### Abstract:

Mycobacterium tuberculosis infections result in a spectrum of clinical outcomes, and frequently the infection persists in a latent, clinically asymptomatic state. The within-host bacterial population is likely to be heterogeneous, and it is thought that persistent mycobacteria arise from a small population of viable, but non-replicating (VBNR) cells. These are likely to be antibiotic tolerant and necessitate prolonged treatment. Little is known about these persistent mycobacteria, since they are very difficult to isolate. To address this, we have successfully developed a replication reporter system for use in M. tuberculosis. This approach, termed fluorescence dilution, exploits 2 fluorescent reporters; a constitutive reporter allows the tracking of bacteria, while an inducible reporter enables the measurement of bacterial replication. The application of fluorescent single-cell analysis to characterise intracellular M. tuberculosis identified a distinct subpopulation of non-growing mycobacteria in murine macrophages. The presence of VBNR and actively replicating mycobacteria was observed within the same macrophage after 48 hours of infection. Furthermore, our results suggest that macrophage uptake resulted in enrichment of non- or slowly replicating bacteria (as revealed by DCS treatment); this population is likely to be highly enriched for persisters, based on its drug tolerant phenotype. These results demonstrate the successful application of the novel dual fluorescent reporter system both in vitro and in macrophage infection models to provide a window into mycobacterial population heterogeneity.
Elucidating population-wide mycobacterial replication dynamics at the single-cell level.

Running title: Mycobacterial replication dynamics at single-cell level.

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Mycobacterium tuberculosis infections result in a spectrum of clinical outcomes, and frequently the infection persists in a latent, clinically asymptomatic state. The within-host bacterial population is likely to be heterogeneous, and it is thought that persistent mycobacteria arise from a small population of viable, but non-replicating (VBNR) cells. These are likely to be antibiotic tolerant and necessitate prolonged treatment. Little is known about these persistent mycobacteria, since they are very difficult to isolate. To address this, we have successfully developed a replication reporter system for use in M. tuberculosis. This approach, termed fluorescence dilution, exploits 2 fluorescent reporters; a constitutive reporter allows the tracking of bacteria, while an inducible reporter enables the measurement of bacterial replication. The application of fluorescent single-cell analysis to characterise intracellular M. tuberculosis identified a distinct subpopulation of non-growing mycobacteria in murine macrophages. The presence of VBNR and actively replicating mycobacteria was observed within the same macrophage after 48 hours of infection. Furthermore, our results suggest that macrophage uptake resulted in enrichment of non- or slowly replicating bacteria (as revealed by DCS treatment); this population is likely to be highly enriched for persisters, based on its drug tolerant phenotype. These results demonstrate the successful application of the novel dual fluorescent reporter system both in vitro and in macrophage infection models to provide a window into mycobacterial population heterogeneity.
INTRODUCTION

Despite recent data indicating a worldwide decline in tuberculosis (TB) mortality and incidence (WHO, 2015), the disease continues to present serious public health challenges. The first key challenge is posed by the large number of individuals with latent *Mycobacterium tuberculosis* infection, an estimated one-third of the world’s population (Hartman-Adams *et al*., 2014). This represents an ongoing infectious disease threat which is particularly relevant in high-incidence settings where HIV co-infection is also common. In HIV co-infected individuals, there is a substantially increased risk of progression to active disease (WHO, 2015). A second significant obstacle lies in the requirement for very long and complex treatment regimens which are required to achieve complete sterilisation of *M. tuberculosis* infections. This is thought to be driven by so-called “persister” or “dormant” populations of mycobacteria, which exhibit a drug tolerant phenotype (Zhang *et al*., 2012). While there are varying definitions of the terms “persister”, here we use it to refer to non- or slowly replicating drug tolerant bacteria.

The existence of drug-tolerant populations of persistent bacteria was first postulated in the 1940s, following the observation that *Staphylococcus aureus* surviving antibiotic treatment were phenotypically and reversibly tolerant, rather than genetically resistant (Bigger, 1944). Since then, the phenomenon has been well described in *Escherichia coli*, and numerous other bacteria have been shown to form persisters (Cohen *et al*., 2013, Helaine and Kugelberg, 2014). Several mediators of persistence have been identified, with toxin-antitoxin modules emerging as key players in formation and maintenance of persister populations (Cohen *et al*., 2013,
Helaine and Kugelberg, 2014, Maisonneuve et al., 2011). Although limited studies have been carried out in *M. tuberculosis*, these are mostly consistent with findings in other bacteria (Dhar and McKinney, 2010, Keren et al., 2011, Manina et al., 2015).

A commonly accepted notion is that bacterial persister populations are non-replicating, which contributes to their drug tolerant phenotype (Helaine and Kugelberg, 2014), although this assumption has been challenged by recent studies (Adams et al., 2011, Wakamoto et al., 2013, Manina et al., 2015). Understanding the physiological state of persister mycobacteria and their role in clinically latent *M. tuberculosis* infection has important implications for TB treatment and prevention. However, our understanding of this physiological state is hampered by a paucity of suitable tools to identify, isolate and characterise non- or slowly replicating mycobacteria.

The nature of bacterial persisters renders them difficult to isolate and characterise. In the first instance, they are likely to undergo very limited (if any) replication. This makes them difficult to recover in their VBNR or non-growing, metabolically active (NGMA) state, while still reflecting relevant physiology. Secondly, based on data from other bacteria, and indirect studies in mycobacteria, they are likely to only be present in very low numbers. We therefore require culture-independent methods which will allow us to enrich for, and isolate, persistent bacteria without perturbing their physiological state. Given the emerging appreciation of the heterogeneous nature of bacterial populations, it is also important to be able to study single cells at the population-wide level. Recent studies have exploited the power of high-resolution technologies such as microfluidics and flow cytometry to enable the rapid
measurement of the physiological state of single bacteria within large populations
(Aldridge et al., 2012, Balaban et al., 2004, Wakamoto et al., 2013, Maglica et al.,
2015, Manina et al., 2015, Roostalu et al., 2008). Helaine et al successfully applied
the latter to measure fluorescence dilution in Salmonella Typhimurium, using a dual
fluorescence replication reporter system (Helaine et al., 2014, Helaine et al., 2010).
These studies revealed that macrophage uptake induces a non-replicating
population of Salmonella, leading to important insights into the characteristics of non-
replicating persistent bacteria in vivo. As we predict that similar mechanisms are
important during M. tuberculosis infection, in this study we aimed to develop and
validate a similar replication reporter system for M. tuberculosis. This has been
applied to provide insights into population-wide mycobacterial replication dynamics
at the single-cell level.
METHODS AND MATERIALS

Bacterial strains and culture. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Bacterial strains utilised in this study are listed in Table 1. Electrocompetent *Escherichia coli* DH10B used for plasmid selection and propagation was obtained from Invitrogen (Carlsbad, CA, USA). *Mycobacterium smegmatis* mc²155 was obtained from the American Type Culture Collection (ATCC 700084). The origin and construction of *Mycobacterium tuberculosis* ΔleuD ΔpanCD was as previously reported (Sampson *et al.*, 2004).

