.
5. Phosphate citrate buffer, pH 6.8: Prepare 600 mL of 0.2 M dibasic sodium phosphate solution then slowly add 0.1 M citric acid solution while stirring and monitoring pH until it reaches 6.8. Add 10 % Tyloxapol to reach a final concentration of 0.05 % w/v and check pH. Filter through 0.22 μm filter.

3 Methods

3.1 Preparation of Starting Culture

1. Aseptically add 10 mL of complete 7H9 broth to 50 mL conical tube
2. Inoculate 50 mL conical tube(s) with test strain(s) of *M. tuberculosis* to theoretical $OD_{590} = 0.02$.
3. Incubate at 37 °C for 2–5 weeks until $OD_{590} = 0.6–1.0$.
4. Aseptically add 100 mL of complete 7H9 broth to a roller bottle.
5. Inoculate roller with 1–2 mL of late log phase culture ($OD_{590} = 0.6$-1.0) of *M. tuberculosis.*
6. Incubate with rolling at 37 °C until late log phase is reached ($OD_{590} = 0.6–1.0$), about 4–6 days (*see* **Note 4**).

3.2 Kill kinetics Against Aerobic, Respirating Bacteria

M. tuberculosis is exposed to varying concentrations of compound, and the viability is monitored over 21 days.

1. For each compound, label six 50 mL conical tubes; 5 with the compound and concentration that will be tested, 1 as DMSO control (*see* **Note 5**).
2. Add 5 mL of complete 7H9 broth to each tube.
3. Dilute the compounds in DMSO to 50× the desired final concentration (*see* **Note 6**).
4. Add 100 µL compound to each tube.
5. Add 100 µL of DMSO to the DMSO control tube(s).
6. Dilute late log-phase culture of *M. tuberculosis* ($OD_{590} = 0.6–1.0$) to $OD_{590} = 0.1$ with complete 7H9 broth.
7. Add 50 µL of culture ($OD_{590} = 0.1$) to each 5 mL sample to obtain ~ 10^5/mL *M. tuberculosis.*
8. Pipette up and down a few times to mix.
9. Incubate cultures at 37 °C standing.
10. Determine the colony forming units (CFU) in each tube on days 0, 7, 14, and 21 by following **steps 11–16** below.
11. Make five tenfold serial dilutions in 7H9 broth (50 µL sample + 450 µL complete 7H9 broth).
12. Plate 50 µL of the undiluted sample and each serial dilution onto one compartment (each) of 7H10 plates to plate to 10^{-6}.
13. Spread bacteria over the surface of the agar using plate spreaders.
14. Incubate plates at 37 °C with liquid on bottom for 24–48 h then flip plates over.
15. Incubate plates for 4–6 weeks at 37 °C, until colonies are large enough to count.

16. Count colonies to determine CFU/mL (*see* **Note 7**).
17. Plot CFU/mL versus time for each culture, including the lower limit of detection (20 for undiluted samples on plates with 2 compartments), and upper limit of detection (10^7).
18. The DMSO control should show growth of >1 log and the starting CFU should be ~10^5.
19. Calculate the MBC as the lowest concentration of compound resulting in 3 logs kill of *M. tuberculosis* over 21 days (*see* **Note 8**).

3.3 Kill Kinetics Against Bacteria Under Nutrient Starvation

M. tuberculosis is starved in PBS–Tyloxapol for 2 weeks prior to the addition of compound; the cell viability is measured over 21 days.

1. For each compound, label six 50 mL conical tubes; 5 with the compound and concentration that will be tested, 1 as DMSO control (*see* **Note 5**).
2. Add 5 mL of PBS–Tyloxapol to each tube (*see* **Note 9**).
3. Remove 5 mL of *M. tuberculosis* from a late log phase roller ($OD_{590} = 0.6–1.0$) (*see* **Note 4**).
4. Harvest cells by centrifuging at 3,725 × *g* for 10 min.
5. Resuspend pellet in 1 mL PBS–Tyloxapol then add 4 mL PBS–Tyloxapol (*see* **Note 10**).
6. Adjust to OD_{590} 0.1 with PBS–Tyloxapol.
7. Inoculate each compound tube and control with 50 μL culture.
8. Incubate at 37 °C standing for 14 days (*see* **Note 11**).
9. Dilute the compounds in DMSO to 50× the desired final concentration (*see* **Note 6**).
10. Add 100 μL compound to each tube.
11. Add 100 μL DMSO to the DMSO control tube.
12. Pipette up and down a few times to mix.
13. Incubate at 37 °C standing.
14. Determine the colony forming units (CFU) in each tube on days 0, 7, 14, and 21 by following **steps 15–20** below.
15. Make five tubes with tenfold serial dilutions in PBS–Tyloxapol (50 μL sample + 450 μL PBS–Tyloxapol).
16. Plate 50 μL of the undiluted sample and each dilution onto a compartment of 7H10 plates to plate to 10^{-6}.
17. Spread bacteria over the surface of the agar using plate spreaders.
18. Incubate plates at 37 °C with liquid on bottom for 24–48 h then flip plates over.
19. Incubate plates for 4–7 weeks at 37 °C, until colonies are large enough to count.

20. Count colonies to determine CFU/mL (*see* **Note 7**).
21. Plot CFU/mL versus time for each culture, including the lower limit of detection (20 for undiluted samples on plates with 2 compartments) and upper limit of detection (10^7).
22. The DMSO control should show ≤1 log drop in viability over 3 weeks, and the starting CFU should be ~10^5 (*see* **Note 12**).
23. Calculate the starvation MBC as the lowest concentration of compound resulting in 3 logs more kill than the DMSO control over 21 days.

3.4 Kill Kinetics Against Bacteria Under Acidic Conditions

M. tuberculosis is incubated in phosphate citrate buffer at pH of 4.5 and pH of 6.8. The cell viability is measured over 21 days.

1. For each compound prepare twelve 50 mL conical tubes (6 tubes for pH 4.5, including a DMSO control and 6 tubes for pH 6.8 including a DMSO control) (*see* **Note 5**).
2. Add 5 mL of phosphate citrate buffer at pH 4.5 to 6 tubes, and 5 mL of phosphate citrate buffer at pH 6.8 to the other 6 tubes (*see* **Note 9**).
3. Dilute the compounds in DMSO to 50× the desired final concentration (*see* **Note 6**).
4. Add 100 μL compound to each tube.
5. Add 100 μL of DMSO to the DMSO control tubes.
6. Measure OD_{590} of a late-log phase roller of *M. tuberculosis* ($OD_{590} = 0.6–1.0$) (*see* **Note 4**).
7. Harvest 10 mL of culture by centrifuging at 3,725 × *g* for 10 min and resuspend in 1 mL phosphate citrate buffer at pH 4.5 (*see* **Note 10**).
8. Harvest another 10 mL of culture by centrifuging at 3,725 × *g* for 10 min and resuspend in 1 mL phosphate citrate buffer at pH 6.8 (*see* **Note 10**).
9. Adjust each culture to $OD_{590} = 1.0$ using the appropriate phosphate citrate buffer
10. Inoculate each compound tube and DMSO control by adding 50 μL of adjusted culture (*see* **Note 13**).
11. Pipette up and down a few times to mix.
12. Incubate at 37 °C standing.
13. Determine the colony forming units (CFU) in each tube on days 0, 7, 14, and 21 by following **steps 14–19** below.
14. Make five tubes with tenfold serial dilutions in the appropriate phosphate-citrate buffer (50 μL sample + 450 μL buffer).
15. Plate 50 μL of the undiluted sample and each dilution onto a compartment of 7H10 plates to plate to 10^{-6}.
16. Spread bacteria over the surface of the agar using plate spreaders.

17. Incubate plates at 37 °C with liquid on bottom for 24–48 h then flip plates over.
18. Incubate plates for 4–7 weeks at 37 °C, until colonies are large enough to count.
19. Count colonies to determine CFU (*see* **Note 7**).
20. Plot CFU/mL versus time for each culture, including the lower limit of detection (20, for undiluted samples on plates with 2 compartments), and upper limit of detection (10^7).
21. The DMSO control should show ≤1 log of drop in viability over 3 weeks in both pH conditions and the starting CFU should be ~10^6/mL (*see* **Note 12**).
22. Calculate the MBC as the lowest concentration of compound resulting in 3 logs kill more than the DMSO control over 21 days. Compare and contrast the MBC of the two conditions tested (pH 4.5 and pH 6.8) to identify if the effect of the compound is pH specific (*see* **Note 14**).

3.5 Interpreting Results

1. Determine if the compound killed at least 3 logs of *M. tuberculosis* in 21 days at concentrations less than or equal to four times the MIC. If yes, the compound is bactericidal under the conditions tested; otherwise the compound is bacteriostatic under the conditions tested (*see* **Note 15**). Figure 1 is an example of a compound that is bacteriostatic and has no MBC under the conditions tested.

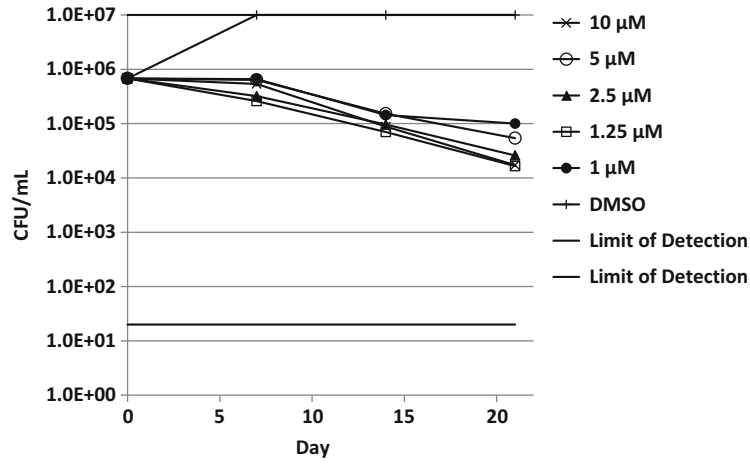

Fig. 1 Example of a bacteriostatic compound. Kill kinetics were determined under standard aerobic growth conditions. The compound minimum inhibitory concentration (MIC) was equal to 1 µM. Notice that the DMSO control grew, while all samples containing compound showed inhibition of growth, and even some kill. None of the concentrations tested show 3 logs of kill over 21 days, making this compound bacteriostatic

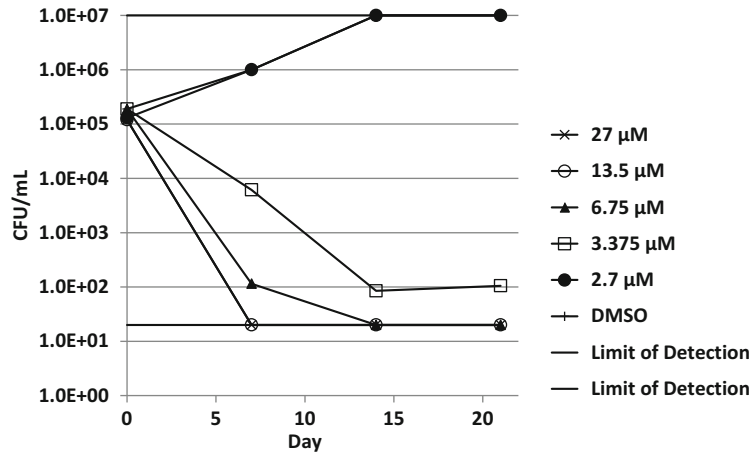

Fig. 2 Example of a compound with concentration-dependent kill. Kill kinetics were determined under standard aerobic growth conditions. The compound minimum inhibitory concentration (MIC) was equal to 2.7 μM. Notice that the DMSO control grew. In this case, the lowest concentration of compound tested also grew, which occasionally happens as MIC values have a twofold to threefold variation (*see* **Note 8**). The lowest concentration to kill 3 logs in 21 days (minimum bactericidal concentration) is 3.375 μM. This compound is bactericidal against *M. tuberculosis*, since it gave 3 logs of kill in 3 weeks, and the MBC is within fourfold of the MIC. This compound is also concentration dependent, as increasing concentrations of compound increased the rate of kill (*see* **Note 16**)

2. If the compound killed *M. tuberculosis*, determine if the rate of kill increases as the compound concentration increases. If yes, the compound exhibited concentration-dependent kill under the conditions tested. Figure 2 is an example of a compound with concentration-dependent kill. If the rate of kill was the same at all concentrations tested, the compound exhibited time-dependent kill. Figure 3 is an example of a compound with time-dependent kill (*see* **Note 17**). Occasionally compounds will kill *M. tuberculosis* so rapidly that it is hard to determine if the kill is time-dependent or concentration-dependent following the protocol described here. For those compounds, repeat the MBC as described except collect and plate for CFU at earlier time points (day 1, day 3, day 5, and day 7).

4 Notes

1. There are other methods for determining MBC with *M. tuberculosis*. Some researchers directly plate for colony forming units (CFU) after 4 (or 7) days of incubation with varying concentrations of compound [10, 11]. A similar method involves making a one to ten dilution from MIC plates containing serial dilutions of compound that are

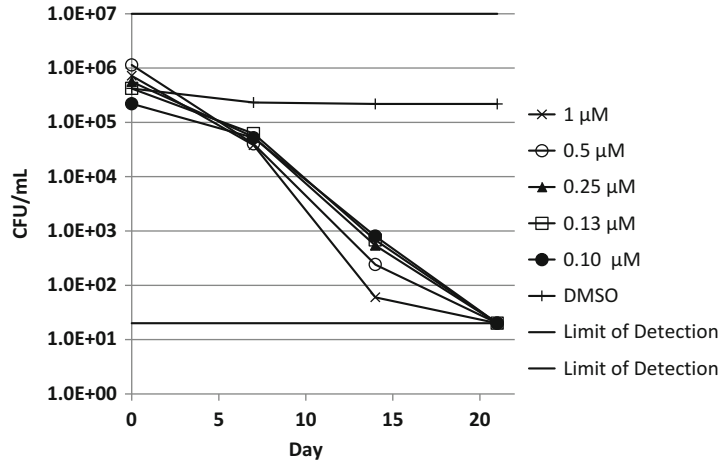

Fig. 3 Example of a compound with time-dependent kill. Kill kinetics were determined under starvation conditions. Notice that the DMSO control shows no growth and very little kill. This compound was bactericidal under these conditions, since it showed 3 logs of kill at concentrations within fourfold of the MIC (MIC=0.10 μM). In fact the MBC is less than or equal to 0.1 μM, the lowest concentration tested. All the concentrations tested show the same rate of kill, making the mode of kill time dependent (*see* **Note 16**)

incubated for 7 days, then plating for CFU. The MBC then corresponds to the concentration of compound in the well that shows three logs of reduced CFU compared to the inoculum [12]. Andreu et al. concluded that using luciferase as a measure of kill was an acceptable method, and potentially even preferred over CFU due to faster turn-around, while detection by resazurin was not reliable [13]. The MBC method presented in this chapter is similar to the method used by Mitchinson's group, except this protocol allows longer (3 weeks vs. 6 days) for the test compound(s) to work [2, 3].

2. This medium does not contain glycerol and is filtered for sterilization. 7H9 can also be autoclaved prior to the addition of OADC.
3. This medium does not contain glycerol. This protocol describes using petri dishes (plates) with two compartments to save space in the incubator, but can also be used with petri dishes that are not split into compartments.
4. If the OD_{590} reaches higher than 1.0, adjust by decanting some culture, and replenishing with fresh medium, then allow to grow for another 1–2 days to ensure that the starting culture of *M. tuberculosis* is in log phase, when it is most susceptible to antibiotics [2, 3].
5. For compounds soluble at 10 mM in DMSO with a MIC <20 μM, we test at 1× MIC, 1.25× MIC, 2.5× MIC, 5× MIC,

and 10× MIC. For compounds that are not soluble in DMSO at 10 mM or that have MIC >20 μM, test the concentrations that do not exceed 2 % DMSO in the samples, as DMSO in excess of 2 % kills *M. tuberculosis* under these conditions. MIC is the minimum inhibitory concentration determined in complete 7H9 broth.

6. This ensures the final DMSO concentration in all samples is 2 %. For example, if the desired final compound concentration is 10 μM in 5 mL, adjust the compound in DMSO to 0.5 mM. Use proper compound handling and storage techniques, avoid freeze thaws, keep away from light, and use fresh DMSO. Other solvents might also work, but would need to be individually tested.
7. The best practice is to be able to count 30–300 colonies per plate to determine CFU.
8. Ideally, the 1× MIC culture should show no growth over 3 weeks, but some variation in MIC is normal (twofold to threefold), so growth in the 1× MIC (and even 1.25× MIC) sample is sometimes observed. Additionally, MICs are usually determined with shorter incubation times than the 3 weeks of incubation used here.
9. Notice that these solutions contain 0.05 % Tyloxapol. Without Tyloxapol, the *M. tuberculosis* clumps, resulting in variation in the starting CFU and unreliable dilutions when plating.
10. Notice that the dispensing agent switches from Tween to Tyloxapol in this step. Tween can be used as a carbon source and is therefore avoided for starvation and low pH MBC *(14)*.
11. While other assays and research groups use longer incubation times for starvation conditions (4 weeks, 6 weeks, and longer), differences in MBC have been seen with as little as 2 weeks of pre-incubation in PBS–Tyloxapol.
12. Some of the *M. tuberculosis* will die with the DMSO control over the course of the assay in starvation and low pH MBC, but not more than 1 log. For that reason, each compound curve must be compared to the DMSO control in order to determine the MBC in comparison to the vehicle control.
13. Notice that the starting bacterial concentration for the low pH MBC is ~10^6 CFU/mL instead of 10^5 CFU/mL like the other assays described here. The recommended starting concentration for MBCs is 10^5 CFU/mL [1]. When this assay was tested without compound with a starting concentration of 10^5 CFU/mL, most of the *M. tuberculosis* died over the course of 3 weeks, suggesting an inoculum-specific dependence for survival.
14. Compounds that are only active at low pH will show kill in the pH 4.5 buffer and not the pH 6.8 buffer. Some compounds may be active in both conditions tested, and those are not pH-specific compounds.

15. Occasionally, some concentrations at the latest time point(s) will show an increase in CFU. This is likely due to growth of spontaneously resistant mutants, but could be due to other factors such as inactivation of the compound. Consider the lowest CFU observed for each concentration when determining if a compound is bactericidal or bacteriostatic.
16. Using the techniques described in this chapter, some compound will be carried over to the 7H10 plate while plating for CFU. This compound carry over can show lower than expected CFU values, even at time 0. We see this most frequently with compounds that have a time-dependent kill. The compound will usually be diluted enough during the plating process to avoid this issue with concentration-dependent compounds. If carryover is causing the decrease in CFU, the DMSO control will not show a decrease in expected CFU. Adding charcoal to the agar may negate compound carryover effects, but does reduce the growth rate.
17. Compounds that kill 3 logs only at the highest concentration tested will not allow you to determine the mode of kill, as no comparison in rate of kill at varying concentrations can be made.

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Chapter 17

Microplate Alamar Blue Assay (MABA) and Low Oxygen Recovery Assay (LORA) for *Mycobacterium tuberculosis*

Sanghyun Cho, Hyung Sup Lee, and Scott Franzblau

Abstract

Throughput in tuberculosis drug discovery was extremely limited prior to the introduction of microplate-based susceptibility assays. The 96-well Microplate Alamar Blue Assay (MABA) allows for the quantitative determination of drug susceptibility against any strain of replicating *Mycobacterium tuberculosis* to be completed within a week at minimal cost. The Low-Oxygen Recovery Assay (LORA) uses a recombinant *M. tuberculosis* expressing luciferase and provides results of drug activity against non-replicating *M. tuberculosis* surviving under hypoxic conditions. Determining activity against non-replicating *M. tuberculosis* is an important factor when developing drug candidates against *M. tuberculosis*. Here we describe a step-by-step procedure for both the MABA and LORA.

Key words Antimicrobial activity, Growth, Aerobic, Hypoxia, Minimum inhibitory concentration, Metabolic activity, Luminescent reporter

1 Introduction

Mycobacterium tuberculosis remains one of the leading infectious causes of mortality worldwide [1]. Along with the need for effective drug treatment for multidrug resistant or persistent *M. tuberculosis*, there is an urgent need for a rapid, sensitive, and cost-effective technique to test drug activity against *M. tuberculosis*. The determination of colony forming units (CFU) takes up to 2 months to obtain results [2, 3]. The BACTEC system, the Mycobacteria Growth Indicator Tubes, and the Etest are simpler and rapider, but the cost is high and it is impractical for clinical use in resource-poor settings [4–6] or for high throughput drug discovery applications. The microplate Alamar Blue assay (MABA) utilizes a 4-week incubation in microplates followed by addition of the redox dye resazurin which is dark blue and nonfluorescent in its oxidized form but becomes pink and fluorescent when reduced to resorufin as a result of cellular metabolism [7]. Therefore, the readout can be visual, colorimetric, or fluorometric. Drug susceptibility studies of

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lab-adapted strains and clinical isolates using resazurin-based assays have shown good correlation among CFU-based assays, the BACTEC 460 system, and MABA [8–11].

While MABA can be used to determine inhibition of actively growing *M. tuberculosis* under aerobic conditions, the Low Oxygen Recovery Assay (LORA) is used to test compound activity against non-replicating *M. tuberculosis* under hypoxic conditions. Non-replicating persisters are responsible for the long duration of treatment required to effect a durable cure in tuberculosis resulting in a focus on new drugs which target this phenotype [12, 13]. Different in vitro models have produced *M. tuberculosis* persisters by exposing them to lethal stress [14–17]. Until recently most drug susceptibility assays for non-replicating *M. tuberculosis* have relied on CFU enumerations [18, 19]. Firefly luciferase reporters have been used in microplate format thus demonstrating the potential for use in HTS applications [20]. The LORA uses a low-oxygen adapted inoculum that is subsequently exposed to test compounds in microplates for 10 days under hypoxic conditions followed by 24–28 h of normoxic "recovery." Viability is assessed by the ability to produce a luminescent signal following the recovery period. The microplate-based LORA is high-throughput screening (HTS)-compatible and is well correlated with CFU obtained immediately at the end of the hypoxic incubation, and thus, it can be used as for primary or secondary screening of compound libraries to quickly assess activity against non-replicating *M. tuberculosis*.

2 Materials

Prepare all solutions using filtered water (Millipore). All media need to be sterilized either by autoclave or filtration with 0.22 μM pore size filters. Prepared media should be protected from direct light and stored at 4 °C.

2.1 MABA Cell Stock Preparation

1. *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294).
2. 20 % w/v Tween 80: Dilute Tween 80 1:5 with dH_2O. Stir the solution until homogenous. Autoclave for 15 min at 121 °C.
3. 50 % v/v glycerol: Dilute glycerol 1:2 in H_2O and mix. Autoclave for 15 min at 121 °C.
4. Middlebrook 7H11 solid medium: Combine 8.4 g of 7H11 Middlebrook agar powder (Becton Dickinson), 360 mL of H_2O, and 4 mL of 50 % v/v glycerol. Autoclave for 15 min at 121 °C. Add 40 mL OADC when the temperature falls below 50 °C.
5. Middlebrook 7H9 broth medium: Combine 1.88 g of 7H9 Middlebrook broth base (Becton Dickinson), 360 mL of H_2O,

1 mL of 20 % w/v Tween 80, 1.6 mL of 50 % v/v glycerol. Autoclave for 15 min 121 °C. When the temperature falls below 50 °C, add 40 mL oleic acid, albumin, dextrose, and catalase supplement (OADC, Becton Dickinson).

6. Nephelo culture flask (500 mL).
7. Klett-Summerson colorimeter.
8. PBS.
9. PBS-Tween 80 (PBST): Mix 100 mL of 10× PBS, 0.5 mL of Tween 80, and 900 mL of H_2O. Autoclave for 15 min at 121 °C.
10. Blood agar plate: Tryptic Soy Agar with 5 % Sheep blood.
11. 8 μM Nucleopore filter membrane.

2.2 MABA

1. 20 % w/v Tween 80: *see* Subheading 2.1, **item 2**.
2. 7H12 medium (1 L): 4.7 g 7H9 Middlebrook broth base (Becton Dickinson), 1 g Bacto Casitone (Becton Dickinson), 5 g Bovine Serum Albumin. 5.6 mg palmitic acid in 1 mL ethanol, 4 mg catalase., 0.22 μm filter-sterilized, store at 4 °C, protect from light.
3. Quality control standard drugs (*see* **Note 1**): rifampin, isoniazid, metronidazole, capreomycin, and streptomycin. Refer to Table 1 for reference ranges.
4. Test compounds: in DMSO or other appropriate solvent at 100× of the highest desired final concentration.
5. Sterile 96-well clear plates.
6. Sonicator; bath or cup-horn type.
7. Blood agar plate: *see* Subheading 2.1, **item 10**.
8. 6-well plates; with each well containing 5 mL 7H11 agar (*see* Subheading 2.1, **item 4**).

Table 1
MABA quality control standard drugs with QC ranges

Compound	Solvent	Stock concentration (mM)	Range of MIC (μM)
Rifampin	DMSO	0.4	0.05–0.1
Isoniazid	H_2O	0.8	0.1–0.3
Metronidazole	DMSO	51.2	>512
Capreomycin	H_2O	3.2	0.4–1.2
Streptomycin	H_2O	1.6	0.3–0.9

9. Alamar Blue® (*see* **Note 2**) or resazurin sodium salt 0.6 mM in PBS: Measure out 13.75 mg of resazurin sodium salt and dissolve in 100 mL of 1× PBS. Use a 0.22 μm syringe filter to sterilize and keep at 4 °C. Prepared resazurin dye solution should be protected from direct light (stable up to 30 days).
10. Microplate fluorometer with 530 nm excitation and 590 nm emission filters.

2.3 LORA Cell Stock Preparation

1. Recombinant *M. tuberculosis* reporter strain harboring pFCA-luxAB [17] (*see* **Note 3**).
2. 20 % w/v Tween 80: *see* Subheading 2.1, **item 2**.
3. 50 % v/v glycerol: 20 % w/v Tween 80: *see* Subheading 2.1, **item 3**.
4. PBS, Tween 80 (PBST): Mix 100 mL of 10× PBS, 0.5 mL of Tween 80, and 900 mL of H_2O. Autoclave for 15 min at 121 °C.
5. Middlebrook 7H9 broth medium: *see* Subheading 2.1, **item 5**.
6. Middlebrook 7H11 solid medium: *see* Subheading 2.1, **item 4**.
7. Dubos Albumin: Dissolve 10 g of Bovine Serum Albumin Fraction V, 15 g dextrose and 1.8 g of NaCl in distilled water to make a final volume of 200 mL. Filter-sterilize, protect from light and store at 4 °C.
8. Dubos Broth Medium: Dissolve 4.75 g of Dubos Broth Base (Becton Dickinson) in 660 mL H_2O in a 1 L Erlenmeyer flask with screw cap (Pyrex 4985) containing a 50 mm Teflon-coated magnetic stirring bar. Autoclave for 15 min at 121 °C. Add 70 mL of Dubos Albumin when the temperature falls below 50 °C, resulting in a total volume of 733 mL.
9. Kanamycin: (20 mg/mL) stock. Add 733 μL of stock to 733 mL of Dubos Broth Medium to make a final concentration of 20 μg/mL.
10. Methylene blue: 500 μg/mL methylene blue in 1× PBS. Add 2.2 mL of stock to 733 mL of Dubos Broth Medium to make a final concentration of 1.5 μg/mL.
11. Blood agar plate: *see* Subheading 2.1, **item 10**.
12. Rubber septa capped Erlenmeyer culture flask (1 L).
13. Magnetic stir plate.
14. Microplate luminometer.

2.4 LORA

1. 7H12 medium: *see* Subheading 2.2, **item 2**.
2. White 96-well plates.
3. Quality control standard drugs (*see* **Note 1**): Rifampin, isoniazid, metronidazole, capreomycin, and streptomycin. Refer to Table 2 for reference ranges.

Table 2
LORA quality control standard drugs with QC ranges

Compound	Solvent	Stock concentration (mM)	Range of MIC (μM)
Rifampin	DMSO	1.6	0.5–2
Isoniazid	H_2O	102.4	>1.024
Metronidazole	DMSO	51.2	100–500
Capreomycin	H_2O	12.8	1–4
Streptomycin	H_2O	1.6	0.3–1.2

4. Test compounds: in DMSO or other appropriate solvent at 100× of the highest desired final concentration.
5. 6-well plates containing 5 mL 7H11 agar/well: *see* Subheading 2.1, **item 4**.
6. Anaerobic indicator strip.
7. Anaerobic jar.
8. Anoxomat.
9. A gas mixture of 10 % H_2, 5 % CO_2, and 85 % N_2.
10. 1 % *n*-decanal solution. Prepare a 10 % *n*-decanal solution in EtOH, and dilute tenfold in PBS to make 1 % *n*-decanal solution.
11. Microplate luminometer.

3 Methods

Carry out all procedures in a sterile environment such as a biological safety cabinet.

3.1 MABA Cell Stock Preparation

1. Prepare inoculum of *M. tuberculosis* H_{37}Rv seed stock (ATCC 27294) by suspending colonies from 7H11 agar in 200 mL 7H9 broth in a 500 ml Nephelo flask.
2. Grow until log phase (40–60 Klett units) (*see* **Note 4**).
3. Transfer to 50 mL conical centrifuge tubes.
4. Centrifuge at 4,000 × *g* for 10 min at 4 °C.
5. Discard supernatant.
6. Add 1 mL of 1× sterile PBS (PBS) into each 50 mL conical centrifuge tube.
7. Resuspend pellet by pipetting.
8. Fill each 50 mL tube with 30 mL of PBS.

9. Resuspend pellet in PBS by vortexing for 5 s.
10. Centrifuge at 4,000 × *g* for 10 min at 4 °C.
11. Discard supernatant.
12. Add 1 mL of PBS into each 50 ml conical centrifuge tube.
13. Resuspend pellet by pipetting.
14. Aliquot 200 μL of suspended cells into 1.5 mL sterile screw-cap micro-tubes.
15. Spare 200 μL for sterility testing on blood agar plate and determine CFU on 6-well plates after serial-dilution (of 10^{-4}–10^{-7}).
16. Label tubes, place in a storage box, and place at −80 °C (*see* **Note 5**).
17. Record Klett-units, tube numbers collected, test results of sterility and CFU, date, and location in freezer (−80 °C). Record on a log sheet (*see* **Note 6**).
18. Maintain seed stocks for up to 1 year.
19. Prepare inoculum of *M. tuberculosis* H_{37}Rv (ATCC 27294) by inoculating the contents of a tube of thawed frozen seed stock into a 200 mL 7H9 broth in a 500 mL Nephelo flask.
20. Grow until log phase (40–60 Klett units).
21. Transfer the culture into 50 mL conical centrifuge tubes.
22. Centrifuge at 4,000 × *g* for 10 min at 4 °C.
23. Discard supernatant.
24. Resuspend pellet in 1 mL PBS and dilute to 30 ml with PBS.
25. Centrifuge at 4,000 × *g* for 10 min at 4 °C.
26. Discard supernatant.
27. Resuspend pellet in 1 mL of PBS with 0.05 % Tween 80 (PBST).
28. Combine resuspended cells into one 50 mL tube by pipetting.
29. Add about 17 mL of PBST for every 100 mL culture.
30. Filter through 8 μM Nucleopore filter membrane; after filtering, cell stock will be clear (*see* **Note 7**).
31. Save about 200 μL for sterility test and plate for CFU after serial dilution of 10^{-4}–10^{-7}.
32. Dispense 500 μL aliquots of the MABA cell stock and store at −80 °C.
33. Record Klett-units, tubes collected, test results of sterility and CFU, date, and location in the freezer (−80 °C).
34. Maintain stock culture for up to 1 year.

3.2 MABA

1. Dispense 200 μL of 7H12 into all outer-perimeter wells of a 96-well plate to minimize evaporation of the medium in the test wells during incubation.
2. Add 200 μL of 7H12 to all wells of column 3.
3. Add 100 μL 7H12 to all other wells.
4. Add 2 μL of listed QC and/or test compounds drugs in columns 1 through 3, rows B through F in a 96-well plate (Table 1).
5. Prepare twofold serial dilution of drugs/compounds from column 3 to 10, remove 100 μL at the end of dilution of column 10.
6. Thaw frozen cell stock and sonicate minimally for 3–5 s at 20 % power to make an even suspension.
7. Dilute the culture with 7H12 to achieve density of between 5×10^4 and 1×10^5 CFU/ml (*see* **Note 8**).
8. Dispense 100 μL of culture into wells from column 2 through 11, rows B through G.
9. Incubate for 7 days at 37 °C in 5 % CO_2, 95 % humidity.
10. Streak the cell stock onto a blood agar plate.
11. Incubate at 37 °C for 1 week to check for contamination.
12. Prepare tenfold serial dilutions up to 10^{-7} and plate 50 μL of each dilution onto 6-well 7H11 agar plates. Incubate at 37 °C in CO_2 incubator for 3 weeks.
13. If bacterial or fungal colonies appear on the blood agar and 7H11 plates within a week, dispose of this batch of cell stock after autoclaving.
14. Count CFU on the 7H11 agar plates after 3 weeks and record.
15. Take out the 96-well plates from the incubator on day 7.
16. Mix in a ratio of 8:5 Alamar Blue (or 0.6 mM resazurin dye) and 20 % Tween 80 (for 1 plate, 2 mL of Alamar Blue (or 0.6 mM resazurin dye) and 1.25 mL 20 % Tween 80 are needed).
17. Add 32.5 μL of Alamar Blue (or 0.6 mM resazurin dye)—Tween 80 mix to all of the wells in the 96-well plates.
18. Incubate the plates for 18–24 h at 37 °C, 5 % CO_2, 95 % humidity.
19. Measure fluorescence at excitation 530 nm and emission 590 nm.
20. MIC_{90} is defined as the lowest concentration effecting a 90 % inhibition of fluorescence relative to the untreated bacterial control.

$$90\%\ \text{inhibition} = 0.1 \times \left(\text{Bacterial Control} - \text{Background}\right)$$

21. *See* Table 1 for Standard Drugs with Quality Control ranges.
22. If MIC of each standard drug falls within the acceptable range, record in the log sheet. If outside the range, repeat MIC QC once again, then dispose of drug stock if it fails to fall within acceptable range (*see* **Note 1**).

3.3 LORA Cell Stock Preparation

1. To prepare an initial seed stock, pick one colony of *M. tuberculosis* reporter strain harboring pFCA-luxAB from 7H11 agar plate and inoculate into 20 mL Middlebrook 7H9 medium plus kanamycin.
2. Incubate at 37 °C with shaking at 150 rpm for 5 days.
3. Transfer 2 mL of culture into 200 mL 7H9 medium plus kanamycin and incubate for 5–7 days.
4. Harvest at logarithmic phase when $OD_{570} = 0.2–0.4$.
5. Aliquot 1 mL of cell suspensions, label tubes, place in a storage box, and store at −80 °C.
6. Plate 100 μL for a sterility test on blood agar plate.
7. Take 100 μL and prepare serial dilutions in range of 10^{-4}–10^{-6} and plate on 7H11 plates.
8. Inoculate 770 mL Dubos broth medium plus kanamycin and methylene blue with an appropriate volume of a seed stock to yield 1×10^5 CFU/mL in an Erlenmeyer culture flask.
9. Close cap tightly with rubber septa cap and stir on magnetic plate at 120 rpm without perturbing the surface at 37 °C.
10. When medium color changes from blue to clear (typically 10–13 days), incubate for another 3–5 days (*see* **Note 9**).
11. Measure RLU by using a luminometer (*see* **Note 10**).
12. Harvest cells by centrifugation at $4{,}000 \times g$ at 4 °C for 10 min (*see* **Note 11**).
13. Resuspend pellet in 1 ml PBS and dilute to 30 mL PBS to each tube.
14. Recentrifuge for 10 min at $4{,}000 \times g$ and discard supernatant.
15. Resuspend pellet in 1 mL of PBS.
16. Dispense seed stock into 500 μL aliquots and maintain at −80 °C.
17. Maintain stock culture for up to 1 year.

3.4 LORA

1. Add 200 μL of 7H12 to all outer perimeter wells of 96-well white plates to minimize evaporation of the medium in the test wells during incubation.
2. Add 200 μL of 7H12 to all wells in column 3.
3. Fill all other wells with 100 μL 7H12.