*E. coli* was cultured in lysogeny broth (LB) with appropriate antibiotic supplementation according to standard protocols at 37°C, with shaking, or on LB-agar at 37°C. Liquid cultures of mycobacterial strains were grown in 7H9 supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC, Becton Dickinson), 0.2% glycerol and 0.05% Tween-80 (7H9-OGT), with appropriate antibiotic supplementation, at 37°C, with shaking. Electro-competent mycobacteria were prepared and transformed as previously described (Snapper *et al.*, 1990). Solid media cultures of mycobacteria were grown on 7H10-agar supplemented with 10% OADC, 1% glycerol and appropriate antibiotics at 37°C. Additionally, for colony forming unit (CFU) enumeration only, *M. smegmatis* was cultured on LB-agar. To induce the expression of proteins under control of the riboswitch-based promoter, theophylline was added at 2 mM.

For testing the stability of fluorescent reporter proteins, cultures were washed, then passaged twice through PBS with 0.05% Tyloxapol and 2 mM theophylline for 24
hours each time. Cultures were then transferred to PBS with 0.05% Tyloxapol (without theophylline) in the presence 30 µg/ml of the protein synthesis inhibitor chloramphenicol.

**Plasmid constructs.** Plasmids used and constructed in this study are listed in Table 1. pST5552 (carrying EGFP under control of the theophylline-inducible riboswitch promoter) (Seeliger et al., 2012) and pCHARGE3 (encoding the far-red fluorescent protein, TurboFP635, under control of the Psmyc promoter) (Carroll et al., 2010), were obtained from Addgene (Table 1). The pSTCHARGE3 plasmid (carrying inducible TurboFP635) and pTiGc plasmid (carrying inducible TurboFP635 and constitutive GFP) were generated in this study. Briefly, the TurboFP635 open reading frame was PCR amplified from pCHARGE3 with primers incorporating EcoRI and HindIII sites (forward primer: 5'-CGATCCGAATTCCAGGAGGTAAC-3', reverse primer: 5'-ATCGATAAGCTTTTACGAGTG-3'). Following EcoRI/HindIII restriction digestion, the TurboFP635 PCR fragment was cloned into the corresponding sites in pST5552, replacing EGFP and inserting TurboFP635 under control of the riboswitch promoter, to generate pSTCHARGE3. The pTiGc plasmid was subsequently generated by exciting hsp60-GFP from pMV306hspGFP (Parker and Bermudez, 1997) with NotI, and cloning into NotI-restricted pSTCHARGE3.

**Mammalian cell culture.** RAW 264.7 cells (ATCC TIB-71) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO₂. Cells were passaged every 2-4 days. For infections, cells were seeded at 5 x 10⁵ cells/well in 24 well plates. Where required for microscopy, 14 mm glass coverslips were sterilised and added to
appropriate wells prior to seeding. The following day, media was replaced with fresh DMEM-10% FCS. To prepare mycobacteria for infection, cultures were filtered through 40 micron cell strainers, then briefly sonicated in an ultrasonic bath (UC-1D, Zeus Automation, South Africa) at 37 kHz for 12 min to disperse clumps (these conditions have previously been shown to reduce clumping without affecting bacterial viability). Bacteria were then washed with PBS-0.05% Tween-80 (PBS-T) prior to centrifugation and resuspension in DMEM-10% FCS. Bacteria were added to macrophages at a 5:1 ratio, and incubated at 37°C in 5% CO₂ for 3 hours. Following the uptake period, cells were washed once with PBS before the media was replaced with DMEM-10% FCS containing 100 U penicillin-streptomycin. The cells were then incubated at 37°C in 5% CO₂ for 1 hour to kill any non-phagocytosed, extracellular bacteria. Cells were washed 3 times before adding fresh DMEM-10% FCS. To recover mycobacteria for CFU determination and flow cytometry, macrophages were lysed by the addition of sterile distilled water followed by pipetting. CFU determination was performed by serial dilution plating of lysates onto LB-agar (for *M. smegmatis*) or 7H10 agar (for *M. tuberculosis*). To induce or maintain the expression of bacterial proteins under control of the riboswitch-based promoter, theophylline was added to mammalian cell cultures at 1 mM. To ensure that dilution of fluorescence only starts intracellularly, after macrophage uptake, macrophages were pre-incubated with 1 mM theophylline, and theophylline was maintained in the cultures up to and including the antibiotic incubation step (this was defined as t=0).

**Persister assay**

To assess whether internalisation of *M. tuberculosis* in macrophages enriches for drug tolerant bacteria (persisters), murine macrophages were infected with pre-
induced *M. tuberculosis* as previously described. Following uptake, penicillin-
streptomycin treatment and PBS washes were performed as previously described. At
specified time points, bacteria were either recovered from infected macrophages and
treated with D-cycloserine (DCS), or DCS-treated while still within macrophages, to
select for drug tolerant persisters. More specifically, intracellular bacteria recovered
from lysed infected macrophages were washed once in PBS and incubated in 7H9
containing 100µg/ml D-cycloserine (DCS) (Keren *et al*., 2011) at 37°C for 4 days.
CFU determination was performed by serial dilution plating of lysates onto 7H10
agar. Untreated controls were included for both CFU and flow cytometry
assessments. The fold increase in drug tolerant bacteria induced by internalisation in
macrophages was quantified as previously described (Helaine *et al*., 2014), by
calculating the ratio between the percentage surviving macrophage-exposed
population and the percentage surviving *in vitro* cultured bacteria. Here, percentage
surviving refers to the bacteria surviving DCS treatment (non-replicating persisters).

To assess population heterogeneity and persister enrichment by flow cytometry,
macrophages with intracellular bacteria were incubated with DMEM-10% FCS
containing 100µg/ml DCS, without theophylline, at 37°C in 5% CO₂ for 4 days.

**Flow cytometry sample preparation, acquisition and analysis.** Bacteria from *in
vitro* culture media (7H9-OGT or DMEM-10%FCS) were gently sonicated as
previously described. Sonicated bacteria from *in vitro* cultures, or bacteria recovered
from lysed macrophages, were pelleted, fixed in 4% formaldehyde for 30 minutes,
then washed twice in PBS-T. Samples not immediately analysed were stored in
PBS-T in the dark at 4°C, then pelleted, resuspended in PBS, and filtered
immediately prior to use. Samples were analysed using a LSRFortessa flow
cytometer (Becton Dickinson). In addition to forward scatter and side scatter, GFP fluorescence intensity was captured by excitation at 488 nm, using a 530/30 filter, and TurboFP635 fluorescence intensity was captured by excitation at 561 nm, using a 610/20 filter. For each experiment, compensation was performed using unlabelled and single colour controls. For samples from *in vitro* liquid cultures, 10,000 to 30,000 events were captured, while 30,000 events were captured for bacterial samples recovered from macrophages.

Flow cytometry data were analysed using FlowJo vX.0.07r2 software. A primary gate was set based on FSC/SSC properties, following which the GFP-positive (live) population was gated, then analysed to determine the geometric mean of the TurboFP635 fluorescence intensity (Fig. S1). To calculate the number of generations based on fluorescence intensity data, the extent of bacterial replication (F = fold replication) was first determined by the ratio $Y_0/Y_t$, where $Y$ is the geometric mean of red (TurboFP635) fluorescence intensity at a specific time. The number of generations were in turn calculated from the formula $F = 2^N$. The number of generations as determined by OD measurements or CFU data were calculated similarly, except that in this case $F = Y_t/Y_0$. Generation times are expressed as mean ± standard deviation.

**Microscopy.** Bacteria from *in vitro* culture medium were sonicated and formaldehyde fixed as described above, then washed with PBS and mounted onto clean microscope slides using Prolong Gold (Invitrogen). Similarly, infected macrophages on coverslips were fixed with 4% formaldehyde for 30 min, washed with PBS and mounted onto clean microscope slides using Prolong Gold.
Cells were observed using a confocal microscope (LSM 780, Carl Zeiss) equipped with a GaAsp detector, using a Plan-Achromat 63x/1.4 Oil DIC M27 objective. Samples were excited with a 488 nm and 561 nm laser, using 490-516 nm and 585-696 nm filters for green and red fluorescence, respectively. Transmitted light images for infected macrophages were collected using the confocal microscope equipped with transmitted light detector. Images were acquired through z-stack acquisition, with an increment of 0.500 µm between image frames, and displayed as maximum intensity projections. Zen imaging software (Zen SP1 2012, Black edition, version 8.1.0.484) was used to view and process images.

**Statistical analysis.** Statistical analysis was carried out using GraphPad Prism V6.04 software.
RESULTS

Construction of replication reporter
The ability to reliably measure bacterial replication dynamics using fluorescence dilution (FD) requires two sufficiently bright and stable, spectrally distinct, fluorescent reporter proteins, and a tightly regulated promoter. This approach relies on the constitutive expression of one reporter protein as a marker of cells, in combination with an inducible reporter protein (Fig. 1a), preferably with a large dynamic range of dilution, which acts as a marker of replication (Fig. 1b). Alternatively, the inducible reporter can be exploited as a proxy for metabolic activity, specifically translational activity (Fig. 1c). To this end, we made use of the green fluorescent protein, GFP (Inouye and Tsuji, 1994), and far-red fluorescent protein, TurboFP635 (Katushka) (Shcherbo et al., 2010). These have been previously shown to be robust reporters in both pathogenic and non-pathogenic mycobacteria in vitro and in vivo (Carroll et al., 2010, Parker and Bermudez, 1997, Zelmer et al., 2012). GFP, under control of the constitutive groEL promoter, was introduced into a plasmid backbone carrying TurboFP635 under control of a riboswitch-based, theophylline inducible promoter (Seeliger et al., 2012), to generate the pTiGc FD plasmid (Fig. 1a). Notably, heat-killing of M. smegmatis (90°C, 30min) resulted in a 15 fold decrease in mean fluorescence intensity when compared to untreated viable bacteria (data not shown), highlighting the suitability of GFP as a marker for cell viability.

The replication reporter protein is tightly regulated and stable
To confirm that the pTiGc plasmid allowed for tightly regulated expression of TurboFP635, we measured green and far-red fluorescence intensity of mycobacteria
carrying this plasmid in the presence and absence of the inducer, theophylline. No growth defect (in comparison to wild type strains) was observed in the presence or absence of theophylline, indicating that expression of the reporter proteins did not incur an *in vitro* fitness cost (data not shown). To facilitate detailed assessment of the fluorescent properties of large numbers of single bacterial cells, we used a combination of flow cytometry and confocal microscopy. As expected, in the absence of induction, the majority of pTiGc-carrying mycobacteria demonstrated green fluorescence, but no red fluorescence above no-reporter control levels (Fig. 2). We confirmed that induction with 2 mM theophylline resulted in high-intensity far-red fluorescence for both *Mycobacterium smegmatis*::pTiGc (Fig. 2a, b) and *Mycobacterium tuberculosis* Δ*leuD* Δ*panCD*::pTiGc (Fig. 2c, d). Confocal microscopy showed normal bacterial morphology and no aggregates for both *Mycobacterium smegmatis*::pTiGc (Fig. 2b) and *Mycobacterium tuberculosis* Δ*leuD* Δ*panCD*::pTiGc (Fig. 2d). Flow cytometry and confocal microscopy therefore confirmed constitutive GFP expression and tightly regulated, strongly inducible TurboFP635 expression in both *M. smegmatis* and *M. tuberculosis*.