4. Add 2 μL of listed QC drugs and/or test compounds in columns 1 through 3, rows B through F in a 96-well plate respectively (Table 2).
5. Twofold serial dilution of drugs/compounds from column 3 to 10, remove 100 μL at the end of dilution of column 10.
6. Thaw cell stock and suspend with minimal use of sonication.
7. Dilute the culture with 7H12 to achieve bacterial concentration between 2×10^5 and 1×10^6 CFU/mL (*see* **Note 8**).
8. Dispense 100 μL of culture into wells in column 2 through 11, rows B through G.
9. Serially dilute the bacterial suspension 10^{-4}–10^{-7} in 7H12 and inoculate 50 μL onto 6-well agar plates.
10. For sterility test, streak the bacterial suspension onto a blood agar plate.
11. Incubate blood agar plate and 7H11 agar plates at 37 °C in CO_2 incubator for 3 weeks.
12. Determine contamination by colony morphology and time of appearance on blood agar plate and determine CFU enumeration on 6-well 7H11 agar plates. If any other bacterial or fungal colonies appear on the blood agar and 7H11 plates within a week, dispose the batch after autoclaving.
13. Place the 96-well plate and an anaerobic indicator strip into an anaerobic jar and generate a hypoxic condition by using an Anoxomat with three cycles of evacuation and filling with an anaerobic gas mixture of 10 % H_2, 5 % CO_2, and 85 %N_2.
14. Incubate the anaerobic jar at 37 °C for 10 days.
15. After 10 days, remove the microplate from the anaerobic jar and transfer to a CO_2 incubator at 37 °C, 5 % CO_2, 95 % humidity for the 28-h normoxic "recovery."
16. Remove the 96-well plate from the incubator on day 11 (10 days of anaerobic incubation + 28 h aerobic recovery phase).
17. Dispense 100 μL of the 1 % *n*-decanal into each well using the pump in the luminometer.
18. Record the luminescence signal values and calculate MIC as in Subheading 3.2, **step 20** and compare to acceptable MIC ranges in Table 2.

4 Notes

1. Metronidazole is not active in MABA but is active in LORA while the opposite is true for isoniazid. So the response to these drugs can confirm that cultures are in replicating or

non-replicating phase, as they should be for MABA and LORA, respectively. All water solubilized drugs should be sterilized by filtration with 0.22 μM pore size filter. Other drugs could be added to the QC drugs listed.

2. Alamar Blue® is a commercial, proprietary reagent based on resazurin that is provided as a sterile, liquid reagent.
3. The recombinant strain of H_{37}Rv harboring pFCA-LuxAB is used in this assay. Other reporter strains can be used for LORA (e.g., luxABCDE) if they produce a sufficient signal to noise ratio [17, 21].
4. If bacterial growth to mid-log phase appears to be accelerated or delayed, there is the possibility of contamination with other bacteria or, low inoculum, or slow adaptation. In any case, QC will determine the quality of each batch. Any batches that do not result in MICs within the stated ranges should be disposed by autoclaving.
5. The volume of culture is sufficient for making inocula for the main culture and preparing seed stocks.
6. Typical fields to be recorded include: Starting culture date, harvest date, stock number or ID, seed stock number or ID, days of culture, sterility test, RFU or RLU (relative fluorescence/luminescence unit), CFU/mL, # of tubes prepared, storage location, and note.
7. Filtration with an 8 μM pore size filter is effective in removing clumps of *Mycobacteria* sp. and in making a single cell suspension [21, 22]. Tween 80 is omitted from 7H12 medium in order to prevent effect on drug activity [23, 24].
8. Target bacterial inocula densities are $5 \times 10^4 - 1 \times 10^5$ CFU/ml for MABA and $2 \times 10^5 - 4 \times 10^5$ CFU/mL for LORA.
9. Premature turbidity in the culture indicates the possibility of contamination, or a loosened cap allowing air into the culture flask. Typically the culture turns turbid after at least a week of incubation. Run QC for the evaluation of contamination if necessary.
10. The RLU level can approximate background noise if the culture is harvested too late. The harvested culture should be evaluated through QC to determine whether the strain adequately produces luminescence after the 28 h recovery phase. If not optimal (less than 500 RLU), it still can be saved as a backup seed stock for future cultures.
11. Storage at −80 °C and harvesting at 4 °C have been used to reduce possible re-adaptation to aerobic condition; however, a brief exposure to oxygen or ambient air does not appear to be sufficient to alter the physiological state of the culture [25].

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Chapter 18

A Multi-stress Model for High Throughput Screening Against Non-replicating *Mycobacterium tuberculosis*

Ben Gold, Thulasi Warrier, and Carl Nathan

Abstract

Models of non-replication help us understand the biology of persistent *Mycobacterium tuberculosis*. High throughput screening (HTS) against non-replicating *M. tuberculosis* may lead to identification of tool compounds that affect pathways on which bacterial survival depends in such states, and to development of drugs that can overcome phenotypic tolerance to conventional antimycobacterial agents, which are mostly active against replicating *M. tuberculosis*. We describe a multi-stress model of non-replication that mimics some of the microenvironmental conditions that *M. tuberculosis* faces in the host as adapted for HTS. The model includes acidic pH, mild hypoxia, a flux of nitric oxide and other reactive nitrogen intermediates arising from nitrite at low pH, and low concentrations of a fatty acid (butyrate) as a carbon source.

Key words *Mycobacterium tuberculosis*, Non-replication, Hypoxia, Acidic pH, Fatty acids, Nitric oxide, Reactive nitrogen species, Phenotypic tolerance, Antibiotics, High-throughput screening

1 Introduction

Multiple lines of evidence indicate that in murine or human tuberculosis (TB), populations of *Mycobacterium tuberculosis* persist in physiologic states that render them phenotypically resistant to most existing chemotherapeutics. Unlike genetically resistant *M. tuberculosis*, progeny of phenotypically resistant bacilli that are refractory to in vivo antibiotic treatment are drug-sensitive when tested in vitro under conditions that sustain replication [1].

Identification of compounds that kill non-replicating *M. tuberculosis* and their targets may shed light on how *M. tuberculosis* survives in a non-replicating state [1–5]. Although there are many ways to put *M. tuberculosis* into a non-replicating state, there is little validation as to which model(s) of non-replicating lead(s) to identification of compounds that kill persistent *M. tuberculosis* in an experimental host or in humans. At low pH, *M. tuberculosis* slows or halts replication and becomes sensitive to pyrazinamide.

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Pyrazinamide is effective in treating TB. The simplest non-replicating model, "Loebel," starves *M. tuberculosis* by prolonged incubation in a nutrient-free buffer such as PBS [6–8]. Starved *M. tuberculosis* are resistant to most known drugs used to treat *M. tuberculosis*, although they can still be killed by rifampicin and the phenothiazines trifluoperazine and chlorpromazine [9–11]. The Wayne model deprives an initially replicating *M. tuberculosis* culture of O_2 in a gradual manner that allows the cells to survive severe hypoxia [12–14]. *M. tuberculosis* rendered severely hypoxic in the Wayne model were resistant to isoniazid, rifampicin, and ciprofloxacin and sensitive to metronidazole and the combination of meropenem and clavulanate [14, 15]. Given metronidazole's activity on hypoxic *M. tuberculosis*, it was predicted that metronidazole would be effective against *M. tuberculosis* in guinea pigs, rabbits, and nonhuman primates, because immunostaining for pimonidazole adducts (a reaction dependent on the compound's reduction by an O_2-inhibited enzyme) indicated that their necrotic granulomas were hypoxic. In contrast, no activity was anticipated in mice, as their non-necrotizing lesions were not hypoxic [16]. As predicted, metronidazole lacked activity against *M. tuberculosis* in mice [17]. In rabbits, metronidazole killed *Mycobacterium bovis* BCG [16], and in cynomolgus macaques it prevented anti-TNF dependent relapse of latent TB [18]. However, metronidazole failed to kill *M. tuberculosis* in guinea pigs [19] and was ineffective in humans [20].

The case for conducting HTS against non-replicating *M. tuberculosis* was made over a decade ago [21] but development of an assay was problematic. The low levels of ATP in non-replicating *M. tuberculosis* [22] make it difficult to use ATP-based readouts as a measure of viability [23, 24]. An early innovation for a non-replicating HTS was the addition of a short outgrowth phase (28 h) after the non-replicating phase to improve the luciferase signal [24]. Building on this concept, we developed a multi-stress non-replicating assay for HTS by splitting the assay into two phases—a non-replicating phase and an outgrowth phase—and extending the duration of the latter (Fig. 1). In the first phase, a logarithmically replicating culture of *M. tuberculosis* is washed in PBS, dispensed into the non-replicating conditions described in Table 1, which are titrated to maintain CFU at a near-constant level, and incubated in the presence of DMSO as a negative control, rifampin or oxyphenbutazone as positive controls, or test compounds. After 3 days, an aliquot of the contents of each well is diluted into a new microplate that is cultured in replication-supporting conditions. *M. tuberculosis* surviving the drug treatment grow during the outgrowth phase, while dead cells fail to increase their biomass. Alternatively, a higher-throughput, less expensive version of the assay limits the dilution to fivefold and carries out both phases in the same plate. In either format, the binary readout is quantified by optical density after a 7- to 10-day outgrowth, allowing one to distinguish inactive from bactericidal compounds.

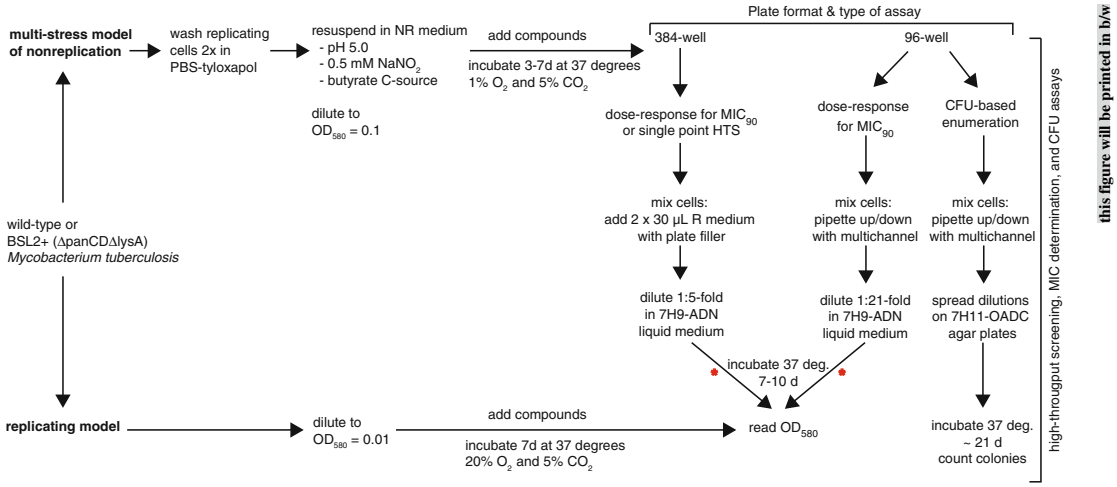

Fig. 1 Overview of the replicating and non-replicating assays. The *red asterisks* indicates the points of the non-replicating assays in which diluted test compound is still present during the replicating outgrowth phase and may result in false-positive hits

There are two drawbacks of this two-stage assay. First, the 3-day exposure to non-replicating conditions, followed by a 7- to 10-day outgrowth in replicating conditions, makes it imperative to limit evaporation and to adhere to strict sterility techniques. Second, compounds that are only active against replicating *M. tuberculosis* but are potent enough to kill *M. tuberculosis* after dilution into the outgrowth phase generate false-positive results. Thus, we typically run two assays in parallel: the two-phase non-replicating assay, and a one-phase assay in the outgrowth conditions alone (replicating assay). To distinguish the true dual-actives, we compare MIC_{90} values and calculate if the compound's potency in the replicating assay is high enough to potentially account for its apparent activity in the non-replicating assay. If this does not appear to be the case, we then confirm non-replicating activity in a two-stage assay conducted in concentration-response format, where the second stage is to conduct serial dilutions in PBS followed by plating on agar for determination of CFU. The extensive dilution preceding and accompanying the plating markedly diminishes the carryover effect of a compound that is only active against replicating *M. tuberculosis.*

The multi-stress non-replicating assay uses 4 conditions, each of which is likely to be pathophysiologically relevant and many of which *M. tuberculosis* is likely to encounter simultaneously (Table 1). (1) Mild acidity (pH 5), mimicking the pH encountered at inflammatory sites or in the phagosome of immunologically activated macrophages [25]. (2) Exposure to reactive nitrogen intermediates [2, 26]. This is conveniently accomplished by adding sodium nitrite (0.5 mM), which is slightly protonated at pH 5 to form nitrous acid, which dismutates and generates a small flux of nitric oxide and other reactive nitrogen intermediates.

Table 1
Comparison of assay conditions and composition of replicating and non-replicating media for the *Mycobacterium tuberculosis* wild-type and Δ*panCD*Δ*lysA* strains

	Replicating medium for *M. tuberculosis* wild-type	Replicating medium for *M. tuberculosis* Δ*panCD*Δ*lysA*	Non-replicating medium for *M. tuberculosis* wild-type	Non-replicating medium for *M. tuberculosis* Δ*panCD*Δ*lysA*
Starting inoculum	0.01	0.01	0.1	0.1
Time of exposure to drug for HTS (days)	7 days	7 days	3 days in non-replicating conditions; 7–10 days at 1/5 or 1/21 initial concentration in replicating medium during outgrowth	3 days in non-replicating conditions; 7–10 days at 1/5 or 1/21 initial concentration in replicating medium during outgrowth
Fold dilution	–	–	1 to 5 (384-well format) or 1 to 21 (96-well format)	1 to 5 (384-well format) or 1 to 21 (96-well format)
Time of replicating outgrowth prior to determining OD_{580}	–	–	7–10 days OG	7–10 days OG
Type of base medium	Middlebrook 7H9	Middlebrook 7H9	Sauton's	Sauton's
pH	6.6	6.6	5.0	5.0
Carbon source	Glycerol (0.2 %); dextrose (0.2 %)	Glycerol (0.5 %); dextrose (0.2 %)	Butyrate (0.05 %)	Butyrate (0.05 %)
Nitrogen source	Glutamate	Glutamate; CAS amino acids (0.5 %)	NH_4Cl and ferric ammonium citrate	NH_4Cl and ferric ammonium citrate
O_2	20 %	20 %	1 %	1 %
CO_2	5 %	5 %	5 %	5 %
Source of nitric oxide and reactive nitrogen intermediates	–	–	0.5 mM $NaNO_2$	0.5 mM $NaNO_2$
Supplements	–	CAS amino acids (0.5 %), OADC supplement, L-lysine (240 μg/mL); pantothenate (24 μg/mL)	–	L-lysine (240 μg/mL); pantothenate (24 μg/mL)
Shared conditions	BSA (0.5 %), NaCl (0.085 %), tyloxapol (0.02 %)			

This mimics not only the action of iNOS [26] but also *M. tuberculosis*'s reduction of nitrate to nitrite [27]. (3) Reduction of gas phase O_2 to 1 %, as *M. tuberculosis*'s environment is hypoxic within human macrophages in vitro [27] and in granulomatous lesions in the human host [16, 28]. (4) Fatty acid as a carbon source (0.05 % butyrate) [29, 30].

Even with this combination of four stresses or restrictions, the non-replicating assay omits other host-relevant conditions that can limit *M. tuberculosis*'s replication, such as deprivation of iron [31–33], magnesium [34], amino acids and vitamins [35, 36], intoxication by copper [37, 38], or exposure to reactive oxygen intermediates [39] or carbon monoxide [40]. The rationale for limiting the number of conditions is as follows. First, some compounds active against non-replicating *M. tuberculosis*, such as thioxothiazolidine inhibitors of dihydrolipoamide acyltransferase, appear to be equally active against *M. tuberculosis* no matter how the non-replicating state is imposed [2]. However, others, such as oxyphenbutazone, depend on one or more of the conditions for their activity [41]. To identify which component of the multi-stress model is important for a compound's activity requires testing the conditions in all combinations, which requires 2^N tests for N conditions (Table 2). Second, exposure to reactive nitrogen intermediates alone induces a transcriptional response in *M. tuberculosis* that encompasses the smaller sets of responses induced by most of the other conditions, including *M. tuberculosis*'s transcriptional response to residence in an activated macrophage [42].

Below we present a detailed description of the multi-stress model of non-replication in 96-well and 384-well formats. HTS conducted with this assay against over 1,000,000 compounds from academic and pharmaceutical libraries has identified hundreds of compounds with selective bactericidal activity against both non-replicating *M. tuberculosis* or with activities against replicating and non-replicating *M. tuberculosis* [41, 43].

2 Materials

2.1 Preparation of Non-replicating M. tuberculosis

1. *M. tuberculosis* wild-type H37Rv or Δ*panCD*Δ*lysA* (*see* **Note 1**).
2. Albumin-Dextrose-NaCl (ADN) 10× stock: mix 5 g BSA (*see* **Note 2**), 2 g D-glucose, 0.85 g NaCl and add ddH_2O to 100 mL. Filter-sterilize and wrap in foil at 4 °C.
3. Replicating medium for *M. tuberculosis* wild-type: dissolve 4.7 g 7H9 powder and 2 mL 100 % glycerol in 900 mL of ddH_2O (7H9 base medium). Autoclave and add 100 mL ADN or oleic acid–albumin–dextrose–catalase (OADC, which is required for the *M. tuberculosis* Δ*panCD*Δ*lysA* and optional for *M. tuberculosis* wild-type) and 10 mL 2 % Tyloxapol.

4. Replicating medium for the *M. tuberculosis* Δ*panCD*Δ*lysA*: To 7H9 complete replicating medium prepared with OADC supplement, mix an additional 6 mL 50 % glycerol in water, 10 mL of 20 % w/v CAS amino acids, 1 mL pantothenate 24 mg/mL and 3.13 mL of 80 mg/mL lysine.
5. Albumin-NaCl (AN) 10× stock: mix 5 g BSA (*see* **Note 2**), 0.85 g NaCl and add ddH_2O to 100 mL. Filter-sterilize and wrap in foil at 4 °C.
6. NH_4Cl 1,000× stock: dissolve 10 g NH_4Cl in 100 mL ddH_2O, and filter-sterilize.
7. $ZnSO_4$ 10,000× stock: dissolve 1 g $ZnSO_4$ in 100 mL ddH_2O, followed by filter sterilization.
8. 1 M $NaNO_2$: dissolve 69 mg $NaNO_2$ in 1 mL ddH_2O. Prepare a fresh stock of $NaNO_2$ the day of the experiment.
9. 2 % v/v Tyloxapol: dissolve 2 mL of 100 % Tyloxapol in 98 mL ddH_2O and filter-sterilize. Wrap in foil at 4 °C.
10. PBS–Tyloxapol (PBS-tyl): dissolve 10 mL of 2 % v/v Tyloxapol in 1 L Dulbecco's PBS without Ca^{2+} and Mg^{2+}. Filter-sterilize and wrap in foil at 4 °C.
11. Modified Sauton's base medium: dissolve 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.05 g ferric ammonium citrate in 900 mL ddH_2O. Prepare fresh and use immediately to prepare "non-replicating complete medium" without filter sterilization.
12. Non-replicating complete medium (without $NaNO_2$) for *M. tuberculosis* wild-type: mix 900 mL of modified Sauton's base medium, 100 mL of AN, 10 mL of 2 % Tyloxapol, 100 μL of 10 % w/v $ZnSO_4$, 1 mL of 10 % w/v NH_4Cl, and 500 μL of 99 % w/v butyrate. Adjust pH to 5.0 with 2 N NaOH. Filter-sterilize (*see* **Note 3**).
13. Non-replicating complete medium (without $NaNO_2$) for *M. tuberculosis* Δ*panCD*Δ*lysA*: supplement 1 L non-replicating medium (*see* Subheading 2.1, **item 12**) with 1 mL of 24 mg/mL pantothenate and 3.13 mL of 80 mg/mL lysine. Adjust pH to 5.0 with 2 N NaOH. Filter-sterilize (*see* **Note 3**).
14. Biospherix hypoxic chamber with ProOx 110 and $ProCO_2$ regulators or an O_2-regulated incubator (Binder GmbH) (*see* **Note 6**).
15. Spectrophotometer.

2.2 Non-replicating Protocol for 384-Well Format

1. *See* Subheading 2.1, **items 1–15**.
2. 384-well black, clear flat bottom, tissue culture treated microplates with lids (*see* **Note 4**).
3. Multidrop Combi Reagent Dispenser with small tube cassette.

4. Plastic containers with clip-lids that hold up to twelve 96- or 384-well lidded microplates in two stacks (approximately 10″ × 7″ × 5″; length × width × height) (*see* **Note 5**).

2.3 Non-replicating Protocol for 96-Well Format

1. *See* Subheading 2.1, **items 1–15**.
2. 96-well, sterile tissue-culture treated microplates with lids.

2.4 Enumeration of Colony Forming Units

1. *See* Subheading 2.1, **items 1–15**.
2. 96-well, sterile tissue-culture treated microplates.
3. 7H11-OADC agar: dissolve 21 g 7H11 powder and 5 mL 100 % glycerol in 900 mL of ddH_2O. Add a large stir bar. Autoclave, place flask on a magnetic stir plate set at medium speed, and allow medium to slowly cool to touch. Aseptically add 100 mL OADC supplement and gently mix to avoid introducing bubbles.
4. Tri-style Petri plates containing 7H11-OADC: In a sterile hood, using a sterile 25 mL pipette, dispense 8 mL of the 7H11-OADC into each section of the tri plate and allow to solidify. Store plates in sterile biosafety hood overnight to dry.

2.5 Comprehensive Non-replicating Stress Deconvolution

1. *See* Subheading 2.1, **items 1–15**.
2. NR deconvolution base medium: Prepare 500 mL NR complete medium as described in Subheading 2.1, **item 12** (for *M. tuberculosis* wild-type) or Subheading 2.1, **item 13** (for *M. tuberculosis* Δ*panCD*Δ*lysA*) with the following modifications: do not adjust pH and omit adding butyrate and $NaNO_2$.
3. NR deconvolution media A–H: Aliquot 40 mL of NR deconvolution base medium into 8 × 50 mL sterile tubes labeled A–H. Add 20 μL 99 % w/v butyrate to appropriate tubes according to Table 2. Adjust pH of all tubes to 5.0 or 7.0 according to Table 2. Filter-sterilize all eight deconvolution media. $NaNO_2$ is added to appropriate deconvolution media at 0.5 mM immediately prior to initiating experiment.

2.6 Identification of $NaNO_2$-Dependent Compounds

1. *See* Subheading 2.4.
2. 100 mM DETA-NO: Dissolve 16.3 mg (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO; CAS 146724-94-9) in 1 mL of 0.01 N NaOH and store at −80 °C. Dilute DETA-NO stock in 7H9 complete medium to prepare 100× stock solutions (10, 20 and 40 mM) prior to addition to *M. tuberculosis*.

2.7 Preparation of M. tuberculosis for Replicating Assays

1. *See* Subheading 2.1, **items 1–4**.

2.8 Replicating Assay Protocol for 384-Well Format

1. *See* Subheading 2.1, **items 1–4**.
2. 384-well black, clear flat bottom, tissue culture treated microplates with lids (*see* **Note 4**).
3. Multidrop Combi Reagent Dispenser with small tube cassette.
4. Plastic containers with clip-lids that hold up to twelve 96- or 384-well lidded microplates in two stacks (approximately 10″ × 7″ × 5″; length × width × height) (*see* **Note 5**).

2.9 Replicating Assay Protocol for 96-Well Format

1. *See* Subheading 2.1, **items 1–4**.
2. 96-well, sterile tissue-culture treated microplates with lids.

2.10 Data Analysis

1. Data analysis software such as Microsoft Excel, Prism 6.0 (GraphPad), or Collaborative Drug Discovery (CDD).
2. *See* Subheading 2.4 for CFU assays.

3 Methods

3.1 Preparation of Non-replicating M. tuberculosis

1. Grow *M. tuberculosis* (*see* **Notes 1** and 7) to mid-log phase ($OD_{580} = 0.4$ to 0.7) in strain-appropriate complete replicating medium in roller bottles.
2. Centrifuge cells at 3,098 × *g* for 8 min at RT at maximum deceleration and acceleration.
3. Resuspend the bacterial pellet in 1 mL PBS–tyloxapol. Add the original culture volume of PBS–tyloxapol. For example, if 45 mL culture volume was washed, the pellet should be resuspended to homogeneity first with 1 mL PBS-tyl, followed by an additional 44 mL PBS-tyl.
4. Wash cells one more time.
5. Resuspend pellet in 1 mL non-replicating medium. Add non-replicating medium to ~¾ of the original culture volume. For a 45 mL original culture, this is approximately 33 mL (*see* **Note 8**).
6. Remove 1 mL aliquot to measure OD_{580}. Adjust optical density of non-replicating-culture to $OD_{580} = 0.1$ using non-replicating medium. If these cells will be used to prepare a single cell suspension (Subheading 3.4, **step 1**), do not dilute the cells until after the slow-speed centrifugation to obtain single cells (*see* **Note 9**).
7. Add $NaNO_2$ to a final concentration of 0.5 mM from freshly prepared 1 M stock solution.
8. Allow cells to sit in non-replicating medium containing $NaNO_2$ for approximately 30 min at RT (*see* **Note 10**).

3.2 Non-replicating Protocol for 384-Well Format

1. Add 15 μL non-replicating *M. tuberculosis* to every well in 384-well assay plates using a MultiDrop Combi plate dispenser and a low volume cassette (*see* **Notes 11**).
2. Dispense 150 nL test compound in 100 % DMSO to achieve 1 % DMSO final (*see* **Notes 12** and **13**).
3. Add 150 nL control compounds oxyphenbutazone (final 100 μM) and/or rifampicin (final 10 μg/mL) or DMSO vehicle control (*see* **Note 14**).
4. Stack up to 3–4 microplates in plastic boxes with snap lids (a total of 6–8 plates) and move to a 37 °C hypoxic chamber set at 1 % O_2 and 5 % CO_2. Put a sterile cotton swab under the lid of the plastic box to prop open one of the four corners. Seal hypoxic chamber door with autoclave tape to ensure a tight seal and no gas leakage.
5. Incubate for 3 days without opening hypoxic chamber (*see* **Note 15**).
6. Remove microplates from hypoxic chamber. Using a plate filler, add 60 μL complete replicating medium by two rounds of 30 μL to initiate the outgrowth phase (*see* **Note 16**).
7. Dip the cotton portion of an individually wrapped wooden cotton swab (Puritan, sterile) into 70 % ethanol (approximately 10 mL in a 15 mL Falcon tube), remove excess ethanol out of the cotton by pushing the cotton against the sterile interior of the Falcon tube, and then use the tip to quickly wipe the entire surface of the 384-well plate. This step will remove any small drops of culture medium that may have sprayed onto the surface (*see* **Note 17**).
8. Stack the microplates in groups of three or four in a plastic container and prop open lid as described in Subheading 3.2, **step 4**. Incubate the outgrowth plates for 7 days at 20 % O_2 and 5 % CO_2.
9. At day 7, remove microplate lids and allow plates to sit uncovered for approximately 20 min (*see* **Notes 18** and **19**).
10. Read the OD_{580} of the entire plate (*see* **Note 20**). Due to the equation used to calculate % inhibition, it is unnecessary to sacrifice a well to blank the plate.

3.3 Non-replicating Protocol for 96-Well Format

1. Using a 12 channel multichannel pipette, add 200 μL non-replicating *M. tuberculosis* at an OD_{580} of 0.1 to a 96-well clear-bottomed, tissue culture treated microplate.
2. Add 2 μL test agents in DMSO (*see* **Note 13**). Mix up/down at least 5–10 times while stirring in a circular motion at the same time to ensure DMSO is adequately dissolved.

3. Add 2 μL control compounds oxyphenbutazone and rifampicin at 100 μM and 10 μg/mL, respectively, and 2 μL DMSO vehicle control (*see* **Note 14**).
4. Replace lid and incubate 3–7 days at 1 % O_2 and 20 % CO_2 (*see* **Note 21**).
5. At day 3 or 7, remove plate(s) from hypoxic chamber.
6. To set up a standard outgrowth (also referred to as a "two-plate assay"), add 200 μL fresh 7H9-ADN (for *M. tuberculosis* wild-type) or 7H9-OADC supplemented with glycerol, CAS amino acids, pantothenate, and lysine (for *M. tuberculosis* Δ*panCD*Δ*lysA*) to every well in a new 96-well microplate (this is called the "outgrowth plate").
7. Mix assay plate containing *M. tuberculosis* and test agents by pipetting 150 μL up and down ten times with a P200 while simultaneously using the tips to stir the well bottoms (*see* **Note 22**).
8. Transfer 10 μL of *M. tuberculosis* from the assay plate to the same well location on the outgrowth plate. Use a 12-channel multichannel pipette to avoid errors.
9. After each transfer, mix up and down 5–6 times using the same pipette tips.
10. Change tips after each set of 12 tips.
11. Place outgrowth plates into a Ziplock or similar food-storage bag with a zipper opening that is cracked open at one end. Incubate at 37 °C at 20 % O_2 and 5 % CO_2 for 7–10 days.
12. At days 7–10, remove plates from the bag in a biosafety hood. Remove lids of the microplates and allow liquid to equilibrate to room temperature for approximately 20 min.
13. Mix cells by pipetting 150 μL up and down five times as described in **step 7**. Pop any large bubbles with sterile tips.
14. Cover plate surface with an optical quality PCR adhesive sticker (Denville) and seal tightly. Read OD_{580} in a spectrophotometer.

3.4 Enumeration of Colony Forming Units

Determination of CFUs is the gold standard to enumerate viable bacteria. This assay is not subject to carryover of compound from an NR assay to the R recovery phase and provides a highly accurate assessment of a compound's activity on NR *M. tuberculosis*.

1. Prepare a single cell suspension of the non-replicating cells by a 10 min centrifugation at 123 × *g* with the brake turned off to maintain a tight pellet. Without disturbing the pellet, carefully transfer the single cell suspension to a new tube and measure the OD_{580} (*see* **Note 9**).

2. Dilute the single cell suspension of NR *M. tuberculosis* to an OD_{580} of 0.1 into NR medium.
3. To determine the starting inoculum, prepare triplicate tenfold serial dilutions from the tube containing non-replicating *M. tuberculosis* at an OD_{580} of 0.1 and spread 10 µL of 10^{-3}, 10^{-4}, and 10^{-5} dilutions on 7H11-OADC agar tri-plates. Incubate for 2.5–3 weeks at 37 °C at 20 % O_2 and 5 % CO_2 and enumerate colonies
4. Using a 12 channel multichannel pipette, add 200 µL of the single cell non-replicating *M. tuberculosis* at an OD_{580} of 0.1 to a 96-well clear-bottomed, tissue culture treated microplate.
5. In triplicate wells, add 2 µL test agents in DMSO (*see* **Note 13**). Mix up/down at least 5–10 times while stirring in a circular motion at the same time to ensure DMSO is adequately dissolved.
6. Add 2 µL control compounds oxyphenbutazone and rifampicin at 100 µM and 10 µg/mL, respectively, and 2 µL DMSO vehicle control (*see* **Note 14**).
7. Replace lid and incubate 3–7 days at 1 % O_2 and 20 % CO_2 (*see* **Note 21**).
8. At day 3 or 7, remove plate(s) from hypoxic chamber.
9. Mix assay plate containing *M. tuberculosis* and test agents by pipetting 150 µL up and down ten times with a P200 while simultaneously using the tips to stir the well bottoms (*see* **Note 22**).
10. Transfer 25–30 µL of triplicates from assay plate to new 96-well plates using wells A1–A12.
11. Make tenfold serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) from rows A to F by serial diluting 10 µL into 90 µL PBS-tyl or 7H9-ADN and mixing five times with a pipette set at 90 µL per dilution. Change tips with each dilution.
12. Spread 10 µL of each dilution on each section of two tri-style 7H11-OADC agar plates as groups of dilutions (10^0, 10^{-1}, 10^{-2} and 10^{-3}, 10^{-4}, 10^{-5}).
13. Incubate agar plates for 2.5–4 weeks at 37 °C at 20 % O_2 and 5 % CO_2 and enumerate colonies (*see* **Notes 23–25**).

3.5 Comprehensive Non-replicating Stress Deconvolution

Deconvolution assays are used to determine which of the stresses in the non-replicating-assay conspired with the test agent to kill non-replicating *M. tuberculosis*. This entails removing stresses one-by-one and inquiring if the test agent retains the ability to kill non-replicating *M. tuberculosis* (Table 2). A complete deconvolution queries 16 variations of the 4 stresses: pH, butyrate, nitrite and oxygen levels.

Table 2
Description of different media used to identify which condition, or combination of conditions, is responsible for an non-replicating-active small molecule to kill *M. tuberculosis* in the multi-stress model of non-replication

Deconvolution medium	pH	Butyrate	Nitrite	Oxygen (%)
a	5.0	Yes	Yes	1
b	5.0	Yes	No	1
c	5.0	No	Yes	1
d	5.0	No	No	1
e	7.0	Yes	Yes	1
f	7.0	Yes	No	1
g	7.0	No	Yes	1
h	7.0	No	No	1
a	5.0	Yes	Yes	20
b	5.0	Yes	No	20
c	5.0	No	Yes	20
d	5.0	No	No	20
e	7.0	Yes	Yes	20
f	7.0	Yes	No	20
g	7.0	No	Yes	20
h	7.0	No	No	20

Alternatively, a compound may kill non-replicating *M. tuberculosis* regardless of the media conditions. A total of 16 tests ($2N$ for N tests; thus, 2^4) are required to deconvolute four media conditions

1. Prepare eight different deconvolution media (a–h) in 40 mL volumes in 50 mL Falcon tubes as shown in Table 2.
2. Grow *M. tuberculosis* (*see* **Notes 1** and **7**) to mid-log phase ($OD_{580} = 0.4–0.7$) in strain-appropriate complete replicating medium in roller bottles.
3. Centrifuge cells at $3{,}098 \times g$ for 8 min at RT at maximum deceleration and acceleration.
4. Resuspend the bacterial pellet in 1 mL PBS–tyloxapol. Add the original culture volume of PBS–tyloxapol. For example, if 45 mL culture volume was washed, the pellet should be resuspended to homogeneity first with 1 mL PBS-tyl, followed by an additional 44 mL PBS-tyl.
5. Wash cells one more time and resuspend cells in PBS-tyl at an OD_{580} of 1.0.

6. Dilute washed cells 1–10 in deconvolution media a–h in 50 mL Falcon tubes to an $OD_{580} = 0.1$.
7. Add 0.5 mM $NaNO_2$ from 100 mM stock to appropriate tubes described in Table 2.
8. For each set of cells in a single deconvolution medium, pour cells into a reagent reservoir and dispense 200 µL cells per well in two 96-well plates using a 12-channel multichannel pipette.
9. Add test agents and DMSO controls as described in Subheading 3.3, **steps 2** and **3** to all 16 microplates.
10. Place one set of duplicate microplates (eight plates, media a–h) into the hypoxic chamber set at 1 % O_2 and 5 % CO_2, and place the other replicate set (eight plates, media a–h) into an incubator set at 20 % O_2 and 5 % CO_2 (Table 2).
11. On day 3, set up standard outgrowths for all plates as described in Subheading 3.3, **steps 6–14**.
12. Read OD_{580} at day 7–10.

3.6 Identification of $NaNO_2$-Dependent Compounds

If the compound's activity on non-replicating *M. tuberculosis* is determined to be $NaNO_2$-dependent (*see* **Note 26**), one can then explore if the compound gains activity against replicating *M. tuberculosis* in the presence of an NO donor at neutral pH, such as DETA-NO. In contrast to the constant flux of NO and reactive nitrogen species formed by $NaNO_2$ at an acidic pH, the short half-life of DETA-NO at neutral pH (~22 h at pH 7.4 at RT) requires daily addition.

1. Prepare a single cell suspension of mid-log *M. tuberculosis* in strain-appropriate 7H9 complete medium pH 6.6 at a density of $OD_{580} = 0.05$ as described in Subheading 3.4, **steps 1** and **2** (*see* **Note 9**). Determine inoculum as described in Subheading 3.4, **step 3** by plating serial dilutions on 7H11-OADC agar plates.
2. Dispense 200 µL cells/well in 96-well plates.
3. Add 2 µL of 100× stock solutions of test compounds and mix well.
4. Add 2 µL of the 100× DETA-NO stock solutions to appropriate wells approximately every 24 h (*see* **Note 27**). DETA-NO concentrations to test are 0, 100, 200 and 400 µM.
5. Add 2 µL of 0.01 N NaOH as a negative control.
6. Add 2 µL of 100× DETA as a negative control (*see* **Note 28**).
7. Plate for CFUs at the desired time points as described in Subheading 3.4, **steps 9–13**. Include 10^{-6} and 10^{-7} dilutions for wells containing vehicle control and those in which there was an obvious increase in optical density.

3.7 Preparation of M. tuberculosis for Replicating Assays

1. Grow *M. tuberculosis* wild-type or Δ*panCD*Δ*lysA* (*see* **Note 2**) to an OD_{580} of 0.4–0.7 in strain-appropriate replicating complete medium.
2. Dilute the exponentially growing culture to an OD_{580} of 0.01 in the same medium.