An important requirement for successful application of the FD technique is stability of the reporter proteins. Specifically, the replication reporter needs to have a sufficiently long half-life such that only bacterial division (and not protein degradation) will result in reduction of signal over the course of the experimental period. To establish if this was indeed the case, we measured reporter protein stability in non-replicating, nutrient-starved cultures in the presence of chloramphenicol, a bacteriostatic drug which blocks *de novo* protein synthesis. Pre-induced *M. smegmatis*::pTiGc cultures were incubated in PBS-0.05% Tyloxapol with 2 mM theophylline, then passaged into
PBS-0.05% Tyloxapol with no theophylline in the presence of 30 µg/ml chloramphenicol for 24 hours (Fig. 3a, b). Similarly, pre-induced *M. tuberculosis*::pTiGc cultures were incubated in media with theophylline, but lacking pantothenate (to restrict growth), before passage into fresh media with no theophylline or pantothenate, but including 30 µg/ml chloramphenicol for up to 4 days (Fig. 3c, d). Subsequent analysis by flow cytometry and confocal microscopy confirmed that non-replicating, chloramphenicol-treated mycobacteria retained high levels of green and red fluorescence intensity. We also demonstrated that the TurboFP635 reporter was stable under acidic conditions, as might be encountered within the macrophage environment (Fig. S2). These results indicate that the TurboFP635 reporter is sufficiently stable to be used as a reliable marker of mycobacterial replication.

**Fluorescence dilution provides a quantitative measure of mycobacterial replication**

We next wished to determine whether FD could provide a reliable quantitative measure of mycobacterial replication comparable to commonly used methods such as optical density measurement. To do this, we passaged pre-induced mycobacteria into media without theophylline, and incubated with shaking at 37°C. In this setting, no new TurboFP635 would be synthesised, and therefore in bacteria undergoing active replication, we would expect the mean far-red fluorescent signal to be reduced with each successive cell division. *M. smegmatis*::pTiGc and *M. tuberculosis* Δ*leuD ΔpanCD*::pTiGc samples were taken at regular intervals for optical density (OD) and/or colony forming unit (CFU) measurements and flow cytometry analysis. Flow cytometry data indicated fluorescence dilution during the course of bacterial growth.
The change in the geometric mean of far-red fluorescence intensity over time (Fig. 3a, b) was then used to calculate the number of generations and compared to the number of generations calculated from OD or CFU measurements. This demonstrated an excellent correlation up to at least five generations, for both *M. smegmatis* (Fig. 4c) and *M. tuberculosis* (Fig. 4d). While we cannot rule out the possibility that degradation of the TurboFP635 reporter may contribute to the loss of fluorescence, our data suggests otherwise. We note that Pearson correlation tests revealed a statistically significant positive correlation between generations calculated based on OD or CFU and those calculated based on FD for *M. smegmatis* (*r* = 0.9394, *N* = 9, 2-tailed *p*-value = 0.0002) and *M. tuberculosis* (*r* = 0.9908, *N* = 6, 2-tailed *p*-value = 0.0001). The calculated average generation time (based on FD measurements) for *M. smegmatis* during logarithmic growth in rich media was approximately 3 h 20 min, corresponding to published replication rates (Gill *et al.*, 2009). Similar results were obtained for *M. tuberculosis ΔleuD ΔpanCD::pTiGc*, although with longer calculated generation times (approximately 18 h 43 min), as expected for this slow growing organism (Gill *et al.*, 2009). These data confirm that FD could be reliably used to monitor mycobacterial replication, for at least 5 generations.

We went on to validate the use of mycobacterial FD to probe mycobacterial replication dynamics under different *in vitro* conditions. We first examined *M. smegmatis* growth during nutrient limitation. Pre-induced *M. smegmatis::pTiGc* cultures were passaged twice in PBS-0.05% Tyloxapol with 2 mM theophylline to remove extracellular nutrients and deplete intracellular nutrient stores. Nutrient-depleted bacteria were washed to remove theophylline, and then added to PBS-
0.05% Tyloxapol (nutrient limited) or standard nutrient-replete growth media (7H9-OGT), in the absence of theophylline. For M. smegmatis, flow cytometry analysis at 0, 6 and 24 h revealed only a small shift in fluorescence intensity in nutrient limited medium (PBS-0.05% Tyloxapol) between 0 and 24 h (Fig. 5a). This shift corresponded to <1 generation (0.93 ± 0.3 at 24 h), contrasting with the large shift in the nutrient-rich (7H9-OGT) medium, which corresponded to 5.34 ± 0.15 generations at 24 h.

We performed a similar analysis for M. tuberculosis, but here we took advantage of the auxotrophic nature of the M. tuberculosis ΔleuD ΔpanCD strain (Sampson et al., 2004). This strain grows normally when exogenously supplemented with leucine and pantothenate, but does not grow in the absence of either of these supplements. M. tuberculosis ΔleuD ΔpanCD::pTiGc was first cultured overnight in 7H9-OGT containing 2 mM theophylline (to induce TurboFP635 expression) and supplemented with leucine and pantothenate (to permit growth). Bacteria were washed, then incubated overnight in 7H9-OGT with no pantothenate (to deplete any intracellular stores of this compound), in the presence of 2 mM theophylline. OD measurements confirmed growth restriction (data not shown). The pre-induced, nutrient-starved bacteria were then passaged into 7H9-OGT, supplemented with leucine, without theophylline and with 0 or 24 µg/ml pantothenate. Samples were taken for OD measurements and flow cytometry at indicated time points (Fig. 5b). As expected, pantothenate depletion restricted growth rates; for pantothenate-depleted cultures after 4 days, FD-calculated generations were 2.78 ± 0.40. In contrast, FD-calculated generations for nutrient replete cultures were 6.15 ± 0.026 at the same time point (96 h). Together, these results demonstrate that FD can be reliably used to monitor
mycobacterial replication, and can provide an accurate measurement of different
growth rates.

**Intracellular mycobacterial replication dynamics**
An important potential application for the FD system is to enable the measurement of
intracellular mycobacterial replication (or lack thereof). To assess whether this was
feasible, we infected murine macrophages with pre-induced *M. smegmatis::pTiGc*.
Infected macrophages were lysed, and recovered mycobacteria were analysed by
flow cytometry or CFU determination (Fig. 6a). In addition, infected macrophages on
coverslips were analysed by confocal microscopy (Fig. 6b). Flow cytometry
demonstrated a shift in the geometric mean of far-red fluorescence intensity of GFP-
positive *M. smegmatis* recovered from macrophages 24 h post-infection (Fig. 6a),
suggesting replication of intracellular *M. smegmatis::pTiGc*. Subsequently, flow
cytometric data were used to quantify *M. smegmatis* replication dynamics during
macrophage infection. When considering the geometric mean of all far-red
fluorescent *M. smegmatis* within the GFP-positive (live) population, the calculated
generation number over 24 h was 1.59 ± 0.38, suggestive of minimal replication of
intracellular bacteria. This was significantly lower than that of *M. smegmatis* cultured
extracellularly in D10 (5.61 ± 1.09). Interestingly, closer analysis of flow cytometric
data revealed the presence of a sub-population (22.0 ± 8.8 %) of bacteria with
reduced TurboFP635 signal at 24 h (Fig. 6a, Fig. S3); this was also evident by
confocal microscopy (Fig. 6b). We observed a clear shoulder on the left of the
asymmetric peak representing intracellular bacteria at 24h, when compared to
intracellular and *in vitro* cultured bacteria at 0h (Fig 6a). When the far-red\textsuperscript{HI} and far-
red\textsuperscript{LO} populations were considered independently (Fig. S3), their generation
numbers were 0.94 ± 0.12 and 3.39 ± 0.16, respectively. M. smegmatis is reported to be rapidly cleared from infected macrophages (Prakash et al., 2010), and CFU results for this experiment are consistent with this (generation number over 24 h <1). However, the flow cytometry data is suggestive of the presence of a sub-population of replicating M. smegmatis::pTiGc within the macrophage culture. No extracellular bacteria were observed by confocal microscopy. We acknowledge that streptomycin can affect both extracellular and intracellular bacteria, however, in this study all intracellular bacteria were subjected to the same streptomycin exposure times; therefore any differences observed between these samples cannot be attributed to the antibiotic. The dual fluorescent reporter system has therefore provided new insights into M. smegmatis-macrophage interaction, highlighting the heterogeneous nature of the intracellular mycobacterial population in this context.

M. tuberculosis population heterogeneity and drug tolerant bacteria (persister) enrichment

We next applied the dual reporter system to interrogate replication dynamics of M. tuberculosis during macrophage infection. RAW264.7 macrophages were infected with pre-induced M. tuberculosis::ΔleuD ΔpanCD::pTiGc as described above (Fig. 6c, d). Flow cytometry was used to compare the bacterial population recovered from macrophages to those grown in vitro for up to 96 h following infection. This revealed that intracellular mycobacteria showed a relatively homogeneous population structure at early time points, with a similar replication rate at 24 h (0.89 ± 0.04) as the in vitro cultured bacteria (0.78 ± 0.15) (Fig. 6c). However, from 48 h onwards, a more heterogeneous population distribution emerged, with a marked slower-growing population at 96 h.
To determine whether the heterogeneous intracellular *M. tuberculosis* population included persister bacteria, we applied a combination of CFU determination and mycobacterial FD in conjunction with D-cycloserine (DCS) treatment to characterise the mycobacteria recovered from macrophages. A previous study demonstrated that internalisation of *Salmonella* by bone-marrow derived macrophages (BMDM) rapidly induced the formation of persisters (Helaine *et al.*, 2014). Similarly, we observed a significant increase in the proportion of drug tolerant bacteria (persisters) following internalisation by macrophages (Fig. 7a). Interestingly, we observed a significant increase in macrophage-induced drug tolerant bacteria (persisters) at later time points, suggesting slow adaptive responses to the intracellular microenvironment. Flow cytometry demonstrated that DCS treatment of intracellular bacteria enriched for a high-intensity far-red population with an evidently slower replication rate from 24 h. (Fig. 7b). This further suggests that the increasing proportion of slow or non-replicating drug tolerant bacteria represent the persisters isolated by CFU survival assays over time following macrophage uptake.
DISCUSSION

A major stumbling block in understanding the biology of latent *Mycobacterium tuberculosis* infection and the phenomenon of drug tolerance in mycobacteria is the paucity of tools to isolate and characterise persistent mycobacteria. These populations are commonly suggested to exist in a non- or slowly replicating state. However, while there is some evidence to support very slow or no growth of *M. tuberculosis* in human samples (Colangeli et al., 2014, Garton et al., 2008, Walter et al., 2015), results from mice (Gill et al., 2009) and nonhuman primates (Ford et al., 2011) have been interpreted to suggest otherwise. Although several studies have linked *M. tuberculosis* drug tolerance to dormancy/lack of replication (Deb et al., 2009, Rodriguez et al., 2014), other data from *in vitro* studies (Wakamoto et al., 2013) and macrophage models (Adams et al., 2011, Raffetseder et al., 2014) suggests that lack of replication is not an absolute requirement for *M. tuberculosis* drug tolerance. These apparently discrepant results have been derived using diverse experimental approaches, highlighting the need for a new, systematic approach to the problem. Understanding the physiological state of “dormant” bacteria, and the cues that induce entry into and exit from a dormant state in clinically latent *M. tuberculosis* infection has important implications for TB treatment and prevention. However, our knowledge of the physiological state of viable, but nonreplicating (VBNR) mycobacteria is severely limited, particularly *in vivo*. This is largely due to lack of suitable tools to identify, isolate and characterise non- or slowly replicating mycobacteria. These populations are inherently difficult to isolate, and new tools to do so are urgently required.
We report here on the development and validation of a powerful new tool for monitoring population-wide mycobacterial replication at the single-cell level. Fluorescence dilution (FD) technology was developed by Helaine et al to monitor Salmonella replication dynamics, and has yielded striking new insights into intracellular replication dynamics of pathogenic bacteria (Helaine et al., 2014, Helaine et al., 2010). Here, we have successfully adapted and applied FD to Mycobacterium smegmatis and M. tuberculosis, demonstrating its utility in both fast-growing saprophytic and slow-growing pathogenic mycobacteria.

The ability to use FD to accurately quantify bacterial replication dynamics has several prerequisites, including sufficiently bright and stable fluorescent reporter proteins. Here, we have demonstrated that the far-red reporter TurboFP635 is sufficiently bright and stable, and thus we selected this for further experiments. Notably, during optimisation of promoter/reporter combinations, we observed that GFP was unexpectedly too unstable to serve as an accurate reporter for mycobacterial replication (data not shown). A second critical feature is that the inducible promoter needs to have undetectable basal activity ( Mitchison and Coates, 2004), but must be expressed at high levels upon induction. We found the recently described theophylline-inducible, riboswitch-based promoter (Seeliger et al., 2012) to be highly suited for this application.