3.8 Replicating Assay Protocol for 384-Well Format

1. Using a MultiDrop Combi plate dispenser and a low volume cassette, add 50 μL of replicating cells in 7H9-ADN or 7H9-OADC (containing appropriate supplements for the *M. tuberculosis* Δ*panCD*Δ*lysA* strain) into a 384-well assay plate.
2. Dispense 500 nL compounds or DMSO controls directly into the wells containing *M. tuberculosis* (*see* **Note 29**).
3. Appropriate controls include those listed for the non-replicating assay in 96-well plates (*see* Subheading 3.3, **step 3**).
4. Place plates into plastic containers as described in Subheading 3.2, **step 8**.
5. Incubate plates for 7 days at 37 °C with 20 % O_2 and 5 % CO_2.
6. At day 7, remove plates from incubator. Remove lids and allow plates to equilibrate to room temperature for 20 min.
7. Replace lids and read OD_{580} (*see* **Note 30**).

3.9 Replicating Assay Protocol for 96-Well Format

1. Using a multichannel pipettor, dispense 200 μL replicating cells in 7H9-ADN or 7H9-OADC (containing appropriate supplements for the *M. tuberculosis* Δ*panCD*Δ*lysA* strain) from a reagent trough into a 96-well plate. Fill well A12 with cell-free R medium for blanking the plate when reading OD_{580} and fill the remaining 95 wells with R Mtb.
2. Add 2 μL compound or DMSO vehicle control to each well and mix up and down five times while and also stirring the tip along the well bottom in a circular motion. Keep columns 1 and 12 compound—and DMSO-free. Column 11 will contain the DMSO vehicle controls (rows A–H).
3. Use multiple positive controls that possess activity on replicating cells such as rifampicin, isoniazid, ethionamide, ethambutol, streptomycin, kanamycin, and PAS.
4. Replace the lid and incubate plates for 7 days at 37 °C with 20 % O_2 and 5 % CO_2. Place plates into plastic containers as described in Subheading 3.2, **step 8**.
5. On day 7, remove plates from incubator. Remove lids and allow plates to equilibrate to room temperature for 20 min.
6. Using a multichannel pipettor, gently mix cells by pipetting up and down, while moving the tip in a circular motion along the well bottom. Pop any bubbles using a clean tip.

7. For the *M. tuberculosis* Δ*panCD*Δ*lysA* strain, replace lids and read the OD_{580}. For the *M. tuberculosis* wild-type strain, replace the lid with an optical quality PCR sticker as described in Subheading 3.3, **step 14** and determine the OD_{580}.

3.10 Data Analysis

1. Convert data from OD_{580} to % growth inhibition by comparing data points to wells containing positive and negative controls. Calculate % inhibition as: $100 - (100 \times (OD_{[TEST\ AGENT]} - OD_{[CONTROL]})/(OD_{[DMSO]} - OD_{[CONTROL]})$ for each well, where: TEST AGENT = compounds being assayed; CONTROL = average optical density of wells containing rifampicin controls; and DMSO = average optical density of wells containing DMSO controls.
2. The minimal inhibitory concentration (MIC_{90}) is the lowest concentration of drug that leads to ≥90 % inhibition of growth.
3. Curve fitting, while not necessary, helps streamline high-throughput analysis of data. Curve fitting software that extrapolates MIC_{90} values includes Prism 6.0 (www.graphpad.com) and Collaborative Drug Discovery (www.collaborativedrug.com).
4. Calculate the ratio of the MIC_{90}-replicating and MIC_{90}-non-replicating (replicating/non-replicating, or, R/NR) (*see* **Note 31**).
5. If the non-replicating MIC_{90} was derived from the 384-well non-replicating assay format, use the following definitions: a replicating active has an R/NR of <0.2; a non-replicating active has an R/NR >5.0; and a candidate dual active R/NR of 0.2–5.
6. If the non-replicating MIC_{90} was derived from the 96-well non-replicating assay format, use the following definitions: a replicating active has an R/NR of <0.05; a non-replicating active has an R/NR >21; and a candidate dual active R/NR of 0.05–21.
7. Test candidate dual actives in a CFU-based assay (Subheading 3.4) to assess the compound's ability to kill non-replicating *M. tuberculosis*. Candidate dual active compounds that fail to kill non-replicating *M. tuberculosis* in a CFU-based readout appeared active in the non-replicating assay as a result of compound carryover in the replicating outgrowth phase of the assay.

4 Notes

1. *M. tuberculosis* wild-type (North or ATCC strains) or *M. tuberculosis* Δ*panCD*Δ*lysA* H37Rv ($mc^2$6220) can be used; the latter can be obtained from Dr. W. Jacobs, Jr. at the Albert Einstein College of Medicine.

2. Record manufacturer, type of BSA (protease-free or not; heat-shock or not) and lot number. The majority of problems we encountered with cell outgrowth have been traced back to BSA quality. We have occasionally observed that cells take longer than 7–10 days to achieve an OD_{580} of >0.4 in the outgrowth phase, or completely fail to outgrow. The one time cells completely failed to outgrow was traced back to the outgrowth medium, 7H9-ADN, which had been prepared with a faulty batch of BSA. We now recommend using Roche BSA Fraction V heat shock or the Middlebrook OADC supplement. OADC supplement is more expensive, but the benefits include slightly faster outgrowth and less variability.
3. Make sure to filter-sterilize medium components as directed in Subheading 2.1. These steps are essential to ensure components do not precipitate during autoclaving.
4. We prefer Greiner black, clear-bottomed tissue culture treated plates, catalogue number 781091. These plates encourage uniform *M. tuberculosis* growth across well bottoms, which is essential to obtain high-quality data when using an optical density readout.
5. Prior to use, wash all plastic containers that will hold 96- and 384-well plates in the incubators with 1 % Vesphene followed by a second rinse with Spor Klenz (Steris). Allow to air-dry.
6. Supplement autoclaved ddH_2O with "Water bath treatment" (Andwin, 190009) fungicide at 4 mL/L and use for water pans for hypoxic chambers and tissue culture incubators.
7. Do not passage strains more than one month to avoid clumping.
8. The exact volume is not essential because cells will ultimately be further diluted.
9. For CFU-based assays, an $OD_{580} = 0.1$ of a single-cell suspension is the equivalent of $\sim 5 \times 10^7$ CFU/mL.
10. Often, this step is unnecessary as the 30 min period overlaps the time spent dispensing the cells in microplates.
11. We have not yet adapted the non-replicating assay to 1,536- or 3,072-well plates but anticipate it may work with significant optimization to solve liquid evaporation.
12. When robotically dispensing compounds using tips, make sure to mix at least one time. Assay ready plates prepared by acoustically dispensing DMSO stocks of compound onto well bottoms of assay plates have worked well for HTS for our group. However, the facility housing the compounds must be maintained under extraordinarily clean conditions to maintain the sterility of the plates. When working with wild-type *M. tuberculosis*, assay plates are processed in a sterile glove box in the BSL3 facility. We always use filter tips to help control mold.

13. Sometimes a compound may precipitate or change color when it is added to the non-replicating *M. tuberculosis* cells, even though the same compound was assumed to be soluble because it had never precipitated in 7H9-ADN. Any time one observes a color change or precipitation in non-replicating-complete medium, the compound is probably chemically modified under these conditions (usually due to the acidic pH and/or reactive nitrogen species from $NaNO_2$).
14. Controls can be added to columns 1, 2, 23 and 24. We prefer columns 1 and 2 to contain DMSO as a vehicle control, while column 23 contains oxyphenbutazone and Column 24 contains rifampicin. One can have less than 16 replicates for each control compound, but a minimum of three wells are required to obtain reasonable Z′ scores. To date, one of the best non-replicating-specific compound controls is oxyphenbutazone, which requires acidic pH and $NaNO_2$ to kill *M. tuberculosis* in the 4-stress non-replicating model [41]. If oxyphenbutazone fails to kill non-replicating *M. tuberculosis*, ensure the pH of the non-replicating medium is 5.0 and that 0.5 mM $NaNO_2$ was added. Because a $NaNO_2$ stock may slowly degrade in water (ddH_2O from some sources may be acidic), it is important to use fresh $NaNO_2$ prepared the same day and kept on ice until ready to use. Alternatively, the pre-weighed $NaNO_2$ powder can be taken to the BSL3 and ddH_2O added immediately before use. Rifampicin can be used as a control for both replicating- and non-replicating-*M. tuberculosis*. Because the non-replicating-activity of rifampicin is significantly less potent than its activity against replicating *M. tuberculosis*, make sure to use rifampicin at concentrations higher than the replicating MIC_{90}. Isoniazid is inactive in the non-replicating model and serves as a negative control for CFU-based assays. Due to its potent replicating activity, INH will have some activity in the non-replicating assay by virtue of killing during the replicating outgrowth phase.
15. In addition to maintaining 1 % O_2, keeping the hypoxic chamber sealed will help prevent evaporation by maintaining high humidity.
16. This is not the same as one round of filling wells with 60 μL. We noticed that the larger volume leads to splashing of media and cells onto the plate surface.
17. The risk of mold contamination increases due to the nature of a long assay that has a 3 day drug exposure and 7–10 day outgrowth. It helps to periodically wipe tissue culture incubators with bleach and ethanol, autoclave plate filler cassettes, wipe outgrowth plate surfaces with 70 % ethanol, use a fungicide in incubator water baths, use 0.2 μm filters on all gas lines, and employ exceptionally sterile technique, including wiping the

surfaces of all reagents and equipment with 70 % ethanol prior to putting into the biosafety hood. We have also noted a higher rate of mold contamination using acoustically dispensed assay ready plates, likely due to the acoustic machine not being housed in a sterile hood.

18. The use of optical stickers for 384-well plates is not recommended for non-replicating assays. We found that removing the sticker to decontaminate the plate simultaneously draws the *M. tuberculosis* culture liquid out of the wells. This is likely related to the liquid of each well approaching the top of the well. This is a serious safety concern for those using wild-type *M. tuberculosis*.
19. Removing the lid, in combination with equilibrating the plate temperature to room air, allows one to replace the lid on the microplate without having condensation on the inner side of the lid. This is essential to read OD_{580}. If this is done with wild-type *M. tuberculosis*, the spectrophotometer has to be in a glovebox in the event of a spill. For *M. tuberculosis* Δ*panCD*Δ*lysA*, the spectrophotometer can be in the BSL2+-rated laboratory without extra precautions.
20. Sometimes we have seen that cells do not grow evenly across the bottom of our 384-well plates and this leads to the formation of a cell layer looking like "doughnuts" on the well bottoms. This is a difficult problem to solve. The best solution is to ensure you use "tissue-culture treated" microplates, which helps distribute the cell growth across well bottoms. Also, sometimes when the replicating outgrowth medium is added (as 2×30 μL), the 384-well microplate can be rotated 180° after the first 30 μL is added in an effort to help mix the cells adhering to the well bottoms. It helps to keep stacks as 3–4 plates to control media wicking to the well surfaces.
21. In setting up a time-kill experiment, please note that in the absence of a test agent, significant, multi-$\log_{10}$ kill may occur after a >7 day incubation under non-replicating conditions. We recommend testing times between 0–7 days; if you are interested in testing time points beyond 7 days, make sure to plate appropriate dilutions that take into account extra killing in control cells not exposed to test agent.
22. Even after vigorous mixing, we have observed cells by light microscopy adhering tightly to the well bottoms of 96-well plates. Extra care must be taken to ensure cells around the well perimeters are adequately mixed.
23. The addition of acidified $NaNO_2$ often results in colonies on agar plates that appear smaller than those that were not previously exposed to $NaNO_2$. Make sure after counting colonies to re-incubate the plates another week to ensure all surviving bacteria had adequate time to grow and be enumerated.

24. If the DMSO-treated cells show multi-$\log_{10}$ killing by day 7 (compared to the inoculum counts) by a CFU assay: (1) confirm the pH of non-replicating medium was 5.0 using a doubly calibrated pH meter (using pH 4.0 and 7.0 standards with a linear range >97 %) because $NaNO_2$ at pH < 5 will produce significantly more nitric oxide and reactive nitrogen species, which can kill *M. tuberculosis*; (2) make sure to use the recommended BSA or OADC; and (3) periodically check the hypoxic chamber calibration.
25. Another challenge in studying non-replicating *M. tuberculosis* is that there are forms called "viable but non-culturable" (VBNC) because they are undetectable as colony-forming units (CFU) on solid media. For example, in what is often called the "Cornell model", immunocompetent mice were infected with wild-type *M. tuberculosis*. After disease progression for 4 weeks, the mice were treated with isoniazid and pyrazinamide for 3 months. At that point no *M. tuberculosis* CFU could be recovered from lungs, liver or spleens in members of the cohort necropsied. Nonetheless, about a third of the remaining cohort eventually relapsed, and nearly all of them relapsed following injection with immunosuppressive corticosteroids [44–46]. The non-replicating assay system we describe does not detect VBNC *M. tuberculosis*. Instead, it deals with *M. tuberculosis* populations that yield nearly the same CFU at the beginning of the assay as at the end, except in the presence of non-replicating-active drugs and compounds.
26. To determine if a compound synergizes with nitric oxide and reactive oxygen species (formed by mixing $NaNO_2$ in acid), instead of using nitrite as a terminal electron acceptor, the activity of a compound should be tested against *M. tuberculosis* in non-replicating medium ±$NaNO_2$ (nitr*ite*) and ±$NaNO_3$ (nitr*ate*).
27. Due to the slightly more acidic pH, the half-life of DETA-NO at pH 6.6 will be shorter that at pH 7.4 (~22 h). To maintain a flux of nitric oxide, DETA-NO should be replenished daily.
28. Alternatively, one can use DETA-NO that was decomposed for 7 days at RT.
29. We favor using filter tips and adding one mix step (~7 μL up/down once) to ensure the compound has been completely dispensed from the tip. Alternatively, one may use microplates that already contain the acoustically dispensed compound on the well bottoms.
30. Alternative readouts may be used as a surrogate for viability, including Alamar blue, strains encoding reports for GFP, luciferase, etc. However, to make an accurate comparison of the activity of a compound against *M. tuberculosis* in replicating and non-replicating conditions, we favor using OD_{580} for both assays.

31. Since a compound is diluted fivefold in the outgrowth phase, a potent replicating-active molecule will appear to kill non-replicating cells during the replicating outgrowth phase (steps highlighted by red asterisks in Fig. 1). We have observed that numerous compounds are modified as a result of incubation in non-replicating medium containing $NaNO_2$. For example, oxyphenbutazone is hydroxylated on the pyrazolidinedione ring at the carbon atom that also serves to attach the butyl chain. The resulting 4-OH-oxyphenbutazone (4-OH-OPB) is an intermediate to forming an electrophilic, reactive species, 4-OH-OPB quinone imine, with a propensity to readily react with an available nucleophile such as *N*-acetyl-cysteine or mycothiol [41]. A medicinal chemist can determine if there are obvious structural alerts (toxicophores) and functionalities that may be reactive in acidic conditions in the presence or absence of reactive nitrogen species. The non-replicating-active molecule can be incubated at 37 °C in non-replicating medium ±0.5 mM $NaNO_2$ and analyzed at 24–72 h by LC-MS to determine what percentage of the starting material remains. One can try to purify any new species (although it is possible a new species does not ionize and may not be detectable) and test its replicating and non-replicating activity, as well as its toxicity to HepG2 cells.

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Chapter 19

Isolation and Characterization of Compound-Resistant Isolates of *Mycobacterium tuberculosis*

Theresa O'Malley and Eduard Melief

Abstract

This chapter describes the isolation and characterization of spontaneous resistant mutants of *Mycobacterium tuberculosis*. The overall objective of resistant mutant isolation is to determine the mode of action and/or cellular targets of new antimycobacterial agents. Whole-genome sequencing of resistant mutants can identify targets of antimycobacterial drugs and mechanisms of resistance, such as efflux, changes in drug permeability, or drug recognition. Mutants allow insight into in vivo biological processes and can help elucidate the number and identity of genes in a given pathway. Resistant mutant characterization can also lay the groundwork for structure/function studies, especially in conjunction with binding studies and X-ray crystallography.

Key words Resistant mutant isolation, Drug resistance, Target identification, Mechanism of action, Whole-genome sequencing

1 Introduction

A drug target is a cellular molecule, such as a protein or enzyme, involved in a particular metabolic pathway or signaling cascade. Target identification is one of the initial steps of modern drug discovery that allows for the development of target-based screens [1]. In addition, target identification can elucidate survival essential cellular pathways within a pathogen, of which multiple enzymes may be targeted [2–4]. A comprehensive understanding of a drug target and its function in the cellular pathway lowers the uncertainty associated with the development of a given drug. Chemistry can be used to tailor a drug to a given target, thereby improving protein binding as well as overcoming toxicity, reducing off-target effects, and improving bioavailability. However, there are relatively few well-characterized drug targets in *Mycobacterium tuberculosis*, and as a consequence, little is known about metabolic vulnerabilities of mycobacteria.

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Drug resistance in *M. tuberculosis* is generally mediated by chromosomal mutations (single substitutions, small insertions, deletions, IS*6110* transposition) that modify either the drug binding site of the target, i.e., *pcnA*, *rpoB*, *inhA* [5–7] or mutations in bacterial enzymes that activate prodrugs, i.e., *katG* [8]. Drug resistance can result from mutations in the target, leading to loss of drug binding affinity, or mutations in transcriptional regulators which lead to upregulation or downregulation of the target [9, 10]. Mutations in membrane proteins leading to decreased membrane permeability or upregulation of efflux pumps are other mechanisms whereby *M. tuberculosis* can acquire drug resistance [11, 12]. Isolation and characterization of resistant mutants has been utilized to identify a range of resistance mechanisms (drug targets, transcription factors, prodrug activators, and efflux) [13].

The ease of generating resistant mutants is tied closely to the compound activity, the concentration at which the compound both permeates the cell and binds to the target. In order to isolate resistant mutants, the minimum inhibitory concentration (MIC) on solid medium of a given compound is first determined by the agar proportion method. Resistant isolates of a wild-type strain are selected by growth on 5× MIC and 10× MIC compound. Resultant colonies are re-streaked onto medium with compound and a shift in MIC is confirmed. Genomic DNA is prepared from at least three resistant clones and analyzed by whole-genome sequencing. A schematic overview of the resistant mutant isolation and characterization process is given in Fig. 1. If mutations seen in all three mutants localize to a particular gene or operon, it is highly suggestive that the gene/operon in question is the target or mechanism of action of a given compound. Additional studies such as complementation or recombineering can be used to confirm that the mutations identified are truly responsible for the drug-resistant phenotype.

2 Materials

M. tuberculosis requires handling in a biosafety cabinet in a Biosafety Level 3 laboratory (*see* **Note 1**).

2.1 Determination of MIC in Agar

1. Middlebrook 7H9 liquid medium: dissolve 4.7 g of 7H9 powder (Difco) in 900 mL of deionized water, add 5 mL of 10 % v/v Tween 80, and autoclave. Add 10 % v/v OADC (oleic acid, albumin, dextrose, catalase) supplement (Becton Dickinson) prior to use.
2. Middlebrook 7H10 solid medium: dissolve 9.5 g of 7H10 agar base (Difco Laboratories, Detroit, MI) in 450 mL deionized water, autoclave, and cool to 50 °C before adding 50 mL OADC enrichment (oleic acid, albumin, dextrose, catalase). Swirl gently to avoid generating bubbles, use immediately or maintain at 50 °C for up to 1 week.

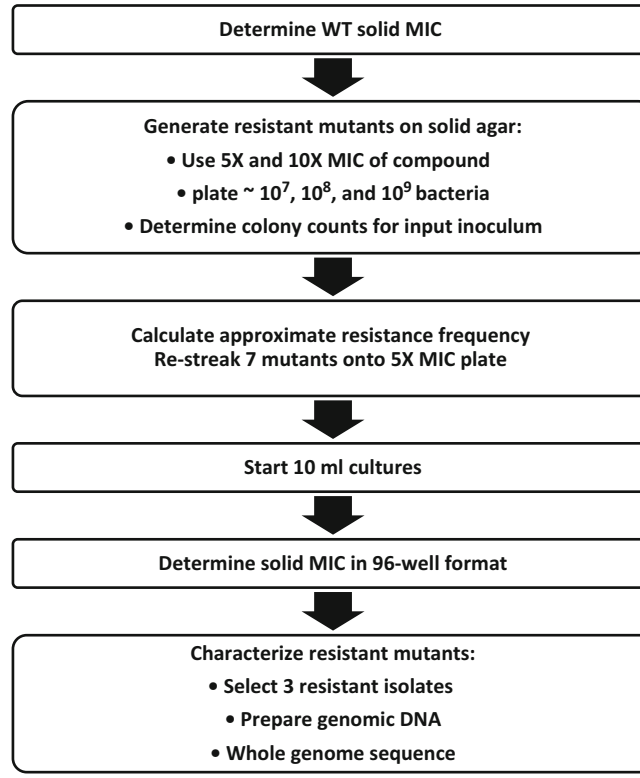

Fig. 1 Schematic overview of the resistant mutant isolation process

3. Compound stocks: 10 mM in DMSO. Dissolve compounds in DMSO and briefly vortex. Inspect for solubilization. If soluble, prepare aliquots and freeze at −20 °C (*see* **Note 2**).
4. Compound working stocks: prepare two working stocks of 1 and 0.1 mM. Prepare stocks by serial dilution of the 10 mM stocks by adding 5 μL stock solution to 45 μL DMSO in 12-tube PCR strip tubes (*see* **Note 3**, Fig. 2).
5. Compound serial dilutions: prepare three further twofold serial dilutions of each stock by adding add 20 μL of compound to 20 μL DMSO (Fig. 2).
6. Compound plates. Prepare a series of plates by adding 10 μL of each compound dilution to 10 mL molten agar in a 50 mL conical tube. Mix gently to avoid making bubbles and pour into one side of a two compartment (also called "I-style" or ½ plate petri dish). Use within 1 week (*see* **Note 4**).

2.2 Isolation of Resistant Mutants

1. DMSO.
2. Compound stocks: 10 mM.
3. Middlebrook 7H9 broth medium (*see* Subheading 2.1, **item 1**).
4. Middlebrook 7H10 solid medium (*see* Subheading 2.1, **item 2**).
5. Full size petri dishes (100 × 15 mm).

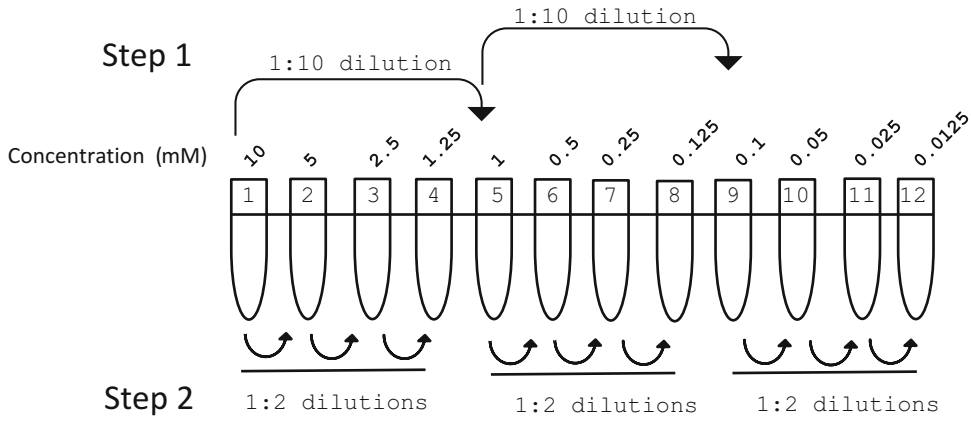

Fig. 2 Preparation of compound plates for use in standard agar assay. (**1**) A 10 mM compound stock is diluted in DMSO to give 1 and 0.1 mM working stocks. (**2**) Each working stock is serially diluted 1:2 in DMSO three times, yielding the indicated concentrations. Each dilution is added to molten agar and poured into I-style petri dishes resulting in a 12-point concentration curve

2.3 Solid MIC in 96-Well Format

1. DMSO.
2. Compound stocks: 10 mM.
3. 1 mM rifampicin in DMSO (*see* **Note 5**).
4. Middlebrook 7H9 broth medium (*see* Subheading 2.1, **item 1**).
5. Middlebrook 7H10 solid medium (*see* Subheading 2.1, **item 2**).
6. Sterile reagent reservoirs.
7. Clear bottom 96-well plates.
8. 15 mL conical tubes.
9. Compound serial dilution plate (staging plate) (*see* **Note 6**). Prepare a serial dilution in agar as follows: dispense 150 μL of molten 7H10-OADC into columns 2–10 of a 96-well plate on a heat block set to 60 °C. Prepare 100 μM compound (or 0.8 μM rifampicin for a rifampicin control plate) in 7H10-OADC; add 150 μL to column 2 and mix well. Serial dilute by transferring 150 μL from column 2 to column 3 and mix well by pipetting three times. Repeat serial dilutions through column 10.
10. Compound MIC plates. Dispense 150 μL of 7H10-OADC into columns 11 and 12 of a 96-well plate. Prepare 100 μM compound (or 0.8 μM rifampicin for a rifampicin control plate) in 7H10-OADC and dispense 150 μL in column 1. Transfer 140 μL of agar from column 10 of the staging plate to column 10 of the final control rifampicin plate. Without changing tips, continue transferring 140 μL from the serial dilution plate to same column of test plate all the way up to column 2 (*see* **Notes 6–8**). Allow agar to solidify overnight in a dark area at room temperature.

2.4 Genomic DNA Isolation

1. Middlebrook 7H9 broth medium (*see* Subheading 2.1, **item 10**).
2. 2 M glycine.
3. 490 cm^2 plastic roller bottles.
4. Screw-cap style 50 mL conical centrifuge tubes (Sarstedt, Sparks, NV, USA).
5. TE: 10 mM Tris, 1 mM ETDA, pH 8.0.
6. 2 mL standard screw cap tubes with O-ring (organic solvent resistant).
7. Chloroform–methanol (2:1).
8. 1 M Tris, pH 9.0.
9. Lysozyme.
10. 10 % w/v sodium dodecyl sulfate (SDS).
11. Proteinase K.
12. Phenol–chloroform–isoamyl alcohol (25:24:1).
13. 3 M sodium acetate, pH 5.2.
14. Molecular biology grade isopropanol.
15. 70 % molecular biology grade ethanol.

3 Methods

3.1 Determination of MIC in Agar

Solid MIC_{99} determinations are performed using the agar proportion method (modified from [14]). A 12-point concentration range starting at 10 μM and ending at 0.0125 μM prepared and inoculated with *M. tuberculosis* (Fig. 1: *see* **Note 9**).

1. Determine the OD_{590} of an actively growing culture of *M. tuberculosis* (*see* **Note 10**).
2. Prepare a cell suspension in Middlebrook 7H9 broth to achieve an OD_{590} to 0.1 (this corresponds to approximately 1×10^7 CFU/mL).
3. Make two dilutions in Middlebrook 7H9 broth to achieve 1×10^5 and 1×10^3 CFU/mL; the dilutions are the test and control suspensions, respectively.
4. Plate 50 μL of the control cell suspension onto compound free plate (this should yield a countable number of colonies).
5. Plate 50 μL of the test suspension onto compound free plate (this should yield a lawn of bacteria).
6. Plate 50 μL of the test suspension onto the compound containing set of I-plates.
7. Place 1–2 plates into sealable plastic bags.
8. Incubate plates at 37 °C for 3–4 weeks or until large colonies form on the compound-free control plates.

9. Record the number of colony forming units as follows:

   No colonies = 0.

   Less than 150 colonies = actual number of colonies.

   Greater than 150 colonies = +.

10. There should be approximately 50 CFU on the control plate and a lawn of growth on the test plate.
11. Calculate the percentage of resistant bacteria, i.e., 100× number of colonies on compound-containing medium divided by the number of colonies on the control plate.
12. The MIC_{99} is the concentration of drug which yields less than 1 % resistant bacteria (*see* **Note 11**).

3.2 Isolation of Resistant Mutants

1. Prepare three plates with 5× MIC and three plates with 10× MIC of compound (*see* **Note 12**).
2. Determine the OD_{590} of an actively growing *M. tuberculosis* culture. An $OD_{590} = 1$ is approximately 4.8×10^8 cells/mL (*see* **Note 13**).
3. Prepare serial ten-fold dilutions from 10^{-1} to 10^{-8} in 7H9 medium.
4. Plate 100 µL of the 10^{-4} through 10^{-8} dilutions onto nonselective medium to determine CFU in the inoculum (*see* **Note 14**).
5. Plate 100 µL of the undiluted suspension and the 10^{-1} and 10^{-2} dilutions onto compound-containing plates.
6. Spread cells evenly on the plates.
7. Incubate plates at 37 °C for approximately 4–6 weeks until colonies form (*see* **Notes 15–19**).
8. Prepare a single standard plate containing 5× MIC of compound (*see* Fig. 3).
9. Draw eight sections onto the underside of the plate.

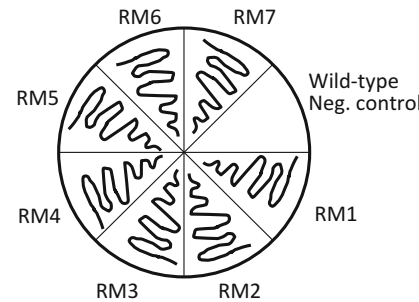

Fig. 3 Confirmation of resistance in solid medium. The wild-type parental strain should not grow on compound containing 7H10 whereas resistant mutants should. Liquid cultures are subsequently prepared from the resistant clones for confirmation and further characterization

10. Streak one section of the plate with the wild-type strain.
11. Streak up to seven resistant mutants in the remaining sections of the plate (*see* **Note 20**).
12. Place plate in sealable bag and incubate at 37 °C for 2–3 weeks.

3.3 MIC Determination in 96-Well Format

Resistant mutant confirmation is performed using a 96-well format in order to minimize compound usage (*see* Fig. 4). Up to seven putative mutants can be compared to wild-type parental control in a single 96-well plate (*see* **Note 8**).

1. Analyze growth on plates. Select mutants where streaks have grown on the 5× MIC plate, but the wild-type has not (*see* Fig. 3).
2. Inoculate 10 mL of 7H9 medium in 50 mL conical tubes with a loopful of the mutants of interest.
3. Incubate at 37 °C for approximately 2 weeks to reach mid to late log phase cultures (OD_{590} 0.5–08). Regular gentle swirling of culture tubes hastens bacterial growth.
4. Dilute to an OD_{590} of 0.1 in 7H9 medium.
5. Dilute the culture further to a theoretical OD_{590} of 0.01—approximately 10^6 CFU/mL.
6. Spot 5 μL of culture into columns 1–11 of each compound agar plate and the rifampicin control plate.
7. Make serial tenfold dilutions of the culture and plate 100 μL onto plain 7H10 agar to determine CFU/mL of the inoculum.
8. Place plates into sealable plastic bags and incubate at 37 °C for approximately 2 weeks.
9. The MIC is the lowest concentration of compound with no growth.

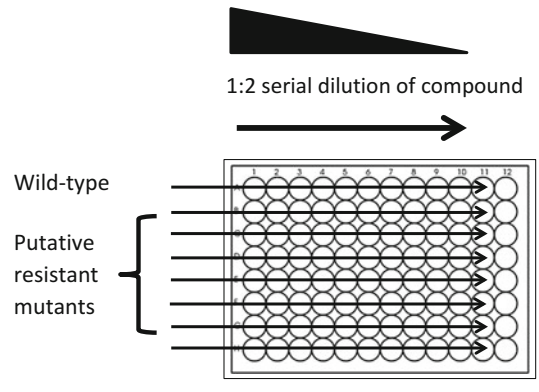

Fig. 4 MIC determination in a 96-well format using agar. A twofold dilution range of compound is prepared in agar in a 96-well plate. The plate is inoculated with the wild-type parental strain and potential resistant mutants. MIC is recorded as the lowest concentration completely inhibiting growth

10. The enumeration plates will take approximately 4 weeks before colonies are ready to count. The starting inoculum should be comparable between strains.

3.4 Genomic DNA Isolation

Select three mutants with a fourfold or greater shift in MIC for whole-genome sequencing (*see* **Notes 21** and **22**).

1. Inoculate 100 mL of 7H9 medium in roller bottle with *M. tuberculosis* strain and incubate until the OD_{590} is 1.0–1.4.
2. Add 10 mL of 2 M glycine and incubate for 16–24 h at 37 °C (*see* **Note 23**).
3. Harvest cells by centrifugation at ~2,600 × *g* for 10 min.
4. Wash cell pellet in 5 mL TE.
5. Store cell pellet at −20 °C for at least 4 h.
6. Thaw and resuspend cells in 0.5 mL TE and transfer to 2 mL screw-cap tube with an O-ring.
7. Add 0.5 mL 2:1 chloroform–methanol (*see* **Notes 24** and **25**) and vortex for 5 min.
8. Centrifuge at 2,500 × *g* for 20 min.
9. Remove both the aqueous and organic layers.
10. Dry the delipidated cells in a heating block at 55 °C for 10–15 min.
11. Resuspend pellet in 0.5 mL TE and transfer to fresh microfuge tube. At this stage no viable bacteria are present.
12. Add 0.1 volume of 1 M Tris, pH 9.0 containing 1 mg/mL lysozyme (do not vortex from this point on).
13. Incubate overnight at 37 °C.
14. Add 0.1 volume of 10 % SDS containing 1 mg/mL proteinase K and incubate at 55 °C for 3 h.
15. Add an equal volume phenol–chloroform–isoamyl alcohol and mix gently for 30 min on a rocking platform.
16. Centrifuge at 12,000 × *g* for 30 min and transfer aqueous (top) phase to fresh tube, extract with equal volume of chloroform.
17. Precipitate DNA with 0.1 volume of 3 M sodium acetate pH 5.2 and 1 volume of isopropanol for 1 h at 4 °C.
18. Centrifuge at 12,000 × *g* for 30 min at 4 °C, carefully decant and discard supernatant.
19. Add an equal volume of 70 % ethanol, centrifuge at 12,000 × *g* for 5 min, and decant/discard liquid.
20. Allow pellets to air-dry completely.
21. Resuspend in 100 μL TE (or buffer compatible with whole-genome sequencing protocols).

22. Determine DNA concentration and spectral readings.
23. Submit DNA samples to whole-genome sequencing facility for analysis (*see* **Note 26**).

4 Notes

1. Personnel require extensive training for handling *M. tuberculosis* and appropriate occupational health screening and monitoring.
2. Sonicate all nonhomogeneous stock solutions at room temperature for 15 min. Vortex and evaluate solubility. If stock solution is still not homogeneous, heat at 55 °C for 15 min, vortex and evaluate solubility. If homogeneity is not yet achieved, reduce concentration to 5 mM by adding equal volume of DMSO. Repeat previous steps if necessary until homogeneous solution is obtained. For some compounds, further dilution of stock solution concentration will be needed.
3. Use of PCR strip tubes for preparing compound working stocks and dilutions in the solid MIC assay simplifies plate preparation.
4. A major challenge with resistant mutant isolation is the quantity of compound used. Compound usage can by minimized by using I-plates for MIC determination sets as opposed to full plates. The solid MIC assay can also be scaled down to use X (quarter-sectioned) plates.
5. As a control, test resistant mutants for changes in sensitivity to rifampicin: a shift in the rifampicin MIC would suggest a general mechanism of resistance; such as upregulation of efflux pumps.
6. The preparation of serial dilutions in 7H10 agar is prone to generating bubbles during the mixing steps. To avoid bubbles in the growth plates, we prepare a serial dilution (staging) plate on a 60 °C heating block. Agar containing the compound is transferred into the final compound plate without carrying over bubbles. It is important to move quickly when making 96-well plates. Molten agar in pipette tips solidifies rapidly. There is no time to change tips between columns, so we begin with the lowest concentration of compound.
7. Beware of introducing bubbles into wells; repeated practice will minimize this issue.
8. The 96-well MIC assay also decreases the amount of time required for mutant confirmation from 4 weeks (standard MIC_{99} assay) to about 2 weeks.

9. Compounds may be chosen based on activity in a liquid MIC assay. However, liquid and solid MIC values do not always correlate; lawns of bacterial growth are sometimes obtained even using 10× liquid MIC selection. For this reason, compounds exhibiting potent activity in the liquid format must be tested for solid MIC prior to resistant mutant isolation.
10. Use an actively growing culture (OD_{590} between 0.6 and 0.9). Cells cultured in roller bottles, 5 mL stirred cultures, or in standing culture have all been used successfully.
11. Given the effort involved to isolate and characterize resistant mutants of *M. tuberculosis*, it is suggested that resistant mutant isolation attempts be limited to compounds with a solid MIC of 10–12.5 μM or less. This rule of thumb limits isolation efforts to the more potent compounds and avoids solubility issues.
12. If compound is limiting, prepare three I-plates with 5× MIC on one side and 10× MIC on the other side. In theory, 12-well compound containing plates could be used for resistant mutant isolation in order to minimize compound usage. However, it is difficult to streak isolated colonies from a 12-well format, thereby increasing the likelihood of isolating siblings. Standard plates are less prone than 12-well plates to drying out during the prolonged incubation necessary for the appearance of the slow growing *M. tuberculosis*. False positives (that fail to grow upon streaking onto compound plates) can also be problematic in 12-well plate selection.
13. Use of a fresh seed or glycerol stock is desirable in order to avoid accumulation of unwanted mutations.
14. Knowledge of the actual inoculum is required in order to calculate resistance frequency. Colonies typically appear within 4–6 weeks. However, some mutations have fitness costs and as such, colonies may take longer to appear.
15. If no colonies are obtained it is possible that mutation of the target is lethal or the compound is a broad spectrum toxin. It is recommended that mutant isolation attempts be repeated three times before abandoning efforts for resistant mutant isolation.
16. Increasing the original inoculum may result in appearance of more resistant colonies, but in our experience higher cell densities yield false positive colonies.
17. Use of mutagenized cells is an option if no colonies are obtained. Spontaneous mutants are preferred as they are less likely to have accumulation of unrelated mutations.
18. Clumping can protect the cells from a bactericidal agent. It may be necessary to dilute the cells prior to plating if the suspension is very dense. If cells are not diluted, it may be difficult to visualize true resistant colonies against a background lawn of sensitive cells.