In this study, we have demonstrated that the pTiGc dual colour reporter plasmid can be successfully applied to monitor in vitro and intracellular mycobacterial replication dynamics by fluorescence dilution. We have shown that we can accurately measure at least 5 bacterial generations. This is comparable to previous work using FD in
Salmonella (Helaine et al., 2010), as well as to studies using microfluidics platforms to monitor single-cell replication dynamics of *M. smegmatis* (Aldridge et al., 2012). Future versions of this system could include a second inducible promoter, an approach that has been successfully applied in *Salmonella* to extend the number of measurable generations up to 10 (Helaine et al., 2010).

The pTiGc reporter system could easily be combined with fluorescence activated cell sorting to isolate different populations for downstream analysis. Until now, this has not been feasible, and previous studies have relied on methods such as preferential lysis of replicating bacteria using antibiotics or other chemical treatment (Canas-Duarte et al., 2014, Keren et al., 2011). Alternatively, analyses have been performed on enriched, but still potentially heterogeneous, populations (Betts et al., 2002, Gengenbacher et al., 2010, Mak et al., 2012, Deb et al., 2009). The dual reporter system described here offers the ability to rapidly and specifically identify non- or slowly replicating mycobacteria in physiologically relevant environments. In combination with fluorescence-activated cell sorting, this system will allow the purification of non-replicating bacteria from other populations, enabling subsequent characterisation of relatively homogeneous populations.

Application of the dual reporter system to characterize intracellular *M. smegmatis* revealed that while the majority of bacteria were non-or slowly replicating, a sub-population of apparently dividing *M. smegmatis* could be detected. Numerous reports indicate that *M. smegmatis* does not survive within murine macrophages (Prakash et al., 2010), with the underlying assumption that it is unable to grow in this environment. However, these studies rely on CFU enumeration as the primary assay.
for mycobacterial survival. This provides only a summative measure of the growth rate, and does not take into account population heterogeneity. As highlighted by Anes et al, even non-pathogenic *M. smegmatis* can undergo periods of intracellular replication (Anes et al., 2006). Our findings support this, and highlight the ability of the dual reporter system to provide a window into unexpected mycobacterial population heterogeneity.

Our dual reporter system also revealed population heterogeneity for intracellular *M. tuberculosis*. Interestingly, early time points showed relatively homogeneous replication rates, very similar to those of *in vitro*-cultured bacteria. However, as the infection proceeded, a more slowly replicating population emerged, suggesting a programmed adaptation to the intracellular environment. This is consistent with a recent study which reported a decrease in replication rate and ribosomal activity of *M. tuberculosis* in the later stages of a murine infection model (Manina et al., 2015). Transcriptional analyses have demonstrated that *M. tuberculosis* undergoes metabolic remodelling upon exposure to the host environment, and during drug treatment of infected hosts (Rodriguez et al., 2014, Walter et al., 2015); the FD system described here provides a powerful tool to explore the heterogeneity of this remodelling and its impact on intracellular growth of mycobacteria. Determination of the physiological state and long-term fate of the slowly replicating population would be of great interest.

We have shown that FD could be applied to demonstrate an increase in the proportion of non- or slowly replicating drug tolerant bacteria (likely persisters) upon exposure to the intracellular macrophage environment, as revealed by DCS.
treatment. This corresponds with a previous study which showed that internalisation of *Salmonella* by BMDM resulted in the formation of persisters. However, in that study a significant increase in macrophage-induced persisters was observed after only 15 min, representing a much more rapid response than seen in the present study. Interestingly, our results suggest that longer internalisation of mycobacteria by macrophages resulted in a significant increase in non-replicating persisters at later time points (72 h and 96 h), compared to earlier time points (24 h and 48 h). A recent study has indicated that mycobacterial phenotypic variation is the result of host immunity and antibiotic stress (Manina *et al*., 2015), while others have reported that physical confinement induces drug tolerance (Luthuli *et al*., 2015). However, the precise mechanisms by which these populations are induced are currently still unknown. Acidification and nutrient deprivation within the *Salmonella*-containing vacuole (SCV) has been shown to play a role in the observed phenotypic heterogeneity and persister formation (Helaine *et al*., 2014). It remains to be determined whether similar signals encountered within the phagolysosomal compartment trigger mycobacterial persister formation. Additionally, the effect of immunological activation of macrophages on *M. tuberculosis* persister formation warrants further investigation. However, we note that the activation of BMDMs with IFN-γ when infected with *Salmonella* did not result in any change in persister proportions (Helaine *et al*., 2014).

Variants of the dual reporter system described here could be combined with other types of mycobacterial biosensors, such as those for monitoring intracellular redox potential (Bhaskar *et al*., 2014), pH and chloride levels (Tan *et al*., 2013), to enable the simultaneous measurement of defined physiological characteristics. In
combination with technologies such as flow cytometry, microfluidics and high-resolution microscopy, this will allow us to probe the impact of bacterial microenvironment on replication dynamics and phenotypic heterogeneity.

In summary, we have demonstrated that FD can be successfully applied both *in vitro* and in macrophage infection models, offering unique opportunities to study how environmental stressors and the innate immune response might impact on phenotypes such as drug tolerance. Ultimately, this system could find application in whole animal infection models, to better understand the host and bacterial signals which lead to mycobacterial persistence.
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Figure Legends:

Fig. 1 Principle underlying the inducible dual reporter system for probing bacterial replication. (a) Schematic representation of pTiGc reporter plasmid, with GFP under control of the constitutive hsp60 promoter, and TurboFP635 under control of the theophylline inducible riboswitch-based reporter (Seeliger et al., 2012). (b) Application of FD to monitor replication dynamics by fluorescence dilution, where inducible fluorescent signal is diluted as bacteria replicate. (c) Application of FD to probe metabolic responsiveness following exposure to a stressor (e.g. antibiotic). In this case, viable bacteria would be expected to exhibit green fluorescence, metabolically active bacteria will exhibit both red and green fluorescence following induction, while dead bacteria will not fluoresce.

Fig. 2 The theophylline-inducible riboswitch-based promoter allows tight regulation of the fluorescence dilution reporter protein, TurboFP635. M. smegmatis::pTiGc was cultured in the presence or absence of 2 mM theophylline, then analysed by (a) flow cytometry or (b) confocal microscopy. Similarly, M. tuberculosis ΔleuD ΔpanCD::pTiGc was cultured in the presence or absence of 2 mM theophylline, then analysed by (c) flow cytometry or (d) confocal microscopy. Representative examples of 3 independent experiments are shown, demonstrating negligible background and significant upregulation of TurboFP635 expression upon theophylline addition. Scale bar = 10 μm. Data shown is representative of 3 independent biological replicates (each with 3 technical replicates).
**Fig. 3** The TurboFP635 is sufficiently stable for application as a replication reporter. (a) and (b) *M. smegmatis::pTiGc* was cultured in 7H9-OGT in the presence of 2 mM theophylline, then passaged twice in PBS-0.05% Tween-80 (PBS-T) + 2 mM theophylline, before inoculating into PBS-T with no theophylline, containing 30 µg/ml chloramphenicol (CHL). Samples were analysed by (a) flow cytometry or (b) confocal microscopy. In (a), fluorescence intensity before and after chloramphenicol treatment is depicted by the brown and orange populations, respectively. For comparison, an actively dividing population, 24 h after theophylline withdrawal, is shown in green. The black population represents unmarked bacteria. (c) and (d) *M. tuberculosis ΔleuD ΔpanCD::pTiGc* was cultured in 7H9-OGT in the presence of 2 mM theophylline, then passaged twice in 7H9-OGT without pantothenate + 2 mM theophylline, before inoculating into 7H9-OGT without pantothenate with no theophylline, containing 30 µg/ml chloramphenicol (CHL). Samples were analysed by (c) flow cytometry or (d) confocal microscopy. In (c), fluorescence intensity before and after chloramphenicol treatment is depicted by the brown and orange populations, respectively. For comparison, an actively dividing population, 96 h after theophylline withdrawal, is shown in green. The black population represents unmarked bacteria. Scale bar = 10 µm. Data shown is representative of 3 independent biological replicates (each with 3 technical replicates).

**Fig. 4** Fluorescence dilution provides an accurate measure of mycobacterial generation time. Mycobacterial cultures were cultured in the presence of 2 mM theophylline, then washed and passaged into fresh medium without theophylline, and growth was monitored by optical density (OD) or colony forming units (CFU) and
fluorescence dilution (FD). (a) and (b) Flow cytometric detection of TurboFP635 fluorescence is shown at selected time points for (a) *M. smegmatis*::pTiGc and (b) *M. tuberculosis* Δ*leuD* Δ*panCD*::pTiGc. (c) and (d) Bacterial generation numbers were calculated from OD (black line) and fluorescence intensity (grey line) measurements for *M. smegmatis*::pTiGc and from CFU (black line) and fluorescence intensity (grey line) measurements for *M. tuberculosis* Δ*leuD* Δ*panCD*::pTiGc as detailed in methods, and are compared for *M. smegmatis*::pTiGc and *M. tuberculosis* Δ*leuD* Δ*panCD*::pTiGc in (c) and (d), respectively. To enable measurement of *M. smegmatis* growth over 24 hours, staggered culture start times were used, with 1 set of cultures being monitored over the 0-12 h period (open symbols) and the second being monitored over the 12-24 h period (closed symbols). Data shown in (c) and (d) is depicted as mean ± SD of 3 technical replicates, and is representative of 3 independent experiments.

**Fig. 5** Fluorescence dilution can be exploited to assess mycobacterial replication dynamics under different *in vitro* conditions. Mycobacterial cultures were grown in the presence of 2 mM theophylline, then washed and passaged into fresh rich medium without theophylline, and growth was monitored by FD. (a) *M. smegmatis*::pTiGc was passaged into either minimal medium (PBS-0.05% Tyloxapol, dark grey and light grey) or rich medium (7H9-OGT, black and green). Samples were taken at 6 h and 24 h. (b) Similarly, *M. tuberculosis* Δ*leuD* Δ*panCD*::pTiGc was passaged into 7H9-OGT with (black and green, 24 h and 96 h, respectively) or without (dark and light grey, 24 h and 96 h, respectively) pantothenate “P”, and flow cytometry samples were taken at 24 and 96 hours following withdrawal of
theophylline. Data shown is representative of 3 independent biological replicates (each with 3 technical replicates).

**Fig. 6** The dual reporter system offers insights into intracellular mycobacterial replication dynamics. RAW264.7 macrophages were infected with *M. smegmatis::*pTiGc (a, b) or *M. tuberculosis* ΔleuD ΔpanCD::*pTiGc (c, d), and intracellular mycobacterial replication was compared to that of *in vitro* cultured mycobacteria, by monitoring TurboFP635 fluorescence. Fluorescence was assessed by flow cytometry (a, c) and confocal microscopy (b, d). Scale bar = 10 μm. Data shown is representative of 3 independent biological replicates (each with 3 technical replicates).

**Fig. 7** Fluorescence dilution allows identification of macrophage-induced persister formation. (a) *M. tuberculosis*::ΔleuD ΔpanCD::*pTiGc cultured *in vitro* or recovered from RAW264.7 macrophages at different time points post-uptake were transferred to 7H9-OGT, containing 100ug/ml DCS and incubated for 96 hours. Formation of macrophage-induced persisters was measured as the ratio of the percentage of the macrophage-exposed population surviving DCS treatment to the percentage of the *in vitro* cultured bacteria surviving DCS treatment. Data shown is depicted as mean ± SD of 3 technical replicates, and is representative of 3 independent experiments. (b) For flow cytometry, RAW264.7 macrophages were infected with *M. tuberculosis*::ΔleuD ΔpanCD::*pTiGc and treated with 100ug/ml DCS for 96h. Intracellular mycobacterial replication was compared to that of untreated intracellular mycobacteria, by monitoring TurboFP635 fluorescence. Data were analysed by using a Student’s t test. *p< 0.05; **p<0.005, ***p<0.0005. Data shown
is representative of 3 independent biological replicates (each with 3 technical replicates).
### Table 1. Plasmids and Strains

| Plasmid/Strain | Description | Source |
|---------------|-------------|--------|
| pST5552      | *hsp60(ribo)-egfp* (inducible EGFP under control of theophylline-inducible riboswitch), Kan<sup>R</sup>, episomal | pST5552 was a gift from Carolyn Bertozzi and Jessica Seeliger (Addgene plasmid number 36255) (Seeliger *et al*., 2012) |
| pCHARGE3     | Psmyc-TurboFP635, Hyg<sup>R</sup>, episomal | pCHARGE3 was a gift from Tanya Parish (Addgene plasmid number 24658) (Carroll *et al*., 2010) |
| pSTCHARGE3   | *hsp60(ribo)-turboFP635* (inducible TurboFP635 under control of theophylline-inducible riboswitch), Kan<sup>R</sup>, episomal | This study |
| pTiGc        | *hsp60(ribo)-turboFP635*, *hsp60-gfp*, Kan<sup>R</sup>, episomal | This study |
| *E. coli* DH10B | Cloning host | Invitrogen |
| *M. smegmatis* mc<sup>2</sup>155 | Non-pathogenic, fast-growing model organism | ATCC, 700084 |
| *M. tuberculosis* ΔleuD ΔpanCD | Double leucine and pantothenate auxotroph | (Sampson *et al*., 2004) |
Figure 1

(a) Inducible → Constitutive

(b) Culture + Inducer → Sub-culture - Inducer

(c) Culture - Inducer + Stressor → Sub-culture + Inducer
Figure 2

(a) Flow cytometry analysis of bacterial populations.