19. Due to the long incubation times required for propagation of *M. tuberculosis* there may be issues with compound stability.
20. Colonies are preferentially chosen from the highest concentration in which they were obtained.
21. For each compound we select three resistant isolates for whole-genome sequencing, allowing cross-validation of the genetic polymorphisms observed.
22. Strains with only a twofold shift in MIC are unlikely to yield useful information on targets and in particular pose constraints on downstream confirmation using recombineering or gene overexpression.
23. Overnight treatment of the 100 mL roller culture with glycine improves the yield of genomic DNA.
24. Phenol is extremely toxic. Wear nitrile gloves when working with phenol.
25. Resuspending the pellet can be difficult. Resuspend as much of the pellet as possible in TE, transferring the suspension to a screw-capped microfuge tube. Add chloroform–methanol to resuspend any residual cell pellet in the original tube, transferring contents to the screw-capped tube.
26. Sample requirements (amount, purity, buffer composition, dry or liquid, shipping method, etc.) for whole-genome sequencing differ for each facility; determine these requirements prior to shipping.

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Chapter 20

Macrophage Infection Models for *Mycobacterium tuberculosis*

Benjamin K. Johnson and Robert B. Abramovitch

Abstract

Mycobacterium tuberculosis colonizes, survives, and grows inside macrophages. In vitro macrophage infection models, using both primary macrophages and cell lines, enable the characterization of the pathogen response to macrophage immune pressure and intracellular environmental cues. We describe methods to propagate and infect primary murine bone marrow-derived macrophages and J774 and THP-1 macrophage-like cell lines. We also present methods on the characterization of *M. tuberculosis* intracellular survival and the preparation of infected macrophages for imaging.

Key words Macrophage, Virulence, Infection model, Mycobacteria

1 Introduction

A hallmark of *Mycobacterium tuberculosis* pathogenesis is the ability to colonize, survive, and replicate within macrophages [1]. Understanding the mechanisms of *M. tuberculosis*–macrophage interactions promises to identify physiological pathways that are central to both bacterial virulence and host immunity. The macrophage niche represents a complex and dynamic environment that cannot be easily replicated by studying extracellular *M. tuberculosis* in bacterial growth media; therefore, several ex vivo *M. tuberculosis*–macrophage infection models have been established. Primary murine bone marrow-derived macrophages (BMDMs) have proven to be a useful model system because they can be easily cultured and expanded to large numbers of cells. Primary macrophages isolated from knockout mice enable the study of *M. tuberculosis* in macrophages lacking specific features of host immunity or to characterize the role of unknown host genes in the control of pathogens. Alternatively, immortalized cell-lines can be used to study *M. tuberculosis*–macrophage interactions, including the J774 and THP-1 cell lines [2, 3]. The response of *M. tuberculosis* to intracellular environments can vary greatly depending on the macrophage cell type

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chosen [4], primary or immortalized, human or murine, therefore, care should be taken when interpreting data from an individual cell type. *M. tuberculosis* macrophage infections can be used for a variety of purposes including characterization of: (1) mutant virulence, (2) antimicrobial efficacy, (3) microbial gene expression in response to immune pressure, (4) intracellular trafficking of microbes and host markers; and (5) the role of macrophage genes in the control of bacterial growth. Additionally, macrophage infection models are amenable to 96-well and 384-well plate formats, enabling high throughput assays of *M. tuberculosis* surivival when using fluorescent or luminescent *M. tuberculosis* reporter strains. In this chapter, we describe: (1) methods to isolate and propagate primary murine BMDMs and the J774 and THP-1 macrophage-like cell lines, (2) methods for infection of macrophages with *M. tuberculosis*, (3) assaying *M. tuberculosis* survival in macrophages, and (4) the preparation of infected macrophages for imaging.

2 Materials

2.1 Isolation of Bone Marrow from Mice

1. Bone marrow macrophage medium (500 mL) (*see* **Note 1**): 335 mL Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, 5 mL of 100 mM sodium pyruvate, 5 mL of 200 mM L-glutamine, 5 mL of 100× penicillin (10,000 IU/mL)–streptomycin (10,000 μg/mL), 50 mL of heat-inactivated, low endotoxin fetal bovine serum (FBS) (e.g., Hyclone™ FBS Thermo Scientific), 100 mL of L-cell conditioned medium (*see* **Note 2**). Sterilize through a 0.22 μm filter.
2. DMEM with 4.5 g/L glucose (no sodium pyruvate or L-glutamine).
3. 100 mm petri dishes.
4. 50 mL sterile conical tubes.
5. 23 gauge × 1" (23 g1) syringe needle.
6. 10 mL Luer lock syringe.
7. Small scissors and forceps.

2.2 Expansion and Passaging of Primary Bone Marrow-Derived Macrophages

1. Bone marrow macrophage medium (500 mL) (Subheading 2.1. **Item 1**).
2. Tissue culture grade phosphate buffered saline (PBS) pH 7.4 without calcium chloride or magnesium chloride.
3. Greiner Bio-One small (18 mm) blade cell scraper (Greiner).
4. INCYTO C-Chip disposable hemacytometer (or similar).
5. 100 mm petri dishes.
6. 50 mL sterile conical tubes.

7. Assay plates. 24-well tissue culture treated plates, or 96/384-well black, clear, flat bottom tissue culture treated assay plates or 96/384-well white, flat bottom, low flange tissue culture treated assay plates.

2.3 Maintenance and Passage of J774 Cells

1. D10 medium (500 mL): 435 mL DMEM with 4.5 g/L glucose, 5 mL of 100 mM sodium pyruvate, 5 mL of 200 mM L-glutamine, 5 mL of 100× penicillin (10,000 IU/mL)–streptomycin (10,000 μg/mL), and 50 mL of heat-inactivated, low endotoxin FBS. Sterilize through 0.22 μm filter.
2. Tissue culture grade PBS pH 7.4 without calcium chloride or magnesium chloride.
3. Greiner Bio-One small (18 mm) blade cell scraper (Greiner).
4. INCYTO C-Chip disposable hemacytometer (Fisher Scientific).
5. 100 mm petri dishes.
6. 50 mL sterile conical tubes.

2.4 Maintenance and Passage of THP-1 Cells

1. RPMI complete medium (500 mL): 445 mL of RPMI 1640 medium, 5 mL of 100× penicillin (10,000 IU/mL)–streptomycin (10,000 μg/mL), and 50 mL of heat-inactivated, low endotoxin FBS. Sterilize through 0.22 μm filter.
2. INCYTO C-Chip disposable hemacytometer (Fisher Scientific).
3. RPMI medium plus 100 nM phorbol 12-myristate 13-acetate (PMA).
4. 100 mm petri dishes (or tissue culture flasks).
5. 50 mL sterile conical tubes.

2.5 Activation of Primary Bone Marrow-Derived Macrophages with IFN-γ and LPS

1. Recombinant Murine Interferon-gamma (IFN-γ), e.g., from PeproTech.
2. Lipopolysaccharide (LPS).

2.6 Infection of Primary Bone Marrow-Derived Macrophages with M. tuberculosis in 24-Well Plates

1. OADC (oleic acid, albumin, dextrose, and catalase) supplement (Becton Dickinson) (*see* **Note 3**).
2. Tween 80 (polysorbate 80).
3. Middlebrook 7H9 medium (Becton Dickinson) supplemented with 10 % v/v OADC and 0.05 % v/v Tween 80.
4. Macrophage infection medium (500 mL): 340 mL of DMEM with 4.5 g/L glucose, 5 mL 100 mM sodium pyruvate, 5 mL of 200 mM L-glutamine, 50 mL of heat-inactivated, low endotoxin FBS, and 100 mL of L-cell conditioned medium (*see* **Note 2**). Sterilize through 0.22 μm filter.

5. Basal uptake buffer (250 mL): 250 mL of tissue culture grade PBS pH 7.4, 1.125 g glucose, 25 mg calcium chloride, 25 mg magnesium chloride, 1.25 g bovine serum albumin (BSA), 250 μL 40–50 % cold fish skin gelatin in water. Sterilize through 0.22 μm filter.
6. Uptake buffer (500 mL): 125 mL basal uptake buffer plus 25 mL heat-inactivated, low endotoxin FBS, 12.5 mL L-cell conditioned medium (*see* **Note 2**), 5 mL 100 mM sodium pyruvate, 332.5 mL DMEM with 4.5 g/L glucose and without sodium pyruvate and L-glutamine. Sterilize through 0.22 μm filter.
7. Wash Buffer (500 mL): 457.5 mL DMEM with 4.5 g/L glucose, 25 mL heat-inactivated, low endotoxin FBS, 12.5 mL L-cell conditioned medium (*see* **Note 2**), 5 mL 100 mM sodium pyruvate. Sterilize through 0.22 μm filter.
8. Tuberculin syringes: 1 mL TB syringe slip tip with BD PrecisionGlide™ Needle 25 G×5/8 (0.5 mm×16 mm, Becton Dickinson, Franklin Lakes, NJ).
9. 24-well tissue culture treated assay plates.
10. 15 and 50 mL sterile conical tubes.

2.7 Infection of Primary Bone Marrow-Derived Macrophages in 96- and 384-Well Plates

1. *See* Subheading 2.6.

2.8 Quantification of M. tuberculosis Survival in Macrophages

1. Lysis buffer: 0.1 % v/v Tween 80 in distilled, deionized water. Sterilize through 0.22 μm filter.
2. Dilution buffer: 0.05 % v/v Tween 80 in 500 mL of PBS. Sterilize through 0.22 μm filter.
3. 7H10 agar medium in quadrant plates: 7H10 agar medium (Becton Dickinson), autoclaved, then supplemented with 10 % v/v OADC and 100 μg/mL cycloheximide (*see* **Notes 3** and **4**). Using a pipette, dispense ~5 mL of medium into each quadrant of 100 mm quadrant petri dishes, cover and stack plates and allow the plates to dry at least 48 h (*see* **Note 4**).
4. 96-well tissue culture treated assay plate.

2.9 Imaging M. tuberculosis Infected Macrophages

1. Round German glass coverslips, 12 mm diameter, #1 thickness (Chemglass Inc.).
2. Premium grade, frosted microscope slides 3″×1″×1 mm.
3. ProLong® Gold antifade reagent (Life Technologies).
4. Tissue culture grade PBS pH 7.4 without calcium chloride or magnesium chloride.

5. 4 % Paraformaldehyde (PFA) in PBS (*see* **Note 5**): Reagent grade, crystalline 4 % paraformaldehyde in tissue culture grade PBS pH 7.4.
6. 24-well tissue culture treated assay plates.
7. 23 g1 syringe needle (*see* **Note 16**).
8. Forceps.
9. Clear, fast drying nail polish.

3 Methods

3.1 Isolation of Bone Marrow from Mice

1. Euthanize the mouse using an approved method, such as carbon dioxide asphyxiation followed by a secondary method of euthanasia, such as cervical dislocation (*see* **Note 6**).
2. Isolate the ilium, femur, and tibia bones and clean the muscle from the bones. Separate the femur from the socket of the ilium and cut the tibia from the femur at the knee with the small forceps and scissors. Take care to not break any of the bones.
3. Move the bones to a new petri dish filled with DMEM and remove any remaining tissue from the bones.
4. From a 50 mL conical tube, fill a 10 mL Luer lock syringe with fresh DMEM and attach a 23 gauge x 1″ (23 g1) needle.
5. Using the small forceps and scissors, cut open both ends of the bone, insert the needle into one end and flush the bone marrow into a clean 50 mL conical tube.
6. Spin down the bone marrow in a swinging bucket, refrigerated centrifuge, at 200 × *g* for 10 min at 4 °C. Remove supernatant and gently resuspend bone marrow in 8 mL of bone marrow macrophage medium pre-warmed to 37 °C.
7. Allow larger particles to settle for approximately 1–2 min.
8. Add 1 mL of resuspended bone marrow to 9 mL bone marrow macrophage medium in a 100 mm petri dish and swirl dish to disperse the cells.
9. Incubate in a humidified incubator at 37 °C and 5 % CO_2.
10. Add 5 mL of fresh bone marrow macrophage medium, prewarmed to 37 °C, to each dish after 3–4 days of incubation. Monitor the presence of adherent cells using an inverted microscope. The macrophage monolayers will be confluent after 5–7 days.

3.2 Expansion and Passaging of Primary Bone Marrow-Derived Macrophages

1. Once the cells are confluent, remove the medium and discard.
2. Add 10 mL of tissue culture grade PBS, prechilled to 4 °C, to each dish.
3. Incubate at 4 °C for 10 min (*see* **Note 7**).

4. Remove the adherent cells using a cell scraper (*see* **Note 8**).
5. Collect the cell suspensions into a 50 mL conical tube.
6. Spin down the cell suspension in a swinging bucket centrifuge at 4 °C, 200 × *g* for 10 min.
7. Discard supernatant and gently resuspend cell pellet in fresh, pre-warmed bone marrow macrophage medium.
8. Remove 10 μL and load onto a hemacytometer for counting the number of cells. A typical confluent 100 mm petri dish will have approximately 1–1.5 × 10^6 cells/mL (*see* **Note 9**).
9. Passage cells: cells can be split up to 1:8. For seeding 24-, 96-, and 384-well assay plates, *see* **Note 10** for appropriate cell concentrations.

3.3 Maintenance and Passage of J774 Cells

1. Thaw a vial of frozen J774 cells in a 37 °C water bath. Add the entire volume of the vial to 10 mL of pre-warmed D10 medium in a 100 mm petri dish.
2. Incubate overnight in a humidified incubator at 37 °C and 5 % CO_2.
3. Remove the medium and replace with 10 mL of fresh D10 medium (to remove any residual DMSO from the freezing medium) and incubate at 37 °C and 5 % CO_2 until the cells become confluent.
4. Passage cells as described above (Subheading 3.2) for the primary murine bone marrow-derived macrophages, with the following differences: (1) use D10 to resuspend scraped cells; and, (2) J774 cells should be split 1:6 and passaged when confluent, approximately every 3 days.

3.4 Maintenance and Passage of THP-1 Cells

1. Thaw a vial of frozen THP cells in a 37 °C water bath. Add the entire volume within the vial to 10 mL of pre-warmed RPMI complete medium in a 100 mm petri dish or T-25 tissue culture flask.
2. Incubate overnight in a humidified incubator at 37 °C and 5 % CO_2.
3. Collect the cell suspensions in a conical tube and spin in a swinging bucket, refrigerated centrifuge, at 200 × *g* for 10 min at 4 °C.
4. Discard supernatant and gently resuspend cell pellet in fresh, pre-warmed RPMI complete medium to remove any residual DMSO from the freezing media.
5. Incubate cells in a humidified incubator at 37 °C and 5 % CO_2 and check dishes or flasks daily for morphology, cell viability, and cell number by observing any changes in media color and counting cells with a hemacytometer.

6. Exchange the medium every 2–3 days. THP-1 cells should be subcultured when a density of 8×10^5 cells/mL is reached and not be allowed to exceed 1×10^6 cells/mL. Passaged cells can be seeded at $2–4 \times 10^5$ cells/mL.
7. To differentiate THP-1 cells into adherent cells with macrophage-like properties, resuspend and seed cells (5×10^5 to 1×10^6 cells/mL) in the desired assay plate in RPMI plus 100 nM PMA for 24 h.
8. After 24 h, wash away non-adherent cells with pre-warmed RPMI complete medium.

3.5 Activation of Primary Bone Marrow-Derived Macrophages with IFN-γ and LPS

1. Macrophages are activated by IFN-γ and LPS in a two step procedure.
2. Scrape macrophages from confluent petri dishes, seed cells into the desired type of assay plate (*see* **Note 10**) and allow to adhere overnight.
3. Add IFN-γ to a final concentration of 100 units/mL and incubate overnight in a humidified incubator at 37 °C and 5 % CO_2.
4. Add LPS at a final concentration of 10 ng/mL and incubate overnight in a humidified incubator at 37 °C and 5 % CO_2.
5. Once activated, the culture medium will turn yellow and the cells will have an altered morphology.

3.6 Infection of Primary Bone Marrow-Derived Macrophages with M. tuberculosis in 24-Well Plates

1. Culture *M. tuberculosis* to mid- to late-log phase (OD_{600} 0.5–1.0) in Middlebrook 7H9 supplemented with 10 % v/v OADC and 0.05 % v/v Tween 80.
2. An OD_{600} of 0.6 represents approximately 1×10^8 bacteria/mL. Add an appropriate volume of bacteria to obtain ~1×10^8 bacteria in 5 mL of pre-warmed uptake buffer.
3. Centrifuge for 10 min at $1{,}800 \times g$.
4. Discard the supernatant and resuspend in 1 mL of pre-warmed basal uptake buffer in a 15 mL conical tube.
5. Place the tube in a 15 mL conical tube rack to provide support and carefully syringe the bacterial solution eight times with a tuberculin syringe (*see* **Note 11**). This procedure will unclump the bacteria into single cells.
6. Add 9 mL of pre-warmed uptake buffer, e.g., a tenfold dilution to approximately 1×10^7 cells/mL.
7. For infection of macrophages in a 24-well plate, calculate the volume of medium needed for 0.5 mL per well and adjust the concentration of the bacteria to 2×10^6 cells/mL (e.g., a five-fold dilution) in warm uptake buffer.
8. Keep the bacteria at 37 °C until ready for the infection.

9. Prior to the day of infection seed wells of 24-well plates with macrophages in macrophage infection medium. If desired, cells may be activated prior to infection (Subheading 3.5). *See* Subheading 3.7 for infection of macrophages in 96- and 384-well plates. *See* **Note 12** for modified methods for infection of J774 and THP-1 cells.
10. On the day of infection, remove the medium and add 0.5 mL of bacterial-uptake buffer suspension, prepared as described above, to each well in a 24-well plate (*see* **Note 13**).
11. Incubate plates for 1 h at 37 °C and 5 % CO_2.
12. Remove bacterial suspension and wash each well twice with 1 mL of pre-warmed wash medium.
13. Add 0.5 mL of fresh, pre-warmed macrophage infection medium.
14. Incubate at 37 °C and 5 % CO_2.
15. To maintain *M. tuberculosis*-infected macrophages, change the medium with fresh, pre-warmed macrophage infection medium every 2 days.
16. Observe macrophages under an inverted light microscope to ascertain cell health. Survival assays can be continued through 14 days post-infection, although survival trends may become variable at time points later than 9 days post infection.

3.7 Infection of Primary Bone Marrow-Derived Macrophages in 96- and 384-Well Plates

1. To infect macrophages with *M. tuberculosis* in a 96- or 384-well plate, follow the recommended volumes and concentrations of macrophages for a specific plate type as described in **Note 10**. *See* **Note 12** for modified methods for infection of J774 and THP-1 cells.
2. Prepare *M. tuberculosis* as described in Subheading 3.6; however, after declumping and resuspending bacteria in uptake buffer, adjust to $OD_{600} = 0.2$ with macrophage infection medium.
3. For a 96-well plate, add 20 μL of the bacterial suspension to each well and for a 384-well plate, add 5 μL of the bacterial suspension per well (do not remove medium prior to infection). The medium in the wells does not need to be changed or washed during the infection, which can reliably proceed for approximately 6 days (*see* **Note 14**).

3.8 Quantification of M. tuberculosis Survival in Macrophages

1. To lyse macrophages, remove medium and add 0.5 mL of lysis buffer to each well.
2. Incubate at 37 °C for 10 min.
3. Observe cells under an inverted light microscope. Macrophages should appear swollen (Fig. 1a) compared to normal, healthy cells (Fig. 1b).

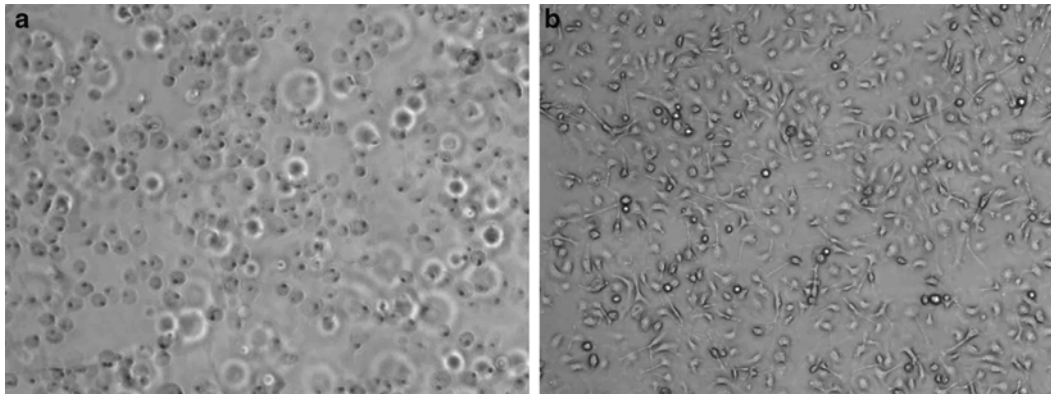

Fig. 1 Primary murine bone marrow-derived macrophages following 10 min in lysis buffer (**a**) and under normal culture conditions (**b**)

4. Using a P1000 (1 mL) pipettor and pipette tip, systematically scrape the well with the pipette tip. Following scraping, pipette the bacterial suspension up and down three times. Utilize a consistent technique for all of the wells.
5. In a 96-well assay plate, serially dilute the lysed macrophage suspension by 10^{-1}, 10^{-2}, and 10^{-3}.
6. Plate 50 μL of the undiluted sample and each dilution onto a quadrant of the appropriate 7H10 agar plate. Spread the lysed macrophage solution onto the agar with either a sterile plate spreader or disposable inoculation loop.
7. Place plates in a bag and incubate at 37 °C. Colonies will begin to appear approximately 2 or 3 weeks after plating, although when they appear can vary considerably. Count colonies at 2, 3, and 4 weeks post-infection to account for all colonies.
8. For quantifying survival of fluorescent *M. tuberculosis* reporters in 96- or 384-well plates, use clear bottom, black-sided plates, and for luciferase reporter assays use solid, white plates.

3.9 Imaging M. tuberculosis Infected Macrophages

1. Autoclave the round glass coverslips in a small beaker lined with tissues.
2. In a biosafety cabinet, add a single coverslip to each well of a 24-well plate (*see* **Note 15**).
3. Seed macrophages in the same manner as described above (Subheading 3.2).
4. Allow macrophages to adhere overnight in macrophage infection medium and infect macrophages as described above (Subheading 3.6).
5. To prepare the coverslips for mounting on microscope slides, remove the medium from each well and wash the wells twice with 1 mL of tissue culture grade PBS.

6. To fix cells, remove PBS and add 1 mL of 4 % paraformaldehyde (PFA) in PBS to each well. Seal the plate in a plastic Ziploc® bag and incubate at 4 °C overnight.
7. Remove the PFA and discard, wash twice with 1 mL of PBS and then add 0.5 mL of PBS to each well.
8. Add 10 μL of ProLong® Gold antifade reagent to the center of a glass microscope slide.
9. Using a hooked syringe needle, draw the coverslip up off the bottom of the well and remove with from the well with forceps (*see* **Note 16**). Take care to note which side of the coverslip is covered with the monolayer. Dip the coverslip into tissue culture grade PBS and remove the excess PBS by touching the side of the coverslip onto a sheet of paper towel.
10. Place the coverslip onto the microscope slide containing the ProLong® Gold antifade reagent, monolayer side down. Avoid capturing air bubbles under the glass. If bubbles are observed, very gently press on the coverslip to push air bubbles to the edge of the coverslip.
11. Allow the ProLong® Gold antifade reagent to cure for 48 h in a biological safety cabinet.
12. Seal the coverslip to the microscope slide by painting the edges of the coverslip and immediately surrounding microscope slide with a clear, fast drying nail polish.
13. Once dry, decontaminate the slides by submerging them in a 10 % bleach solution for 10 min or other approved decontamination procedure and remove materials from the BSL-3 laboratory.

4 Notes

1. When preparing tissue culture reagents be sure to use endotoxin-free plasticware to avoid activation of the macrophages. Measure reagents using disposable plastic pipettes, or for larger volumes sterile, 50 mL conical tubes or the volume gradations on the top portion of the filtration unit.
2. To prepare L-cell conditioned medium, quickly thaw a tube of L929 fibroblasts (ATCC) at 37 °C. Gently pipette thawed cells into a conical tube with 5 mL of D10 medium and centrifuge at 300 × *g* for 5 min. Remove the supernatant and resuspend in 7 mL of D10 medium and transfer to a T-25 tissue culture flask. Incubate at 37 °C and 5 % CO_2 until cells are confluent, about 4–5 days. Once confluent, remove the supernatant, wash once with sterile PBS, and then add 5 mL of PBS with 0.25 % trypsin (w/v) and 0.53 mM EDTA and incubate for 5–10 min until the cells detach. Centrifuge cells at 300 × *g* for

5 min and resuspend in 10 mL of D10. Use this culture to seed 1 mL of culture into 30 mL of D10 into a T-150 flask and incubate flask at 37 °C and 5 % CO_2 until cells are 90 % confluent (approximately 4–5 days). Passage cells as described above (but using 10 mL of PBS/Trypsin/EDTA to detach cells) and resuspend in 4 mL of D10. Use 1 mL of cells to seed a triple layer flask (e.g., Nunc Triple flask 500 cm^2 with vent cap) containing 180 mL of modified D10 medium with reduced antibiotics (a final concentration of 10 IU/mL of penicillin and 10 μg/mL of streptomycin). Incubate the triple layer flasks at 37 °C and 5 % CO_2 for approximately 1 week, mixing the flasks every other day. To harvest L-cell conditioned medium, pour supernatants into sterile endotoxin-free tubes (e.g., 50 mL conical tubes) and centrifuge at 1,800 × *g* for 10 min. Pour the supernatants into the fresh 50 mL conical tubes and freeze at –20 °C. The highest level of macrophage differentiating cytokines will be obtained shortly before the cells begin to die and the medium turns orange. Harvest the supernatant as soon as the color of the medium begins to change.

3. To prepare the OADC supplement dissolve 8.5 g NaCl and 50 g of BSA fraction V in 900 mL of ddH_2O. We have found it useful to gently heat and stir the solution as the BSA tends to clump and go into solution slowly. Dissolve 20 g D-dextrose, 40 mg catalase, and 0.5 g oleic acid into the solution. Bring the final volume up to 1 L. Aliquot and centrifuge the solution in 500 mL bottles for 30 min at 1,800 × *g*, 4 °C. Sterilize supernatants with a 0.22 μm filter sterilization unit and aliquot into 50 mL conical tubes. Store at 4 °C. The OADC supplement is also available for purchase.
4. Do not add OADC or cycloheximide to 7H10 agar medium before autoclaving. Warm the OADC and cycloheximide to 55 °C and add to the 7H10 agar cooled to 55 °C. Mix thoroughly and dispense agar into quadrant plates. To dry plates, cover and stack plates and leave in a closed biological safety cabinet for 2 days.
5. *Hazardous*: Prepare 4 % PFA in a chemical fume hood. Heat a glass beaker of stirred PBS on a hot-plate to approximately 60 °C. Add PFA to heated PBS, however, it will not dissolve into solution. Add 1 N NaOH until the PFA goes into solution. Allow solution to cool, bring it up to volume with PBS, and filter. Check the pH of the PFA and if necessary adjust it to pH 7.4 (using pH strips instead of pH meter). Freeze aliquots of PFA for single use. Do not freeze and thaw PFA multiple times.
6. It is essential that one is in compliance with the specific Institutional Animal Care and Use Committee (IACUC) approved methods for the care and euthanasia of mice.

7. Be careful to not incubate the cells at 4 °C for more than 10 min because cell viability may decrease.
8. It is particularly important to scrape along the edges because cells tend to accumulate at the edges of the dish.
9. When counting cells, observe and count four, 1 mm × 1 mm squares (e.g., the four corners of the hemacytometer). Take the average number of cells and multiply by 10^4 to obtain cells/mL.
10. When seeding 24-, 96-, or 384-well plates, addition of the following amounts of cells will result in confluent wells within 24 h. For 24-well plates, add 0.5 mL of cells at $1–1.5 \times 10^6$ cells/mL to each well; for a 96-well plate, add 80 μL of cells at approximately 2×10^6 cells/mL per well; and for a 384-well plate, add 20 μL of cells at approximately 2×10^6 cells/mL per well. When preparing 384-well plates, after the addition of macrophages, centrifuge the assay plates at $100 \times g$ for 5 min to ensure cells are in contact with the bottom of the well.
11. Working with sharps in the BSL-3 represents a potential safety hazard and requires training prior to use. Blunt needle syringes are available and can be substituted for safety purposes.
12. For infection of J774 or THP-1 cells, perform experiments as described for primary murine bone marrow-derived macrophages, but substitute macrophage infection medium with D10 medium or RPMI complete medium, respectively, and omit the antibiotics from both media.
13. It is important that if multiple 24-well plates are being infected, each plate is allowed a similar amount of time for bacterial infection. Take note of the time each plate was infected and initiate at least the first wash step for each plate following 1 h of infection.
14. To reduce position effects associated with evaporation, we have found it useful to stack the plates and place an empty plate on the top and bottom of the stack. Additionally, for a 6 day infection, we place the plates in an open plastic Ziploc® bag with a moist paper towel to act as a local humidifier. For consistent data, avoid using the two outside rows and columns in a 384-well plate and one outside row and column in a 96-well plate as these wells are the most prone to evaporation and edge effects.
15. To maintain the sterility of the coverslips, we have found it useful to use a vacuum to transfer single coverslips to each well of a 24-well plate. In a biological safety cabinet, attach a tube to a vacuum source and a sterile 1 mL pipette tip to the other end of the tube. Apply the vacuum and the pipette tip will hold a single coverslip that can be transferred to a well in a 24-well plate.

16. We have found it useful to use a small pair of pliers to bend the end of a syringe needle (23 g1) into a small hook. The hook can be used to gently break the surface tension between the glass slide and the plastic bottom of the well. This step requires special care to avoid breaking coverslips.

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Chapter 21

Infection of Human Neutrophils to Study Virulence Properties of *Mycobacterium tuberculosis*

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Abstract

Polymorphonuclear neutrophils (PMN) are professional phagocytes and the first line of defense against invading microbes. Upon infection with *Mycobacterium tuberculosis,* PMN are attracted to the site of infection along an interleukin 8 gradient. In patients with active tuberculosis, PMN comprise the predominant population in the lung and carry the main mycobacterial load suggesting a minor role for PMN in protective host defense against *M. tuberculosis* but rather in pathology. Therefore, better understanding of PMN biology in tuberculosis is of pivotal importance to develop novel immune modulating measures and host directed therapies. Virulent *M. tuberculosis* escape the otherwise microbicidal armamentarium of PMNs by inducing necrotic cell death through the PMN's own reactive oxygen species. Studying the interactions between PMN and different *M. tuberculosis* strains, and virulence factors thereof, is vital to comprehend tuberculosis pathogenesis. Working with PMN is challenging as these cells are non-adherent, motile and—with a half-life of 6–12 h in vitro—rather short-lived. Here, we provide an isolation and infection protocol that is tailored to study mycobacterial infection in human PMN regarding the intracellular fate of mycobacteria and host cell responses, such as cell death and release of microbicidal effectors.

Key words Polymorphonuclear, Neutrophil, Phagocytosis, Cell death, *Mycobacterium tuberculosis*

1 Introduction

Being professional phagocytes, polymorphonuclear neutrophils (PMN) are the first cells which arrive at sites of infection. After sensing and chasing the bacterial invaders, PMN engulf pathogens in a membrane-enclosed compartment termed phagosome. PMN carry a highly potent repertoire of antimicrobial effectors to kill and eliminate bacteria but also to disrupt tissue integrity. The PMN's armory contains NADPH-oxidase and myeloperoxidase (MPO), which generate reactive oxygen species (ROS), lysozyme, proteases (such as elastase, gelatinase, collagenase, cathepsins), antimicrobial peptides (AMP) (such as cathelicidin and α-defensins) as well as iron-sequestrating proteins (lipocalin 2 and lactoferrin (LF)) [1]. PMN granules are differentiated into three types: azurophilic (primary), specific (secondary) and tertiary granules.

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Azurophilic and specific granules mainly contain antimicrobial effectors, such as AMP, LF, lysozyme, MPO as well as cathepsins and proteinase-3 [2]. Azurophilic granules release their contents into the forming phagosome, whereas specific granules fuse with both the phagosome and the plasma membrane to release their contents extracellularly, including enzymes degrading extracellular matrix proteins (such as collagenase and gelatinase) [3]. Tertiary granules contain enzymes for the disruption of the extracellular matrix, such as matrix metalloproteinase 9 (MMP-9) [4]. Furthermore, upon encountering microbes, PMN release DNA to form NETs (*n*eutrophil *e*xtracellular *t*raps), which bind microbes and contribute to their killing. NET release has been described as a targeted process, which seems to be associated with cell death (termed NETosis) and requires elastase as well as MPO activity and ROS production [5].

In contrast to other phagocytes, such as macrophages, closing of the phagosomal cup in PMNs is often concomitant to fusion of the phagosome with acidic granules, accelerating maturation and fusion with lysosomes, which is essential for bactericidal activity by PMN.

Alveolar macrophages are the first cells encountering inhaled *Mycobacterium tuberculosis*, which triggers initial inflammatory responses, including interleukin (IL) 8 release. IL-8 attracts PMN and causes a massive influx of these cells to the site of infection, thereby exacerbating local inflammation [6]. PMN are efficient in phagocytosing mycobacteria [7]. Consequently, PMN represent the predominant cell population and main carriers of *M. tuberculosis* in cells retrieved by bronchoalveolar lavage from lungs of patients with active tuberculosis (TB) [8]. This is in contrast to the widely used experimental model of chronic infection in C57BL/6 and BALB/c mice, where PMN only appear in the lung in the first 2 weeks of infection, with a putative early function in protection, but disappear from the lesions at later stages [9]. Certain inbred mouse strains such as C3HeB/FeJ mice develop structured granulomas with central necrosis similar to human TB, but succumb much earlier to the disease compared to C57BL/6 mice [10]. In these mouse strains, PMN are maintained in the lung throughout the infection, and treatment with ibuprofen inhibiting the key enzymes in prostaglandin E2 synthesis, cyclo-oxygenases (COX), reduce lesion size and mycobacterial loads [11].

The high susceptibility of C3HeB/FeJ mice to infection together with predominance of PMN in active TB patients indicates that PMN have a minor function in protection against *M. tuberculosis*. In our studies PMN fail to eliminate *M. tuberculosis*, but quickly succumb to necrosis in a ROS-dependent manner [12]. Thereby, *M. tuberculosis* reduces the exposure time to antimicrobial effectors. In addition, necrosis of PMN may also help mycobacteria to avoid cross-presentation of *M. tuberculosis* antigens by apoptotic bleb formation of the host cell and transfer to

bystander antigen-presenting cells [13, 14]. The induction of necrosis of infected PMN depends on the *M. tuberculosis* RD1 locus, which encodes important virulence factors including ESAT-6, CFP-10, and the ESX1-5 secretion system [15, 12].

The methods described here provide the opportunity to study pathogen-specific virulence mechanisms in human PMN. We present a tailored protocol to isolate PMN cells from human peripheral blood by density gradient centrifugation and to infect them with serum-opsonized mycobacteria in cell culture. In our hands, uptake of *M. tuberculosis* by PMN was most efficient, when mycobacteria were opsonized with complement. We provide a selection of different assays to study interactions between mycobacteria and PMN including measurement of ROS production and cytokine profiles of infected host cells, as well as determination of necrotic vs. apoptotic cell death. In addition, we present a protocol to analyze the phagosomal fate of intracellular mycobacteria and characterisation of phagosomes by immunocytochemistry and subsequent laser-scanning microscopy. Finally, we provide a protocol to determine colony-forming units (CFU) of mycobacteria in PMN.

Due to the fact that PMNs are non-adherent, motile and cannot be stored as frozen stocks, they require special handling. More importantly, unlike other commonly investigated cell populations, for instance macrophages, PMNs have a half-life of only 6–12 h in vitro, which prohibits culture for more than 2 days [16]. Furthermore, collecting supernatants and performing washing steps to remove mycobacteria or tracers for cell labeling is more elaborate, and carryover or loss of PMNs during such procedures has to be avoided.

2 Materials

Store all solutions and buffers at 4 °C unless indicated otherwise. All solutions, buffers, and materials used in Subheadings 2.1, 2.2 and 2.6 must be kept sterile.

2.1 Purification of Human PMNs from Whole Blood

1. 50 mL heparinized human peripheral blood, freshly drawn from arm vein.
2. Histopaque 1119.
3. Phosphate-buffered saline (PBS).
4. Percoll gradient: 100 % Percoll made isotonic by addition of 1/10 volume of 10× PBS. 65, 70, 75, 80, and 85 % Percoll dilutions with 1× PBS.
5. Complete medium for PMN culture: RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine.
6. Cell counting chamber, e.g., Neubauer chamber.

2.2 Mycobacterial Culture and Infection (See Note 1)

1. Frozen stock of mycobacterial strain of interest, e.g., *M. tuberculosis* H37Rv (*see* **Note 2**).
2. Middlebrook 7H9 liquid medium: 4.7 g 7H9 Middlebrook, 500 μL Tween 80, add 900 mL H_2O, autoclave, let the broth cool down before you add 1 mL OADC supplement (oleic acid, albumin, dextrose, catalase: Beckon Dickinson) per 10 mL medium. Do not store complete medium longer than 4 weeks.
3. Phosphate-buffered saline.
4. 27G needle.
5. 1 mL syringes.
6. Paraformaldehyde.

2.3 Measurement of Reactive Oxygen Species

1. Cells of interest, e.g., *M. tuberculosis*-infected human PMNs.
2. FACS tube.
3. Phosphate-buffered saline.
4. Dihydrorhodamine 123.
5. 4 % paraformaldehyde in PBS.
6. Flow cytometer, e.g., BD FACS Calibur.

2.4 Measurement of Cytokine Profiles

1. Cell culture supernatant, e.g., from cultured, *M. tuberculosis-infected* human PMNs.
2. Spin-X centrifuge tube filter, 0.22 μm, cellulose acetate (Corning) or similar.
3. Cytometric bead array (CBA) kit, e.g., BD human inflammatory cytokines kit.
4. Flow cytometer, e.g., BD FACS Canto II.
5. CBA analysis software, e.g., BD FCAP array software.

2.5 Detection of Necrosis by Measurement of Lactate Dehydrogenase Activity

1. Cell culture supernatant, e.g., from *M. tuberculosis*-infected human PMNs.
2. Total cell lysate, e.g., from *M. tuberculosis*-infected human PMNs.
3. Lysis buffer: 10 % (v/v) Triton X-100 in PBS.
4. Spin-X centrifuge tube filter, 0.22 μm, cellulose acetate, or similar (Corning Incorporated).
5. Lactate dehydrogenase activity kit, e.g., Roche Cytotoxicity detection kit.
6. ELISA reader, e.g., Opsys MR ELISA reader (Dynex Technology).

2.6 Measuring Necrosis and Apoptosis by Flow Cytometry

1. Cells of interest, e.g., *M. tuberculosis*-infected human PMNs.
2. FACS tubes.
3. Recombinant human Annexin V coupled to phycoerythrin (Molecular Probes).
4. Annexin V binding buffer (Becton Dickinson).
5. Live/dead cell stain (Molecular Probes).
6. 4 % paraformaldehyde in PBS.
7. Flow cytometer, e.g., BD FACS LSR II.

2.7 Determination of the Phagosomal Fate of Mycobacteria by Immunocytochemistry and Confocal Laser-Scanning Microscopy

1. Cells of interest, e.g., *M. tuberculosis*-infected human PMNs.
2. Paraformaldehyde.
3. Phosphate-buffered saline.
4. Permeabilization buffer: 0.05 % (v/v) Triton X-100 in PBS.
5. Blocking buffer: 10 % normal goat serum in PBS.
6. Primary antibody directed against protein of interest, e.g., mouse anti-human lysosomal associated membrane protein 1 (Lamp-1).
7. Fluorochrome-coupled secondary antibody directed against the primary antibody, e.g., rat anti-mouse IgG coupled to Cy5.
8. 4′,6-Diamidino-2-phenylindole (DAPI).
9. Mounting medium, e.g., Micro-Tech-Lab Confocal-Matrix.
10. Confocal laser scanning microscope, e.g., Leica SP5.

2.8 Measuring Mycobacterial Viability by Colony Forming Units

1. Mycobacterial species such as *M. tuberculosis*.
2. Cells of interest, e.g., human PMNs.
3. Lysis buffer: 0.1 % (v/v) Triton X-100 in PBS.
4. Dilution buffer: 0.05 % (v/v) Tween 80 in PBS.
5. 7H11 agar plates: dissolve 21 g Middlebrook 7H11 agar, 1 g asparagine, and 5 mL glycerol in 900 ml of H_2O and autoclave. Let the solution cool down to 56 °C. Add 10 % heat-inactivated bovine serum and pour 20 mL into each petri dish. Let cool down to room temperature until it is solidified.

3 Methods

Keep in mind that human PMNs are a very short-lived cell population with a half-life of 6–12 h in vitro and therefore need to be used immediately after isolation. Additionally, PMNs are non-adherent and motile. Therefore, loss of cells during washing steps and carryover of cells when taking supernatants of the wells containing the PMNs needs to be avoided (*see* **Note 1**).

3.1 Purification of Human PMNs from Whole Blood

Perform all steps and use all solutions and buffers at room temperature unless indicated otherwise (*see* **Note 3**).

1. Carefully layer 25 mL of heparinized fresh human peripheral blood onto 20 mL Histopaque 1119 in a 50 mL conical tube and centrifuge for 20 min at 800 × *g*, with acceleration and brake off. After centrifugation, the blood will be separated into serum, peripheral blood monocytes, Histopaque 1119, PMN cells mixed with some erythrocytes, and erythrocytes (from top to bottom) (*see* Fig. 1).
2. Collect serum and PMN in separate 50 mL tubes.
3. Fill the tubes containing PMNs up to 30 mL with PBS and centrifuge for 5 min at 485 × *g*.
4. Discard supernatant carefully and resuspend the cells in 2 mL PBS (*see* **Note 4**).
5. Layer PMNs on top of an isotonic Percoll gradient consisting of 65, 70, 75, 80, and 85 % dilutions (from top to bottom) in PBS in a 15 mL conical tube.
6. Centrifuge for 20 min at 800 × *g* with acceleration and brake off.
7. Harvest PMNs from the interface between the 75 and 70 % layer and transfer the cells into 50 mL tubes.
8. Add PBS to 30 mL and centrifuge for 5 min at 485 × *g*.

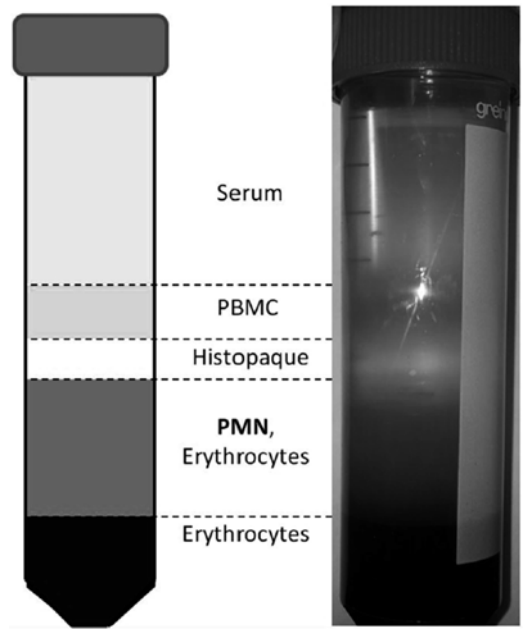

Fig. 1 Histopaque gradient after centrifugation of freshly drawn, heparinized human whole blood

9. Discard supernatant and resuspend pellet in 1 mL complete medium for PMN culture.
10. Determine PMN cell numbers using a cell counting chamber (*see* **Note 5**).
11. Calculate the desired total PMN cell number according to your personal experimental layout. For example, calculate to seed 4×10^5 PMNs per well in a 24-well plate.
12. Resuspend the required numbers of PMN, e.g., 4×10^6 in 1-2 mL complete medium in a 50 mL conical tube to prepare them for mycobacterial infection within the tube. Keep cells at 37 °C and 5 % CO_2.

3.2 Mycobacterial Culture and Infection (See Note 1)

Perform all steps and use all solutions and buffers at room temperature unless indicated otherwise

1. One week before planned infection, thaw a vial of 1 mL of frozen mycobacteria (*see* **Notes 1** and **2**), e.g., *M. tuberculosis.*
2. Incubate 1 mL stock into an appropriate culture flask, e.g., 75 cm^2 flask, containing 9 mL Middlebrook 7H9 medium; add antibiotics, if needed (*see* **Note 6**).
3. Incubate mycobacterial cultures at 37 °C for 3–4 days, flask lying flat.
4. Maintain mycobacterial culture by transferring 1–2 mL into 9–8 mL fresh Middlebrook 7H9 medium and incubate for additional 3–4 days at 37 °C (*see* **Note 7**).
5. On day of infection, harvest mycobacterial cells in a 15 mL conical tube by centrifuging at $3{,}500 \times g$ for 10 min.
6. Discard supernatant, resuspend pellet in 10 mL PBS, and centrifuge at $3{,}500 \times g$ for 10 min.
7. Discard supernatant and resuspend pellet in 1 mL PBS.
8. Prepare a single cell suspension by passing the mycobacteria five times through a 27G needle using a 1 mL syringe.
9. Transfer 200 µL of the mycobacterial suspension into a cuvette, add 800 µL of 4 % paraformaldehyde in PBS (i.e., a 1 in 5 dilution of your original mycobacterial suspension) and close with a cap.
10. Measure OD_{580} of the mycobacterial suspension.
11. Calculate the total number of mycobacteria in the original sample. An OD_{580} of 0.1 is approximately 5×10^7 bacteria per mL (remember to account for the dilution factor). A typical multiplicity of infection is 1 mycobacterium per PMN.
12. For opsonization, transfer the mycobacteria in a fresh 1.5 mL tube and add the same amount of autologous serum isolated in Subheading 3.1, **step 1**.
13. Incubate for 30 min at room temperature.

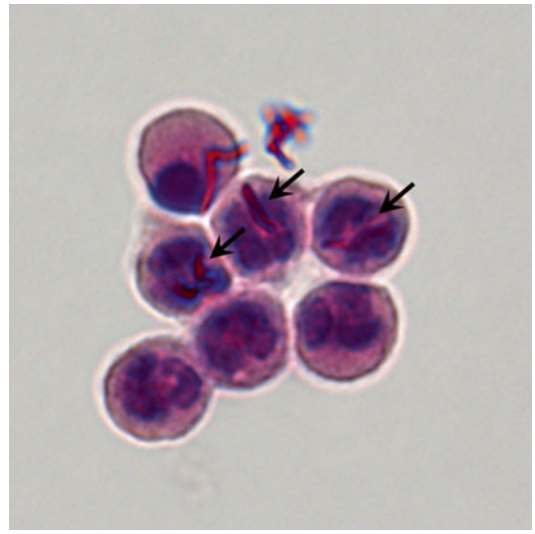

Fig. 2 Human polymorphonuclear neutrophils isolated from peripheral blood and infected with *Mycobacterium tuberculosis* for 2 h. Cells were spun onto a glass slide using cytocentrifugation and stained with acid-fast stain (*M. tuberculosis*, *pink*, *arrows*) and hemalaun

14. Add the opsonized mycobacteria Subheading 3.2 to the PMNs isolated from Subheading 3.1 and mix by pipetting.
15. Incubate for 2 h at 37 °C and 5 % CO_2.
16. Centrifuge at 485 × *g* for 5 min at room temperature to remove extracellular mycobacteria.
17. Discard supernatant and resuspend in appropriate volume of complete PMN culture medium, e.g., 4×10^5 infected PMN in 1 mL per well.
18. Seed infected PMN in plates and keep them at 37 °C with 5 % CO_2.
19. At desired time points after infection, e.g., 2, 6, 24 h, continue with one or more of the readouts (*see* Fig. 2).

3.3 Measurement of Reactive Oxygen Species

1. Collect content of each well and transfer into a fresh 5 mL FACS tube.
2. Centrifuge at 485 × *g* for 5 min at room temperature, discard supernatant and resuspend pellet in 1 mL PBS.
3. Centrifuge at 485 × *g* for 5 min at room temperature and discard supernatant.
4. Resuspend pellet in 1 mL PBS and add 30 µL dihydrorhodamine 123 (DHR123) (*see* **Note 8**).
5. Incubate for 5 min at 37 °C with 5 % CO_2.
6. Centrifuge at 485 × *g* for 5 min at room temperature, discard supernatant and resuspend pellet in 1 mL PBS.

7. Centrifuge at 485 × *g* for 5 min at room temperature and discard supernatant.
8. Resuspend pellet in 200 μL 4 % paraformaldehyde in PBS and measure immediately by using a flow cytometer, e.g., BD LSR II.

3.4 Measurement of Cytokine Profiles

1. Collect content of each well and transfer into a fresh 1.5 mL tube.
2. Centrifuge at 485 × *g* for 5 min at 4 °C to pellet PMNs.
3. Carefully collect supernatant and transfer up to 800 μL into a SpinX tube (*see* **Note 9**).
4. Centrifuge at 3,500 × *g* for 2 min at 4 °C.
5. Discard SpinX column and transfer flow-through into a fresh 1.5 mL tube.
6. Perform sample preparation for cytokine measurement by cytometric bead array kit according to manufacturer's protocol (*see* **Note 10**) as follows.
7. Add 10 μL of each capture bead solution (provided), for each sample to be analyzed including controls and standards (provided), into a 15 mL conical tube and vortex thoroughly.
8. Add 50 μL of the capture bead mixture, 50 μL of the PE detection reagent (provided), and 50 μL of the test sample or standard into a 1.5 mL tube. Mix carefully by pipetting.
9. Incubate for 3 h at room temperature, protected from light.
10. Add 1 mL of wash buffer (provided) to each assay tube and centrifuge at 200 × *g* for 5 min.
11. Carefully discard the supernatant by aspiration and resuspend the bead pellet in 300 μL of wash buffer for each assay tube.
12. Perform data acquisition using a flow cytometer, e.g., BD FACS Canto II.
13. Perform data analysis by using BD FCAP software. This software differentiates the bead–cytokine populations according to the individual cytokines and calculates cytokine concentrations in unknown samples by using known standards.

3.5 Detection of Necrosis by Measurement of Lactate Dehydrogenase Activity

1. Collect content of each well and transfer into a fresh 1.5 mL tube.
2. Centrifuge at 485 × *g* for 5 min at 4 °C to pellet PMNs.
3. Carefully collect supernatant and transfer up to 800 μL into a SpinX tube (*see* **Note 9**).
4. Centrifuge at 3,500 × *g* for 2 min at 4 °C.
5. Discard SpinX column and transfer flow-through into a fresh 1.5 mL tube.
6. Perform the cytotoxicity assay (*see* **Note 11**).
7. Perform data acquisition by measurement of the absorbance at OD_{490} using an ELISA reader (*see* **Note 12**).

3.6 Measuring Necrosis and Apoptosis by Flow Cytometry

1. Collect content of each well and transfer into a fresh 5 mL FACS tube.
2. Centrifuge at 485 × *g* for 5 min at room temperature, discard supernatant, and resuspend pellet in 100 µL Annexin V–PE solution.
3. Add 3 µL live/dead cell stain to the same tube.
4. Incubate for 15 min at room temperature in the dark (*see* **Note 13**).
5. Add 400 µL annexin V binding buffer and centrifuge at 485 × *g* for 5 min at room temperature.
6. Discard supernatant and resuspend cells in 4 % (v/v) paraformaldehyde and measure immediately by using a flow cytometer, e.g., BD LSR II.

3.7 M. tuberculosis-Containing Phagosome Characterization by Microscopy

1. Collect content of each well from Subheading 3.2 and transfer into a fresh 1.5 mL tube.
2. Centrifuge at 485 × *g* for 5 min at room temperature, discard supernatant, and resuspend pellet in 1 mL pre-warmed (37 °C) 4 % paraformaldehyde in PBS.
3. Incubate for 20 min at 37 °C.
4. Centrifuge at 485 × *g* for 5 min at 4 °C, discard supernatant and resuspend pellet in 200 µL PBS.
5. Attach cells to a glass slide by centrifugation at 300 × *g* for 3 min.
6. Permeabilize cell membranes by incubation in 50 µL permeabilization buffer for 10 min at room temperature in a humidity chamber.
7. Wash with 100 µL PBS.
8. Add 100 µL blocking buffer and incubate for 30 min at room temperature in a humidity chamber.
9. Remove blocking buffer by aspiration and add 100 µL of primary antibody diluted in blocking buffer (*see* **Note 14**), e.g., mouse anti-human Lamp-1, diluted 1:200.
10. Incubate for 1 h at room temperature in a humidity chamber.
11. Wash three times with 100 µL PBS.
12. Add 100 µL of secondary antibody diluted in blocking buffer, e.g., rat anti-mouse IgG coupled to Cy5, diluted 1:200.
13. Incubate for 45 min at room temperature in the dark in a humidity chamber.
14. Wash with 100 µL PBS followed by sterile filtered H_2O.

15. Add 100 µL DAPI, diluted 1:1,000 in sterile filtered H_2O and incubate for 5–7 min at room temperature in the dark in a humidity chamber.
16. Wash two times with 100 µL sterile filtered H_2O and mount glass slides with a cover slip using mounting medium.
17. Analyze using a confocal laser-scanning microscope.

3.8 Measuring Mycobacterial Viability by Colony Forming Units

1. Add 10 µL lysis buffer per 1 mL PMN culture, so the final concentration of Triton X is 0.1 %. Mix by pipetting.
2. Prepare tenfold serial dilutions in dilution buffer, e.g., 1:10, 1:100, 1:1,000, and 1:10,000.
3. Plate 100 µL of each serial dilution onto Middlebrook 7H11 agar plates supplemented with 10 % heat-inactivated bovine serum.
4. Incubate for 3–4 weeks at 37 °C.
5. Count the colonies of the plates and calculate the colony forming units for each well.

4 Notes

1. *Biosafety cautions*: Experiments with *Mycobacterium tuberculosis* strains must be performed in a biosafety laboratory according to local regulations. In all procedures, avoid the generation of aerosols. Please contact your local biosafety officer and make yourself familiar with local regulations and permissions, and all necessary biosafety requirements that may apply, before you start any experiments.
2. Store mycobacterial strains in Middlebrook 7H9-OADC medium plus 10 % v/v glycerol at –80 °C.
3. Human PMN are extremely sensitive to cold temperatures.
4. Since the PMN cultures still contain some erythrocytes, the pellet is often soft. Use caution when discarding the supernatant.
5. Do not use Trypan blue for dead cell exclusion since it damages PMNs and produces false positive signals.
6. Genetically modified mycobacteria often require antibiotics.
7. Mycobacteria are pre-cultured and split to reduce numbers of dead bacteria due to freezing/thawing and to use them at log phase of growth for infection.
8. DHR 123 is uncharged and can cross the membrane barrier. Reactive oxygen species (ROS) like superoxide and hydrogen peroxide oxidize DHR 123 to the cationic and fluorescent rhodamine 123.

9. SpinX tubes contain a column with a sterile filter in it. This step assures that no mycobacteria are carried over.
10. All solutions, buffers, antibodies, beads, standards, etc. needed are provided with the CBA kit. Briefly, the method is based on the simultaneous detection of up to six cytokines within one sample. The cytokines are captured by specific antibodies coupled to beads of known, distinct fluorescence intensity for each cytokine (e.g., APC fluorochrome). A detection antibody and a standard series of known cytokine concentrations are used to determine the amount of cytokine within the sample. Other kits are available, but this one was used successfully.
11. All solutions needed are provided with the LDH cytotoxicity kit. Briefly, lactate dehydrogenase activity is measured by a change of color, when the enzyme processes the substrate. Lactate dehydrogenase is strictly located intracellular within the cytoplasm. Detection within the supernatant represents cell membrane disintegration and therefore necrosis.
12. To express necrosis rates in percentage terms, 0 % and 100 % values for LDH activity are needed. OD measurement of the supernatant of uninfected PMN represents the 0 % value. To obtain a 100 % value, lyse PMNs by adding 10 μL lysis buffer per 1 mL PMN culture, so the final concentration of Triton X is 0.1 % and mix by pipetting.
13. The characteristic flip of phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet during apoptosis is detected by binding of Annexin V coupled to a fluorophore such as PE. Since the membrane integrity is preserved, apoptotic cells are not positive for live/dead cell stain, which is not membrane permeable. Live/dead cell stain recognizes amino residues, which occur more often at the inner plasma membrane leaflet than at the outer. Therefore, necrotic cells are found to be double positive for both live/dead cell stain and Annexin V–PE. Since the membrane integrity is no longer maintained after necrosis, Annexin V–PE is able to bind phosphatidylserine at the inner membrane leaflet as well.
14. Primary antibodies to detect any proteins of interest can be used. Dilute and adjust protocol according to the manufacturer's data sheet.

Acknowledgements

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Chapter 22

Isolation of Bead Phagosomes to Study Virulence Function of *M. tuberculosis* Cell Wall Lipids

Anna C. Geffken, Emmanuel C. Patin, and Ulrich E. Schaible

Abstract

Following pathogen recognition by macrophages, the causative agent of human tuberculosis, *Mycobacterium tuberculosis*, is internalized by receptor-mediated phagocytosis. Phagosomes containing nonpathogenic bacteria usually follow a stepwise maturation process to phagolysosomes where bacteria are eliminated. However, as a hallmark of *M. tuberculosis* virulence, pathogenic mycobacteria inhibit phagosome maturation in order to generate an intracellular niche for persistence and replication in resting macrophages. In contrast, activation by interferon gamma and tumor necrosis alpha activates microbicidal effectors of macrophages such as nitric oxide synthase, NO-mediated apoptosis and LRG-47-linked autophagy, which drives *M. tuberculosis* into phagolysosomes. Glycolipid compounds of the mycobacterial cell wall have been suggested as virulence factors and several studies revealed their contribution to mycobacterial interference with phagosome maturation. To study their effect on phagosome maturation and to characterize phagosomal protein and lipid compositions, we developed a reductionist mycobacterial lipid-coated bead model. Here, we provide protocols to "infect" macrophages with lipid-coated magnetic beads for subsequent purification and characterization of bead phagosomes. This model has been successfully employed to characterize the virulence properties of trehalose dimycolate, as one of the cell wall glycolipids essential for inhibition of phagosome maturation.

Key words Phagocyte, Macrophage, Phagosome, Lipid, Bead, Proteome

1 Introduction

Pathogenic mycobacteria such as the agent of human tuberculosis (TB), *M. tuberculosis*, are facultative intracellular microbes converting the macrophage (MPH) into a hospitable host environment. Internalization into macrophages facilitates mycobacterial escape from humoral immune factors and promotes microbial survival and growth [1]. The MPH plays a key role in the first line of defense against pathogenic invaders by initiating phagocytosis and other innate antimicrobial programs. Phagocytosis involves formation of the phagocytic cup and subsequent enclosing of engulfed bacteria within a phagosome [2]. Usually, a particle-containing phagosome follows a programmed path through the endosomal

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system, which is controlled by fusion and fission events eventually leading to phagolysosome fusion and elimination of the particle. However, pathogenic microbes have developed strategies to either avoid phagocytosis or deviate phagosome biogenesis to survive and thrive within host cells.

Mycobacteria engage different germ-line encoded pattern-recognition receptors (PRR), including C-type lectins such as the mannose receptor and DC-SIGN, which recognize cell wall glycolipids to initiate phagocytosis. Upon uptake, pathogenic mycobacteria arrest phagosome maturation at an early endosomal stage. This stage is characterized by an almost neutral pH of 6.3 due to paucity of the proton-pumping vesicular ATPase and low levels of LAMP-1 and active lysosomal hydrolases [1, 2]. The mycobacterial phagosome, however, carries actin-binding coronin 1 and enriches with iron-bound transferrin which is delivered through the transferrin receptor, most probably to supplement the mycobacterial need for iron [3]. Although the early endosomal niche of *M. tuberculosis* seems to suit its needs for survival and proliferation, virulent *M. tuberculosis* can withstand lysosomal conditions [4]. *M. tuberculosis* has also been observed escaping into the cytoplasm [5] but those findings were critically discussed [6]. Numerous virulence factors, which potentially interfere with phagosome maturation, have been described for *M. tuberculosis*. Those include the protein phosphatase PtpB, the eukaryote-like lipid phosphatase SapM as well as the serine/threonine kinase PknG [7]. Components of the complex mycobacterial cell wall have also been identified as being able to deviate the phagosome biogenesis, including mannose-capped lipoarabinomannan, phosphatidyl-inositol mannosides, and the cord factor, trehalose-6,6-dimycolate [3, 8–11].

To characterize the function of purified mycobacterial cell wall lipids, simplified lipid-coated bead models have been developed [3, 11, 12]. Bovine-serum-albumin (BSA) was covalently linked to magnetic polystyrene beads (Dynabeads, Dynal) via ester-bonds facilitated by the tosyl-activated surface of the beads. The lipid-binding capacity of BSA was employed to coat the beads with purified cell wall lipids of *M. tuberculosis* such as TDM or control lipids. These beads were used to "infect" murine macrophages. After different time points, bead phagosomes were isolated, purified and analyzed using three different types of assays to characterize the maturation stage, i.e., enzyme tests (Fig. 1), FACS (Fig. 2), and western blot (Fig. 3).

Therewith, we were able to show that TDM exposed on the particle surface can interfere with bead phagosome maturation [3]. Furthermore, using this reductionist model, we revealed that the virulence function of TDM, i.e., decelerating phagosome maturation, is abolished in interferon gamma activated MPH by nitric oxide [3]. The lipid-coated bead model as detailed below is a useful approach to study effects of mycobacterial (and other pathogen-derived) glycolipids on phagosome biogenesis and other innate responses of MPH and other phagocytes.

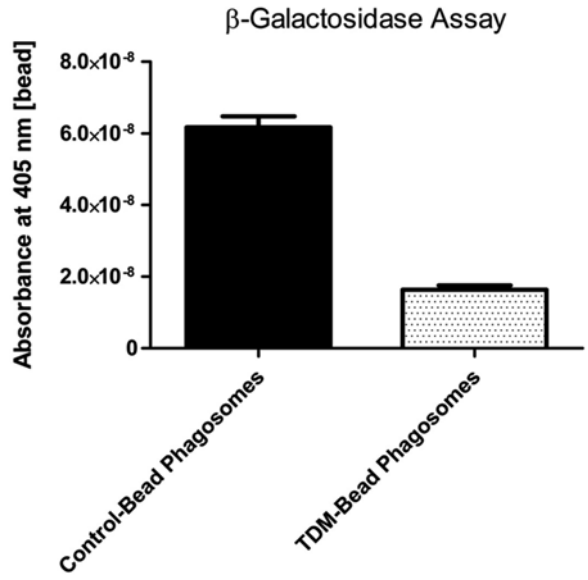

Fig. 1 TDM inhibits the acquisition of a lysosomal enzymes in bead phagosomes. 3× 10 μL of isolated control and TDM bead phagosomes were incubated with 150 μL β-Galactosidase reaction mix in a 96-well plate for 18 h at 37 °C. The reaction was stopped by addition of 150 μL 0.5 M Na_2CO_3. The enzyme activity was determined by measuring absorbance at 405 nm and finally normalized with the number of beads in each sample. Data are expressed as mean of β-Galactosidase activity for technical triplicates ± SD

2 Material

2.1 Cell-Culture of RAW 264.7

1. RAW 246.7 cell line (ATCC, Wesel, Germany).
2. RAW-medium: DMEM (Dulbecco's modified eagle medium) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 1 % L-glutamine, and 1 % penicillin/streptomycin. Sterilize through a 0.2 μm filter.

2.2 Materials for Coating of Dynabeads

1. Phosphate buffered saline (PBS).
2. Dynabeads (M-280 Tosyl-activated, Life Technologies).
3. DynaMag-2 Magnet (Life Technologies).
4. Bovine serum albumin (BSA), 1 % in PBS.
5. Sterilize BSA-solution through a 0.20 μm filter prior use.
6. TDM solution: dissolve 10 mg Trehalose-6,6-dimycolate (TDM) (BioClot GmbH or non-commercial sources) in 2 mL chloroform (*see* **Note 1**).
7. Water-bath sonicator, e.g., Sonorex Super AK 255H (Bandelin Electronic).

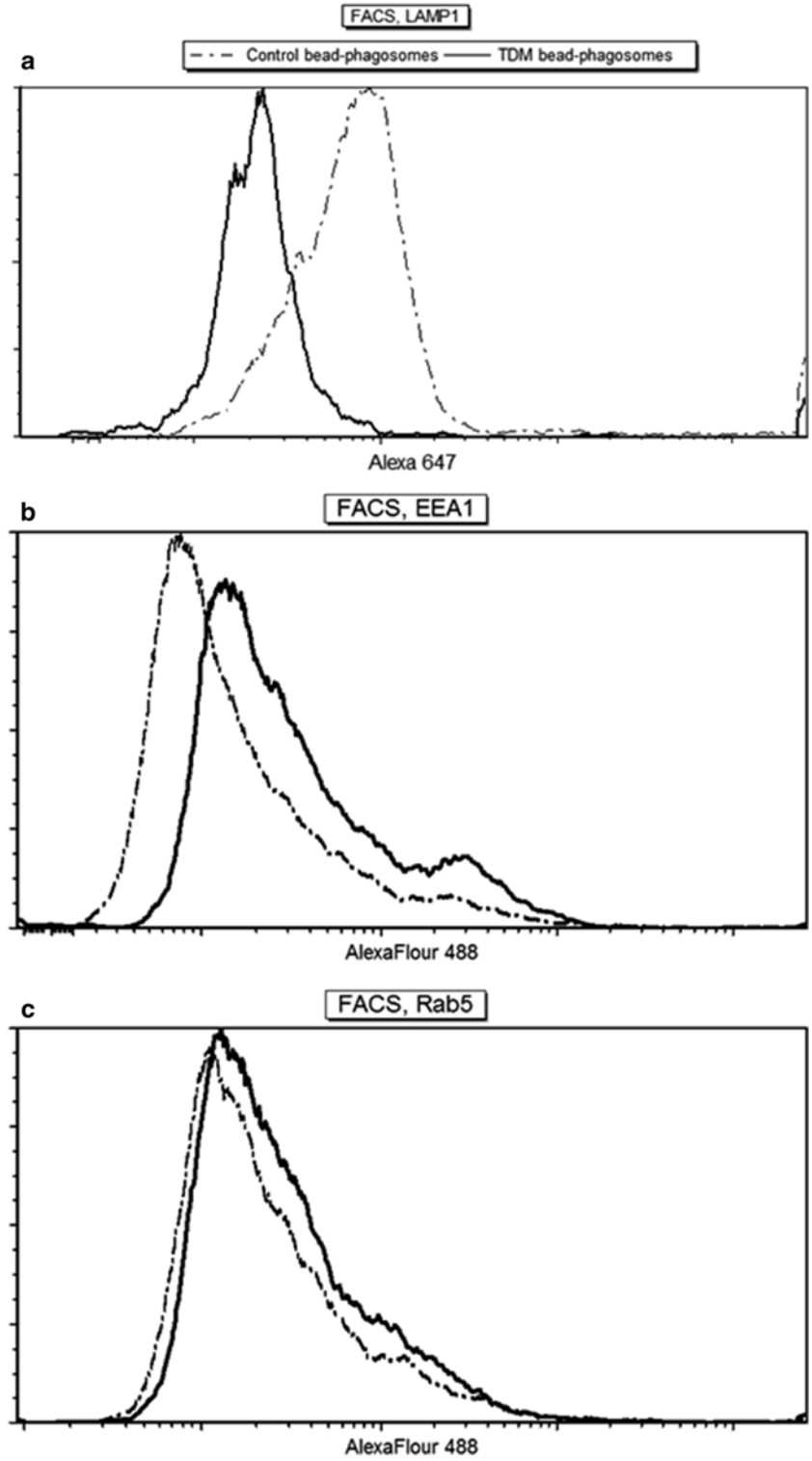

Fig. 2 TDM inhibits the acquisition of LAMP-1 but retains EEA1 and Rab5 in bead phagosomes. FACS analysis of control and TDM-bead phagosomes previously stained for LAMP-1 (**a**), EEA1 (**b**), and Rab5 (**c**). 100 μL of isolated control and TDM-bead phagosomes were blocked and subsequently incubated with antibodies to LAMP-1, EEA1, or Rab5 for 30 min at 4 °C. After washing, bead phagosomes were subsequently incubated with a secondary antibody coupled to $AlexaFlour_{647}$ for 20 min at 4 °C. Bead phagosomes were finally fixed with 1 % PFA and analyzed by FACS using the LSR II Flow Cytometer

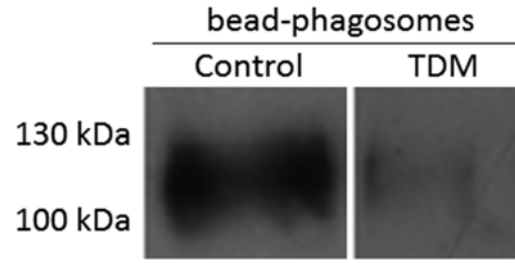

Fig. 3 TDM inhibits the acquisition of LAMP1 in bead phagosomes. 0.25 μg protein from isolated control and TDM-bead phagosomes were loaded onto a 10 % SDS-PAGE and subsequently blotted on a PVDF-membrane. LAMP1 was detected using a specific antibody via secondary antibody coupled to HRP and detected by chemiluminescence

2.3 Materials for Isolation and Purification of Bead Phagosomes

1. RAW 246.7 cell line (ATCC) (*see* **Note 2**).
2. RAW-medium: DMEM supplemented with 10 % heat-inactivated FCS, 1 % L-glutamine, and 1 % penicillin/streptomycin. Sterilize complete medium through a 0.2 μm filter.
3. PBS.
4. D10-medium: DMEM is supplemented with 10 % heat-inactivated sterile FCS.
5. Homogenization-buffer: 250 mM sucrose, 0.1 % w/v gelatine, 20 mM HEPES pH 7. Store at 4 °C. Add protease inhibitors, e.g., Protease Inhibitor Tablets (Roche) before use (*see* **Note 3**).
6. Metal Douncer (Wheaton).
7. DNase.
8. DynaMag-2 Magnet (Life Technologies).
9. 15 % Ficoll gradient: dissolve 1.5 mg Ficoll in 10 mL homogenization buffer (*see* **Note 4**).

2.4 β-Galactosidase Assay

1. 1 M citrate buffer pH 3.5: dissolve 8.4 g citric acid and 2.94 g sodium citrate dehydrate in 100 mL deionized water.
2. Lysosomal β-Galactosidase reaction mix: add 0.04515 g ONPG (*p*-nitrophenyl-β-D-galactopyrannoside), 140 μL Triton X-100, and 3 mL citrate buffer to 16.86 mL deionized water.
3. Stop-Solution: dissolve 10.6 g $NaCO_3$ in 200 mL deionized water.
4. ELISA-Reader, e.g., Tecan Sunrise (Tecan Group Ltd).
5. Neubauer improved counting chamber, e.g., Paul Marienfeld GmbH & Co. KG.

2.5 FACS

1. Neubauer improved counting chamber.
2. Cytofix/Cytoperm (BD Bioscience).

3. Perm-Wash-Puffer (BD Bioscience).
4. Antibody to LAMP1 (Hybridoma Bank): dilute 1:500 in 100 µL Perm-Wash-Puffer.
5. Antibody to EEA1 (Early endosomal antigen-1, GeneTex): dilute 1:100 in 100 µL Perm-Wash-Puffer.
6. Antibody to Rab5a (GeneTex): dilute 1:100 in 100 µL Perm-Wash-Puffer.
7. Alexa Fluor 647-conjugated F (ab′) 2 fragment goat anti-rabbit IgG: dilute 1:1,000 in Perm-Wash-Puffer.
8. 10 % PFA: dissolve 5 g in 50 mL PBS (*see* **Note 5**).
9. LSR II Flow Cytometer (BD Biosciences).