- M. smegmatis::pST5552 (no TurboFP635)
- M. smegmatis::pTiGc - Theo
- M. smegmatis::pTiGc + Theo

(b) Fluorescence microscopy images of bacterial populations.

- Theo

+ Theo
M. tuberculosis ∆leuD ∆panCD::pST5552 (no TurboFP635)
- M. tuberculosis::pTiGc - Theo
- M. tuberculosis::pTiGc + Theo

(d)

- Theo

+ Theo
Figure 3

(a) Scatter plot showing fluorescence intensity distribution of different bacterial strains.

- **M. smegmatis** (unmarked)
- **M. smegmatis::pTiGc, 7H9-OGT No CHL, 24 h**
- **M. smegmatis::pTiGc, PBS-T 30 CHL, 0 h**
- **M. smegmatis::pTiGc, PBS-T 30 CHL, 24 h**

(b) Fluorescence images showing GFP, TurboFP635, and Overlay for different conditions:

- 7H9-OGT No CHL 24 h
- PBS-T 30 CHL 0 h
- PBS-T 30 CHL 24 h
M. tuberculosis (unmarked)

M. tuberculosis ΔleuD ΔpanCD::pTiGc, 7H9-OGT No CHL, 96 h

M. tuberculosis ΔleuD ΔpanCD::pTiGc, PBS-T 30 CHL, 0 h

M. tuberculosis ΔleuD ΔpanCD::pTiGc, PBS-T 30 CHL, 96 h
Figure 4

(a) 0 h 3 h 6 h 9 h 12 h 15 h 18 h 21 h 24 h

(b) 0 h 24 h 48 h 72 h 96 h 120 h

(c) Generations vs. Time (hours)

(d) Generations vs. Time (hours)
Figure 5

(a) 0 h, minimal
(b) 6 h, rich
(c) 24 h, rich

(b) 0 h, - P
(b) 24 h, + LP
(b) 96 h, - P
(b) 48 h, + LP
Figure 6

(a)

In vitro cultured bacteria, 24h
Intracellular bacteria, 6h
Intracellular bacteria, 0h
Intracellular bacteria, 24h
In vitro cultured bacteria, 24h

(b)

GFP
TurboFP635
Overlay

0 h

6 h

24 h
In vitro cultured bacteria,

Intracellular bacteria, 0h
Intracellular bacteria, 24h
Intracellular bacteria, 96h

In vitro cultured bacteria, 96h

(c)

(d)

|                | GFP       | TurboFP635 | Overlay   |
|----------------|-----------|------------|-----------|
| 0 h            | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| 72 h           | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| 96 h           | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
Figure 7

(a) Untreated intracellular bacteria, 72h
DCS-treated intracellular bacteria, 0h
DCS-treated intracellular bacteria, 72h

Time in macrophages (hours) before DCS treatment

(b) DCS-treated intracellular bacteria, 72h
DCS-treated intracellular bacteria, 0h
Untreated intracellular bacteria, 72h
**Supplementary Material:**

**SUPPLEMENTARY FIGURE LEGENDS:**

**Fig. S1 Gating strategy.** All analysis of flow cytometry data was conducted using FlowJo V10. (a) Initial gating was carried out based on forward scatter (FSC) and side scatter (SSC) properties of the particles, using a polygon gate to capture particles representing single bacteria, typically with SSC-A between 5.0 x 10^3 and 1.4 x 10^4 and FSC-A between 6.9 x 10^3 and 3.0 x 10^4. (b) Next, the far-red and green fluorescent properties of the bacterial population were assessed, with a second rectangular gate set on the GFP-positive population, representing live bacteria. (c) The geometric mean of the far-red fluorescence was used to determine number of generations. A typical series of histogram plots is shown.

**Fig. S2 The TurboFP635 and GFP reporters are stable under acidic conditions.** To test whether the selected reporters would be stable under conditions potentially encountered within a macrophage, M. smegmatis::pTiGc was cultured in 7H9-OGT in the presence of 2 mM theophylline, then passaged twice in PBS-0.05% Tyloxapol + 2 mM theophylline, before inoculating into PBS-0.05% Tyloxapol with no theophylline, containing 30 μg/ml chloramphenicol (CHL), at (a) pH 6.4, (b) 5.5 and (c) 4.5, and incubating for 24 h. Samples were analysed by flow cytometry.

**Fig. S3 Sub-population of M. smegmatis with reduced red fluorescence recovered from macrophage cultures.** Flow cytometric analysis of M. smegmatis::pTiGc recovered from RAW264.7 macrophage cultures, showingTurboFP635 fluorescence of bacteria recovered at 24 h post-infection, indicating far-redLO and far-redHI gates.
Fig. S1 Gating strategy

(a) Gating strategy for bacteria using FSC-A vs. SSC-A flow cytometry parameters.

(b) Cytometry dot plots showing different conditions: Unlabelled, TurboFP635 only, pTiGc, 0 h, pTiGc, 6 h, pTiGc, 12 h, pTiGc, no theophylline.

(c) Histogram showing bacterial number (% M.I.O.) by TurboFP635.
Fig. S2 The TurboFP635 and EGFP reporters are stable under acidic conditions.

(a) M. smegmatis (unmarked)
(b) M. smegmatis::pTiGc, 7H9-OGT No CHL, 24 h
(c) M. smegmatis::pTiGc, PBS-T + CHL, 0 h

■ M. smegmatis::pTiGc, PBS-T + CHL, 24 h
Fig. S3 Sub-population of *M. smegmatis* with reduced red fluorescence recovered from macrophage cultures.