2.6 Western Blot

1. Protein assays, e.g., 660 nm Protein Assay (Thermo Fisher Scientific Inc.).
2. BSA.
3. PBS.
4. ELISA plate reader, e.g., Tecan Infinite M200 (Tecan Group Ltd).
5. Tecan Infinite M200 Software Mangellan 6.0 (Tecan Group Ltd).
6. 30 % Acrylamide/Bis (37.5:1).
7. 20 % SDS.
8. TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylendiamine).
9. Ammonium persulfate (APS).
10. Gel electrophoresis system, e.g., Mini Protean Tetra Cell (Bio-Rad Laboratories Inc.).
11. Separating gel buffer: 1.5 M Tris–HCl, pH 8.8.
12. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8.
13. 5× Laemmli Sample Buffer: Mix 9.4 mL of 1 M Tris–HCl pH 6.8, 3 g SDS, 15 mL glycerine, 15 mg bromophenol blue, and 5.6 mL deionized water. Add 100 mM DTT before use.
14. Protein markers, e.g., PageRuler Prestained Protein Ladder (Thermo Fisher Scientific).
15. Running buffer: 25 mM Tris, 190 mM glycine, 0.1 % w/v SDS (pH 8.3).
16. PVDF membrane.
17. Blotter for Western transfer, e.g., Bio-Rad Criterion Blotter (Bio-Rad Laboratories Inc.).
18. Transfer buffer: 48 mM Tris, 38.5 mM glycine, 0.04 % w/v SDS, 20 % v/v methanol.

19. Tris-Buffered Saline with Tween 20 (TBST): 2.423 g Tris, 8,006 g NaCl, 1 mL Tween 20 in 1 L distilled water, pH 7.6.
20. Blocking Buffer: 5 % milk powder, 2 % goat serum in TBST.
21. Antibody to LAMP1 (Hybridoma Bank): dilute 1:500 in blocking buffer.
22. IgG HRP-conjugated goat anti-rat: dilute 1:1,000 in blocking buffer.
23. ECL advance chemiluminescence kit.
24. Hyperfilm MP.

3 Methods

3.1 Cell Culture of RAW 264.7

Prepare RAW 264.7 macrophages 1 day prior to bead infection. Work under sterile conditions.

1. Seed 10^7 Raw 264.7 macrophages per T-75 flasks in 20 mL RAW-medium.
2. Incubate overnight at 37 °C, 7.5 % CO_2.

3.2 Coating of Magnetic Beads with Lipids

Prepare control and TDM-coated beads 1 day prior bead-infection. Work under sterile conditions (*see* **Note 6**).

1. Wash Dynabeads (5–10 beads/cell) three times with 1 mL PBS using the DynaMag-2 Magnet.
2. Coat Dynabeads by incubating with 1 % BSA in 20 mL PBS for 3 h at room temperature with constant overhead shaking.
3. Pellet BSA-coated beads by centrifugation at 300 × *g* for 5 min at RT.
4. Discard supernatant and wash Dynabeads three times with 1 mL PBS using the DynaMag-2 Magnet.
5. For coating beads with glycolipids, dissolve TDM in chloroform using a glass vial (50 μg per 10^7 beads).
6. Evaporate chloroform by blowing nitrogen gas into the glass-vial (*see* **Note 7**).
7. Add BSA-coated beads to the dried TDM in a total volume of 500 μL PBS. In parallel, add control beads to an empty glass-vial.
8. Sonicate control and TDM beads in a water-bath sonicator for 90 min at full speed and store overnight at 4 °C prior to use (*see* **Note 8**).

3.3 Isolation and Purification of Bead Phagosomes

Work under sterile conditions.

1. Discard RAW-medium from RAW 264.7 macrophages.
2. Wash cells with 5 mL warm PBS.

3. Prepare suspensions of control and TDM-coated beads in 5 mL ice-cold D10-medium at a concentration to achieve a multiplicity of "infection" of 5–10 beads per macrophage.
4. Pour beads onto the monolayer of macrophages and incubate for 5 min at 4 °C followed by 5 min at 37 °C and 7.5 % CO_2 (this will synchronize uptake).
5. Remove medium and wash cells with 5 mL warm D10-medium per flask to remove excess beads.
6. Add 10 mL warm D10 medium per flask and allow phagocytosis to proceed for the required amount of time (usually 30 min) in D10 media at 37 °C and 7.5 % CO_2.
7. Discard medium and wash with 5 mL PBS.
8. Scrape macrophages into ice-cold PBS to stop phagocytosis and phagosome maturation.
9. Transfer cells to a 50 mL tube and centrifuge at 300 × *g* for 5 min at 4 °C.
10. Remove supernatant and resuspend macrophage pellet in 1 mL of homogenization buffer.
11. Isolate bead phagosomes from macrophages by disruption using a metal Douncer placed in ice. Check the level of disruption regularly using light microscopy during douncing until 90–95 % of the macrophages are lysed (*see* **Note 9**).
12. Break up the DNA in the bead phagosome samples by five passages through a 23 gauge needle.
13. Digest the DNA in the bead phagosome samples by incubation with 50 u/mL DNase for 10 min at 37 °C. Repeat this step if sample is still sticky.
14. Wash the bead phagosomes three times in 1 mL homogenization buffer using the DynaMag-2 Magnet.
15. Layer bead phagosomes onto a 15 % Ficoll cushion and centrifuge at 450 g for 15 min at 4 °C. Recover the bead phagosome containing pellet.
16. Repeat the Ficoll gradient step (*see* **Note 10**).
17. Remove supernatant and resuspend the bead phagosome pellet in 1 mL homogenization buffer and wash bead phagosomes three times in 1 mL homogenization buffer using the DynaMag-2 Magnet.
18. Resuspend bead phagosomes in 1 mL ice-cold PBS and store at –20 °C until further use.

3.4 β-Galactosidase Assay

1. Add 10 µL control or TDM-bead phagosome samples to 150 µL lysosomal β-galactosidase reaction in triplicate in a 96 well plate.
2. Mix and incubate for 1 h at 37 °C.

3. Add 150 µL stop-solution and incubate for 5 min at RT (*see* **Note 11**).
4. Measure the absorbance at 405 nm (A_{405}) using an ELISA reader.
5. Count the number of bead phagosomes using a Neubauer improved counting chamber and light microscopy.
6. Normalize the enzyme activities to the total number of bead phagosomes.
7. Dilute 10 µL control of TDM-bead phagosome samples in 990 µL water and shake vigorously for 30 min (*see* **Note 12**).
8. β-Galactosidase Activity equals the mean A_{405} per number of bead phagosomes.

3.5 FACS

1. Determine number of beads/mL in control and TDM-bead phagosome samples by counting with a Neubauer improved counting chamber slide using light microscopy.
2. Dilute 10 µL control or TDM-bead phagosome samples in 990 µL water and shake vigorously for 30 min (*see* **Note 12**).
3. Aliquot 10^7 control or TDM-bead phagosomes and centrifuge for 10 min at 900 × *g* at 4 °C (*see* **Note 13**).
4. Resuspend the pellet in 250 µL Cytofix/Cytoperm and incubate for 20 min at 4 °C.
5. Add 750 µL 1× Perm-Wash-buffer and centrifuge at 900 × *g* for 10 min at 4 °C.
6. Resuspend pellet in 1 mL 1× Perm-Wash-buffer and centrifuge for 10 min at 900 × *g* at 4 °C.
7. Resuspend pellet in 100 µL of 1× Perm-Wash-buffer containing the primary antibody for either LAMP-1 (Fig. 2a), EEA1 (Fig. 2b), or Rab5a (Fig. 2c) and incubate for 30 min at 4 °C (*see* **Note 14**).
8. Add 1 mL 1× Perm-Wash-Puffer and centrifuge for 10 min at 900 × *g* at 4 °C.
9. Resuspend pellet in 100 µL 1× Perm-Wash-Puffer containing the corresponding secondary antibody Alexa Fluor 647-conjugated F (ab′) 2 fragment goat anti-rabbit or anti-rat IgG and incubate for 20 min at 4 °C.
10. Add 1 mL 1× Perm-Wash-buffer and centrifuge for 10 min at 900 × *g* at 4 °C.
11. Resuspend the pellet in 300 µL 1 % paraformaldehyde in PBS.
12. Analyze samples using a flow cytometer as LSR II Flow Cytometer.

3.6 Western Blot

1. Incubate 10 µL of control or TDM-bead phagosomes with 150 µL of the Pierce 660 nm protein reagent in triplicate and incubate for 5 min at RT.
2. Prepare standards by dilutions of a 2 mg/mL BSA stock in PBS to 1.5, 1, 0.75, 0.5, 0.25, 0.125, and 0.0625 mg/mL.
3. Incubate 10 µL of standards with 150 µL of the Pierce 660 nm protein reagent in duplicate and incubate for 5 min at RT.
4. Measure the A_{660} of standards and samples using an ELISA plate reader.
5. Calculate protein concentrations using the ELISA-reader software.
6. For the separating gel, mix 8.2 mL deionized water, 6.6 mL 30 % acrylamide, 5 mL separating gel buffer, 200 µL 10 % SDS, 10 µL TEMED, and 100 µL 10 % APS and cast gel in the Mini-Protean Tetra Cell.
7. For the stacking gel, mix 6.1 mL deionized water, 1.3 mL 30 % acrylamide, 2.5 mL stacking gel buffer, 100 µL 10 % SDS, 10 µL TEMED, and 50 µL 10 % APS and cast gel in the Mini-PROTEAN Tetra Cell. Insert a 10-well gel comb immediately without introducing air bubbles.
8. Take 0.25 µg total protein from control and TDM-bead phagosome samples, add 5× Laemmli Sample Buffer and heat for 5 min at 95 °C.
9. Load samples along with 3 µL of the protein ladder PageRuler Prestained Protein Ladder to the 10 % SDS-gel and run gel for 80 min at 120 V in running buffer using the Bio-Rad Mini Protean Tetra Cell.
10. Disassemble the Bio-Rad Mini Protean Tetra Cell and rinse gel with deionized water.
11. Cut a PVDF membrane to the size of the gel and immerse in methanol. Rinse twice in distilled water and once with transfer buffer.
12. Assemble gel and PVDF membrane in the Bio-Rad Criterion Blotter and transfer proteins for 45 min at 100 V in transfer buffer (*see* **Note 14**).
13. Disassemble Bio-Rad Criterion Blotter and block membrane in blocking buffer for 1 h at RT.
14. Wash membrane three times in TBST for 10 min.
15. Incubate membrane with the primary antibody LAMP1 in blocking buffer at 4 °C ON shaking.
16. Wash membrane three times in TBST for 10 min.

17. Incubate membrane with the secondary antibody IgG HRP-conjugated goat anti-rat in blocking buffer for 3 h at RT shaking.
18. Wash membrane three times in TBST for 10 min and rinse membrane once with deionized water.
19. Mix 1 mL ECL Detection Reagent 1 with 1 mL ECL Detection Reagent 2 and pour onto the membrane. Incubate 1 min at RT and wash membrane carefully with 3 mL deionized water.
20. Detect signals using Hyperfilm MP (*see* **Note 15**).

4 Notes

1. Keep in mind, that TDM is an inflammatory agent. Hence, avoid contact with skin.
2. RAW 264.7 cells were recently upgraded to biosafety level 2 organisms.
3. Gelatin dissolves best when the homogenization buffer is heated in a water bath at 50 °C.
4. Weight and dissolve Ficoll powder in a 50 mL tube by vortexing. Transfer Ficoll solution to a 15 mL tube and cool on ice.
5. The samples can be stored in 10 % PFA for 1 day, if the FACS-analysis is not performed the same day.
6. The DynaMag-2 Magnet is a magnetic Eppendorf-tube stand. Hence, Eppendorf-tubes are used for samples containing beads.
7. Chloroform is inflammable and therefore be careful when evaporating it by blowing nitrogen into the vial. Evaporate chloroform from the bottom and not from the wall of the glass vial, otherwise TDM sticks to the wall where it does not get into contact with the bead suspension.
8. In some sonication devices, sonication waves are weaker or stronger depending on the location in the water bath. Here glass vials containing beads are fixed with adhesive tape to hold them in the right spot.
9. When the stamp of the Douncer is moved up and down, avoid formation of air bubbles.
10. The Ficoll gradient must be ice cold.
11. After addition of the stop solution, the wells of the 96-well plate are completely full. Avoid sealing the plate with its lid afterwards, because excess fluid may drip into other wells.
12. Dilution of the bead phagosome samples and vortexing heavily is important, since bead phagosomes tend to stick together in highly concentrated phagosome samples.

13. The pellet can be very small and hence hardly visible.
14. The protein ladder used herein is pre-stained, and the red and green bands should be visible on the PVDF-membrane after successful blotting.
15. The ECL-System used herein is very strong. Hence, exposure of the Hyperfilm MP to the membrane must be very quick (5–10 s).

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Chapter 23

Live Imaging of *Mycobacterium marinum* Infection in *Dictyostelium discoideum*

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Abstract

The *Dictyostelium discoideum–Mycobacterium marinum* host–pathogen system is a recently established and powerful model system for mycobacterial infection. In this chapter, two simple protocols for live imaging of *Dictyostelium discoideum* infection are described. The first method is used to monitor the dynamics of recruitment of GFP-tagged *Dictyostelium discoideum* proteins at single time-points corresponding to the main stages of the infection (1.5–72 h post infection). The second method focuses at the early stages of the establishment of an infection (0–3 h post infection). In addition, several procedures to improve the imaging of the bacterium-containing compartment are described. Basic bacterial parameters such as bacterial growth and the recruitment of host proteins to the bacterium-containing compartment can be easily and precisely quantified using macros for ImageJ. These methods can be adapted to monitoring mycobacteria infection in other systems using mammalian cells.

Key words *Dictyostelium discoideum*, Mycobacteria, Intracellular infection, Live microscopy, Fluorescence

1 Introduction

The social amoeba *Dictyostelium discoideum* is a well-established model system to study a wide range of cellular processes, including cell adhesion, chemotaxis, cell migration, cytokinesis, and phagocytosis (reviewed in ref. [1]). This is due to the simple laboratory handling of *D. discoideum* and to its haploid genome, which is fully sequenced and annotated (www.dictybase.org). Mutants are generated by homologous recombination or random Restriction Enzyme-Mediated Integration (REMI) mutagenesis, and new techniques as targeted gene insertion have been successfully developed [2]. Besides, *D. discoideum* is easily tractable for biochemistry and live imaging methods.

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In its natural environment, the forest soil, *D. discoideum* feeds on bacteria by phagocytosis. Interestingly, phagocytosis is a highly conserved process between *D. discoideum* and human phagocytes, such as macrophages and neutrophils. Moreover, it was shown that a high proportion of the phagosomal proteome of *D. discoideum* is orthologous to the one of insects and vertebrates [3]. Therefore, *D. discoideum* has become a powerful model to investigate phagocytosis.

Recently, given the excellent time and spatial resolution of such techniques, live imaging has become invaluable in gaining new insights into the phagosomal dynamics of *D. discoideum*. For instance, it has been shown using GFP- and RFP-tagged proteins that the vacuolar H^+-ATPase is retrieved by vesicles just before exocytosis [4] and that WASH-driven actin polymerization drives retrieval of the vacuolar H^+-ATPase [5]. Although GFP and RFP are the most common fluorophores, many others are available for use in *D. discoideum* [6]. These fluorophores are often used to track fusion proteins spatially and temporally. They can be constitutively expressed using actin promoters, or inserted downstream of endogenous promoters to monitor transcriptional activity, thus serving, for example, as cell fate tracker during differentiation.

During the last decade, *D. discoideum* has also emerged as a model system to study host–pathogen interactions of several bacterial and fungal pathogens, due to its high conservation of innate immunity related genes and pathways. Pathogens studied in *D. discoideum* include *Salmonella typhimurium* [7], *Pseudomonas aeruginosa* [8], *Cryptococcus neoformans* [9], *Vibrio cholerae* [10], *Legionella pneumophila* [11], and mycobacterial species [12, 13]. For example, new insight into alterations of phosphoinositide and membrane dynamics upon *Legionella* infection were gained by live imaging of *D. discoideum* cells [14].

Here, we describe *D. discoideum* as a host for *Mycobacterium marinum*. *M. marinum* is a genetically close relative of *Mycobacterium tuberculosis* [15], the causative agent of human tuberculosis. Although *M. marinum* cannot cause a systemic infection in humans, it causes tubercular diseases in its natural hosts, frogs and fish, and has become a model to study the mechanism of pathogenicity of *M. tuberculosis* (reviewed in ref. [16]). Moreover, virulence genes are highly conserved between both species and their intracellular fate is very similar in their respective hosts (reviewed in ref. [17]). *M. marinum* presents several advantages for laboratory handling as compared to *M. tuberculosis*, such as safer manipulation and a faster replication rate.

Mycobacterial infection is especially interesting to study because of the drastic manipulation of the host phagocytic pathway. This is achieved by several mechanisms, including the arrest of phagosomal maturation [18]. Furthermore, the compartment loses its phagosomal identity and becomes a permissive replication niche. Phagosomal phosphoinositides and RabGTPases, the major regulators of the phagocytic pathway, play an important role in this subversion [19, 20].

This chapter covers relevant methods to monitor and quantify *M. marinum* infection in *D. discoideum* by live imaging. Specifically, these methods enable to study the dynamics of GFP-tagged proteins that localize to the phagosome of pathogenic and nonpathogenic bacteria during infection, such as Rab5a, Rab7a, or Vacuolin A. Comparison with nonpathogenic conditions is a very valuable method to unravel the manipulation of the phagosome dynamics by mycobacteria. The first method is a general protocol that allows to follow the dynamics of GFP-tagged proteins at single time-points during the whole course of infection (1.5–72 h post infection), including the establishment of the niche and the escape of the bacterium into the cytosol [12, 13]. The second protocol described focuses on the events occurring immediately after addition of the bacteria, such as bacteria uptake and early recruitment or retrieval of phagosomal proteins (0–3 h post infection). Moreover, we describe a procedure to quantify the dynamics of recruitment of GFP-tagged proteins in the vicinity of the bacteria, as well as bacterial growth. This can be achieved using simple macros of the free software ImageJ, which can be easily customized. Obviously, all these methods can also be valuable to monitor the intracellular fate of various bacterial pathogens in almost any phagocytic cell.

2 Materials

2.1 Cell Culture

1. *D. discoideum* wild-type strain AX2(Ka) [21].
2. *D. discoideum* GFP-expressing variants, e.g., GFP-Rab5a (pDX-GFP-Rab5a), GFP-Rab7a (pDXA-GFP-Rab7a), and VacA-GFP (pDM323-VacA-GFP). The first two strains are available from the authors. The VacA-GFP strain was generously provided by Dr Monica Hagedorn.
3. Hl5c medium including glucose supplemented with vitamins and microelements (ForMedium): resuspend 26.55 g of powder in 1 L of deionized water. Filter-sterilize (*see* **Note 1**).
4. Penicillin/streptomycin 100× stock solution, 10,000 U and 10,000 µg/mL, respectively.
5. Geneticin (G418): prepare a 1,000× stock solution by dissolving 10 mg of powder in 1 mL deionized water. Filter-sterilize and store at −20 °C.
6. 10 cm cell culture dishes, tissue-culture treated polystyrene.

2.2 Mycobacterial Culture

1. *M. marinum* M strain (ATCC BAA-535). Other mycobacterial species, such as *M. smegmatis* or *M. tuberculosis*, can be used as well.
2. 7H9 medium (Difco): dissolve 4.7 g of Middlebrook 7H9 powder in 900 mL of deionized water. Add 500 µL Tween 80

and 2 mL of glycerol and autoclave. Let the medium cool down and add 10 % v/v of Middlebrook OADC supplement (Becton Dickinson). Filter-sterilize and store at 4 °C.

3. Kanamycin: prepare a 1,000× stock solution by dissolving 30 mg of powder in 1 mL deionized water. Filter-sterilize and store at −20 °C.
4. Hygromycin: prepare a 1,000× stock solution by dissolving 100 mg or powder in 1 mL deionized water. Filter-sterilize and store at −20 °C.
5. Glass beads, 5 mm diameter.

2.3 Preparation of Cells for Infection

1. 10 cm cell culture dishes, tissue-culture treated polystyrene.
2. μ-dish, 35 mm high (ibidi GmbH).
3. Hl5c medium: *See* Subheading 2.1 **item 3**.
4. LoFlo medium (Low fluorescent medium, ForMedium): resuspend 16.8 g of powder in 1 L of deionized water and adjust to pH 6.5. Filter-sterilize (*see* **Note 1**).

2.4 Preparation of an Agarose Overlay

1. UltraPureTM Agarose.
2. Hl5c medium: *See* Subheading 2.1 **item 3**.
3. LoFlo medium: *See* Subheading 2.3 **item 4**.
4. Glass plate (approximately 10 × 7 cm).
5. Kimwipe paper.
6. Spatula or razor blade.

2.5 Long-Term Infection Assay

1. Hl5c medium: *See* Subheading 2.1 **item 3**.
2. Blunt needle 25 G × 3/4″; 0.5 × 18.
3. 3 mL syringe.
4. 6-well plates.
5. Penicillin/streptomycin: *See* Subheading 2.1 **item 4**.
6. Cell culture inverted microscope with a 40× phase contrast objective.
7. Parafilm.
8. μ-dish, 35 mm high (ibidi GmbH).
9. Spinning Disc Confocal Microscope (*see* **Note 2**).
10. Widefield microscope (*see* **Note 3**).

2.6 Early Infection Assay

1. Hl5c medium: *See* Subheading 2.1 **item 3**.
2. Blunt needle 25 G × 3/4″; 0.5 × 18.
3. 3 mL syringe.

4. μ-dish, 35 mm high (ibidi GmbH).
5. Cell culture inverted microscope with a 40× phase contrast objective.
6. LoFlo medium: *See* Subheading 2.3 **item 4**.
7. Thin metal plate.
8. Parafilm.
9. Spinning Disc Confocal Microscope (*see* **Note 2**).
10. Widefield microscope (*see* **Note 3**).

2.7 Quantification and Data Analysis

1. ImageJ software.

3 Methods

3.1 Cell Culture

1. Recover *D. discoideum* cells from frozen spore stocks stored in liquid nitrogen.
2. Culture *D. discoideum* AX2(Ka) [21] in HL5c medium containing penicillin/streptomycin (100 U and 100 μg/mL respectively) in adherent culture at 22 °C to a maximum density of 5×10^7 cells/10 cm dish.
3. Culture GFP-Rab5a (pDX-GFP-Rab5a), GFP-Rab7a (pDXA-GFP-Rab7a) and VacA-GFP (pDM323-VacA-GFP) strains in HL5c medium containing 10 μg/mL G418.
4. The doubling time of *D. discoideum* is around 12 h. New cultures should be started from frozen cell aliquots about every 4–6 weeks.

3.2 Mycobacterial Culture

1. Recover mycobacteria (*see* **Note 4**) from −80 °C glycerol stocks and inoculate in 5 mL of 7H9 medium (plus appropriate antibiotics depending on the bacterial strain) (*see* **Notes 5** and **6**).
2. Incubate with shaking culture (150 rpm) at 32 °C.
3. Inoculate 25 mL of 7H9 medium in flasks containing 5 mm glass beads (to minimize clumping).
4. Incubate with shaking until OD_{600} of 1 is reached (approximately 1.8×10^8 bacteria/mL).

3.3 Preparation of Cells for Infection

1. Plate *D. discoideum* cells in 10 cm dishes for long-term infection assays (*see* Subheading 3.5), or in μ-dishes for early infection assays (*see* Subheading 3.6) in HL5c medium without penicillin/streptomycin. Dishes should be 20 % confluent.

2. Incubate for 1 day 22 °C. The plates should be 80 % confluent the day of infection.
3. For early infection assays, exchange the medium from HL5c to LoFlo 2 h prior to infection (*see* **Note 7**).

3.4 Preparation of an Agarose Overlay

An agarose overlay is used to improve imaging (*see* **Note 8**).

4. Dissolve 0.15 g of agarose in 10 mL of Hl5c or LoFlo (*see* **Note 7**) by heating in a microwave.
5. To obtain a 2 mm-thin agarose sheet, pour the solution on a 10 cm by 7 cm glass plate and wait until the agarose has solidified.
6. Cut 1.5 × 1.5 cm agarose slices with the help of a spatula or razor blade and keep them in a petri dish filled with Hl5c or LoFlo until use to avoid drying.
7. Before use, fish an agarose slice from the petri dish with the help of a spatula.
8. Remove the excess of Hl5c or LoFlo from the agarose slice with a tissue.
9. Aspirate carefully the medium from the μ-dish, leaving a thin film over the cells.
10. Deposit the agar slice gently by sliding it from the spatula in the middle of the μ-dish.

3.5 Long-Term Infection Assay

1. Measure the OD_{600} of the mycobacterial culture and calculate the volume necessary for a multiplicity of infection (MOI) of 10.
2. Centrifuge the mycobacteria at 18,000 × *g* for 4 min.
3. Wash the pellet twice with 1 mL Hl5c.
4. Syringe the suspension ten times through a blunt needle to break up bacteria clumps.
5. Aspirate the medium from the *D. discoideum* culture (*see* Subheading 3.3) and add the bacterial suspension in 5 mL of Hl5c medium without penicillin/streptomycin.
6. Seal the 10 cm dish with Parafilm and centrifuge twice at 500 × *g* for 10 min. Turn the dish 180° between spins to redistribute the bacteria homogenously.
7. Incubate for 10–20 min to allow phagocytosis.
8. Wash off the extracellular bacteria with 7 mL of Hl5c several times (3–8 repeats). Add and remove the medium gently to avoid detaching *D. discoideum* cells from the dish (*see* **Note 9**).
9. Resuspend the *D. discoideum* cells to a density of 1×10^6 cells/mL in Hl5c. Add penicillin/streptomycin (at a final concentration of 5 μg/mL streptomycin, 5 U/mL penicillin) to prevent extracellular growth of bacteria [22].

10. Dispense about 5 mL of infected cells suspension into multiple wells of a 6-well plate.
11. Incubate with shaking at 130 rpm at 25 °C for the required time.
12. Take samples at different hours post infection. Standard time-points are 1.5, 3, 6, 12, 24, 36, 48, and 72 h post infection. For each time-point, calculate the density of *D. discoideum* cells in the suspension and plate 4×10^6 cells in a final volume of 2 mL Hl5c into a μ-dish.
13. Allow *D. discoideum* cells to adhere to the μ-dish for at least 30 min.
14. Once at the microscope, take 1 μm Z-stacks with the 488 nm channel to monitor the GFP-labeled *D. discoideum*, the 561 nm channel to monitor the mCherry- or DsRed-expressing mycobacteria and phase contrast to detect *D. discoideum* cell contours (*see* Figs. 1 and 2). For time-lapse microscopy, it is recommended to perform single plane imaging instead of collecting Z-stacks, which can be easily achieved using an agarose overlay (*see* Subheading 3.4).

3.6 Early Infection Assay

The long-term infection assay (*see* Subheading 3.5) can be used to monitor the infection up to 72 h post infection. However, this protocol is not adequate to focus on events that happen immediately after the addition of the bacteria, such as bacterial uptake or early recruitment and retrieval of proteins to the niche. This is due to several time-consuming steps in the long-term infection protocol, such as the 20 min incubation time to allow the *D. discoideum* cells to phagocyte bacteria and the washing steps to remove the extracellular bacteria afterwards. Therefore, a different protocol is required to observe the events that happen early during infection.

1. Grow the mycobacteria culture until OD_{600} reaches 1 (~1.8×10^8 bacteria/mL).
2. Harvest 5×10^8 bacteria at 18,000 × *g* for 4 min.
3. Wash the pellet twice with 1 mL of Hl5c.
4. Syringe the suspension ten times through a blunt needle to break up bacteria clumps.
5. Cool down *D. discoideum* cells by placing the 80 % confluent μ-dish (*see* Subheading 3.3) on a thin metal plate in a styrofoam laboratory tray filled with ice for 10 min. This synchronizes *D. discoideum* phagocytosis of mycobacteria (*see* **Note 10**). From this step, *D. discoideum* cells should remain on the cooled metal plate as long as possible until visualization at the microscope.

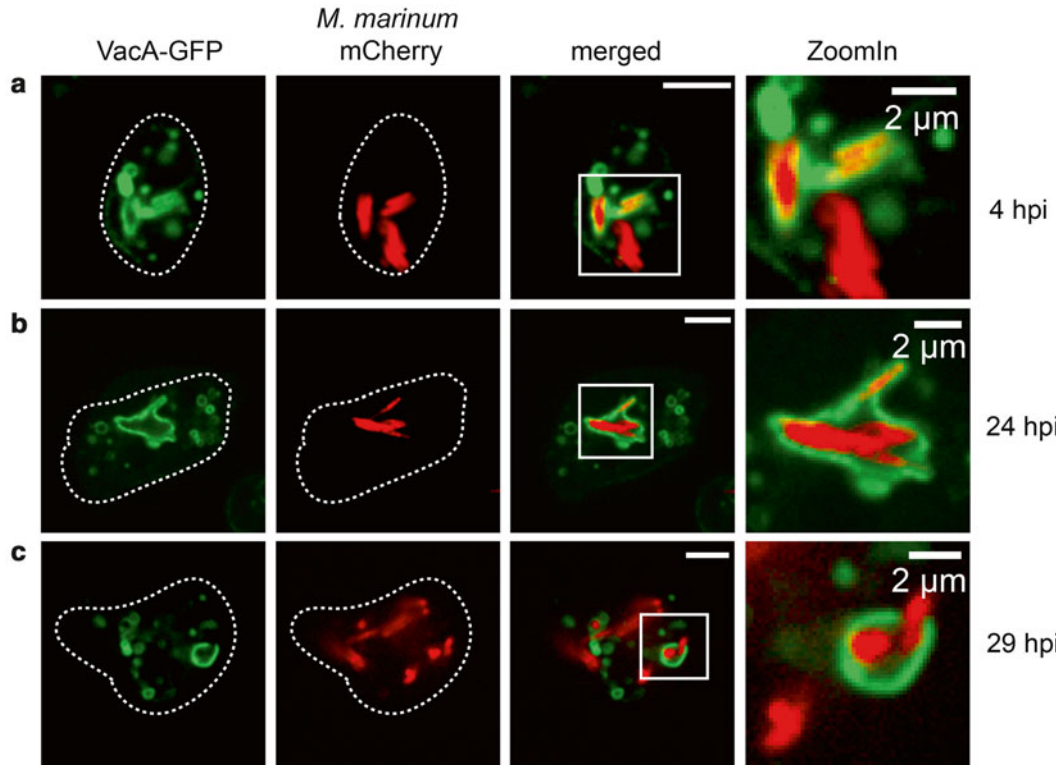

Fig. 1 Monitoring the different stages of *M. marinum* infection using Vacuolin A-GFP expressing *D. discoideum* cells. Confocal images of Vacuolin A-GFP expressing *D. discoideum* cells infected with mCherry *M. marinum* bacteria at different time-points. (**a**) The bacterium-containing phagosomes are partially decorated with Vacuolin A-GFP. (**b**) Bacteria are in a Vacuolin A-GFP positive compartment, revealing that it has some late endosomal characteristics in *D. discoideum* [12]. (**c**) The bacterium-containing compartment is broken and bacteria can access the cytosol. The scale bars are 5 µm if not indicated otherwise

6. Gently aspirate the medium of the *D. discoideum* culture from the cooled µ-dish and add 40 µL of the mycobacteria suspension in 1 mL of LoFlo medium (*see* **Note 7**) to obtain an MOI of 2.
7. Seal the µ-dish with Parafilm and centrifuge it at 500 × *g* for 2 min at 4 °C. In order to centrifuge the µ-dishes, place them inside 10 cm culture dishes ringed by some towel paper to prevent their movement.
8. Remove the medium carefully with a 1 mL pipette.
9. Place an agarose overlay on top of the cells (*see* Subheading 3.4).
10. Once at the microscope (*see* **Note 11**), use the 488 nm channel to monitor the GFP-labeled *D. discoideum*, the 561 nm channel to monitor the mCherry or DsRed expressing mycobacteria and the phase contrast to detect *D. discoideum* cell contours (Fig. 3).

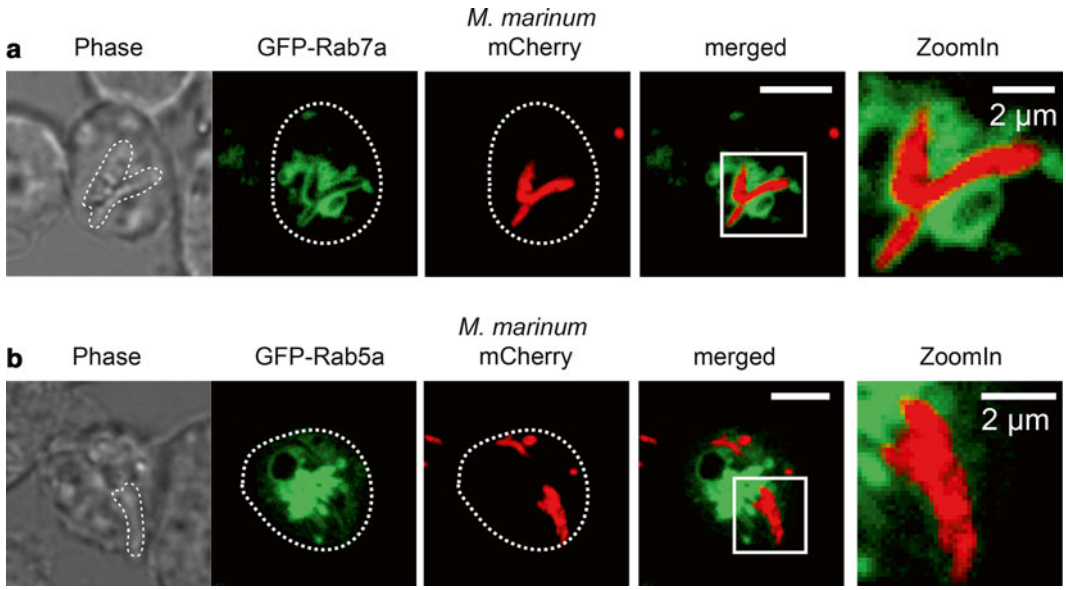

Fig. 2 Monitoring the late stages of infection in *D. discoideum* cells expressing GFP-Rab7a and GFP-Rab5a. Confocal images of GFP-Rab7a and GFP-Rab5a expressing *D. discoideum* cells infected with mCherry *M. marinum* bacteria, at 24 h post infection. (**a**) Bacteria are in a GFP-Rab7a-decorated compartment, underlining its late endosomal characteristics. (**b**) Consistently, bacteria are in a compartment that is not labeled by the early endosomal marker GFP-Rab5a. The scale bars are 5 µm if not indicated otherwise

11. Take 2D time-lapse movies with intervals of 5 s. One plane imaging is recommended instead of Z-stacks in order to reduce phototoxicity during long-time recordings. In order to maintain the same imaging plane, use the autofocus function offered by the imaging software (*see* **Note 12**).

3.7 Quantification and Data Analysis

ImageJ is a free Java software that can be used to visualize and analyze the images acquired at different microscopes [23]. The wide range of tools and plugins available enable customized combinations to perform the appropriate analysis in each case. Large sets of images can be easily and quickly processed using macros and the Batch Processing tool. Fiji (Fiji Is Just ImageJ) is a distribution of ImageJ that contains many useful plugins that are uploaded and updated automatically [24].

The dynamics of the recruitment of *D. discoideum* proteins to the mycobacterium-containing compartment can be measured by the increase in fluorescence intensity of GFP-proteins in the vicinity of the bacterium over time. ImageJ tools enable the precise quantification of this recruitment, when combined in the appropriate pipeline. However, automated tracking of the bacterium of interest is difficult and frequently inaccurate, and often becomes the most challenging of the analysis steps. To circumvent this problem we first track the bacterium manually using a simple plugin

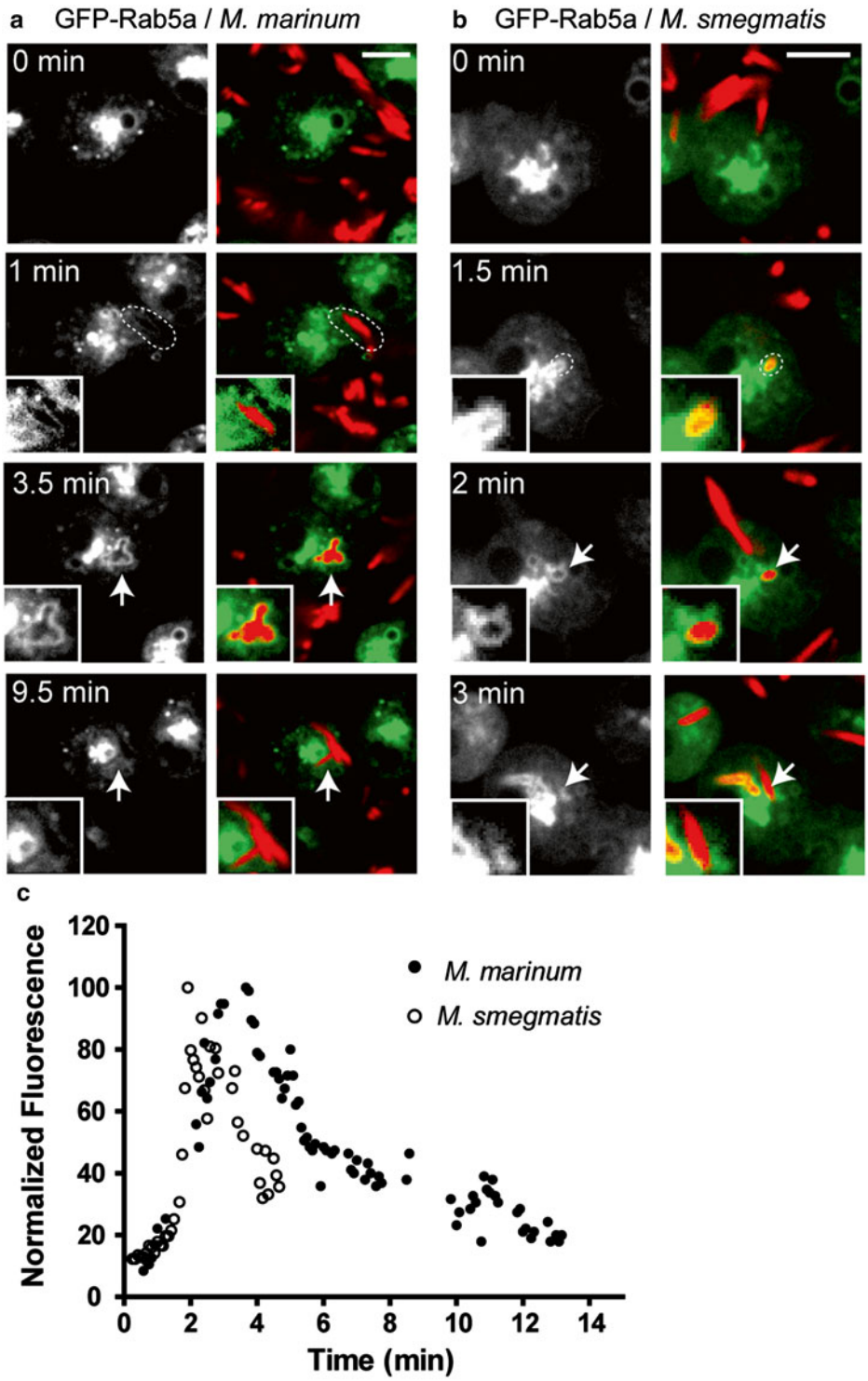

Fig. 3 Monitoring the dynamics of GFP-Rab5a recruitment to the *M. marinum* and the *M. smegmatis* containing compartments in *D. discoideum*. Time-lapse imaging of GFP-Rab5a expressing *D. discoideum* cells infected with (**a**) mCherry *M. marinum* bacteria or (**b**) DsRed *M. smegmatis* bacteria showing the retrieval of Rab5a approximately at 9.5 min and 3 min, respectively. Time-lapse was recorded using 5 s intervals. Dashed circles correspond to the first time at which bacteria first colocalize with GFP-Rab5a after uptake. Arrows point to intracellular bacteria. Insets show events of interest with higher contrast. (**c**) Quantification of GFP-Rab5a dynamics after uptake of *M. marinum* (*black circles*) and *M. smegmatis* (*white circles*). The scale bars are 5 μm

that automatically recenter the "clicked" bacterium of interest in a recalculated image field for further visualization and analysis (*see* **Note 13**). Then, a pipeline that crops the field around the selected bacterium to avoid interference from surrounding bacteria and that performs the subsequent analysis can be applied. As an example of such a pipeline, the following macro has been developed and can be applied to time-lapse RGB stacks (Fig. 3).

```
macro "Measurement of host markers at the bacterium-containing
    compartment"{
    title = getTitle
    x = getNumber("Crop width?",100);
    y = getNumber ("Crop length?",100);
    a = getWidth;
    b = getHeight;
    makeRectangle((a/2)-(x/2), (b/2)-(y/2), x, y);
    run("Crop");
    run("Split Channels");
    c = getString("channel of bacteria?", "red");
    selectWindow(title + " (" + c + ")")
    run("Threshold…");
    d = getNumber ("Min threshold?",100);
    e = getNumber ("Max threshold?",250);
    setThreshold(d, e);
    run("Convert to Mask");
    run("Close-");
    run("Fill Holes");
    run("Analyze Particles…", "size = 0-Infinity circularity = 0.00-
        1.00 show = [Overlay Outlines] exclude add in_situ stack");
    f = getString("channel to analyse?", "red");
    g = title + " (" + f + ")";
    h = getNumber("band width?", 1);
    for (i = 0 ; i < roiManager("count"); i++) {
        roiManager("select", i);
        run("Make Band…", h);
        run("Set Measurements…", "area mean standard min
            perimeter integrated median area_fraction stack dis-
            play add redirect = [" + g + "] decimal = 3");
        run("Measure");
    }
}
```

This macro is composed of the combination of tools described below:

1. "Crop" (Image > Crop) the center of the image to isolate the bacterium of interest. This step simplifies the analysis, since only the bacterium in the center of the image will be considered for the subsequent processing (*see* **Note 13**).
2. "Split channels" (Image > Color > Split channels) to separate the channels composing the image.
3. "Threshold" (Image > Adjust > Threshold) to create a binary image of the channel where the bacterium can be detected (*see* **Note 14**).
4. "Close" (Process > Binary > Close) and "Fill Holes" (Process > Binary > Fill Holes) in order to improve the detection of the bacterium.
5. "Analyze Particles" (Analyze > Analyze particles), in order to identify the bacterium of interest as "object" or "particle" (*see* **Note 15**). Select "Add to Manager" to import those "objects" to the "ROI Manager". The "ROI Manager" enables the subsequent management of the identified "objects".
6. "Make Band" (Edit > Selection > Make band) and selecting the appropriate width to fit to the membrane of the compartment.
7. "Measure" (Analyze > Measure) or (ROI Manager > Measure) to obtain the integrated intensity for the band around each identified bacterium in the "ROI Manager" (*see* **Note 16**). The results will be displayed in a new window and can be easily imported to other programs, such as Excel.

3.8 Analysis of Mycobacteria Growth Over Time

Basic parameters related to the bacterium, such as intracellular or extracellular growth, can be quantified with ImageJ in a similar manner as described in Subheading 3.7. For example, the growth of red fluorescent mycobacteria can be measured by the increase in fluorescence intensity over time. The representation of the integrated intensity of the (group of) bacteria over time reflects the increase of bacterial mass (Fig. 4).

The following macro has been developed as an example of pipeline to quantify bacteria growth during time-lapse microscopy (*see* **Note 17**):

```
macro "Measurement of bacterium growth"{
    title = getTitle
    x = getNumber("Crop width?",100);
    y = getNumber ("Crop length?",100);
    a = getWidth;
```

```
    b = getHeight;
    makeRectangle((a/2)-(x/2), (b/2)-(y/2), x, y);
    run("Crop");
    run("Split Channels");
    c = getString("channel of bacteria?", "red");
    selectWindow(title + " (" + c + ")")
    run("Threshold…");
    d = getNumber ("Min threshold?",100);
    e = getNumber ("Max threshold?",250);
    setThreshold(d, e);
    run("Convert to Mask");
    run("Close-");
    run("Fill Holes");
    run("Analyze Particles…", "size = 0-Infinity circularity = 0.00-
        1.00 show = [Overlay Outlines] exclude add in_situ
        stack");
    f = getString("channel to analyse?", "red");
    g = title + " (" + f + ")";
    for (i = 0 ; i < roiManager("count"); i++);
    run("Set Measurements…", "area mean standard min perim-
        eter integrated median area_fraction stack display add redi-
        rect = [" + g + "] decimal = 3");
    roiManager("Measure");
}
```

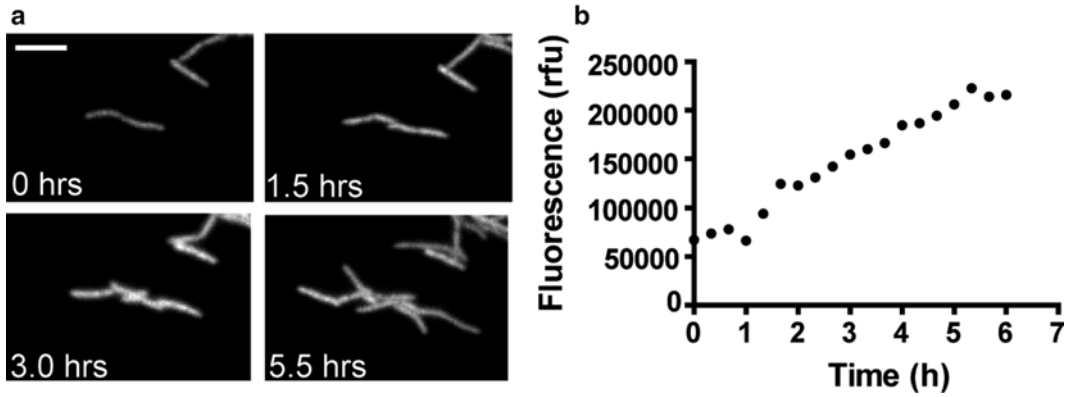

Fig. 4 Measurement and quantification of bacterial growth. (**a**) Images showing *M. smegmatis* growth in 7H9 in a μ-dish with an agarose overlay on top. Time-lapse was recorded using 20 min intervals. (**b**) Quantification of bacteria growth. The scale bar is 5 μm

4 Notes

1. It is better to use filter-sterilized medium for microscopy experiments, because most autoclaved media show high autofluorescence, likely due to Maillard chemical reactions between amino acids and reducing sugars that increase with temperature.
2. A Spinning Disc Confocal Microscope (Intelligent Imaging Innovations Marianas SDC mounted on an inverted microscope (Leica DMIRE2)) is used for visualization of rapid events. It is equipped with two lasers with lines at 488 and 560 nm for green and red fluorophores, respectively. Both fluorescence channels can be excited sequentially to avoid possible interferences in their detection. The Slidebook version 6.0 (Intelligent Imaging Innovations) software version 5.0 can be used for precise control of Neutral Density and Exposure of the lasers as well as Intensification and Gain of each individual signal. High resolution is given by the 100× oil objective (NA 1.4) and the sensitivity of the Evolve EMCCD Camera from Photometrics®.
3. Leica AF6000 LX is a widefield microscope used for long term experiments that can last for several days. It is coupled to a thermo-box that enables accurate temperature regulation (from 22 to 37 °C) and stability. The GFP and the I3 (FITC/RFP) filters can be used in combination with phase contrast. Both fluorescence channels can be excited sequentially to avoid possible interferences in their detection. The 100× oil objective (NA 1.47) and the sensitivity of the Cascade II 512 Camera from Photometrics® provide high resolution. The Leica LAS AF Lite software can be used to control different positions and intervals in time-lapse experiments, as well as the autofocus function (*see* **Note 12**).
4. *M. marinum* is a fish and frog pathogen that cannot cause systemic infection in humans because of growth restriction above 30 °C. However, it may produce skin lesions and granuloma in the extremities. Therefore, special handling and laboratory equipment are required (gloves, lab coat, blunt needles, etc.).
5. *M. marinum* is classified as a Biosafety Level 2 (BSL2) microorganism. Red fluorescent *M. marinum* bacteria expressing the pCherry10 plasmid [25] are grown in 7H9 supplemented with 100 μg/mL hygromycin at 32 °C. Under these conditions, the doubling time of *M. marinum* is approximately 10 h.
6. *M. smegmatis* is a nonpathogenic mycobacterial strain and is used as a control. Red fluorescent *M. smegmatis* harboring the Msp12::DsRed plasmid (kind gift of Dr L. Ramakrishnan, WASHU, Seattle) is grown in 7H9 supplemented with 50 μg/mL kanamycin at 37 °C. *M. smegmatis* doubles each 3 h [26].

7. LoFlo is a low fluorescence medium and is less phototoxic than Hl5c. Therefore, it is more appropriate for time-lapse recordings lasting up to a few hours. However, LoFlo medium is very poor in nutrients, so for longer recordings it is more appropriate to add 50 % Hl5c.
8. An agarose overlay is used to improve imaging for two reasons. First, it reduces *D. discoideum* cell movement so it prevents their escape from the imaging field. Second, it slightly squeezes *D. discoideum* cells, so vesicles and other organelles become concentrated in the XY plane and are consequently in the best orientation to acquire relevant data. It has to be taken into consideration that mechanical pressure induces autophagy in *D. discoideum* [27]. Therefore, addition of an agarose overlay is not recommended when visualizing autophagy-related events and markers.
9. Control the efficiency of the washing step by checking the presence of extracellular bacteria with an inverted cell culture microscope.
10. Low temperature inhibits cell motility and phagocytosis in *D. discoideum* [28, 29]. This property can be used to synchronize phagocytosis of mycobacteria with a temperature switch from 4 to 22 °C under the microscope. The frequency of uptake events will increase during the first minutes of imaging, which is especially important when monitoring the early time-points of infection.
11. The temperature switch from 4 to 22 °C occurs when removing the μ-dish from the cold metal plate and placing it onto the microscope stage.
12. Several microscopy programs enable autofocusing, which consists in the automatic selection of the most contrasted plane in a Z-stack. This is especially important during long-term imaging, when a significant shift in the *Z* axis over time could lead to a subsequent loss of the appropriate focal plane.
13. Best results are obtained when the bacterium or the bacterium-containing compartment of interest is in the center of the image. This can be done manually by cropping each image individually, or in a semi-automated way using specific plugins. In this case, the *CenterOnClick* plugin (Nicolas Roggli, nicolas.roggli@unige.ch, unpublished) was used. As a result of clicking on a pixel/object of interest, this plugin recalculates the image and recenters it on that pixel/object.
14. Applying the appropriate parameters in the Thresholding tool is important to correctly define the bacterium as "object". Therefore, it is recommended to do several tests, varying the parameters in the box that appears once this tool is selected. Please note that there are additional tools to further define and correct the "objects" included in (Process > Binary).

15. In the "Analyze Particles" tool, select the adequate pixel size range and circularity of the "object" (bacterium) of interest. The "objects" that do not fulfill these parameters will be excluded from the following steps.
16. Select the parameters to be quantified in "Set Measurements" (Analyze > Set Measurements). For fluorescent measurements, it is recommended to measure the "Area", the "Mean" and the "Integrated density", but other options are also available. It is important to select the name of the image to be measured in the "Redirect to" display menu.
17. This macro is very similar to the one described in Subheading 3.7. The main difference is that it lacks **step 6**. Therefore, instead of measuring the fluorescence in the vicinity of the bacterium, what is measured is the fluorescence of the bacterium itself.

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Chapter 24

Testing Chemical and Genetic Modulators in *Mycobacterium tuberculosis* Infected Cells Using Phenotypic Assays

Vincent Delorme, Ok-Ryul Song, Alain Baulard, and Priscille Brodin

Abstract

Mycobacterium tuberculosis is able to colonize host cells, and it is now well admitted that the intracellular stage of the bacteria contributes to tuberculosis pathogenesis as well as to making it a persistent infection. There is still limited understanding on how the tubercle bacillus colonizes the cell and what are the factors impacting on its intracellular persistence. Recent advances in imaging technique allow rapid quantification of biological objects in complex environments. Furthermore, *M. tuberculosis* is a microorganism that is particularly genetically tractable and that tolerates the expression of heterologous fluorescent proteins. Thus, the intracellular distribution of *M. tuberculosis* expressing fluorescent proteins can be easily quantified by the use of confocal microscopy. Here we describe high-content/high-throughput imaging methods that enable tracking the bacillus inside host settings, taking into account the heterogeneity of colonization.

Key words *Mycobacterium tuberculosis*, High-content/high-throughput screening, Chemogenomics, Drug discovery, siRNA library, Automated confocal microscopy, Image-based analysis

1 Introduction

There is a strong interplay between *M. tuberculosis* and the host at various levels, including entry, intracellular trafficking and antigen presentation [1]. Once internalized by alveolar phagocytic cells, *M. tuberculosis* prevents the acidification of the phagosomal compartment, thereby allowing its survival as it is sheltered from host defenses. In contrast with other intracellular pathogens, the colonization of cells by *M. tuberculosis* is usually slow: a typical experiment lasts 5–7 days, but host cells can be maintained in infection condition up to 2 weeks. However, intracellular replication of *M. tuberculosis* can vary depending on the virulence of the strain or clinical isolate used, leading to different levels of cytopathogenicity of the host cell being investigated. There is still much to be investigated on the difference in the type of cells that can be colonized, the impact of the strain virulence on the host and the overall dynamic of the process, and the molecular determinants important

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for those processes in the various settings are far from being fully understood. In this context, the identification of small molecule inhibitors active inside the host cell, siRNA, or molecular genetic interventions interfering with the pathological cycle of the bacteria would help to elaborate new efficient weapons against this scourge.

Antibacterial compounds identified during the last five decades were mainly revealed by whole cell phenotypic screens. Equally, recent studies of mycobacterial survival within host cells in *in vitro* models usually rely on CFU (colony forming units) determination or RLU (relative light units) titration which is labor intensive and time-consuming. More importantly, in the context of intracellular pathogens, such approaches do not take into account individual host cell parameters from the whole sample. Thus, *M. tuberculosis*-colonized macrophage assays have been developed, miniaturized, and automated to allow for the screening of large libraries of compounds, host pathway genetic modulators, or mutants. Done in parallel to axenic assays, this methodology classifies compounds or genes as involved on intracellular bacterial survival or specifically on extracellular *M. tuberculosis* multiplication.

1.1 Experiment Outline

Here we provide high-content/high-throughput image-based methods to investigate quantitatively and qualitatively the impact of various genetic or chemical modulators on *M. tuberculosis* infected host cells (Fig. 1) [2–5]. The following protocol details the preparation of various types of professional phagocytic cells as well as type II pulmonary epithelial cell model to monitor host—*M. tuberculosis* interactions at the single cell level, thereby adding a considerable value over the CFU-based methods that fail to take into account individual host cell parameters. The high-throughput screening (HTS) assays presented here are based on the adaptation of cellular imaging to automation, which consists of acquiring automated images from a large set of samples. The acquisition device used in this method is an automated confocal fluorescence microscope recording several fields per well of 96– or 384– well microplate. Thanks to high resolution images that can now be obtained with such modern devices, not only quantification of the bacterial survival can be obtained, but also phenotypical changes of either the bacteria or the host cell can be observed. The methodology presented here was developed to quantify intracellular *M. tuberculosis* by detecting enhanced-GFP (eGFP)-expressing bacteria inside fluorescently labelled phagocytic or non-phasocytic cells.

The first part of the procedure explains the preparation and the use of the constituents required for the assay. The method describes how macrophages are prepared and infected at a low Multiplicity of infection (MOI) with an eGFP expressing *M. tuberculosis* strain prior to incubation with compounds. Special attention is given in the description of bacterial culture methods that allow for the growth of non-clumping bacteria, as this parameter is crucial for successful and reproducible infections of macrophages.

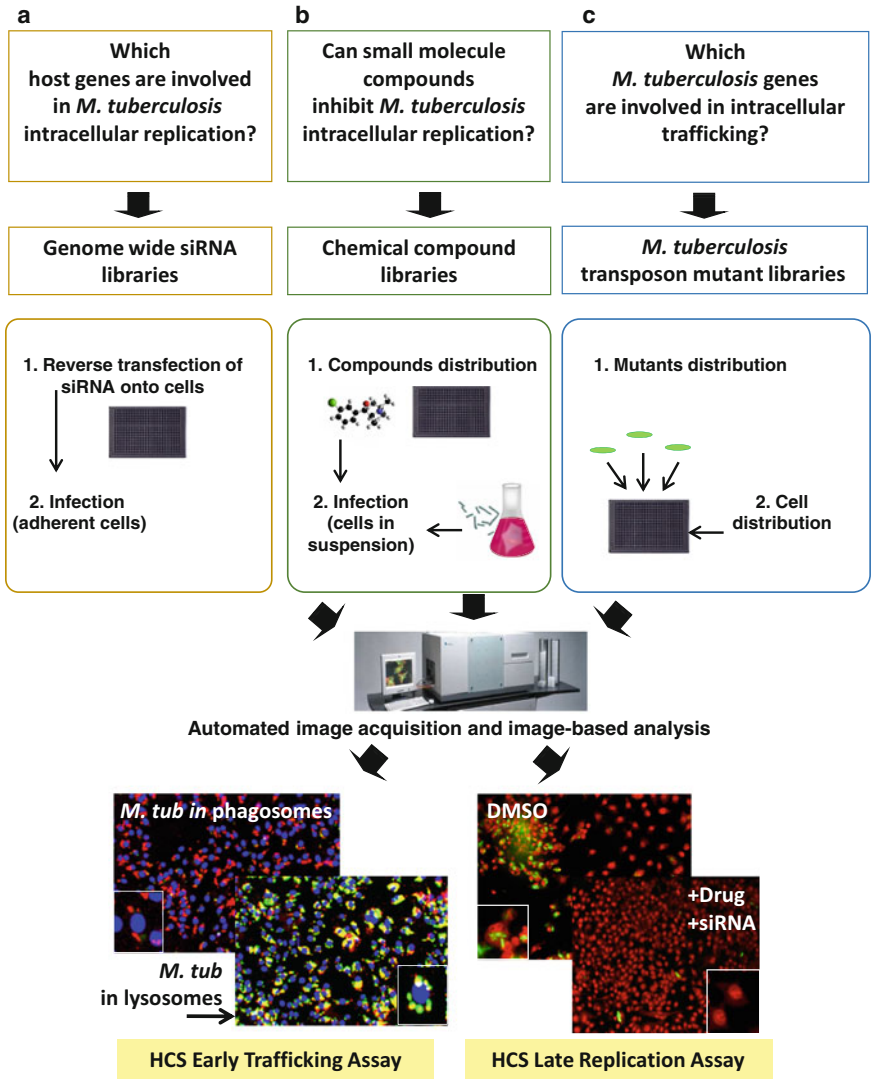

Fig. 1 Overall scheme for the different assays described in this chapter. Adapted from ref. [4]

Then, culture methodologies of selected cell lines or primary cells successfully used for *M. tuberculosis* infection in our laboratory are given. The robustness and rapid growth of murine macrophage cell line Raw264.7 make this cell line a good candidate for large-scale screens. More specific assays have been efficaciously adapted in our laboratory to other cell lines such as the human monocytic cell line THP-1, the A549 human type II pneumocyte-like cell line, murine bone marrow derived macrophages or dendritic cells, and human peripheral blood derived macrophages.

We also describe protocols for preparing and dispensing all components involved in the various screening methodologies, which include distribution of library compounds directly on bacteria or on infected cells, distribution and transfection of siRNA and

distribution of wild type (WT) or mutated mycobacteria for cell infection. Special attention is paid on the initial MOI using 2-week precultures of mycobacteria as a critical parameter of the assay [3].

Whereas quantification of intracellular *M. tuberculosis* is measured using eGFP-expression, cell quantification requires nucleus and/or cytosol staining. We describe cell staining procedures, either for end point studies when kinetic data are not needed or for dynamic studies requiring image acquisition during minimum 3 days.

Finally, we outline basic image parameters that can best depict the impact of the intervention (compounds, siRNA, or genetic alterations) on the bacterial survival or virulence.

2 Materials

2.1 Growth of Mycobacterium tuberculosis

1. Middlebrook OADC enrichment, containing oleic acid, bovine albumin, dextrose, catalase, and NaCl. Store at 4 °C in the dark.
2. 7H11 agar: Add 18 g of Middlebrook 7H11 and 5 mL of glycerol to 900 mL of deionized water; autoclave for 10 min at 121 °C. Cool to 55 °C and add 100 mL of OADC. Complete with 50 mg/mL hygromycin B or 25 mg/mL kanamycin, as required (*see* **Note 1**).
3. 5 % w/v Tyloxapol: Add 5 g of Tyloxapol to 100 mL of warm deionized water (60–70 °C). Filter-sterilize through a 0.22 μm membrane. Store at room temperature in the dark.
4. 20 % w/v Tween 80: Add 20 g of Tween 80 in 100 mL of deionized water. Filter-sterilize through a 0.22 μm membrane.
5. Hygromycin B: 50 mg/mL stock in PBS. Store at 4 °C. Add to media at a final concentration of 50 μg/mL.
6. Kanamycin: 50 mg/mL stock in PBS. Store at –20 °C in 500 μL aliquots.
7. Complete 7H9 medium (7H9-OADC-Tw): Add 4.7 g of 7H9 medium and 5 mL of glycerol to 900 mL of deionized water; autoclave for 10 min at 121 °C. After cooling at room temperature, add 100 mL of OADC and 2.5 mL of 20 % w/v Tween 80. Complete with 50 mg/mL hygromycin B or 25 mg/mL kanamycin, as required (*see* **Note 1**).
8. Sterile 50 and 200 mL bacterial culture flasks.
9. Sterile 25 cm^2 tissue culture flasks.
10. Sterile 50 mL tubes.
11. PBS.
12. 384-well plates

13. Fetal bovine serum (FBS): heat-inactivate at 56 °C for 30 min. Store at –20 °C in 50 mL aliquots.
14. Complete RPMI medium: add 50 mL of FBS to a 500 mL bottle of RPMI 1640 medium supplemented with glutamine. Store at 4 °C for up to 1 month.
15. Complete Iscove's Modified Dulbecco's Medium (IMDM) medium: add 50 mL of FBS and 500 μL of 50 mM β-mercaptoethanol to a 500 mL bottle of IMDM medium supplemented with 25 mM HEPES and L-glutamine. Store at 4 °C for up to 1 month.
16. Microplate fluorescence reader, e.g., Victor 3 (Perkin Elmer) or any other microplate fluorescence reader equipped with emission and excitation filters at 488 and 520 nm, respectively.

2.2 Macrophage and Epithelial Cell Line Culture

1. Cell lines (*see* **Note 2**).
2. Complete RPMI medium: add 50 mL of heat-inactivated FBS to a 500 mL bottle of RPMI 1640 medium supplemented with glutamine. Store at 4 °C for up to 1 month.
3. Phorbol 12-myristate 13-acetate (PMA) stock (50 mg/mL): dissolve 10 mg of PMA in 200 μL of DMSO. Store at –20 °C in the dark.
4. 1× Versene solution: store at 4 °C and use within 1 month after opening.
5. Dimethylsulfoxide (DMSO): store at room temperature.
6. Sterile 75 and 175 cm^2 tissue culture flasks.
7. Sterile 50 mL tubes.
8. Counting chamber.

2.3 Murine Primary Macrophage and Dendritic Cell Culture

1. Murine cells (*see* **Note 2**).
2. 10× Red cells lysis buffer: Add 15.6 g of NH_4Cl, 2 g of $NaHCO_3$ and 74 mg of EDTA in 200 mL of deionized water. Adjust the pH to 7.3 using HCl and filter-sterilize through a 0.22 μm membrane. Store at –20 °C.
3. 1× Red cells lysis buffer: dilute 5 mL of the 10× solution with 45 mL of deionized water. Store at 4 °C.
4. Complete RPMI medium: add 50 mL of FBS to a 500 mL bottle of RPMI 1640 medium supplemented with glutamine. Store at 4 °C for up to 1 month.
5. Complete Iscove's Modified Dulbecco's Medium (IMDM) medium: add 50 mL of FBS and 500 μL of 50 mM β-mercaptoethanol to a 500 mL bottle of IMDM medium supplemented with 25 mM HEPES and L-glutamine. Store at 4 °C for up to 1 month.

6. 0.1 mg/mL Murine Macrophages-Colony Stimulating Factor (M-CSF) stock: resuspend 100 µg of lyophilized M-CSF n 1 mL of sterile water. Store at −20 °C in 100 µL aliquots. Avoid more than three freeze–thaw cycles.
7. 0.1 mg/mL Murine Granulocyte macrophage-Colony Stimulating Factor (GM-CSF) stock: in sterile condition, resuspend 100 µg of lyophilized M-CSF or GM-CSF in 1 mL of sterile water. Store at −20 °C in 100 µL aliquots. Avoid more than three freeze–thaw cycles.

2.4 Human Primary Macrophage Cell Culture

1. Human blood.
2. 1 % FBS in PBS: add 5 mL of FBS to 500 mL of PBS. Store at 4 °C for up to 1 month.
3. 10 % DMSO in complete RPIM (*see* Subheading 2.1 **Item 14**).
4. Ficoll-Paque solution. Store at room temperature in the dark.
5. PBMC-buffer: dissolve 29.2 mg of EDTA in 45 mL of PBS. Filter-sterilize through a 0.22 µm PTFE membrane. Add 5 mL of FBS and sonicate for 30 min. Store at 4 °C.
6. 0.1 mg/mL Human Macrophages-Colony Stimulating Factor (*h*M-CSF) stock: in sterile condition, resuspend 100 µg of lyophilized *h*M-CSF in 1 mL of sterile water. Store at −20 °C in 100 µL aliquots. Avoid more than three freeze–thaw cycles.
7. $CD14^+$ beads: magnetic micro-beads conjugated to monoclonal anti-human CD14 antibodies (Miltenyi Biotec).
8. Magnet, column adaptors, and columns for magnetic separation (Miltenyi Biotec).

2.5 Preparation of Plates for SiRNA

1. RNase AWAY Spray Bottle.
2. RNAase-free water.
3. siRNA buffer. Prepare 1× siRNA buffer by mixing 1 volume of 5× siRNA buffer (Thermo Dharmacon) with 4 volumes of RNAase-free water (*see* **Note 3**).
4. Transfectant: Lipofectamine RNAiMAX Transfection Reagent, Lipofectamine LTX Reagent, or HiPerFect Transfection Reagent.
5. Sterile 384-well assay plates with lids, compatible with cell culture and laser-scanning confocal microscope (*see* **Note 4**).
6. Microplate breathsealing film (Greiner) or any equivalent adhesive breathable sealing film suitable for your 384-well plates.
7. Automatic Plate Washer adapted to your 384-well plates.
8. 50 mg/mL amikacin stock: dissolve 500 mg of amikacin in 10 mL of PBS. Filter-sterilize through a 0.22 µm PTFE membrane and aliquot. Store at −20 °C.

9. Complete RPMI medium supplemented with amikacin: add 1 volume of 50 mg/mL amikacin to 1,000 volumes of complete RPMI medium.
10. Scramble siRNA solution: dilute ON-TARGETplus Non-targeting Pool (Thermo Dharmacon) or siGENOME Non-Targeting siRNA Pool #2 (Thermo Dharmacon) with 1× siRNA buffer to reach a final concentration of 20 μM. Store stock solutions at −20 °C avoiding thawing–freezing cycles.
11. Automatic Liquid Dispenser able to accurately dispense volumes of 5–50 μL, adapted to your 384-well plates.
12. Sterile 50 mL polystyrene reservoirs.
13. CSF. *See* Subheading 2.3 **items 6** and 7, and Subheading 2.4 **item 6**.

2.6 Preparation of Compound Plates

1. Echo (Labcyte) or any other High Precision Automatic Liquid Dispenser able to accurately dispense volumes of 2.5–500 nL, adapted to your 384-well plates.
2. Dry DMSO: distil under reduced pressure. Store over activated 4 Å molecular sieve under an inert atmosphere.
3. Sterile 50, 100, and 200 mL bacterial-culture flasks.
4. Microplate aluminum sealing film (Corning) or any equivalent adhesive aluminum sealing film suitable for your 384-well plates.
5. Tissue culture medium: RPMI or IMDM (Subheading 2.3, **items 4** and **5**).
6. CSF. *See* Subheading 2.3 **items 6** and 7, and Subheading 2.4 **item 6**.
7. Tissue culture medium: RPMI or IMDM. *See* Subheading 2.3, **items 4** and **5**.
8. CSF. *See* Subheading 2.3 **items 6** and 7, and Subheading 2.4 **item 6**.
9. Microplate breathsealing film.

2.7 Preparation of Plates for Screening Mutants

1. Sterile 96-well plates.
2. Automatic Plate Washer

2.8 Staining of Cell Nuclei

1. Hoechst 33342 stain stock solution: 10 mg/mL in water. Store at 4 °C and protect from light.
2. 33× Hoechst 33342 solution in PBS (10 μg/mL): prepare freshly by diluting 20 μL of the stock solution in 20 mL of the appropriate complete medium. Freshly prepare 1× Hoechst solution (300 ng/mL) by adding 1 volume of 33× Hoechst solution to 32 volumes of the appropriate complete medium.
3. Microplate breathsealing film.

2.9 Live Cell Staining

1. 33× Hoechst 33342 solution in PBS (10 μg/mL): prepare freshly by diluting 20 μL of the stock solution in 20 mL of the appropriate complete medium. Freshly prepare 1× Hoechst solution (300 ng/mL) by adding 1 volume of 33× Hoechst solution to 32 volumes of the appropriate complete medium.
2. 1,000× Syto 60 (Life Technologies) stock (5 mM) in DMSO. Store at −20 °C and protect from light. Freshly prepare 1× Syto 60 (5 μM) by diluting 20 μL of the stock solution in 20 mL of the appropriate complete medium.
3. 1,000× CellMask (Life Technologies) stock (5 mg/mL) in DMSO. Store at −20 °C and protect from light. Freshly prepare 1× CellMask (5 μg/mL) by adding 1 volume of stock solution in 1,000 volumes of the appropriate complete medium.
4. 400× LysoTracker (Life Technologies) stock (1 mM) in DMSO. Store at −20 °C and protect from light. Freshly prepare 1× LysoTracker by adding 1 volume of stock solution in 400 volumes of the appropriate complete medium.
5. Microplate breathsealing film

2.10 Cell Fixation

1. Formalin solution (4 % w/v formaldehyde): store at room temperature and use within 1 month.
2. Microplate breathsealing film.
3. PBS.
4. Plate washer.
5. 1 % FBS in PBS.

2.11 Cell Permeabilization

1. 0.1 % w/v Triton X-100 solution: add 1 mL of 10 % Triton X-100 to 100 mL of PBS. Filter-sterilize through a 0.22 μm membrane. Store at 4 °C.
2. PBS.

2.12 Fixe Cell Staining

1. 4′,6-diamidino-2-phenylindole (DAPI) stain: Store powder at 4 °C and protect from light. Prepare 200× DAPI stock (1 mg/mL) by mixing 10 mg of DAPI in 10 mL of Wata. Filter-sterilize through a 0.22 μm membrane. Store in 500 μL aliquots at −20 °C. Freshly prepare 1× DAPI (5 μg/mL) by diluting 45 μL of 200× solution in 9 mL of PBS per 384-well assay plate to stain.
2. 2,000× HCS LipidTox Far-Red or Green neutral lipid (Life Technologies) stock in DMSO. Store at −20 °C and protect from light. Freshly prepare 1× HCS LipidTox solution by adding 1 volume of stock solution in 2,000 volumes of PBS.
3. 50× Alexa Fluor 660 phalloidin solution: reconstitute 10 nmol of Alexa Fluor 660 phalloidin (Life Technologies, 300 Units) in 1.5 mL of methanol. Store at −20 °C and protect from light.

Freshly prepare 1× Alexa Fluor 660 phalloidin by adding 1 volume of stock solution in 49 volumes of PBS.

4. 1 % FBS in PBS.
5. Microplate breathsealing film.

2.13 Image Acquisition and Analysis

1. Automated laser-scanning confocal microscope: Opera (Perkin Elmer) or similar instrument adapted for 384-well plates, equipped with 405, 488, 561 and 640 nm excitation lasers as well as 20× and 60× water objectives.
2. Image analysis software (*see* **Note 5**).

3 Methods

3.1 Growth of Mycobacterium tuberculosis

N.B. Handling of *M. tuberculosis* strains requires a biosafety cabinet and a dedicated biosafety laboratory adapted for pathogens of class 3. A strain expressing a fluorescent protein is required (*see* **Note 1**).

1. Grow *M. tuberculosis* on fresh 7H11 plates containing the appropriate antibiotic for 2–3 weeks (*see* **Note 1**).
2. Inoculate *n. tuberculosis* into 5 mL of 7H9-OADC-Tw plus required antibiotic (*see* **Note 1**).
3. Incubate *n. tuberculosis* at 37 °C, 5 % CO_2 without shaking for 7 days.
4. Inoculate *n. tuberculosis* into 50 mL of fresh 7H9-OADC-Tw plus required antibiotic (*see* **Note 1**), to reach a starting OD_{600} of 0.1.
5. Incubate for 7 days at 37 °C with shaking at 200 rpm.
6. Inoculate 200 mL of fresh 7H9-OADC-Tw plus required antibiotic (*see* **Note 1**) to a starting OD_{600} of 0.1 and incubate for 7 days at 37 °C with shaking at 200 rpm.
7. Pellet bacteria by centrifugation at 4,000 × *g* for 10 min at room temperature and resuspend in 35 mL of PBS. **Steps 8–11** are optional as they are used for the titration of the bacteria. For cellular infection, directly go to **step 12**.
8. Prepare series of ten-fold dilutions of bacteria in PBS using a 384-well plate (dilutions from 1- to 10^{-10}-fold).
9. Record fluorescence levels (RFU) of each dilution using a microplate fluorescence reader.
10. Plate dilutions on 7H11 agar plates and grow for 2–3 weeks at 37 °C.
11. Count CFU on plates from the different dilutions. Plot CFU as a function of RFU and fit the curve using a linear regression to obtain parameters for the CFU = *f*(RFU) relationship. Same

parameters can be reused for all aliquots of the same stock, but a new regression should be established for each stock of bacteria.

12. Wash bacteria once more with 35 mL of PBS.
13. Resuspend bacteria in 35 mL of freshly prepared complete RPMI medium.
14. Centrifuge at 700 rpm for 2 min (*see* **Note 6**).
15. Calculate the bacterial concentration by measuring the fluorescence (RFU) of the sample using the microplate fluorescence reader, referring to the CFU = *f*(RFU) linear relationship previously established (**steps 8–11**).
16. Dilute bacteria in complete RPMI for macrophages and epithelial cells or IMDM for dendritic cells to reach a concentration of 2×10^6 CFU/mL (*see* **Note 7**).

3.2 Macrophage and Epithelial Cell Line Culture

N.B. All cell-culture work should be carried out in a dedicated laboratory under a type 2 biosafety cabinet.

1. Establish Raw264.7 macrophages monolayer by plating 5×10^6 cells in 20 mL of complete RPMI medium per 75 cm^2-tissue culture flasks or 1×10^7 cells in 40 mL of complete RPMI medium per 175 cm^2-tissue culture flasks (*see* **Note 8**).
2. Incubate for 2 days at 37 °C, 5 % CO_2.
3. Remove the medium and carefully wash the monolayer twice using pre-warmed (37 °C) PBS.
4. Incubate cells with 4 mL (75 cm^2 flasks) or 8 mL (175 cm^2 flasks) of 1× Versene solution at 37 °C and 5 % CO_2 for 20 min.
5. Collect cells in a sterile 50 mL tube.
6. Centrifuge cells at $400 \times g$ for 10 min at room temperature.
7. Resuspend cells in pre-warmed complete RPMI medium.
8. Count cells using a counting chamber and adjust to 1×10^6 cells/mL.
9. **Steps 1–8** can be repeated to maintain and passage Raw264.7 cells every 2 days, for a maximum of ten passages.
10. Establish a THP-1 cell suspension at 8×10^5 cells/mL in 20 mL of complete RPMI medium per 75 cm^2-tissue culture flasks (*see* **Note 8**).
11. To maintain and passage undifferentiated THP-1 cells, repeat **step 10** every 2 days, for a maximum of ten passages.
12. Add PMA at a final concentration of 50 ng/mL to differentiate cells.
13. Incubate for 3 days at 37 °C, 5 % CO_2.
14. Collect cells in a sterile 50 mL tube.
15. Centrifuge cells at $400 \times g$ for 10 min at room temperature.
16. Resuspend cells in pre-warmed complete RPMI medium.

17. Count cells using a counting chamber and adjust to 1×10^6 cells/mL.
18. Establish A549 human type II pneumocyte-like cells monolayer by plating 1×10^6 cells in 20 mL of complete RPMI medium per 75 cm^2-tissue culture flasks (*see* **Note 8**).
19. Incubate 2 days at 37 °C, 5 % CO_2.
20. Remove the medium and carefully wash the monolayer twice using pre-warmed (37 °C) PBS.
21. Incubate cells with 4 mL (75 cm^2 flasks) or 8 mL (175 cm^2 flasks) of a 1× Versene solution at 37 °C and 5 % CO_2 for 20 min.
22. Collect cells in a sterile 50 mL tube.
23. Centrifuge cells at $400 \times g$ for 10 min at room temperature.
24. Resuspend cells in pre-warmed complete RPMI medium.
25. Count cells using a counting chamber and adjust to 1×10^6 cells/mL.
26. To maintain and passage A549 cells, repeat **steps 20–26** every 2 days, for a maximum of ten passages.

3.3 Murine Primary Macrophage and Dendritic Cell Culture

Murine macrophages and dendritic cells are differentiated from monocytes purified from mice bone-marrow (*see* **Note 9**).

1. Collect bone marrow in complete RPMI (macrophages) or IMDM (dendritic cells) medium, centrifuge at $400 \times g$ for 5 min and discard the supernatant.
2. Resuspend cells in 1 mL of 1× Red cells lysis buffer and incubate for 1 min exactly.
3. Immediately dilute with 49 mL of complete RPMI or IMDM medium.
4. Centrifuge at $400 \times g$ for 5 min and discard the supernatant.
5. Resuspend cells in 10 mL of complete RPMI or IMDM medium and count the cells using a counting chamber.
6. Repeat **step 4** and resuspend the pellet in pre-warmed (37 °C) complete RPMI or IMDM medium to reach a concentration of 5×10^5 cells/mL for cell culture or 2×10^7 cells/mL for freezing. While freezing, complement the medium with 10 % DMSO.
7. Establish macrophage monolayer by plating 5×10^5 cells in 20 mL of complete RPMI medium containing 40 ng/mL M-CSF per 75 cm^2-tissue culture flasks, or 1×10^6 cells in 40 mL of medium per 175 cm^2-tissue culture flasks.
8. Establish dendritic cell monolayer by plating 5×10^7 cells in 100 mL of complete IMDM medium containing 40 ng/mL GM-CSF per 500 cm^2-petri dish.
9. Incubate for 4 days at 37 °C, 5 % CO_2.

10. Remove culture medium and replace with fresh complete RPMI medium containing 40 ng/mL M-CSF (macrophages) or IMDM containing 40 ng/mL GM-CSF (dendritic cells).
11. Incubate cells at 37 °C, 5 % CO_2.
12. Collect macrophages on day 7 by repeating **steps 3–8** from Subheading 3.2.
13. On day 8, add 50 mL of complete IMDM medium containing 40 ng/mL GM-CSF on the dendritic cell monolayer.
14. Incubate dendritic cells for 3 days at 37 °C, 5 % CO_2.
15. Gently remove the culture medium from the petri dish using a 25 mL pipette.
16. Wash the cell monolayer with 50 mL of pre-warmed (37 °C) PBS. Gently remove the supernatant using a 25 mL pipette.
17. Add 50 mL of pre-warmed 2 mM EDTA in PBS.
18. Incubate cells for 5 min at 37 °C, 5 % CO_2.
19. Gently collect cell supernatant in a sterile 50 mL tube using a 25 mL pipette.
20. Centrifuge at 400 × g for 10 min and discard the supernatant.
21. Resuspend cells in pre-warmed complete IMDM medium. Count cells using a counting chamber.
22. Adjust to 1×10^6 cells/mL using pre-warmed complete IMDM medium containing 40 ng/mL GM-CSF.

3.4 Human Primary Macrophage Cell Culture

Human macrophages are $CD14^+$ differentiated monocytes, isolated from healthy volunteer peripheral blood samples (*see* **Note 10**).

1. Dilute 1 volume of blood in 1 volume of 1 % FBS in PBS.
2. In a 50 mL tube, carefully deposit 30 mL of diluted blood over 15 mL of Ficoll-Paque solution. Do not mix the tube.
3. Centrifuge at 400 × *g* for 20 min with braking speed of the centrifuge set to minimum.
4. Collect monocytes by carefully pipetting the white ring located at the interface between the plasma (top) and the Ficoll-Paque (bottom) layer.
5. Wash monocytes three times by centrifugation at 400 × *g* for 10 min at room temperature and resuspension of the pellet in 50 mL 1 % FBS in PBS.
6. Pool monocytes in one 50 mL tube. Count cells using a counting chamber. At this step, monocytes can be stored at –80 °C in complete RPMI medium containing 10 % DMSO.
7. Centrifuge monocytes at 400 × *g* for 10 min.
8. Resuspend cell pellet in 80 µL of cold (4 °C) PBMC-buffer per 10^7 total cells.

9. Add 20 µL of $CD14^+$ magnetic beads per 10^7 total cells and incubate for 15 min at 4 °C.
10. Dilute cells in 50 mL of cold PBMC-buffer. Centrifuge at 400 × *g* for 10 min at 4 °C and resuspend the pellet in 2 mL of cold PBMC-buffer.
11. Apply cell suspension onto a column attached to the magnet and rinse twice with 3 mL of cold PBMC-buffer. Wash the column three times with 3 mL of cold PBMC-buffer.
12. Remove the column from the magnet and elute $CD14^+$ monocytes in a clean 15 mL tube by firmly flushing out the column with 5 mL of cold PBMC-buffer.
13. Centrifuge cells at 400 × *g* for 10 min and resuspend in 5 mL of complete RPMI medium containing 40 ng/ml *h*M-CSF.
14. Count cells using a counting chamber.
15. Seed cells at 1×10^6 cell/mL and incubate for 4 days at 37 °C, 5 % CO_2.
16. Replace the medium with fresh complete RPMI medium containing 40 ng/mL *h*M-CSF and incubate cells for 2 days at 37 °C, 5 % CO_2.
17. To collect cells, repeat **steps 3–8** from Subheading 3.2.

3.5 Preparation of Plates for SiRNA

The overview diagram of the following steps is shown in Fig. 1A. The infection procedure (infection of adherent cells) is detailed in Fig. 2a.

1. Clean all equipment to work in RNAse-free condition (*see* **Note 3**).
2. Add siRNA to reach the recommended final concentration (Table 1).
3. Prepare transfection reaction by mixing together PBS and transfectant. Vortex briefly. Refer to Table 1 for the appropriate transfectant and volumes needed for each cell type.
4. Incubate 3 min at room temperature.
5. Distribute 10 µL of the transfection reaction per well for each plate (*see* **Note 11**).
6. Incubate 30 min at room temperature.
7. Prepare cells as required (*see* Subheadings 3.2–3.4).
8. Add the appropriate number of cells per well (Table 1).
9. Incubate for 3 days at 37 °C, 5 % CO_2.
10. Prepare a bacterial suspension at 2×10^6 CFU/mL (*see* Subheading 3.1 and **Note 7**). You will need 20 mL of bacterial suspension per plate.
11. Remove the supernatant from plates using a multichannel pipette.

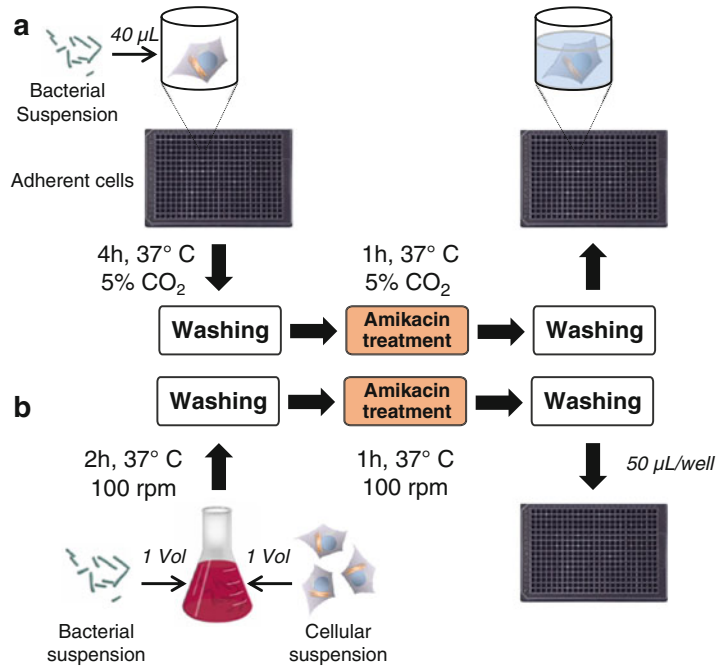

Fig. 2 Overall scheme for (**a**) infection of plated cells and (**b**) infection of cells in suspension

Table 1
Recommended conditions for siRNA transfection in 384-well microplate

Cell type	Transfectant name	Transfectant volume/well (µL)	PBS volume/ well (µL)	siRNA final concentration (nM)	Cells/ well	Medium (µL)
Raw264.7	LTX	0.1	9.9	80	2,000	40[a]
BMDC	LTX	0.1	9.9	50	40,000	40[b]
PBMC CD14+	HiPerFect	0.9	9.1	80	20,000	40[c]
A549	RNAiMAX	0.1	9.9	20	1,500	40[a]
THP-1	RNAiMAX	0.1	9.9	50	40,000	40[a]

[a]Complete RPMI medium
[b]Complete IMDM medium containing 40 ng/mL of murine GM-CSF
[c]Complete RPMI medium. Supplement with 40 ng/mL of *h*M-CSF 6 h after cell plating

12. Add 40 µL of bacterial suspension per well for each 384-well plate (*see* **Note 11**).
13. Seal plates using breathsealing films and centrifuge at 300×*g* for 1 min.
14. Incubate for 4 h at 37 °C, 5 % CO_2.
15. Remove the breathsealing films.

16. Wash the cells using an automatic plate washer or by carefully pipetting up and down 50 µL of the appropriate complete medium (RPMI or IMDM) in each well using a multichannel pipette. Finish by removing the supernatant from the plates.
17. Add 50 µL of complete medium supplemented with 50 µg/mL amikacin per well for each plate.
18. Incubate for 1 h at 37 °C, 5 % CO_2.
19. Remove the supernatants from the plates using a multichannel pipette.
20. Repeat **step 16** twice.
21. For each plate, add 50 µL per well of the appropriate complete medium (RPMI or IMDM) containing 40 ng/mL M-CSF, GM-CSF or *h*M-CSF for primary murine macrophages, murine dendritic cells and human macrophages, respectively. Cell lines do not require supplementation with growth factors.
22. Seal plates using breathsealing films and incubate for 1–5 days at 37 °C, 5 % CO_2.

3.6 Preparation of Compound Plates

The overview diagram of the following steps is shown in Fig. 1B. The infection procedure (infection of cells in suspension) is detailed in Fig. 2b.

1. Prepare compound stock solutions at 10 mM by dissolving pure compounds in dry DMSO (*see* **Note 12**). Store stock solutions at –20 °C.
2. Design the source and assay plate layout. An example of plate layout is given in Fig. 3.
3. Transfer stock solutions into the 384-well source plates.
4. Prepare assay plates by transferring compounds, either directly from the source plate, or using intermediate dilution plates, to reach the final concentration needed (*see* **Note 11**). The final amount of DMSO in the assay plate should be <1 % v/v for each well.
5. Store assay and source plates at –20 °C, sealed with an adhesive aluminum sealing film.
6. Prepare a bacterial suspension at 2×10^6 CFU/mL (*see* Subheading 3.1 and **Note 7**). You will need 10 mL of bacterial suspension per plate.
7. Prepare a cellular suspension as required (*see* Subheadings 3.2–3.4). You will need 10 mL of cellular suspension per plate.
8. Mix 1 volume of cells with 1 volume of bacteria in bacterial-culture flasks of appropriate volume.
9. Incubate the suspension with mild shaking (100 rpm) for 2 h at 37 °C.

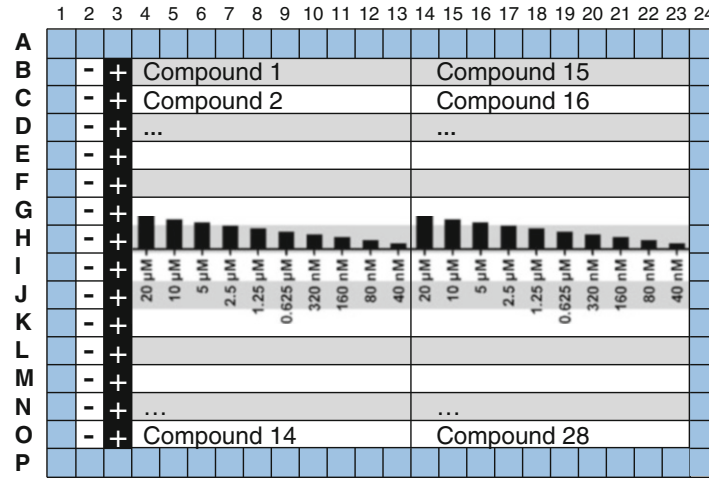

Fig. 3 Example of a 384-well assay plate layout. In this design, all wells located at the border (in *blue*), are filled with medium only, to limit side effect during incubation. Positive (+) and negative (–) controls are added in columns 2 and 3, respectively. Each compound is assayed in a dose–response format including a total of ten concentrations, starting from 20 µM (columns 4 and 14) down to 40 nM (columns 13 and 23). Using this layout, 28 different compounds (*lines B* to *O*) can be tested in a single plate

10. Distribute the suspension equally in sterile 50 mL tubes.
11. Wash the infected cells by centrifugation at 400 × *g* for 10 min and resuspend in 40 mL of medium (complete RPMI for macrophages and pneumocytes; complete IMDM for dendritic cells).
12. Repeat **step 6** and resuspend infected cells in the appropriate medium (complete RPMI or complete IMDM) supplemented with 50 µg/ml amikacin.
13. Incubate the suspension with mild shaking (100 rpm) for 1 h at 37 °C. This step is required to kill remaining extracellular bacteria.
14. Wash the infected cells three times.
15. Resuspend in the appropriate complete medium (RPMI or IMDM) containing 40 ng/mL M-CSF, GM-CSF or *h*M-CSF for primary murine macrophages, murine dendritic cells and human macrophages, respectively. Cell lines do not require supplementation with growth factors.
16. Dispense 50 µL of infected cells per well for each assay plate prepared (*see* **Note 11**). If needed, fill empty wells with medium to limit evaporation and border-effects.
17. Incubate the plates for 5 days at 37 °C, 5 % CO_2.

3.7 Preparation of Plates for Screening Mutants

The overview diagram of the following steps is shown in Fig. 1C.

1. Prepare bacterial suspensions of mutants at 2×10^6 CFU/mL (*see* Subheading 3.1 and **Note 7**).
2. Design the assay plate layout. A layout similar to that used for compound is possible (Fig. 3). In this case, prepare two-fold dilutions of each mutant in PBS using intermediate 96-well plates.
3. Distribute 20 µL of bacterial suspensions into 384-well assay plates (*see* **Note 11**).
4. Prepare a cellular suspension as required (*see* Subheadings 3.2–3.4). You will need 12 mL of cellular suspension per plate.
5. Distribute 30 µL of cellular suspension in the 384-well assay plates containing the mutants.
6. Incubate for 2 h at 37 °C, 5 % CO_2.
7. Wash the cells using an automatic plate washer or by carefully pipetting up and down 50 µL of the appropriate complete medium (RPMI or IMDM) in each well using a multichannel pipette. Finish by removing the supernatant from the plates.
8. For each plate, add 50 µL per well of the appropriate complete medium (RPMI or IMDM) containing 20 ng/mL M-CSF, GM-CSF or *h*M-CSF for primary murine macrophages, murine dendritic cells and human macrophages, respectively. Cell lines do not require supplementation with growth factors.
9. Seal plates using breathsealing films and incubate for 1–5 days at 37 °C, 5 % CO_2.

3.8 Staining of Cell Nuclei

When kinetic data are required, cells can be stained at the beginning of the experiment using Hoechst 33342 stain. Plates can be kept for 3 days at 37 °C without cell toxicity.

1. Prepare 20 mL of 1× Hoechst solution (300 ng/mL) per plate.
2. Remove the supernatant from the plates using a multichannel pipette.
3. Add 50 µL per well of 1× Hoechst solution (*see* **Note 11**).
4. Seal plates using breathsealing films and incubate for 30 min at 37 °C, 5 % CO_2.
5. Read plates at regular time points using the high-content confocal microscope (*see* Subheading 3.13).

3.9 Live Cell Staining

For live cell imaging, fixation is not required and you can directly proceed with cell staining (*see* Fig. 4a).

1. Remove the supernatant from the plates using a multichannel pipette.
2. For each plate, add 50 µL per well of 1× Hoechst (Nuclei staining), 1× Syto 60 (Nuclei and Cytoplasm staining),

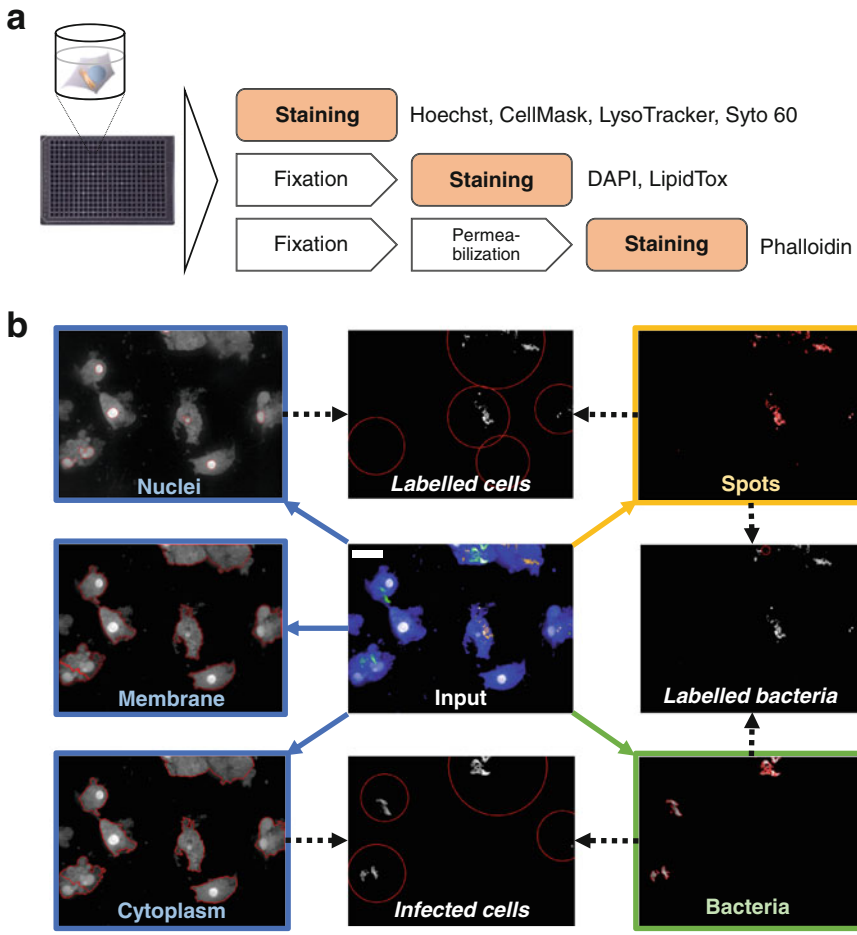

Fig. 4 (**a**) Workflow of the staining process for all dyes listed in this chapter. (**b**) Workflow of the image analysis process. Cells, nuclei, and membranes (cellular populations) are first segmented from the blue channel. Bacteria and organelles (*spots*) are then segmented from the green and red channel, respectively. Finally, labelled cells, labelled bacteria, and infected cells are deduced by combining populations together: *spots* with cells, *spots* with bacteria, and bacteria with cells, respectively. The scale bar represents 20 μm

1× CellMask (membrane staining), 2 μM LysoTracker red DND-99, or 2 μM LysoTracker green DND-26 (Lysosomal compartments staining) solution to the plates (*see* **Note 11**).

3. Seal plates using breathsealing films and incubate for 30 min at 37 °C, 5 % CO_2.
4. Read plates using the high-content confocal microscope (*see* Subheading 3.13).

3.10 Cell Fixation

If you do not want to proceed immediately with staining, for immunofluorescence assay and for specific dye like DAPI, a fixation step is required prior to staining (*see* Fig. 4a).

1. Remove the supernatant from the plates using a multichannel pipette.
2. Add 50 µL per well of 4 % Formalin solution to the plates (*see* **Note 11**).
3. Incubate for 30 min at room temperature.
4. Wash cells twice by pipetting up and down 50 µL of PBS with a multichannel pipette or using an automatic plate washer. Remove the supernatant from the plates using a multichannel pipette.
5. At this step, plates can be stored for up to 1 month by adding 50 µL per well of 1 % FBS in PBS, sealing and keeping in the dark at 4 °C.

3.11 Cell Permeabilization

These steps should only be performed with staining that requires a chemical treatment to permeabilize cells, like F-actin staining using Alexa Fluor 660 phalloidin.

1. Remove the supernatant from the plates using a multichannel pipette.
2. Add 50 µL of 0.1 % Triton-X-100 solution per well and incubate for 3 min at room temperature (*see* **Note 11**).
3. Wash the cells twice with PBS using a multichannel pipette or an automatic plate washer.
4. Proceed immediately with staining (*see* Subheading 3.12).

3.12 Fixed Cell Staining

For F-actin staining, a permeabilization step is needed prior to staining (*see* Subheading 3.11).

1. Remove the supernatant from the plates using a multichannel pipette.
2. For each plate, add 50 µL per well of either 1× DAPI (Nuclei staining), 1× HCS LipidTox (Lipid Droplet staining) or 1× Alexa Fluor 660 phalloidin (F-actin staining) solution to the plates (*see* **Note 11**).
3. Incubate 30 min at room temperature in the dark.
4. For Lipid Droplet staining, go directly to **step 7**. For F-actin staining, wash the cells twice with PBS using a multichannel pipette or an automatic plate washer.
5. Remove supernatant from plates using a multichannel pipette and add 50 µL per well of 1 % FBS in PBS.
6. Seal plates using breathsealing films.
7. Read plates using the high-content confocal microscope (*see* Subheading 3.13).

3.13 Image Acquisition and Analysis

For the determination of the ratio of infected cells, host cell viability or bacterial load, a 4×, 10× or 20× magnification should be used, as it allows the recording of a larger number of cells. For the study of bacterial intracellular trafficking, a 60× magnification is required. Bacteria are detected using the green channel, obtained through the GFP fluorescence. Cell nuclei and cytoplasms are detected using the blue (DAPI, Hoechst) or the far-red (Syto 60) channels. Cell membranes are detected using the far-red channel. Organelle labelling (spots) are detected using the green (LipidTox green, LysoTracker green), red (LysoTracker red) or far-red (LipidTox far-red, LysoTracker far-red) channels (Fig. 4b). Once cells, bacteria and spots have been segmented and listed, it is possible to analyze the extent of infection/labelling of the cells/bacteria (Fig. 4b).

1. Choose the objective from the microscope.
2. Define wells to be read and the number of fields to acquire from each well. Typically, four fields are sufficient to allow the detection of about 1,000 cells with a 20× magnification, while ten fields are sufficient to allow the detection of about 100 cells at a 60× magnification.
3. Set the exposure parameters and acquisition time. Refer to Table 2 for excitation and emission wavelength of common dyes.
4. Proceed with plates reading and collect images.
5. Set the intensity threshold that will be used for bacteria detection. Pixels with an intensity lower than the threshold are discarded.

Table 2
Excitation and emission wavelength for common fluorophores

Fluorophore	Excitation (nm)	Emission (nm)
GFP	488	520
DAPI	405	450
Syto 60	640	690
Hoechst 33342	405	450
LysoTracker green	488	530
LysoTracker red	561	600
CellMask	640	690
Alexa Fluor 660 phalloidin	640	690
HCS LipidTox Deep Red	640	690
HCS LipidTox Green	488	520

6. Cluster the remaining adjacent pixels to generate a list of objects. Calculate the area of each object.
7. Remove objects with a size lower than 3 pixels. Divide the object list in two: objects with a low area (isolated objects) and with a high area (clumped objects).
8. Cut clumps into single objects: detect intensity maxima inside clumped bacteria and grow regions step-by-step using maxima as centers.
9. You have now three distinct bacterial populations in hand: isolated bacteria, clumped bacteria and bacteria from clumps. Use the different populations to calculate all bacteria-related parameters. For screening purposes, the total bacterial area, expressed in pixels, is the most useful parameter to determine the extent of bacterial growth (Fig. 5a).

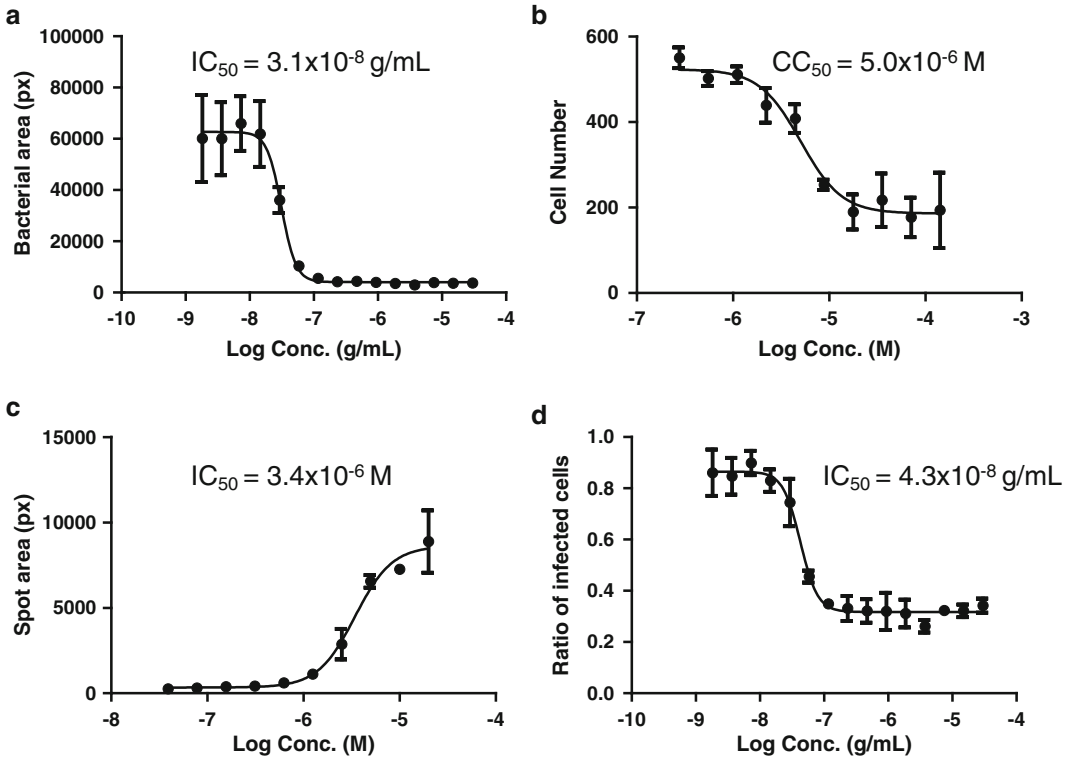

Fig. 5 Examples of dose–response curves, obtained using two-fold dilutions of various chemical modulators, on (**a**) the bacterial growth, determined by the area of bacteria (in pixel), (**b**) the cellular viability, determined by the number of cells, (**c**) the extent of labelling, determined by the area of spot (in pixel), and (**d**) the extent of infection, determined by the ratio of infected cells. All graphs represent the average of three experiments ± SD. Fitting were obtained by least squares regression of a sigmoidal dose–response (variable slope) equation, using the GraphPad Prism software

10. Set parameters for the cell/nucleus/membrane detection methods to yield the best detection in your staining condition (*see* **Note 13**). This could be achieved by manually checking that the cells/nuclei/membranes are correctly segmented, using 2–3 representative pictures.
11. Host cells can be defined from the association of up to three populations: a population of nuclei, a population of cytoplasm and a population of membranes. Depending on the staining condition, some populations may be lacking.
12. Use the different cellular populations to calculate all host-related parameters. For screening purposes, the total cellular area (expressed in pixels) and the total number of cells proved to be the most useful parameters to determine the cellular viability (Fig. 5b).
13. Set parameters for the spot detection method to yield the best detection in your staining condition (*see* **Note 13**). This could be achieved by manually checking that the organelles are correctly segmented, using 2–3 representative pictures.
14. Calculate mass center coordinates of each spot in the picture.
15. Step through the list of identified cellular/bacterial objects to determine which spot is located within each object. Store the data in arrays or lists.
16. Divide your cellular/bacterial populations into different subpopulations accordingly to their extent of labelling.
17. Use the different populations to calculate all trafficking or organelle-related parameters. For screening purposes, the total spot area (expressed in pixels), the total number of cells/bacteria labelled, and the average area of spots in cells/bacteria (expressed in pixels) proved to be the most useful parameters to determine the extent of labelling (Fig. 5c).
18. Calculate mass center coordinates of each identified bacteria.
19. Step through the list of identified cellular objects to determine which bacterium is located within each object. Store the data in arrays or lists.
20. Divide your cellular populations into different subpopulations accordingly to their extent of infection.
21. Use different populations to calculate all infection-related parameters. For screening purposes, the total number of infected cells, the average area of bacteria in infected cells (expressed in pixels), and the ratio of infected cells proved to be the most useful parameters to determine the extent of infection (Fig. 5d).

4 Notes

1. For *M. tuberculosis* H37Ra strains, it is recommended to use tyloxapol instead of tween 80. To prepare complete medium for H37Ra strain (7H9-OADC-Ty), add 100 mL of OADC and 400 μL of 5 % w/v tyloxapol. Complete with 50 mg/mL hygromycin B or 25 mg/mL kanamycin, as required. For antibiotic selection: *M. tuberculosis* H37Rv-GFP and *M. tuberculosis* H37Ra-GFP are recombinant strains bearing an integrative plasmid (based on Ms6) carrying a GFP gene constitutively expressed from the pBlaF promoter [2] and conferring resistance to hygromycin B. *M. tuberculosis* H37Rv-RFP is a recombinant strain bearing a replicative plasmid (based on pMV261) carrying a pDSRed1-1 (Clontech) gene under the Hsp60 promoter (kind gift from Sang Hyun, Cho) and conferring resistance to kanamycin. *M. tuberculosis* Beijing GC1237-mCherry and related transposon mutants are recombinant strains bearing the pCherrry10 plasmid [6], conferring resistance to hygromycin B. The transposon mutants were transduced with MycoMarT7, conferring an additional resistance to kanamycin.
2. The following host cells have been used in our assays: Raw264.7 cells: a mouse, tumor-derived macrophage cell line [7] available from the American Type Culture Collection (ATCC, TIB-71). THP-1 cells: Human monocyte cell line [8] available from the American Type Culture Collection (ATCC, TIB-202). A549 cells: Human type II pneumocyte-like cell line [9] available from the American Type Culture Collection (ATCC, CCL-185). Murine bone-marrow derived macrophages (BMDM): isolated from 6- to 10-week-old C57BL6 mice. Murine bone-marrow derived dendritic cells (BMDC): isolated from 6- to 10-week-old C57BL6 mice. Human monocytes: isolated from the $CD14^+$ fraction of peripheral blood mononucleated cells (PBMC).
3. Working in RNAse-free conditions: always wear clean lab coat and gloves. Clean the workbench, the PSM and all equipment with RNase AWAY solution prior to the experiment. Use certified RNAse-free tips, pipettes, Eppendorf, and tubes.
4. Routinely, we used either PerkinElmer CellCarrier-384 (Optically Clear Bottom, tissue culture treated, black, sterile with Lid); or Greiner Bio-One, Cat 781091 (384-well polystyrene cell culture microplates, Flat Bottom, μClear, black, sterile with Lid).
5. You can develop image analysis routines by yourself using any object-oriented computer language, e.g., C++, Java, Python, but it is advised to use a dedicated software featuring predefined detection algorithms. We used the Acapella Studio (Perkin Elmer) software to develop our own routines with the best flexibility.

6. As the presence of aggregates in the mycobacterial inoculum could lead to a heterogeneous invasion process, it is important to care about the preparation of the bacteria. To remove bacterial aggregates, centrifuge at 700 rpm for 2 min and observe the supernatant under a light microscope. You should see a well dispersed suspension without aggregates. You may repeat the centrifugation step if needed.
7. You can adapt the bacterial concentration to reach higher or lower multiplicity of infection (MOI), as required. The value given here (2×10^6 bacteria/mL) yields a MOI of 2, which is generally used with macrophage cell lines. A MOI of 1 is generally preferred with human and murine primary cells, but MOI of 5–20 are recommended for the infection of A549 epithelial cells.
8. Cells can be stored in liquid nitrogen for long-term storage. Typically, 1 mL aliquots containing 1×10^7 (Raw264.7), 8×10^5 (THP-1), or 5×10^6 (A549) cells in complete RPMI medium containing 10 % DMSO are made. After thawing the aliquot and ten-fold dilution into pre-warmed (37 °C) RPMI medium, cells are pelleted by centrifugation for 5 min at $400 \times g$ at room temperature and resuspended in fresh pre-warmed complete RPMI medium to remove DMSO.
9. Undifferentiated monocytes can be stored at –80 °C for several months and in liquid nitrogen for longer term storage.
10. While monocytes can be stored at –80 °C for several months, it is not recommended to freeze the isolated $CD14^+$ fraction as it could strongly decrease the yield of the overall purification due to cell mortality.
11. The use of an automatic plate washer/dispenser is recommended at this step, to ensure fast, reproducible and homogenous washing/distribution.
12. We recommend using borosilicate amber glass vials with hermetic polypropylene PVDF or PTFE-coated screw caps. Always work in a fume hood, wearing a lab coat and gloves. As DMSO is very hygroscopic, it is also advised to work under a light flow of nitrogen to avoid hydration of the solvent.
13. The easiest algorithm for a fast cellular or organelle detection is the threshold-based method, similar to that used for bacteria detection. However, this method usually cannot yield a list of individualized objects, especially when objects are too close to each other's, which generally happens at low magnification (20×). In this case, more precise algorithms based on contrast, shape and/or intensity have to be developed. The use of already available algorithms is then recommended, as *de novo* development can be time-consuming.

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